



Physicochemical characterization of different vegetative stages of *Ateleia glazioviana*

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[Caracterização físico-química de diferentes estágios vegetativos de *Ateleia glazioviana*]

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ABSTRACT

Ateleia glazioviana is an important poisonous plant in southern Brazil, responsible for important economic losses in livestock. The aim of this work was to identify the amount of potential toxic active components in different vegetative stages. Plant samples were collected at different maturity stages and identified for registration in the SISGEN system. An aqueous extract was obtained from the leaves to evaluate the predominant compounds present in the samples (200g/500mL). Chemical detection of toxic compounds was performed using gas chromatography. Subsequently, the most expressive chemical groups were analyzed and compared with structures of toxic potential, with phenolic/ketonic groups being found (spectral region between 4,600 and 4,700cm⁻¹), suggesting substances already identified as *Glaziovianin A* or *Rotenone*. The amount of toxic compound in fresh plant was determined to be 0.0013mg/kg. There was no difference in the quantity of the toxic compound in relation to the maturity degree of the plant or season. The results of toxicological tests for the degree of irritation and cellular damage were positive. Due to the structural similarities of these molecules, further analyses are necessary to characterize the compounds more accurately.

Keywords: gas chromatography, poisonous plant, ruminant, toxicology

RESUMO

A *Ateleia glazioviana* é uma planta tóxica importante no sul do Brasil, que ocasiona perdas econômicas em ruminantes. O objetivo deste estudo foi identificar possíveis componentes ativos tóxicos em diferentes estágios de maturação da planta. Um extrato aquoso foi obtido das folhas para avaliar os compostos predominantes nas amostras (200g/500mL). A detecção química de compostos tóxicos foi realizada utilizando-se o método de cromatografia gasosa. Posteriormente, os grupos químicos mais expressivos foram analisados e comparados com estruturas de potencial tóxico, sendo encontrados grupos fenólicos/cetônicos (região espectral entre 4.600 e 4.700Cm⁻¹), o que pode sugerir que sejam substâncias já identificadas como *Glazioviana A* e *Rotenona*. A quantidade de composto tóxico de planta fresca foi determinada em 0,0013mg/kg. Não houve diferença na quantidade do composto tóxico em relação ao grau de maturidade da planta ou à época do ano. Os testes toxicológicos para o grau de irritação e o dano celular apresentaram resultados positivos. Devido às similaridades estruturais dessas moléculas, são necessários estudos adicionais para detalhar os compostos com maior precisão.

Palavras-chave: cromatografia gasosa, planta tóxica, ruminante, toxicologia

INTRODUCTION

In Brazil, livestock farming for beef and milk production systems is carried out on pasture (native or planted), which occupy about 21.2%

of the land in Brazil (Síntese..., 2018). Although this grazing system has economic advantages, there is a potential risk for ingestion of poisonous plants. According to Pessoa *et al.* (2013) the number of toxic plants in 2013 was 131 species

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and 79 genera, and as indicated by these authors, the total amount is constantly increasing.

Since 1996, a disease that affects cattle has been observed, initially in the west of Santa Catarina and later in the northwest of Rio Grande do Sul state, Brazil. It is characterized by clinical manifestations related to the nervous system such as apathy, lethargy and blindness and to the cardiovascular system such as “sudden death” and chronic heart failure. According to Gava *et al.* (2001), both conditions are caused by the ingestion of *Ateleia glazioviana*, a poisonous plant.

Stigger *et al.* (2001) described the clinical and histopathological effects of poisoning on the myocardium of sheep. Almeida (2006) observed clinical signs of depression and incoordination resulting from *A. glazioviana* poisoning in sheep.

García *et al.* (2004) administered leaves of *A. glazioviana* Baill. to cows at different stages of gestation, and among the results obtained, abortions and cardiac fibrosis were observed. In the Rio Grande do Sul state, *A. glazioviana* represented 10.31% of poisoning in cattle between 1990 and 2005 (Rissi *et al.*, 2007).

