Conjecturas

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Cytotoxicity and potential anti-inflammatory activity of Libidibia ferrea L. extract on RAW264.7 macrophages

Citotoxicidade e potencial atividade anti-inflamatória do extrato de Libidibia ferrea L. em macrófagos RAW264.7

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RESUMO

Libidibia ferrea L, is extensively used in traditional medicine due to its many pharmacological benefits. This study investigated the non-cytotoxic in in-vitro concentration and the anti-inflammatory effects of the hydroalcoholic extract of the bark of *L. ferrea* in LPS (lipopolysaccharide from Escherichia coli) stimulated RAW 264.7 cells. Different concentrations of *L. ferrea* (100 µg/mL; 50 µg/mL; 12,5 µg/mL; 6,25 µg/mL; 3,12 µg/mL; 1,56 µg/mL), medium culture (negative control), LPS (positive control) and dexamethasone (standard drug) was applied to 1 µg/mL LPS-induced RAW 264.7 macrophages in vitro for 24 h. Cytotoxicity was investigated using MTT (3-(4.5-dimethylthiazol-2yl|-2.5diphenyltetrazolium bromide) colorimetric assay. The production of nitric oxide (NO) was determined using the Griess Reaction. The extract showed no significant cytotoxicity at concentrations from 1,56 to 100 µg/mL (cell viability>80%) in the MTT cell viability assay after 24 h of treatment. The minimum anti-inflammatory concentration of *L. ferrea* extract based on the release of NO by macrophages was 50 and 100 µg/mL ($p<0.05$), showing better results than the dexamethasone (standard drug) ($p<0.05$). This study suggests *L. ferrea* anti-inflammatory properties.

Palavras-chave: Inflammation; Nitric Oxide; Cytotoxicity; Herbal Medicine.

ABSTRACT

Libidibia ferrea L, é amplamente utilizada na medicina tradicional devido aos seus muitos benefícios farmacológicos. Este estudo investigou a concentração in vitro não citotóxica e os efeitos antiinflamatórios do extrato hidroalcoólico da casca de *L. ferrea* em células RAW 264.7 estimuladas por LPS (lipopolissacarídeo de Escherichia coli). Diferentes concentrações de *L. ferrea* (100 µg/mL; 50 µg/mL; 12,5 µg/mL; 6,25 µg/mL; 3,12 µg/mL; 1,56 µg/mL), meio de cultura (negativo controle), LPS (controle positivo) e dexametasona (droga padrão) foram aplicados em 1 µg/mL de macrófagos RAW 264.7 e induzidos por LPS in vitro por 24 h. A citotoxicidade foi investigada pelo ensaio colorimétrico MTT (brometo de 3-(4,5-dimetiltiazol-2il|-2,5difeniltetrazólio) e a produção de óxido nítrico (NO) foi determinada pela Reação de Griess. O extrato não apresentou citotoxicidade significativa nas concentrações de 1,56 a 100 µg/mL (viabilidade celular >80%) no ensaio de MTT após 24 h de tratamento. A concentração anti-inflamatória mínima do extrato de *L. ferrea* com base na liberação de NO pelos macrófagos foi de 50 e 100 µg/mL

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(p<0,05), mostrando melhores resultados que a dexametasona (medicamento padrão) (p<0,05). Este estudo sugere propriedades anti-inflamatórias de *L. ferrea.*

Keywords: Inflamação; Óxido nítrico; Citotoxicidade; Fitoterapia.

INTRODUCTION

The inflammatory reaction is one of the primary host defense responses against tissue aggression like infectious agents, and physical trauma (Dong et al., 2017). When properly regulated, the inflammatory response assists repair and restoration of tissue functions (Jia et al., 2017). However, exacerbated and persistent contact results in pain, heat, flushing, edema, and loss of function which is characterized as the disease state, such as oral lesions of inflammatory origin. Therefore, the control of the inflammatory process is key to avoid significant tissue damage (Park et al., 2016).

