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Phytochemical Analysis, Antioxidant And Cytotoxic Activities Of Sida Rhombifolia Linn.

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Abstract:
Background*: The species Sida rhombifolia Linn, popularly known as "guanxuma", has several popular therapeutic uses. Therefore, this study aimed to characterize the ethanolic extract of the species collected in southern Brazil.*
Materials and Methods*: The extract was subjected to qualitative and quantitative tests regarding the content of secondary metabolites, using spectroscopic and liquid and gas chromatographic methods to trace its profile of chemical compounds. The study also investigated the antioxidant potential using the DPPH and ABTS methods. Cell viability was also analyzed using the MTT method.*
Results*: Total polyphenol content was 134.0±0.25 mg/g, total flavonoids 14.46±0.05 mg/g and condensed tannins 30.7±0.11 mg/g of ESR. In the HPLC-MS analysis, the compound with the highest concentration was isoquercetrin (3.00±0.077 mg/g), followed by the results of apigenin (2.02±0.01 mg/100g) and results of kaempferol (135. 87 ±0.01mg/g). In the CG-DIC analysis, specific for fatty acids, the highest concentrations were palmitic, linoleic and alpha-linolenic acids, respectively. The DPPH method presented an IC50 value of 1554.92 ± 0.18 µg/mL and ABTS the FRS% value was 101.8. The MTT results do not contain concentrations of cytotoxic effects.*
Conclusion: *The data observed in this research show therapeutic potential in the species as S. rhombifolia has good antioxidant potential.*
Key Word*: Sida rhombifolia, Malvaceae, Guanxuma, Biological activity, Antioxidant activity.*

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I. Introduction

The species Sida rhombifolia L. is known as a fortifier, diuretic, antidiarrheal, antiseptic, antibiotic, tonic and anti-inflammatory, among which it is used to treat diabetes [1,2.] Sida rhombifolia is one of 200 species of the genus Sida, belonging to the Malvaceae family. It grows in tropical and warm regions and is distributed throughout the tropics [1, 2, 3]. The original genus Sida is native to Brazil, where has a wide distribution in the Northeast and South and to a lesser extent in the North, Midwest and Southeast of the country [2]. Also known as 'guanxuma', 'mata pasto', 'vassourinha', 'relógio', it is a plant native to the American continent, occurring intensively in South America. It also occurs to a lesser extent in the south of the United States. The genus Sida is considered native to Brazil and has 103 species according to Bovini (2015) in the List of species of the Flora of Brazil [4]. The phytochemical investigation of this genus has resulted in the identification of around 142 chemical constituents, among which alkaloids, flavonoids and ecdysteroids [5,6] are the predominant groups. The crude and isolated extracts have shown a broad spectrum of pharmacological effects in vitro and ex vivo [7].
Plants have developed enzymatic and non-enzymatic defense mechanisms capable of neutralizing the cytotoxicity of reactive oxygen species (ROS) [8]. Therefore, the aim of this study was to conduct a preliminary phytochemical analysis and identify the main classes of secondary metabolites and the antioxidant activity of the species, as well as to evaluate the cytotoxic effect of the extract in vitro.

II. Material And Methods

Botanical material and extract preparation: Samples of Sida rhombifolia were collected in São Gabriel (30º 18' 21S and 54º 19' 41W), Rio Grande do Sul, from December (2018) to February (2019) (summer). They were then authenticated and deposited in the herbarium of the Federal University of Santa Maria, exsiccate number SMDB 17.237. This research was registered in the National System for the Management of Genetic Resources and Associated Traditional Knowledge (SisGen - AFB6706). The aerial parts and roots of the plant were separated and dried in an oven (from 40 to 50ºC) and pulverized in a mill. The 70% hydroethanolic extract was obtained by cold maceration with solvent renewal until the plant was completely exhausted. The organic solvent was then evaporated, and the extract was freeze-dried. The final freeze-dried product was stored in a freezer until the biological tests.

Identification and characterization of the chemical matrix: The phytochemical classes [9,10,11] researched in the aerial parts of the Sida rhombifolia plant were saponins [12], tannins [13,12], phenolic compounds and flavonoids [12], coumarins [12] and anthocyanin heterosides [14,13,15].

Dosage of total polyphenols, flavonoids, and condensed tannins: Total polyphenols were determined using the Folin-Ciocalteau [16], while flavonoids were determined using the methodology proposed by RIO (1996)[17] , and condensed tannins were determined by Agostini Costa et. al. (1999)[18] and Agostini Costa et. al. (2003)[19].

