

In Vitro Cytotoxicity and Genotoxicity Evaluation of an Orabase *Libidibia ferrea* L.

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ABSTRACT

Objective: To evaluate the cytotoxic and genotoxic effects of a phytotherapeutic ointment containing 2% *L. ferrea* L extract. **Material and Methods:** *Libidibia ferrea* L. barks were used as raw material. The extract was prepared by decoction under reflux in a hydroalcoholic solution. The extract concentration in the orabase formulation was determined based on the minimum inhibitory concentration. Biocompatibility was assessed by cell culture tests from MRC5 human fibroblasts. The Alamar Blue test evaluated cytotoxicity; the Trypan Blue Solution trial evaluated cell viability. The comet assay on alkaline and neutral pH assessed genotoxicity. Test results were tabulated and analyzed through descriptive and inferential statistics, with ANOVA Test ($p \leq 0.05$). **Results:** No cytotoxic activity or morphological induction of cell death for the test formulation. Regarding genotoxicity, the Juca formulation could not induce DNA damage in MRC-5 at 25 and 50 $\mu\text{g/mL}$ concentrations through the comet assay at neutral or alkaline pH. **Conclusion:** The tested formulation is biocompatible, and no cytotoxic or genotoxic effects were observed under test conditions, demonstrating viability for future *in vivo* tests.

Keywords: Phytotherapy; Oral Ulcer; Plant Extracts.

■ Introduction

With the strengthening of Public Policies aimed at valuing plant-derived products and their introduction as a therapeutic resource within the Health System, the use of medicinal plants has evolved significantly, supported by the results of scientific studies, which indicate their use as effective and safe therapeutic agents [1]. The population's acceptance of herbal medicine leads to excellent prospects in the product market, including products for dental use [2].

In Dentistry, various materials used in clinical practice can contact oral mucosa cells, gums, or dental tissues. Thus, biocompatibility, carcinogenic, and mutagenic tests of all materials used in the oral cavity are recognized. At first, an *in vitro* cytotoxic test is required, and only from satisfactory results in this first stage can *in vivo* tests be carried out to evaluate the biological behavior of the material [3].

The *Libidibia ferrea* L. species is native to the Brazilian Amazon region and is known as juca. This plant is widely used in folk medicine for many purposes, such as inflammation and pain. The extract concentration can improve wound re-epithelialization, reducing healing time [4].

It was verified that the main constituents of *Libidibia ferrea* Martius fruits were hydrolyzable tannins (gallic acid and ellagic acid) using spectrophotometry and chromatography. From the qualitative and quantitative analysis, it was possible to separate these markers and quantify phenolic compounds by ultraviolet and visible light [5].

Ulcers are common oral cavity lesions, and treatment alternatives are frequently searched for. They are characterized by loss of integrity in the cutaneous or mucosal tissue, changing the anatomical structure, or loss of physiological function of compromised tissues. Aphthous stomatitis is one of the most common pathologies among ulcerated lesions affecting oral mucosa. The prevalence in the general population is reported from 5% to 66%, and its pathogenic hypotheses are numerous and varied for different patients [6].

The disease etiology is not entirely known, and all clinical conditions are symptomatically treated, seeking to reduce inflammation and relieve pain by administering topical or systemic medications [7].

Treating aphthous ulcers is based on anesthetic therapies, protective bio-adhesive products, and periodic topical medications that reduce the frequency or intensity of attacks. Isolated lesions are commonly treated with triamcinolone acetonide or covered with 0.05% clobetasol gel, and in resistant cases, systemic steroids are used.

This study aimed to assess the safety and risk of orabase *Libidibia ferrea* L. ointment by evaluating its cytotoxic and genotoxic capacity in cell culture studies and visualizing its future *in vivo* use in Dentistry.

■ Materials and Methods

Plant Material and Extract Preparation (*L. ferrea* L.)

Libidibia ferrea L. barks were used as raw material. Samples were collected, and voucher specimens (228.022) were deposited at the National Institute of Amazon Research (INPA). Barks were processed at the School of Pharmaceutical Sciences, Federal University of Amazonas (UFAM), Brazil, according to the methodology previously described [8].

Samples were submitted using two procedures: first, the material prepared in the laboratory was air-dried in the shade for 48 hours. Subsequently, the material was air-dried at 40°C in an air-flow oven until residual moisture stabilized, and then the material was milled in a rotary knife mill. The extract was prepared by decoction under reflux in a hydroalcoholic solution: 7.5% ethanol (m/v), 1:1 (v/v). Aseptic principles were used during extract preparation to preserve the material's quality. The method for obtaining the extract followed the established standard, which determined the concentration at 7.5% as the best result.

