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Estudo *in vivo* dos potenciais tóxico, citotóxico e genotóxico do extrato etanólico da casca do caule de *Cenostigma gardnerianum* Tul. (Caneleiro)

In vivo study on the toxic, cytotoxic, and genotoxic potentials of stem bark ethanolic extract of *Cenostigma gardnerianum* Tul. (Caneleiro)

Estudio *in vivo* de los potenciales tóxico, citotóxico y genotóxico del extracto etanólico de la corteza del tallo de *Cenostigma gardnerianum* Tul. (Caneleiro)

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Resumo

Muitas espécies de plantas medicinais vêm sendo utilizadas como alternativa terapêutica no tratamento de enfermidades ao redor do mundo, bem como promovendo avanços na produção de medicamentos fitoterápicos. Contudo, esse uso muitas vezes é feito de forma indiscriminada e leva a sérias consequências no organismo de quem as utiliza. Portanto, estudos sobre os potenciais tóxico, citotóxico e genotóxico de espécies de plantas medicinais são necessários para seu uso seguro e eficaz. *Cenostigma gardnerianum* Tul, popularmente conhecida como Canela-de-velho ou Caneleiro, é largamente utilizada pela população por suas propriedades anti-inflamatórias, antiespasmolíticas, antitussivas, antifúngicas, entre outras. O objetivo da presente pesquisa foi estudar e analisar possíveis toxicidade, citotoxicidade e genotoxicidade do extrato etanólico da casca do caule de *Cenostigma gardnerianum* Tul. por meio do bioensaio *in vivo* de Allium cepa. Dividiu-se o extrato em quatro concentrações, divididas nos grupos A, B, C e D, respectivamente: 0,5mg/ml, 1,0mg/ml, 2,0mg/ml e 5,0mg/ml, além do Controle Negativo (água sem cloro), Controle Positivo (dimetilsulfóxido) e Branco (água destilada). Realizou-se a medição das raízes para avaliação da toxicidade, calculou-se o Índice Mitótico (IM) para pesquisa de citotoxicidade e, por fim, fez-se a contagem de micronúcleos para estudo da genotoxicidade de cada concentração. O baixo crescimento dos meristemas indicou presença de toxicidade; os índices mitóticos encontrados foram estatisticamente menores do que no controle negativo, revelando citotoxicidade; e a alta contagem de micronúcleos mostrou presença de genotoxicidade. A partir dos resultados obtidos, concluiu-se que o extrato etanólico da casca do caneleiro apresenta atividade tóxica, citotóxica e genotóxica, com valores estatisticamente mais significativos para concentrações a partir de 2mg/ml.

Palavras-chave: Bioensaio; Caneleiro; Allium cepa.

Abstract

Many species of medicinal plants have been used as a therapeutic alternative in the treatment of diseases around the world, as well as promoting advances in the production of herbal medicines. However, this use is often done indiscriminately and leads to serious consequences

in the body of those who use them. Therefore, studies on the toxic, cytotoxic, and genotoxic potentials of medicinal plant species are necessary for their safe use. *Cenostigma gardnerianum* Tul, popularly known as Caneleiro or Canela-de-velho, is widely used by the population for its anti-inflammatory, antispassmolytic, antitussive, antifungal properties, among others. The aim of this research was to study and analyze a possible toxicity, cytotoxicity, and genotoxicity of the ethanolic extract from the bark of the stem of Cenostigma gardnerianum Tul. by means of the Allium cepa in vivo bioassay. The extract was divided into four concentrations, divided into groups A, B, C and D, respectively: 0.5mg/ml, 1.0mg/ml, 2.0mg/ml and 5.0mg/ml, in addition to the Negative Control (dechlorinated water), Positive Control (dimethyl sulfoxide) and Blank control (distilled water). The roots were measured for toxicity evaluation, the Mitotic Index (MI) was calculated for cytotoxicity research, and finally micronuclei count was performed to study the genotoxicity of each extract concentration. The low growth of the meristems indicated the presence of toxicity; the mitotic indices found were statistically lower than in the negative control, revealing cytotoxicity; and the high micronucleus count showed the presence of genotoxicity. From the results obtained, it was concluded that the ethanolic extract of the Caneleiro bark presents toxic, cytotoxic, and genotoxic activity, with statistically significant values for concentrations starting from 2mg/ml.

Keywords: Bioassay; Caneleiro; Allium cepa.

