

Cytotoxicity and antimicrobial activity of mouthwash obtained from the extract of *Libidibia ferrea* Mart**Citotoxicidade e atividade antimicrobiana de enxaguatório bucal obtido do extrato de *Libidibia ferrea* Mart**

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ABSTRACT

The aim of this research was to evaluate the *in vitro* cytotoxicity and antimicrobial activity of a new mouthwash containing the extract of juca (*Libidibia ferrea* Mart.) against oral microorganisms. The dry plant extract was obtained by decoction of juca dried fruits in a hydroalcoholic solution (1:1, v/v) and submitted to a spray dryer appliance. The mouthwash formulations containing 1.0 % (w/v) of the dry extract were prepared with vehicle composed for: distilled water, sodium benzoate, saccharine, glycerin, Tween, mint essence and 10% sodium hydroxide. Chlorhexidine (0.12 %) and the vehicle were used as positive and negative control, respectively. The oral rinsing formulations from juca were tested at baseline (day 0), 30 and 60 days after the preparation. The cytotoxicity of these formulations was evaluated by hemolysis (2 % (w/w) erythrocytes from *Mus musculus* mice) and cell culture (MRC-5) tests. The antimicrobial activity was evaluated by microdilution against *Streptococcus mutans* (ATCC 25175), *Streptococcus salivarius* (ATCC 7073), *Lactobacillus casei* (ATCC 7469) and *Candida albicans* (DPUA 1706). The mouthwash from juca extract did not promote cytotoxic effect in human fibroblasts. This oral rinsing formulation presented antimicrobial activity against bacteria and fungi common from oral cavity. The mouthwash was considered efficient and safe for using as product of oral health under the conditions studied.

Keywords: Juca, Oral health, Phytotherapy, Biological activity.

RESUMO

O objetivo deste trabalho foi avaliar a citotoxicidade *in vitro* e a atividade antimicrobiana de um enxaguatório bucal contendo o extrato de jucá (*Libidibia ferrea* Mart.) contra micro-organismos orais. O extrato seco da planta foi obtido por decocção de frutos secos de jucá em solução hidroalcoólica (1: 1, v / v) e submetido a um aparelho de secagem por atomização. As formulações bucais contendo 1,0% (p / v) do extrato seco foram preparadas com água, conservante, surfactante e solução tampão. O enxaguatório bucal de clorexidina (0,12%) e o veículo foram utilizados como controle positivo e negativo, respectivamente. As formulações de enxaguante de jucá foram testadas no início (dia 0), 30 e 60 dias após a preparação. A citotoxicidade dessas formulações foi avaliada por hemólise (eritrócitos a 2% (p / p) de camundongos *Mus musculus*) e cultura de células (MRC-5). A atividade antimicrobiana foi avaliada por microdiluição contra *Streptococcus mutans* (ATCC 25175), *Streptococcus salivarius* (ATCC 7073), *Lactobacillus casei* (ATCC 7469) e *Candida albicans* (DPUA 1706). O enxaguatório bucal do extrato de jucá não promoveu efeito citotóxico em fibroblastos humanos. Esta formulação de enxaguante bucal apresentou atividade antimicrobiana contra bactérias e fungos comuns da cavidade oral. O enxaguatório bucal foi considerado eficiente e seguro para uso como produto de saúde bucal nas condições estudadas.

Palavras-chave: Jucá, Saúde oral, Fitoterapia, Atividade biológica.

1 INTRODUCTION

The main species of *Libidibia* (*Caesalpinaceae* family) genus are native from Brazil, and they can be found in different regions of the country. In Brazilian Amazon region, *Libidibia* (*Caesalpinia*) *ferrea* var. *parvifolia* (Mart. ex Tul.) L. P. Queiroz (*L. ferrea*), is known as iron wood or juca. This plant is widely used by folk medicine for many purposes (Ferreira and Soares, 2015). Different parts of the plant have been already evaluated for several therapeutic properties such as: anti-inflammatory, analgesic, antimicrobial, antipyretic and wound healing (Sampaio et al., 2009; De Araujo et al., 2014).