Recently, Gava *et al.* (2021) described epidemiological data and pathological conditions related to spontaneous intoxication by *A. glazioviana* (leg. Papilionoideae) in sheep and goats in the western of Santa Catarina state. The mortality rate observed was 18%. According to Perosa *et al.* (2024), between 2013 and 2022, the number of diagnoses issued in Santa Catarina state due to *A. glazioviana* poisoning were 0.4% in ovine and 0.29% in bovine.

Despite several studies describing the epidemiological and pathological aspects caused by the consumption of *A. glazioviana* in Brazil, there is still a lack of knowledge about the toxicity of this plant at different vegetative stages. In this context, this article presents a chromatographic and toxicological evaluation of exsiccates of young shrubs and adult plants of *Ateleia glazioviana*.

MATERIALS AND METHODS

Parts of *Ateleia glazioviana* were collected from cattle production properties, previously selected by professionals from the Veterinary Pathology Laboratory of the *Instituto Federal Catarinense*

(IFC) Concórdia, with geographic location coordinates registered in SISGEN as number A2D0D81, in the following months of 2019: January, May, September and December.

Leaves of two different degrees of maturity (young shrubs and adult plants) were collected. After being properly separated and identified, the leaves were used in the preparation of extracts used as samples for the study. For each month, two extracts were prepared, called Extract A (Ea) and Extract B (Eb), which are composed of young leaves and mature leaves, respectively.

The leaves of samples of *Ateleia glazioviana* were previously washed with running water. Afterwards, 200g of leaves were added to a volume of 500 mL of distilled water and subjected to exhaustive maceration, followed by rest for one hour and then filtered under reduced pressure with the aid of a fine mesh Buchner funnel (400 mesh). The process was carried out for the young and mature leaves, resulting in Extract A and Extract B, and both were stored under refrigeration (5 - 8°C), protected from light.

The qualitative determination of the major components was performed by Gas Chromatography (GC), using a sample volume for injection of 1µL (the same for the standard and solvent – Methyl-Ethyl-Ketone) followed by 6 washes. Carrier gas pressure (Nitrogen) was 4 psi. The gas flow to the column was 0.2 ml/min., composed of Hydrogen and Synthetic Air. Column pressure was adopted at 1.8 psi. To determine the resulting peaks, an FID detector (Agilent 7820A) was used at a temperature of 425°C in the FID, and an oven temperature of 144°C. For the flow of the sample components, a heating ramp from 40 to 144°C was used, for a time of 80 min. Chromatographic analysis was performed in duplicate for each sample.

Fertile fresh white Lohmann eggs (Lohmann selected Leghorn, LSL) were used in the HET-CAM test (ICCVAM, 2010) to perform the chorioallantoic membrane chicken egg test (HET-CAM). They were kept under optimized incubation conditions (temperature between 38 to 39°C and humidity between 55 and 60% at 10 days). On day 10, the eggshell surrounding the airspace was carefully removed with a rotary tool (Dremel, WI). A volume of 0.3mL of each substance was then added to each egg (negative

control - 0.9% saline solution; positive control - 0.1M NaOH solution; and samples from Extract A and Extract B). The observation of the irritating effect was observed in 30 seconds, 2 minutes and 5 minutes after the application of each substance. The result of the irritation index

$$II = \left\{ \left[\frac{(301 - ht)}{300} \right] * 5 \right\} + \left\{ \left[\frac{(301 - lt)}{300} \right] * 7 \right\} + \left\{ \left[\frac{(301 - ct)}{300} \right] * 9 \right\}$$

II: irritation index, ht: hemorrhage time, lt: lysis time, ct: coagulation time.

To assess toxicity in red blood cells from sheep by measuring free hemoglobin, a red cell suspension was first obtained by blood centrifugation and washing with 0.85% NaCl. Afterwards, aliquots of the suspension of 2% red blood cells were removed for addition with aliquots of extract A and B diluted successively in the proportion of 1/1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, in buffer solution. After incubation (30 minutes at 37°C) the formation of buds at the bottom of each well was evaluated. Saline solution and 0.01% KCN solution were used as negative and positive controls, respectively.