Inflammation is an important response to different stimuli (chemical, biological or physical). During this process, several molecular and cellular effects are responsible for the initiation and regulation of the interactions between cells (Guo et al., 2012; Germolec et al., 2018). In inflammation, the damaged tissue is infiltrated by cells such as macrophages, neutrophils, and lymphocytes, that produce mediators responsible for the process of exacerbation (Park et al., 2016).

Macrophages play a key role in inflammation since they produce free radicals such as nitric oxide (NO) and proinflammatory cytokines (Jia et al., 2017; Park et al., 2016; Wang et al., 2017). Several studies have shown that the excessive activity of macrophages results in inflammatory disorders (Kolaczkowska and Kubes, 2013; Mocsai, 2013). Therefore, the inhibition of the production of pro-inflammatory factors by heath cells has been considered promising for inflammatory disease control. The oral mucosa act as a physical barrier to the outside world is covered with stratified squamous epithelium, which provides a flexible covering of the underlying structures as well as a barrier to pathogens (Qin et al., 2017). Inflammatory diseases of the oral mucosa, associated or not with the presence of microorganisms, are part of several health problems in Dentistry. A few examples are gingivitis and periodontitis, which are prevalent in adults (Michaud et al., 2017).

Herbal medicine is the study of medicinal plants and their applicability in the treatment of diseases in general (Venâncio et al., 2015). For therapeutic purposes, the main anti-inflammatory agents used in dentistry are no steroidal and steroidal compounds where the prolonged usage of these compounds can cause adverse effects (De Oliveira et al., 2014). In this context, phytotherapy enabled new paths of study within dentistry, as new therapeutic methods for the treatment of oral diseases.

Medicinal plants and herbal medicines are effective therapeutic agents for the treatment and prevention of numerous pathologies because of their easy access, low adverse effects, and high acceptance. Thus, many studies have been implemented for search of new therapeutic properties and the determination of their safety (Oliveira et al., 2014). In dentistry, several studies have been performed to evaluate plant species, like *Libidibia ferrea* as new agents that can provide biocompatible products with lower toxicity. Natural products that combat the process of oral diseases, such as caries periodontal diseases, and oral candidiasis, could be used as important therapeutic agents (Marreiro et al., 2014).

L. ferrea var. parvifolia (Mart. ex Tul.) L. P. Queiroz (Leguminosae), basionym Caesalpinia ferrea Mart. ex Tul. (Caesalpiniaceae) is a medium-sized angiosperm native to Brazil that is commonly known as "jucá" or ‘’pau-ferro’' and found throughout Brazil, mainly in the northeast and north regions (Freitas et al., 2012). This plant has demonstrated great potential for the future due to its anti-inflammatory properties (Carvalho et al., 1996, Freitas et al., 2012, Ferreira et al., 2019; Falcão et al., 2019). This study aimed to analyze the in vitro extract the cytotoxic effect of its extract in culture and to evaluate the extract minimum anti-inflammatory concentration on macrophages cell culture. The hypothesis was that the extract does not affect cellular viability and has promising anti-inflammatory properties since it reduces NO production.

METHODOLOGY

The bark samples of the botanic species *L. ferrea* was supervised by an ethnobotanist, carried out in the city of Manaus in the National Institute of Amazon Surveys (INPA), and registered under number 228-022. These samples were transported to the Faculty of Pharmaceutical Sciences (FCF) of the Federal University of Amazonas (UFAM) and processed following Venâncio et al. (2015) methodology. The extractive hydroalcoholic solution of L. ferrea was prepared by infusion under the concentration of

7.5% (w/v), using water and alcohol as extracting liquids. Then, the material was filtered and removed to Spray Dryer appliance (MSD 1.0, Labmaq, São Paulo, Brazil) to obtain the powdered extract by spray drying to maintain stability. The dry extract solution was prepared based on the minimum inhibitory concentration defined in previous research (Oliveira et al., 2013).