High performance liquid chromatography (HPLC) - Quantification of phenolic compounds: The detection and quantification of phenolic compounds in the extract of S.ida rhombifolia was conducted following a validated method [20] using a High Performance Liquid Chromatography (HPLC) coupled to a Photodiode Array (PDA) Detector. The separation was conducted on a reversed-phase C-18 column (particle size 5 µm, 150 mm, 4.6 mm) at 38°C. The injection volume was 20 µL and the mobile phases consisted of 5% (v/v) methanol in acidified water (0.1% (v/v) formic acid) and 0.1% (v/v) formic acid in acetonitrile. The phenolic compounds were identified by comparison with the retention time of authentic standards and the spectral data obtained from UV-visible absorption spectra. For quantification purposes, chromatograms were obtained at 280 nm, 320 nm and 360 nm.

Gas chromatography coupled with mass spectrometry: Specific Analysis of Fatty Acids: The analyses were conducted on an Agilent Technologies 6890 gas chromatograph equipped with a flame ionization detector (CG-DIC) and auto-injector. The temperature remained constant at 280 ºC. The FA separation was conducted on a silica capillary column model number 2560, with a length of 100 m, an internal diameter of 0.25 m and a stationary phase thickness of 0.20 µm. Nitrogen was used as the carrier gas, under an initial pressure of 15 psi. The flow rate was kept constant at 0.3 mL min-1. The furnace temperature (T°) programming was furnace initial T°C = 115°C, maximum T°C: 260°C Tacha = 2.00, T° = 240°C and End time = 7 min, Run time = 62.50 min. The compounds were identified and quantified by comparing the retention times of the peaks found in the samples with the individual retention times of the fatty acid standards under study, under the same working conditions, as well as by comparing the abundance of the majority ions obtained from the reference standards with the ions found in the sample.

Antioxidant capacity in vitro

DPPH radical sequestration assay: For the DPPH (2,2-diphenyl-1-picrylhydrazyl) test, the solution containing the extracts was prepared from 5g of crude S. rhombifolia extract in 30mL of methanol and diluted to the final concentrations of 1.30; 2.60; 5.20; 10.41; 20.8; 41.6; 83.3 and 166.7 µg/mL. DPPH added to methanol was used as a negative control. Reading was conducted on a UV/VIS spectrophotometer at a wavelength of 517 nm. The IC50 was determined by linear regression, using the average values of the duplicates [21]. The average absorbance of the crude extract was used to determine the percentage of total radical scavenging activity using the formula [(A0 - As) /AS] × 100, where 'A0' is the absorbance of the control and 'As' is the absorbance of the sample. A graph of the percentage of total radical scavenging activity versus concentration was plotted to obtain the value of half the maximum effective concentration (IC50).

ABTS radical sequestration assay: The percentage sequestering activity of the extract was determined using the ABTS assay (Re et al., 1999). The ABTS solution was prepared by mixing 5 mL of 7 mM ABTS in water with 88 µL of 140 mM sodium persulfate, and this solution was kept in the dark at room temperature for 12 hours. The solution was then diluted in 10 mM phosphate solution pH 7.0 to obtain 42.7 µM ABTS in the final solution. The extract was diluted to concentrations of 5, 10, 20, 30 and 40 µg/mL. The samples (75 µL) were placed in 96-well plates with the ABTS solution (150 µL) in water and kept in the dark at room temperature for 30 min. The absorbance was measured at 734 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific, Shanghai, China) (Sample Abdomen). The interference of the extract's turbidity (Blank Abs) was determined by preparing the sample solutions with 150 µL of water instead of the ABTS solution. The negative control (Abs negative control) was evaluated by mixing the ABTS solutions with 75 µL of water. The results were given as a percentage of free radical scavenging activity.

Assessment of cell viability

Materials: Dimethyl sulfoxide (DMSO); 3-(4,5-Dimethyl- 2-thiazolyl)- 2,5-diphenyl- 2H-tetrazolium bromide bromide 2,5-diphenyl-3,-(4, 5-dimethyl-2-thiazolyl) tetrazolium (MTT), phosphate buffered saline (PBS), trypsin-EDTA solution (170,000 U/L trypsin and 0.2 g/L EDTA), fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), supplemented with L- glutamine (584 mg/L) and antibiotic solution (10,000 units of penicillin and 10 mg/mL streptomycin) were obtained from Sigma-Aldrich (São Paulo, SP, Brazil).