Juca extract solution was dried in a spray dryer device (MSD 1.0, Labmaq do Brasil, Ribeirão Preto, SP, Brazil).

Orabase Ointment Formulation (*Libidibia ferrea* L.)

The extract concentration in the orabase formulation was determined based on the minimum inhibitory concentration (37.5 mg/mL) determined in the first phase of studies with Juca, tested against oral cavity microorganisms [9]. Following the phytotherapy principles, the ointment, which emphasizes using vegetable raw material without isolates, has 2% *Libidibia ferrea* L. extract and final presentation in a 10g tube. The formulation is composed only of the oil phase represented by petrolatum (80%), mineral oil (17%), carboxymethyl cellulose (0.5%), and propylparaben (0.15%).

Cell Assay

MRC-5 cell fibroblasts were cultured in culture bottles containing Dulbecco's Modified Eagle Medium (DMEM) culture medium supplemented with 10% fetal bovine serum (FBS) and 1% Ampicillin-streptomycin antibiotic. Cells were kept in an incubator with a 5% CO₂ atmosphere at 37 °C [10].

Test Substance Solubilization

The final concentration of the stock solution was 20 mg/ml. For assays, the stock solution (diluted ointment) was diluted in culture medium at 25, 50, and 100 µg/mL concentrations to prepare test substances.

Cytotoxicity Assays

Alamar Blue

The previously cultured cells were transferred to a 0.5 mL Square 96-well microplate and stored in an oven at 37°C with a 5% CO₂ atmosphere. After 24 hours, cells were treated with 25.50 and 100 µg/mL test substances. DMSO (0.2%) was used as a negative control, and as a positive control, Doxorubicin at a concentration of 5.0 µg/mL. The treatment period was 72 hours, with the plate in an oven at 37°C with 5% CO₂. After this period, 10 µL of Alamar blue (0.02%) was added, and plates were subsequently read in an ELISA reader at excitation wavelengths of 530 nm and emission of 590 nm [10].

Trypan Blue

Cells were plated at a density of 1.5×10^4 cells per well in 24-well plates to perform the assay. After 24 h, the medium was removed, and then test substances (juca orabase ointment formulation) were added at concentrations of 25, 50, and 100 µg/mL, negative control DMSO 0.2%, and positive control Doxorubicin 10 µM. After the 72-hour treatment period, cells were harvested and centrifuged at 1500 rpm for 3 minutes [11].

The supernatant was discarded, and the cell pellet was resuspended with 90 µL of complete DMEM medium. After 2 minutes, 10 µL of the cell suspension was transferred to a Neubauer Chamber, 10 µL of Trypan Blue was added, and viable and non-viable cells were counted [11].

For evaluation of morphological changes caused by *L. ferrea* L. ointment, cells were cultivated at a density of 3.0×10^4 cells/mL in 24-well plates and treated with test solution at concentrations of 25, 50, and 100 µg/mL, negative control DMSO 0.2%, and positive control Doxorubicin 10 µM [11].

The supernatant from wells and cells (after trypsinization) was collected in microtubes after 72h. Then, the solution was centrifuged at 3000 rpm for 5 minutes, the supernatant was discarded, and the pellet was resuspended in 200 µL of PBS. After this process, an aliquot of 100 µL was removed and centrifuged for 5 minutes at 1000 rpm. After centrifugation, cells adhered to slides were fixed and stained with the LaborClin® fast panoptic stain kit. Slides were then analyzed under an optical microscope to assess morphological changes by comparing treated and untreated cells [11].

Genotoxicity Test - Comet Assay

MRC-5 cell fibroblasts were treated for 3 hours with the orabase *Libidibia ferrea* L. (Juca) formulation at concentrations of 25, 50, and 100 µg/mL, negative control DMSO 0.2%, and positive control Doxorubicin 10µm [12,13].

After treatment, cells were resuspended in a culture medium with the aid of trypsin and centrifuged for 5 minutes at 1500 rpm. The supernatant was discarded, and the cell pellet was incorporated into a low-melting-point agarose (LMP) solution. Then, the content was transferred to microscope slides previously covered with Normal melting point agarose (NMP) [12,13].

After gelling agarose at a temperature of 4°C, slides were placed in lysis solution (Figure 2) for at least 1 hour for cell and nuclear wall denaturation, leaving the cell's DNA. Slides were then removed from the lysis solution and horizontally placed in the electrophoresis vat filled with electrophoresis solution for 20 minutes to unpack the DNA [12,13].