Resumen

Muchas especies de plantas medicinales han sido utilizadas como alternativa terapéutica en el tratamiento de enfermedades en todo el mundo, así como promoviendo avances en la producción de medicamentos fitofármacos. Sin embargo, este uso a menudo se hace de forma indiscriminada y tiene graves consecuencias en el organismo de quien las utiliza. Por consiguiente, los estudios sobre los potenciales tóxicos, citotóxicos y genotóxicos de las especies de plantas medicinales son necesarios para su uso seguro. *Cenostigma gardnerianum* Tul, popularmente conocida como Canela-de-velho o Caneleiro, es ampliamente utilizada por la población por sus propiedades anti-inflamatorias, antiespasmolíticas, antitússico, antifúngicas, entre otras. El objetivo de esta investigación era estudiar y analizar la posible toxicidad, citotoxicidad y genotoxicidad del extracto etanólico de la corteza del tallo de Cenostigma gardnerianum Tul. por medio del bioensayo in vivo de Allium cepa. Se dividió el extracto en cuatro concentraciones, divididas en grupos A, B, C y D, respectivamente: 0,5mg/ml, 1,0mg/ml, 2,0mg/ml y 5,0mg/ml, además del Control Negativo (agua sin cloro),

Control Positivo (dimetilsulfóxido) y Blanco (agua destilada). Se realizó la medición de las raíces para la evaluación de la toxicidad, se calculó el Índice Mitótico (IM) para la investigación de la citotoxicidad y, finalmente, se realizó el recuento de micronúcleos para el estudio de la genotoxicidad de cada concentración. El bajo crecimiento de los meristemas indicó presencia de toxicidad; los índices mitóticos encontrados fueron estadísticamente menores que en el control negativo, revelando citotoxicidad; y el alto recuento de micronúcleos mostró presencia de genotoxicidad. A partir de los resultados obtenidos, se llegó a la conclusión de que el extracto etanólico de la corteza del Canelero presenta una actividad tóxica, citotóxica y genotóxica, con valores estadísticamente más significativos para concentraciones a partir de 2mg/ml.

Palabras clave: Bioensayo; Caneleiro; Allium cepa.

1. Introduction

Since the dawn of humanity, the use of the most diverse types and species of plants in the treatment of innumerable illnesses has been increasing and becoming more important in today's society. A medicinal plant is any plant which, when administered to humans or animals, has therapeutic properties, producing active ingredients capable of restoring body homeostasis (Lopes et al., 2005).

The use of medicinal plants for the treatment of diseases has historical records dating from 4.000 BC, says Duarte (2006), travelling from the first existing medical record in the Pennsylvania Museum, from 2,100 BC, through the Egyptian manuscript "Ebers Papyrus" from 1.500 B.C, with 811 prescriptions and 700 drugs registered, and the first Chinese text on medicinal plants, from 500 B.C, until getting in the present day, where their use increases exponentially every day.

According to Silva et al. (2015), the knowledge generated about the therapeutic properties of plants is passed from generation to generation and, therefore, becomes an indispensable instrument for the pharmaceutical industry, in the elaboration of new medicines and new prototype molecules, for example.

Cenostigma gardnerianum Tul., popularly known as "caneleiro" or "canela-de-velho", is a species belonging to the genus Cenostigma Tul. and widely distributed in the Northeast and can be found in the regions of the Brazilian Caatinga and Cerrado (savannah). There are reports on the use of leaves, flowers and bark of the stem of species of this genus as

spasmolytic, in addition to antiulcerogenic, anti-inflammatory, hepatoprotective and antibacterial activities verified by their leaf extract (Sousa et al, 2007).

In an experiment with *C. gardnerianum*, Alves et al. (2012) isolated bergenin from the bark extract of this species and confirmed the antinociceptive activity of this substance in an abdominal contortion test induced by 0.8% acetic acid in mice. Previous *in vivo* pharmacological studies had already shown the antiasthmatic, antitussive, anti-inflammatory, and antifungal potential of bergenin, as well as its anti-HIV and anti-hepatotoxic action in *in vitro* tests (Ye et al., 2004).

The use of this and other medicinal plants is often done by people who have difficult access to medical and pharmaceutical care, in which this practice tends to become their only available resource (Silva et al, 2015). This indiscriminate use often leads not to the alleviation of symptoms or cure of the illnesses, but to serious side effects/toxic and even to the death of those who use them.

Due to this wide use, the evaluation of the cytotoxic and genotoxic potential of these plants is necessary, contributing to their knowledge and consequent safe and effective use. This evaluation can be done initially with the *Allium cepa* test. This is one of the most efficient tests, because of its low cost and easy handling, as well as the rapid growth of meristem and abundant number of cells in division (Silva et al., 2015).

