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ABCTRACT
The effects of Bacterial Nanocellulose (BNC) loaded with *Libidibia ferrea* (Lf) hydroalcoholic extract were investigated on the healing process of burn in diabetic and non-diabetic animals. *In vivo* assay was performed with 36 male rats, with streptozotocin-induced diabetes and burns induced by contact. Animals were divided into Nd-BNC (Non-diabetic + Bacterial nanocellulose membranes); Nd-BNC-Lf (Non-diabetic + Bacterial nanocellulose membranes + *Libidibia ferrea*); D-BNC (Diabetic + Bacterial nanocellulose membranes); D-BNC-Lf (Diabetic + Bacterial nanocellulose membranes + *Libidibia ferrea)*. Wounds were evaluated for 28 days histologically. Lf extract and BNC-Lf extract showed antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. The severe degree of infection, granulation and inflammation observed after 14 days in diabetic rats (exposed or not to Lf extract), disappeared after 21 days. On the 28th day, there was no histological difference among the groups. BNC-Lf extract demonstrated to have antimicrobial activity, however as an wound dressing, both BNC or BNC-Lf extract were effective in the healing of second-degree burn wounds.

Key words: Bacterial Nanocellulose, *Libidibia férrea*, Wound Healing, Antimicrobial Activity.

RESUMO

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Os efeitos da nanocelulose bacteriana (BNC) carregada com extrato hidroalcoólico de *Libidibia ferrea* (Lf) foram investigados no processo de cicatrização da queimadura em animais diabéticos e não diabéticos. O ensaio in vivo foi realizado com 36 ratos machos, com diabetes induzida por estreptozotocina e queimaduras induzidas por contato. Os animais foram divididos em Nd-BNC (membranas de nanocelulose bacteriana + não diabética); Nd-BNC-Lf (não diabético + membranas de nanocelulose bacteriana + *Libidibia ferrea*); D-BNC (membranas de nanocelulose bacteriana + diabética); D-BNC-Lf (membranas de nanocelulose bacteriana diabética + + *Libidibia ferrea*). As feridas foram avaliadas histologicamente por 28 dias. Os extratos Lf e BNC-Lf apresentaram atividade antimicrobiana contra Staphylococcus aureus, Pseudomonas aeruginosa e Escherichia coli. O grau severo de infecção, granulação e inflamação observado após 14 dias em ratos diabéticos (expostos ou não ao extrato de Lf) desapareceu após 21 dias. No 28º dia, não houve diferença histológica entre os grupos. O extrato de BNC-Lf demonstrou ter atividade antimicrobiana, no entanto, como curativo, tanto o extrato de BNC quanto o de BNC-Lf foram eficazes na cicatrização de feridas de queimadura de segundo grau.

Palavras-chave: Nanocelulose bacteriana, Libidibia férrea, Cicatrização de feridas, Atividade antimicrobiana.

1 INTRODUCTION

The *Libidibia ferrea* Mart. (*Libidibia ferrea* Martius ex. Tulasne var. *leiostachya* Benth; botanical synonym: *Caesalpinia ferrea*) belongs to the Family of Leguminosae (Fabaceae Lind.), and the Subfamily: Caesalpinioideae (1). The chemical compounds described for Lf are terpenes (lupeol and α-amyrin), tannins and phenolic acids, C-glycosylated flavonoids (isoorientin, orientin and vitexin), lectins and steroids (2,3).

Bacchi and Sertie cite that *Libidibia ferrea* Mart. has long been used in folk medicine as a healing resource and in the treatment of gastroduodenal ulcer (4). Storey and Salem, in an ethnopharmacological study conducted in the state of Amazonas, Brazil, reported Lf was one of the thirteen most used plants for the treatment of tuberculosis (5). Traditionally, Lf is used for symptomatic treatment of cutaneous and mucosal lesions (6). Aqueous extract of the stem bark of Lf, orally, reduced blood glucose levels in rats with streptozotocin-induced diabetes (7). The hypoglycemic activity was also observed by Souza and colleagues following oral administration of aqueous extracts of the stem bark to alloxan-induced diabetic rats (8).

Acute toxicity studies indicated that aqueous extract, when administered orally, did not induce clastogenic or cytotoxic effect (9); and the LD_{50} assay of *Libidibia ferrea* performed intraperitoneally indicated low toxicity (10). Wyrepkowski and co-workers demonstrated that the plant had no mutagenic effect (11). Freitas et al. observed that crude extract of this variety did not present cytotoxic or antitumor action in cell cultures NCI-H292 (human lung mucoepidermoid carcinoma cells), HEp-2 (human larynx epidermoid carcinoma cells) and Sarcoma 180 (12).

