REVISTA DE ODONTOLOGIA DA UNESP

Rev Odontol UNESP. 2015 Mar.-Apr.; 44(2): 118-124
Doi: http://dx.doi.org/10.1590/1807-2577.1064

Herbal mouthwash based on *Libidibia ferrea*: microbiological control, sensory characteristics, sedimentation, pH and density

Enxaguatório bucal fitoterápico à base de Libidibia ferrea: controle microbiológico, características organolépticas, sedimentação, pH e densidade

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Resumo

Introdução: A Fitoterapia é o estudo de plantas medicinais e suas aplicabilidades para a cura de doenças em geral, constituindo um método terapêutico que pode ser utilizado para a prevenção e tratamento de doenças bucais. Dentre as plantas estudadas, a *Libidibia ferrea*, conhecida como jucá ou pau ferro, é bastante utilizada na medicina popular por apresentar propriedades terapêuticas anti-inflamatória, analgésica, antimicrobiana e antitérmica. **Objetivo:** Avaliar, *in vitro,* a estabilidade farmacológica de um enxaguatório bucal fitoterápico à base do extrato de *Libidibia ferrea* (228.022 - INPA). **Material e método:** Foi realizado o controle microbiológico do enxaguatório através da determinação do número total de microrganismos de *Salmonella sp*., *Escherichia coli, Pseudomonas aeruginosa* e *Staphylococcus aureus*; foram analisadas as características organolépticas (cor, odor, brilho e consistência), sedimentação (centrífuga), aferição do pH (peagâmetro) e densidade (picnômetro). **Resultado:** O enxaguatório mostrou-se ausente de microrganismos e não foram observadas alterações das características organolépticas e sedimentação. Os valores médios de pH foram de 6,21, 6,15 e 5,85 nos tempos de armazenamento de 0, 30 e 60 dias, respectivamente, e de densidade 1,029, 1,033 e 1,035 g/ mL, respectivamente, porém sem interferência na característica final da formulação. **Conclusão:** O enxaguatório à base de *Libidibia ferrea* apresentou condições de estabilidade e qualidade farmacológicas.

Descritores: Fitoterapia; odontologia; higiene bucal.

Abstract

Introduction: Phytotherapy is the study of herbal medicines and their applicability to cure diseases in general, being a therapeutic method which can be used for the prevention and treatment of mouth diseases. Among the herbal studied, the *Libidibia ferrea*, known as jucá or ironwood, is widely used in folk medicine by presenting anti-inflammatory, analgesic, antimicrobial and antipyretic therapeutic properties. **Objective:** To evaluate in vitro pharmacological stability of the *Libidibia ferrea* extract's mouthwash (INPA - 228 022). **Material and method:** It was held the mouthwash microbiological control by determining the total number of microorganisms and *Salmonella* sp, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*; stability characteristics (color, odor, brightness and consistency), sedimentation test (centrifuge), the pH measurement (pH meter) and density evaluation (pycnometer) were analyzed. **Result:** The mouthwash showed 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 1.029, 1.033 and 1.035 g/ mL density values, respectively, without interfering with the final characteristic of the formulation. **Conclusion:** The mouthwash presented pharmacological stability and quality conditions.

Descriptors: Phytotherapy; dentistry; oral hygiene.

INTRODUCTION

Dental caries and periodontal disease, the most common diseases affecting humanity, involve the adherence of bacteria and development of biofilm on both natural and restored tooth surfaces[1].

Control of biofilm and the pathologies results from its presence may be achieved by means of mechanical and chemical processes, or diet. Mechanical control is the most effective method for the prevention and removal of biofilm, but it is not always adequately performed. Thus, antimicrobial substances have been used for chemical control of dental biofilm, as a supplementary manner to mechanical procedures[1-3].