This *in vivo* bioassay has good acceptance, for the onion roots are in direct contact with the infusions, thus allowing the study of different concentrations of the substance examined (Bagatini et al., 2007). Its toxicity can be evaluated through root growth, the cytotoxicity through the Mitotic Index (MI) calculation and the mutagenicity/genotoxicity through the observation and quantification of micronuclei and chromosomal aberrations (Figueiredo, 2014).

Therefore, the objective of this research was to study the toxic, cytotoxic, and genotoxic potentials of stem bark ethanolic extract of *Cenostigma gardnerianum* Tul. (Caneleiro) by means of the *in vivo* test of *Allium cepa*.

2. Methodology

2.1 Scope of the research

The present research is in the experimental scope, characterizing a qualitative and quantitative analysis of meristem cells of *Allium cepa* roots.

2.2 Sample acquisition and extract preparation

The stem bark of *C. gardnerianum* Tul. were collected in November 2019 in urban environment, in the neighborhood Morada do Sol, located in the city of Teresina -PI. Then there was the production of exsiccates, followed by the deposit in the Graziela Barroso Herbarium of the Universidade Federal do Piauí (UFPI) to confirm the species, being under the registration number 32111.

The bark samples collected were sent to the biochemistry laboratory of the Centro Universitário UniFacid | Wyden, where they were dried at room temperature and protected from sunlight for 10 days, for total loss of moisture, further grinded to a larger contact surface. The crushed sample and the ethanol solvent (P.A ethyl alcohol) were added in a 1,000ml beaker, being left in contact for 14 days, with discontinue agitations every 24 hours, in order to avoid saturation.

The ethanol solution was then filtered on filter paper to separate the solid part and the resulting solution. Finally, the ethanol was evaporated in an evaporator by which the pure dry extract was obtained.

The calculations required were performed to prepare the dilutions, obtaining concentrations of 0.5 mg/ml (group A), 1.0 mg/ml (group B), 2.0 mg/ml (group C) and 5.0 mg/ml (group D), all diluted in 250 ml of distilled water, in addition to the negative control (dechlorinated water), positive control (dimethylsulfoxide - DMSO) and blank (distilled water).

2.3 Test on *Allium cepa*

The acquisition of the *Allium cepa* specimens necessary for the bioassay was made in the city of Teresina-PI in a popular mark of the Piçarreira Neighborhood, having as inclusion criteria small, healthy, same origin and non-germinated onions.

The bulbs had their tips peeled and were submerged in water for a period of 2 hours to eliminate any impurities that could compromise the subsequent root growth (Neto, 2011). Then, 10 replicates were used for each extract concentration, including the blank group, the negative and positive controls, placing the bulbs to germinate with the lower part in contact with the solutions in vials for seven days, at room temperature and protected from any light.

After the established time, the roots of all bulbs were measured with the aid of a ruler, disregarding the noticeably short or long ones, and the average length for toxicity evaluation was calculated. Then, the meristems were collected with the aid of a scalpel and placed in eppendorfs containing Carnoy fixative solution for 24 hours, followed by immersion in 70% ethanol and storage in the refrigerator until the moment the slides were made.

At the time of the slide's preparation, using forceps, a root of each eppendorf was removed and placed on a watch glass, in which distilled water was added. After 10 minutes, the roots were transferred to another clock glass, in which drops of the acetic carmine dye 2% were added – until the superposition of the roots -- and warmed up in a Bunsen Burner until the appearance of small clouds of smoke, to obtain good penetration of the dye in the roots.

In continuity, the root tips were transferred to histological slides, in which the coifs (apical portion of the root) were separated from the meristem regions and discarded, leaving only the meristems for analysis. A drop of glacial acetic acid was then applied to each root to obtain a good crushing and a coverslip was placed on each slide.

The squash technique of the roots was then made with the thumb finger, applying enough pressure to spread the cells and keep the coverslip intact. The ready-made slides were taken under the microscope, in which 100x lens and 10x eyepiece (1000x magnification) were used.

The analysis of 1000 cells per slide was performed and the cytotoxicity was investigated by calculating the Mitotic Index (MI), in which the number of cells in mitosis for each concentration was divided (prophase + metaphase + anaphase + telophase) by the total number of cells (interphase + cells in division), multiplying all this by 100 (Pires et al., 2001). In addition, micronucleus research and counting in meristem cells was performed for genotoxicity analysis, with also 1000 cells per slide

2.4 Statistical Analysis

The statistical and mean analyses of the data were made through the Analysis of Variance (ANOVA) and Tukey Test, using the GraphPad Prism software (8.2.1 Version) and and considering as statistically significant values $*p \leq 0.05$.

