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RESEARCH ARTICLE

EFFECT OF NANOSTRUCTURED EXTRACT MORINDA CITROFOLIA L. (NONI) IN INFECTED WOUNDS IN RATS

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ABSTRACT

Morinda citrifolia L. (Rubiaceae), popularly known as Noni, looked at the scientific community because of their anti-inflammatory and immunomodulatory effects. This study explores the potential of Noni to ameliorate inflammation and infection in open infected skin wounds of rats. Fourteen Wistar rats weighing 285±12g were used. The study was done in a group whose open infected skin wounds were treated with topical application formulated nanostructured base hydroalcoholic extract Morinda citrifolia L. (Noni) 5mg/mL/Kg (Noni group (N), n=6) and the second group with wounds treated with saline 0.9 % (Control group (C), n=6). A bacteriological exam of the wounds fluid for gram positive and gram-negative bacteria, the tecidual expression of TNFa and IL-1a by immunohistochemical technique, and histological analysis by HE stain were performed. The expression of TNFa could be clearly demonstrated in lower degree in skin wounds treated with Noni (N) than in Saline group (C). In comparison, wound tissue from (N) group displayed leukocyte infiltration significantly lower than that observed in Saline group (C) ($p<0.05$). Culture results of the samples taken from wound fluid on third post-treatment day revealed wound infection in only one rat of group, where Proteus mirabilis, Escherichia coli and Enterobacter sp were isolated. In the rats whose wounds were treated with Saline (C), polymicrobial infection with more than 100,000 CFU/g was detected in all the wounds. In addition to its anti-inflammatory properties, the protective effects of Morinda citrifolia L. (Noni) in infected skin wounds were able to reduce infection and have antibacterial action.

INTRODUCTION

The healing of open skin wounds involves the epithelium and underlying stroma. Processes such as angiogenesis, activation, migration, and proliferation of fibroblasts, myofibroblasts and endothelial cells; formation of granulation tissue; and wound contraction are needed to close these defects [1,2]. Some wounds are also frequently inflamed and, in general, stromal involvement and inflammation greatly increase the risk of subsequent complications [3,4]. When a tissue is injured, there is a cascade of signaling, molecular, and cellular interactions, in an attempt to restore the shape and function of the compromised area. In connective tissues, this repairing process is mediated by the formation of granular tissue, richly vascularized containing inflammatory cells and fibroblasts [3-5].Open wounds are those where a break in the integrity of the skin results from trauma. Within this concept, there is a classification regarding the type of injury according to the object that causes the trauma (incisions or surgical, contusive, lacerated and perforating) [5]. The healing process has a sequence of phases, where local reactions occur in the skin structure [6]. Among the repair phases of a wound, we can mention: inflammatory phase (lasts about 3 days), proliferative phase that includes reepithelialization, extracellular matrix synthesis and neovascularization (can last from the third to the 20th day after the injury) and stage of maturation that occurs after the 20th day (can last about 1 year). Each of these stages presents important characteristics for the complete/partial restitution of function and tissue form [6-8].

The repair process begins immediately after injury by various growth factors, cytokines, and low molecular weight compounds. Disruption of blood vessels also leads to the formation of blood clot, which is composed of cross-linked fibrin, and extracellular matrix proteins such as fibronectin, tenascin, and thrombospondin [9]. Wound infection develops in 2% to 5% of patients undergoing surgical procedures each year in most hospitals worldwide and continues to be considered one of the most important problems in surgical wards nowadays. It is one of the main factors that alter the physiologic evolution of the wound healing [10,11] The

bacteria inhibit angiogenesis, secrete plasminogen activators, and proteolytic enzymes that may affect the extracellular matrix, blocking the wound contraction [12]. Several substances have been used to treat infected skin wounds, like honey, sugar, antibiotics and phytotherapeutics agents [5,10].
Medicinal plants influence the health conditions of the people, in part due to the increase of studies with phytotherapeutics, leading to a confirmation of the therapeutic action of several popular plants, a fact that proves Phytotherapy as part of the culture of the population, being used and widespread for many generations [13]. In Brazil, the use of medicinal herbs has its bases in the indigenous practice, which is influenced by the African and Portuguese culture, generated a vast popular culture [12-14].

