Genotoxicity assessment of saline extract from *Pilosocereus gounellei* (Cactaceae) and its chemopreventive effect against cyclophosphamide-induced DNA damage

Alisson Macário de Oliveira, Anderson Felipe Soares de Freitas, Patrícia Maria Guedes Paiva, Thiago Henrique Napoleão*

Departamento de Bioquímica, Centro de Ciências, Universidade Federal de Pernambuco, Recife, Pernambuco, Brasil

**ARTICLE INFO**

**Keywords:**
- Cactus
- Micronuclei
- Comet assay
- Anti-Genotoxic effect
- Biological sciences
- Metabolite
- Pharmaceutical science
- Natural product
- Biochemistry
- Toxicology

**ABSTRACT**

*Pilosocereus gounellei* (Cactaceae) is used to treat wounds and inflammation. In this study, we evaluated whether the saline extract from its stem would have genotoxic or anti-genotoxic effects. In the genotoxicity evaluation, mice received the extract (500, 1,000, or 2,000 mg/kg) orally while negative and positive controls were treated with saline solution (0.9% NaCl) *per os* and cyclophosphamide (CPA, 80 mg/kg *i.p.*), respectively. In the anti-genotoxicity assay, using other animals, treatments were carried out by administering the extract (500, 1,000 or 2,000 mg/kg) or saline solution (negative control) *per os* and then CPA (80 mg/kg *i.p.*) 1 h later. Genotoxic effects were evaluated by micronucleus test and comet assay using peripheral blood and bone marrow cells. Oral administration of only the extract at 500 and 1,000 mg/kg did not result in genotoxicity. A slight increase in the incidence of micronucleus was observed at the highest dose (2,000 mg/kg). Administration of the extract before CPA reduced the micronucleated polychromatic erythrocytes (MNPCE) number by 49.07–71.43%, and DNA fragmentation in peripheral blood (85.04–94.44%) and bone marrow (87.43–92.70%) cells also decreased. In conclusion, when administered orally at the tested doses, the extract is genotoxically safe, being cautious in doses above 1,000 mg/kg, and has a protective effect against CPA-induced DNA damage.

1. **Introduction**

Genotoxicity assessments are performed to detect whether a compound can induce direct or indirect DNA damage that may result in sequence alterations, loss of heterozygosity, and chromosome disorders (Silva et al., 2013; Tolentino et al., 2015). These events play an important role in many malignancies and induceheritable effects leading to congenital disorders (Shah, 2012; Kasper et al., 2018). The evaluation of potential genotoxic effects is essential in the risk/benefit analysis of new drugs as well as for preparations already used in traditional medicine (Prinsloo et al., 2018; Fatch et al., 2019). International regulatory agencies have validated and recommend various genetic tests for genotoxic assessment, such as comet and micronucleus assays (Llana-Ruiz-Cabello et al., 2015).

The micronucleus test is based on the detection of DNA breaks that result in chromosome fragments which are not included in the nucleus during mitosis, forming an additional nucleus (OECD, 2016). The comet assay is a technique used for detecting the presence of single strand breaks of DNA at alkali-sensitive sites or sites of incomplete excision repair in mammalian cells (OECD, 2016). These tests have been used to analyze the toxicity (Araújo et al., 2015; Oliveira et al., 2016; Ramos et al., 2019) as well as anti-genotoxic, anti-mutagenic, and anti-carcinogenic effects (Lemes et al., 2017; Makhuvele et al., 2018) of extracts and isolated compounds from plants.

*Pilosocereus gounellei* (Cactaceae), popularly known in Brazil as xique-xique, is a plant used in traditional medicine to treat perforations in the upper and lower limbs, as well as to produce anti-inflammatory preparations (Agra et al., 2008; Roque et al., 2010). Acute toxicity evaluation of a saline extract from *P. gounellei* stem showed that it did not promote adverse effects on survival, motor coordination, or the hematological, biochemical, and histological parameters of mice. This extract also did not present significant toxic effects when administered over 28 consecutive days and was reported to have antinociceptive and antipyretic activities (Oliveira et al., 2018, 2019). Nonetheless, no scientific studies are available to date that rule out the genotoxic potential of *P. gounellei*. Thus, in this study, we aimed to evaluate the genotoxicity of the saline extract...
from *P. gounellei* through micronucleus and comet assays, as well as investigate its possible chemopreventive action against cyclophosphamide-induced DNA damage.