The hydroalcoholic extract of *L. ferrea* was solubilized in methanol at 1 mg ml-1 and subjected to analysis by high-performance liquid chromatography (HPLC) on the chromatograph Accela system (Thermo Scientific, Waltham, USA), to find phenolic compounds. The separation occurred in the Luna C18 Columm (5 µm, 150 × 4.6 mm d.i.) (Phenomenex, Torrance, CA, USA), with an injection volume of 10 µl. The movable phase was composed of a solution of 1% acid formic (A) and methanol (B) with a flow rate of 1 ml min-1 . The elution took place in gradients mode, starting in a variation from 20% (B) to 80% (B) for 10 min. The chromatograph was equipped with diode array detectors (DAD) Where spectra were obtained in the triple quadrupole mass spectrometer detector model TSQ Quatum Access (Thermo Scientific, Waltham, MA USA), using an interface with Atmospheric Pressure Chemical Ionization (APCI) in negative mode and ion fragmentation. The presence of gallic acid was defined by comparison with retention time (Rt) of the standard and ions observed in the mass spectrum and other peaks observed in the chromatogram were identified by comparison data in the literature.

The murine macrophage cell line, RAW 264.7 (ATCC® TIB-71™), was obtained from the Faculty of Pharmaceutical Sciences, the Federal University of Amazonas and maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco® Invitrogen, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco® Invitrogen, Grand Island, NY, USA) and penicillin/streptomycin (100 IU/ml/ 100 µg/ml; Gibco® Invitrogen, Grand Island, NY, USA) in a humidified atmosphere at 37°C under 5% CO2. The cells were sub-cultured every 2 days.

Cell viability was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) assay. This assay is used to detect living cells, and the signal generated depends on the degree of cell activation (Mosmann, 1983). Cells were seeded into 96-well plates at a density of 1 × 106 cells/well and maintained in a humid incubator (5% CO2/ 95% air, 37°C) for 24 h. Then cells were treated with the *L. ferrea* extract

(1.56, 3.12, 6.25, 12.5, 50 and 100 µg/ml) in the presence of lipopolysaccharide from Escherichia coli (LPS, 1 µg/ml) and incubated (5% CO2/ 95% air, 37°C) for 24 h. The DMEM medium and LPS were negative and positive control groups, respectively. The dexamethasone (20 µg/ml) was used as a standard group. The cell medium was then discarded, the MTT (5 mg/ml, 10 µl/well; Sigma Chemical Co., St. Louis, MO, USA) was added with DMEM (50 µl/well) and incubated for 3 h (5% CO2/ 95% air, 37°C). 100 µl dimethyl sulfoxide (DMSO) was then added to dissolve the formazan crystals released by the metabolically active cells. The optical density was assessed at 560 nm by a microplate reader (Spectrophotometer DTX - 800 Beckman Coulter, USA), and the relative cell viability was calculated.

The production of nitric oxide (NO) was measured using the Griess method described by Green et al. (1982) with modifications. Oxide nitric detection in the experimental samples, nitrite (NO2) production, was measured in the supernatants using the Griess assay. 100 µl of the supernatant sample was incubated with an equal volume of the Griess reagent (Sigma Chemical Co., St. Louis, MO, USA) at room temperature for 30 min. The absorbance was measured at 540 nm by a microplate reader (Spectrophotometer DTX-800 Beckman Coulter, USA). The NO2 concentration (µmoles) was determined by comparison with a standard curve for NaNO2 at a concentration range from 1.5 to 200 µM.

All data were expressed as mean ± standard deviation (SD) and the normal distribution was confirmed by the Kolmogorov-Smirnov test. The results were analyzed by one-way ANOVA test using the statistical software package, GraphPad Prism 5.0 software, followed by Tukey's and Dunnett's tests. $p<0.05$ was considered statistically significant.

RESULTS

The phytochemical profile detected the presence of innumerable compounds (tannins, flavonoids, polyphenols), and among them gallic acid, ellagic, orientin, dihydroxybenzoate diglycoside, and isorhamnetin according to the chromatogram shown (Figure 1 and Table 1).

Figure 1 - Cromatogram of the dry extract of the stem bark of *L. ferrea*.

