Cytogenotoxicity of aqueous extracts of *Vochysia divergens* Pohl (Vochysiaceae) Bark

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Recebido em 17 de junho de 2024.
Aceito em 13 de setembro de 2025.
Publicado em 30 de dezembro de 2025.

Resumo

Vochysia divergens Pohl, conhecida popularmente no Brasil como cambará, é usada para tratamento de infecções, asma e distúrbios digestivos. Apesar de ser muito utilizada no pantanal, não há estudos sobre sua citogenotoxicidade que comprovem a segurança de seu uso. Nesse sentido, este trabalho objetivou avaliar o potencial tóxico, citotóxico e genotóxico de extratos aquosos da casca de *V. divergens*, por meio do teste *Allium cepa* L. A avaliação foi realizada a partir de dois tipos de extratos aquosos, infuso e decocto, com cinco concentrações distintas (12,5 mg mL^{-1}, 25 mg mL^{-1}, 50 mg mL^{-1}, 100 mg mL^{-1} e 200 mg mL^{-1}) e dois controles, um negativo (água destilada) e um positivo (sulfato de cobre penta-hidratado). Foram avaliados o crescimento do sistema radicular, índice mitótico e a frequência de alterações cromossômicas e/ou anormalidades nas fases da divisão celular. Os dois extratos aquosos avaliados apresentam potencial tóxico, citotóxico e genotóxico sobre as células do meristema apical de *A. cepa*, com exceção da concentração de 200 mg mL^{-1} do extrato tipo infuso, a qual não apresentou aumento ou inibição da mitose. O extrato aquoso infuso foi o que causou menos alterações na divisão celular, sendo o mais recomendado para utilização como fitoterápico.

Palavras-chave: Cambará. Plantas medicinais. Fitoterapia. Pantanal.

Citogenotoxicidad de extractos acuosos de corteza de *Vochysia divergens* Pohl (Vochysiaceae)

Resumen

Vochysia divergens Pohl, conocida popularmente en Brasil como cambará, se utiliza para tratar infecciones, asma y trastornos digestivos. A pesar de ser ampliamente utilizado en el Pantanal, no existen estudios sobre su citogenotoxicidad que demuestren la seguridad de su uso. En este sentido, este trabajo tuvo como objetivo evaluar el potencial tóxico, citotóxico y genotóxico de extractos acuosos de la corteza de *V. divergens*, mediante la prueba de *Allium cepa* L. La evaluación se realizó utilizando dos tipos de extractos acuosos, infusión y decocción. con cinco concentraciones diferentes (12,5 mg mL^{-1}, 25 mg mL^{-1}, 50 mg mL^{-1}, 100 mg mL^{-1} y 200 mg mL^{-1}) y dos controles, uno negativo (agua destilada) y otro positivo (cobre sulfato pentahidratado). Se evaluó el crecimiento del sistema radicular, el índice mitótico y la frecuencia de cambios cromosómicos y/o anormalidades en las fases de división celular. Los dos extractos acuosos evaluados tienen potencial tóxico, citotóxico y genotóxico sobre las células del meristemo apical de *A. cepa*, a excepción de la concentración de 200 mg mL^{-1} del extracto tipo infusión, que no mostró un aumento o inhibición de la mitosis. El extracto acuoso infundido provocó los menores cambios en la división celular, siendo el más recomendado para su uso como medicina herbaria.

Palabras clave: Cambará. Plantas medicinales. Fitoterapia. Pantanal.

Cytogenotoxicity of aqueous extracts of *Vochysia divergens* Pohl (Vochysiaceae) Bark

