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Cytotoxic Action of the Lyophilized Infusion of *Aristolochia triangularis* Leaves on *Saccharomyces cerevisiae*

Ação citotóxica da infusão liofilizada das folhas de Aristolochia triangularis sobre Saccharomyces cerevisiae

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ABSTRACT

The use of native plants in folk medicine has been increasing in Brazil, highlighting the need to study these plants and their bioactive compounds. *Aristolochia triangularis*, or "cipó-mil-homens," is one such plant, raising concerns due to the possible presence of toxic compounds like aristolochic acids. This study aimed to evaluate the effect of the lyophilized infusion of *A. triangularis* leaves at different concentrations on cell growth and mortality rate in *Saccharomyces cerevisiae*-Fleischmann®. The infusion of A. triangularis leaves was prepared and lyophilized. Concentrations of 1.25, 2.50, and 5.00 $\mu g.L^{-1}$ were prepared and used to evaluate the interaction of the yeast with the extract at 3 and 6 hours, with tests for cell growth capacity and mortality rate using methylene blue. The results showed that the *A. triangularis* extract inhibited yeast growth, depending on the concentration and exposure time. The 5.00 $\mu g.L^{-1}$ concentration had a higher rate of dead cells at 3 hours, while the 1.25 $\mu g.L^{-1}$ concentration increased mortality after 6 hours of exposure. These findings underscore the importance of assessing the toxicity of medicinal plant extracts.

Keywords: Native plants, Folk medicine, Bioactives**.**

RESUMO

O uso de plantas nativas na medicina popular tem aumentado no Brasil, destacando a necessidade de estudar essas plantas e seus bioativos. *Aristolochia triangularis*, ou cipó-mil-homens, é uma dessas plantas, que levanta preocupações devido à possível presença de compostos tóxicos, como os ácidos aristolóquicos. Com isso, o estudo visou avaliar o efeito do infuso liofilizado das folhas de *A. triangularis* em diferentes concentrações no crescimento celular e na taxa de mortalidade em Saccharomyces cerevisiae- Fleischmann®. O infuso das folhas de *A. triangularis* foi preparado e liofilizado. Foram preparadas concentrações de 1,25, 2,50 e 5,00 $\mu g.L^{-1}$, e utilizados para avaliar a interação da levedura frente a ao extrato nos tempos de 3 e 6 horas, sendo realizados os testes da capacidade de crescimento celular e taxa de mortalidade utilizando azul de metileno. Os resultados mostraram que o extrato de *A. triangularis* inibiu o crescimento da levedura, dependendo da concentração e do tempo de exposição. A concentração de 5,00 $\mu g.L^{-1}$ apresentou uma maior taxa de células mortas em 3 horas, enquanto a concentração de 1,25 $\mu g.L^{-1}$ aumentou a mortalidade após 6 horas de exposição. Estas descobertas destacam a importância de avaliar a toxicidade de extratos de plantas medicinais.

Palavras chave: Plantas nativas, Medicina popular, Bioativos

1. INTRODUCTION

The relentless pursuit of answers about human life and health has led scientists to explore a wide variety of organisms, from the simplest to the most complex. In this context, the yeast *Saccharomyces cerevisiae* has gained prominence as a model organism for biological investigation. Moreover, these yeasts share many genes and cellular processes with more complex organisms, including humans (LIDZBARSKY et al., 2018).

One of the most notable characteristics of this microorganism is its short life cycle, combined with an extremely rapid growth rate. These traits allow for experiments to be conducted in short periods, significantly expediting results (ELEUTHERIO et al., 2018). Another aspect that makes the use of *S. cerevisiae* attractive is its non-pathogenic nature, meaning that its study and manipulation pose no significant risks. Furthermore, this microorganism possesses stress response and cellular damage mechanisms, making it a useful model for investigating different diseases (DAHIYA et al., 2020).

These mechanisms enable the observation of various fundamental physiological responses, such as changes in viability and growth rate, alterations in metabolism with the production of different metabolites, oxidative stress, and more severe damage to deoxyribonucleic acid (DNA) (ANJU; SIDDHARDHA; DYAVAIAH, 2020), among other relevant parameters. Thus, they provide indicators of the compound's toxicity degree and its potential genotoxic and mutagenic effects (ČANADI JUREŠIĆ et al., 2021). Such information is crucial for assessing the safety of various compounds before their widespread application, potentially advancing the development of more efficient therapies and treatments.

