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Original Article

Phytochemical analysis, toxicity and evaluation of antioxidant and antimicrobial activities of leaves of *Dipteryx alata* Vogel

Análise fitoquímica, toxicidade e avaliação das atividades antioxidante e antimicrobiana de folhas de *Dipteryx alata* Vogel

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Abstract

In this study, our objective was to conduct a comprehensive phytochemical analysis, determine toxicity levels, and assess the antioxidant and antimicrobial properties of extracts derived from the leaves of *Dipteryx alata* Vogel, a native species of the Brazilian cerrado flora. Three distinct extracts were prepared utilizing assisted ultrasound and the Soxhlet apparatus, namely, Ultrasound Crude Extract (UCE), Soxhlet Crude Extract (SCE), and the Soxhlet Ethanol Extract (SEE). The phytochemical analysis revealed the presence of flavonoids, tannins, phytosterols, and saponins in all extracts. Additionally, alkaloids were specifically identified in the SCE and SEE extracts. In the analysis using LC-DAD, the compounds gallic acid, rutin, quercetin, luteolin and kampefrol were determined in higher concentrations in the SCE, followed by the SEE and UCE, respectively. The GC-MS analysis revealed the presence of campesterol, stigmasterol and β-sitosterol in all extracts, with UCE and SCE showing a higher concentration of β-sitosterol. SCE showed the highest concentration of all identified compounds. In the analysis of antioxidant activity by DPPH• and ABTS•$^{+}$, SEE showed greater efficiency (IC_{50} = 2.98 ± 2.92 and 6.57 ± 0.89 µg/mL, respectively). In the toxicity test with *Allium cepa*, all extracts stimulated root growth at 50 g/mL; UCE and SEE stimulated root growth at 250 g/mL; and SEE inhibited root growth at 750 g/mL. In the *Artemia salina* toxicity, all extracts were non-toxic. Antibacterial activity was identified in the microorganisms *S. aureus* and *S. mutans*; however, the extracts did not show antifungal action against the strain of *C. albicans*. The extracts of *D. alata* have therapeutic potential for applicability in dentistry.

Keywords: *baru*, chemical characterization, phenolic compounds, antibacterial, oral health.

Resumo

Neste estudo o objetivo foi realizar a análise fitoquímica, determinar a toxicidade, e avaliar as atividades antioxidante e antimicrobiana dos extratos da folha da *Dipteryx alata* Vogel, uma espécie nativa da flora do cerrado brasileiro. Three extracts were prepared using assisted ultrasound and the Soxhlet apparatus, namely, Crude Ultrasound Extract (UCE), Crude Soxhlet Extract (SCE), and the Ethanol Soxhlet Extract (SEE). A análise fitoquímica identificou a presença de flavonoides, taninos, fitoesteróis e saponinas em todos os extratos; alcaloides foram identificados nos extratos SCE e SEE. Na análise using LC-DAD os compostos ácido gálico, rutina, quercetina, luteolina e kampefrol foram determinados em maior concentração no SCE, seguido pelo SEE e UCE, respectivamente. Já a análise by GC-MS revelou a presença de campesterol, estigmasterol e β-sitosterol em todos os extratos, sendo que o UCE e o SCE apresentaram maior concentração de β-sitosterol. O SCE apresentou a maior concentração de todos os compostos identificados. Na análise da atividade antioxidante por DPPH• e ABTS•$^{+}$, o SEE apresentou maior eficiência (IC_{50} = 2,98 ± 2,92 e 6,57 ± 0,89 µg/mL, respectivamente). No teste de toxicidade com *Allium cepa*, todos os extratos estimularam o crescimento radicular na concentração de 50 g/mL; UCE e SEE estimularam o crescimento radicular a 250 g/mL; e o SEE inibiu o crescimento radicular a 750 g/mL. Na análise de toxicidade usando *Artemia salina*, todos os extratos foram atóxicos. Foi identificada atividade antibacteriana nos microrganismos *S. aureus* e *S. mutans*; entretanto, os extratos não apresentaram ação antifúngica contra a cepa de *C. albicans*. Os extratos da *D. alata* apresentam potencial terapêutico para aplicabilidade em odontologia.

Palavras-chave: *baru*, caracterização química, compostos fenólicos, antibacteriano, saúde bucal.

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1. Introduction

The characterization of bioactive compounds from Cerrado plants holds significant relevance in the pursuit of alternative therapies that encompass desirable attributes such as antioxidant, antimicrobial, anticarcinogenic, antidegenerative, and anti-aging properties. The development of new products with these characteristics is of interest to various industries including food, pharmaceuticals, and cosmetics (Kumar and Pandey, 2013; Panontin et al., 2022).

Dipteryx alata Vogel (D. alata) is a fruit species indigenous to the Brazilian cerrado, belonging to the Leguminosae family, commonly known as baru. Its wood, fruits, pulp, almonds, and oil are all fully utilizable, constituting a source of income for the regional population, while also boasting high nutritional and technological value (Santiago et al., 2018; Lima et al., 2022). The oil extracted from its seeds is highly unsaturated, a characteristic attributed to the presence of oleic acid, linoleic acid, and α-tocopherol (Lima et al., 2022).