Abstract

Vochysia divergens Pohl, popularly known in Brazil as "cambará", is used to treat infections, asthma and digestive disorders. Despite being widely used in the Pantanal, there are no studies on its cytogenotoxicity that prove the safety of its use. In this sense, this work aimed to evaluate the toxic, cytotoxic and genotoxic potential of aqueous extracts from the bark of *V. divergens*, using the *Allium cepa* L test. The evaluation was carried out using two types of aqueous extracts, infusion and decoction, with five different concentrations (12.5 mg mL^{-1}, 25 mg mL^{-1}, 50 mg mL^{-1}, 100 mg mL^{-1} and 200 mg mL^{-1}) and two controls, one negative (distilled water) and one positive (copper sulfate pentahydrate). The growth of the root system, mitotic index and the frequency of chromosomal alterations and/or abnormalities in the phases of cell division were also evaluated. The two aqueous extracts evaluated have toxic, cytotoxic and genotoxic potential in the cells of the apical meristem of *A. cepa*, with the exception of the concentration of 200 mg mL^{-1} of the infusion-type extract, which did not show an increase or inhibition of the mitosis. The infused aqueous extract caused the least changes in cell division and is the most recommended for use as an herbal medicine.

Keywords: Cambará. Medicinal plants. Phytotherapy. Pantanal.

Introduction

Phytotherapy is the use of medicines obtained from plants or herbs to treat or prevent health conditions. The use of plants for medicinal purposes is a very ancient practice and, for many communities, it symbolizes the only therapeutic means used to treat various illnesses (Viana and Ramos 2019).

The use of plant species for the prevention and treatment of diseases has been transmitted over generations, and the traditional formula is passed on based solely on its beneficial effects, without knowledge of the properties, constitution or chemical composition of these medicinal plants, which in some cases can cause adverse effects (Souza et al. 2020; Pedroso and Pires 2021). This makes it necessary to carry out studies that validate this traditional knowledge by evaluating the risks and benefits so that these medicinal properties present in certain plant species can be used appropriately (Pedroso and Pires 2021).

Among the various medicinal plants used as herbal medicine is *Vochysia divergens* Pohl, popularly known in Brazil as "cambará", which is used in folk medicine to treat infections, asthma and digestive disorders (Hess et al. 1995; Guarim Neto et al. 2006). Using its leaves, tea is prepared for the treatment of asthma and flu, and tea made from the bark is used to treat ulcers and in known for its anti-inflammatory properties (Guarim Neto et al. 2006; Jesus et al. 2009). A syrup can also be made from the bark for the treatment of appendicitis. (Guarim Neto et al. 2006).

Teas are widely used for medicinal purposes; therefore, the number of studies that ensure the safety of these forms of treatment has been increasing. Most aim to evaluate the possible risks and effects that these plants can cause to DNA (Cabaravdic 2010). The *Allium cepa* L. test is one of several study protocols that exist for this purpose, as it allows one to evaluate the toxic and cytotoxic effects of aqueous plant extracts through the reduction of root growth and the reduction of the mitotic index, respectively (Almeida et al. 2019), as well as genotoxic effects through analysis of the presence of micronuclei or anaphase-telophase abnormalities (Fiskesjö and Levan 1994).

This test is considered to be a bioindicator of the possible effects of chemicals, plant extracts and complex mixtures on cellular activity (Hister et al. 2017), and is recommended for evaluating levels of mutagenicity and toxicity in medicinal plants (Bagatini et al. 2007). Medicinal plants, even when used in the form of aqueous extracts, may contain mutagenic or toxic substances and, therefore, pose health risks (Bochner et al. 2012).

The use of the *A. cepa* test for studies with medicinal plants is advantageous as it is low cost, and has high sensitivity and reliability, in addition to being able to be correlated with other tests, including those carried out on mammals (Grant 1982; Fiskesjö 1985; Leme and Marin-Morales 2009).