Electrophoresis was conducted without light for 30 minutes, at 20 Volts for the comet at Alkaline pH and 25 Volts for the comet at Neutral pH. Subsequently, slides were placed in containers containing neutralizing solution for 5 minutes. Finally, the dry slides were fixed in absolute ethanol and stored for later reading. Slides were analyzed under a fluorescence microscope using 40 µL of Syber Green solution (1:10000) [12,13].

Fifty comets were counted per slide (n=2 per exposure in triplicate), being classified according to the amount of fragmented DNA in the comet's tail, indicating the degree of DNA breakage (0 - no damage, 1 - tail smaller than the nucleoid diameter, 2 - tail of the same size as the nucleoid diameter, 3 - tail larger than the nucleoid diameter, and 4 - tail two or more times the nucleoid diameter) [12,13].

As for the DNA damage classification, it could be inferred that Grade 0 has no tail (no damage); Grade 1 has a small tail, smaller than the head diameter; Grade 2 has a tail length twice as long as the head diameter; Grade 3: tail greater than twice the head diameter; Grade 4: Long, fan-shaped, spread tail [12,13]. The results were expressed as damage index and damage frequency.

Statistical Analysis

Experiments were performed in triplicate for cytotoxicity assays against MRC-5 cells, and values obtained were used for IC50 calculations from the dose-response concentration curves, with 95% confidence intervals (95% CI). The statistical analyses of the other tests were performed by one-way analysis of variance (ANOVA), followed by the Tukey and Bonferroni tests for comparison between groups. Values with p<0.05 were considered statistically significant. All experiments were performed in biological triplicate, and data were analyzed using the GraphPad Prism 6.0 software.

■ Results

According to the in vitro alamar blue assay results, one sample did not show high cytotoxicity up to the highest concentration tested. The IC₅₀ value is >50 µg/mL in the MRC-5 strain, with an average percentage of 81.25% cell viability (Figure 1).

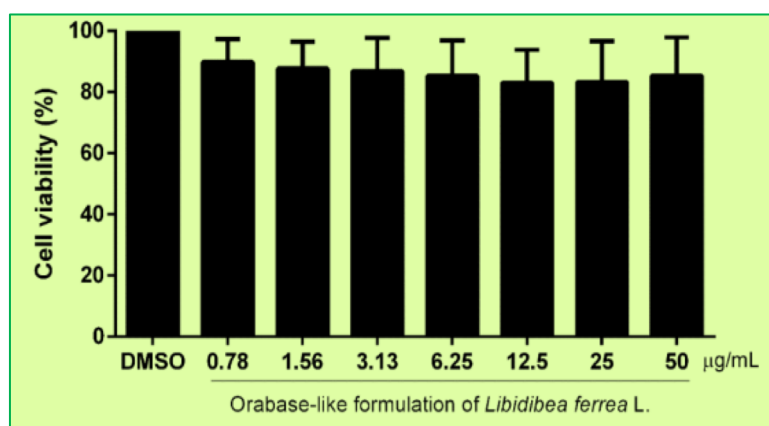


Figure 1. Cytotoxic effect of jucá ointment on human (MRC-5) cell fibroblasts using the Alamar blue assay at 72h treatment time.

As for the cell viability assay, only treatment with the positive control (Doxorubicin) reduced cell viability compared to the negative control ($p < 0.05$). *Libidibia ferrea* L. solutions did not reduce cell viability at the different concentrations tested, as shown in the figure below (Figure 2).