The use of microorganisms in in vitro tests allows for control of the experimental environment, providing a detailed analysis of cellular responses to toxic compounds under study, such as industrial chemicals, pesticides, environmental contaminants, and drugs (ROSCINI et al., 2019; HE et al., 2020). Thus, the use of *S. cerevisiae* as an experimental biological model offers an efficient and ethical approach for evaluating potentially toxic compounds, enabling tests that assess the mutagenic effect of a substance and provide information about its effects on genetic material (COÊLHO et al., 2022). *S. cerevisiae* assays are economically viable, as it is a microorganism with rapid growth and low nutritional demands (9. MAGISTRATI et al., 2023). For this reason, toxicity assessment of medium-

term or chronic exposure can be evaluated with rapid responses and prolonged exposure in these cells.

In the last decade, there has been an expansion in the number of studies with alternative model organisms. The main models used include zebrafish, nematodes, and fruit flies for toxicity studies. Considering that these are organisms that offer logistical convenience and productivity in in vitro assays (ANKLEY et al., 2021; LEBRE et al., 2022). According to Bondue et al. (2023), when proposing the use of alternative model organisms, structural and biological similarities between the most used models and the alternatives should be considered. However, evaluating this biochemical network is a complex but necessary matter, especially for proposing new insights and thereby enhancing knowledge.

The use of S. cerevisiae as an alternative model organism is based on the similarity of these cells to mammalian cells, including macromolecules, organelles, various proteins, and their orthologous genes related to human diseases (PLOGER et al., 2000; VANDERWAEREN et al., 2022; WINKLER et al., 2022). Thus, these microorganisms can be used as a bioindicator of toxicity for assays using different compounds, serving as an option to assess the toxicity of medicinal plant extracts, which still lack information on their efficiency of use.

Some plants used in traditional medicine are well-documented in the literature. However, others have few studies confirming their therapeutic potential, even though they are widely used in traditional medicine. One such plant is *Aristolochia triangularis*, belonging to the *Aristolochiaceae* family, Piperales order, popularly known as "cipó-mil-homens" and "jarrinha" (MADANI et al., 2022; OJO et al., 2022). This family has different species containing a variety of chemical compounds with therapeutic potential (IGNÀCIO et al., 2020).

The Aristolochia genus has various applications, used in the treatment of different ailments due to its chemical properties (ALEGRANSI et al., 2021). However, some species have toxic compounds, such as aristolochic acids, which, when ingested in high concentrations, can cause adverse health effects (LERMA-HERRERA et al., 2022). A. triangularis has broad application potential due to the presence of phytochemicals in its composition. However, it is important to note that both medicinal plants and herbal medicines can cause adverse effects, including interactions with other drugs when used concurrently, sometimes resulting in intoxications (LIN et al., 2020; FANG et al., 2021). In this sense, toxicity assays play a fundamental role, being essential for understanding

adverse effects resulting from the interaction between chemical substances and biological systems (KREWSKI et al., 2020; SCHUIJT et al., 2021).

Therefore, the analysis of adverse effects and the understanding of interactions with biological systems become essential elements, significantly contributing to providing relevant and well-founded information about the use of medicinal plants. Continuous research and comprehensive studies are imperative for the assessment and assurance of safety in the use of plants as therapeutic resources. Thus, this study aimed to evaluate the action of different concentrations of the lyophilized infusion of A. triangularis leaves on the cellular growth and mortality rate in Saccharomyces cerevisiae-Fleischmann®.

2. MATERIAL AND METHOTODS

2.1 Study Development Location

This study was conducted at the Laboratory of Toxicological Assays (LETOX) of the Federal University of Grande Dourados – UFGD and the Laboratory of Biotechnology, Biochemistry, and Biotransformation of the Center for Studies in Natural Resources – CERNA of the State University of Mato Grosso do Sul – UEMS.

2.2 Plant Material and Plant Identification

Leaves of A. triangularis were collected in Parque Victelio de Pellegrin (W 22°23’17.2”, S 54°84’20.2”), Jardim Novo Horizonte, Dourados/MS, Brazil, in May 2021. These were fragmented into strips of 1–3 cm and then stored in a freezer at -20 °C until the extraction moment. The plant was identified by Dr. Joelcio Freitas, and an exsiccata (MBML53232) was deposited in the herbarium of the Museum of Biology Prof. Mello Leitão (MBML), located in Santa Teresa, Espírito Santo, Brazil. IBAMA authorization number: 51842. SisGen/MMA registration code: A1F6637.

2.3 Infusion Preparation

The sample (IFLAT extract) whose cytotoxicity was analyzed in this research was prepared as detailed in a previous publication by De Araújo et al. (2023). In brief, section-divided in natura leaves of A. triangularis (40.0 g) were subjected to extraction by infusion in pre-heated distilled water at 95 °C for 15 min. Immediately afterward, the infusion to 10% (w/v; ~4 liters) thus obtained was hot filtered, frozen, and lyophilized, successively, to give

the freeze-dried aqueous extract (IFLAT; 17.5 g) of A. triangularis leaves, which was kept in a desiccator until bioassay initiation.

