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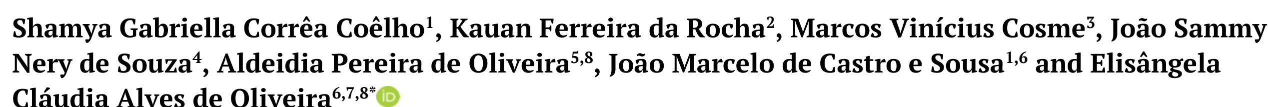
GENETICS

Toxicogenetic evaluation of the ethanolic flower extract of *Pityrocarpa moniliformis* (Benth.) in preclinical studies



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ABSTRACT. The species *Pityrocarpa moniliformis* (Benth.), commonly known as angico-de-bezerro or catanduva, is traditionally used in folk medicine for the treatment of prostate inflammation and is known to possess important biological properties, such as antioxidant and antimicrobial activities. However, despite its therapeutic applications, the genotoxicological profile of this species remains poorly understood. This study aims to evaluate the toxicity, cytotoxicity and mutagenic potential of the ethanolic extract of flower of *Pityrocarpa moniliformis* (Benth.) using the *Artemia salina* bioassay, MTT assay and *Allium cepa* L. test. The extract was tested at concentrations ranging from 0.1 to 1000 μg mL^{-1}. The *A. salina* and MTT assays demonstrated a decrease in cell viability at concentrations starting from 243 μg mL^{-1}. In the *A. cepa* test, no cytotoxic effects were detected; however, a significant increase in chromosomal alterations was observed at 500, 750 and 1,000 μg mL^{-1}. Our findings, therefore, suggest that the ethanolic flower extract of *P. moniliformis* exhibits toxicogenetic effects at higher concentrations, necessitating further investigation in preclinical studies.

Keywords: cytotoxicity; medicinal plants; mutagenicity; toxicity.

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Introduction

Pityrocarpa moniliformis (Benth.) species, also known as angico-de-bezerro and catanduva (Rocha et al., 2019), is an endemic species from Northeast Brazil, often found in Caatinga phytogeographic domain (Sousa et al., 2014). It has been traditionally used to treat prostate inflammation, typically being prepared as a liqueur, infusion, or tea. The inner bark of *P. moniliformis* is the most commonly used part of the plant for medicinal purposes (Silva et al., 2015). Secondary metabolites, such as phenolic compounds (Silva et al., 2011), tannins (Trentin et al., 2015), besides terpenes, saponins and flavonoids were found in *P. moniliformis*. According to Silva et al. (2011), these phenolic compounds may have an antioxidant activity, mainly due to their redox properties. Additionally, Trentin et al. (2015) demonstrated that the tannins of this plant possess antimicrobial activity.

Due to the indiscriminate use of medicinal plants, there is a continuous scientific interest in investigating not only their pharmacological effects but also their potential harmful effects on human health, such as toxic and deleterious effects to the organism, including toxicity at the cellular and/or genetic level (Vaz et al., 2016). In this sense, toxicological and genotoxicological studies of plant extracts and herbal medicines, including *P. moniliformis*, are essential (Silva et al., 2021).

In this scenario, the *Artemia salina* (Leach) assay represents an efficient starting point for cytotoxicity assessment, as well as for general toxicity screening of new products. It is a valuable tool for the preliminary toxicity evaluation of compounds with potential biological activities (Filipe et al., 2022). The MTT assay, in turn is characterized as an appropriate method to assess cytotoxicity and *in vitro* cell proliferation (Van et al., 2011; Skrzydlewski et al., 2022). Another widely used test is the *Allium cepa* L., which is considered a reliable tool for evaluating the toxicity, cytotoxicity, genotoxicity and mutagenicity of different chemical compounds

or plant extracts (Camilo-Cotrim et al., 2022). This is a standard test characterized by high sensitivity, easy execution, low cost, and high reproducibility (Leme & Marin-Morales, 2009; Ozkara et al., 2015).

Together, these assays are valuable tools for elucidating putative cytogenotoxic effects of *P. moniliformis*, giving that existing toxicity data remains insufficient to validate its safe use as a medicinal plant by the population, especially in Brazilian Northeast. The objective of the present study was to evaluate the toxic, cytotoxic and mutagenic effects of the ethanolic extract of *P. moniliformis* flower, using standardized *in vitro* and *in vivo* assays.