Fruits of *L. ferrea* are important sources of polyphenols. The total phenol content in hydroalcoholic extracts of fruits ranges from 7.3 up to 28.0 %, reaching values of 460 mg/g of gallic acid². Condensed and hydrolysable tannins were frequently identified¹ and ellagic acid, gallic acid and methyl gallate were already isolated supporting that a variety of phenolic compounds can be found in *L. ferrea* fruits (Ueda et al., 2001; Pedrosa et al., 2016).

The antimicrobial activity of polyphenols from medicinal plants against oral pathogens has particularly interest (Jeon et al., 2011). If a direct destructive effect does not occur it is expected that polyphenols interfere on many bacterial regulatory mechanisms (e.g. quorum sensing) with or without a measurable effect on bacterial growth (Silva et al., 2016). Thus, there is a good chance to obtain pharmaceutical formulations based on polyphenols from plants for future clinical trials.

In the study with a mouthwash formulation containing plant extract to control dental biofilms was tested its stability under several parameters (pH, density, sedimentation, and time stability up to 60 days) (Marreiro et al., 2014a). The organoleptic properties were also monitored and the minimum inhibitory concentration (MIC) values against *Streptococcus mutans* (*S. mutans*) and *Streptococcus oralis* (*S. oralis*) were 4.3 and 3.7 µg/mL, respectively (Venâncio et al., 2015).

The purpose of this research was to evaluate the cytotoxicity and antimicrobial activity of a new mouthwash containing extract of *L. ferrea* against oral microorganisms.

2 MATERIAL AND METHODS

Samples of *L. ferrea* dry fruit were collected and voucher specimens (228.022) were deposited at the National Institute of Amazon Research (INPA). The fruits were processed at the School of Pharmaceutical Sciences, Federal University of Amazonas (UFAM), Brazil.

The extraction process was carried out according to the methodology described by Venâncio et al. (2015). A sample of 7.5 g of the plant was submitted to decoction in a hydroalcoholic solution (1:1, v/v) for 15 minutes. The plant extract was cooled, filtered and dried in a spray dryer appliance (MSD 1.0, Labmaq, Ribeirão Preto, São Paulo, Brazil).

The characterization of *L. ferrea* extract by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) was previously performed and published by Pedrosa et al. (2016).

2.1 MOUTHWASH FORMULATION

The components of the product were: distilled water, *L. ferrea* extract, saccharin, glycerin, mint essence, sodium benzoate, surfactants and sodium hydroxide. The plant extract ranged a final concentration of 1 % in the mouthwash.

The mouthwash was subjected to a natural aging process under controlled environmental conditions (room temperature of 24°C - 26°C) for 30 and 60 days. A standard mouthwash solution was subjected to immediate evaluation starting at day 0 (baseline).

A solution of 0.12 % chlorhexidine digluconate (Sigma-Aldrich, St. Louis-MO, USA) and a mouthwash solution containing only the vehicle components were used as positive and negative control, respectively. The plant extract without any components of the mouthwash was also tested. Finally, the six testing groups were: chlorhexidine mouthwash as positive control (PC), mouthwash components as negative control (NC), *L. ferrea* hydroalcoholic extract (Lf-E), *L. ferrea* mouthwash day 0 (Lf-0), *L. ferrea* mouthwash day 30 (Lf-30) and *L. ferrea* mouthwash day 60 (Lf-60).

2.2 CYTOTOXICITY ASSAYS

2.2.1 Hemolysis Test

The hemolysis test was performed as described by Fischer et al. (2003). A solution of erythrocytes (2.0 %, v/v) was prepared using blood samples (1 mL) collected from *Mus musculus* mice (EAEC-UFAM 116/2012). Then, 1 mL of blood was added to 10 mL of saline solution (0.85 % sodium chloride and 10 mM calcium chloride) and centrifuged (700 g / 5 min) (5804R Centrifuge / Eppendorf / AG / Germany). The supernatant was removed, resuspended in 10 mL of saline solution and centrifuged at every cycle. At the end of 3 cycles, the plasma (supernatant) was removed and 2 mL of the red blood cells were transferred (infranatant) to 98 mL of phosphate buffer solution.