Baru is cited in popular medicine for its purported anti-rheumatic, cicatrizing, and tonic properties. It is utilized in the treatment of reproductive disorders and is indicated as a menstrual regulator (Rambo et al., 2020; Nazato et al., 2010; Puebla et al., 2010). Researchers have reported its potential use in treating metabolic diseases, cancer, microbial infections, chronic kidney disease (CKD), antiophidian effects, and oxidative stress, among other conditions (Lima et al., 2022; Araújo et al., 2017; Oliveira-Alves et al., 2020; Santos et al., 2017; Schincaglia et al., 2020; Ferraz et al., 2012, 2014; Siqueira et al., 2012). Studies have also highlighted baru as an excellent raw material for the cosmetic and oleochemical industries (Monteiro et al., 2022).

Furthermore, *baru* exhibits recognized therapeutic activities such as anti-inflammatory, antimicrobial, antioxidant, chemopreventive, cytoprotective, antimutagenic, antiestrogenic, and antiangiogenic effects. These properties are attributed to the presence of phenolic compounds, which are frequently encountered in cerrado plants, rendering them ideal candidates for bioprospecting endeavors in dentistry (Monteiro et al., 2022).

Esteves-Pedro et al. (2012) identified components such as triterpenoids, isoflavonoids, and phenolic acids in *D. alata* extract. The latter two constituents belong to phenolic compounds, a term that encompasses a wide range of plant substances, many of which are of significant biological importance.

Given the common presence of microorganisms in the oral cavity, antimicrobial substances hold great interest in oral health. The oral "ecosystem" is believed to comprise 500 to 700 species, including aerobic microorganisms, facultative anaerobes, and strict anaerobes (Santos et al., 2017).

Consequently, the pursuit of low-cost antibacterial products that are convenient for use and application in the routines of healthcare professionals and patients underscores the exploration for accessible antibacterial substances (Santos et al., 2017; Campidelli et al., 2022).

Therefore, the objective of this study was to perform a chemical screening of the crude hydroalcoholic extract from the leaves of *Dipteryx alata* Vogel and assess its antioxidant and antimicrobial activities against pathogenic oral bacteria, along with evaluating its toxicity.

2. Material and Methods

2.1. Plant material

The leaves of *D. alata* were collected in the municipality of Palmas, Tocantins, Brazil in August 2020, at the geographical coordinates 10°15'07"S, 48°19'46"W. Botanical identification was performed by the Herbarium of the State University of Tocantins – HUTO by the exsiccate No 8056. The project is registered in SISGEN with the No A475DDE. To prepare the leaves for analysis, they were first sanitized using a 2% sodium hypochlorite solution. Subsequently, the leaves were dried in an oven with air circulation at 50 °C for 48 hours. Once dried, they were ground using a Willey-type knife mill (Star FT 50 model, Forenox brand).

2.2. Preparation of extracts

The extraction of *D. alata* leaf extracts was carried out utilizing two distinct methodologies. The Ultrasound Crude Extract (UCE) was prepared by mixing 5g of plant material with 80 mL of 70% ethanol. The mixture was then subjected to an ultrasound bath (USC1600, ultrasonic cleaner, frequency 40 kHz, 135 W) for cycles of 1 hour at room temperature. This process was repeated five times, with the supernatants combined after each extraction.

For the extraction conducted using the Soxhlet apparatus, 5 g of plant material powder was utilized along with 200 mL of different solvents. The Soxhlet Crude Extract (SCE) was obtained using 70% ethanol as the solvent. In the case of the Soxhlet Ethanol Extract (SEE), the same methodology as SCE was followed, with the addition of a prior degreasing step using hexane solvent. Once degreasing was completed, the cartridge with the sample was dried at room temperature for 24 h, followed by extraction with 70% Ethanol solvent. Following the extractions, all solutions were solvent removed using a rotary evaporator (Fisaton 801, Brazil) at 45 °C. The extracts were then lyophilized and stored in an amber bottle and kept in a desiccator until analysis.

3. Phytochemical Screening

The phytochemical screening was conducted through qualitative tests, relying on observations of color changes and/or precipitate formation upon chemical reactions with specific reagents tailored for detecting flavonoids, tannins, phytosterols/triterpenoids, quinones, saponins, and alkaloids (Saraiva et al., 2018).

4. Chemical Composition

4.1. Determination of the content of phenolic compounds

The total phenolic content was determined using the Folin-Ciocalteu method, as described by Amorim et al.

(2012), with modifications, using gallic acid as a standard. It was mixed with 0.2 mL of methanolic solutions of *D. alata* leaves extracts (1 mg/mL) or the standard gallic acid (2-100 g/mL), 1 mL of sodium carbonate (7.5%, w/v), 0.5 mL of Folin-Ciocalteu reagent (10%, v/v) and 8.3 mL of distilled water, shaken gently and kept for 30 min in the dark. The absorbance was measured at 760 nm in a spectrophotometer (Global Analyzer, model GTA-96) and using water as a blank.

The total phenolic content was determined by interpolating the absorbance of the samples against a calibration curve constructed with different concentrations of gallic acid in methanol. The experiment was conducted in triplicate for each sample. The result was expressed as mg of gallic acid equivalents (GAE) per gram of *D. alata* leaves extract (mg GAE/g).