2.4 Experimental Conditions

For the study, experimental groups were categorized as follows: a negative control group (C-), which was devoid of stressor compounds, and a positive control group (C+), containing hydrogen peroxide at a concentration of 5% ($v.v^{-1}$).

The concentrations of the lyophilized infusion of A. triangularis leaves (ILFAT): (1.25, 2.50, and 5.00 $\mu g.L^{-1}$) were prepared and diluted in a solution of glucose with autoclaved distilled water 5% ($w.v^{-1}$) and added 0.1 g of freeze-dried yeast Saccharomyces cerevisiae – Fleischmann® composing the reaction mixture.

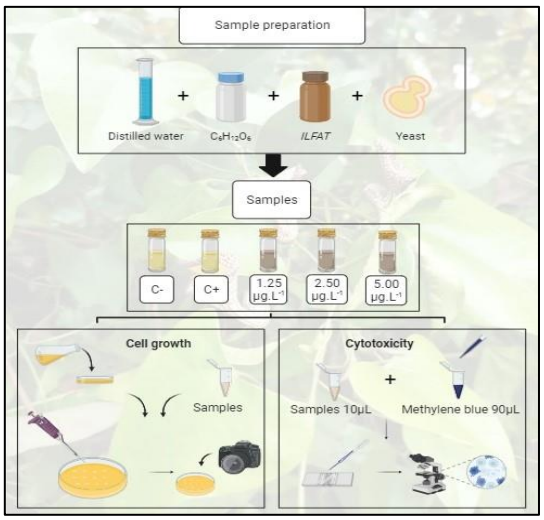
2.5 Cell Growth Capacity Test

The cell growth capacity test was adapted from the methodology of Mueller et al. (2020). Thus, the reaction mixture was incubated on an orbital shaker at 200 rpm at a temperature of 30 °C. At exposure times of 3 and 6 hours, 0.3 µL aliquots were collected with the aid of a micropipette and dripped onto Petri dishes, previously prepared with solid Sabouraud Dextrose Agar. The plates were incubated at 30 °C for a period of 48 h or until colony growth. After this period, the colonies were photographed and analyzed qualitatively. The experiments were conducted in triplicate.

2.6 Cytotoxicity Assay

To assess the mortality rate, i.e., whether different concentrations of ILFAT are toxic to yeast cells. Aliquots of 100 µL of the sample were collected and added to 900 µL of methylene blue dye according to the methodology described by Sarabia et al. (2019) (Figure 1).

Figure 1 – Flowchart of analysis development stages.



2.7 Statistical Analysis

The data were plotted and analyzed for mean and standard deviation using Microsoft Excel 16 software, and the graphs were generated using the Graph Pad Prism program.

3. RESULTS AND DISCUSSION

It was observed that the Fleischmann® yeast exhibited cell growth capacity at all tested concentrations. However, in comparison to the positive control group, minimal cell growth was evident. Additionally, there was inhibition that varied according to the dose and exposure time (Table 1). In general, concentrations of 1.25 and 2.50 ($\mu g.L^{-1}$) resulted in yeast growth patterns similar to those observed in the negative control group. However, as cells were exposed to the higher concentration of 5.00 ($\mu g.L^{-1}$), this inhibition became more pronounced, especially after 6 hours of exposure.

Table 1 – Cell growth of Fleischmann® yeast at different concentrations of *Aristolochia triangularis* leaf infusion.

	Concentrations (µg. L $^{-1}$)				
Time (h)	C-	C+	1.25	2.50	5.00
3					
6					

Source: Data from research.

Studies conducted by Tavares et al. (2022), to assess the toxicity of compounds in yeast employed the cell growth capacity test with the Fleischmann® yeast as a model microorganism/bioindicator. In this study, the yeast was exposed to different concentrations of 2,4-dichlorophenoxyacetic acid (2.0, 4.0, and 6.0 µg.L^{-1}) and incubated for 30, 60, and 90 minutes. After 72 hours of incubation at 30 °C, researchers observed a severe inhibition of cell growth at the highest concentration and longer exposure time. Conversely, Khan et al. (2017), studies, investigating the action of the aqueous extract of Butea monosperma flowers (EABMF) on the growth of S. cerevisiae, revealed a notable increase in the growth, latency, and logarithmic phases of yeast cells compared to the control group. No changes were detected in the stationary and death phases, suggesting that the extract had a significant impact on the growth behavior of *S. cerevisiae*. Yeasts, eukaryotic organisms, can be employed as models for cytotoxicity assessment, which involves studying the harmful effects of substances on cells. These microorganisms are widely distributed in different natural environments and play significant ecological roles (GILBERT; STEPHENS, 2018; VOOLSTRA; ZIEGLER, 2020).