Materials and methods

Botanical material

The botanical material used for the study was the flower of the species *Pityrocarpa moniliformis* (Benth.), collected on April 1, 2013, at the *Universidade Federal do Piauí*, *Campus* Professora Cinobelina Elvas, located in the city of Bom Jesus, Piauí (Lat. 9 °5'00.1'S Long. 44°19'38.1'W). A voucher specimen was deposited in the Herbarium Graziela Barroso-UFPI/Teresina on 08/03/2013, under registration number 28.828.

Plant extract

Pityrocarpa moniliformis flowers were dried in a forced air circulation oven at 40°C for 48 hours. Subsequently, the dried material was placed in a sealed glass container, and absolute ethanol was added until the plant material was completely submerged. The plant material was kept at rest for 72 hours. After this period, it was subjected to *vacuum* filtration to obtain the organic solution. Then, the resulting solution was evaporated using a rotary evaporator under reduced pressure and temperature of 60°C to obtain dry ethanolic extract. Finally, the ethanolic extract obtained from the flower of the species *Pityrocarpa moniliformis* (Benth.) was weighed. This extraction procedure was performed following the methodology described by Matos (2009).

To prepare the stock solution, 0.05 g of the ethanolic extract was dissolved in 50 mL of distilled water, resulting in a 1 mg mL^{-1} solution. To this solution, 20 μL of *Tween* 80 was added to enhance solubility. The resulting solution was placed in a falcon tube, covered, protected from light and stored in a freezer (-10 °C). Subsequently, the test samples used were prepared from the stock solution. The following concentrations were defined to perform the tests: 0.1; 3; 9; 27; 81; 243; 500; 750 and 1000 μg mL^{-1}.

Artemia salina Leach bioassay

The *A. salina* bioassay, used to determine the toxicity of the ethanolic extract of the flower of *Pityrocarpa moniliformis* (Benth.), was performed according to the protocol proposed by Meyer et al. (1982), with modifications. The authors also established a relationship between the degree of toxicity and the average lethal dose, LC_{50}, exhibited by plant extracts on *A. salina* larvae. Based on this relationship, extracts may be classified as highly toxic ($LC_{50} \leqslant 100$); moderately toxic (LC_{50} between 100 and 500); slightly toxic (LC_{50} between 500 and 1000) or non-toxic (LC_{50} above 1000) (Meyer et al., 1982). *Artemia salina* cysts were incubated in 500 mL of distilled water (pH 8), supplemented with 18 g of sea salt, under constant lighting and aeration for 48 hours to promote hatching. After this period, the newly hatched nauplii were collected using Pasteur pipettes, and groups of 10 larvae were transferred into test tubes containing the different concentrations of the ethanolic extract. Each test tube contained 3 mL of the sample at the respective concentrations, with aerated saline water used for the dilution. The larvae were exposed to the sample solutions for 24 hours under constant illumination. Positive and negative controls were included in the assay. The positive control consisted of distilled water, while the negative control used saline water; both were kept under the same conditions as the test samples. After exposure for 24 hours, the number of dead larvae in each group were counted. All tests were conducted in quintuplicate. The limits established by the literature were taken into account. The classification of toxicity followed the criteria established by Meyer et al. (1982), in which LC_{50} values below 100 μg mL^{-1} indicate high toxicity, values between 100 and 500 μg mL^{-1} indicate moderate toxicity, values between 500 and 1000 μg mL^{-1} indicate low toxicity, and values above 1000 μg mL^{-1} are considered non-toxic.

Cell lineage

RAW 264.7 cells (murine macrophage strain from the Rio de Janeiro Cell Bank, Brazil) were cultured in 25 cm cell culture flasks containing DMEM medium (EARLE, Cultilab), 10 mL of 100 mM sodium pyruvate (Gibco)

and 1.0 mL of antimycotic antibiotic solution (10,000 IU mL^{-1} penicillin/10 mg mL^{-1} streptomycin, Cultilab), supplemented with 10% fetal bovine serum (FBS; Gibco) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. A 0.25% Trypsin/EDTA solution was used to detach and harvest the cells prior to the experiments. The culture medium was replaced after 48 hours of incubation.