4.2. Determination of tannin content

The tannin content was determined using a modified version of the Folin-Ciocalteu Method, following the procedure described by Amorim et al. (2012). Each extract (1 mL, 1 mg/mL) was mixed with casein (0.1 g) and distilled water (5 mL), followed by vigorous stirring until homogenized. The mixture was vigorously stirred until homogenized. The solution was left for 3 h and then centrifuged at 1358 rpm × g for 10 min at 10 °C. In the supernatant, the non-tannin phenolic constituents were determined similarly to the total phenolic content. The amount of tannins was calculated as the difference between the total phenolic and non-tannin phenolic content in the extract and was expressed in milligram equivalents of gallic acid (GA) per gram of lyophilized extract of *D. alata* (mg GAE/g). The experiment was conducted in triplicate for each sample.

4.3. Determination of flavonoids content

The flavonoids content was measured based on the method reported by Peixoto-Sobrinho et al. (2011), with modifications (Soares et al., 2017). Then 0.5 mL of extract (1mg/mL) was mixed with 0.5 mL of Aluminum chloride (20 mg/mL), followed by 1.5 mL of sodium acetate (50 mg/mL). The mixture was incubated at room temperature for 2.5 h. Absorbance was measured by spectrophotometer at 440 nm. The total flavonoid content was determined by interpolating the absorbance of the samples against a calibration curve constructed with different concentrations of rutin in methanol (10-400 µg/mL, w/v). The experiment was conducted in triplicate for each sample.

4.4. Determination of flavonols

The flavonols content was determined using the method described by Miliauskas et al. (2004) using rutin as a standard. Methanolic solutions (0.5 mL) of *D. alata* extracts (1 mg/mL, w/v) or rutin standard (10-400 mg/mL, w/v), aqueous acetic acid solution (0.5 mL at 60%, v/v), aluminum chloride (1 mL at 5%, w/v), methanolic pyridine solution (2 mL at 20%, v/v) and 6 mL of distilled water. The blank was prepared by replacing aluminum chloride with methanol. The mixtures were gently shaken, kept in the dark for 30 min and their absorbance measured at 420 nm in a UV-Vis spectrophotometer. The total flavonol content was expressed in milligrams of rutin equivalents (RE) per gram of dry extract (mg RE/g) (Miliauskas et al., 2004). The experiment was conducted in triplicate for each sample.

4.5. Evaluation of antioxidant activity

Two methods were used to evaluate the antioxidant activity: evaluation of the blocking effect of the stable free radical 2,2-dipheninl-1-picryl-hydrazyl (DPPH•) and evaluation of the elimination of the radical cation 2,2'-azinobis-3- ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$).

For the DPPH• evaluation, the method described by Peixoto-Sobrinho et al. (2011) was used, with the rutin pattern as a positive control. In triplicates, 0.5 mL of different concentrations of extracts or standards (10-200 µg/mL, w/v) were mixed with a methanolic solution of DPPH• (3 mL to 40 µg/mL). The blank was prepared by replacing the DPPH• with methanol in the reaction medium. The reaction complex and the blank were stirred and kept in the dark for 30 min. and the absorbances were measured at 517 nm in a spectrophotometer calibrated with methanol. As a negative control, a 40 µg/mL DPPH• solution was used. Free radical scavenging activity or antioxidant activity (AA) was expressed as the percentage of inhibition determined by the Equation 1:

$$\%AA = ABSnc - \left(ABSsample - ABSblank\right) / ABSnc \times 100 \quad (1)$$

where % AA is the percentage of antioxidant activity; ABS_{nc}, the absorbance of the negative control; ABS_{sample}, the absorbance of the sample; ABS_{blank}, the absorbance of the blank.

The IC_{50} (amount of sample needed to decrease the initial concentration of DPPH• by 50%), expressed in µg/mL was calculated using the calibration curves obtained by plotting the different concentrations against the % AA.

The antioxidant activity was also evaluated by the ability to eliminate the $ABTS^{\bullet+}$ radical cation, according to the methodology described by Chen et al. (2019) and modified by Rabêlo et al. (2014). $ABTS^{\bullet+}$ solution was prepared by mixing 7 mM $ABTS^{\bullet+}$ (5 mL) and 2.45 mM (88 µL) potassium persulfate, followed by incubation at room temperature in the dark for 16 h. Afterwards, it was diluted in 100% ethanol to obtain an absorbance of 0.700 ± 0.005 at 734 nm. $ABTS^{\bullet+}$ solution (2.7 mL) was carefully mixed with 0.3 mL of test samples (10-400 µg/mL, w/v). The reaction mixture was left to rest at 30 °C for 30 min, followed by the measurement of absorbance at 734 nm in a spectrophotometer (Global Analyzer model GTA-96). The percentage of inhibition AA (%) was calculated as previously described. The IC_{50} value was also calculated to measure the concentration of a sample needed to decrease the absorbance by 50%. IC_{50} was expressed in µg/mL.

4.6. Characterization by Liquid Chromatography with Diode Array Detection (LC-DAD)

The extracts were solubilized in water: methanol (7:3 v:v) and evaluated in an analytical LC-6AD Shimadzu chromatograph column (LC-6AD Shimizu, Kyoto, Japan)

with the aid of a photodiode array detector (PDA) system monitored between wavelengths λ = 200-800 nm. A Thermo Electron Corporation ODS HYPERSIL column (C-18, 150 mm long × 4.6 mm diameter, Thermo Electron Corporation) was employed in the analysis.