### 2. Materials and methods

#### 2.1. Plant material

The stem of *P. gounellei* was collected at Limoeiro, Pernambuco, Brazil, under the authorization (number 36301) of the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio). The collection was recorded (ADF1667) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen) and a voucher specimen (no. 82,853) was deposited at the herbarium “UFPE – Geraldo Mariz” in the *Universidade Federal de Pernambuco* (UFPE). The stem had its thorns separated from the core (marrow), which was then maintained at 28 °C for 72 h. Next, the dried core (20 g) was crushed and homogenized in 150 mM NaCl (200 mL) for 16 h at 28 °C by magnetic stirring. The suspension was then centrifuged (3,000 g, 15 min, 25 °C) and the supernatant corresponded to the saline extract (Oliveira et al., 2018).

#### 2.2. Animals

All the experiments were approved by the Ethics Committee on Animal Use of UFPE (process no. 23076.0481175/2015-12). Male Swiss albino mice (*Mus musculus*) weighing 30–35 g were obtained from the vivarium of the *Laboratório de Imunopatologia Ketzo Asami* of UFPE and used in experiments after a one-week adaptation period in the laboratory of animal experimentation at the *Departamento de Bioquímica* of UFPE, where they were kept under a 12/12 h light/dark cycle at 22 °C. Water and food (Labina, Purina, Brazil) were available ad libitum.

#### 2.3. Evaluation of the genotoxicity of *P. gounellei* stem extract

##### 2.3.1. Treatments

The mice were divided into experimental groups of 10 animals each. The extract was dissolved in saline solution (0.9% NaCl) and administered by gavage in a single dose of 0.1 mL at 500, 1,000 and 2,000 mg/kg body weight (b.w). These doses were selected based on the previous acute toxicity study (Oliveira et al., 2018) and following the limit recommended by OECD (2001). The negative control group received only saline solution *per os* and the positive control group received an intraperitoneal injection of cyclophosphamide (CPA; Sigma-Aldrich, USA), dissolved in sterile deionized water, at 80 mg/kg b.w (Exresson, 2005).

##### 2.3.2. Micronucleus test

The assay was carried out following the protocol described by Maistro (2014). Five mice of each group were euthanized by cervical dislocation 24 h after the treatment and the other five after 48 h. Saline solution (2 mL) was used to collect the bone marrow from the femur. After centrifugation (7 min), 10 μL of bone marrow or blood was placed on three slides and stained with acridine orange and the presence of micronuclei (Figure 1) was evaluated using a fluorescence microscope (Olympus BX 50). For each slide, 2000 polychromatic erythrocytes were counted by a blinded researcher (6,000 cells/animal). The number of micronucleated polychromatic erythrocytes (MNPCE) was determined, as well as the ratio between polychromatic (PCE) and normochromatic (NCE) erythrocytes.

##### 2.3.3. Comet assay

Four or twenty-four hours after the treatment, samples of peripheral blood from the orbital plexus were collected from five mice. Bone marrow samples of ten mice per group were also collected after 24 h and analyzed. A hemocytometer was used to count the cells, which had their viability determined by trypan blue exclusion assay. Cells were only used if viability was >90%. The comet assay was then carried out according to Terrazas et al. (2013). The cells were evaluated in the fluorescence microscope (400× magnification) with excitation filter set at 515–560 nm and emission filter at 590 nm. In a blind analysis, individual nucleoids were scored, examining 200 randomly selected and non-overlapping cells per animal. The tail size and the diameter of the head (nucleus) were observed and used to score the cells according to Hartmann and Speit (1997): class 0 – no tail; class 1 – tail shorter than the head diameter; 2 – tail length measuring 1–2 times the head diameter; 3 – tail length measuring more than twice the head diameter. Total score for 200 comets was calculated by multiplying the number of cells in each class by the respective class number (0, 1, 2 or 3).

#### 2.4. Evaluation of the anti-genotoxic effect of *P. gounellei* stem extract against cyclophosphamide-induced DNA damage

Anti-genotoxicity evaluation was performed using animals other than those from the genotoxicity test. Treatments were carried out by administering to the mice (n = 10 per group) the extract (500, 1,000 or 2,000 mg/kg *per os*) or saline solution (negative control) and then CPA (80 mg/kg i.p.) 1 h later. The micronucleus and comet assays were then performed as described above. The anti-genotoxic effect (% reduction) in the micronucleus assay was evaluated according to Waters et al. (1990) using the equation:

\[
\% \text{Reduction} = \frac{PC - T}{PC - NC} \times 100
\]

where *PC*, *T*, and *NC* correspond to the mean number of micronucleated cells in the positive control (only CPA), treatment (saline extract), and negative control, respectively.

In the comet assay, the percentage of reduction in genotoxic effect was calculated by the ratio between the score for the extract group and the score for the only CPA group.

