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Phytochemical profile and cytotoxicity assessment of the extract from *crescentia cujete* l. leaves

Perfil fitoquímico e avaliação do potencial citotóxico do extrato das folhas de *crescentia cujete* L.

Perfil fitoquímico y evaluación del potencial citotóxico del extracto de las hojas de crescentia cujete L.

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Milena Emanuela Nascimento Lima
ORCID: https://orcid.org/0000-0002-6007-6459
Faculdade de Integração do Sertão, Brasil
E-mail: milenax.nas@gmail.com
Bruna Miranda Januário
ORCID: https://orcid.org/0000-0003-1547-6901
Faculdade de Integração do Sertão, Brasil
E-mail: buhmirandaj@gmail.com
Pedro Erian de Andrade Barros
ORCID: https://orcid.org/0000-0003-4530-2398
Faculdade de Integração do Sertão, Brasil
E-mail: pedroerian29@gmail.com
Jamilly Moura Feitosa
ORCID: https://orcid.org/0000-0002-2900-2785
Faculdade de Integração do Sertão, Brasil
E-mail: Jamillymouraf@outlook.com
Viviane da Silva Lima
ORCID: https://orcid.org/0000-0003-2734-8851
Faculdade de Integração do Sertão, Brasil
E-mail: limmavivianne@gmail.com
Gabriela Cavalcante da Silva
ORCID: https://orcid.org/0000-0003-3390-6645
Faculdade de Integração do Sertão, Brasil
E-mail: gcavalcante1988@gmail.com

Abstract
Introduction: over the years, plant species have become a therapeutic alternative for a large part of the population, even with limited scientific research that ensures their effectiveness and safety. Among these, there is the Crescentia cujete, an arboreal species, with great distribution in the Northeast of Brazil. Known as the local population as a coité or gourd tree, it is used for different therapeutic purposes, such as analgesic, anti-tumor, spasmolytic and some gynecological disorders. Objective: to evaluate the possible cytotoxicity and to carry out the phytochemical characterization of the hydroalcoholic extract of Crescentia cujete. Methodology: the extract was obtained through homogenization with hydroalcoholic solution. The evaluation of cytotoxicity was based on the methodologies of erythrocyte osmotic fragility and lethality against Artemia salina L. Results: The presence of promising phytochemicals such as tannins, saponins and coumarins were detected. The cytotoxic assays showed CL_{50} 1186.77µg / mL, considered low cytotoxic potential compared to Artemia salina L. regarding hemolytic activity, the extract obtained a percentage of 60.67%. Conclusion: therefore, it is encouraged to carry out new research in vivo and in vitro that seeks to characterize its toxic potential, as well as further studies for the quantification of phytochemicals for possible application in the development and discovery of drugs.
Keywords: Phytochemistry; Cytoxicity; Crescentia Cujete.

Resumo
Introdução: com passar dos anos as espécies vegetais passaram a ser alternativas terapêuticas para grande parte da população, mesmo com restritas pesquisas científicas que assegurem sua eficácia e segurança. Dentre essas, encontra-se a *Crescentia cujete*, uma espécie arbórea, com grande distribuição no Nordeste do Brasil. Conhecida população local como coité ou árvore de cabaça, é utilizada para diferentes finalidades terapêuticas, como analgésico, antitumoral, espasmolítica e alguns distúrbios ginecológicos. Objetivo: avaliar a possível citotoxicidade e realizar a caracterização fitoquímica do extrato hidroalcoólico da *Crescentia cujete*. Metodologia: o extrato foi obtido através de homogeneização com solução hidroalcoólica. A avaliação da citotoxicidade se baseou nas metodologias de fragilidade

osmótica eritrocitária e letalidade frente *à Artemia salina* L. Resultados: foram detectados a presença de promissores fitoquímicos como taninos, saponinas e cumarinas. Os ensaios citotóxicos demonstraram CL_{50} 1186,77µg/mL, considerado baixo potencial citotóxico frente à *Artemia salina* L. quanto ao percentual hemolítico, o extrato obteve percentual de 60,67%. Conclusão: portanto, fomenta-se a realização de novas pesquisas *in vivo e in vitro* que busque caracterizar seu potencial tóxico, como também mais estudos para quantificação dos compostos fitoquímicos para possível aplicação no desenvolvimento e descoberta de fármacos.
Palavras-chave: Fitoquímica; Citotoxicidade; *Crescentia Cujete.*