The flow and injection volume were, respectively, 1 mL/min and 10 µL in each analysis. All chromatographic analysis took place at a temperature of 25 °C second methodology developed by by Cardoso et al. (2022) and described by Augusco et al. (2023). Eluent A was composed of a binary mobile phase of water with acetic acid (6%) and 2 mM sodium acetate, and eluent B, composed of acetonitrile. A gradient elution profile was employed as follows: 0 min with 5% B, 20 min with 15% B, 30 min with 60% B, and 40 min with 100% B.

To identify the compounds present in the samples, standards were used as references, and their absorption spectra and retention times were compared using a DAD scanning detector within the spectral range of 200-800 nm (Cardoso et al., 2022). Compounds found in extracts were unambiguously identified by constructing calibration curves using linear regression with LC. The linearity of the standards was evaluated within 10 concentration ranges, yielding high coefficients of determination (R^2). Specifically, caffeic acid and gallic acid demonstrated an R2 value of 0.9994, whereas rutin, quercetin, luteolin, and kaempferol, along with quercetin, exhibited an R2 value of 0.9996. Standards of these compounds (Sigma, 98%) were prepared in water-methanol solution at a concentration of 1000 µg/mL. All standards were procured from Sigma (Sigma, 98%, St. Louis, MO, USA).

4.7. Characterization by Gas Chromatography Coupled with Mass Spectrometry (GC-MS)

GC-MS analysis was performed using GC-2010 Plus equipment (Shimadzu, Kyoto, Japan), equipped with a mass spectrometry detector (GC-MS Ultra 2010), using LM-5 (5% phenyl methylpolysiloxane), capillary column of fused silica (15 m length × 0.2 mm diameter, and 0.2 µm thick film). To prepare the extract for GC-MS analysis, 2 mL of water was added to 100 mg of the extract and solubilized using ultrasound for 1 min. Then 2 mL of hexane was and the mixture was subjected to ultrasound for 2 min. After the phase formation the hexane fraction was separated from the aqueous fraction. To the aqueous fraction was added 2 mL of hexane and the process was repeated. After the two extractions, the hexane fractions were dried e and suspended in 1000 mL of hexane. Prior to GC-MS analysis, the solution was filtered through a 0.45 µm Ultrafilter.

The analysis was conducted under the following conditions: helium carrier gas with a purity of 99.999% and a flow rate of 1 mL/min. A 1 µL injection volume was utilized with a split ratio of 1:20. The initial oven temperature was set at 150 °C, followed by heating at a rate of 15 °C/min until reaching 280 °C, where it was held for 15 minutes. Both the injector and detector temperatures were maintained at 280 °C.

For mass spectrometry (MS) scanning, electronic impact ionization was applied at a voltage of 70 eV. The mass range was set from 45 to 600 nm m/z, with a scan interval of 0.3 seconds.

All standards utilized in the analysis were procured from Sigma (Sigma, ≥ 98%, St. Louis, MO, USA). The linearity of the standards was evaluated across 5 concentration ranges, and the concentrations of compounds were determined via external calibration. The mean standard errors for the peak areas of replicate injections (n = 5) were determined to be less than 2%, indicating excellent repeatability of the calibration curve. Furthermore, the coefficient of determination (R^2) was calculated to be 0.9996 for campesterol, stigmasterol, and β-sitosterol.

5. Antimicrobian Activity

To determine the antimicrobial activity, reference microorganisms including *Streptococcus mutans* ATCC 25175, *Staphylococcus aureus* ATCC 6538 and *Candida albicans* ATCC 90028 provided by the Microbiology Laboratory of University of São Paulo (USP – Bauru) were used. These strains were stored at -70 °C in Brain Heart Infusion (BHI) broth with 20% glycerol. Upon reactivation, the pathogen suspension was prepared and the turbidity was adjusted using the McFarland scale of 0.5. Spectrophotometric readings were conducted at 625 nm until absorbance values between 0.08 and 0.10 (1.0×10^8 CFU/mL) were obtained. Subsequently, the suspension was diluted to a concentration of 1.0×10^7 CFU/mL (NCCLS, 2003).

5.1. Well diffusion test

The detection of antimicrobial activity was performed by the well diffusion method, using extracts UCE, SCE and SEE from *D. alata* leaves diluted in 10% dimethyl sulfoxide (DMSO) and adjusted for concentrations (200, 100 and 50 µg/mL). Positive controls were 0.12% Chlorhexidine Gluconate for bacteria, Nystatin 100,000 iu/mL for yeast and the negative control was 10% DMSO solution (Oliveira et al., 2016).

The plates were incubated for 24 h at 37 °C in microaerophilia. The results were analyzed by measuring the halos with a digital caliper (Starret 799 model) and the diameters compared with the halos obtained in the controls.

5.2. Determination of the Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) was determined for the extracts that presented the best results in the well diffusion test, using the broth microdilution technique following the methodology described in the M7-A6 standard of the National Committee for Clinical Laboratory Standards (NCCLS, 2003). The concentrations of the extracts used were 100; 50; 25; 12.5; 6.25; 3.12; 1.56 and 0.781 µg/mL.

The wells containing culture medium, 1% DMSO solvent and pathogen suspension at the predetermined concentration were considered negative control. Wells comprising culture medium, 0.12 percent chlorhexidine gluconate, and pathogen suspension at the predetermined concentration were used as positive controls. Additionally, wells with culture medium and the suspension of the pathogen at the predetermined concentration were employed as growth controls. For sterility control of the medium (m-h broth), wells containing only the broth were utilized. The developer solution used was 0.03% resarzurin.

