Physicochemical assessment of an orabase formulation of *Libidibia ferrea* L

Avaliação físico-química de uma formulação orabase de *Libidibia ferrea* L

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ABSTRACT
The aim of the study was to control the physicochemical quality of an orabase formulation of *L. ferrea* L. An experimental, *in vitro*, controlled study was carried out as follows: centrifuge tests, pH, density, rheological behavior at room temperature (25.0 ± 2.0 °C), microbiological control by determining the total number of aerobic microorganisms, yeast growth, and organoleptic characteristics. The storage conditions were as follows: light room (±25.9°C), dark room (±28.8°C), refrigerator (± 2 to 8°C), and the experimental periods were 0, 30 and 60 days. In the centrifuge test, the separation of 1 oily phase (liquid and transparent) was observed in all storage conditions and times tested; in the pH test, the formulation remained stable with pH variations between 6.01 and 6.67, but there was no statistically significant difference at all times and conditions tested. The rheological behavior in terms of viscosity revealed that time and storage conditions did not alter the samples and the mean standard values related to rotation were 6 mPa.SNa (5111.9 ±11), 12 mPa.S (2555.2 ±7), 30 mPa.S (1023.9 ±2) and 60 mPa.S (512.5 ±4). The assessment of contaminants revealed no growth of contaminants in the storage conditions and times tested. As for the organoleptic characteristics, the samples showed no changes throughout the time and storage conditions. The formulation is physically and chemically stable under all storage conditions and times tested, proving to be suitable for extemporaneous formulations.

Palavras-chave: quality control, phytotherapy, ointments, dentistry.

RESUMO
O presente estudo teve como objetivo realizar o controle de qualidade físico-químico de uma formulação em orabase de *L. ferrea* L. Tratou-se de estudo experimental, *in vitro*, controlado, onde foram realizados testes de centrifugação, pH, densidade, comportamento reológico, em temperatura ambiente (25,0 ± 2,0 °C), controle microbiológico pela determinação do número total de microrganismos aeróbios e

pesquisa de levedura, e caracteres organolépticos. As condições físicas de armazenamento testadas foram: temperatura ambiente claro (±25,9°C), temperatura ambiente ao abrigo da luz (±28,8°C) e geladeira (± 2 a 8°C), nos períodos experimentais, 0, 30 e 60 dias. No teste centrifugação, observou-se a separação de 1 fase oleosa (líquida e transparente) em todos os ambientes de armazenamento e tempos testados; no teste do pH, a formulação manteve-se estável com variações de pH entre 6,01 a 6,67, sem houve diferença estatisticamente significantes; para avaliação da viscosidade, as amostras se mantiveram constantes não variando em relação ao tempo de estudo e ambiente de armazenamento, com valores padrões médios correspondentes a rotação 6 mPa.S (5111.9 ±11), 12 mPa.S (2555.2 ±7), 30 mPa.S (1023.9 ±2) e 60 mPa.S (512.5 ±4). Na avaliação de contaminantes não houve crescimento de contaminantes em todas condições de armazenamento e tempo testados. Quanto as características organolépticas, as amostras não apresentaram alterações durante todo tempo e condições de armazenamento. A formulação apresentou estabilidade físico-química em todas as condições de armazenamento e tempo testadas mostrando-se favorável para formulações magistrais.

Palavras-chave: controle de qualidade, fitoterapia, pomadas, odontologia.

1 INTRODUCTION

The Amazon flora has the one of the largest genetic diversity of medicinal plants. According to ethnobotanical studies, several plants from this region and from the Brazilian northeast, Espírito Santo and Rio de Janeiro are used in dentistry. Among the species, the *Libidibia ferrea* L., *Caesalpinia ferrea* L. ex, also known as jucaina, jucá, pau ferro-verdadeiro or birá-obi, is a species of the Leguminosae family and it one of the largest families among the dicotyledons with approximately 650 genera and more than 18 thousand species (CAVALHEIRO et al., 2009; MARREIRO et al., 2014; da COSTA, GUILHON-SIMPLICIO and SOUZA, 2015; MACEDO et al., 2020).