Resumen
Introducción: a lo largo de los años, las especies vegetales se han convertido en alternativas terapéuticas para gran parte de la población, incluso con escasas investigaciones científicas para asegurar su eficacia y seguridad. Entre estos, se encuentra la Crescentia cujete, una especie arbórea con amplia distribución en el Nordeste de Brasil. Conocida como población local como coité o jícara, se utiliza para diferentes fines terapéuticos, como analgésicos, antitumorales, espasmolíticos y algunos trastornos ginecológicos. Objetivo: evaluar la posible citotoxicidad y realizar la caracterización fitoquímica del extracto hidroalcohólico de Crescentia cujete. Metodología: el extracto se obtuvo mediante homogeneización con una solución hidroalcohólica. La evaluación de la citotoxicidad se basó en las metodologías de fragilidad osmótica y letalidad eritrocitaria frente a Artemia salina L. Resultados: se detectó la presencia de fitoquímicos prometedores como taninos, saponinas y cumarinas. Los ensayos citotóxicos arrojaron CL_{50} 1186.77µg / mL, considerado bajo potencial citotóxico contra Artemia salina L. en cuanto al porcentaje hemolítico, el extracto obtuvo un porcentaje de 60.67%. Conclusión: por tanto, fomenta la realización de nuevas investigaciones in vivo e in vitro que busquen caracterizar su potencial tóxico, así como estudios adicionales para cuantificar compuestos fitoquímicos para su posible aplicación en el desarrollo y descubrimiento de fármacos.
Palabras clave: Fitoquímica; Citotoxicidad; Crescentia Cujete.

1. Introduction

The use of plants for therapeutic purposes has been widespread among different ethnicities and cultures since antiquity. Observations from the population on the use of medicinal plants were and remain essential for the evolution of studies on plant drugs, allowing the advance in the elucidation of pharmacologically active natural compounds with therapeutic potential (Colalto, 2018). In this context, Brazil holds the greatest plant diversity in the world, with several cataloged species, which has a tradition of use related to popular knowledge that transcends generations (Lima et al., 2020).

Numerous medicinal plants do not have considerable studies in the literature to prove the safety of their use by the population, which may cause impacts on consumer health (Alcantara et al., 2015). The large portion of the population that makes concomitant use of conventional medicines and medicinal plants are mainly adults and elderly people who use medicines as the main treatment of chronic diseases and generally believe that the use of medicinal plants is a therapeutic alternative free of harmful effects and unable to cause drug interactions. However, the interaction of medicinal plants with synthetic drugs can cause antagonistic and/or synergistic effects as a result of the interaction of several active chemical compounds, at various sites of action, in different organs and tissues (Texeira, 2011; Simões, 2008).

It is common to observe these days, in public markets, the open trade of these plant species, where most times scientific knowledge is vague, so their real effects, whether harmful or beneficial, are not confirmed (Vilas-Boas et al., 2018). According to data from the Toxic-Pharmacological Information System (Sinitox), cases of poisoning due to the misuse of medicinal plants were recorded, reaching about 1,207 cases of human poisoning by plants in the country (year of reference: 2016). Several factors lead to this occurrence, such as the lack of knowledge about the cultivation, the inadequate identification of the plant, adverse reactions, drug interaction, concentration, and frequency of use of the herbal medicine (Sinitox, 2016; Silva et al., 2017).

The Bignoniaceae family comprises 82 genera and about 827 species with pantropical distribution (Lohmann et al., 2011). Brazil is one of the centers of dissemination and diversity of the family with the equivalent of 32 genera and 391 species (Lohmann, 2012). Several chemical components are referred to this family, such as flavonoids, terpenoids, quinones, but mainly naphthoquinones and aromatic compounds (Zuntini, 2014). Among the different genera of the family, Crescentia is

among the most significant members because of its medicinal properties.

Inserted in the genus Crescentia, is the species *Crescentia cujetia*, which is a small tree reaching 5 to 10 meters in height, with alternate leaves, yellowish-green flowers, bipartite calyx, tubular corolla, unilocular ovary with many eggs, ovoid or globose with many seeds, and hard fruits in the form of a berry (Madhukar et al., 2013).