To evaluate toxicity in red blood cells from sheep by measuring lactate dehydrogenase, a red cell suspension was first obtained by blood centrifugation and washing with 0.85% NaCl. Afterwards, aliquots of the red blood cell suspension were removed for addition with aliquots of extract A and B diluted successively in the proportion of 1/1, 1/4 and 1/16 in a buffer solution. Afterwards, the working reagent (1mL) was pipetted and the absorbance (A) of each tube was measured at 340 nm during an interval of 1 minute. The concentration of Lactate Dehydrogenase (LD) was performed according to the equation $(LD \text{ (U/L)} = [A \text{ final} - A \text{ initial} / 2] \times 8095)$ (Lactate Dehydrogenase – Labtest, Minas Gerais, Brazil). As a negative control, saline solution was used and 1% hemolyzed sheep blood was used as a positive control.

Statistical analysis was performed using SPSS v.29.0.2.0 software (IBM). The Kolmogorov–Smirnov test was applied to decide whether distributions were parametric. The results between groups were compared using the student *t*-test given that equal variances were assumed. A 99% CI of the difference was used, with $p \leq 0.05$ considered significant.

(II) was given according to the equation below, on a scale of 0 to 4.9 denoted as non-irritating (or practically no irritation) and 5.0 to 21 denoted as irritating (moderate, severe, or extreme irritation) (The Hen's..., 2010):

RESULTS AND DISCUSSION

The chemical characterization of an active principle contained in natural species is a great challenge, as it is dispersed among several other chemical compounds, often with similar chemical structures. Figure 1 shows the chromatograms highlighting the peaks of the major compounds present in each of the extracts, with A1 and A2 being duplicates of Extract A; and B1 and B2 for Extract B, respectively.

In this analysis, in addition to the isometric form of the selected peaks resulting between the samples of the two extracts, it was possible to identify the clear presence of the solvent that was used in the determination process in the upper part of the chromatograms, corresponding to the peaks in the region from 4,200 to 4,600 cm^{-1} indicating the presence of the ketone carbonyl group (Methyl-ethyl-ketone). This indicates the non-occurrence of chemical reaction - formation of azeotropes - between the solvent and the analyte, reinforcing the integrity of the species contained.

As for the lower part of the chromatograms, it was possible to identify the presence of peaks in the spectral region between 4,500 and 4,700 cm^{-1} for both extracts and, comparing with the literary standard of 4,200 to 4,900 cm^{-1} , it can be suggested that chemical species belonging to the classes of phenolic and/or ketone substances (whose large chemical class is Flavones) are present in the samples, corroborating the results obtained by Yokosuka *et al.* (2007). These attribute the toxic potential arising from *Ateleia glazioviana* to *Glaziovianin A* and classify it as a Flavonoid (a new isoflavone). Furthermore, after calculating the retention time of the substances in the column (R_f), values of 6.4; 6.43; 7.23 and 8.96 min were verified, also referring to the same chemical classes.

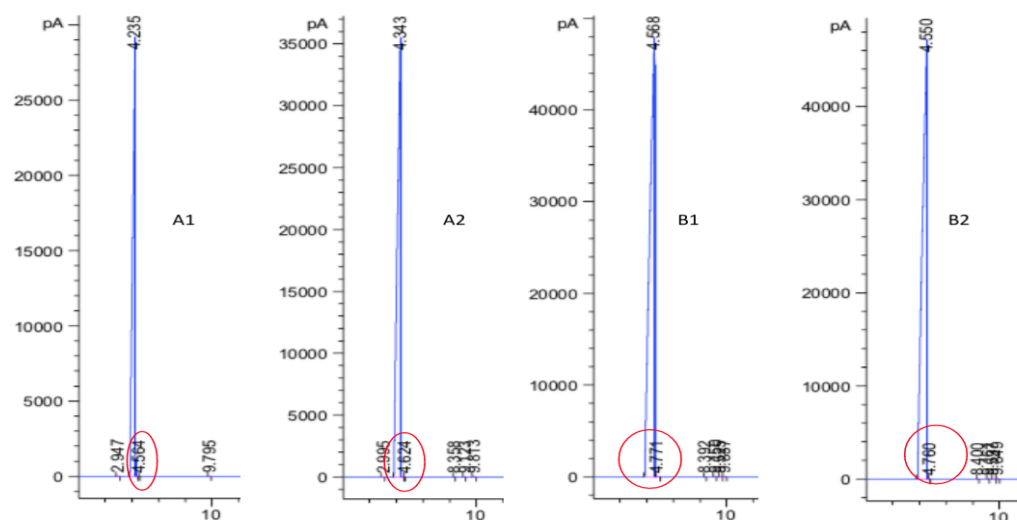


Figure 1. Chromatograms of the evaluated extracts.