Libidibia ferrea L. powder is widely used in folk medicine due to its anti-inflammatory, analgesic, antimicrobial, and antipyretic therapeutic properties. Different parts of the plant are used and commercialized, such as the bark flour, which has aroused great interest of researchers in the field of biotechnological studies (CAVALHEIRO et al., 2009; SAMPAIO et al., 2009; OLIVEIRA et al., 2013; MARREIRO et al., 2014; da COSTA, GUILHON-SIMPLICIO and SOUZA, 2015; MACEDO et al., 2020; ALMEIDA et al., 2021). Moreover, it can be used to treat wounds, bruises, asthma, chronic cough, and gastric ulcers. It has cardiotonic, antihistamine, antiallergic and anticoagulant properties as well as antimicrobial activity against microorganisms present in dental biofilm (ALVES et al., 2009; CAVALHEIRO et al., 2009; DROZINO et al., 2017; AMERICO et al., 2020).

Preliminary studies on the phytochemical profile from the hydroethanolic extract of the leaves and stem bark of *Libidibia ferrea* L. have confirmed the presence of substances such as flavonoids, saponins, tannins, coumarins, steroids, and phenolic compounds. The assessment of its chemical compounds have shown that the polyphenols, particularly the gallic and ellagic acid, are the possible substances responsible for part of the biological and therapeutic properties of the pod (da COSTA, GUILHON-SIMPLICIO and SOUZA, 2015; KOBAYASHI et al., 2015; MACEDO et al., 2020).

In vitro studies of a mouthwash formulation from the hydroalcoholic extract of 1% *Libidibia ferrea* L. proved its antimicrobial activity against microorganisms present in the dental biofilm. In the pre-clinical and pharmacological tests of the mouthwash, cytotoxicity was assessed using hemolysis and cell culture tests, and the microhardness test (Knoop hardness) was used to assess its erosive potential and ability to stain teeth. The *L. ferrea* L. mouthwash showed to be stable, and no contaminants were found in the product. It also showed bactericidal and fungicidal activity against dental biofilm microorganisms, and the hemolysis and cell culture tests revealed that it was not cytotoxic. No changes were observed in the microhardness of dental enamel, but enamel color changed after prolonged use (VENÂNCIO et al., 2015).

Scientific evidence of the use of natural products and the development of new pharmacological technologies offer new treatment options, extemporaneous formulations, improvement of existing drugs on the market, and new effective active ingredients. Despite increasing industrial production of medications, products isolated from plants or synthesized from them have been gaining market as they promise curative discoveries /palliative treatments (BÔAS and GADELHA, 2007; AKKARI et al., 2016; BARKAT et al., 2016).

The quality control of medications/herbal products is essential to ensure their efficacy and safety. Therefore, a series of analyses of the raw material, such as pre-clinical pharmacological tests (physicochemical and microbiological analyses) and clinical trials, and of the formulation are imperative to understand the biological and toxic effects. Although several studies have demonstrated the need to ensure the safety of each plant product, the application and validation of analytical methods for plant-based raw materials are still scarce in the literature (SIMÕES et al., 2007; BRASIL, 2020).

The aim of the study was to control the quality of an oral pharmaceutical formulation based on *Libidibia ferrea* L. and to assess its stability according to its physicochemical properties.

2 METHODS

The collection of the stem bark of *Libidibia ferrea* L. was carried out in the city of Manaus at the National Institute for Research in the Amazon (INPA). The research was registered under number 228.022 and the stem bark was processed at the School of Pharmaceutical Sciences of the Federal University of Amazonas (UFAM) following the methodology proposed (VENÂNCIO et al., 2015). During the preparation of the extract, asepsis was used to ensure the quality of the material was preserved. The extract was obtained through the extraction decoction technique using a drug:solvent ratio of 7.5% (m/v) with distilled water and ethanol in a proportion of 1:1. The material extracted was cooled and filtered in a 1,000 mL volumetric flask and the jucá extract solution was dried in the Spray Dryer (LabMaq – MSD 1.0 model) to obtain the spray-dried extract.