With the technological advances of allopathic medicine and the pharmaceutical industry in recent years, herbal medicines have been placed in the background, being something allied to popular belief and without scientific bases [13]. However, due to side effects and the high cost of medicines, Phytotherapy is again highlighted and scientific studies with medicinal plants are being summarized [14]. Among the species for herbal treatment highlights the Morinda citrifolia L. (Rubiaceae), popularly known as Noni. Information on its therapeutic benefits has gone through the world causing great demand as a medicinal product [15,16]. Scientific studies attest biological activities of Morinda citrifolia L (Noni) described by the polynesians as antioxidant, anti-inflammatory, analgesic, antibacterial and anti-tumor [16]. In this paper, we present the results of anthraquinones, triterpenes, iridoids, among others [17]. The anti-inflammatory activity of Noni has been studied *in vivo* and *in vitro* by inhibiting the activity of COX-1 enzymes and COX-2, and the release of chemical mediators from macrophages (NO) and prostaglandin 2 (PGE-2) [18]. Morinda citrifolia L. (Rubiaceae), popularly known as Noni appears as the high healing power agent, whose empirical knowledge of the population has made the fruit is constantly present in the diet [19,20]. Despite the great success and international demand for Noni products, Brazil has reduced the amount of research carried out to obtain more data on this plant species, although scientific articles and papers mention that Morinda citrifolia L (Noni) has phytotherapeutic activity of analgesic, antimicrobial, antitumor, anti-inflammatory and antioxidant effects [21,22].

In this sense, products using biotechnology in the form of nanoparticles or nanostructured compounds, can have excellent results, since, due to their reduced diameter, the substance can be used in smaller doses, avoiding the toxic effect of the plant and maintaining its phytotherapic action.
Morinda citrifolia L. (Noni) might improve the healing of infected skin wounds in a rat model. We have approached the question of whether topical treatment with formulated nanostructured base hydroalcoholic extract.

MATERIALS AND METHODS

Experimental design

The experimental protocol was approved by the Ethics Committee on the Use of Animals - *POTIGUAR UNIVERSITY - LAUREATE INTERNATIONAL UNIVERSITIES*, number 012/2016, Brazil. Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996. The Institutional Committee on Ethics in the Use of Animals approved the research project under protocol - Brazilian College of Animal Experimentation (Law 11.794 / 2008 - CONCEA - Brazilian College of Animal Experimentation).

Animals

Wistar rats *(Rattus norvegicus albinus Rodentia* mammalia) weighing 285±12g were used. Surgeries were carried out in the experimentation area of the Potiguar University Animal Bioterium (UnP), where the animals were randomly separated, placed in individual cages, lined with pinewood. The animals were then placed on ventilated shelves (Model: ALE02; Mark ALESCO/BRASIL 2007®) for adaptation, remaining one week in acclimatization period, being evaluated daily under a standard controlled temperature of 20°C ± 2°C, humidity in Around 45% ± 15% and ambient lighting, with a reversed 12h light-dark cycle. The environment remained under noise below 60dB.They were polypropylene housed in cages and maintained under controlled temperature conditions on a 12h light-dark cycle and allowed access to commercially available rat chow (Labina, Purina®) and water *ad libitum.*

Experimental groups

The animals were randomly divided into 2 groups with 6 animals each. The control group was treated with 0.9% saline solution control group (C) and the experimental group submitted to a single treatment containing Noni nanoemulsion extract at a dose of 5mg/ mL/Kg (N), both topically.