Based on the chromatograms obtained, it was possible to identify (first in a qualitative way) the existence of a majority peak. But in quantitative terms, the peak resulted in a concentration of 1.3×10^{-4} mg/kg, with an accuracy of 95.47%. It was also possible to determine the exact amount of the only toxic compound identified in the plant. It should be noted that both forms of maturation, adult and young trees, presented the same amount of toxic product throughout the year. This result is important, as there are reports in the literature that young forms of the plant would be more toxic to ruminants (Gava *et al.*, 2021). To assess this observation with our results, it is important to consider the ease of ingestion of a younger plant, given the height of the adult tree,

or even due to the animal preference for ingesting more tender leaves.

However, it is not prudent to state, based solely on the chromatographic parameters, that Glaziovianin A is the toxic active compound of *Ateleia glazioviana*. There are similarities in the chemical structure, literary patterns, including mechanisms of poisoning, with other chemical substances. Andrade *et al.* (2015), reported on the chemical species Rotenone (C₂₃H₂₂O₆, and molar mass of 394.41g/gmol), which has similarity in the chemical structure and the intoxication mechanism with *Glaziovianin A*. The chemical structures can be compared in Figure 2.

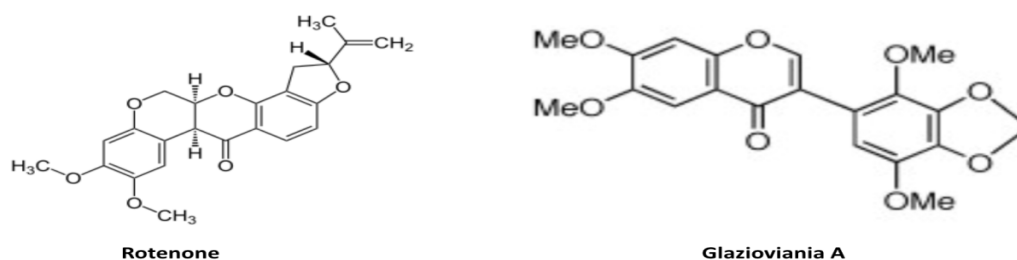


Figure 2. Chemical structures of Rotenone and *Glaziovianin A* species.

Source: Yokosuka *et al.*, 2017.

In the toxicological evaluation, the hemolytic activity of extracts A and B was determined by red cell lysis. According to our results, cell lysis was reached at a concentration of 1/4 of Extract A and 1/1 of Extract B (Fig. 3). The analysis of

the cytotoxicity of crude *Ateleia glazioviana* extracts was investigated in sheep erythrocytes. The hemolytic action of different plant compounds is attributed to a series of non-specific mechanisms, e.g. surfactant compounds

that produce their hemolytic effect by solubilization of the erythrocyte plasma membrane, or osmotic lysis, which promotes changes in the permeability of the red blood cell plasma membrane (Aparicio *et al.*, 2005). This is the only result where a difference between

extracts was observed. Since we identified the same amount of toxic compound on both extracts, we speculate that the hemolytic effect is due to another chemical substance present on the plant, in different amount between both extracts.