The phytochemical characterization was performed to identify the phenolic substances. The extract was stabilized in methanol at 1 mg mL^{-1} and analyzed using high-performance liquid chromatography (HPLC) in the Accela HPLC system (Thermo Scientific, Waltham, MA, USA). Separation was achieved by Luna C18 column (5 µm, 150 x 4.6 mm i.d.) (Phenomenex, Torrance, CA, USA) at injection volume of 10 µL. The mobile phase consisted of a solution of 1% formic acid (A) and methanol (B) at a flow rate of 1 mL min^{-1}. Gradient elution began with a variation from 20% B to 80% (B) for 14 min and isocratic elution at 80% (B) for 10 min. The chromatograph-triple quadrupole mass spectrometer (TSQ Quantum Access, Thermo Scientific, Waltham, MA, USA) was equipped with diode array detectors (DAD), with an ultraviolet spectrum range from 200 nm to 400 nm, and an interface using atmospheric pressure chemical ionization (APCI) in negative mode and ion fragmentation. The presence of gallic acid was established by comparing the standard retention time and ions in the mass spectrum. The other peaks observed in the chromatogram were identified by comparing them with the literature.

The concentration of the orabase formulation was determined based on the result of the minimum inhibitory concentration test (37.5mg/ml), determined at the initial phase of the *Libidibia ferrea* L. studies, and tested against microorganisms in the oral cavity (OLIVEIRA et al., 2013). The active principle in the formulation was *Libidibia ferrea* L. and its composition presented an oily phase in which the excipient products (solid vaseline, mineral oil, carboxymethylcellulose, propylparaben) were placed in a water bath (SL 155/10) and heated at 75°. A mercury thermometer was used to measure and control the temperature after the solid components were included into the liquid. The dry extract was then added and homogenized until total dissolution.

The formulation was stored at different temperatures and times. According to the standardization procedure described in the Brazilian Pharmacopoeia (BRASIL, 2020), the storage conditions were as follows: bright room (±25°C), dark room (±25°C), and refrigerator (2 to 8°C). The pre-established experimental storage periods were: 0, 30 and 60 days, according to the previous results of the stability tests (MATOS, 2016).

The tests for the characterization of the formulation were selected according to the Brazilian Pharmacopoeia (BRASIL, 2020): centrifuge test, determination of apparent pH, determination of mass density and relative density, microbiological evaluation to search for contaminants and pathogenic microorganisms, rheological behavior, and evaluation of organoleptic characteristics, as described below. All tests were performed in triplicate, and the results were the mean values of each sample.

To verify the viability and initial stability of the formulation, 5g of Libidibia ferrea L. orabase was placed in Falcon® tubes and centrifuged (KASVI) at 3000 rpm for 30 minutes at room temperature to observe possible phase separation. The results were recorded as YES (if positive) or NO (if negative).

The pH was measured using a potentiometer (TEC2, TECNAL, Piracicaba, SP, Brazil). The device was calibrated at pH 4.0 and 7.0 buffer solutions (DINÂMICA, Brazil). The measurements were performed directly from the formulation and in triplicates, and the result was the mean values of the readings.

The samples (50 mL) were transferred to a clean, dry, and previously calibrated metal pycnometer (50 mL) with a lid (ASTM D1475) (determination of the mass of the empty pycnometer and the mass of the pycnometer with water, freshly distilled and boiled at 20ºC), and the temperature was adjusted to 20ºC. When necessary, the excess of the product was removed and weighed. The weight of the samples was determined through the mass difference of the full and empty pycnometer. The relative density was calculated using the formula: $p(^{sample}) = d(^{water}) \times d(^{sample}) + 0.0012$.

The microbiological control of the orabase formulation consisted of determining the total number of aerobic microorganisms of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and yeast growth, as recommended by the Brazilian Pharmacopoeia (BRASIL, 2020).

The sample was prepared in a 1:10 proportion using a proportion of the formulation (100 µL) for 900 µL of peptone water, pH 7.2, then diluted and homogenized in proportions 1:10; 1:100; 1:1000. For plate counting of microorganisms, 10 µL of each

sample and dilution were placed on a petri dish containing either Casein-soy agar or Sabouraud-dextrose agar. The analysis was done in triplicates. The Casein-soy agar plates were incubated for 24 and 48 hours at 35ºC to determine total aerobic microorganisms and the Sabouraud-dextrose agar plates were incubated for 5 to 7 days at 25ºC to determine filamentous fungi and yeast colonies. After this period, if there was colony growth, the number of CFU/mL was calculated.