Cell viability assay

For cytotoxicity studies, the colorimetric method 3-(4,5-Dimethylthiazol-2-oyl) 2,5-Diphenyl Tetrazoylium Bromide (MTT) described by Mosmann (1983) was used. Cells were seeded in 96-well plates and incubated for 24 hours in an oven at 37°C in a humidified atmosphere containing 5% of CO_2. Cells were incubated with different concentrations of the ethanolic extract from the flowers of Pityrocarpa moniliformis (Benth). After incubation, 10 μL of MTT at 5 mg mL^{-1} was added to each well. The plate was then incubated for another 3 hours in a humidified incubator under standard culture conditions. After this period, the supernatant was carefully removed, and 100 μL of 10% DMSO was added to each well to solubilize the formazan crystals. The plate was wrapped with aluminum foil and stored in an incubator at 37°C overnight. Optical density was measured using a spectrophotometer at 570 nm. The negative control consisted of cells maintained in 1% DMEM culture medium, while the positive control was treated with Triton X-100.

Allium cepa assay

The *Allium cepa* cytogenotoxicity test was performed according to the protocol described by Guerra e Sousa (2002), with some modifications. Initially, the bulbs were cultivated in distilled water, completing the volume of the samples every 24 hours to a final volume of 5 mL. The bulbs were kept under these conditions for 48 hours at approximately 25°C prior to treatment with the ethanolic extract of *Pityrocarpa moniliformis* flowers. For each extract concentration, five *Allium cepa* bulbs were used and treated for 24 hours. After the treatment period, the root tips were collected and fixed in Carnoy's solution (3:1 v/v ethyl alcohol: acetic acid) for 24 hours. The fixed meristems were then washed with distilled water and hydrolyzed in 1N HCl for 10 minutes. Following hydrolysis, the root tips were macerated and stained with 1% acetic orcein. The prepared slides were observed under a light microscope at 400× magnification. A total of 1,000 cells were counted in each of the 5 treated bulbs for each concentration tested. The following parameters were evaluated: mitotic index MI = [(number of dividing cells/total cells analyzed) x 100], number of cells in interphase, number of cells undergoing division, including the distribution across the different phases of the cell cycle, and frequency and types of chromosomal aberrations (CA).

Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) with a fixed factor, followed by Tukey's multiple comparison test, considering a significance level of $p < 0.05$. All analyses were conducted using GraphPad Prism software, version 8.0.

Results

Toxicity of *Pityrocarpa moniliformis* flower ethanolic extract identified by *Artemia salina* test

The observed viability of *Artemia salina* nauplii was 62% at the concentration of 243 μg mL^{-1}, decreasing sharply to 4% at 500 μg mL^{-1}. At the two highest concentrations tested, 750 μg mL^{-1} and 1000 μg mL^{-1}, nauplii mortality reached 100%, demonstrating toxicity comparable to that of the positive control (Table 1). Based on these results, the ethanolic extract of *Pityrocarpa moniliformis* flowers was classified as moderately toxic, with an LC_{50} value of 269.0 μg mL^{-1}.

Cytotoxic and mutagenic effects of *Pityrocarpa moniliformis* flower extract by MTT and *Artemia cepa* tests

Cells exposed to concentrations starting at 243 μg mL^{-1} exhibited a significant reduction in viability compared to the negative control in MTT test. The viability decreased by 49, 50, 52, and 60% at concentrations of 243, 500, 750, and 1000 μg mL^{-1}, respectively (Figure 1). Compared to the positive control, no statistically significant differences were observed at concentrations of 500, 750, and 1000 μg mL^{-1}, further confirming the cytotoxic effects of the extract. The extract exhibited a 50% inhibitory concentration (IC_{50}) equal to 164 μg mL^{-1} (data not shown).

Table 1. Toxicity of the Ethanolic Extract of *Pityrocarpa moniliformis* Flower in *Artemia salina* test.