Chlorhexidine, a cationic bis-biguanide biocide with high substantivity and broad spectrum of action against Gram positive bacteria, yeasts and dermatophytes, is among the oral antiseptics most used[4]. However, it has adverse effects when used for a prolonged period of time, such as staining the teeth, changes in taste and increase in the formation of supragingival biofilm. Therefore, its use is limited to situations in which mechanical oral hygiene is compromised[5].

The quest for increasingly simple oral hygiene methods, capable of minimizing the appearance of local side effects on the patient, led to the search for oral mouth washes based on natural chemical substances. Phytotherapeutic products have satisfactory effects, and have shown to be a complementary alternative, contributing to improving the population's access to care with prevention and treatment of oral diseases[6-8].

Among the phytotherapeutic products of interest for use in Dentistry, *Libidibia ferrea* (*L. ferrea*) popularly known as ironwood, has been extensively studied. Anti-inflammatory, analgesic and antimicrobial properties have been demonstrated, causing interest in continuing with analysis of its pharmacological and therapeutic characteristics[9-12].

Studies with *L. ferrea* have demonstrated promising results, and there is a perspective for its use as a mouth wash for dental biofilm control, because it has antibacterial activity against the microorganisms present in the oral cavity[10,12].

However, in order to perfect new products used in Dentistry, and to prove their efficacy, they need to be submitted to diverse tests, seeking to visualize their clinical performance when used in the oral cavity, in order to make the use of the product feasible in daily clinical routine[13,14].

Therefore, the aim of this research was to evaluate the *in vitro* pharmacological stability of a phytotherapeutic mouth wash based on *L. ferrea* extract with regard to the microbiological parameters of control, organoleptic characteristics, sedimentation, pH and density.

MATERIAL AND METHOD

The botanical species *L. ferrea* (228.022 - INPA) was collected at the National Research Institute of Amazonia ["Instituto Nacional de Pesquisa da Amazônia (INPA)] and processed at the Pharmaceutical Science Faculty of the Federal University of Amazonas (Figure 1).

The manipulation of *L. ferrea*, and the other tests performed in the research are described according to the flow diagram (Figure 2).

The extractive solution of *L. ferrea* was prepared with 7.5 grams of ironwood beans in 500 mL of distilled water and 500 mL of alcohol at 96 °C in decoction for a period of 15 minutes in heat insulation and under reflux. After this period, the material was removed, cooled and filtered, then taken to the Spray Dryer appliance (MSD 1.0, Labmaq, Ribeirão Preto, São Paulo, Brazil) in order to obtain the dry extract by spray drying to the concentration of 7.5% (m/v), with the purpose of obtaining the powder to maintain stability (Figure 3).

Figure 1. A – L. *ferrea* tree; B – *L. ferrea* Fruits (pods).