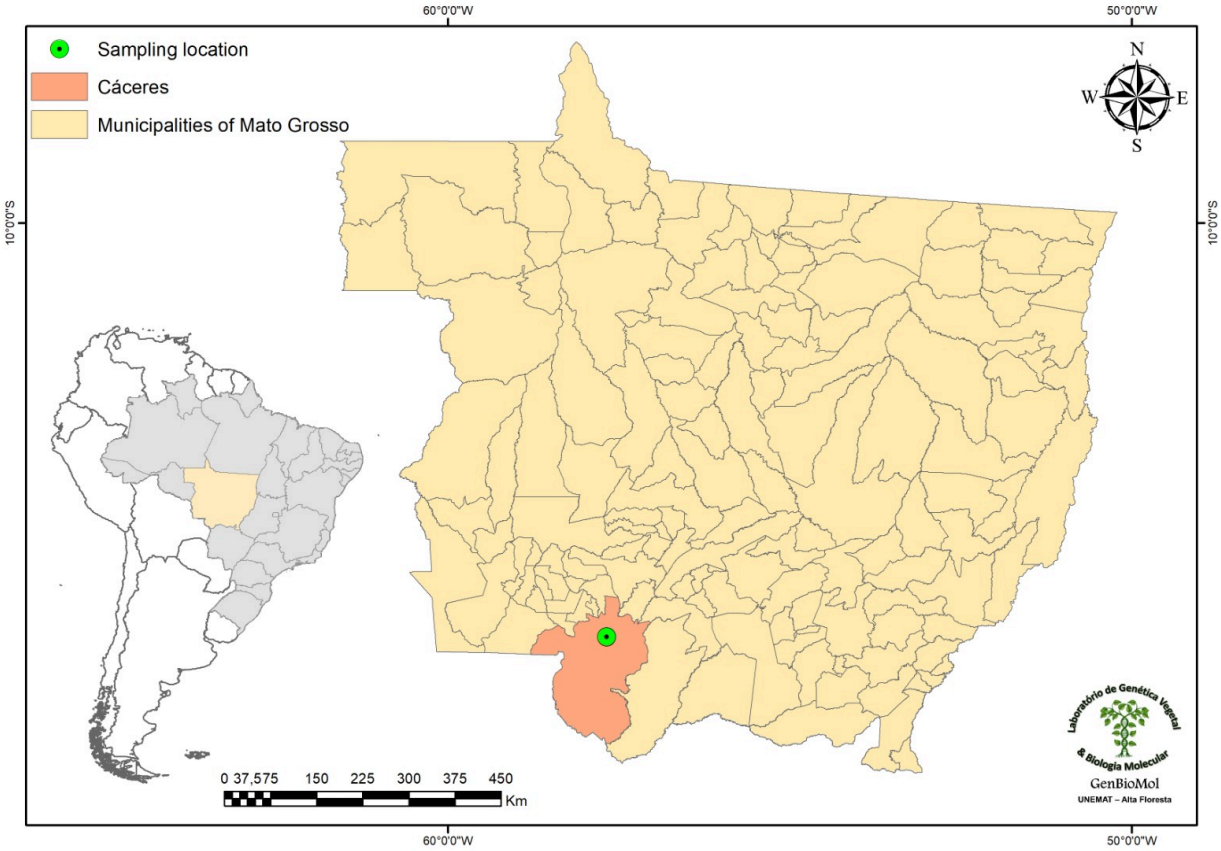
Considering that cambará is widely used by the populations that reside in the Pantanal for medicinal purposes and that there is a need for more studies to verify its safety, this work aimed to evaluate the toxic, cytotoxic and genotoxic potential of aqueous extracts from the bark of *V. divergens* via the *A. cepa* test.

Materials and Methods

Sampling location

Samples of stem bark were collected from adult individuals of *V. divergens* in Baía do Malheiros, located in the municipality of Cáceres, Mato Grosso (Figure 1). The samples were dried at room temperature and taken to the Plant Genetics and Molecular Biology Laboratory (GenBioMol) at UNEMAT, Alta Floresta University Campus, to carry out the *A. cepa* test.

Figure 1. Map showing the sampling location of V. divergens bark in Baía do Malheiros, in the municipality of Cáceres, Mato Grosso, Brazil. Source: Authors.



Aqueous extracts

Two types of aqueous extracts were prepared using the bark of *V. divergens*, infusion (AEI) and decoction (AED), following the guidelines of the Resolution of the Collegiate Board (RDC) No. 26/2014 (BRASIL 2014). For each of the extracts, five concentrations were evaluated (12.5 mg mL^{-1}, 25 mg mL^{-1}, 50 mg mL^{-1}, 100 mg mL^{-1} and 200 mg mL^{-1}). To obtain the extract of the highest concentration, 1,500 mL of distilled water and 300 g of *V. divergens* stem bark were used. The remaining concentrations were obtained from the dilution of the most concentrated extract.