Treatment	% of viable nauplii
CP	0.0 ± 0.0
CN	100 ± 0.0
0.1 μg mL^{-1}	92.0 ± 13.03#
3 μg mL^{-1}	86.0 ± 16.73#
9 μg mL^{-1}	86.0 ± 13.41#
27 μg mL^{-1}	80.0 ± 23.45#
81 μg mL^{-1}	76.0 ± 15.16#
243 μg mL^{-1}	62.0 ± 13.03*#
500 μg mL^{-1}	4.0 ± 5.47*
750 μg mL^{-1}	0.0 ± 0.0*
1000 μg mL^{-1}	0.0 ± 0.0*
LC_{50} (μg mL^{-1})	269.0
IC	229.2 – 315.7
r^2	0.811

Data expressed as mean ± SD. The '*' indicates statistical significance compared to the negative control, and the '#' indicates statistical significance compared to the positive control, using the variance test ANOVA with a fixed factor, followed by Tukey's multiple comparisons test, for $p < 0.05$. LC_{50}: Lethal concentration in μg mL^{-1}. CI: Confidence interval; r^2: Determination of coefficient.

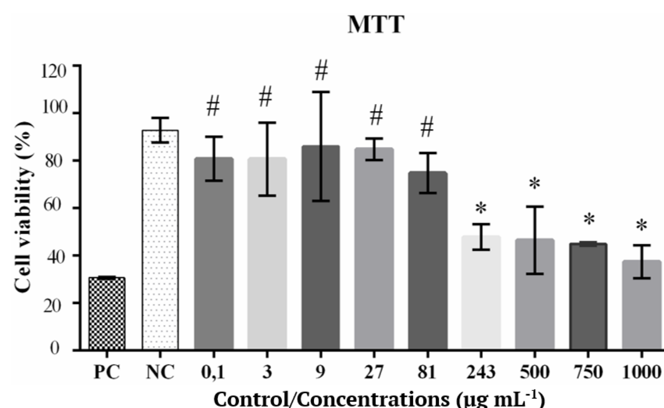

Figure 1. Effect of the ethanolic extract of the *Pityrocarpa moniliformis* flower on the cell viability of macrophages of the RAW 264.7 lineage in the period of 24 hours. PC: Positive control; NC: Negative Control. The '*' indicates statistical significance compared to the negative control, and the '#' indicates statistical significance compared to the positive control, using the variance test (ANOVA with a fixed factor), followed by Tukey's multiple comparisons test, $p < 0.05$.

No significant difference was observed between concentrations tested and negative control by using the mitotic index (MI) parameter in *Allium cepa* test. The analysis of the number of meristematic cells in the different phases of the cell cycle revealed a significant decrease in the number of cells in prophase, at concentrations of 243 μg mL^{-1}, 500 μg mL^{-1} and 750 μg mL^{-1} (Table 2). Considering the number of chromosomal aberrations as an indicator of mutagenicity, a significant increase in chromosomal damage was observed at the higher concentrations tested 500 μg mL^{-1}, 750 μg mL^{-1}, and 1000 μg mL^{-1} (Table 3). The predominant chromosomal alterations detected were anaphase bridges and delays, mainly at the concentration of 1000 μg mL^{-1} (Figure 2).

Table 2. Analysis of cytotoxicity in *Allium cepa* roots treated with the ethanolic extract of the flower of *Pityrocarpa moniliformis* (Benth.).

Treatment	Interphase	Cells in division	Prophase	Metaphase	Anaphase	Telophase	MI (%)
NC	2.773 ± 3.64	2.227 ± 3.64	2.129 ± 3.11	41 ± 1.64	25 ± 1.58	32 ± 2.07	44.54 ± 0.36
0.1 μg mL^{-1}	2.778 ± 7.05	2.222 ± 7.05	2.142 ± 4.72	33 ± 2.96	26 ± 2.86	21 ± 4.08	44.44 ± 0.70
3 μg mL^{-1}	2.779 ± 5.06	2.221 ± 5.06	2.147 ± 2.19	41 ± 2.16	23 ± 1.94	10 ± 0.70	44.42 ± 0.50
9 μg mL^{-1}	2.790 ± 3.53	2.210 ± 3.53	2.116 ± 1.30	34 ± 2.16	29 ± 1.92	31 ± 3.03	44.20 ± 0.35
27 μg mL^{-1}	2.793 ± 4.03	2.207 ± 4.03	2.130 ± 1.58	30 ± 1.22	37 ± 1.51	10 ± 0.70	44.14 ± 0.40
81 μg mL^{-1}	2.797 ± 1.51	2.203 ± 1.51	2.106 ± 1.30	38 ± 1.14	32 ± 1.67	27 ± 1.14	44.06 ± 0.15
243 μg mL^{-1}	2.799 ± 4.81	2.201 ± 4.81	2.097 ± 1.14*	48 ± 2.30	30 ± 1.0	26 ± 2.77	44.02 ± 0.48
500 μg mL^{-1}	2.799 ± 4.38	2.201 ± 4.38	2.083 ± 2.07*	42 ± 0.89	36 ± 1.78	40 ± 1.58	44.02 ± 0.43
750 μg mL^{-1}	2.801 ± 1.09	2.199 ± 1.09	2.077 ± 1.81*	46 ± 1.30	35 ± 1.87	41 ± 1.30	43.98 ± 0.10
1000 μg mL^{-1}	2.803 ± 2.30	2.197 ± 2.30	2.117 ± 4.72	33 ± 3.39	26 ± 1.58	21 ± 2.0	43.94 ± 0.23