The assays were performed in a 96-wells plate. In each well were added 100 µL of the extracts test and 100 µL of the erythrocyte solution. For this assay, 0.2 % of Triton™ X-100 (PBST, Sigma-Aldrich, St. Louis-MO, USA) was used as positive control and DMSO (Dimethyl Sulfoxide) was used as negative control. All the substances were tested in triplicate. The plate was incubated (1 h / 25°C) under constant agitation and centrifuged (900 g / 10 min). After the visual reading, according to the intensity of color in the supernatant, it was transferred to another plate and subjected to a spectrophotometer at 540 nm (Multimode Detector DTX 800 - Beckman Coulter). Less than 10 % of hemolysis was considered as non-toxic effect.

2.2.2 Fibroblast Cells Assay

The fibroblasts MRC-5 cells were grown in DMEM medium supplemented with 10 % of fetal bovine serum, 2 mM glutamine, 100 mg/mL streptomycin and 100 U/mL of penicillin. They were incubated at 37 °C with a 5 % CO₂ atmosphere.

The Alamar Blue assay for measuring the cytotoxicity was performed for all testing groups according to Ahmed et al. (1994). The MRC-5 cells were seeded at a density of 1×10^4 in 100 µL of DMEM per well in a 96-well microplate. After 24 hours of seeding, the cells were treated with 100 µL of the extracts (250-1.95 µg/mL) during 24 hours. For this test, doxorubicin was used as positive control (25-0.19 µg/mL) and PBS (phosphate buffer solution) was used as negative control. After the treatment period, 10 µL of Alamar Blue (0.02 %) was added to each well for 3 h. Then, the fluorescence was measured in an ELISA reader (Thermofisher, Synergy HT. Detector, Biotek, São Paulo, Brazil).

2.3 ANTIMICROBIAL ACTIVITY

The following microorganisms were used in the microbiological assay: *S. mutans* (ATCC 25175), *S. salivarius* (ATCC 7073), *Lactobacillus casei* (*L. casei*) (ATCC 7469) and *Candida albicans* (*C. albicans*) (DPUA 1706).

The bacteria strains were provided by the National Institute of Health Quality Control of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) and the yeast culture from the DPUA Culture Collection, UFAM. The bacteria were cultivated in brain heart infusion medium (BHI) (Difco™, MI, USA) and *C. albicans* in Sabouraud-dextrose (Difco™, MI, USA). All microorganisms were incubated under aerobic conditions at 37 °C. They were reactivated in nutrient broth BHI (37 °C / 24 h) under aerophilic conditions for *S. salivarius* and *L. casei*, and microaerophilic conditions for *S. mutans*. For the yeast, the reactivation was done on Sabouraud-dextrose broth (37 °C / 48 h) in aerophilic conditions.

The inoculum of the microorganisms was prepared under aseptic conditions: for bacteria, culture aliquots from BHI broth were inoculated into 5 mL of sterile saline solution. For *C. albicans*, the inoculum was prepared from young colonies with the aid of a sterile platinum loop (5 mm diameter) in a test tube containing sterile saline solution. The suspensions were vigorously mixed in a vortex mixer until they became homogeneous.

The standardization of the inoculum was performed according to the 0.5 McFarland scale to provide a pattern of 10^8 CFU.mL⁻¹ for bacteria and 10^6 cells.mL⁻¹ for the yeast.

2.4 MINIMUM INHIBITORY CONCENTRATION (MIC)

The MIC was determined using the resazurin microdilution broth assay in microplates (Sampaio et al., 2009; CLSI, 2011; Andrews 2001).

Sterile 96-well microplates (U-bottom shape) were used for dilution, totalizing 100 μ L of final volume in each plate. A standard volume of inoculum (20 μ L) was added in the wells, except in controls for sterility of the testing groups (plant extract or mouthwash + medium without inoculum). A solution of 0.12 % chlorhexidine digluconate (Sigma-Aldrich, St. Louis-MO, USA) was used as positive control (PC). A test for the viability of microorganisms (medium + inoculum without any test product) was also prepared.

