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In vitro cytotoxic and genotoxic effects of *Cissus verticillata* and *Sphagneticola trilobata* used for treatment of Diabetes Mellitus in Brazilian folk medicine

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ABSTRACT. *Cissus verticillata* and *Sphagneticola trilobata* have been used in Brazilian folk medicine for Diabetes Mellitus treatment, although their pharmacological and toxicological profile has not been clearly established. Thus, the aim of this study was to evaluate the preclinical toxicity of the aqueous extracts of *C. verticillata* and *S. trilobata*. The main groups of secondary metabolites were investigated, and the species differed by the presence of coumarins in *C. verticillata* and by tannins in *S. trilobata* extracts. The highest contents of phenolic compounds and flavonoids were quantified in *C. verticillata* infusion with 2.594 ± 0.04 mg equivalents of gallic acid g⁻¹ of extract and 1.301 ± 0.015 mg equivalents of catechin g⁻¹ of extract, respectively. While the extract of *S. trilobata* showed minimum values of these compounds, with 0.002 ± 0.001 mg equivalents of gallic acid g⁻¹ extract and 0.005 ± 0.0004 mg equivalents of catechin g⁻¹ of extract, respectively. These differences implied the results of *in vitro* antioxidant activity evaluated using ferric reducing antioxidant power (FRAP), in which the sample of *C. verticillata* at 5 mg mL⁻¹ showed a value of 122 µM ferrous sulfate equivalents (FSE), while *S. trilobata* showed 0.93 µM FSE at the same concentration. With respect to cytotoxic assay with murine fibroblast cell line (3T3) only *S. trilobata* exhibited cytotoxic effects measured by MTT and Sulforhodamine B assays, evidenced by the cell viability value of approximately 16%, in both tests after 24 and 72 hours of exposure of the cells to 5 mg mL⁻¹ of the extract. Comparatively, at 5 mg mL⁻¹ the *C. verticillata* extract showed cell viability of 142% and 95%, respectively, after 24 hours of cell exposure. On the other hand, both species showed genotoxic profiles evidenced by chromosomal aberrations by *Allium cepa* bioassay, observed by the higher percentage values of chromosome bridges, chromosome loss, and disturbed anaphase for all concentrations of both extracts than those of the negative control. The results support the characterization of the toxicological profile for both species and create an alert regarding the use of *S. trilobata*, which should be avoided.

Keywords: *Wedelia paludosa*; *Cissus sicyoides*; Phytochemistry; Cytotoxicity; Cytogenotoxicity; Medicinal plants.

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Introduction

Medicinal plants, until the 20th century, were the main way to treat and prevent diseases (Dutra, Campos, Santos, & Calixto, 2016). Although the drug development and new health technologies have been observed, about 80% of the world's population still uses plant species for therapeutic purposes (Corrêa, Rodrigues, & Barbano, 2016). In this way, the Ministry of Health in Brazil has encouraged the use of medicinal plants and herbal medicines in the Unified Health System (SUS), through the implementation of the National Policy on Integrative and Complementary Practices (PNPIC) (Corrêa, Rodrigues, & Barbano, 2016). Therefore, in 2017, it was instituted the State List of Medicinal Plants of interest of the Unified Health System and its Complementary List (REPLAME) in Rio Grande do Sul in the south of Brazil. Among their assignments, is the State Guide of Health Research Priorities, focusing on the use of native species of regional flora.

In this sense, Campos, Mineto, Raasch, Suyenaga, & Perassolo (2018) conducted a study about the use of medicinal plants in the adjuvant treatment of chronic diseases, in the integrated health center of a Brazilian Institution. “Insulin”, that is a common name attributed to *Cissus verticillata* and *Sphagneticola trilobata* species, was among the medicinal plants mentioned as complementary to the conventional treatment of chronic disease.

Cissus verticillata (L.) Nicolson & C.E. Jarvis (*Syn* = *C. sicyoides*) is a climbing plant of the Vitaceae family that can be easily found in several regions of Brazil and is popularly known as “*Insulin*, *Anil-trepador*, *Cortina-japonesa*, and *Uva-brava*”. Different effects have already been scientifically attributed to this species such as antitumor, anxiolytic, anticonvulsant, antilipemic, anti-inflammatory, and antidiarrheal (Almeida, Rafael, Couto, & Ishigami, 2009; Beserra, et al. 2016; Almeida, Oliveira, Lucena, Soares, & Couto, 2006; Lucena, et al. 2006).

On the other hand, *Sphagneticola trilobata* (L.) Pruski (*Syn* = *Acmela brasiliensis*, *Wedelia paludosa*), is a native species from the Brazilian flora that belongs to the Asteraceae family. This plant is popularly known as “*Pseudo-arnica*, *Margaridão*, *Pingo-de-ouro*, *Arnica-do-mato*, *Picão-da-praia*, and *Vedélia*” and can be found in many regions of Brazil. Despite being considered an ornamental plant owing to the presence of yellow flowers, it has been used in the folk medicine (Batista, Brandão, Braga, & Oliveira, 2009). Some studies have demonstrated different activities for *S. trilobata* extracts, such as anti-inflammatory, antifungal, trypanosomicidal, antimicrobial, and hypoglycemic (Chiari, et al. 1996; Block, Scheidt, Quintão, Santos, & Cechinel-Filho, 1998; Block, et al. 1998; Bresciani, et al. 2004).