The extracts with antioxidant, anti-inflammatory, antimicrobial properties can be embedded in natural films, such as bacterial cellulose. Bacterial cellulose is a polysaccharide excreted

extracellularly by several microorganisms including *Agrobacterium, rhizobium, Pseudomonas, Sarcin* and *Acetobacter* (13,14). Its nanofibrilar nature and the ability to be molded into three-dimensional structures during their synthesis, making it an ideal matrix for medical devices and skin replacement grafts or as dressings in the treatment of injuries, burns and ulcers (13). Bacterial nanocellulose also helps relieve the pain caused by wounds, protects against infections and speeds up the healing process (15).

For this reason, this work evaluated the association of the *Libidibia ferrea* properties with the bacterial nanocellulose, focusing on a cicatrizing product, mainly applied in the treatment of burns in healthy and diabetic rats.

2 MATERIAL AND METHODS

Obtaining the *Libidibia ferrea* extract

Bark was collected from a voucher specimen deposited at the Universidade Federal do Rio Grande do Norte (UFRN) – Brazil - herbarium under the number 12181 and identified as *Libidibia ferrea* C. Mart ex Tul var ferrea. A small portion of the stem bark was scraped from the stem. The samples were stored in plastic bags at -4 ^{o}C and transported to the Laboratory for the Toxicological Research (Lapetox), University of Sorocaba. Bark was dried in an oven with circulating air at 40 ^{o}C for 72 hours and it was ground to a thin powder with a knife and hammer mill (Marconi® - MA340, Brazil). The crude extract was prepared from the powdered barks of *L. ferrea* with an alcohol-water solution (7:3 v/v). The preparation was concentrated in a Rotavapor (Büchi R-153, Switzerland) under reduced pressure and then lyophilized (Thermo Fisher Scientific, USA).

Obtaining the bacterial nanocellulose membrane

Bacterial nanocellulose membranes (BNC) were produced by culturing *Gluconacetobacter xylinus* ATCC 53582 in Hestrin & Schramm medium. The cultivation and preparation of the membranes were performed following Jozala et al (14).

Incorporation and Release of the extract in BNC

The incorporation and release assays were performed in BNC 24-well plates immersed in Lf extract (10 mg. mL^{-1}) (pH 6.8). The plates were placed in a bench-rotating shaker at 25°C at 100 rpm for 24 and 48 hours. After the periods, the evaluation of the Lf extract regarding incorporation and release was conducted following the microbiological methodology described by Ataide et al (13).

Microbiologic Assays

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For the Minimum Inhibitory Concentration (MIC) assays, *Staphylococcus aureus* (ATCC 10390), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeuroginosa* (ATCC 9721) were used. The MIC methodology was adapted in 96-well microplates (13,16). The microplate was wrapped in the oven at 37°C overnight. After this period, 5 μL of each well were collected and dripped onto petri dishes containing *Tryptone Soya Agar* (TSA) for activity analysis. The petri dishes were kept in the oven at 37°C overnight**.**

For the antimicrobial activity evaluation tests, the same MIC microorganisms were used through agar diffusion. Cultivation was performed in TSB broth (100rpm/37ºC/24h). An aliquot of the previously diluted suspension containing 10^6 CFU/mL was transferred, homogenized in 250 mL of TSB agar (TSB broth with 0.8% w/v bacteriological agar) and inverted into Petri dishes (± 20 mL in each plate). The membranes were placed in the center of each plate (one membrane by plate) for the evaluation of the activity (14,17).

Physical-chemical characterization

The characterization of the specific chemical groups of Lf extract, BNC and BNC-Lf extract was performed in Fourier Transform Infrared Spectroscopy (FTIR) (Shimadzu, FTIR IRAffinity-1S, Kyoto, Japan) using transmittance modes. The membranes were kept in an oven at 30ºC, after drying, they were ground in grail. Approximately 2 mg mass of the sample was mixed with 300 mg KBr for tablet formation. The spectra were obtained in the wavelength range of 4000 to 400 cm^{-1} after 64 scans, with a resolution of 4 cm^{-1}. The spectra were normalized and vibration bands were attached to the main chemical groups.