3. Results and Discussion

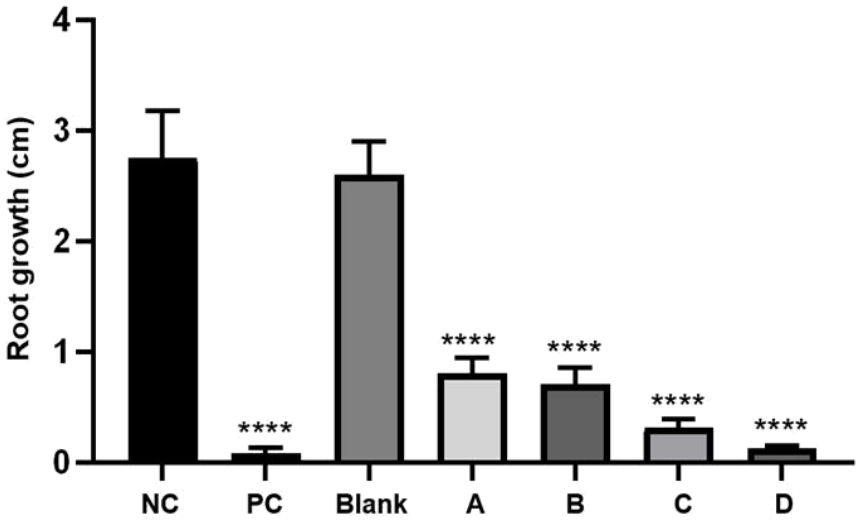
Medicinal plants, important components of biodiversity, are constantly used in the manufacture of home remedies in traditional communities, being essential raw material to produce herbal medicine and new therapeutics (Leo et al., 2007).

The importance of the use of the *Allium cepa in vivo* bioassay in the study of the genotoxic potential of plant extracts and medicinal infusions is highlighted by several research and experiments (Fachinetto et al, 2007). In this research, the ethanolic extract of *Cenostigma gardnerianum* Tul. has investigated its toxic, cytotoxic, and genotoxic potential in a pioneering study using the *Allium cepa* test system.

3.1 Toxicity

The toxic evaluation of *C. gardnerianum Tul.* was performed based on the comparison of root growth means of the concentrations used (groups A, B, C and D) and negative control, discarding exceptionally long roots. Figure 1 shows that in the negative control group (NC), the mean growth was 2.75 ± 0.75 cm, while group A showed a growth of 0.81 ± 0.19 cm, group B 0.71 ± 0.29 cm, group C 0.31 ± 0.19 cm, and group D 0.11 ± 0.08 cm. Blank group (distilled water) and positive control (Dimethylsulfoxide) are also shown in Figure 1.

Figure 1: Graphical representation of the root growth mean of *Allium cepa* in the different concentrations of the *Cenostigma gardnerianum* Tul. bark extract.



NC = Negative Control. PC = Positive Control. ****Highly significant values ($p<0.0001$) compared to negative control (ANOVA - Tukey Test). Source: Authors.

From Figure 1**,** it is noteworthy that the extract concentrations used (A, B, C, D) influenced and impaired root growth to a high degree, when compared to the growth in the negative control bulbs (NC). This difference varied according to the dosage, showing that the higher the concentration of the extract used, the more affected was the rooting growth in *Allium cepa* and, clearly, the smaller the root size, as seen in group D, in which root development, 0.12 ± 0.08, was almost equal to the positive control (PC), 0.08 ± 0.12 cm.

In addition, it was observed that the Blank treatment (distilled water) was not impaired in concerning to developing the roots (2.6 ± 0.4 cm), which means that the distilled water used in dilutions of the extract concentrations had no influence on the inhibition and low growth rate of the roots, thus showing the presence of toxicity of *Cenostigma gardnerianum* Tul. at the concentrations used, with highly significant values (****) compared to negative control (NC), mainly at 2,0 mg/ml and 5,0 mg/ml.

3.2 Citotoxicity

The cytotoxicity of the extract was evaluated by calculating the Mitotic Index (MI), given in percentage, after the count and analysis of the cell cycle of 1000 cells per bulb. Group D (5mg/ml) was discarded, for its roots were shorter than the established 0,2 cm lower limit.

Table 1: Results of the Mitotic Index calculation of the meristems exposed to concentrations of 0.5, 1.0, 2.0 and 5.0 mg/ml of *Cenostigma gardnerianum* Tul. bark extract.