The microplates were sealed and incubated (37 °C / 24 h) in aerophilic conditions for *S. salivarius*, *L. casei* and *C. albicans*, and microaerophilic conditions for *S. mutans*. After the incubation period, 30 μ L of 0.01 % resazurin aqueous solution was placed. Readings were carried out after 1 h of incubation (Andrews 2001). The *L. ferrea* mouthwash was distributed in log dilution according to extract concentration (mg/mL).

The color change (blue to pink) represented the presence of viable cells after exposition to the tested product. For confirmation of the MIC aliquots of each well were taken and incubated in BHI agar distributed in Petri dishes (*S. mutans*, *S. salivarius* and *L. casei*), and in Sabouraud agar (*C. albicans*). The bacteria and yeast cultures were incubated as described to confirm the growth of microorganisms.

2.5 STATISTICAL ANALYSIS

The results of hemolysis tests and antimicrobial activity were tabulated and described by descriptive statistics and for the cell culture test results were obtained by non-linear regression and IC₅₀ values with 95 % confidence intervals (95 % CI).

3 RESULTS

L. ferrea dry extract composition was analyzed by LC–MS/MS. This method evidenced the presence of phenolic acids and flavonoids as the substances of higher amount and, in minor proportions, were determined the presence of organic acids, secoridoids and phytohormones. The main components were kaempferol, gallic acid and quinic acids, respectively (Pedrosa et al., 2016). The results of hemolysis test are shown on Table 1.

Table 1: Hemolysis Test results after statistical analysis of EC₅₀ (µg / mL)

Codes	Products	EC ₅₀ values (µg/ mL)
PC	0,12 % Chlorhexidine	75.14 (69.83 - 80.86)
Lf-0	<i>L. ferrea</i> mouthwash (time 0)	> 2000
Lf-30	<i>L. ferrea</i> mouthwash (30 days)	> 2000
Lf-60	<i>L. ferrea</i> mouthwash (60 days)	> 2000
Lf-E	<i>L. ferrea</i> extract	> 2000
NC	Vehicle of mouthwash	300.5 (259.1 - 348.6)

The test in fibroblasts cells by Alamar Blue™ method showed that none of the samples tested was able to induce cytotoxic effect with IC₅₀ values (Inhibitory Concentration) above 50 µg / mL (Table 2).

Table 2: Alamar Blue Cytotoxicity Test results

Codes	Products	IC ₅₀ values (µg/mL)
PC	0.12 % Chlorhexidine	> 50
Lf-0	<i>L. ferrea</i> mouthwash (time 0)	> 50
Lf-30	<i>L. ferrea</i> mouthwash (30 days)	> 50
Lf-60	<i>L. ferrea</i> mouthwash (60 days)	> 50
Lf-E	<i>L. ferrea</i> extract	> 50
NC	Vehicle of mouthwash	> 50

IC₅₀ – Inhibitory Concentration
Statistical analysis and IC₅₀ values obtained by a nonlinear regression/
GraphPad Prism 5.0 program.

Table 3 shows the MIC and Minimal Bactericidal Concentration (MBC) values of the mouthwash from *L. ferrea* extract demonstrating its antimicrobial activity against all the microorganisms tested. The results of MIC values were 300.0 µg/mL against *S. mutans* and *S. salivarius*, corresponding to 22.0 µg/mL of the extract used. The Minimal Fungicidal Concentration (MFC) of the *L. ferrea* mouthwash was 250.0 µg/mL against *C. albicans*.

Table 3. Minimal Inhibitory Concentration (MIC), Minimal Bactericidal Concentration (MBC) and Minimal Fungicidal Concentration (MFC)

Microorganisms	<i>L. ferrea</i> mouthwash		<i>L. ferrea</i> extract		CHX 0,12 %	PC	Vehicle of mouthwash	
	Lf-0	Lf-E	Lf-0	Lf-E	PC	NC	MBC/	MBC/
	MIC	MBC/	MIC	MBC/	MIC	MBC/	MIC	MBC/
		MFC		MFC		MFC		MFC
<i>S. mutans</i>	300.0	550.0	22.0	41.0	-	300.0	-	-
<i>S. salivarius</i>	300.0	650.0	22.0	48.0	-	300.0	59400.0	-
<i>L. casei</i>	-	250.0	-	18.0	-	300.0	29700.0	49500.0
<i>C. albicans</i>	-	250.0	-	18.0	-	300.0	-	24750.0

The other components tested did not show any antimicrobial activity.