However, the acute and chronic toxicological effects of both species are still unclear and do not support its safe use. In this sense, the investigation of the toxicological potential of medicinal plants contributes to ensuring the health of the population who use them. In addition, the cytotoxic and genotoxic potentials of plant species and xenobiotics should be checked in the preclinical stage, ensuring safety for subsequent stages such as a clinical trial. Therefore, the aim of this study was to perform the phytochemical screening and evaluate the *in vitro* antioxidant capacity of *C. verticillata* and *S. trilobata* aqueous extract, as well as to investigate their *in vitro* cytotoxicity and genotoxicity.

Material and methods

Solvents, reagents, and reference substances

In this study were utilized the P.A. grade solvents: acid glacial, ethyl alcohol absolute, ethyl acetate, dimethyl sulfoxide, formic acid, and hexane from Merck (Darmstadt, Germany) or Synth (Diadema, SP, Brazil). The reagents buffer phosphate saline (PBS), Dulbecco’s Modified Eagle Medium, fetal bovine serum (FBS), orcein-acetic, sodium hydroxide, sodium nitrite, aluminum chloride, *Folin-Ciocalteu* reagent, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, sodium carbonate, sulforhodamine B, trichloroacetic acid (TCA), and Tris buffer were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The reference substances catechin, gallic acid, kaempferol, luteolin, quercetin, and rutin were also purchased from Sigma.

Plant material

Aerial parts of *C. verticillata* and *S. trilobata* were collected in Novo Hamburgo, RS (South of Brazil) in 2018 (27°35’08.78” S and 48°31’16.26” W). Botanical identification was carried out at Herbarium of *Universidade Feevale* by an expert botanist and the voucher specimens were deposited under the numbers HEFE 454 and HEFE 456, respectively. The scientific names were checked in *The Plant List* database (<http://www.theplantlist.org/>, accessed in November 2018).

Preparation of extracts

For analysis on Thin Layer Chromatography (TLC), determination of total phenolic compounds, total content of flavonoids, and kaempferol, extracts from each species were obtained by maceration process, at 25%, for 3 days, with hydroalcoholic solution (80% v/v). The plant: solvent ratio was 1:1 and the extracts were kept protected from light until analysis.

For the investigation of the potential antioxidant effect and *in vitro* toxicological evaluation, were obtained infusions of each species at 80 °C, for 15 minutes. For the analyzes, serial dilutions were obtained from the infusions, according to the work concentrations described in the in the following *in vitro* methods (Agência Nacional de Vigilância Sanitária [ANVISA], 2019).

Phytochemical screening

Presence of the main groups of secondary metabolites was investigated: total phenolic compounds, tannins, flavonoids, cardiotonic heterosides, saponins, alkaloids, coumarins, anthraquinones, and anthocyanins (Costa, 2001).

Based on qualitative colorimetric reactions, the phenolic compounds were determined by reaction with ferric chloride, flavonoids with metal magnesium, in acid medium and anthocyanins in the presence of alkaline and acid substances. In addition, the presence of anthraquinones was verified with toluene and potassium hydroxide and cardiotonic heterosides through Keller-Kiliani (deoxyglucose), Kedde (lactonic ring), and Salkowsky reaction (steroid nucleus).

Determination of tannins and alkaloids was based on the principle of reactions with precipitate formation, with application of gelatin solution for tannins and reagents of Bertrand, Mayer, Dragendorff, and Bouchardat for alkaloids. Saponin research was based on the afrogenic property of this group, therefore, its determination was evaluated through the capacity of foam formation by the sample. Then coumarins were evaluated for their characteristic of fluorescence in alkaline medium and observed under ultraviolet light (365 nm) (Costa, 2001).

Determination of total content of phenolics compounds in hydroalcoholic extract

Total content of phenolics compounds was determined by *Folin-Ciocalteu* spectrophotometric method, according to Meda, Lamien, Romito, Millogo, and Nacoulma (2005) and Singleton, Orthofer, and Lamuela-Raventós (1999). Briefly, 500 μL of the extracts or standard solutions were added to 2.5 mL of 0.2 M *Folin-Ciocalteu* reagent. The solution was vortexed and equilibrated for 6 min. Then, 2 mL of sodium carbonate solution (75.0 g L^{-1}) were added. After 2 hours of incubation in the dark at room temperature, the absorbance was measured at 760 nm using a UV/Vis spectrophotometer (Shimadzu 2600, Japan). The phenolic content was expressed as mg of gallic acid equivalent (GAE) g^{-1} d.w. from the calibration curve of gallic acid standard solutions. All determinations were carried out in triplicate.

Determination of total flavonoids content in hydroalcoholic extract

Total flavonoids content was measured following the methodology described by Zhishen, Mengcheng, and Jianming (1999). In a 10 mL volumetric flask was added 1 mL of the hydroalcoholic extract, 2 mL of ultrapure water, 300 μL of 5% sodium nitrite and allowed to stand for 5 min. Then 300 μL of 10% aluminum chloride was added and allowed to stand for 6 min. Subsequently, 2 mL of 1 M sodium hydroxide was added, and the volume was made up with ultrapure water. Analyzes were carried out in a spectrophotometer at a wavelength of 510 nm. Total flavonoids content was expressed in mg catechin equivalent (CE) g^{-1} d.w. All experiments were performed in triplicate.