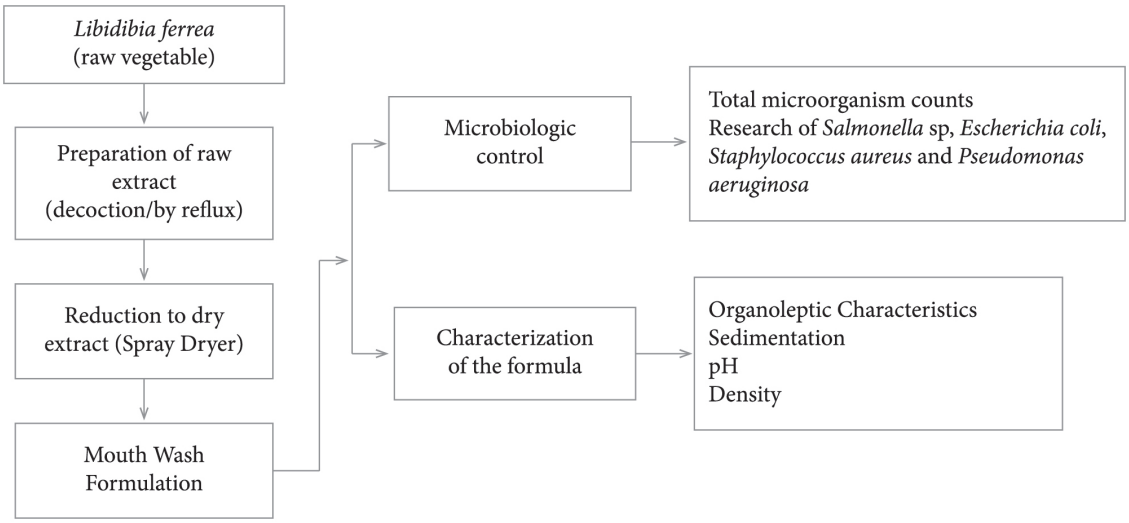

Figure 2. Flow Diagram - Study Methodology.

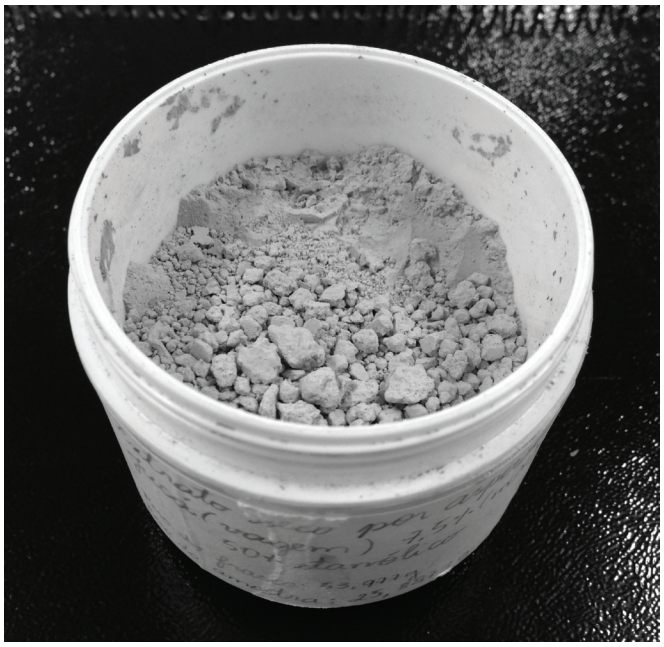

Figure 3. Extract from *L. ferrea* bean.

Figure 4. *L. ferrea* Mouth Wash.

Formulation of the mouth wash (Figure 1) was done in accordance with the methodology adapted from Zanin et al.[15]. The components were: sodium benzoate, saccharine, glycerin, *L. ferrea extract* 7.5% (m/v), 80% Tween, 20% Tween, distilled water, mint essence and 10% sodium hydroxide (Figure 4).

Microbiological control of the *L. ferrea* mouth wash consisted of determining the total number of microorganisms and presence of *Salmonella* sp., *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus,* as recommended in Brazilian Pharmacopoiea[16] for the microbiological analysis of non sterile products, as follows:

Total Microorganism Counts

Aseptically, 1 mL of the *L. ferrea* mouth wash was transferred to 9 mL of phosphate buffer solution pH 7.2 (Synth, Diadema, São Paulo, Brazil), for total microorganism counts. Samples in the proportions of (1:10; 1:100; 1:1000 e 1:10.000) were submitted to agitation for 10 min. After homogenization, 1,0 mL of each sample was pipetted and added to 20.0 mL of thioglycollate agar (DIFCO™) for bacteria and Sabouraud agar (DIFCO™) for yeasts, in Petri dishes, which were placed in an oven at 35 °C for 24 h and at 25 °C for 7 days to research bacteria and fungi, respectively. After this period, if there were suspect colonies, the number of colonies would be counted with the aid of a colony counter, calculating the number of colony forming units (CFU/mL).