The mucoadhesive strength of BNC and BNC- Lf extract was assessed by the force required to remove the membrane from a mucin disc using the TAXTPlus (Stable Micro Systems, UK) texture analyzer. The specimens were fixed in a water bath with the temperature set at 37°C. The mucin discs were prepared by compression (Lemaq, rotary compressor machine, Mini Express LM-D8, Diadema, BR). The surface of the mucin disc was hydrated and the disc fixed at the lower end of the analytical probe. The mucin disc was compressed apically → on the basal surface of the samples, with a compression force of 0.1 N. The contact time of the disc with the surface of the samples was 200s. The removal of the analytical probe was at the rate of 0.5 mm. s^{-1}. The force required to separate the surfaces was determined by the time *x* force ratio ($n = 3$).

Vertebrate animal study

For vertebrate animal study was used Male Wistar rats (*Rattus norvegicus* var. Albinus) weighting from 160 to 180 g were purchased from Anilab (*Laboratório de Animais*, Paulínia, SP,

Brazil). We allowed animals to habituate to the Alesco® microenvironment isolation cages under standard environmental conditions (22°C, 12:12 h dark/light cycle) while providing commercial diet (Purina®, São Paulo, Brazil) and tap water *ad libitum*. The experimental protocol was approved by the Animal Use Ethics Committee of the University of Sorocaba (Process no. 057/2015), in compliance with the International Guideline ARRIVE (Animal Research: Reporting of *in Vivo* Experiments). For euthanasia method all animals were submitted to anesthesia and neuromuscular block (ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (6 mg/kg)) followed by diaphragm perforation. There were no survivors. (18,19).

Diabetes induction and Preparation of rat skin

Diabetes was induced by streptozotocin administration (65 mg/kg, diluted in 0.1 mol/L citrate buffer, pH 4.5) intraperitoneally. 2 days later, after fasting (6 hours), blood glucose was dosed in the Freedom Lite Freestyle (Abbott) equipment. Animals with glycemia above 150 mg/dL were considered diabetic (20,21).

Thirty-six (36) animals were randomly allocated (by a random number table) into four groups (n=9/group):

I) Nd-BNC (Non-diabetic + Bacterial nanocellulose membranes);

II) Nd-BNC-Lf (Non-diabetic + Bacterial nanocellulose membranes + *Libidibia ferrea*);

III) D-BNC (Diabetic + Bacterial nanocellulose membranes);

IV) D-BNC-Lf (Diabetic + Bacterial nanocellulose membranes + *Libidibia ferrea)*.

All animals were submitted to anesthesia and neuromuscular block prior to the burn (ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (6 mg/kg)). After the anesthesia, an area of a 3x3 cm on the animals' backs was shaved and the shaved area was sanitized with 1% polyvinylpyrrolidone-iodine (PVP-I). The burns were induced symmetrically in shaved areas by contact with the aluminum bar (R = 12 mm), preheated at 100°C, for 10 seconds (22,23).

After induction of the lesion and upon awakening, the animals received oral dipyrone daily at 20 mg/kg of body weight for post-burn analgesia. Lesions were observed for 28 consecutive days. Dermal exposure to BNC and BNC-Lf extract was performed daily along the period (Figure 1).

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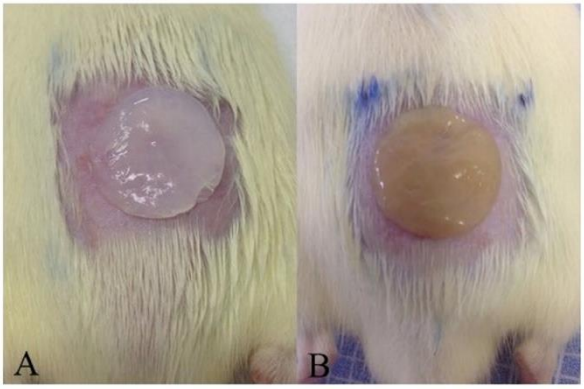


Histological evaluation

For histological evaluation, on 14th, 21st and 28th day, 03 rats from each group were randomly anesthetized with ketamine (100 mg/kg) and xylazine hydrochloride (6 mg/kg) and euthanized (22). The skin of the burned area was dissected; the fragments were dehydrated in ethyl alcohol, diaphanized by xylol, impregnated and included in paraffin (24). Slides were examined by a blinded pathologist (single blind condition).

The histological parameters analyzed were: inflammation cells (neutrophil), degree of infection, granulation tissue degree, angiogenesis, organization of the collagen, and reepithelization. The semi-quantitative analysis of the anatomopathological aspects was done. In this way, results were scored as: (+) Presence if there is signal of the analyzed characteristic; (-) Absence if there is no signal of the analyzed characteristic. In the presence of the analyzed parameter, the following scores were assigned: + Slight; ++ Moderate; +++ Severe.