The infused extract was obtained by heating water to boiling point (100 ºC) and pouring it over the stem bark. The container was closed and left to stand for 10 minutes and then filtered and diluted to obtain the five concentrations.

For the decoction-type extract, the samples of stem bark were placed in a container with water and then brought to the boil for five minutes, counted after the start of boiling. Upon reaching room temperature, the extract was filtered and diluted to the concentrations to be evaluated.

Allium cepa **L. test**

The *A. cepa* test was performed according to the methodology proposed by Fiskesjö (1985) and Babich et al. (1997). The cataphylls and dry roots were carefully removed from the bulbs, which had been previously placed in distilled water for root emission and, 48 h later, transferred to the aqueous extracts to be tested.

The *A. cepa* bulbs were subjected to five concentrations of AEI and AED obtained from the bark of *V. divergens*. Distilled water was considered as a concentration of 0 mg mL^{-1} and was used as the negative control (NC) and copper sulfate pentahydrate ($CuSO_4.5H_2O$) at a concentration of 0.64 mg mL^{-1} as the positive control (PC). Copper sulfate pentahydrate is a toxic chemical compound that causes damage to cellular structure and is cited in the literature for cytogenotoxicity and mutagenicity bioassays using *Allium cepa* (Cardoso et al. 2022; Soares et al. 2022; Messias et al. 2021).

The experiment was conducted in a completely randomized design (CRD) and the bulbs were organized in a 2 x 5 + 2 scheme, with two types of extract (AEI, AED), five concentrations and two control treatments (NC and PC) with 10 replications each. The bulbs were kept in a BOD germination chamber, where they remained at a controlled temperature (25 °C ± 2) and in the absence of light for 48 hours, according to the methodology proposed by Babich et al. (1997). After this period, the bulbs were removed from the germination chamber to carry out cytogenotoxicity assessments.

Assessment of toxic, cytotoxic and genotoxic potential

To evaluate the toxic potential, root system growth (RSG) was measured using a digital caliper (Mitutoyo). For this evaluation, 20 roots from each concentration of AEI, AED, and the NC and PC were randomly selected, totaling 100 roots from each extract and 20 from each control.

The cytotoxic potential was obtained using the mitotic index (MI) and the genotoxic potential was evaluated by verifying of the presence and frequency of chromosomal alterations and/or abnormalities in the phases of cell division.

For the analysis of the cytotoxic and genotoxic potential, the roots of *A. cepa* bulbs were collected from each concentration of both extracts. The material was placed in a fixing solution (3:1, ethanol: acetic acid (v/v)) for 24 hours at room temperature, then transferred to 70% ethanol and kept under refrigeration (± 4 °C) until the preparation of the slides.

The MI was obtained through equation "A" proposed by Pires et al. (2001), the frequency of chromosomal alteration f(A) was calculated using equation "B", while the chromosomal alteration index (AI) was obtained using the equation "C":

$$\text{A)}\ \ MI = \frac{\text{number of cells in mitosis}}{\text{number of cells observed}} \times 100$$

$$\text{B)}\ \ f(A) = \frac{\text{number of cells with alterations}}{\text{number of dividing cells}} \times 100$$

$$\text{C)}\ \ AI = \frac{\text{number of cells with alterations}}{\text{total number of cells observed}} \times 100$$

Slide preparation and cell counting

For the preparation of the slides, the methodology proposed by Souza et al. (2005) was used. The radicles were washed in distilled water for five minutes, hydrolyzed in 1N hydrochloric acid (HCl) for 15 minutes and again washed in distilled water for five minutes. For the preparation of the slides, the apical meristem of one to three roots was used, and this was stained with approximately 100 µL of aceto-orcein 2%. With the aid of a glass rod, the meristem was lightly pressed and subsequently covered with a coverslip.

Eight slides per concentration of each of the extracts evaluated were prepared, as well as for the NC and PC. A total of 250 cells per slide were analyzed, totaling 2,000 cells for each treatment and 10,000 cells per extract. The slides were observed under an optical microscope (BIOVAL), at a magnification of 400x, using the scanning method, with the number of cells in each phase of the cell cycle (interphase, prophase, metaphase, anaphase, telophase) being recorded for each slide, as well as when changes in any of them were present. The photographic record was performed using a CMOS color digital camera (1.3 MP) coupled to the microscope and for image capture, and editing were performed in the TSview software.