Thin Layer Chromatography

For the analyses in TLC, silica gel GF₂₅₄ plates (Merck) were used as stationary phase. The solvent systems consisted of ethyl acetate: hexane (3:1 v: v); hexane: ethyl acetate (1:1 v: v), and hexane: ethyl acetate (3:1 v: v) (Beltrame, et al. 2001). Flavonoids (kaempferol, luteolin, quercetin, and rutin) were used as reference substances. Spots were applied to the plate using a thin glass capillary enough to apply a neat spot. After the sample separation in chromatography vessel, nebulization with aluminum chloride in methanol was performed for visualization of the bands by ultraviolet light at 365 nm. Finally, the retention factors were calculated (R_f).

Determination of kaempferol content in hydroalcoholic extract

High Performance Liquid Chromatography (HPLC) analysis was performed on a Shimadzu UFLC system, equipped with LC 20A quaternary gradient pump using SPD-M20A PDA diode array detector (DAD). The data was acquired on the liquid chromatography solution administrator data system using BETASIL C18 column (150 x 4.6 mm, 3 μm) and an isocratic mixture of methanol HPLC. and water containing 0.1% v/v formic acid in the ratio of 80:20. Always before used, the mobile phase was filtered through a 0.22 μm Millipore filter and degassed by sonication for 30 min. The flow rate was adjusted to 0.4 mL min^{-1} . Injection volume was adjusted to 5 μL and detection was made at wavelength 258 nm (Al-Rifai, Aqel, Awaad, & ALOthman, 2015).

Determination of total antioxidant activity using FRAP (Ferric Reducing Antioxidant Power) assay in aqueous extract

Aqueous extracts were obtained at concentrations of 2.5, 5.0 and 10.0 mg mL⁻¹ by infusion previously described in *Preparation extract Section*. Ferric reducing antioxidant power assay (FRAP) was measured according method described by Benzie and Strain (1996) in infusions of *C. sicyoides* and *S. trilobata*. The results were expressed as μ M of ferrous sulfate equivalents (FSE), based in a standard curve elaborated with FeSO₄ solutions.

Evaluation of cytotoxicity, mutagenicity, and genotoxicity using *Allium cepa* bioassay in aqueous extract

For the preliminary evaluation of cytotoxicity, genotoxicity, and mutagenicity the *A. cepa* bioassay was performed based on Fiskesjo (1985). Infusions were previously obtained (*Preparation extract Section*) from leaves of *C. verticillata* and *S. trilobata* at concentrations 2.5, 5.0 and 10.0 mg mL⁻¹. Thirty small bulbs of *A. cepa* were immersed in distilled water for 24 hours to stimulate the root growth of the meristem. Then, the roots were exposed at three concentrations of infusions, the negative control (distilled water) and the positive control (acetaminophen 800.0 mg L⁻¹) for 48 hours. After exposure period, root growth was evaluated by measuring the length of three roots of each bulb to calculate the general cytotoxicity index by the inhibitory effect of the extract. Subsequently, several root tips were removed from the bulbs, fixed in 3:1 (v/v) ethyl alcohol: glacial acid acetic PA. Roots were stored overnight at 4 °C. Then the roots were placed in ethanol 70% and refrigerated until use. For slide preparation, the roots were hydrolyzed in HCl 1 N for 5-6 minutes at 60 °C and rinsed in distilled water. Afterwards, the portion of the root with cell division were washed again in distilled water, stained with 1% orcein-acetic solution on microcopy slides for 1 hour. For macroscopic evaluation of cytotoxicity, the root length predicting the general cytotoxicity index was evaluated for the inhibitory effect caused by the extract. Identification parameters of (a) cytotoxicity, (b) mutagenicity, and (c) genotoxicity were: (a) Mitotic Index (MI); (b) Micronuclei (MN) per 1000 cells and presence of (c) Chromosomal Aberrations (CA) was evaluated in 200 anaphases and telophases respectively. CA were classified such as: anaphase with chromosome bridge and chromosome loss, disturbed anaphase, telophase with bridge and chromosome loss. Cells were analyzed by optical microscopy 100x.

Evaluation of cytotoxicity in cell culture

Cell culture and exposure medium

Fibroblasts Balb/c 3T3 cell line (purchased from the Rio de Janeiro Cell Bank, Brazil) were seeded in DMEM (Dulbecco's Modified Eagle Medium) with 10% v/v of fetal bovine serum and maintained at 37 °C in a humid atmosphere at 5% of CO₂. For cytotoxicity assays, the cells were seeded at a rate of 2×10^4 cells/well in a 96-well polystyrene microplate with 100 μ L of low glucose DMEM. Then, the microplates were maintained for 24 hours, at same atmosphere conditions until exposure.

Aqueous extracts (*S. trilobata* and *C. verticillata*) prepared by infusion were used as an Exposure Medium (EM). Bicarbonate, Hepes, DMEM, and 1% FBS reagents were added to each extract. EM were filtered by filter 0.22 μ m and the extracts were diluted with DMEM medium to obtain the different concentrations: for *S. trilobata* were tested 0.5 mg mL⁻¹, 1.0 mg mL⁻¹, 2.0 mg mL⁻¹, 3.0 mg mL⁻¹, 3.5 mg mL⁻¹, 4.0 mg mL⁻¹, 5.0 mg mL⁻¹, and 6.0 mg mL⁻¹, and for *C. verticillata* were tested 1.0 mg mL⁻¹, 5.0 mg mL⁻¹, 10.0 mg mL⁻¹, 15.0 mg mL⁻¹, and 30.0 mg mL⁻¹. These concentrations were determined based on the results of *Allium cepa* bioassay. The EM were exchanged each 24 hours. The evaluation times were 24 and 72 hours, corresponding to acute and chronic cytotoxicity, respectively. For negative controls, the cells were maintained only with DMEM 1% FBS, while for positive control, the cultures were exposed to H₂O₂ 1% for 1 hour before the cytotoxicity assays.