Fruits in Brazil are used in popular medicine for respiratory and skin problems, wound healing, insect repellent, anemia, and also as an abortifacient inducer (Aguirre-Dugua et al., 2017). The tea from its leaves is used to treat headaches, hypertension, as a diuretic, and in the treatment of bruises (Das et al., 2014). Given the scarcity of research proving the biological activities and cytotoxicity of this species, it is relevant to study it for the feasibility of contributing to the scientific community for future research on the therapeutic application of this genus.

2. Methodology

2.1 Plant material and extract preparation

For the study, leaves of *Crescentia cujete* L., collected in October 2019 at Fazenda Laranjo Velho, Bom Nome, district of São José do Belmonte, State of Pernambuco, Northeastern Brazil, were used. The leaves of Crescentia cujete L. were collected and sent to the Faculdade de Integração do Sertão-FIS, Serra Talhada – PE, washed in running water and submitted to a drying process in an oven at an average temperature of 36ºC for 5 days. After completing the removal of moisture, the leaves were crushed until sprayed and then subjected to extraction by dynamic maceration in a hydroalcoholic solution at 80% (w/v) for 12 hours at room temperature. Afterward, the solution was filtered and submitted to a rotary evaporation process at reduced pressure to remove the solvent, until obtaining a pasty concentrate, which was later kept in a desiccator at room temperature.

2.2 Qualitative phytochemical screening of hydroalcoholic extract from leaves of Crescentia cujete L.

The preliminary phytochemical screening was performed seeking to research the classes of metabolites present in the extract, based on precipitation reactions and coloring of the extracts diluted in solution and specific reagents for each test according to the methodology of Radi, et al (2007).

2.3 Tannin Research

Triplicate tests were performed to identify and confirm the presence of tannins in the sample. At least two of the three reactions performed must show a positive result. In the test tube, 10 mg of the hydroalcoholic extract were dissolved in 2 ml of methanol, and 5 ml of distilled water was added, proceeding with filtration of the solution and addition of 5 drops of the 10% ferric chloride solution. The presence of condensed tannins is indicated by the green color of the sample.

2.4 Coumarins Research

The research to identify coumarins was carried out according to Kloss (2016). In the test tube, 10 mg of the hydroalcoholic extract diluted with a methanolic solution was added, and this was covered with filter paper wet with a 10% sodium hydroxide solution. Afterward, the tube was left in a water bath for 10 minutes. The filter paper was examined under ultraviolet (UV) light. Fluorescence indicates the presence of coumarins.

2.5 Quinones Research

For investigation of quinones, 10 mg of the extract were weighed and 2 ml of methanol were added to dissolve, then 5 ml of chloroform were added and stirred. Afterward, it was left to rest for 15 minutes. The chloroform phase was collected by

transferring to a test tube and 1 mL of 5% aqueous sodium hydroxide solution was added. The appearance of purple color is indicative of the presence of quinones.

2.6 Saponin Research

For identification of saponins, there were used 10 mg of the extract dissolved in 2mL of ethanolic solution and then 5 mL of boiling distilled water was added. After cooling, it was stirred vigorously, leaving to rest for 20 minutes. The presence of saponins is indicated by the formation of foam.

2.7 Alkaloid Research

The alkaloid group was evaluated as described by Kloss et al. (2016). Ten milligrams of the extract were diluted in 2 mL of ethanol in the test tube and 2 mL of 10% hydrochloric acid was added, the solution was heated in a water bath for 10 minutes. After that, the extract was cooled to room temperature and using a Pasteur pipette, 8 drops of Dragenodorff's reagent were added. The presence of alkaloids is observed by the orange to red coloration.

2.8 Determination of LC_{50} through a bioassay with *Artemia salina L.*

The lethality test in *Artemia salina L.* was performed according to the methodology described by Meyer (1982). After the eggs hatch, ten Artemia nauplii were collected with the aid of a Pasteur pipette and transferred to each of the test tubes. Contained 5ml of seawater. Soon after, concentrations of 250, 500, 750, 1000, 1250, 1500 µg/mL of the hydroalcoholic extract of leaves of *Crescentia cujete* were prepared. The nauplii were incubated in contact with the extract at their respective concentrations at room temperature, and during this contact period, readings were taken at 12 and 24 hours to record the number of live nauplii, this test was performed in triplicate.