NC: Negative Control; MI: Mitotic Index. (Data expressed as mean ± SD). The '*' indicates statistical significance of the concentrations tested in comparison to the negative control, using the test of variance (ANOVA with a fixed factor), followed by Tukey's multiple comparisons test, for $p < 0.05$.

Table 3. Analysis of chromosomal aberrations in *Allium cepa* roots treated with the ethanolic extract of the flower of *Pityrocarpa moniliformis* (Benth.).

Treatment	Total chromosomal aberrations
CN	8.0 ± 3.67
0.1 μg mL^{-1}	8.2 ± 3.03
3 μg mL^{-1}	7.2 ± 2.04
9 μg mL^{-1}	8.0 ± 1.22
27 μg mL^{-1}	8.0 ± 2.12
81 μg mL^{-1}	8.8 ± 2.16
243 μg mL^{-1}	9.6 ± 2.07
500 μg mL^{-1}	14.0 ± 1.22*
750 μg mL^{-1}	14.6 ± 2.70*
1000 μg mL^{-1}	15.2 ± 2.16*

NC: Negative Control. (Data expressed as mean ± SD). The '*' indicates statistical significance of the concentrations tested in comparison to the negative control, using the test of variance (ANOVA with a fixed factor), followed by Tukey's multiple comparisons test, for $p < 0.05$.

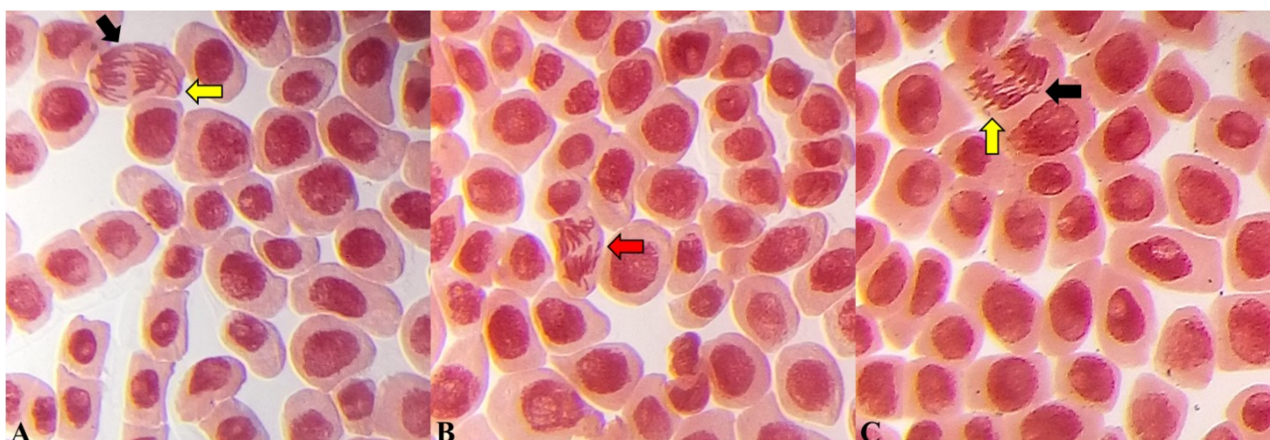

Figure 2. Cells obtained from *Allium cepa* roots treated with different concentrations of ethanolic extract from the *Pityrocarpa moniliformis* flower at 400x magnification. A- Bridge and delay in anaphase. B- Fragment in metaphase. C-Delay in anaphase. Arrows: yellow (delay); black (bridge); red (fragment).