6. Toxicological Assays

The evaluation of extract toxicity was conducted utilizing the *Allium cepa* (onion) method and the *Artemia salina* assay, as described by Meneguetti et al. (2012).

Initially, the stock solution was prepared by weighing 0.5g of each crude extract, and diluted in 10 mL of mineral water. From the stock solution, dilutions were performed to obtain the extracts at concentrations: 750, 250 and 50 µg/mL, which were used in the toxicity experiment. The *Allium cepa* (onion) specimens employed were of uniform size, non-germinated, and healthy, obtained from a supermarket in the city of Palmas, Tocantins.

The experimental design was completely randomized in a factorial scheme, arrangement (4 × 3) with 9 treatments: the control, three concentrations of each extract and three replications per treatment. The Relative Growth Index (RGI) was determined through the root growth of the control and extracts obtained by Equation 2:

$$RGI = RLS / RLC \quad (2)$$

where RLS is the root length sample and RLC is the root length control. The effect of the extract in relation to the control was determined according to the RGI, which is subdivided into 3 categories:
1- RGI < 0,8: Growth inhibition (I);
2- 0,8 ≤ RGI ≤ 1,2: Same control effect (SC);
3- RGI > 1,2: Growth stimulus (G).

The toxicity assay using *Artemia salina* was performed using a solution containing sea salt at a concentration of 35 g/L, pH between 8.0 and 9.0 was used to hatch the eggs of *Artemia salina* and to prepare the other dilutions. About 10 brine shrimp nauplii were transferred to test tubes containing 5mL of saline solution and aqueous extracts of *D. alata* leaves at concentrations of 50, 500, 1,000 and 5,000 mg/L (McLaughlin et al., 1998). The extract classification followed the criteria established by Ntungwe et al. (2020).

7. Statistical Analysis

The experiments were performed in triplicate, and the results expressed as mean ± standard deviation. The data obtained were analyzed using the AgroEstat program and GraphPad Prism. Analysis of variance was used to compare the mean values obtained in the study, and p values < 0.05 were considered statistically significant by Tukey's test with DMS (5%) = 0.7817.

8. Results and Discussion

8.1. Preliminary phytochemical screening

The phytochemical screening of the classes of secondary metabolites presents in the extracts of *D. alata* leaves showed that the UCE, SCE and SEE extracts have positive results for flavonoids, tannins, phytosterols/triterpenoids and saponins, a result similar to that obtained by Sanchez (2014) when carrying out a phytochemical study of *Baru* pulp.

The identified metabolites are of significant interest in health due to their diverse actions in disease prevention and treatment (Schincaglia et al., 2020).

Flavonoids have antioxidant, antibacterial, hepatoprotective, analgesic, anti-inflammatory, antifungal and anticancer properties. According to Ucella-Filho et al. (2012), tannins have antitumor, anthelmintic and antimicrobial activity, acting on tissue repair and regulating enzymatic activity. Phytosterols provide immunological protection and cholesterol level control, as well as cytotoxicity on tumor cells is associated with the presence of saponins. In the SCE and SEE extracts, both obtained by extraction in a Soxhlet apparatus, the presence of alkaloids was detected, metabolites with recognized anesthetic and anti-inflammatory action, which helps to prevent bacterial biofilm (Augusco et al., 2023).

8.2. Content of total phenolic compounds, tannins, flavonoids and flavonols

Among the *D. alata* leaf extracts analyzed, the UCE presented the lowest content of total phenolics, tannins, total flavonoids and flavonols (Table 1). This outcome can be attributed to the extraction method employed, as UCE was the only extract obtained using assisted ultrasound. The SEE extract demonstrated a significantly higher quantity of total phenolics, total flavonoids, and flavonols ($p < 0.05$), indicating that the prior degreasing process was conducive to the extraction of these compounds.

A higher amount of tannins was also observed in the SCE, which suggests the temperature as a potentiating factor for the extraction of phenolic compounds.

Table 1. Quantification of total phenolics, tannins, flavonoids, and flavonols in extracts from *D. alata* leaves obtained via ultrasound-assisted and Soxhlet apparatus.

	Extracts		
	UCE Mean ± SD	**SCE Mean ± SD**	**SEE Mean ± SD**
Total Phenolics (mg GAE/g)	50.00 ± 2.50^{c}	66.22 ± 6.53^{b}	77.37 ± 4.01^{a}
Tannins (mg GAE/g)	18.27 ± 0.38^{b}	20.19 ± 0.38^{a}	18.65 ± 0.38^{b}
Flavonoids (mg RE/g)	12.12 ± 5.92^{c}	53.88 ± 3.42^{b}	144.93 ± 3.88^{a}
Flavonols (mg RE/g)	21.60 ± 0.30^{c}	32.82 ± 2.49^{b}	87.74 ± 0.53^{a}

GAE = Gallic acid equivalent; RE = routine equivalent; SD = Standard deviation; UCE = Ultrasound Crude Extract; SCE = Soxhlet Crude Extract; SEE = Ethanol Extract. Values followed by the same letter indicate significant similarities in the same column ($p < 0.05$, ANOVA followed by Tukey's test). Values represent the mean followed by the standard deviation (Mean ± SD).