Statistical analysis

The determination of the significance of the mucoadhesive properties was possible through the analysis of variance (ANOVA). All tests were run with a 95% confidence interval ($p < 0.05$). For the analysis of the results with the animals, Students' t-Test was used for the parametric data, with a significance level of 5%. The Bartlet test was used to evaluate the homoscedasticity of the data.

3 RESULTS

Microbiological assays

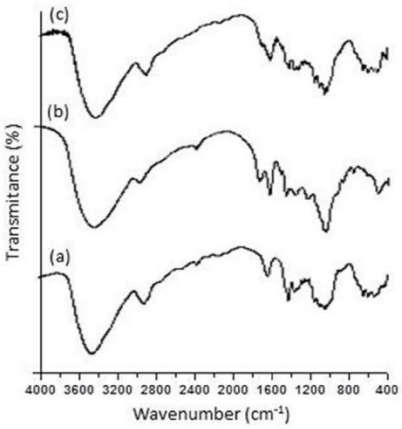
The MIC results showed the Lf extract has antimicrobial activity for *S. aureus* (0.39 $mg.mL^{-1}$), *P. aeruginosa* (0.79 $mg.mL^{-1}$) and *E. coli* (0.19 $mg.mL^{-1}$). By the Agar Diffusion methodology, it was possible to observe the BNC-Lf extract has an antimicrobial action against all the

microorganisms studied, proving Lf extract is released from BNC through the formation of the inhibition halo.

The mean halo formed by the zone of inhibition against *S. aureus* (Gram-positive) was approximately 36 mm. With the Gram-negative ones under study, the formation of the halo was 29 mm on average. This difference may be related to the type of microorganisms studied. A test was also run with only BNC, and it showed absence of antimicrobial activity.

Physical-chemical characterization

The characteristic stretches of *Libidibia ferrea* and BNC without and with Lf are shown in Figure 2. The BNC with Lf extract showed displacements of the stretches at 1448 cm-1 and 1508 cm-1 at the rings (COOH) and 817 cm-1 for the carbonyl (CO) group (25,26). The stretching 1618 cm-1 for conjugated carbonyl (C = O) and 1230 cm-1 for glycosides (C-O) belonging to *Libidibia ferrea* are present in BNC (26).



The mucoadhesive property can be assessed by means of a tensile test. The maximum strength (Fmax) to separate the BNC without and with Lf from the mucin disc were 0.493 ± 3.6 and 0.407 ± 10.7 N, respectively. Mucosadhesion may occur by increasing system viscosity or by molecular interactions such as ionic interactions or formation of hydrogen bonds. In this way, the mucoadhesion analysis evaluates the interaction between the membrane and the surface of the mucosa at 37ºC (27-29).

Although the nominal values of Fmax are higher in BNC-Lf extract, there is no significant difference ($p > 0.05$), that means Lf extract incorporation did not interfere in the mucoadhesive property (27). No studies on the evaluation of the mucoadhesive properties conducted with BNC were found in the literature.

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Evaluation of the wound

Non-diabetic rats treated with BNC-Lf extract presented an improvement in the wound, with epithelialization already on the 14th day, while all other animals presented this epithelization after 21 days (Table 1). On the 28th day, there was no histological difference among the groups, that is, all of them evolved in a similar way. The severe degree of infection, granulation and inflammation observed on the 14th day in diabetic rats (with or without Lf extract), disappeared on the 21st day.

In general, the BNC with or without Lf extract, proved to be effective in the treatment of lesions induced by thermal contact.

Table 1. Histological evolution of wounds in Non-diabetic (Nd) and Diabetic (D) rats 14, 21, and 28 days after topical application of Bacterial Nanocellulose membrane (BNC) with or without *Libidibia ferrea* (Lf).

	Inflammation cells (Neutrophil)			Degree of Infection			Granulation degree			Angiogenesis			Collagen organization			Epithelization		
	14	21	28	14	21	28	14	21	28	14	21	28	14	21	28	14	21	28
Nd-BNC	+	+	-	-	´-	-	++	-	-	++	-	-	++	++	+++	-	+	+
Nd-BNC-Lf	++	-	-	-	-	-	++	-	-	++	-	-	++	++	+++	+	+	+
D-BNC	++++	-	-	+++	-	-	+++	-	-	+++	-	-	-	+++	+++	-	+	+
D-BNC-Lf	++++	-	-	+++	-	-	+++	-	-	+++	-	-	+	+++	+++	-	+	+

Scores: + Slight presence; ++ Moderate presence; +++ Severe presence; - Absence.