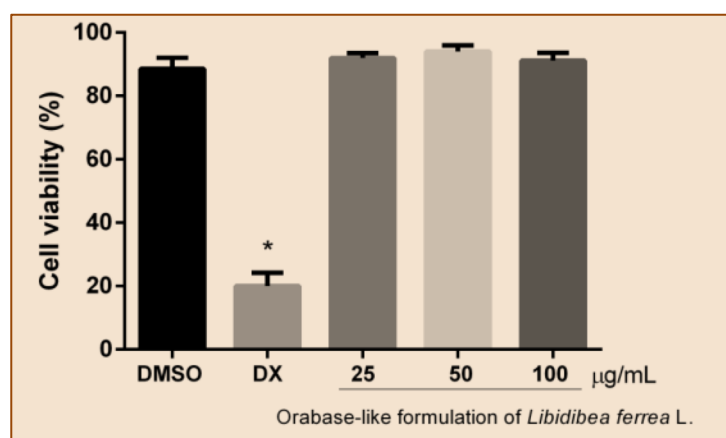
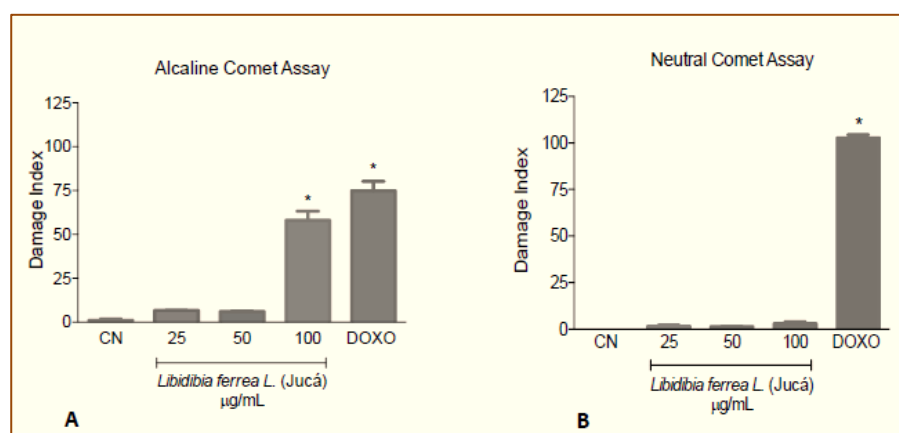


Figure 2. Viability of MRC-5 cells after treatment with *Libidibia ferrea* L. orabase ointment within 72 hours. DX- Doxorubicin (10 µg/mL).

The adopted methodology evaluated genotoxicity using the single-cell comet test at alkaline and neutral pH. Substances of 25, 50, and 100 µg/mL were tested to assess their ability to cause DNA damage in non-neoplastic cells using alkaline pH (Figure 3A) and neutral pH (Figure 3B). The DNA damage index (DI) was used for comparative analysis. This parameter reflects the extent of DNA damage.

When tested at a concentration of 100 µg/mL, it induced DNA damage in MRC-5, with a damage index of 58 on the comet assay in alkaline pH, with the most frequent type of damage being grade 1 (Figure 4). The most frequent types of damage in the test at alkaline pH, after treatments with test substances, were grades 0 and 1. When Doxorubicin was used (10 µg/mL), grades 2 and 3 were the most frequent damage.



Bars represent the mean $\pm$ SD of three independent experiments; *Differences are significant when compared to the control in (dimethyl sulfoxide, used as solvent) at $p < 0.001$; with Tukey's multiple comparison test at one-way ANOVA–Tukey's multiple comparison test.

Figure 3. Evaluation of genotoxicity of *Libidibia ferrea* treatment for three hours using the comet assay in MRC5 cells at alkaline pH (A) and neutral pH (B). The effect of the treatment with *Libidibia ferrea* for three hours at 25, 50, and 100 µg /g induced DNA damage in MRC5 cells using the comet assay.

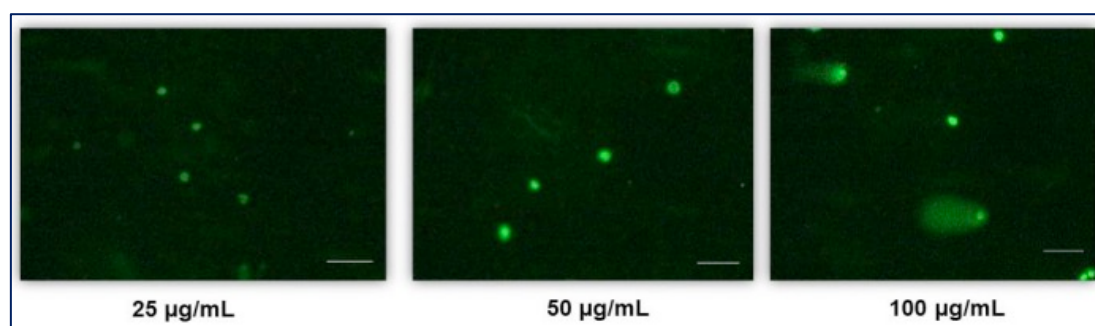


Figure 4. Photomicrographs of the treatment effect of the formulation in orabase of *Libidibia ferrea* L. (jucá) in alkaline pH showing the induction of DNA damage at a concentration of 100µg/mL, with the most frequent type of damage being grade 1.

Under the test conditions of the comet assay at neutral pH, none of the tested concentrations was capable of causing DNA damage (Figure 5), with no statistically significant difference between treatments when compared to the control.