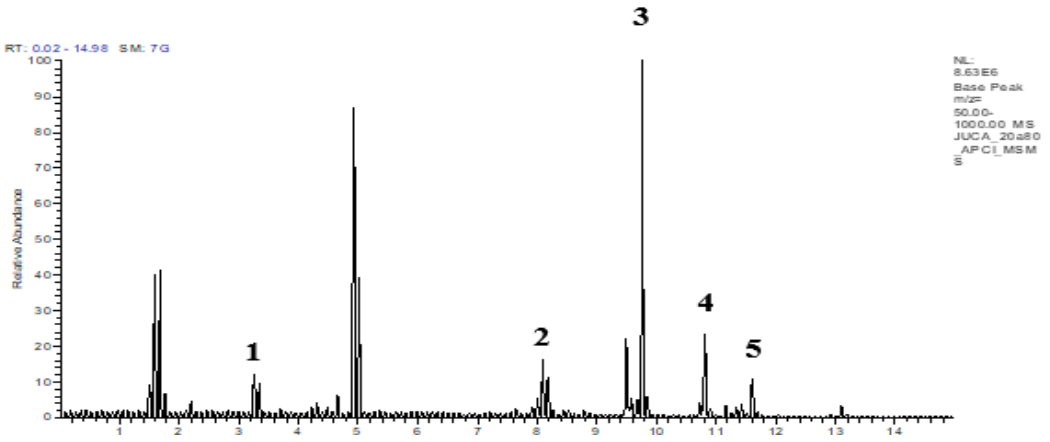
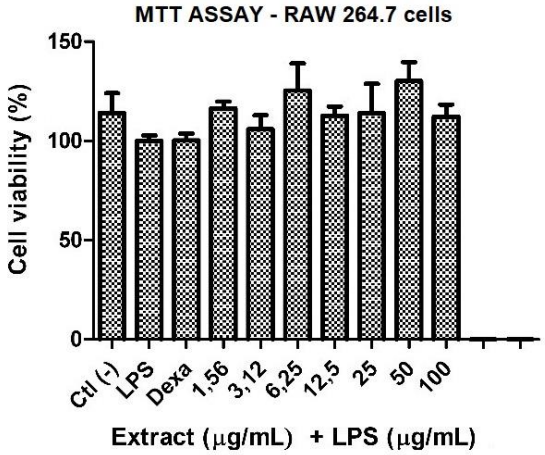


Table 1- Substances characterized by LC-MS / MS

Peak	Time Retetion	[M-H]⁻	Fragmentations	Substances	λ_{max}/nm	References
1	3,34	169	-	Gallic acid	216, 272	(PEDROSA et al., 2016)
2	8,07	447	357; 285	Orientin	217, 256,364	(SPRENGER., 2011)
3	9,76	447	315; 207; 137; 109	Dihydroxybenzoate diglycoside	210, 248, 364	(ROSA et al., 2021)
4	10,79	301	284; 229; 201; 185	Ellagic acid	209, 253,367	(SILVA et al., 2019)
5	11,6	315	301; 109	Isorhamnetin	209, 246, 364	(PEDROSA et al., 2016)

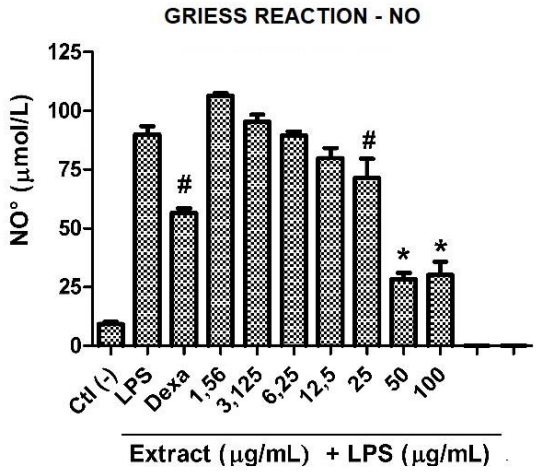
In this study, we reported the cytotoxic and antiinflammatory effects of *L. ferrea* to extract on macrophages in an in vitro culture. To determine the appropriate concentration range for the investigation of anti-inflammatory activity, we initially assessed cytotoxicity and found that cell viability was not affected. The potential cytotoxicity of *L. ferrea* on RAW 264.7 cells was evaluated by MTT assay. The results show that extract concentrations between 1.56 and 100 µg/ml had no cellular toxicity on RAW 264.7 cells, maintaining the cell viability nearly 100% for 24 h (Figure 2).