Cell culture: For the test, cell lines 3T3 (murine swiss albino fibroblasts) and HT-29 (human colon adenocarcinoma) were grown in DMEM medium supplemented with 10% (v/v) FBS (v/v), L-glutamine (584 mg/L) and antibiotics (10,000 units of penicillin and 10 mg/mL streptomycin), at 37°C, 5% CO2. The cells were routinely grown in culture flasks of 75 cm2 and were harvested using trypsin-EDTA when the cells reached approximately 80% confluence.

Essay: The cytotoxic effects of the extract were evaluated by the MTT viability assay on 3T3 and HT-29 cells, used as tumor and non-tumor cell models. The MTT endpoint is a measure of cell metabolic activity and is based on the protocol first described by Mossmann (1983). Initially, 3T3 and HT-29 cells were seeded in 96-well plates at a density of 1 x 10 5 cells/mL and incubated for 24 h under 5% CO2 at 37°C. After this period, the spent medium was replaced with 100 µL of fresh medium supplemented with 5% (v/v) FBS containing the extract in the concentration range of 3.9 to 500 µg/mL. After 24 h (for 3T3 cells) or 72 h (for HT-29), the effects of the treatment on the cell cultures were investigated by assessing cell metabolism (MTT assay). One hundred µL of MTT tetrazolium salt (0.5 mg/mL dissolved in FBS-Free Medium) were added to each well. The plates were further incubated for 3 h to allow purple formazan crystals to form. These crystals were then solubilized by adding 100 µL of DMSO to each well, followed by shaking the plate for 10 min at room temperature.

The absorbance of the solutions was measured at 550 nm on a microplate reader (Thermo Microplate Photometer Scientific™ Multiskan™ FC). Cell viability was finally calculated as the percentage reduction of tetrazolium salt by viable cells for each sample. Viability values were normalized to control cells, which were defined as 100% cell viability.

Statistical analysis

The results were analyzed using one-way analysis of variance (Anova), followed by the Tukey *posthoc* test (Graphpad Prism software). The data are expressed as mean ± standard deviation (S.D.), with differences between groups being considered statistically significant when $p<0.05$.

III. Results

The crude extract of Sida rhombifolia (ESR) had positive results to anthocyanin heterosides, saponins, hydrolysable tannins and flavonoids (table 1). However, the coumarins had a negative reaction, which differs from the study by Chaves et al. (2017)[22], which identified coumarins, scoporone and scopoletin. Genetic factors, seasonality, light and temperature can alter the synthesis of secondary metabolites [23]. In other phytochemical tests, the following classes of metabolites have been described as present in *S. rhombifolia* extract: alkaloids, glycosides, tannins, saponins, terpenoids, polyphenols, flavonoids, carbohydrates, oils and fats, resin and mucilage, steroids [24, 25, 26,1].

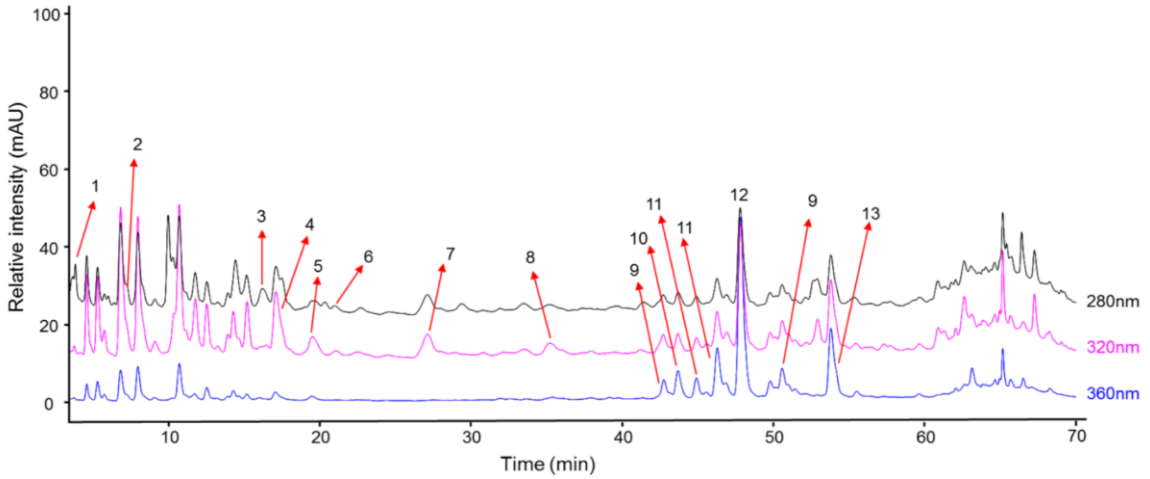
Table no 1: Phytochemical evaluation of the aerial parts of Sida rhombifolia