#### 2.5. Statistical analysis

Normal distribution was verified employing the Kolmogorov–Smirnov test. The data from the comet assay were submitted to one-way analysis of variance (ANOVA) and Tukey’s test. The data obtained from the micronucleus assay were submitted to ANOVA and linear regression. All tests were performed using the GraphPad Prism 8 software (version 8.0.1) and the significance level was set at *p* < 0.05.
3. Results and discussion

The saline extract of *P. gounellei* stem used here was previously evaluated by thin layer chromatography (TLC) and high-performance liquid chromatography and it was reported the presence of flavonoids at 0.054 g% (including kaempferol derivatives), reducing sugars and a polysaccharide content of 17.17 g%. Alkaloids, coumarins, cinnamic acid derivatives, tannins, terpenes, and steroids were not detected (Oliveira et al., 2018, 2019).

The balance between the therapeutic and toxicological effects is an essential measure to determine the usefulness of a pharmacological drug (Munari et al., 2010). Some plant substances such as alkenyl benzenes, alkaloids and anthraquinone glycosides are known to be potentially carcinogenic and teratogenic (Kristanc and Kreft, 2016). The in vivo micronucleus and comet assays are both considered appropriate for genotoxicity tests in a standard battery for testing pharmaceuticals (OECD, 2014).

The clastogenic/aneugenic potential of the *P. gounellei* stem extract was investigated by the micronucleus test (Table 1). The results showed that treatment with the extract at 500 and 1,000 mg/kg for 24 h did not significantly increase (*p > 0.05) the mean number of MNPs in bone marrow in comparison with the negative control. On the other hand, a small but significant increase (*p < 0.05) was observed in the MNPs in samples collected at 24 h from animals treated with the dose of 2,000 mg/kg compared to the negative control; however, the values were significantly (**p < 0.05**) lower than those in the positive control.

In vivo genotoxicity of saline extract from *Pilosocereus gounellei* stem extract by comet assay in mice peripheral blood and bone marrow: mean frequency of damaged cells, average distribution per class and average damage score.

| Sample          | Collection time | Treatments                  | Frequency* | Damage class | Score |
|-----------------|-----------------|-----------------------------|------------|--------------|-------|
|                 |                 | Negative control            | 1.76 ± 0.72| 98.24 ± 1.50| 1.85 ± 0.44 |
|                 |                 | Extract (per os)            |            |              |       |
| Peripheral blood| 4 h              | 500 mg/kg                   | 2.00 ± 0.65| 98.00 ± 0.75| 1.90 ± 0.65 |
|                 |                  | 1,000 mg/kg                 | 2.03 ± 0.32| 97.97 ± 0.47| 2.04 ± 0.25 |
|                 |                  | 2,000 mg/kg                 | 2.16 ± 0.86| 97.84 ± 0.75| 2.10 ± 0.86 |
|                 |                  | CPA 80 mg/kg i.p.           | 50.92 ± 3.09| 49.08 ± 3.27| 61.49 ± 2.41 |
|                 |                  | CPA 80 mg/kg i.p.           | 14.42 ± 4.18| 13.33 ± 0.58| 86.37 ± 4.04 |
| Bone marrow     | 24 h             | Negative control            | 1.88 ± 0.41| 98.40 ± 0.58| 1.96 ± 0.22 |
|                 |                  | Extract (per os)            |            |              |       |
|                 |                  | 500 mg/kg                   | 2.00 ± 0.63| 98.00 ± 0.63| 2.00 ± 0.63 |
|                 |                  | 1,000 mg/kg                 | 2.13 ± 0.76| 97.87 ± 0.22| 2.01 ± 0.76 |
|                 |                  | 2,000 mg/kg                 | 2.22 ± 0.54| 97.78 ± 0.34| 2.13 ± 0.50 |
|                 |                  | CPA 80 mg/kg i.p.           | 70.48 ± 2.49| 70.81 ± 1.07| 88.37 ± 4.04 |

In negative control, mice received saline per os. (*) Significantly different from the negative control (*p < 0.001). (*) Total number of damaged cells (class 1 + 2 + 3). CPA: cyclophosphamide.
in the incidence of micronucleus was observed at the highest dose (2,000 mg/kg). In addition, the cell viability observed in the Trypan blue staining protocol was over 90% in all treatments (data not shown), which confirms the absence of cytotoxicity found by PCE/NCE ratio analysis in the micronucleus test (Table 1).