4 DISCUSSION

The plant extract mouthwash was effective in reducing plaque and gingivitis. This natural product may be considered as a good alternative for using like oral rinsing due the chlorhexidine mouthwash promotes many side effects (staining of the teeth and restorations, mucosa desquamation, and taste changes), which limit its acceptability and long-term use (De Araújo et al., 2018).

A possible limitation of this new and natural product is the color. It can decrease the prolonged use of this mouthwash. This is what already happens with the use of chlorhexidine. However, empirical observations as the use of infusion and decoction by the local population show that the use of the plant does not result in significant tooth staining.

However, in order to improve the use of *L. ferrea* mouthwash in Dentistry and to confirm its efficacy, it is necessary to subject the product to diverse tests. One of them is the performance when used in the oral cavity and then use the product in daily clinical routine (Venâncio et al., 2015; Oliveira et al., 2011).

In this research, the use of a new herbal mouthwash from extracts of *L. ferrea*, is considered ecological correct, because it does not compromise the nature, since the *L. ferrea* plant is not destroyed and only the fruits are used. This plant has been utilized by folk medicine and is composed of phenolic compounds as condensed tannins, catechins and hydrolysable tannins such as gallic acid and ellagic acid (Ferreira and Soares, 2015; Vasconcelos et al, 2011).

The isolation of active components from *L. ferrea* extract by successive high performance liquid chromatography was performed. The results showed the presence of two compounds that were identified as gallic acid and epicatechin by retention time and spectral comparisons of respective standards (Pedrosa et al., 2016).

The crude extract contains anthraquinones, alkaloids, depsides, depsidones, flavonoids, lactones, saponins, sugars, sesquiterpenes and triterpenes, but tannins are regarded as the major component (Souza et al., 2006). The aqueous and acetone water extracts are active against all Gram-positive bacteria and against some Gram-negative bacteria (De Araújo et al., 2014).

The mouthwash was evaluated according to pharmacological stability and quality conditions, showing to be absent from microorganisms and no changes were observed in the organoleptics and sedimentation characteristics. The average pH values were 6.21, 6.15 and 5.85 at 0, 30 and 60 days, respectively, and density values of 1.029, 1.033 and 1.035 g/mL, respectively, without interfering in the final characteristics of the formulation (Venâncio et al., 2015). Souza et al. (2020) analyzed the aforementioned formulation with the objective of evaluating its stability, in three storage conditions and in six time intervals, demonstrating favorable conditions of stability and absence of microorganisms.

In the process of evaluation and validation of a product, it is necessary to prove the benefits of its use, its effectiveness as drug and its risks to human health, animals and the environment. Therefore, it is essential to conduct toxicological tests to evaluate the safety and the risk / benefit of possible therapeutic use of this new product (Evans, 2009).

Thus, this study is justified as a way to use a medicinal herb extract in dentistry therapeutic practice. The analysis of the mouthwash formulation based on *L. ferrea* extract using cytotoxicity tests is important because herbal solutions are more affordable and have wide drug benefits.

In the hemolysis test it was found that the *L. ferrea* mouthwash showed no hemolytic activity at the times studied. However, the vehicle and the 0.12 % chlorhexidine mouthwashes caused hemolysis with EC₅₀ of 75.14 and 300.5 µg / mL, respectively. This result is considered satisfactory since the concentration of vehicle in the mouthwash formulation is lower than the effective concentration of hemolysis.

According to the Alamar BlueTM method for cell culture test it was found that no tested sample was able to induce a cytotoxic effect in contact with the cells. The values of IC₅₀ above 50 µg / mL demonstrated the absence of cytotoxicity under the tested conditions.

The results obtained by Marreiro et al. (2014b), using the same cytotoxicity assay methodology of this study, showed that both formulations (mouthwash and vehicle of *L. ferrea*) were cytotoxic causing the fibroblasts death. The mouthwash presented feasibility cell of 8 % and the

vehicle of 7 % when compared to the positive control. However, when the study was carried out *in vivo*, the toxicity of the product showed no inflammatory changes.