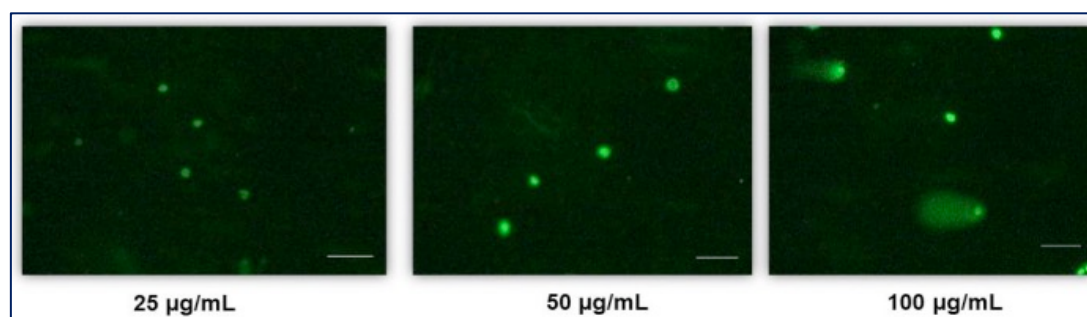


Figure 5. Photomicrographs of the treatment effect of the formulation in orabase of *Libidibia ferrea* L. (jucá) in neutral pH showing no induction of DNA damage.

■ Discussion

Folk medicine has adopted herbal medicine as a common practice, spreading knowledge used by populations over several years and reaching many generations, thus proving the therapeutic potential of several

specimens commonly used. Even though medicinal plants have been part of people's daily lives for many years, there has been a significant increase in interest in recent decades among researchers and health professionals [14–16].

Studies referring to pharmaceutical technological innovation have increased in recent decades due to the popularity of herbal products; therefore, further research to ensure the efficacy and safety of these formulations is extremely important. However, for a plant to become a finished pharmaceutical product, several protocols related to product extraction, guaranteeing the stability of pharmacological properties, *in vitro* studies, and *situ* studies are needed [17]. In this sense, the present study evaluated the *in vitro* cytotoxicity and genotoxicity of an orabase *Libidibia ferrea* L. ointment to promote its safe use in the future, preventing cell and tissue damage.

The Alamar blue stain was used according to the methodology previously described [10] to test different orabase ointment concentrations. Blue indicates non-fluorescent and non-viable cells, and pink indicates fluorescent and viable cells. According to the Alamar blue *in vitro* assay results, the sample did not show high cytotoxicity up to the highest concentration tested. The IC_{50} value is $>50 \mu\text{g/mL}$ in the MRC-5 lineage, with a mean cell viability percentage of 81.25%.

The trypan blue exclusion was another method used to measure cell viability [18]. In this test, the staining or non-staining of the cell is observed for the interpretation of the test. Viable cells can expel trypan blue from their interior, while non-viable cells remain bluish [19].

In other studies with *Libidibia ferrea* L., the group had already obtained favorable results concerning cytotoxicity [20] and evaluated the cytotoxicity through alamar blue, 7.5% ethanolic extract of the Juca stem bark, and no sample analyzed showed toxic effect when in contact with cells (MRC-5), confirming the results of the present study.

The comet assay was used to assess the *in vitro* genotoxicity, in which fragmentation of the nucleus was measured through the movement of DNA, forming the tail when the genetic material was submitted to an electric current in an alkaline or neutral solution. Analysis at alkaline pH can detect different types of DNA damage, and analysis at neutral pH makes it possible to identify the mechanism of DNA strand breakage [21].

In the genotoxicity studies, the first two concentrations tested, 25 and 50 $\mu\text{g/mL}$, could not induce DNA damage in alkaline and neutral comets, proving to be dose-dependent. Results have shown that at a concentration of 100 $\mu\text{g/mL}$, it was able to induce DNA damage in MRC-5, with grade 1 being the most frequent type of damage, and when Doxorubicin (10 $\mu\text{g/mL}$) was used, the most frequent types of damage were grades 2 and 3.

Under these conditions, treatment with the test substances, not even at the highest concentration tested, caused DNA damage; there was no statistically significant difference in any of the treatments when compared to the control. After treatments with test substances, the most frequent types of DNA damage were grades 1 and 2. Doxorubicin (10 $\mu\text{g/mL}$) caused a higher frequency of damage in grades 2, 3, and 4.

■ Conclusion

The orabase *Libidibia ferrea* L. formulation was not considered cytotoxic and genotoxic at the concentrations tested, being a formulation with good safety perspectives for use.

Authors' Contributions

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All authors declare that they contributed to the critical review of intellectual content and approval of the final version to be published.			

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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