A proportion of 100 µL of the orabase formulation and 900 µL of casein-soy broth was homogenized and incubated for 18-24 hours at 350°C to determine growth of *Escherichia coli*. After this period, 100 µL of casein-soy broth was transferred to tubes containing 900 µL of MacConkey broth and incubated for 24-48 hours at 43ºC ± 1ºC. The subculture was seeded on MacConkey agar plate and incubated for 18-72 hours at 35°C. Colony growth and characteristics were observed. In case of suspicious colonies, they would be seeded on eosin blue methylene agar (EMB) and incubated for 24 hours at 35ºC.

A proportion of 900 µL of casein-soy broth and 100 µL of the orabase formulation was homogenized and incubated at 35ºC for 18–24/48 hours to determine growth of *Pseudomonas aeruginosa*. After this period, the culture was seeded on Cetrimide agar and incubated for 18-72 hours at 35ºC. A proportion of the formulation and 900 µL of casein-soy broth was homogenized and incubated for 18 to 24 hours at 35ºC to determine growth of Staphylococcus aureus. After this period, the culture was seeded on mannitol salt agar and incubated for 18-72 hours at 35ºC.

The rheological characteristics were assessed using the digital rotational viscometer Brookfield Marte MVD-5, using a spindle number 2. The analyses were based on the protocol of the Brazilian Pharmacopoeia (BRASIL, 2020) and Marafon et al. (2016). After adjusting each speed factor (6, 12, 30 and 60 rotation speed), the viscosity and shear stress values were read at a temperature of 25°C ±2°C.

Regarding color and brightness, the analyses were performed under daylight and the color of the sample was compared with the color of the standard reference material. The characteristics of consistency were analyzed using touch sensitivity and the results were complemented by the previously described viscosity test. A small sample of the orabase formulation was placed on the palm of a sanitized hand or in a glass container and slowly inhaled to identity the odor. Therefore, the odor intensity was classified as follows: none, weak, distinct, or strong. The sensation caused by the odor was aromatic, fruity, moldy, or rancid (BRASIL, 2020). The analyses on the sample of the orabase

formulation were also performed in triplicates and the result was the mean values of the analyses.

In the quantitative data analysis, as the hypothesis of normality was rejected using the Shapiro-Wilk test, the median and quartiles (Q_i) were calculated using the box plot graph method and the nonparametric Kruskal-Wallis test was used to compare the medians. The software used in the analysis of the data was the Minitabe program, version 18, and the level of significance of the statistical tests was 5% (VIEIRA, 2004).

3 RESULTS AND DISCUSSION

After qualitative analysis of the phytochemical profile of the plants, the presence of 5 phenolic compounds were confirmed, as described in Figure I and Table I.

Figure I. Chromatogram of the hydroalcoholic extract of 7.5% *L. ferrea* L.