Data analysis

The Shapiro-Wilk and Levene tests were performed to verify the normality and homogeneity of the data, respectively. The root length data was transformed into square root. Then, the data were submitted to analysis of variance (ANOVA) and the significant means were compared via Tukey's Ttest at the level of 5% probability. Statistical analyses were carried out with the aid of the R program (RStudio, 2021).

Results and Discussion

Toxic effect of aqueous extracts of *V. divergens*

The analysis of variance (ANOVA) revealed a significant interaction between the concentrations and aqueous extracts of the stem bark of *V. divergens* on the growth of the root system (Table 1).

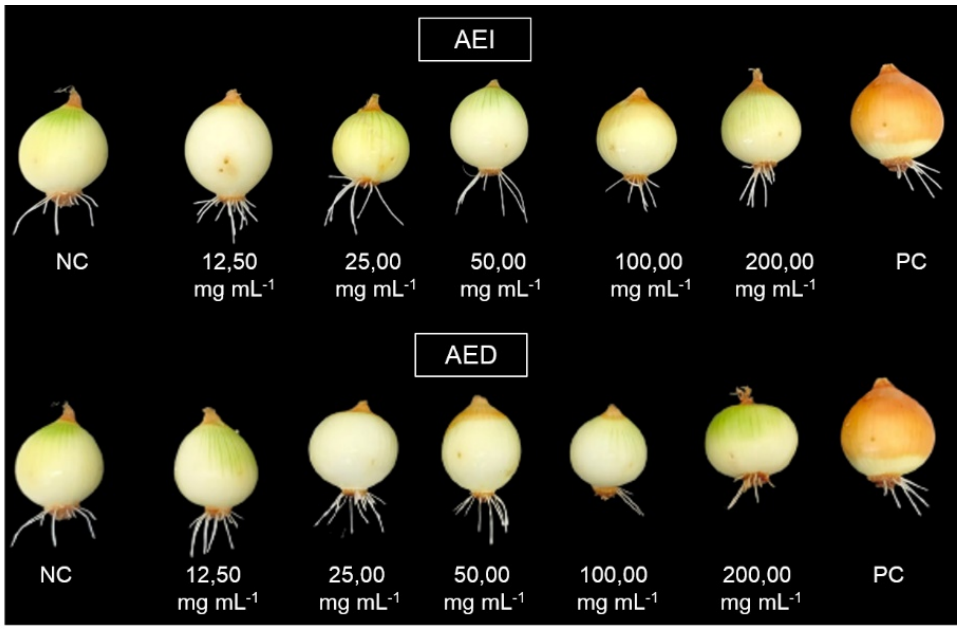
Table 1. Summary of the analysis of variance (ANOVA) for the effect of Vochysia divergens extracts and their concentrations on Allium cepa root length

Source of variation	df	MSR
Extract	5	20.72**
Concentration	1	6.03**
Extract x concentration	5	3.61**
Mean	-	3.99

df: degrees of freedom, MSR: mean square residual, ** significant at 1% probability. Standard deviation 0.86.

It was possible to observe variations in root lengths according to the concentrations of the extracts (Figure 2).

Figure 2. Root length of Allium cepa bulbs after exposure to different concentrations (mg/mL) of aqueous extracts of the stem bark of Vochysia divergens. Aqueous extract of the infusion type (AEI) and aqueous extract of the decoction type (AED). NC: negative control, distilled water (0 mg mL^{-1}) and PC: positive control, 0.64 mg mL^{-1} copper sulfate pentahydrate.



The mean root length at all concentrations of the AEI differed statistically from the NC, and stimulated the RSG; whereas, for the AED, the concentrations of 25.00 and 50.00 mg mL^{-1} stimulated the RSG, and the concentrations of 12.50; 100.00 and 200.00 mg mL^{-1} showed a similar response to the NC (Table 2). Most of the concentrations tested stimulated RSG. For Bezerra et al. (2016), the growth of the root system is linked to the intense mitotic activity that occurs in the meristematic tissue. Thus, the concentrations tested showed a toxic effect in both extracts with the exception of the concentrations of 12.50; 100.00 and 200.00 mg mL^{-1} of AED.