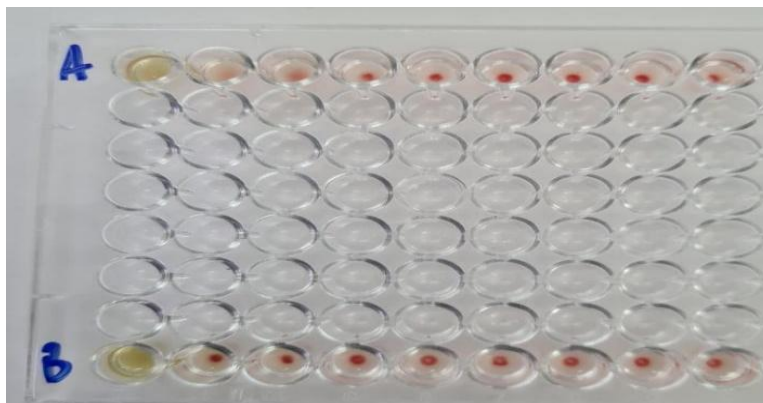


Figure 3. Verification of red cell button formation on a microplate. Extracts A and B were tested at concentrations 1/1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 and 1/256.

However, cells treated with extracts A and B showed hemolysis, in which the extent of cell damage was proportional to the concentration of the respective extracts. Thus, cell viability progressively decreases with increasing concentration of extracts. Different levels of cytotoxicity can be related to the biochemical parameters of the cells involved, such as the composition of the plasma membrane and metabolic activity, the time of exposure to the toxic agent, and the toxicity assay used (Papo and Shai, 2005).

Figure 4 illustrates the release of LD in blood cells treated with extracts A and B at concentrations (1/1, 1/4 and 1/16 (v/v)). There was no statistical difference ($p > 0.05$) comparing extract A and B.

One of the indicators of cell death is the release of intracellular content into the extracellular medium, and the quantification of LD in the cell culture medium supernatant indicates damage to the cell membrane and consequent cell death (Decker and Lohmann-Mattes, 1988). The LD assay, therefore, indicates the loss of plasma membrane integrity of treated cells in a similar degree on both extracts.

Figure 5 shows the relationship between II and the logarithms of the concentration of extracts A and B. This relationship is represented by equation 1 as a function of the log of concentration for this substance. The II for extracts A and B were 6.29 and 5.81 respectively, with both extracts tested being considered irritating, according to the methodology. There was no statistical difference ($p > 0.05$) comparing extract A and B.

Cytotoxicity studies are based on the relationship between the dose and the chemical structure of compounds. Changes in the chemical structure, due to the maturation stage of the plant, showed alterations in the cytotoxic activity. Thus, toxicological studies using *in vitro* models such as HET-CAM, allow to determine the tissue irritability profile of these compounds, and discriminate the different levels of toxicity by calculating the II. HET CAM is a very sensitive test to determine toxicological parameters, therefore, the use of this methodology is acceptable, becoming an alternative to other *in vivo* essays (The Hen's..., 2010; Bubalo *et al.*, 2015; Tsarpali *et al.*, 2015).

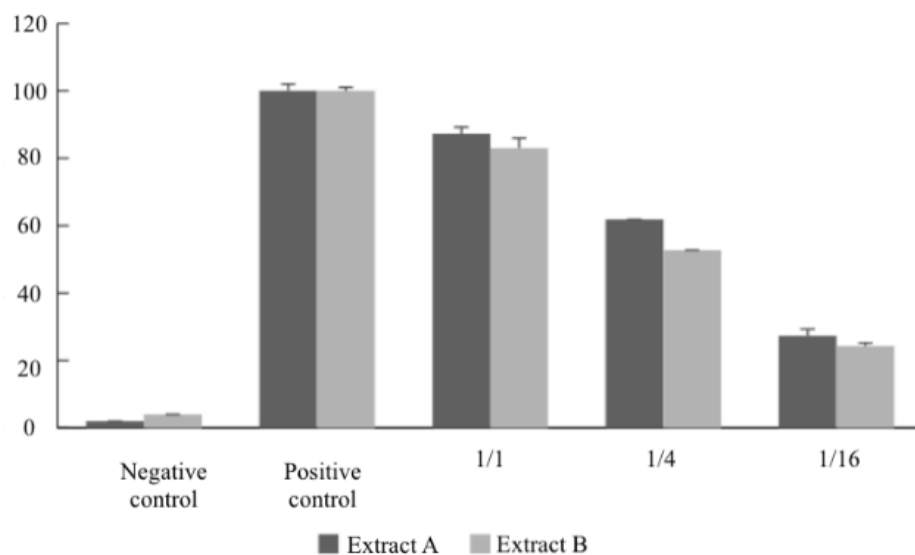


Figure 4. Release of intracellular LD from red blood cells subjected to extracts A and B (at concentrations 1/1, 1/4 and 1/16).