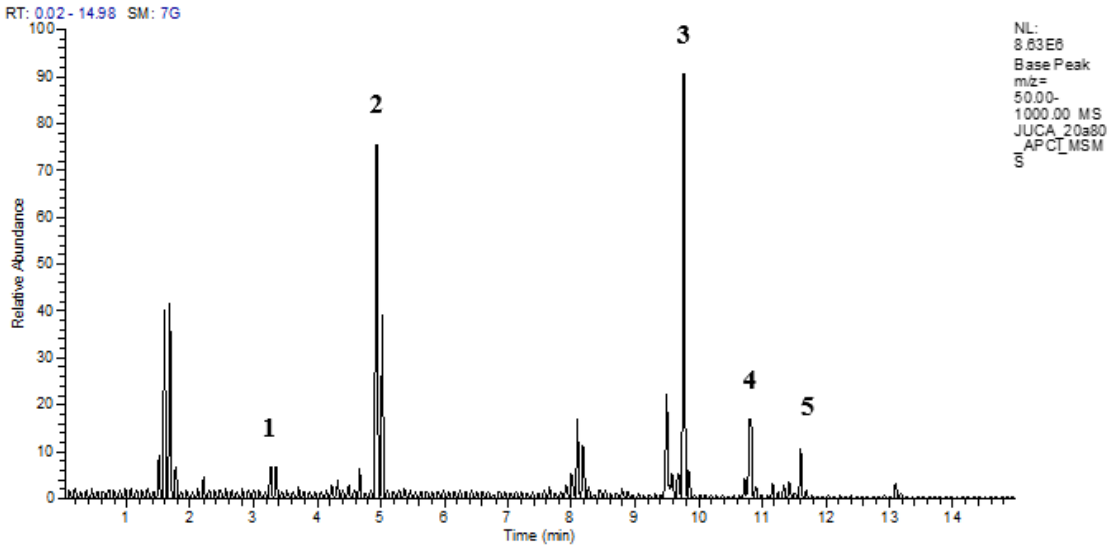


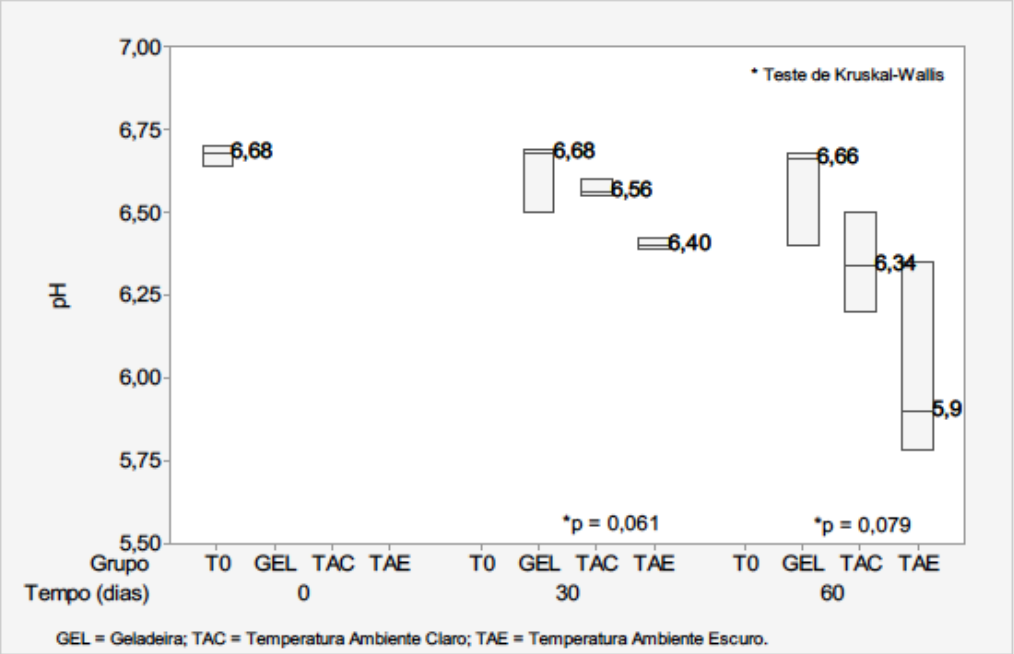
Table I. Substances characterized by LC-MS/MS in hydroalcoholic extract of 7.5% *L. ferrea* L.

Peak	R.T	$[M-H]^-$	Dillutions	Substance	λ_{max}/nm
1	3.34	169	-	Gallic acid	216, 272
2	8.07	447	357; 285	Orientin	217, 256,364
3	9.76	447	315; 207; 137; 109	Dihydroxybenzoate diglycoside	210, 248, 364
4	10.79	301	284; 229; 201; 185	Ellagic acid	209, 253,367
5	11.6	315	301; 109	Isorhamnetin	209, 246, 364

In the centrifuge test, performed in triplicates, the separation of 1 small oily phase from the formulation of *L. ferrea* L. was observed at all times (0, 30 and 60 days) and storage conditions. The presence of precipitate was observed in the samples from the dark room after 60 days.

The results of the determination of the pH of the *L. ferrea* L. ointment are shown in Table II and figure II. There was no statistical difference when comparing the pH values with times and storage conditions (T30 p= 0.061 and T60 P=0.079).

Figure II. Comparison of the median pH and groups at 30 and 60 days, Manaus - AM.