In this study, in addition to the increase in RSG, a change in coloration at the tips of some of the roots was observed. According to Fiskesjö (1985), these observations are macroscopic parameters used as indicators of toxicity in the *A. cepa* test.

Table 2. Test of the means of root length of Allium cepa subjected to different concentrations of aqueous extracts of Vochysia divergens stem bark

Concentration	Extract	
	Infusion	Decoction
00.00 mg mL^{-1}	11.69 bA	11.69 bcA
12.50 mg mL^{-1}	21.24 aA	16.68 abB
25.00 mg mL^{-1}	18.86 aA	18.22 aA
50.00 mg mL^{-1}	21.21 aA	18.09 aA
100.00 mg mL^{-1}	21.18 aA	12.20 bcB
200.00 mg mL^{-1}	19.97 aA	8.94 cB

Means followed by the same uppercase letters in the row and lowercase letters in the column do not differ statistically from each other according to the Tukey test, at 5% probability. 0.00 mg mL^{-1} = NC (negative control).

Cytotoxic effect of aqueous extracts of *V. divergens*

For the MI, analysis of variance (ANOVA) revealed a significant interaction between the concentrations and aqueous extracts of *V. divergens* stem bark (Table 3).

Table 3. Summary of the analysis of variance (ANOVA) for the effect of Vochysia divergens stem bark extracts and their concentrations on the mitotic index of Allium cepa meristematic cells

Source of variation	df	MSR
Extract	1	345.00**
Concentration	5	833.20**
Extract x concentration	5	77.6**
Mean	-	28.72

df: degree of freedom, MSR: mean square residual, ** significant at 1% probability. Standard deviation 7.99.

For the AEI, all the concentrations stimulated mitosis, with the exception of 200.00 mg mL^{-1}, which showed a similar response to the NC. However, for the AED, all the concentrations stimulated mitosis, with the exception of 200.00 mg mL^{-1}, which inhibited mitosis (Table 4). According to Smaka-Kinc (1996), the reduction in mitosis is a reliable way to determine the presence of cytotoxic substances. On the other hand, the increase in mitosis indicates that there was an induction of cell division, which can be considered harmful to cells, since it can lead to the appearance of tumors in living beings (Grippa et al. 2010). The MI can be used as an indicator of an adequate cell proliferation, and the cytotoxic effects of certain substances can be evidenced by the increase or decrease in the MI (Leme and Marin-Morales 2009; Tedesco and Laughinghouse 2012).

Therefore, the increase and decrease in mitosis observed in the present study indicate the cytotoxic potential of the aqueous extracts of *V. divergens* with the exception of the concentration of 200.00 mg mL^{-1} of the AEI.

Table 4. Test of the means for the mitotic index (MI) of Allium cepa meristematic cells subjected to different concentrations of aqueous extracts of Vochysia divergens stem bark

Concentration	Extract	
	Infusion	Decoction
00.00 mg mL^{-1}	21.85 cA	21.85 cA
12.50 mg mL^{-1}	36.85 aA	33.90 aA
25.00 mg mL^{-1}	38.45 aA	28.10 bB
50.00 mg mL^{-1}	35.85 abA	28.60 abB
100.00 mg mL^{-1}	31.35 bA	32.75 abA
200.00 mg mL^{-1}	19.35 cA	15.75 dA

Means followed by the same uppercase letters in the row and lowercase letters in the column do not differ statistically from each other according to the Tukey test, at 5% probability. 0.00 mg mL^{-1} = NC (negative control).