The results from the comet assay are shown in Table 2. For peripheral blood samples collected after 4 h, the cells in the class 0 were predominant in all groups, but the CPA-treated group showed a significantly lower (p < 0.05) number of undamaged cells and a higher score than the other groups. In the blood samples collected after 24 h, there were no significant (p > 0.05) differences between the negative control and extract treatments; but CPA genotoxicity was evident, with a damage score of 88.37. With respect to the bone marrow samples, there were also no significant (p > 0.05) differences in the score between the negative control and the extract group, while the positive control group showed a score of 80.25. The saline extract of *P. gounellei* exhibited antinociceptive and antiinflammatory activities at doses ranging from 125 to 500 mg/kg b.w (Oliveira et al., 2018, 2019) and the data obtained in this study showed that the extract has no genotoxic effect at these doses and even at higher doses.

Plants used in traditional medicine have also gained attention due to their potential for the development of chemopreventive drugs (Farombi and Owoeye, 2011; Niwa et al., 2016). Anti-genotoxic properties have been reported for several plant preparations, suggesting their putative protective effect against, for example, dietary carcinogens and inflammatory agents as well as in conjunction with genotoxic chemotherapeutics (Prajitha and Thoppil, 2016; Seo et al., 2019). Our study also investigated the anti-genotoxic potential of the saline extract from *P. gounellei* against cyclophosphamide (CPA), a chemotherapeutic agent that causes genetic damage (Magosso et al., 2016).

In the evaluation of the anti-genotoxic effect by the micronucleus assay, the oral administration of different doses of the extract before the CPA injection led to a significant reduction in the frequency of MN in bone marrow compared to that in mice only treated with CPA (Table 3). The damage reduction ranged from 49.07 to 65.74% in samples collected after 24 h and 54.28%–71.43% in those collected after 48 h. A gradual increase in the extract concentration result in a proportional decrease in the frequency of MNPCE in bone marrow compared to that in mice only treated with CPA (Table 3).

The comet assay was also used to investigate the ability of *P. gounellei* stem extract to prevent CPA genotoxic effects (Table 4). The administration of the saline extract before CPA substantially inhibited the damage (p < 0.05). The DNA fragmentation in peripheral blood cells decreased by 90.53–93.41% in samples collected after 4 h and 85.04–94.44% after 24 h, while in bone marrow the decrease was between 87.43% and 92.70% after 24 h.

This ability of *P. gounellei* stem extract to prevent CPA genotoxic effects can be associated to the presence of antioxidants such as flavonoids. The antioxidant properties of flavonoids can lead to inhibition of lipid peroxidation and consequently prevent the generation of reactive oxygen species (ROS) that are involved in the induction of DNA damage.
peroxidation, chelation of redox-active metals, and inhibition of reactive oxygen species (Tsa0, 2010). The presence of flavonoids can even elevate the levels of antioxidant enzymes (Sooodabeh et al., 2014). Zorgui et al. (2008) reported that Opuntia ficus-indica cladeo extract was able to protect Balb/c mice against genotoxicity induced by zearalenone, according to the micronucleus and comet assays. The antioxidant activities of cladeo extracts from O. ficus-indica have been attributed to significant amounts of polysaccharides, as well as flavonoids and phenolic acids (Rocchetti et al., 2018).

Although the exact mechanism of P. gounellei extract in the inhibition of genotoxicity induced by CPA is not completely elucidated, we hypothesize that the antioxidant properties of the extract may explain the protective effects (Oliveira et al., 2019). It is possible that treatment of animals with the P. gounellei extract could improve their antioxidant system, scavenging reactive oxygen species and free radicals generated by CPA and, consequently, reducing chromosome aberrations in somatic cells.

4. Conclusion

The present study was the first in vivo evaluation of the genotoxicity of an extract from P. gounellei. From our results, it can be stated that P. gounellei stem extract, when administered orally at the tested doses, was not genotoxic in peripheral blood erythrocytes and bone marrow cells of mice by the comet assay, but presented aneugenic/clastogenic effects of V79 cells by the comet assay. P. gounellei extract also plays a role in inhibiting CPA-induced genotoxicity, including other previously described activities (antioxidant, antipaptyric) which may be related to the main components of the extract and its possible antioxidant activity. Thus, future studies are needed to clarify the mechanisms of action to certify their biological safety and improve human health.

Declarations

Author contribution statement

T. Napoleao: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. A. Oliveira: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. A. Freitas: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. P. Paiva: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Funding statement

The authors express their gratitude to the Conselho Nacional de Desenvolvimento Científico e Tecnológico(CNPq) for financial support (407192/2018-2) and investigator research grants (PMGP and THN). We are also grateful to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior(CAPES; Finance Code 001) and the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco(FACEPE; APQ-0108-2.08./14) for financial support. AMO would like to thank FACEPE (BPBG-0467-2.08/15) for the graduate scholarship.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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