The *Aristolochias* have traditionally been employed in folk medicine, noted for their numerous therapeutic pharmacological properties, as evidenced in recent research (MUELLER et al., 2022). Various parts, including stems, leaves, and roots, are widely used to treat a variety of conditions such as stomach disorders, gout, asthma, fever, and seizures, as indicated by Lorenzi and Matos (2008). However, due to their global distribution and the dissemination of uses primarily based on anecdotal reports, exercising caution is crucial when employing these medicinal plants (HEINRICH et al., 2022). In an ethnobotanical study conducted by Battisti et al. (2021), in the municipality of Palmeira das Missões, RS, it was revealed that Aristolochia triangularis is widely used in the form of infusions and decoctions.

The root is used as a laxative agent, branches to relieve abdominal pain, the vine and roots to thin the blood, and it offers benefits for headaches, menopausal symptoms, and liver problems.

Species of the genus Aristolochia contain aristolochic acids, compounds that induce adverse effects in humans (YOUNIS et al., 2021). This family of nitrophenanthrene carboxylic acids has various well-known purposes such as antibacterial, anti-inflammatory, analgesic, and antitumor actions. Additionally, some Aristolochia plants with low AA content are adopted for curative processes of pulmonary pathologies. Although their recommended maximum daily human dose is restricted to 9 g, the maximum content of aristolochic acid I (AAI) has not been defined. It's worth noting that these products are prohibited, especially for at-risk groups such as pregnant women, lactating individuals, and those with renal insufficiency (WANG et al., 2021).

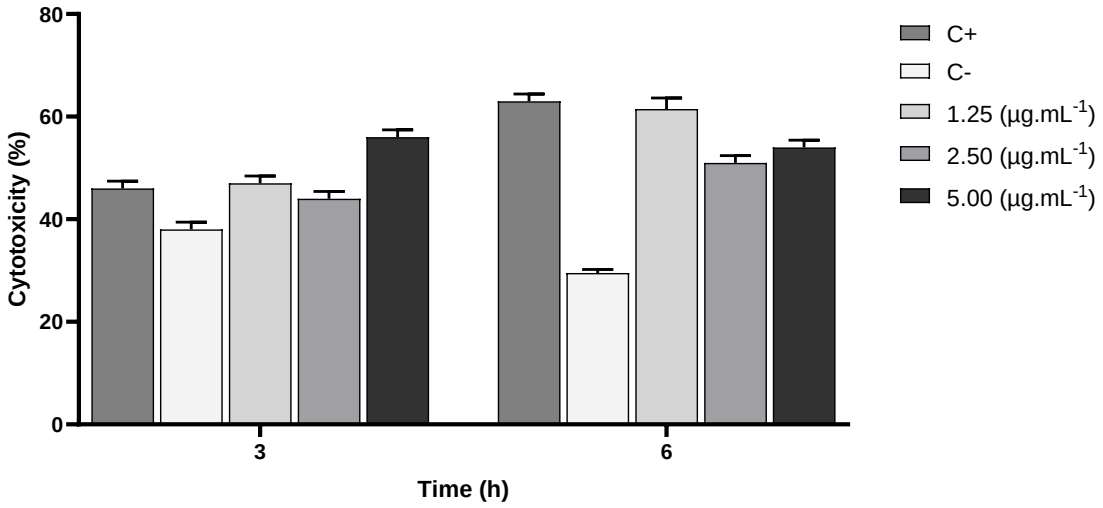
Given this, it is imperative to explore this archetype, shedding light on new scientific discoveries, considering contradictions regarding the availability of secondary metabolites, especially aristolochic acids, from plants of this genus. Wilkinson et al. (2014), analyzing NMR and LC-MS of hydro-methanolic extracts of A. triangularis leaves cultivated in the Botanical Garden of the Dresden University of Technology, found Aristolactam I (AL I) and Aristolochic Acids (AAs) I, II, C, and D in concentrations ranging from 1 to 633 µg.mg-1, with AAs I (82-633 µg.mg-1) and II (0-157 µg.mg-1) predominating. On the other hand, Araújo et al. [25] found, among the five mentioned derivatives (AL I, AAS I, II, C, and D), only AL I for the F1 fraction obtained from the liquid-liquid extraction procedure. However, Caballero et al. (2014), did not identify AAs I and II in the ethanolic extract of *A. triangularis* leaves collected in Nova Santa Rita, RS, with HPLC equipment.