Genotoxic effect of aqueous extracts of *V. divergens*

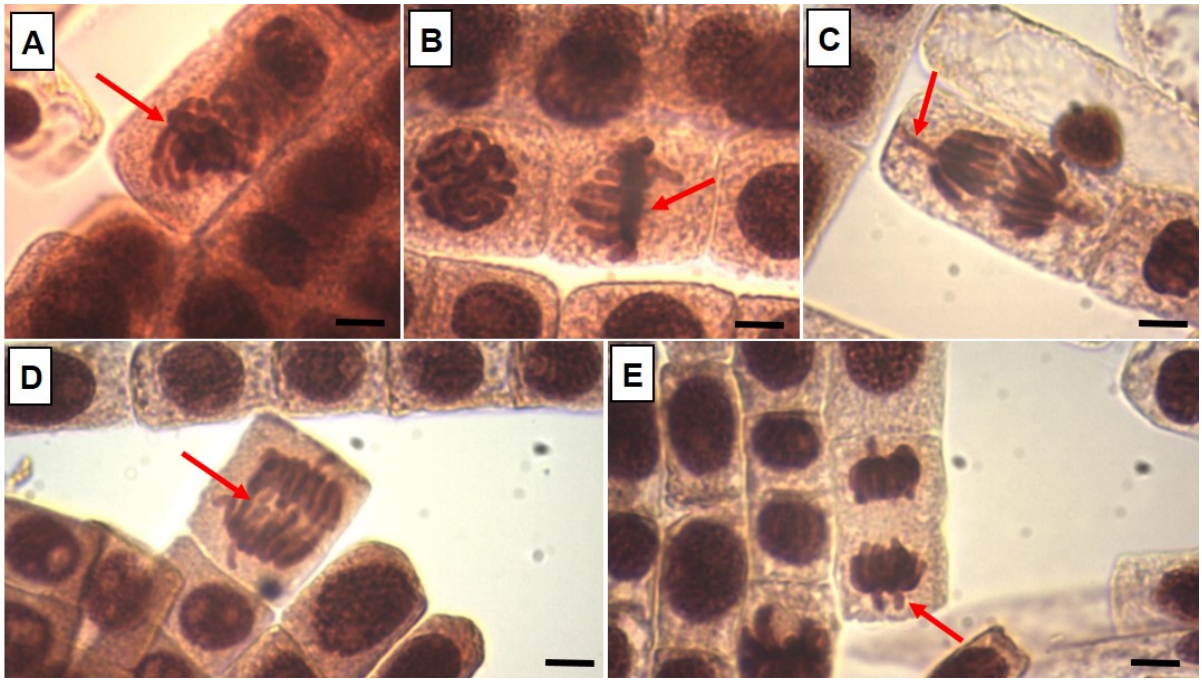
Chromosomal alterations were observed in all concentrations (Table 5) and irregular metaphase and anaphase with isolated chromosome were the alterations that appeared most frequently in the concentrations tested. The changes can be seen in Figure 5.

Table 5. Chromosomal alterations observed during cell division of Allium cepa cells submitted to aqueous extracts of Vochysia divergens stem bark

				AEI				
Concentrations (mg mL^{-1})	**IP**	**IM**	**EAC**	**ACB**	**ETC**	**TA**	**f(A)**	**AI**
12.50	12	64	67	0	22	165	22.39%	8.25%
25.00	13	64	61	1	10	149	19.38%	7.45%
50.00	14	41	63	1	8	127	17.71%	6.35%
100.00	3	20	6	1	0	30	4.78%	1.50%
200.00	8	1	1	0	0	10	2.58%	0.50%
				AED				
12.50	0	28	43	0	4	75	11.06%	3.75%
25.00	0	46	44	1	22	113	20.11%	5.65%
50.00	1	55	51	0	9	116	20.28%	5.80%
100.00	5	65	56	0	13	139	21.22%	6.95%
200.00	0	6	12	0	0	18	5.71%	0.90%
NC	0	0	0	0	0	0	0.00%	0.00%
PC	1	15	8	1	9	34	29.06%	1.70%

AEI= aqueous extract infusion; AED= aqueous extract decoction; NC= negative control; PC= positive control; IP= irregular prophase; IM= irregular metaphase; EAC= early anaphase chromosome; ACB= anaphase with chromosomal bridge; ETC= early telophase chromosome; TA= total cells with alteration; f(A)= frequency of chromosomal alteration; AI= chromosomal alteration index.

Figure 5. Chromosomal changes observed during cell division of Allium cepa roots. (A) irregular prophase, (B) irregular metaphase, (C) early anaphase chromosome, (D) anaphase with chromosomal bridge, (E) early telophase chromosome. 20 µm bar.