- Research of *Salmonella sp* and *Escherichia coli*

A quantity of 1 mL of *L. ferrea* mouth wash was transferred to 9 mL of lactose broth, to research *Salmonella sp* and *E. coli*, incubated at 35 °C for 24 and 48h. After this period, 1 mL of

lactose broth (DIFCO™) was transferred to two tubes containing tetrathionate broth (DIFCO™) and selenite cystine broth (DIFCO™), which were incubated at 35 °C for 24h. After this period, the sample from the tetrathionate broth was seeded in a tube containing brilliant green agar (DIFCO™) and two Petri dishes containing Xylose lysine deoxycholate agar (XLD agar) (DIFCO™) and bismuth sulfite agar (DIFCO™). The same procedure was performed with the sample inoculated in the selenite cystine broth, transferring it to the three previously mentioned media, which were incubated at 35 °C for 24h. The characteristics and growth of colonies were observed. If there were suspect colonies, they would be seeded with a bacteriological needle in a tube containing triple sugar- iron agar (TSI) (DIFCO™) and incubated at 35 °C for 24h.

In the research of *E. coli*, 1 mL of the lactose broth was transferred to the plate containing MacConkey agar (DIFCO™) and incubated at 35 °C for 24h. If there were suspect colonies, these would be seeded in eosin methylene blue agar (DIFCO™) and incubated at 35 °C for 24h.

- Research of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

A quantity of 1 mL of *L. ferrea* mouth wash was aseptically transferred to 9 mL of soyabean casein broth, to research *S. aureus* and *P. aeruginosa*, and incubated at 35 °C for 24 to 48h. After this period, it was seeded on Vogel Johnson agar (DIFCO™), for the research of *S. aureus* and Cetrimide agar (DIFCO™), or the research of *P. aeruginosa* at 35 °C for 24 h.

The organoleptic characteristics (color, odor, brightness and consistency) (Figure 1) were evaluated by means of sensory analysis (characteristics detectable by the sensory organs), in comparison with a standard sample to analyze changes under controlled environmental conditions, for each experimental time interval (0, 30 and 60 days), in accordance with the Collegiate Board Resolutions ("Resolução Diretoria Colegiada" - RDC) 211/05[17] and RDC 45/12[18].

The sedimentation test (Figure 1) was based on the rotary speed of falcon tubes (Kasvi, K19-0050, Curitiba, Paraná, Brazil) containing 35 mL of the mouth wash, in triplicate, in the time intervals of 0, 30 and 60 days, using a centrifugal (Centrifuge 5804R, Eppendorf, AG, Germany) at 900 *g* for 5 minutes, to check for a possible separation of the phases of the solution[16].

The pH of the *L. ferrea* mouth wash (Figure 1) was measured by means of a previously calibrated pH meter (pHmeter TEC-2/ Tecnal/Piracicaba/São Paulo, Brazil). pH was determined in triplicate in the time intervals of 0, 30 and 60 days, by the mean value of the potential between the measurements of approximately 10 mL of the solution[16].

The density of the mouth wash (Figure 1) was determined according to Brazilian Pharmacopoeia[16]. The densities were defined in the 25 mL pycnometer (empty, with distilled water and with the mouth wash), in triplicate, in the time intervals of 0, 30 and 60 days, using an analytical balance (0.01mg/Shimadzu AY220/China).

The results obtained in the evaluation of microorganisms, organoleptic and sedimentation characteristics were tabulated and described by descriptive statistics. In the evaluation of pH and density, the data were presented by means of tables and graphs, in which the mean and standard deviation (SD) were calculated. For the data that presented normal distribution the Analysis of Variance (ANOVA) and Tukey tests were applied[19,20].

RESULT

In this study, under the conditions and culture media tested, there was no indication of the presence of microorganism (bacteria, fungi or yeasts) in the *L. ferrea* mouth wash, with the product showing absence of contamination.

Evaluation of the organoleptic characteristics indicated that no changes in color, odor, brightness or consistency of the *L. ferrea* mouth wash occurred in the time intervals tested (0, 30 and 60 days), with the mouth wash having a wine brown color, pleasant odor and homogeneous aspect.