2.9 Measurement of hemolytic activity

The assay was performed with concentrations of 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml, 750 µg/ml, 1000 µg/ml. After adding sheep blood, the samples were kept at room temperature for 15 minutes. Then the samples were centrifuged at a rotation of 3500 rpm for 15 minutes. The reading of the supernatant was done with an Oleman® spectrophotometer, using a 540 nm filter, and thus the absorbance values of each sample should be obtained. The hemolytic percentage was established with the absorbance of the positive control being designated as 100%. The percentage of hemolysis is established based on the formula: % = Sample absorbance x 100/Control absorbance (DARCIE et al, 1975), and these assays were performed in triplicate.

2.9.1 Statistical Analysis

The software used was GraphPad Prism version 8.0, to calculate the means, standard deviations, as well as to construct the graphs and dose-response curve.

3. Results and Discussion

The hydroalcoholic extract of dark green *Crescentia cujete L.* leaves presented an average yield percentage of 4.59% concerning the dry weight of the crushed sample and the weight obtained after the preparation of the extract, as described in (Table 1).

Table 1. Percentage of hydroalcoholic extract yield

Crescentia cujete L.	Dry weight (g)	Extract weight (g)	Yield (%)
Leaves	100 g	4,596g	4,59%

Source: Authors.

The preliminary phytochemical screening carried out in the present study allowed us to trace the chemical profile of the compounds present in the leaves of *C. cujete*, which indicated the presence of classes of metabolites such as coumarins, saponins, and condensed tannins. The results are expressed in (Table 2) and were obtained through qualitative analysis of chemical reactions.

Table 2. Preliminary phytochemical analysis of the hydroalcoholic extract of *Crescentia cujete* L leaves.

Metabolite classes	*Crescentia cujete L.*
Alkaloids	-
Coumarins	+
Quinones	-
Condensed tannins	+
Hydrolyzable tannins	-
Saponins	+

Source: Authors.

In all samples analyzed, the presence of saponins and tannins was observed, similar to the result found by Das et al. (2014) on ethanol extracts from leaves of *Crescentia cujete*. The non-detection of alkaloids in the species under study corroborates the data obtained in the study by Paulo (2016), and by Das et al. (2014) on ethanol extracts of *Crescentia cujete* leaves using three reagents, but none were positive. However, the work carried out by Martins et al. (2014) with ethanol extract of the fruit of the species in question resulted in positivity for alkaloids. In the study carried out by Viana (2015) using the dichloromethane extract of the species *Sparattosperma leucanthum* of the Bignoniaceae family, only the presence of tannins was observed.

This divergence may be related to environmental conditions such as seasonality, temperature, pathogen attacks, and degree of maturation, among other factors, as they interfere in the synthesis of phytochemicals. In the same sense, there may be a distinct distribution of metabolites by plant organs, which may affect the total amount of metabolites produced (Paulo, 2016).

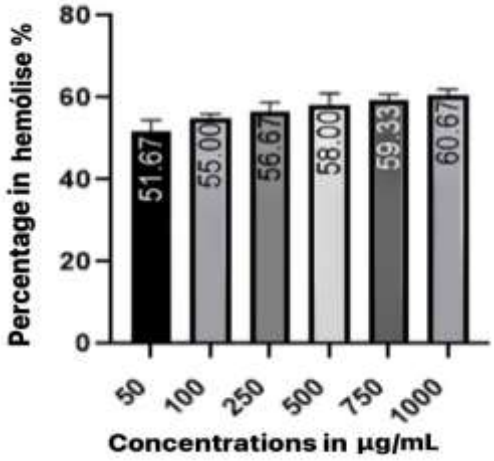
Coumarins are a class of metabolites derived from cinnamic acid, widely distributed in the plant kingdom, and can also be found in fungi and bacteria (Kloss et al, 2016). This class of metabolites is attributed to a wide variety of therapeutic activities, such as antioxidant, anti-inflammatory, an inhibitor of platelet aggregation, and stimulant of venous circulation

(Silva et al, 2016).

Condensed tannins are distributed in several families of the plant kingdom, especially woody plants, to which therapeutic activities are described as an antioxidant, and potential application as a preventive and therapeutic agent against various types of neoplasms, especially skin cancer (Couto et al, 2010).

The assay to determine the hemolytic capacity on erythrocytes showed that at the concentration of 1000 µg/mL the extract caused a percentage of hemolysis of 60.67% (Graph 1), considered high since values above 40% are already referred to as high degree hemolysis, and low degree when values are below 10% (Nofiani, 2011).