Legend: geladeira = refrigerator (GEL); temperatura ambiente claro = light room (TAC); temperatura ambiente escuro = dark room (TAE). Kruskal-Wallis test.

Table II. pH values after ointment formulation (T0), after 30 (T30), and 60 days (T60) at different storage conditions, Manaus - AM.

	T0	T30			T60		
	T0	Geladeira (2 a 8°C)	Temp. ambiente claro (25,9°C)	Temperatura ambiente escuro (28,8°C)	Geladeira (2 a 8°C)	Temperatura ambiente claro (25,9°C)	Temperatura ambiente escuro (28,8°C)
1ª Leitura	6,64	6,68	6,55	6,40	6,66	6,50	5,90
2° Leitura	6,68	6,69	6,56	6,42	6,68	6,34	5,78
3ª Leitura	6,70	6,50	6,60	6,39	6,40	6,20	6,35
Média	**6,68**	**6,68**	**6,56**	**6,40**	**6,66**	**6,34**	**5,90**

Legend: 1ª, 2 ª e 3 ª leitura = 1st reading; 2nd reading; 3rd reading; media = mean geladeira = refrigerator; temperatura ambiente claro = light room; temperatura ambiente escuro dark room.

The density of the *L. ferrea* L. formulation was determined at different storage conditions after 0, 30 and 60 days. At time 0, a mean of 0.958g/cm^3 was obtained. After 30 days of formulation, the mean densities were: refrigerator (0.971g/cm^3), light room (0.976g/cm^3) and dark room (0.975g/cm^3). After 60 days of formulation, the mean

densities were: refrigerator (0.935g/cm^3), light room (0.974g/cm^3) and dark room (0.968g/cm^3). The values indicate stability of the formulation during the 60 days of quality control, and no differences between groups in the storage conditions were observed.

The microbiological tests did not reveal the presence of contaminants (bacteria and/or fungi) at any time or storage conditions.

The results described in Table III show that the viscosity of the orabase formulation ranged when a shear stress was applied as it did not obey Newton's law of viscosity, in which viscosity remains constant. Thus, we can consider that the orabase formulation of *L. ferrea* L. is non-Newtonian and it has a possible pseudoplastic behavior, that is, viscosity decreases as stress increases, and it becomes more fluid.

Table III. Comparison of median viscosity in relation to groups at 0, 30 and 60 days according to rotation speed. Manaus - AM.

	Grupos								
Tempo/	T0		Geladeira		TAC		TAE		
Rotação	Med	d_i	Med	d_i	Med	d_i	Med	d_i	p*
T0									
6	5107,2	1,9	-	-	-	-	-	-	
12	2553,1	2,0	-	-	-	-	-	-	
30	1022,5	5,3	-	-	-	-	-	-	
60	512,4	3,0	-	-	-	-	-	-	
T30									
6	-	-	5117,4	2,3	5112,0	11,6	5107,2	11,0	0,393
12	-	-	2554,3	17,9	2552,5	6,4	2553,0	0,6	0,550
30	-	-	1020,5	1,3	1023,9	5,8	1022,0	0,3	***0,026***
60	-	-	512,6	2,9	512,5	4,1	511,2	1,0	0,561
T60									
6	-	-	5113,8	16,0	5111,6	7,8	5107,5	91,9	0,301
12	-	-	2557,1	16,5	2556,9	1,8	2554,4	0,6	0,292
30	-	-	1028,6	8,7	1026,0	3,2	1022,2	0,2	0,288
60	-	-	512,9	1,5	512,0	3,4	511,0	0,1	0,059

Med = mediana; d_i = desvio interquartil; * Teste não paramétrico de Kruskal-Wallis.

Legenda: Grupos = groups; Geladeira = refrigerator; Tempo/rotação = time/rotation speed; Med/mediana = median; d_i interquartile deviation; * Kruskal-Wallis nonparametric test.

During the assessment of the organoleptic characteristics, the color, odor, brightness, and consistency were analyzed. The samples showed no color changes in the formulation between times and storage conditions. The color was characterized as light brownish (Date), which was determined using the Coral® color scale. The formulation had a strong and aromatic odor and a shiny and smooth consistency without granulations.