Preparation of the vegetable extract

The hydroalcoholic extract of Morinda citrifolia L. was prepared from the aerial parts (stem, leaves, and fruits) of fresh adult plants. The collected material was placed at room temperature; Then crushed and placed in the oven for 24h at a temperature ranging from 45°C to 50°C to remove moisture. Then the material was ground to obtain the powder, weighed and deposited in a glass vessel with the addition of 70% hydroalcoholic solution in the ratio of 1:3 of the powder. The resulting mixture was allowed to stand for 12h and stirred for five minutes every two hours under two simple filtration procedures under reduced pressure to give the crude extract which was concentrated in a rotary evaporator under reduced pressure at a temperature between 55°C and 60°C, for total solvent elimination. The product obtained after concentration in paste was diluted in distilled water until the hydroalcoholic extract at 5mg/mL/Kg was obtained in the refrigerator and kept at 10°C until it was used.

Surgical model

All the animals were submitted to the same operative procedure, carried out in the surgical center of the Bioterium of the Potiguar University (UnP). For induction of anesthesia, Zoletil® 50 was used at a dose of 50mg/Kg, an injectable anesthetic composed of hydrochloride tiletamine and hydrochloride zolazepam in a 1:1 ratio. The anesthetic was administered intramuscularly into the right quadriceps region with 1mL disposable syringes and 26G needle 0:45x13 1/2" Descarpack®.Having established the full anesthesia mouse flow pressure test was made and antisepsis shaving the dorsal region of the animal with chlorhexidine gluconate 2% of spray NEBA-SEPT®brand in operative area and protection with sterile surgical field. For preparation of the wound, we used a digital caliper ("ZAAS precision"-8/200mm /2015 / Brazil®), to standardize the size of the lesion 1cm^2. All surgical instruments were sterilized by autoclaving

(BRASMED 21Lts-2014/Brazil®). The skin incision length was 1cm^2 with a quadrangular shape (figure 1). Immediately after the surgical procedure, the wounds were contaminated with topical application of 0.5 mL of multibacterial solution prepared with 1g of fresh feces and 10 mL of saline. In the following day, the infected wounds of the Noni group (N) (n = 6) were topically treated with topical application formulated nanostructured base hydroalcoholic extract Morinda citrifolia L. (Noni) 5mg/mL/Kg - 0.2mL once a day. The wounds of group saline (C) (n = 6) rats were treated with 0.2 mL of saline solution daily (Figure 2). When the wounds were totally epithelialized, the healing time was recorded and the resection of the scar was performed under anesthesia. The healed tissues were used for histopathological study and immunohistochemical dosage of tumor necrosis factor-α (TNF-α) and interleukine-1 (IL-1β).

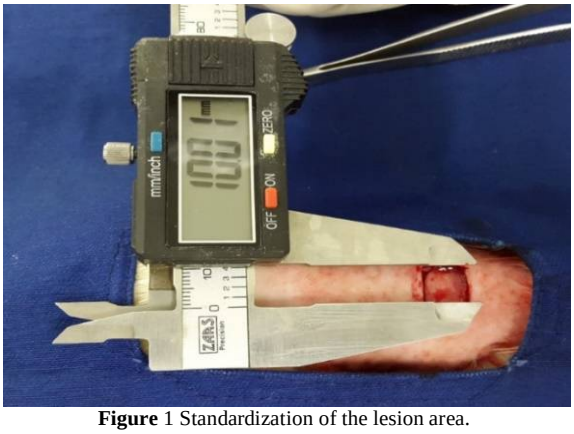


Figure 1 Standardization of the lesion area.

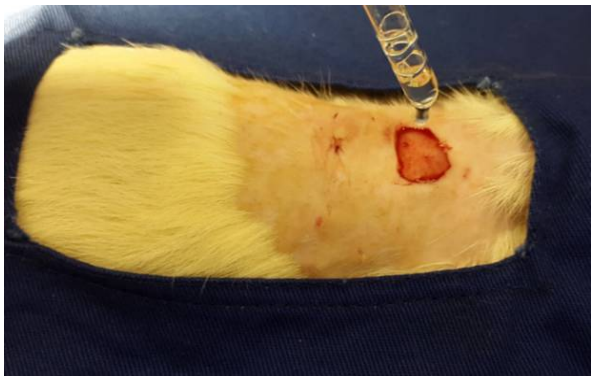


Figure 2 Administration of formulations

The animals were placed in individual cages with your registration number and packed in ventilated shelves (Model ALE02; ALESCO Brand, 200/ Brazil®), with water supply and food *ad libitum*. Throughout the postoperative period, analgesia was performed to treat postoperative pain, by administering Dipirone at a dose of 500mg/mL/Kg, 6/6h, through a gavage probe.