Graphic 1. Hemolytic percentage of the hydroalcoholic extract of *Crescentia cujete* L. leaves at different concentrations.



Source: Authors.

In the search for new substances that present promising therapeutic activity and that do not cause harmful effects to organisms, tests such as this one is constantly used to investigate the capacity of natural products to cause hemolytic activity, increasing the permeability or disintegrating the erythrocyte cell membrane (Junior-Birth, 2016).

Among the classes of metabolites found in the extract under study, saponin is known in the literature for its surface-active activity capable of causing damage to the cell membrane. This effect is a result of its ability to interact with the components of the cell membrane, mainly with sterol molecules such as cholesterol, causing deformation in the membrane leading to the extravasation of intracellular content (Nunes, 2015).

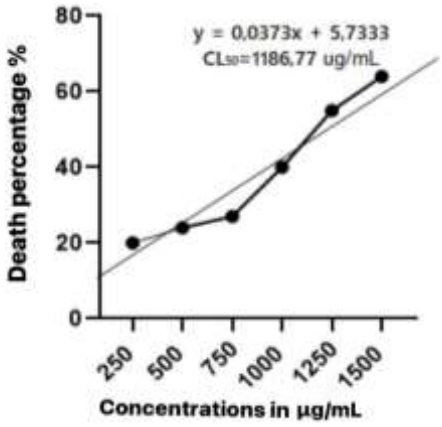
This hemolytic action of saponins is part of the defense mechanism of the plant species against attacks from predators such as fungi, insects, bacteria, and viruses, being linked to antibacterial, antiviral, and antifungal activities presented by several plants. In addition, it is the target of great interest by the pharmaceutical industry because it is used as an adjuvant and active ingredient in formulations (Simões et al., 2016).

Gonçalves (2011), analyzing the action of tannins isolated from the plant species *Mimosa arenosa*, reported hemolytic activity on human blood cells. Tannins can interact with macromolecules, thus conferring toxic activities and binding agents (Carvalho et al., 2012). In this case, in addition to saponins, it can be said that tannins may have contributed to the hemolytic effect of the species under study.

The bioassay against *Artemia salina* is considered a useful preliminary test to investigate cytotoxicity, which

has shown a good correlation with several biological activities, such as antitumor, antibacterial, and antifungal activities (KARCHESY et al., 2016). According to Meyer et al. (1982) plant extracts with LC_{50} less than 1000 µg/mL are considered potentially toxic. As shown in (Graph 2), the plant extract presented a LC_{50} value of 1186.77 µg/mL, which demonstrates low cytotoxicity.

Graph 2. Cytotoxicity of hydroalcoholic extract of *Crescentia cujete* L. leave against *Artemia salina*.



Source: Authors.

The result obtained in this study corroborates the result obtained in the work by Sousa et al. (2019), which evaluated the cytotoxicity against *A. salina* of the ethanol extract of the leaves of *Pithecoctenium crucigerum* of the Bignoniaceae family, which demonstrated a value of LC_{50} >1000 µg\mL, thus considering the extract to be nontoxic.

However, Cansian (2010) in his study evaluated the toxicity against *Artemia salina* with hydroalcoholic extract 20% of the root and stem of the species *Tynanthus micranthus* (Bignoniaceae), resulting in cytotoxic activity for both extracts, detecting LC_{50} 63.88 µg/mL and 42.99 µg/ml, respectively. Elevated cytotoxic activity was also demonstrated in the study by Viana (2015), who evaluated the toxicity against *A. saline* of the dichloromethane extract from the stem of the species *Sparattosperma leucanthum* (Bignoniaceae) obtaining LC_{50} 289.4 µg\mL.

Such results diverge from the results obtained in this work, possibly because of the higher concentration and different metabolites extracted from the plant species, as well as the use of solvents in different concentrations. When analyzing the results of cytotoxicity and associating them with the phytochemicals of the extract, it can be said that *C. cujete* leaves do not have metabolites that induce cytotoxicity against *Artemia salina*, or are in lower concentration in the leaves.

4. Conclusion

According to the results, it can be concluded that the LC_{50} value obtained was considered low cytotoxic potential; however, it presented a high hemolytic percentage. These results, associated with the phytochemical screening of the extract, make it relevant to continue further future phytochemical studies to investigate possible therapeutic potentials correlated with the demonstrated phytochemicals, as well as in vivo toxicity tests.

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