Cytotoxicity assay using MTT reduction and Sulforhodamine B

In order, to evaluate the acute and chronic cytotoxicity using the mitochondrial activity parameter, a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used as described by Mosmann (1983). After exposure for 24 hours, the medium was discarded and the well was rinsed with buffer phosphate saline pH 7.4 (PBS), 8 μ L of MTT (5 mg mL⁻¹) in 200 μ L of DMEM was add to the well and the plates are incubated for two hours at 37°C. After incubation, the medium was removed and 200 μ L of dimethyl sulfoxide (DMSO) was added to each well for formazan crystals solubilization. All samples were transferred to a 96-well plate and the absorbance

was measured at 570 nm with a microplate spectrophotometer Spectramax M3 (Molecular Devices®), using 200 µL of DMSO for the blank. The same assay was performed after 72 hours of exposure.

Sulforhodamine B (SRB) assay was performed according Skehan et al. (1990), and this method is based on the ability of the dye to bind on the cell proteins that were fixed with trichloroacetic acid (TCA). After 24 hours of exposure, the exposure medium was discarded, and the cells were rinsed with 100 µL PBS. After, 50 µL of 1% trichloroacetic acid were added to the wells and maintained for 1 hour at 10°C. Then, the contents were discarded, and 5 rinses were performed with ultrapure water. Subsequently, 50 µL of SRB was added and incubated for 30 minutes at room temperature (25 °C). SRB was discarded and 4 rinses with 1% acetic acid were performed. Finally, 200 µL of Tris pH 10.8 buffer was add in each well and the content was transferred to the reading plate. The absorbance was measured by microplate spectrophotometer at 564 nm, using. 200 µL of Tris buffer for the blank. The same assay was performed after 72 hours of exposure.

For each test, two independent studies were performed, all of them in quadruplicates. Data obtained were expressed as mean and standard deviation.

Statistical analyses

Statistical analyses were performed by GraphPad Prism software (version 6.0), with Kruskal-Wallis followed by Dunn *post hoc* test and ANOVA-two way followed by Bonferroni *post hoc* test. The significance level was set at 5.00%.

Results and discussion

Phytochemical screening, thin layer chromatography, and determination of total phenolics compounds and total flavonoids in hydroalcoholic extracts

In the phytochemical screening of *C. verticillata* extract flavonoids, phenolic compounds, coumarins, saponins, and alkaloids were identified. And in the *S. trilobata* flavonoids, phenolic compounds, tannins, saponins, and alkaloids were identified. Therefore, both species showed similarity in phytochemical screening with moderate presence of phenolic compounds and flavonoids, differing by the presence of tannins in *S. trilobata* and coumarins in *C. verticillata*. In the same way, Viana et al. (2004) and Dias et al. (2017) identified steroids, tannins, phenolic compounds, coumarins, flavonoids, and saponins in the extract of *C. verticillata* leaves. And previous studies reported the presence of terpenic compounds, steroidal compounds, flavonoids, and absence of alkaloids in *S. trilobata* (Novaes, et al., 2001; Batista, et al., 2009).

Three eluent systems of different degrees of polarity were utilized for quercetin, rutin, luteolin and kaempferol reference substances. Through this analysis, no bands corresponding to these substances were observed in the *S. trilobata* extract. Kaempferol was characterized only in the extract of *C. verticillata*, observed through the bands that presented the same retention factors (Rf) in relation to the kaempferol reference substance, according to what is presented in Table 1. An HPLC method was performed to quantify the kaempferol content in *C. verticillata*, confirming the presence of 45.8 µg mL⁻¹. In addition, other flavonoids (lutein, quercetin and rutin) were not characterized in the sample of *C. verticillata* in these eluent systems used (Table 1).

With regards to the moderate presence of phenolic compounds and flavonoids in both extracts, the total contents of these compounds were measured, and the results are shown in Table 2. In both assays, the extract of *C. verticillata* exhibited higher concentrations of phenolic compounds and flavonoids than *S. trilobata*, superior to 1000-fold and 260-fold, respectively. Phenolic compounds have high antioxidant activity, being responsible for capturing reactive oxygen species and preventing cellular damage caused by oxidative stress in humans, thus preventing the development of diseases (Kasote, Katyare, Hegde, & Bae, 2015; Lin et al., 2016; Tungmunthum, Thongboonyou, Pholboon, & Yangsabai, 2018).

Sulphur homeopathic medicine showed potential to inhibit *S. sclerotiorum* growth in all dynamizations, as shown in Figure 1. In the first evaluation - day 4 - it was possible to verify that the growth of the control treatment was greater than the fungus submitted to treatment with Sulphur, and statistically significant with Sulphur 200CH and 1000CH. The best suppressive effect over the fungus was observed with the dynamization of 1000CH, which has inhibited more efficiently the *S. sclerotiorum* growth during the experiments compared to control treatment.

Table 1. Results of Thin Layer Chromatography.