The tests used for the quality control of herbal products are the same used for industrial medications, in which the analyses are carried out to obtain data on the

biological, chemical, physical-chemical, botanical, and microbiological aspects of the raw material of the plant and the technological adjuvants, packaging materials, and the processing and final stages of the standardized pharmaceutical form (GIL, 2007; SIMÕES et al., 2007; BRASIL, 2020).

The literature describes some of the therapeutic properties of the chemical compounds found in *L. ferrea* L. In the present study, several chemical compounds were found, and the three major ones were: gallic acid, ellagic acid and rutin, corroborating the findings (SAMPAIO et al., 2009; da COSTA, GUILHON-SIMPLICIO and SOUZA, 2015; KOBAYASHI et al., 2015; MAGALHÃES et al., 2015; MACEDO et al., 2020).

The orabase formulation of *L. ferrea* L. proved to be easy to manipulate and handle. These types of pharmaceutical preparations can be applied on the skin or on mucous membranes for a local effect of the pharmacological compounds. These compounds must be physically and chemically compatible with the active principles, packaging, and environmental factors to ensure their preservation. The orabase formulation proposed has oily components. Vaseline and mineral oil were used as the oily phase of the formulation, and it provides an emollient action on the tissue. To facilitate its adherence to the mucosa, prolonging its availability at the site of action, carboxymethyl cellulose (CMC) was used as it is a mucoadhesive polymer compatible with the other components in the formulation. Studies have reported that although CMC is a great polymer for mucosal adhesion, there are other components that can be used in oily formulations, such as maltodextrin (VILLANOVA, ÓREFICE and CUNHA, 2010; SOUZA, 2016; BRASIL, 2020).

For the production of a semi-solid pharmaceutical formulation, the following aspects must be considered during quality control tests: organoleptic characteristics, including visual aspects such as transparency, color, and sensory aspects; rheological behavior by testing spreadability, consistency, penetrability, plasticity and gloss, coalescence, homogeneity, pH, and drug dosage (BRASIL, 2020). The organoleptic characteristics are simple indications, mainly in semi-solid formulations, to determine possible alterations and acceptance of the product by the consumer (BRASIL, 2020). Therefore, based on the results obtained for the formulation proposed, the quality control tests showed a lack of physical stability due to the small phase separation, absence of microbiological or fungal contaminants for the period proposed in the present study, a pleasant appearance and color, and a pleasant consistency to touch, which remained unchanged throughout the period studied. These findings differ (MATOS, 2016), whose

formulation showed bacterial/fungal contamination within 60 days, which require a quantification test of chemical markers to prove the apparent physical and chemical stability reported in our results.

The determination of relative density and mass is extremely important to evaluate the stability of a pharmaceutical formulation. Adequate density allows for uniform mixing of the active ingredient and a higher content of the active ingredient, without loss of therapeutic properties (JUNIOR et al., 2010), which corroborates the results found in the present study, suggesting that the formulation was stable throughout the times and storage conditions tested.

The determination of pH may suggest possible degradation of the formulation with storage, requiring other complementary tests to assess stability. The pH of the formulation must be compatible with the physiological region of treatment (OLIVEIRA et al., 2013; MARAFON et al., 2016). In the present study, the average pH found at different times and storage conditions showed that the formulation was stable with pH values between 6.01 and 6.67. Our results were more stable and satisfactory than those reported[17]. The determination of pH is an important functional indicator. The oral cavity has a slightly acidic pH, close to neutrality (around 7), which provides bacterial and fungal protection and maintains salivary buffering and healing capacity, since the pH close to neutrality is directly related to the tissue regeneration process (LEONARDI, GASPAR and CAMPOS, 2002; BALBINO, PEREIRA and CURI. 2005; MARREIRO et al., 2014; VENÂNCIO et al., 2015).

4 CONCLUSION

The results indicate that the *Libidibia ferrea* L. extract has three major compounds: gallic acid, ellagic acid and rutin. The formulation showed apparent physical-chemical stability at all times and storage conditions, did not change color, odor, or consistency, presented high viscosity, and absence of bacterial and/or fungal contaminants, proving to be suitable for extemporaneous formulation for a 60-day validity period.

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