Regarding the cytotoxicity cell assay through the mortality rate assay, it was observed that the concentration of 5.00 µg.L-1 showed a higher number of dead cells in 3 hours of exposure compared to the C+ control. Concerning the exposure time of 6 hours, the group with the highest cell death rate was the 1.25 µg.L-1 compared to C+. Possibly, the lower concentration was metabolized more efficiently by the yeast regarding the 3-hour exposure time. Additionally, it was observed that the group of 2.50 µg.L-1 exhibited a lower cell mortality rate against S. cerevisiae-Fleischmann® in the two studied times (Figure 2).

For in vitro analyses, it is essential to maintain precise control of cell viability rates, and for this, it is important to employ rapid and reliable methods. Among the available options, staining using methylene blue stands out, a widely adopted method. In this procedure, live

cells remain colorless, while dead cells acquire a blue color. This approach allows for an effective evaluation of the percentage of viable cells in a given population (HAINES, 2017; EVANS; FOSTER, 2011; FAN; SHEN, 2019; BELIGA ETAL., 2020).

Figure 2 – Evaluation of the cytotoxicity of different concentrations of *Aristolochia triangularis* infusion on Fleischmann® yeast.



Additionally, cell viability estimation is crucial for various assays, spanning from pharmaceutical toxicity, malignant transformation, and mutagenesis to apoptosis analysis and cellular pathology, and plays a fundamental role in understanding carcinogenic processes. However, to conduct this assessment effectively, colorimetric tests are often employed, utilizing compounds whose colors are exclusively altered by viable cells, remaining unchanged in non-viable cells (GOMES ET AL., 2020).

There are no studies in the literature investigating the effect of aqueous extracts obtained by infusion of *Aristolochia triangularis* leaves on *S. cerevisiae* – Fleischmann®. However, other biological models have been tested with plant extracts. An example is the study conducted by Mcbride et al. (2016), which provides relevant insight as they investigated the toxicity of the aqueous extract of Aristolochia ringens root using *Artemia salina*, a widely employed method to assess the ability of a compound to induce mortality and determine the biological activities of natural products. The results of this study indicated

that as the extract concentration increased to 1000 µg.mL^{-1}, there was a significant rise in mortality rate, suggesting a potential cytotoxicity associated with the extract.

Furthermore, during tests to investigate the biological and chemical potentials of *Aristolochia triangularis*, CABALLERO et al. (2014), discovered that aristolactam BII, an example of a phenanthrene alkaloid found in this and other *Aristolochiaceae* species, demonstrated antifungal action against *Candida krusei*. Nevertheless, De Araújo et al. (2023), studying the infusion of its leaves, found the abundance and diversity of its secondary metabolites. It is precisely this plurality that complicates the understanding of its chemical composition in biological activities, although it is a rich field with gaps to be filled. Moreover, they identified 61 undescribed chemical constituents. They also found significant amounts of lignoids, alkaloids, and glycosylated flavonoids, which may be related to bioactivities. However, they did not evidence the association of these compounds with biological activities. Thus, they recommend caution in the use of the plant, especially concerning its association with other herbs, and encourage further studies to mitigate therapeutic actions and integrate them with its metabolites.

In this study, we highlighted that higher concentrations and longer exposure times induced a physiological adjustment of the yeast concerning its cellular response due to the exposed conditions. They did not respond in the parameter of cell growth entirely efficiently, and consequently, there was induction of cell death. In this sense, studies focused on the use of chemical compounds, especially those from plants used in folk medicine without scientific foundation, should always be carefully evaluated regarding the interaction with living organisms. Responses are crucial for understanding the effects concerning dose and exposure time, as well as the interaction between chemical substances and biological systems.

4. CONCLUSION

The aqueous extract of *Aristolochia triangularis* exerts inhibitory properties on the growth of Fleischmann yeast, dependent on both concentration and exposure time. In the 3-hour exposure, the concentration that most affected live cells was 5.00 µg.L^{-1}, and in the 6-hour exposure, it was 1.25 µg.L^{-1} when compared to the positive control. Conversely, the dose of 2.50 µg.L^{-1} showed the lowest rate of cell mortality against *Saccharomyces cerevisiae*-Fleischmann® at both studied time points.

These results are crucial for understanding the biological processes occurring in the interaction between biological systems and potentially toxic compounds, providing valuable insights for future research with other eukaryotic organisms. Therefore, further studies are needed to explore the potential of this plant, widely used in southern Brazil.

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