Mobile phase	Sample	UV coloration	Rf* value
Ethyl acetate : hexane (50:50%)	Kaempferol	Blue-green	0.67
	Luteolin	Yellow	0.30
	Quercetin	Blue	0.80
	Rutin	Light blue	0.20
	<i>C. verticillata</i>	Blue-green	0.67
	<i>S. trilobata</i>	Red	0.89
Ethyl acetate : hexane (70:30%)	Kaempferol	Light yellow	0.75
	Luteolin	Light yellow	0.15
	Quercetin	Dark yellow	0.96
	Rutin	Dark yellow	0.17
	<i>C. verticillata</i>	Light yellow	0.75
	<i>S. trilobata</i>	Red	0.83
Ethyl acetate :hexane (30:70%)	Kaempferol	Light yellow	0.14
	Luteolin	-	-
	Quercetin	Blue	0.40
	Rutin	-	-
	<i>C. verticillata</i>	Light yellow	0.14
	<i>S. trilobata</i>	Red	0.72
		Red	0.47

(-): not applicable; *Retention factor.

Table 2. Quantification of total phenolic compounds and flavonoids in the hydroalcoholic extracts of *Cissus verticillata* and *Sphagneticola trilobata* leaves

Samples	Total phenolic compounds (mg GAE g ⁻¹)	Total flavonoids (mg CE g ⁻¹)
<i>C. verticillata</i>	2.5940 ± 0.0400	1.3010 ± 0.0150
<i>S. trilobata</i>	0.0020 ± 0.0008	0.0050 ± 0.0004

Values expressed as mean ± standard deviation; GAE: gallic acid equivalents; CE: catechin equivalents.

Antioxidant activity using FRAP assay of aqueous extracts

Antioxidant activity for both plant species is presented in Table 3. As it is shown, both extracts at concentrations of 2.5 mg mL⁻¹, 5.0 mg mL⁻¹, and 10.0 mg mL⁻¹ implied a proportional increase in antioxidant potential. *Cissus verticillata* showed higher antioxidant capacity than *S. trilobata*. Additionally, the presence of coumarins in *C. verticillata* infusion could contribute to the antioxidant effect of this species owing to their electronegative groups (Borges Bubols et al., 2013; Al-Majedy, Al-Amiery, Kadhum, & BakarMohamad, 2016).

Table 3. Evaluation of *in vitro* antioxidant activity of aqueous extracts of *Cissus verticillata* and *Sphagneticola trilobata* by FRAP assay

Plant	Concentration		
	2.5 mg mL ⁻¹	5.0 mg mL ⁻¹	10.0 mg mL ⁻¹
<i>C. verticillata</i>	91.70 µM FSE	122.00 µM FSE	340.40 µM FSE
<i>S. trilobata</i>	0.74 µM FSE	0.93 µM FSE	1.92 µM FSE

FSE: ferrous sulfate equivalent.

Studies have shown that the concentration of phenolic compounds and flavonoids is related to their antioxidant capacity. Song et al. (2010) observed a positive correlation between the antioxidant capacity and the total polyphenol content in 56 plant species. In addition, important antidiabetic properties have been attributed to these metabolites, performed by normalizing blood glucose levels, glucose uptake, insulin secretion, and immune function modulation DM (Lin et al., 2016; Panche, Diwan, & Chandra, 2016). Some reviews, which summarize the beneficial effects of antioxidants in DM, highlight the ability to reduce the oxidative stress and inflammation process generated by this chronic metabolic disease. By stabilizing free radicals, antioxidant compounds help to reduce complications of DM, such as vascular ones (Bajaj & Khan, 2014; Rajendiran, Packirisamy, & Gunasekaran, 2018). Among flavonoids, it has been reported that kaempferol reduces glucose levels, glycated hemoglobin, and improves insulin resistance. This information supports the antidiabetic effect of *C. verticillata* in a preliminary study (Viana et al., 2004).

Cytotoxicity, mutagenicity, and genotoxicity of aqueous extracts using *Allium cepa* bioassay

Regarding toxicity, tests using the *Allium cepa* bioassay have been used as a platform to test different plant species. According to Tedesco and Laughinghouse IV (2012), *A. cepa* bioassay is an excellent indicator of chromosomal alterations caused by exposure to agents such as plants extracts. In addition, Fiskesjo (1985) defined this bioassay as an important parameter for the identification of cytotoxicity and cytogenetic damages in the cell cycle by evaluation of the parameters root length, mitotic index (MI), micronuclei, and chromosomal aberrations.

Table 4 shows the results of micronuclei frequency, mitotic index, and root length at different concentrations of aqueous extracts of *C. verticillata* and *S. trilobata* leaves. No reduction was observed in root length between treatments ($p > 0.05$). On the other hand, the positive control group was significantly different from the other treatments for micronuclei parameter. The MI results showed a significant reduction ($p < 0.001$) by exposure to 10.0 mg mL^{-1} de *C. verticillata* when compared with other treatments. This result predicts a cytotoxic effect for *C. verticillata* infusion at 10.0 mg mL^{-1} . Based in mutagenicity analysis by *A. cepa*, the different extracts concentration of *C. verticillata* did not show micronuclei (MN).