No studies were identified quantifying the contents of total phenolics, tannins, flavonoids, and flavonols in extracts of *D. alata* leaves. However, Barizão et al. (2021) analyzed hydroalcoholic extracts of the pulp and peel of *D. alata* fruit, finding higher values of phenolic compounds (3.15 ± 0.14 and 2.98 ± 0.01 mg GAE/g) for ethanol and methanol solvents, respectively. These values were lower than those presented by the UCE (50.00 ± 2.5 mg GAE/g), which was the lowest result obtained in this study.

Flavonoids, considered the most important class of polyphenols, play a crucial role in plant defense mechanisms against external agents. In pharmacology, they exhibit antimicrobial, anti-inflammatory, and antioxidant properties (Carvalho et al., 2021).

8.3. Evaluation of antioxidant activity

The results of the evaluation of antioxidant activity (AA%), expressed in terms of IC_{50}, of leaf extracts obtained by ultrasound-assisted and Soxhlet apparatus determined by DPPH• and ABTS•$^+$, are shown in Table 2.

The SEE extract showed superiority in the results both in the DPPH• and ABTS•$^+$ assays, surpassing, in some tests, the rutin and gallic acid standards. These results are in agreement with the results obtained in the chemical quantification of phenolic compounds, flavonoids and flavonols in the extracts of leaves *of D. alata*. The SCE extract showed lower antioxidant capacity by the DPPH• assay, however, in the ABTS•$^+$ assay, it showed antioxidant capacity similar to the gallic acid standard ($p < 0.05$). The difference in results is explained by the different mechanisms of antioxidant action of each method. A similar result regarding the variation of results between the DPPH• and ABTS•$^+$ methods was reported (Silvério et al., 2013) during a study with ethanolic extract from the leaves of *D. alata.*

In dentistry, antioxidants are related to the manifestation of periodontal disease, from its installation in the oral cavity to its progression to chronic periodontitis, as demonstrated Chen et al., (2019) when analyzing the biomarkers of oxidative stress present in saliva.

8.4. Analysis by LC-DAD and GC-MS chromatography

Table 3 presents a summary of the compounds identified by LC-DAD in the leaf extracts of *D. alata*, while Figure 1 depicts the corresponding chromatograms obtained from the extracts.

Quercetin was the compound found in greater quantity in the three extracts analyzed, followed by kampefrol, luteolin, gallic acid, caffeic acid and rutin (Table 3). All compounds were determined in higher concentration in the SCE, followed by the SEE and UCE, respectively, which suggests that the extraction by Soxhlet apparatus was more efficient for the extraction of these compounds. In addition, there was no statistical difference between the SCE and SEE extracts.

The presence of the flavonoids quercetin, luteolin, kampferol and rutin confer antithrombotic, vasodilatory, analgesic, anti-inflammatory, cytoprotective, antibacterial and vasoprotective properties (Chen et al., 2004; Oliveira et al., 2020; Augusco et al., 2023)

Table 2. Assessment of antioxidant activity (DPPH• and ABTS•$^+$) in extracts from *D. alata* leaves obtained through ultrasound-assisted and Soxhlet apparatus, alongside positive controls rutin and gallic acid.

	DPPH•	ABTS•$^+$
	IC_{50} (μg /mL) Mean ± SD	IC_{50} (μg /mL) Mean ± SD
UCE	80.68 ± 0.06 ^{b}	11.43 ± 0.35 ^{c}
SCE	85.26 ± 1.37 ^{c}	7.60 ± 1.27 ^{b}
SEE	2.98 ± 2.92 ^{a}	6.57 ± 0.89 ^{a}
Rutin	11.92 ± 0.47 ^{a}	
Gallic Acid		6.75± 0.01^a

SD = Standard deviation; UCE = Ultrasound Crude Extract; SCE = Soxhlet Crude Extract; SEE = Ethanol Extract. Values followed by the same letter indicate significant similarities in the same column ($p < 0.05$, ANOVA followed by Tukey's test). Values represent the mean followed by the standard deviation (Mean ± SD).

Table 3. Quantification of chemical compounds in *D. alata* leaf extracts by LC-DAD (mg/g ± SD) obtained via ultrasound-assisted and Soxhlet apparatus methods.

Compound	Concentration (mg/g)		
	UCE Mean ± SD	SCE Mean ± SD	SEE Mean ± SD
Gallic Acid	57.7 ± 0.2^d	77.4 ± 0.3^d	72.8 ± 0.4^d
Caffeic Acid	35.3 ± 0.1^e	44.3 ± 0.2^e	43.1 ± 0.2^e
Rutin	20.1 ± 0.1^f	22.6 ± 0.1^f	22.2 ± 0.1^f
Quercetin	112.3 ± 0.4^a	151.7 ± 0.6^a	145. 3 ± 0.6^a
Luteolin	63.1 ± 0.1^c	79.4 ± 0.2 ^{c}	78.7 ± 0.2 ^{c}
Kampefrol	67.2 ± 0.2^b	83.6 ± 0.2^b	82.8 ± 0.1^b

SD = Standard deviation; UCE = Ultrasound Crude Extract; SCE = Soxhlet Crude Extract; SEE = Ethanol Extract. Values followed by the same letter indicate significant similarities in the same column ($p < 0.05$, ANOVA followed by Tukey's test). Values represent the mean followed by the standard deviation (Mean ± SD).