Figure 2. Cellular cytotoxicity of *L. ferrea* extract. Percentage of viable macrophage cells after in vitro challenge with different extracts of *L. ferrea*, LPS and dexamethasone during the 24 h period. ANOVA, Tukey and Dunnett Test.



Once the *L. ferrea* non-cytotoxicity effect was confirmed, its anti-inflammatory effect was then evaluated through cellular NO production. The result of the study showed that *L. ferrea* had a significant impact on NO production in LPS-stimulated RAW 264.7 cells, with a better result than that of dexamethasone corticosteroid that is considered the gold standard within the anti-inflammatory classes. Compared with the CTRL/Medium, LPS significantly increased the production of NO ($p<0.05$). *L. ferrea* extracts concentrations of 50 and 100 µg/mL significantly inhibited the NO production compared to the CTRL/LPS and other *L. ferrea* concentrations ($p<0.05$). As indicated, *L. ferrea* (50 and 100 µg/ml) significantly suppressed the NO production in a greater rate that of dexamethasone ($p<0.05$). Moreover, the extract reduced the NO production by macrophages in a dose-dependent manner at 6.25-100 µg/ml (Figure 3).

Figure 3. Potential anti-inflammatory effects of *L. ferrea* extract. Production of nitric oxide (NO) by macrophages after challenge with different concentrations of *L. ferrea*, LPS, and dexamethasone extract during 24 h. (*) p <0.05 when compared with all other groups. (#) p <0.05 when compared to LPS. ANOVA, Tukey and Dunnett Test.



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DISCUSSION

The literature describes some therapeutic properties of the chemical compounds found in *L. ferrea*, and through pharmacological tests, demonstrated antimicrobial, as well as anti-inflammatory and healing activity (Carvalho et al., 1996; Sampaio et al., 2009; Falcão et al., 2019). Kobayashi et al. (2015) and Leandro et al. (2019) analyzed the phytochemical profile of *L. ferrea* revealing the presence of organic acids, saponins, phenols, and tannins, sesquiterpene, lactones, and anthraquinones, among others.

The profile analyzes of the hydroalcoholic extract of *L. ferrea* were performed by HPLC-DAD-MS / MS. Five peaks were identified, substance 1 (Rt 3.34 min), the one that expressed only the molecular ion m / z 169 corresponding to the deprotonated gallic acid (Pedrosa et al., 2016). Substance 2 (Rt 8.07 min) indicates glycosylated luteolin whose deprotonation generated [MH +] 447 and two fragments m / z 357 (base peak) at / z 285 indicator flavonoid peak, thus orienting towards the substance known as orientin (Sprenger and Cass, 2013). Substance 3 was responsible for the most intense peak with Rt 9.76 min and [MH +] 447 fragmented in m / z 315 base peak, m / z 207, m / z 137 indicating the loss of hydroxyl of the benzoate portion in / z 109 referring to CO2 loss, being compatible with glycosylated acid, also found in the study by Rosa et al. (2021). Substance 4 indicated in the analysis was ellagic acid having the molecular ion and base peak m / z 301 and the fragments m / z 284, m / z 229, m / z 201 in / z 185, in that order

of intensity, corroborating the peaks with the structure of the molecule found by Silva et al. (2019). Finally, peak 5 (Rt 11.6 min) was indicated as isorhamnetin, which presented the molecular ion m / z 315 with fragmentations of m / z 301 equivalent to the loss of methyl and the fragment m / z 109, enabling the presence of the flavonoid, and corroborating with Pedrosa et al. (2016). These results were compared to those in the literature, which indicate the antiinflammatory capacity of the aforementioned substances.