All the concentrations tested showed genotoxic potential, with the percentage of chromosomal alterations ranging from 2.28% to 22.39% (Table 5). For Russel (2002), chromosomal alterations are characterized by modifications in the chromosomal structure or in the total number of chromosomes, which can occur spontaneously or as a result of exposure to physical or chemical agents.

The AED was the extract that caused the greatest number of cellular alterations, despite being the most recommended extractive method for parts of plant with a rigid consistency, such as bark (Brasil 2014). This fact may be related to the gradual increase in water temperature during decoction, which favors the extraction of substances with different polarities (Manahan 2012). Due to a decrease in the initial temperature during the infusion period, the infusion ends up reducing the extraction of the compounds present in the plant material (Ramalho et al. 2013).

The results of this study revealed a cytogenotoxic effect of the aqueous extract of the bark of *V. divergens* on the roots of *A. cepa*. According to Galindo et al. (2012), toxic substances may not show acute effects on exposed organisms, but may cause tissue damage, genetic damage to somatic and germ cells, accumulation of contaminants, and neoplasms. Peron et al. (2009) indicate that alterations in cell division and genetic material can have severe consequences on human health.

Therefore, aqueous extracts that have cytogenotoxic potential in bioassays, such as the *A. cepa* test, can be indicate that the chemical factor tested may present a health risk (Belcavello et al. 2012; Carvalho et al. 2017).

Hess et al. (1995) studied the presence of compounds in the stem bark of *V. divergens* with biological and pharmacological activities, and described three chemical substances, β-sitosterol, betulinic acid and sericic acid, the latter being the main active constituent isolated, which has an antibacterial action. β-sitosterol has antimicrobial, anti-inflammatory, anticancer, angiogenic,

antioxidant, immunomodulatory, antidiabetic (Ambavade et al. 2014), antiulcer (Ling and Jones 1995) and gastroprotective activities (Navarrete et al. 2002). Betulinic acid also has anticancer and anti-inflammatory activities (Fulda 2009; Potze et al. 2014), in addition to antiviral, antibacterial, antimalarial and antitumor ones as well (Hsu et al. 2015). The fact that these chemicals have anti-inflammatory action may explain, in part, the popular use of this plant for the treatment of infectious diseases.

β-sitosterol exhibits toxicity (Ambavade et al. 2014) and betulinic acid is cytotoxic (Pisha et al. 1995; Tiwari et al. 2014; Luo et al. 2016). Despite the low toxic and cytotoxic potential of these chemicals, they may have contributed in some way to the results obtained in the present study. Further research is needed on the chemical composition of the *V. divergens* bark in order to check for other substances that may have a higher toxic, cytotoxic or genotoxic potential.

Despite the benefits, the chemical substances present in these secondary metabolites, known as active ingredients, can in certain quantities present toxic effects, i.e., they end up causing damage to the body instead of benefits (Hister 2021).

Conclusions

The aqueous extracts from the infusion and decoction of the bark of the stem of *V. divergens* in the concentrations tested showed a toxic and cytotoxic effect on *Allium cepa* cells, which was evidenced by the increase/inhibition of root system growth and mitotic index, respectively.

All the concentrations of both extracts (infusion and decoction) showed genotoxic potential that was proven by the presence of chromosomal alterations. The AEI was the extract that caused the least alterations in cell division and is the most recommended for use for preparing the herbal medicine since it presents a lower health risk.

This result provides basic data regarding the cytogenotoxicity of *V. divergens*; however, it is necessary to carry out further studies with other types of tests that verify its chemical substances that have toxic and mutagenic potential. This can contribute to the safe treatment of diseases treated by the species via folk medicine, as well as the production of drugs by the pharmaceutical industry.

Conflict of Interest: the authors declare that there are no conflicts of interest.

Thanks: To the Coordination for the Improvement of Higher Education Personnel (CAPES), for the financial support. To the State University of Mato Grosso (UNEMAT), the Postgraduate Program in Genetics and Plant Improvement (PGMP) and the Long-Term Ecological Research Program (PELD) – Pantanal for research support.

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