Metabolites	***Sida rhombifolia aerial parts***	**Test and Reagents**	**Positive result**
Anthocyanin heterosides	+	Sulfuric acid and sodium picrate	Brown red coloring
Saponins	+	Foam index	Persistent foam formation
Hydrolyzable tannins	+	$FeCl_3$	The blue color indicates the possible presence of hydrolyzable tannins
Flavonoids	+	Shinoda/Cyanidin Test	Orange or red coloring
Coumarins	-	Observation under ultraviolet light	Bright blue or green fluorescence

HPLC-PDA analysis of the hydroethanolic extract of S. rhombifolia identified some of the phenolic compounds present (Figure 1). The combination of spectral data from the recorded chromatogram and information from the literature allows the provisional identification of 8 phenolic acids and 5 flavonoids . The compound with the highest concentration found was isoquercetrin (300.86 ± 7.79 mg/100g), followed by apigenin derivatives

(202.02 ± 1.23 mg/100g) and kaempferol derivatives (135.87 ± 1 .50 mg/100g), Isoquercitin was the main phenolic identified by Ferro et al. (2019) [27] in extracts from their study.

Figure 1. Chromatogram obtained by injection of the ethanolic extract of *Sida rhombifolia*, profile of phenolic compounds.



Chromatograms acquired at 280nm, 320 nm, and 360 nm. Peak 1: Gallic acid; Peak 2: 3,4-Dihydroxybenzoic acid; Peak 3: Vanillic acid; Peak 4: Caffeic acid; Peak 5: Chlorogenic acid isomer; Peak 6: Syringic acid Peak 7: *p*-Coumaric acid; Peak 8: *t*-Ferulic acid; Peak 9: Apigenin derivatives; Peak 10: Quercetin derivative; Peak 11: Kaempferol derivatives; Peak 12: Isoquercetrin; Peak 13: Myricetin.

The results of the specific analysis of fatty acids (FA) (table 2) are confirmatory of a higher incidence of palmitic acid (C16:0), long-chain FA omega 6: linoleic (C18:2n6) essential, and long-chain FA omega 3: alpha-linolenic (C18:3n3) essential.

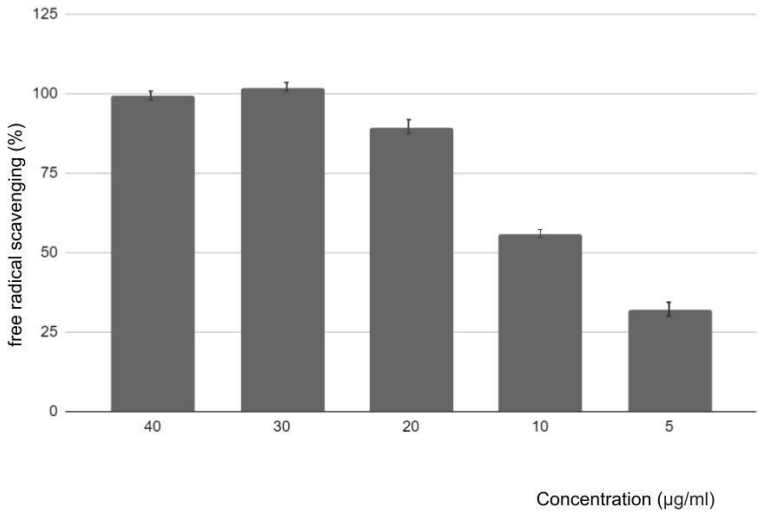
Table no 2: Gas chromatography coupled to flame ionization detector (GC-DIC): specific analysis of fatty acids.