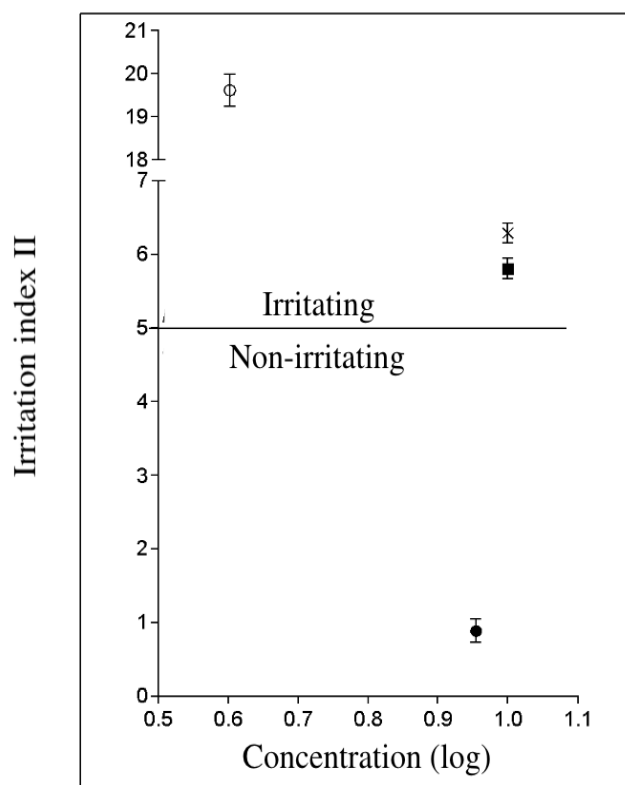


Figure 5. Dose-response relationship for extracts A and B. NaOH (0.1 M) (○), 0.9% NaCl (●), Extract A (×), Extract B (■). Each dot represents an experiment (n = three eggs). Concentrations were logarithmically transformed.

However, in the extant literature, there are few studies regarding toxicological characteristics. In addition, several factors, such as seasonality, temperature, water availability, ultraviolet radiation, addition of nutrients, atmospheric pollution, mechanical damage, and pathogen attack can interfere with the content of secondary metabolites (Gobbo-Neto and Lopes, 2007).

Therefore, further studies must be carried out to understand the complexity of the mechanism of action and the structure-activity relationship of the active compounds present in the extracts. Such studies should involve molecular interactions and characterization of chemical compounds present in the extracts with cell membrane receptors.

CONCLUSION

The Gas Chromatography process was useful in identifying major substances present in a plant extract of *Ateleia glazioviana*. However, the GC system should not be used as the only experimental technique for identifying and quantifying high molecular weight substances, such as organic compounds, as they are homogenized among similar ones in the matrix. It was possible to determine the amount of toxic compound in the plant, both young and adult fresh leaves presenting 0.0013mg/kg. The *in vitro* toxicological evaluation tests of HET-CAM and toxicity in red cells (cell lysis and lactate dehydrogenase) showed good correlation with the molecules elucidated in GC and can be good techniques (together with other identification techniques, or even individually) for routine assessments in toxicology laboratories, in addition to the potential for complementing poisoning diagnoses, as they are easy to conduct and of low cost.