The cell viability was measured based on MTT assay, which is a widely used approach for measuring cell viability/drug cytotoxicity (Stepanenko and Dmitrenko, 2015; Guerra et al., 2017; Falcão et al., 2019). A study evaluated the *L. ferrea* effect on cell viability in vitro by MTT assay in a mouse embryonic fibroblast cell line (3T3 cells), as well as its anti-inflammatory and antinociceptive activities (Falcão et al., 2019). As in our study, *L. ferrea* crude extract and its fractions maintained the cell viability for 24 h. Also, the authors confirmed significant fibroblast viability up to 72 h, reinforcing our results regarding the *L. ferrea* non-cytotoxicity for long periods (Falcão et al., 2019). In the study carried out by Guerra et al. (2017) similar results were found, where crude *L. ferrea* extracts were successful in exerting a protective effect on the HEK 293 non-tumor strain, showing high rates of cell proliferation and viable cells.

LPS induced expression of iNOS trigged the NO production (Dong et al., 2017). The effects *of L. ferrea* on iNOS were evaluated by Griess analysis. It was found that 50 and 100 µg/mL of *L. ferrea* extract significantly inhibited LPS-stimulated iNOS, characterizing the minimum anti-inflammatory concentrations of the extract. It is also noteworthy that this anti-inflammatory profile surpassed the dexamethasone corticosteroid, considered the gold standard within the anti-inflammatory classes used.

NO is of great interest to medicine because of its evidence in inflammatory signaling pathways (Serafim et al., 2017). The production of free radical's reactive oxygen species (ROS), as well as the NO, may cause exacerbated alterations in the tissues (Pedrosa et al., 2016). One of the advantages of using natural products is their safety and antioxidant effectiveness. When compared to synthetics, natural products help protect tissues through different mechanisms and one of them is reduction of NO, which when produced in large quantities can harm to tissues (Pedrosa et al., 2016). Studies on NO have made clear its biological principles, such as the action on the immune system and

inflammatory processes. Diseases may be associated with high or low levels of NO in the body (Brito et al., 2011). NO can be considered a mediator of the inflammatory process that activates angiogenic phenomena and determines the synthesis of mucosal chemokines (Franceschelli et al., 2019).

In this study, it was shown that extract reduces NO secretion, with an effect greater than that of Dexamethasone. A member of the glucocorticoid group, dexamethasone is widely used in the treatment of inflammatory, autoimmune, and tumoral diseases due to its potentiated anti-inflammatory and immunosuppressive effects. Despite its extensive clinical use, this drug under prolonged use can lead to undesirable side effects such as osteonecrosis and reduced immune defense. Thus, the superior *L. ferrea* effect to the Dexamethasone standard further enhances its anti-inflammatory property. The anti-inflammatory biological effect, in addition to the antioxidant and antinociceptive *L. ferrea* extract fractions properties are probably related to the presence of gallic and ellagic acid, as already identified in phytochemical studies by this research group (Falcão et al., 2019; Pedrosa et al., 2016).

The anti-inflammatory properties of some Fabaceae family members are described in the literature (Freitas et al., 2012; De Araújo et al., 2014; Falcão et al., 2019 and Pedrosa et al., 2016). Several studies have shown that tannins, the main *L. ferrea* constituents, exert protective activity in normal cells (Engelke et al., 2016; Hsu et al., 2016). Among them, those that are strongly present in *L. ferrea* are gallic acid and acid ellagic, so it is believed that these are the main components responsible for these effects (Falcão et al., 2019).

The *L. ferrea* extract anti-inflammatory properties, as well as its non-cytotoxicity, added to its antimicrobial properties, identified in a previous study by this research group, strengthens its biological effects and possible clinical use. Venâncio et al. (2015) made the in vitro evaluation of the *L. ferrea* pharmacological stability mouthwash and there was the absence of microorganisms in its presence. Therefore, this study validate previous research, provide evidence to the development of larger studies in vivo, its expansion to clinical studies and, in the future the use of a product in the dental area for the treatment of inflammatory diseases that affect an oral cavity.

CONCLUSION

The results demonstrated that *L. ferrea* extract tested on macrophages did not exert a cytotoxic effect for these cells. At the same time a minimum anti-inflammatory concentration of *L. ferrea* extract based on the release of nitric oxide (NO) by macrophages is 100 and 50 µg/ml.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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