Table 4. Results of aqueous extracts of *Cissus verticillata* and *Sphagneticola trilobata* leaves to the bioassay in *Allium cepa* in relation to micronuclei frequency, mitotic index, and root growth

Plant	Treatment	MN/1000 cells	MI/1000 cells (%)	Root growth (cm)
<i>C. verticillata</i>	PC	1.1 ± 1.3^a	0.4 ± 0.3^a	2.4 ± 0.3^a
	NC	0.0 ± 0.0^b	4.7 ± 2.0^b	2.3 ± 0.5^a
	2.5 mg mL^{-1}	0.0 ± 0.0^b	3.0 ± 1.8^b	2.1 ± 0.4^a
	5.0 mg mL^{-1}	0.0 ± 0.0^b	3.2 ± 1.5^b	2.1 ± 0.6^a
	10.0 mg mL^{-1}	0.0 ± 0.0^b	1.2 ± 0.5^a	1.9 ± 0.3^a
	P	0.012	0.001	0.120
<i>S. trilobata</i>	PC	-	-	1.3 ± 0.5^a
	NC	0.0 ± 0.0	4.7 ± 0.7^a	2.5 ± 0.5^b
	2.5 mg mL^{-1}	0.0 ± 0.0	2.2 ± 1.2^b	1.6 ± 0.8^a
	5.0 mg mL^{-1}	0.0 ± 0.0	2.4 ± 0.9^b	1.8 ± 0.3^a
	10.0 mg mL^{-1}	0.0 ± 0.0	2.9 ± 1.0^{ab}	1.4 ± 0.4^a
	P	1.000	0.007	0.002

Negative Control (NC): distilled water; Positive Control (PC): acetaminophen 800 mg L^{-1} ; (MN): Micronuclei; (MI): Mitotic Index. Values with different letters (^{ab}) shows significant difference (Kruskal-Wallis followed by Dunn post hoc test, $p < 0.05$); (-) Analyzed cells were not enough for the statistic.

For *S. trilobata*, MI values was lower than that of the negative control group, indicating decreased cell division. The average root length for all replicas was greater than the negative control. In contrast to the results found for the aqueous extract of *C. verticillata* in the *A. cepa* bioassay, the extract of *S. trilobata* showed an effect of cytotoxicity evidenced by the reduction of MI in the concentrations of 2.5 mg mL^{-1} , 5.0 mg mL^{-1} and 10.0 mg L^{-1} ($p = 0.007$). In addition, the root length of *A. cepa* was shorter after application of the three concentrations, compared to the negative control ($p = 0.002$).

The results of chromosomal aberrations in *A. cepa* root after exposure to infusions of *C. verticillata* and *S. trilobata* are shown in Table 5. With respect to *C. verticillata* infusion there was no statistical difference for chromosomes with bridges ($p = 0.190$) and chromosomes with loss ($p = 0.200$) presented in anaphases and telophases. However, at 5.0 mg mL^{-1} and 10.0 mg mL^{-1} it was possible to observe a high percentage of disturbed anaphases, characteristic of genotoxic effect.

Likewise, in the study conducted by Vicentini, Camparoto, Teixeira, & Mantovani (2001), to evaluate the cytotoxicity of extract *C. verticillata* at 0.07 and 0.70 mg mL^{-1} over 24 hours did not evidence alterations in the meristematic cells using the *A. cepa* bioassay. In another study, Sáenz, García, Quilez, & Ahumada (2000) demonstrated that the aqueous extracts from *Agave intermixta* (0.5 g mL^{-1}) and *C. verticillata* (0.5 g mL^{-1}), in combination showed a mitodepressive effect on the rate of cell division in *Allium cepa* root meristems and no chromosomal aberrations was observed.

Allium cepa bioassay also showed a pronounced toxic effect caused by *S. trilobata* extract when compared with the negative control owing to large occurrence of chromosomal aberrations such as anaphases and telophases with chromosome bridge, anaphases, and telophases with chromosome loss as well as disturbed anaphases. This result suggests a genotoxic effect of *S. trilobata* extract at all concentrations tested. The Figure 1 illustrates some of the chromosomal aberrations after exposing the roots to the extracts.

Table 5. Results of chromosomal aberrations of infusions of *Cissus verticillata* and *Sphagneticola trilobata* by *Allium cepa* bioassay.

Plant	Treatment	Chromosome bridges (%)	Chromosome loss (%)	Disturbed Anaphase (%)
<i>C. verticillata</i>	PC	-	-	-
	NC	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0 ^a
	2.5 mg mL ⁻¹	2.3 ± 3.8	3.2 ± 3.5	0.0 ± 0.0 ^a
	5.0 mg mL ⁻¹	1.9 ± 2.6	1.0 ± 1.1	4.6 ± 6.1 ^b
	10.0 mg mL ⁻¹	7.4 ± 10.2	0.5 ± 0.9	4.2 ± 4.8 ^b
	<i>P</i>	0.190	0.200	0.020
<i>S. trilobata</i>	PC	-	-	-
	NC	0.3 ± 0.5 ^a	6.7 ± 5.0 ^a	0.0 ± 0.0 ^a
	2.5 mg mL ⁻¹	8.6 ± 6.0 ^b	15.4 ± 3.3 ^b	14.4 ± 7.3 ^b
	5.0 mg mL ⁻¹	10.2 ± 7.9 ^b	7.7 ± 2.2 ^a	7.9 ± 4.5 ^b
	10.0 mg mL ⁻¹	8.3 ± 6.3 ^b	19.1 ± 8.9 ^b	13.8 ± 12.0 ^b
	<i>P</i>	0.020	0.011	0.033

Negative Control (NC): distilled water; Positive Control (PC): acetaminophen 800 mg L⁻¹. Values with different letters (^{ab}) shows significant difference (Kruskal-Wallis followed by Dunn post hoc test, $p < 0.05$). (-) Analyzed cells were not enough for the statistic. Analysis in 200 cells.