REFERENCES

- ALMEIDA, M.B. *Avaliação das lesões cardíacas e encefálicas induzidas pelas intoxicações por Ateleia Glazioviana e Tetrapteryx multiglandulosa em ovino*. 2006. 54f. Dissertação (Mestrado em Ciências) - Programa de Pós-graduação em Veterinária, Universidade Federal de Pelotas, Pelotas, Rio Grande do Sul, RS.
- ANDRADE, J.A.; COSTA NETO, E.M.; BRANDÃO, H. Using ichthyotoxic plants as bioinsecticide: a literature review. *Rev. Bras. Plantas Med.*, v.17, p.649-656, 2015.
- APARICIO, R.M.; GARCÍA, C.M.J.; VINARDELL, M.P. *et al.* In vitro studies of the hemolytic activity of microemulsions in human erythrocytes. *J. Pharm. Biomed. Anal.*, v.39, p.1063-1067, 2005
- BUBALO, M.C.; RADOŠEVIĆ, K.; SRČEK, V.G. *et al.* Cytotoxicity towards CCO cells of imidazolium ionic liquids with functionalized side chains: preliminary QSTR modeling using regression and classification based approaches. *Ecotoxicol. Environ. Saf.*, v.112, p.22-28, 2015.
- DECKER, T.; LOHMANN-MATTHES, M.L. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J. Immunol. Methods*, v.115, p.61-69, 1988.
- GARCÍA, Y.; SANTOS, M.C.; SCHILD, A.L. *et al.* Lesões perinatais em bovinos na intoxicação experimental por *Ateleia glazioviana* (Leg.Papilionoideae). *Pesqui. Vet. Bras.*, v.24, p.178-184, 2004.
- GAVA, A.; BARROS, C.S.L.; PILATI, C. *et al.* Intoxicação por *Ateleia glazioviana* (Leg. Papilionoideae) em bovinos. *Pesqui. Vet. Bras.*, v.21, p.49-59, 2001.
- GAVA, A.; MOLOSSI, F.A.; OGLIARI, D. *et al.* Spontaneous poisoning by *Ateleia glazioviana* (leg. Papilionoideae) in sheep and goats in the West region of Santa Catarina. *Pesqui. Vet. Bras.*, v.41, p.e06724, 2021.
- GOBBO-N, L.; LOPES, N.P. Plantas medicinais: fatores de influência no conteúdo de metabólitos secundário. *Quím. Nov.*, v.30, p.374-381, 2007.
- PAPO, N.; SHAI, Y. Host defense peptides as new weapons in cancer treatment. *Cell. Mol. Life Sci.*, v.62, p.784-790, 2005.
- PEROSA, F.F.; GRIS, A.H.; PIVA M.M. *et al.* Diseases diagnosed by the Veterinary Pathology Laboratory in the decade 2013–2022. *Bol. Diag. Lab. Patol. Vet.*, v.4, p.16-35, 2024

PESSOA, C.R.M.; MEDEIROS, R.M.T.; RIET-CORREA, F. Importância econômica, epidemiologia e controle das intoxicações por plantas no Brasil. *Pesqui. Vet. Bras.*, v.33, p.752-758, 2013.

RISSI, D.R.; RECH, R.R.; PIEREZAN, F. *et al.* Intoxicações por plantas e micotoxinas associadas a plantas em bovinos no Rio Grande do Sul: 461 casos. *Pesqui. Vet. Bras.*, v.27, p.261-268, 2007.

SÍNTESE ocupação e uso das terras no Brasil. Brasília: Embrapa, 2018. Disponível em: <https://www.embrapa.br/car/sintese>. Acessado em: 28 jun. 2021.

STIGGER, A.L.; BARROS, C.S.L.; LANGOHR, I.M. *et al.* Intoxicação experimental por *Ateleia glazioviana* (Leg.Papilionoideae) em ovinos. *Pesqui. Vet. Bras.*, v.21, p.98-108, 2001.

THE HEN'S Egg Test–Chorioallantoic Membrane (HET-CAM) test method. *Res. Triangle Park: National Toxicology Program*, 2010. (Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVMA)).

TSARPALI, V.; BELAVGENI, A.; DAILIANIS, S. Investigation of toxic effects of imidazolium ionic liquids, [bmim][BF₄] and [omim][BF₄], on marine mussel *Mytilus galloprovincialis* with or without the presence of conventional solvents, such as acetone. *Aquat. Toxicol.*, v.164, p.72-80, 2015.

YOKOSUKA, A.; HARAGUCHI, M.; USUI, T. Glaziovianin A, a new isoflavone, from the leaves of *Ateleia glazioviana* and its cytotoxic activity against human cancer cells. *Bioorg. Med. Chem. Lett.*, v.17, p.3091-94, 2007.