In the sedimentation test, no separation of phases or precipitation of sediments were observed in the *L. ferrea* mouth wash, in any of the three experimental time intervals tested (0, 30 and 60 days) when the product was submitted to centrifugation.

The *L. ferrea* mouth wash presented mean pH values of 6.21, 6.15 and 5.85 in the time intervals of 0, 30 and 60 days, respectively. The mean pH values were stable in the time intervals of 0 and 30 days, however, there was a statistically significant difference in the time intervals of 0 and 60 days and 30 and 60 days, at the level of 5%, when the Tukey test was applied (Table 1).

With regard to density, the values obtained were 1.29, 1.33 and 1.35 g/mL in the experimental time intervals of 0, 30 and 60 days, respectively. There was statistical difference over time, when the Tukey test was performed, only in the periods of 0 to 60 days, however, without interfering in the final characteristics of the formulation (Table 1).

DISCUSSION

The use of antimicrobial mouth washes as adjunct treatment to mechanical means of dental biofilm and gingival inflammation control has been well established[1,21,22].

Table 1. Distribution according to the mean pH and density of the *L. ferrea mouth wash* in the time intervals of (0, 30 and 60 days)

Variable/Time	n	Mean	S.D.	p*
pH				0.001
0 Days	3	6.21a	0.07	
30 days	3	6.15a	0.09	
60 days	3	5.85b	0.03	
Density g/mL				<0.001
0 Days	3	1.029a	0.00	
30 days	3	1.033b	0.00	
60 days	3	1.035b	0.00	

SD - Standard Deviation. *ANOVA. Different letters indicate statistical difference at the level of significance of 5% (Tukey Test).

In spite of plant-based products becoming increasingly popular world-wide, Kunle et al.[23] have emphasized that one of the obstacles to their acceptance is the lack of quality control, because the profile of the end product constituents have implications in efficiency and safety. In this context, Zangh et al.[24] stated that in order to attain overall improvement in quality, efforts must be made to enhance methodological techniques of researches and improve the regulation of phytotherapeutic medications.

Therefore, the National Agency for Sanitary Vigilance ("Agência Nacional de Vigilância Sanitária - ANVISA"), by means of Resolution RDC 13 of 15/03/2013[25], established that all phytotherapeutic medication must be submitted to formulation stability tests. Production operations must follow operational procedures with clearly defined and approved standards, in conformity with the notification or registration of Traditional Phytotherapeutic Products with the competent sanitary agency. The final goal is to obtain products that are within the quality standards demanded.

The purpose of microbiologic control is to determine the total number of microorganisms present in non sterile preparations, cosmetics and vegetable drugs, and thus there are standards pre-establish by the World Health Organization that make contamination by bacteria and fungi acceptable, provided this is at a level that is not very high, and the absence or reduced presence of microorganisms with pathogenic potential. Therefore, it is of the utmost importance to identify pathogenic microorganisms such as *Salmonella* sp., *E. coli, S. aureus* and *P. aeruginosa,* which must not be present, thus assuring products of good quality, whatever their origin may be[14,25]. Therefore, as demonstrated in the results of this study, the *L. ferrea* mouth wash was shown to be free of contamination for the tested microorganisms and is within the standards of safety demanded for its use.

The organoleptic characteristics of the product remained unaltered and satisfactory in the time intervals tested. The mouth wash was analyzed as regards color, odor and aspect, in accordance with the parameters described by Brazilian Pharmacopoeia[16]. According to Isaac et al.[26], the aspect of a phytocosmetic, as regards homogeneity and coloring is important from a commercial point of view, because these may influence purchase by the consumer, who would not feel attracted by the appearance of the product. Furthermore, they affirmed that changes in odor may be related to microbial contamination, even when there is no visual alteration, thus emphasizing the importance of the development of a protocol for studying the physical-chemical stability of phytocosmetics.