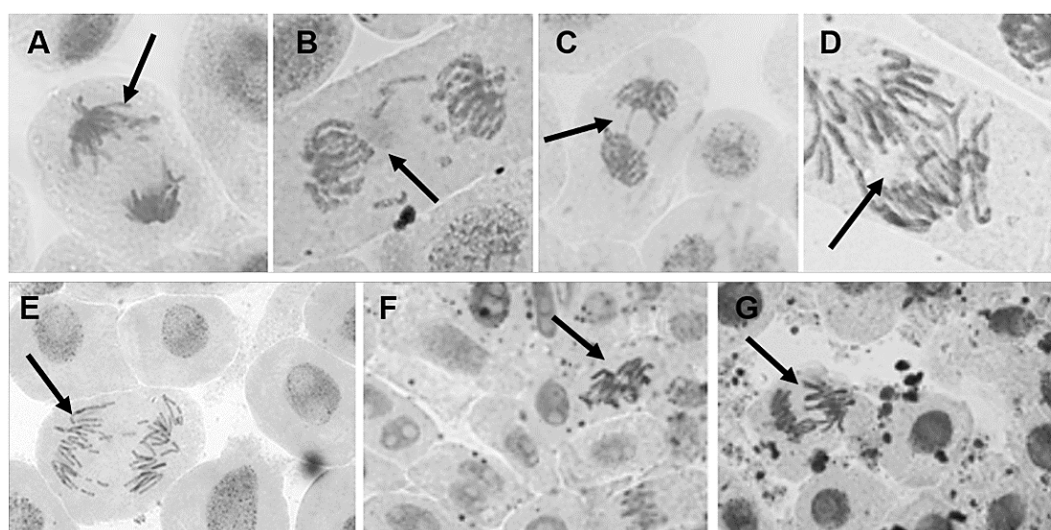


Figure 1. Chromosomal aberrations visualized in the meristem root of *Allium cepa* exposed to *Cissus verticillata* aqueous extract: (A) anaphase with chromosome loss; (B) telophase with chromosome loss; (C) anaphase with chromosome bridge; (D) disturbed anaphase; and exposed to *Sphagneticola trilobata* aqueous extract: (E) anaphase with chromosome loss; (F) disturbed anaphase; (G) anaphase with chromosome bridge. The arrows indicate the respective chromosomal aberrations.

Cytotoxicity assay using MTT reduction and Sulforhodamine B

Results of cytotoxicity assay presented in Figure 2 demonstrated a dose-dependent increase in the cell viability in 3T3 cells treated to *C. verticillata*. Comparing acute (24 hours) and chronic (72 hours) cytotoxicity of *C. verticillata*, mitochondrial activity (MTT assay) was above negative control, indicating cell proliferation (Figure 2 A). Corroborating these results, it was verified by Sulforhodamine B (SRB) assay an increase in the cell protein density (Figure 2 B) and analyzes of the images from microscope also indicate an increase in the number of cells. This result suggests a possibility of cell proliferation of 3T3 cells in the presence of the extract. None of the concentrations of this species showed cytotoxic profile for this cell line in MTT and SRB assays after exposure period of 24 and 72 hours.

However, in the same comparison for *S. trilobata* a significant reduction started between concentrations 1.0 and 2.0 mg mL⁻¹ at 72 hours. The cytotoxic effect resulted in mitochondrial activity below 70% of negative control representing a cytotoxic profile. In MTT assay (Figure 2 C) there was a significant difference in concentrations 2.0 mg mL⁻¹ ($p < 0.01$), 4.0 mg mL⁻¹ ($p < 0.01$) and negative control ($p < 0.5$) when comparing exposure between 24 and 72 hours at different concentrations. Similarly, the SRB assay (Figure 2 D) showed statistical difference in concentrations 0.5 mg mL⁻¹ and 2.0 mg mL⁻¹ ($p < 0.01$) by analyzing the same variables. For MTT and SRB assay, *S. trilobata* exhibits a dose-dependent cytotoxicity profile beginning in 2.0 mg mL⁻¹ at 72 hours of exposure when concentrations showed a cell viability rate equal or inferior to 70%. The same toxicity profile is not present at concentrations 0.5 mg mL⁻¹, 1.0 mg mL⁻¹, and 2.0 mg mL⁻¹ for 24 hours of the extract exposure suggesting no toxicity at these concentrations in 3T3 cells.