The antimicrobial activity of *L. ferrea* fruit extract in the research of Sampaio et al. (2009) was carried out against the same oral pathogens of this study, using the microdilution method for planktonic cells (MIC) and a multispecies biofilm model. The results showed in MIC values of 40.0 µg/mL, 100.0 µg/mL, 66.0 µg/mL, 66.0 µg/mL and 25.0 µg/mL for *S. mutans*, *S. oralis*, *S. salivarius*, *L. casei* and *C. albicans*, respectively. For the biofilm assay, chlorhexidine and plant extract showed no growth at 10^{-4} and 10^{-5} microbial dilution, respectively. The study concluded that *L. ferrea* fruit extract can inhibit *in vitro* growth of oral pathogens in planktonic and biofilm models supporting its use against oral infections.

Although this study did not tested multispecies biofilms, it has great perspective to be efficient *in vivo*, since the extract *in vitro* had antimicrobial activity against oral microorganisms. About this topic, Moreira et al. (2013) affirmed that *in vitro* studies have limitations conditions, no matter how much they approximate to real clinical situations they do not reproduce the real oral conditions, which are known to be extremely complex. However, *in vitro* researches have their values as initial screening tests, which may suggest valuable future clinical trials or observational studies.

Jeon et al. (2011) and Moreira et al. (2013) affirmed that bacteria in classical MIC/MBC assays are in suspension, whereas the oral bacteria associated with caries are enmeshed in the plaque biofilm matrix. There is a plethora of evidence showing that biofilm cells are more resistant to antimicrobials than cells in suspension. Thus, describing antibacterial activity against planktonic oral bacteria with constant exposure to an agent at a high concentration over a period of 24 hour is relevant to know how oral bacteria would respond in the mouth.

Marreiro et al. (2014a) evaluated the *in vitro* antimicrobial activity of a mouthwash containing *L. ferrea* extract to control oral biofilm using the same methodology of this study. The results showed MIC values against *S. mutans*, *S. oralis* and *L. casei* in concentrations of 4.37 µg/mL, 3.75 µg/mL and 4.37 µg/mL, respectively.

Candida albicans was the most susceptible microorganism in this study since all tested compounds had fungicidal action forward to this yeast in all tested concentrations. This result is important because Canela et al. (2018) showed that *Candida* spp. is responsible for 80 % of all systemic fungal infections and is associated with high mortality rates. *C. albicans* was the predominant specie (44 %) involved.

According to Alavarce et al. (2015) with the increasing development of antifungal resistance, there is a persistent requirement in research for newer effective agents. Thus, our results suggest the

potential use of *L. ferrea* extract in the treatment of infectious conditions such as candidiasis and denture stomatitis.

The mouthwashes used to control plaque biofilms may be very important since these agents can remove these biofilms and/or kill disease-associated with bacteria; they can also even inhibit at sub-lethal levels the expression of pathogenic traits (Marsh, 2010). In this way, the beneficial activities of the resident oral microbiota will be retained and the risk of dysbiosis occurring will be reduced (Marsh; Head; Devine, 2014).

Studies of antimicrobial activity have been carried out with several medicinal plants indicating great potential for combating several diseases (Antunes et al., 2020; Martins and Casali, 2019).

Jeon et al. (2011) and Jain et al. (2015) affirmed that natural products remain a largely unexplored source of effective antibiofilm molecules with potentially low toxicity that could be used in alternative or adjunctive anticaries therapies.

This study suggests that *L. ferrea* mouthwash would be a future alternative choice for prevention of caries and oral candidiasis. For that reason, further investigation must be conducted to allow the formulation of this product as an adjuvant to oral health maintenance.

5 CONCLUSION

L. ferrea mouthwash (1%, v/v) is not cytotoxic in human fibroblasts and has antimicrobial activity against some oral pathogenic microorganisms. According to the *in vitro* tests, the results support the use of *L. ferrea* extract for oral health due its biofilm inhibition and related diseases as dental caries and candidiasis. Clinical trials with this antimicrobial product are warranted to the determination of its efficacy and safety parameters *in vivo*.

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