The results obtained in the sedimentation test are in agreement with those of the study of Oliveira Marreiro et al.[10], in which a formulation based on *L. ferrea* extract was used, and there was no separation of the phases of the product, therefore, obtaining the same results as those in the present study.

There was no statistically significant difference in the mean pH values in the time intervals of 0 and 30 days. However, there was difference in the times of 0 and 60 days and 30 and 60 days, without however compromising the characteristics of the formulation, since the pH was maintained at around 6.0, a value considered satisfactory, because 5.5 is the pH considered critical for enamel demineralization[6]. This result can be explained by the addition of 20% NaOH to the formulation of the *L. ferrea* mouth wash, acting as an alkalizing and buffering agent. This achieved the objective of leaving the pH compatible with the use of the formulation as a mouth wash, as proposed by Rowe et al.[27], since it provided a formulation resistant to variations in pH. This proposal was followed by Zanin et al.[15], who developed a mouth wash based on the hydroalcoholic extract of *Salvia officinalis* L. and used 20% NaOH in their formulation, which functioned as an alkalizing agent, stabilizing the pH at around 6,3. The concentration used in the present study was 10% NaOH , due to the fact that 20% NaOH had caused darkening of the *L. ferrea* mouth wash, because it was more concentrated, and there was no loss of its action, and achieved the objective of not changing the color of the mouth wash. The concentration of 10% NaOH was also used by Andreolli, Lara[28] in an *in vitro* study about the remineralizing potential of mouth washes.

Cavalcanti et al.[29] evaluated the pH of ten brands of mouth washes sold in Brazil, and the values ranged from 3.56 (Peroxyl®) to 7.43 (Cepacol®), in which three brands presented pH values below 5,5. These results corroborate the findings of the studies of Hannan et al.[30], who analyzed the pH of fluoridated mouth washes commercially available in the city of Manaus – AM, and verified that two presented a pH of around 5,5, considered potentially erosive: Johnson & Johnson Reach® Zoodent, with pH 5.14 and Colgate Plax® Kids, with pH 4.75. Whereas Marinho, Araújo[31] affirmed that mouth washes with acid pH were shown to be more effective as regards reduction in fermentation and production of extracellular polysaccharides by the microorganisms, thus influencing the metabolism of dental biofilm.

The density values obtained increase in the experimental time intervals of 0, 30 and 60 days. There was statistical difference over time only between 0 to 60 days, with the mean density of the *L. ferrea* mouth wash being around 1.32 g/ mL. Isaac et al.[26] , in their research stated that the variation in the density values were probably as a result of the loss of water or even volatility of the mouth wash, thus explaining the increase in density of the product over the tested time intervals, however, without interfering in the final characteristic of the formulation.

Leite* developed a mouth wash based on the extract of essential oil f *Baccharis dracunculifolia* leaves, and obtained a final pH equal to 5.89 and density of 1.04 g/ mL, values close to those found in the present study.

Oriqui et al.[32] proposed a guide for determination of the stability of chemical products, emphasizing the need for establishing the profile of stability for the product for at least the entire period of validity to be proposed. Therefore, the times chosen for the tests in the present study were 0, 30 and 60 days, as a way of obtaining

* Leite MF. Desenvolvimento e caracterização de microemulsões para enxaguatório bucal com extrato de Baccharis dracunculifolia [tese]. Ribeirão Preto: Faculdade de Ciências Farmacêuticas da USP; 2009.

initial parameters with reference to the study of stability of the *L. ferrea* mouth wash, as recommended in RDC 45/12[18].

CONCLUSION

Based on the results of the methodologies used in this study, it could be concluded that the *L.ferrea* mouth wash presented conditions of stability (organoleptic, sedimentation, pH and density characteristics) and absence of microorganisms.

ACKNOWLEDGEMENTS

Amazonas State Foundation for Research Support (FAPEAM - 450.002.001.002.001/019.451-4).

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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Received: May 16, 2014
Accepted: October 27, 2014