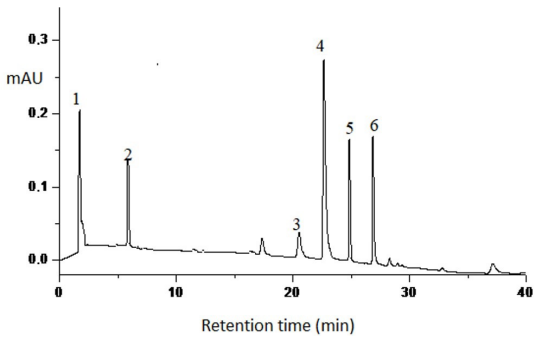

Figure 1. Representative Chromatogram (LC-DAD) of *D. Alata* leaf extracts. Peak 1: gallic acid; peak 2: caffeic acid; peak 3: rutin; peak 4: quercetin; peak 5: luteolin; peak 6: kampefrol.

No studies were found on the chemical characterization of *D. Alata* leaves. However, according to Nguyen et al. (2021), in a study involving mice, flavonoids were found to damage the cell membrane of *C. albicans* strains resistant to traditional drug therapy, exhibiting antifungal activity against the tested strains.

According to Zhang et al. (2021), quercetin stimulates oral epithelial cells and reduces the release of pro-inflammatory cytokines, thereby exerting a blocking effect on the pathological processes of radiation-induced oral mucositis. This suggests that quercetin may be a promising therapeutic agent for managing adverse events of head and neck radiotherapy.

Additionally, Mooney et al. (2021) suggest that quercetin promotes periodontal tissue homeostasis and creates an oral microenvironment conducive to symbiotic microbiota.

Yang et al. (2017) identified that the presence of quercetin in dentin adhesives resulted in a decrease in the metabolic activity of *S. Mutans*, as well as the ability to promote the cross-linking of collagen present in dentin, enhancing its union with the restorative material, prolonging the half-life of adhesive restorations. The inhibitory activity of kampferol against oral pathogens when associating with the compound the rupture of the cell wall and cytoplasmic membrane of Gram-positive bacterial cells of *S.*

Table 4 presents the chemical composition of extracts analyzed by GC-MS. It is evident that both the Ultrasound Crude Extract (UCE) and the Soxhlet Crude Extract (SCE) exhibited a higher concentration of β-sitosterol compared to the Soxhlet Ethanol Extract (SEE). Moreover, the SCE displayed a higher concentration of all identified compounds. These findings suggest that the hot extraction process favored the extraction of the compounds, and the preceding degreasing step (SEE) may have negatively influenced their quantification.

Few studies have been conducted with extracts from the leaves of *D. alata*. However, a similar study conducted by Gomes (2019) focused on baru almond oil, identifying the compounds campesterol (1.27 mg/g), stigmasterol (1.85 mg/g), and β-sitosterol (3.07 mg/g).

Phytosterols have hypocholesterolemic activity, anti-inflammatory, antioxidant and anticarcinogenic, and β-sitosterol has estrogenic action. Therefore, the identification of these compounds justifies the traditional use of baru in folk medicine as a regulator for menstrual and anti-rheumatic conditions. Also, phytosterols such as β-sitosterol and stigmasterol have antitumor, antifungal, analgesic and anti-inflammatory properties. These activities are useful in the dental field as they largely affect oral diseases and the formation of bacterial oral flora (Alves et al., 2010).

Table 4. Quantification of chemical compounds in *D. alata* leaf extracts by GC-MS (mg/g ± SD) obtained via ultrasound-assisted and Soxhlet apparatus methods.

Compound	Concentration (mg/g)		
	UCE Mean ± SD	SCE Mean ± SD	SEE Mean ± SD
Campesterol	20.7 ± 0.1^{c}	91.8 ± 0.3^{a}	10.1 ± 0.1^{c}
Estigmasterol	25.7 ± 0.2^{b}	86.5 ± 0.2^{b}	13.3 ±0.19^{b}
β-sitosterol	60.1 ± 0.2^{a}	81.3 ± 0.2^{c}	22.7 ± 0.1^{a}

SD = Standard deviation; UCE = Ultrasound Crude Extract; SCE = Soxhlet Crude Extract; SEE = Ethanol Extract. Values followed by the same letter indicate significant similarities in the same column ($p < 0.05$, ANOVA followed by Tukey's test). Values represent the mean followed by the standard deviation (Mean ± SD).

8.5. Antimicrobial activity

8.5.1. Well diffusion test

The average of the inhibition zone (mm) of the antimicrobial activity of the microorganisms *Staphylococcus aureus* (*S. aureus*) and *Streptococcus mutans* (*S. mutans*), are presented in Table 5.

The best inhibition response, when comparing all extracts against all microorganisms, occurred in the 200 µg/mL SEE extract against *S. aureus*. All extracts, at all concentrations analyzed against *S. aureus* showed a good activity, with the exception of SEE 50 µg/L.

Aleluia et al. (2017), present tannin as the main component related to activity in the fight against gram-positive and gram-negative bacteria constituting the oral biofilm, increasing bacterial lysis, as well as interfering in the mechanism of bacterial adherence to the surface of the teeth, inhibiting the formation of the bacterial biofilm.

No antifungal activity was observed for SCE, UCE and SEE extracts at the concentrations tested on *C. albicans*, so the Minimum Inhibitory Concentration test was not performed for this microorganism.

8.6. Minimum Inhibitory Concentration (MIC)

The crude extracts that exhibited antimicrobial activity against pathogens in the well diffusion test underwent determination of the minimum inhibitory concentration.