4 DISCUSSION

The study was based on a dressing to diabetic wound care and antimicrobial activity attributed to Lf extract once the described reports suggest the use of this plant for symptomatic treatment of skin and mucous lesions (6). In addition, BNC has excellent properties such as tissue regeneration, faster healing properties, shape stability and wound dressing (30).

In the microbiological assays, the minimum inhibitory concentrations for *S. aureus*, *P. aeruginosa* and *E. coli* attributed to Lf extract solubilized in PBS were very low. Oliveira and co-workers used the bark of Lf stem for MIC assays and they observed inhibition of *Streptococcus mutans* (ATCC 25175, *Streptococcus salivarius* (ATCC 25175) and *Streptococcus oralis* (ATCC 10557), Gram-positive species and oral pathogens, at 37.9 mg. mL^{-1} of Lf. A MIC of 18.7 mg. mL^{-1} was found for *Candida albicans* (INCQS 40040), an oral pathogenic yeast (31). These concentrations were higher than the MIC found in our study, showing the *Libidibia ferrea* extract obtention process was very efficient.

Pavan et al. performed *in vitro* antimicrobial activity assays with Lf and defined the MIC at 4 mg. mL^{-1} for *Mycobacterium tuberculosiss* (32). Sampaio et al. observed the aqueous extract of the fruit was able to inhibit the growth of oral pathogenic organisms *Candida albicans* ATCC 36232, yeast, *Streptococcus mutans* ATCC 25175; *Streptococcus oralis* ATCC 10557; *Streptococcus*

salivarius ATCC 7073, *Lactobacillus casei* ATCC 7469, all Gram-positive microorganisms (33). Additionally, agar diffusion test showed that BNC without Lf has no antimicrobial activity. The use of polymeric compounds has proven there is no antimicrobial activity, and this activity would be related to the insertion of active bio-molecules in the materials (13,34,35).

Our research group characterized this same Lf bark extract by HPLC and showed a material rich in ellagic acid (36). Ellagic acid has antimicrobial activity and explains the antimicrobial barrier created. Oliveira et al. observed that, for Gram-positive pathogenic oral species, the bark of Lf stem had a halo of 13.5 mm on average, and a halo of 21.5 mm for the yeast species *C. albicans* (31). In our studies, BNC loaded with Lf presented a halo of inhibition of 36 mm in diameter when in contact with Gram-positive pathogen, and a similar antimicrobial efficiency among Gram-negative microorganisms.

Kobayashi and co-workers induced a wound in rats and evaluated skin healing after exposure to two concentrations of hydroalcoholic extract of Lf fruits (50 and 12.5%), topically. The animals receiving 50% Lf extract showed delay in epithelialization and in contraction of the wounds. However, the group Lf 12.5% showed good re-epithelialization, indicating the concentration of the extract may influence the treatment result (37).

Another study evaluated the healing capacity of a polysaccharide extracted from Lf bark in an excisional cutaneous wound model in rats. Four concentrations of Lf polysaccharides (0.025 to 0.1%) were evaluated and the plant polysaccharides accelerated healing and stimulated proliferation of dermal fibroblasts and keratinocytes (38,39). Lf polysaccharides accelerate the cutaneous wound healing by controlling the inflammatory phase and attenuates hypernociception through the modulation of inflammatory mediators (40).

In our study, on the 28th day, all studied groups (with or without Lf extract) had total lesion epithelization. Then, it is suggested despite Lf has good antimicrobial activity it does not stimulate epithelization in topical treatment.

5 CONCLUSION

The Lf extract and BNC-Lf extract showed antimicrobial activity against Gram-positive and Gram-negative microorganism. Loading the Lf into BNC would create an ideal wound dressing, since it aligns the beneficial characteristics of both components.

Os curativos com Lf, para o grupo dos ratos não diabéticos, se mostraram levemente mais eficientes no processo de cicatrização das feridas, pois apresentaram menor grau inflamatório no 21 primeiro dia e epitelização no 14 dia de aplicação. Já para os grupos diabéticos o BNC com ou sem Lf o comportamento foi o mesmo. Moreover, the total epithelization on the 28th day was equivalent for all groups.

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FIGURE REFERENCE

Figure 1. Dermal exposure to Bacterial Nanocellulose (BNC) membrane dressing (A) and BNC + *Libidibia ferrea* dressing (B).

Figure 2. Fourier Transform Infrared Spectroscopy spectra of *Libidibia ferrea* (a), Bacterial Nanocellulose membrane (BNC) (b), BNC with *Libidibia ferrea* (c).