Groups	MI ± SD
NC	7,5 ± 1,40
Blank	7,1 ± 1,32
A	4,4 ± 1,12
B	3,5 ± 0,86*
C	1,2 ± 1,43*

NC = Negative Control. MI = Mitotic Index. SD = Standard Deviation. *Significant values ($p<0.0005$) compared to negative control (ANOVA - Tukey Test). Source: Authors.

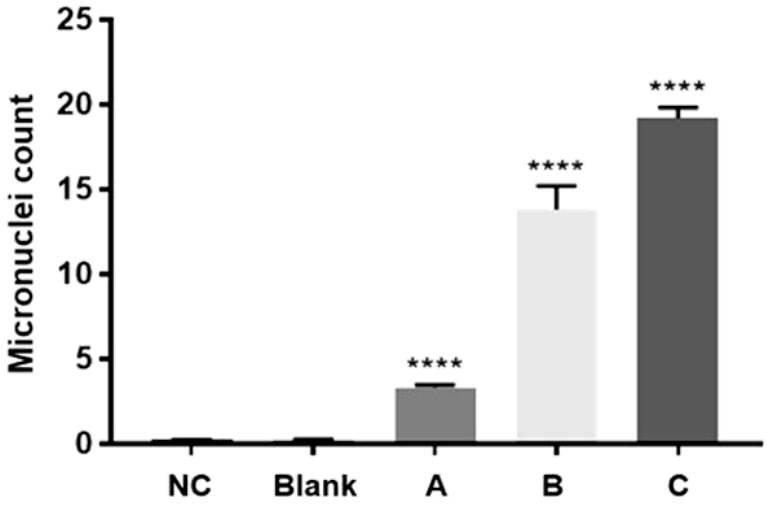
The results obtained in Table 1 varied according to the concentrations used, showing a reduction of the Mitotic Index (MI) in groups A (0.5mg/ml), B (1.0mg/ml) and C (2.0mg/ml) when compared to Negative Control, having greater statistical significance in the concentrations of groups B and C.

Leme & Marin-Morales (2009) comment that significant decays in the Mitotic Index compared to Negative Control may point to the existence of chemicals that negatively affect cell proliferation (mitosis) and may cause changes in the growth and development of exposed organisms. Therefore, from 1mg/ml, the extract of *C. gardnerianum* Tul. has shown a high degree of cytotoxicity, making it unsafe for consumption at higher concentrations.

3.3 Genotoxicity

As highlighted by Silva et al. (2020), studies on genotoxic effects contribute to the determination of toxicity of products with pharmacological potential, evaluating the capacity of a compound in damage human genetic material (DNA). Figure 2 presents a graphic representation of the micronuclei mean found in the meristems of groups A, B and C, as well as the Negative Control (NC), for comparison and analysis on the genotoxic potential of *C. gardnerianum* Tul.

Figure 2: Incidence of micronuclei in every 1000 meristem cells analyzed in different concentrations of the ethanolic extract of *Cenostigma gardnerianum* Tul.



NC = Negative Control. ****Highly significant values ($p<0.0001$) compared to negative control (ANOVA - Tukey Test). Source: Authors.

The data obtained in the graphic of Figure 2 shows an increase in the frequency of micronuclei in groups A (3.2 ± 0.39), B (12.2 ± 0.29) and C (20 ± 0.19) when compared to the results found in the negative control (0.2 ± 0.25), being in greater evidence groups B and C. Furthermore, the Blank group (distilled water) had a low presence of micronuclei, showing that the distilled water used for the extract dilutions had no role in the higher frequency of micronuclei found in the concentrations used in the study.

Micronuclei consist of small amounts of DNA located in the cellular cytoplasm, arising when chromosome fragments without centromere, chromatids or whole chromosomes are not incorporated within the nucleus of the daughter cell during mitosis, may appear more than once in each cell (Schmid, 1975).

Therefore, the bark extract of *C. gardnerianum* Tul. showed great genotoxic potential in all concentrations used, with values of high statistical significance (****), evidencing risks to those who make use of this plant.

4. Conclusion

From the results obtained in this research with the *Allium cepa* bioassay, it is concluded that the ethanolic extract from the bark of the stem of *Cenostigma gardnerianum* Tul. (Caneleiro) has high toxic, cytotoxic and genotoxic potential, with evidence in the concentrations of groups C and D.

Therefore, the use of this plant species is not safe and, consequently, not recommended, and should be used with caution by the population. However, further studies on this plant are necessary to confirm its toxicity, cytotoxicity, genotoxicity, and its risk for popular use, as well as its use by the pharmaceutical industry.

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