	Nomenclature	Concentrations mg/g
1	**C4:00**	14,6
2	**C6:00**	21,2
3	**C12:00**	10,4
4	**C13:00**	7,3
5	**C14:00**	11,8
6	**C15:00**	4,3
7	**C16:00**	**368,2**
8	**C16:1**	3,3
9	**C17:00**	9,9
10	**C18:00**	56,3
11	**C18:1n9c**	70,3
12	**C18:2n6c**	**318,8**
13	**C18:3n3**	**94,4**
14	**C20:00**	8,8
15	**C21:00**	5,1
16	**C22:00**	14,2
17	**C20:5n3**	2,9
18	**C23:00**	4,3
19	**C24:00**	3,5

Nomenclature	Concentrations mg/g
Total	1029,6

The antioxidant capacity was evaluated by ABTS and DPPH assays. Using the DPPH method, the extract showed dose-dependent antioxidant activity with an IC50 value (50% inhibitory concentration) of 1554.92 ± 0.18 µg/mL when observed on the ascorbic acid standard (considered to be significantly different when $p < 0.05$), a higher result than that found by Laili et al. (2022)[2] who quantified IC 50 at 547.44 µg/mL in the crude extract of the species. In the ABTS method, ESR showed promising antioxidant activity (figure 2), and ABTS IC50, the percentage of free radical scavenging (FRS%) value was 101.8.

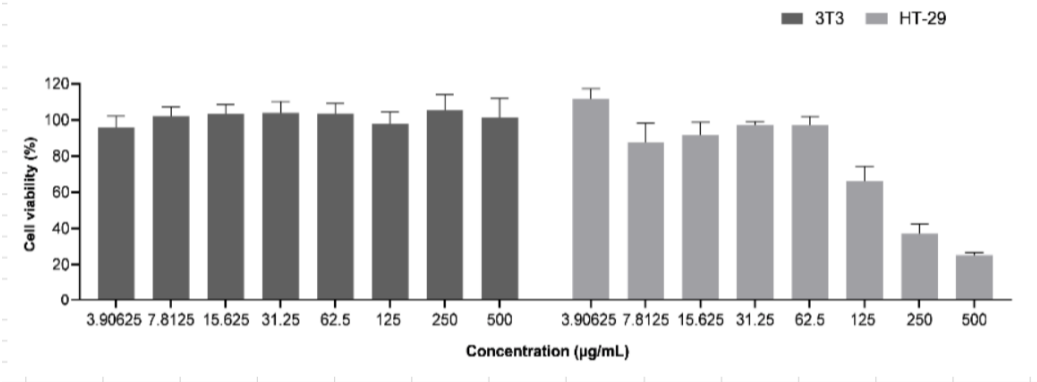
Figure 2. Essay regarding the radical ABTS.



Phenolic compounds belonging to plants are found in a variety of structures, such as phenolic acids, coumarin derivatives, tannins and flavonoids, which can act as reducing agents, free radical scavengers, metal chelators or singlet oxygen deactivators [28, 29, 30]. Initially, analyses of total phenolic content (CFT) and total flavonoid content (FT) were conducted as indicators of compounds with antioxidant activity [27].

The MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a cell proliferation test that aims to assess both cell viability, in this study with the 3T3 cell line (24 hours) and the cytotoxicity of ESR in tumor cells (HT-29 line) for 72 hours. The extract did not present a cytotoxic effect at any concentration tested (figure 3). Thounaojam et al. (2011)[1] also evaluated the influence of the aqueous extract of the leaves on the viability of 3T3L1 mouse preadipocyte cells and there were no changes in cell viability from 10 to 1000 µg/mL. It was possible to determine the minimum inhibitory concentration necessary to obtain 50% of viable or non-viable cells due to the cytotoxicity promoted by the tested agent [31, 32] , with the IC50 HT-29 being 138.7± 3.02.

Figura 3. MTT- ESR toxicity against the 3T3 and HT-29 cell lines, expressed by the percentage of cell viability. Note: Data are presented as mean ± standard error. Where $p<0.05$.



IV. Discussion

The leaves and stems of S. rhombifolia have anti-inflammatory, antinociceptive and antioxidant activities due to alkaloids, flavonoids and phenolic constituents in the plant [33]. In this study, we confirmed the presence of flavonoids and phenolic compounds among other chemical compounds. The composition and concentration of extracted metabolites can be affected by many factors, one of them being solvent extraction [34]. Differences in the composition and concentration of these metabolites would be directly related to the level of their biological activity [35]. Furthermore, the large number of metabolites found in the S. rhombifolia extract may result in synergistic interactions and antagonistic between metabolites, influencing the biological activity obtained [36].

V. Conclusion

The results obtained with conventional methodologies for determining total polyphenols, flavonoids and tannins and antioxidant activity by the DPPH, ABTS methods suggest considerable antioxidant activity related to the presence of polyphenolic compounds, such as flavonoids and tannins quantified in this study. The species also presents relevant levels of fatty acids, suggesting that further studies can deepen its applicability. The results of our work allowed us to conclude that the different concentrations of S. rhombifolia tested were not cytotoxic in cell culture.

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