According to CLSI (2009), plant extracts are classified on the following scale: MICs <100 µg/mL are considered highly active antimicrobial agents, MICs ranging from 100 to 500 µg/mL are classified as active, and MICs ranging between 500 and 1000 µg/mL are considered moderately active. MICs ranging from 1000 to 2000 µg/mL are considered to have low activity, and MICs greater than 2000 µg/mL are classified as inactive. Our findings showed that both UCE and SEE extracts were active against *S. aureus*, with bactericidal action at a concentration of 1.56 µg/mL (active extract). Similar antimicrobial activity was observed against *S. aureus* by Sousa et al. (2017). However, Gomes (2019) didn't identify any antimicrobial activity of *baru* oil against *S. aureus*, *E. coli*, *S. typhimurium* and *S. pyogenes* strains.

Both UCE and SEE exhibited bactericidal activity against *S. aureus* and *S. mutans*, microorganisms commonly found in the oral cavity, that caused bacterial plaque and periodontal disease, respectively.

8.7. Toxicity analysis

The *in vitro* evaluation of the toxicity of the extracts of the leaves of *D. alata* against the root of *Allium cepa*, in

Table 5. The antimicrobial activity of extracts from the leaves of *D. alata* was evaluated using both ultrasound-assisted and Soxhlet apparatus extraction methods. The study focused on assessing their effectiveness against microorganisms such as *S. aureus* and *S. mutans*. The positive control was established using the well diffusion technique.

Extracts	Concentration (μg/mL)	Microorganisms	
		S. aureus **Mean ± SD**	***S. mutans*** **Mean ± SD**
		Zone of inhibition (nm)	
UCE	50	11.02 ± 0.39^{g}	8.86 ± 0.04^{f}
	100	11.50 ± 0.04^{b}	9.27 ± 0.23^{e}
	200	11.37 ± 0.73^{d}	11.36 ± 0.14^{b}
SEE	50	9.66 ± 0.16^{i}	8.51 ± 0.09^{g}
	100	11.16 ± 0.32^{f}	9.59 ± 0.20^{d}
	200	12.52 ± 0.36^{a}	12.16 ± 0.32^{a}
SCE	50	11.46 ± 0.65^{c}	7.71 ± 0.75^{h}
	100	10.82 ± 0.06h	7.17 ± 0.15^{i}
	200	11.26 ± 0.26^{e}	10.89 ± 0.12^{c}
Chlorhexidine (+)	+	10.65 ± 0.86	12.56 ± 0.41
DMSO (-)		0.00 ± 0.00	0.00 ± 0.00

UCE = Ultrasound Crude Extract; SEE = Ethanol Extract; SCE = Soxhlet Crude Extract. Values represent the mean followed by the standard deviation (Mean ± SD), SD = standard deviation. (+) positive control; (-) negative control.

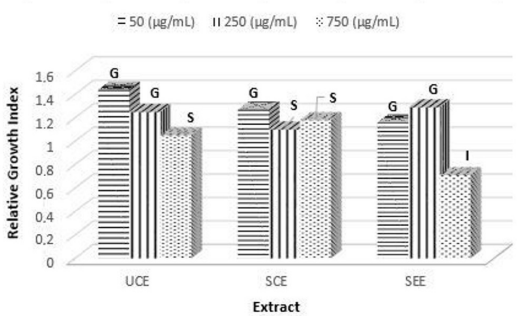

Figure 2. Comparison of root growth by concentration of *D. alata* leaf extracts, obtained by ultrasound assisted and Soxhlet apparatus, where: I = Growth Inhibition; S = Same control effect; G = Growth stimulus; UCE = Ultrasound Crude Extract; SCE = Soxhlet Crude Extract; SEE = Ethanol Extract.

the concentrations 50, 250 and 750 and the control group with mineral water are presented in Figure 2.

All the analyzed extracts presented a stimulus to root growth at a concentration of 50 μg/mL and at a concentration of 250 μg/mL, only the SCE presented the same root growth as the control group, while the UCE and the SEE presented a stimulus of root growth when compared to the control group (mineral water). At a concentration of 750 μg/mL, only SEE inhibited root growth, while the other extracts showed similar performance to the control.

The dose-response curve obtained by the toxicity test (IC_{50}) using the *Artemia salina* test of *D. alata* leaf extracts, showed low toxicity, since the mean lethal concentration (IC_{50}) was not reached even at a concentration of 1000 μg/mL.

No toxicity studies were found in the literature with extracts of *D. alata* leaves against *Allium cepa* root and *A. salina* nauplii.

9. Conclusion

The extracts from the leaves of *D. alata* exhibited phenolic compounds including gallic acid, caffeic acid, quercetin, kaempferol, luteolin, and others in lower concentrations. The quantity of these compounds obtained is directly correlated with the extraction method utilized.

In vitro bactericidal activity of SEE and UCE was evidenced against *S. aureus* with parameters similar to those of the reference chlorhexidine. The extracts obtained from the leaves of *D. alata* showed low toxicity in the methodologies tested.

The potential for expanding the utilization of *D. alata* is promising, as it not only promotes the preservation of the species in native areas but also encourages its use in phytoremediation efforts. However, further research and in vivo assays are necessary to evaluate the feasibility of utilizing *D. alata* for the treatment of oral disorders. These studies will help provide a comprehensive understanding of its efficacy and safety profile in clinical applications.

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