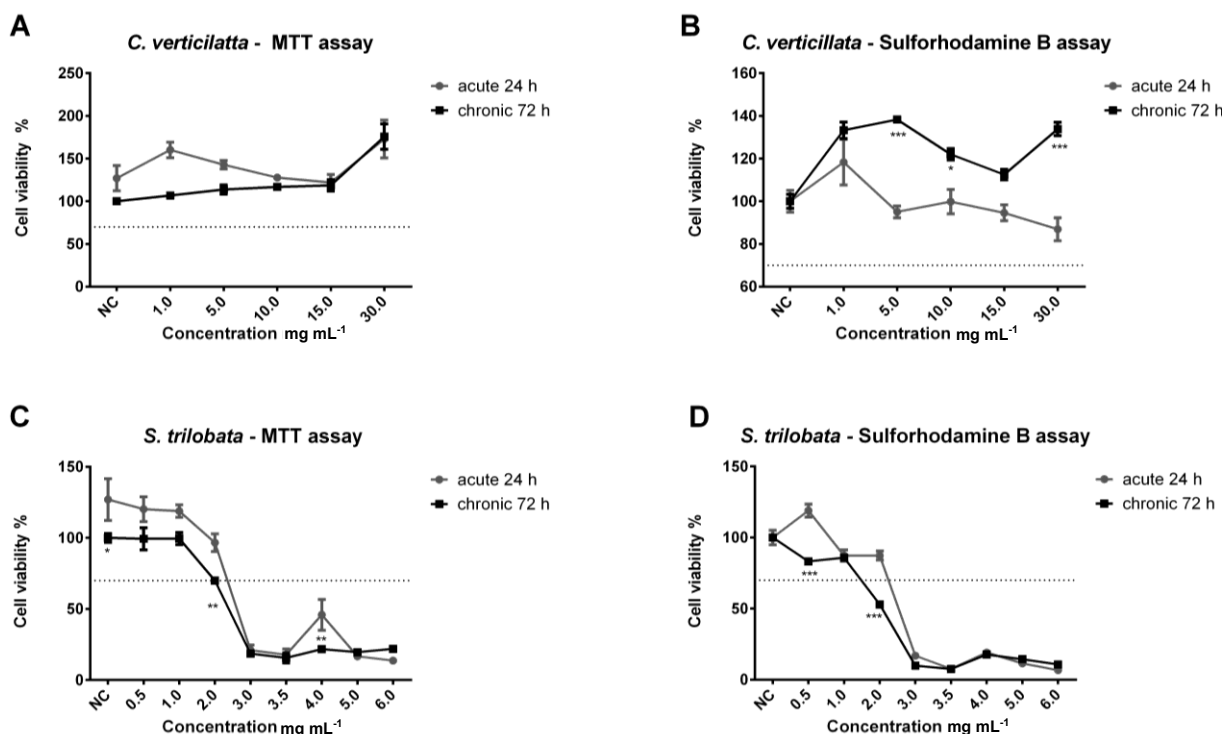


Figure 2. Cytotoxicity of leaves of *Cissus verticillata* (A, B) and *Sphagneticola trilobata* (C, D) in 3T3 cells. The dashed line presents 70% of the control value (NC – negative control). Values below the dashed line are considered cytotoxic. Two-way ANOVA followed by Bonferroni post hoc test, comparing cell exposure for 24 and 72 hours under the same concentrations as the extracts (** $p < 0.001$; * $p < 0.01$; ** $p < 0.05$).

In both assays, 3.0 mg mL^{-1} of *S. trilobata* showed high cytotoxicity characterized by cell viability percentage below 20% of negative control. A reduction in protein expression can be observed in the cells exposed at 24 hours and 72 hours to *S. trilobata* extract. Exposure time is directly correlated to the cell damage and this is reproduced in cell viability percentages shown in SRB assay.

Qualitative evaluation performed through microscopic observation after 24, 48, and 72 hours of exposure showed dose-dependent morphological alterations in all concentrations of *S. trilobata* extract. The evaluation revealed retraction, cellular detachment, rounding of cell border, and intercellular spaces increase as shown in Figure 3.



Figure 3. Cytotoxic effect of *Sphagneticola trilobata* extract (2.0 mg mL^{-1}) on morphology of 3T3 cells comparing: (A) Negative Control - 0 h; (B) acute exposure (24 hours); (C) chronic exposure (72 hours) exposure. The arrows indicate morphological alterations and cell death.

Few studies support the toxicological potential of *S. trilobata*. Batista et al. (2009), found cytotoxic activity of *S. trilobata* aerial parts demonstrated by kaurenoic acid and grandiflorenic acid isolated from the organic fraction of its extract by *Artemia salina* assay. However, this effect was not evidenced in the aqueous fraction. Another study carried out by Bürger et al. (2005) evaluated the toxicity of a hydroalcoholic extract of *S. trilobata* aerial parts in Swiss mice after extract administration (500.0 , 1000.0 , 2000.0 , and $4000.0 \text{ mg kg}^{-1}$) by protocols of acute and subacute toxicity. In the first protocol, it was obtained a LD₅₀ (lethal dose 50 %) higher than 4000 mg kg^{-1} . Whereas, in subacute treatment, alterations in body weight and hematological parameters were not observed, suggesting the plant was free from toxic effects. Toxic effects may be suggested by the presence of tannins in aqueous extract of *S. trilobata* leaves.

Tannins are part of the phenolic compounds group, because they are highly hydroxylated, differing from the other compounds by their reactivity with proteins and enzymes. This affinity is related to the hydrogen bonds that occur between the phenols of the tannins and the carbonyl group of the peptide of proteins (Field & Lettinga, 1992). Among related effects, mutagenic and carcinogenic were observed (Chung, Wong, Wei, Huang, & Lin, 1998). Another study conducted by Eck-Varanka et al. (2015) demonstrated the toxic effect of tannins by micronuclei assay.

Conclusion

The results suggest the *in vitro* toxicity of *S. trilobata* infusion using *A. cepa* bioassay and cytotoxic assay. In this regard, patients and the population should be advised of the risks associated with the use of this medicinal plant. In contrast, *C. verticillata* despite exhibiting genotoxic potential at high concentrations, showed relevant and promising antioxidant potential for the management of diabetic patients. Future experiments will be conducted to identify the compounds related to the toxicological and hypoglycemic effects of both species.

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