Discussion

The *Artemia salina* and MTT tests showed similar results, with toxicity and cytotoxicity effects beginning at 243 μg mL^{-1}. These effects are likely attributable to the high concentration of secondary metabolites present in *Pityrocarpa moniliformis*, including phenolic compounds, flavonoids, and tannins. Rocha et al. (2019) reported cytotoxicity in cyclohexane, ethyl acetate, and methanolic leaf extracts of *P. moniliformis*, while Trentin et al. (2015) observed similar cytotoxic effects with leaf methanolic extracts of the species. Both studies correlated the cytotoxic activity of this specie with the presence of its bioactive compounds. Oliveira Teixeira et al. (2003) showed that tannins may be primarily responsible for blocking cell division in the meristematic cells of *Allium cepa*, as they act through protein precipitation and enzymatic inhibition. Similarly, Castro et al. (2020) highlighted that flavonoids, at high concentrations, can also inhibit cell division. However, the literature reveals some divergences regarding the toxicity attributed to phenolic compounds such as flavonoids and tannins. Sousa et al. (2007), evaluated the antioxidant activity of the ethanolic extract of five medicinal plants: *Terminalia brasiliensis* (leaf and bark); *Terminalia fagifolia* (leaf); *Cenostigma macrophyllum* (leaf); *Qualea grandiflora* (leaf) and *Copernicia prunifera* (root), showing that most of these species demonstrated DPPH radical scavenging activity, and attributed these properties to the total phenol content (Sousa et al. 2007). Ślusarczyk et al. (2009) also associated the presence of phenolic compounds and flavonoids to antioxidant properties.

Alves et al. (2014), evaluating the cytotoxicity of the hydroalcoholic, hexane, ethyl acetate and diclomethane extracts obtained from the leaves, fruits, fruit peels and seeds of *P. moniliformis* by the MTT assay, did not observe cytotoxic activity on the tested human tumor cell lines even at the highest concentration tested (25 μg mL^{-1}). On the other hand, our results diverged from these findings, which may be attributed to differences in the cell lines tested, the types of extracts, the plant parts analyzed, and mainly, the concentrations used in each assay.

It is important to emphasize that the protective or cytotoxic effects of any species may be related to several factors, such as the different solvents used to obtain the extract, the cell lineage or organism studied, besides the concentration of the extract used and the time of exposure to the treatment. Bulbovas et al. (2005) also pointed out that the concentration of certain secondary metabolites can vary significantly in response to seasonal changes. The discrepancies observed in cytotoxicity results can be also explained by the difference in sensitivity of the *in vitro* and *in vivo* tests (Chapdelaine, 2010).

Our results demonstrated that exposure to the ethanolic extract of the flower of *Pityrocarpa moniliformis* (Benth.) led to a decrease in the number of cells in prophase at concentrations of 243, 500, and 750 μg mL^{-1}, accompanied by an increase in the number of cells in interphase. Furthermore, it was possible to detect a significant increase in the frequency of chromosomal aberrations, mainly bridges and chromosomal delays in anaphase (500 μg mL^{-1}), when compared to the negative control. This suggests that cells may remain longer in interphase, progressing more slowly through the cell cycle phases to allow the DNA repair machinery sufficient time to assemble during the G_2 phase and correct such damage. Therefore, the toxicity and cytotoxicity evidenced in this work can be attributed to the mutagenic potential of the ethanolic extract of the flower of *Pityrocarpa moniliformis* (Benth.), shown by the *Allium cepa* test. It is suggested that the DNA lesions induced by the treatment are sufficiently severe to trigger cell death pathways, such as apoptosis or necrosis, since genetic damage often leads to cellular demise.

Taken together, our results demonstrate the toxicogenetic potential of the ethanolic extract of *Pityrocarpa moniliformis* flower at higher concentrations, reinforcing the need for caution in the use of this species. These findings highlight the need for more detailed studies investigating the interactions between this extract and genetic material.

Conclusion

The ethanolic extract of *Pityrocarpa moniliformis* flower exhibited toxicity in *Artemia salina* and cytotoxicity in the RAW264.7 cell line. Furthermore, the extract demonstrated mutagenic potential in *Allium cepa* test at higher concentrations. Therefore, *in vivo* studies focused on the antioxidant and toxicological analysis are recommended to better characterize the toxicogenetic profile of this species.

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