Immunohistochemical staining of TNF-α and IL-1β.

Immunohistochemical staining for TNF-α and IL-1β was performed on tissue samples obtained from the healing skin. These samples were fixed in 4% paraformaldehyde, processed in routine technique, cut into 5mm-thick frozen sections, and dried at room temperature. Absolute methanol containing 3% hydrogen peroxide was added to block endogenous peroxidase. After three washings with a phosphate-buffered solution (PBS) for 5min each, these sections were treated with 1% polyoxyethylene-10-octylphenyl ether in PBS for 20min at room temperature. After washing in the same way, these were reacted with 100mL of biotinylated anti-rat TNF-α monoclonal antibody (Pharmingen®,San Diego,CA/USA) or biotinylated anti-rat IL-1β monoclonal antibody(Pharmingen®,San Diego,CA/USA), diluted in 9mL of PBS and 1mL of whole goat serum at room temperature in a moist chamber for 2h. After washing, the solutions were incubated with two drops each of avidin solution and biotinylated peroxidase solution in 4.5mL of PBS and 0.5mL of 5% skim milk for 2h at 37°C. After PBS rinsing, diaminobenzidine and nickel were applied for 8min to achieve permanent color change. Six views were randomly selected for each section and observed under a light microscope (x100). The mean number of reactive cells in the six views was regarded as the data for each sample. Sequential images of microscopic sections were photographed within 72hours after immunostaining, by a digital camera (Sony®, Tokyo, Japan) mounted on a lightmicroscope (Olympus® B-50, Tokyo, Japan) at a magnification of 100x, and saved in jpg file format. Images were analyzed then in Image-Pro Plus software (Media Cybernetics®, LP, USA). Briefly, the entire area marked by cytokines was marked, and the total marked area was calculated. The area stained by the antibody of interest was identified and calculated by using the software color algorithm. The integrated optical areas stained by antibodies were then recorded. The score was calculated for each of the antibodies and it was averaged.

Histopathology

The biopsies of skin wounds were processed following the routine and stained with hematoxylin and eosin (HE) for histological analysis of the inflammatoryreaction, using the optical microscope (Olympus® B-50, Japan, Tokyo). The quantification of cells, fibers and elements of the inflammatory reaction was performed by the Image Pro-Plus Media (Cybernetics® software, LP, USA).

Bacteriological examination

At the 3th postoperative day, exudate was collected from the wounds for microbiology and for quantitation of bacterial population. MacConkey's agar, blood agar and salt mannitol agar. Were The agar plates are incubated at 37°C and examined for growth after 24, 48 and 72 hours. Any growth in the plates of bacteria of the same biotype.

The cultured in the wounds was considered positive and expressed colony-forming units per gram of tissue (CFU/g). All procedures were performed under laminar air flux.

Statistical analysis

Data are presented as mean ± standard deviation. Results were analyzed with ANOVA and Student t test. Statistical significance was assumed at p <0.05.

RESULTS

Tumor necrosis factor alpha (TNFα)

The expression of TNFα could be clearly demonstrated in lower degree in skin wounds treated with Noni (691.6 ± 67.4ìm^2) than in saline (2320.0 ± 338.1ìm^2) treated wounds, as can be shown in table 1. Only the distinct decrease of tissue reactivity occurred when the simvastatin microemulsion was applied to the infected wounds (Table 1).

Table 1 Mean and statistical analysis of optical density related to the expression of cytokines in tissues of skin infected wounds treated with Noni and saline (μm^2).

GROUPS	TNF-α (pg/mL)	IL-1β(pg/mL)	*p*-value
Saline (C)	2320.0 ± 338.1**	721.6 ± 68.9**	0.00001
Noni (N)	691.6 ± 67.4	424.8 ± 47.2	0.00001

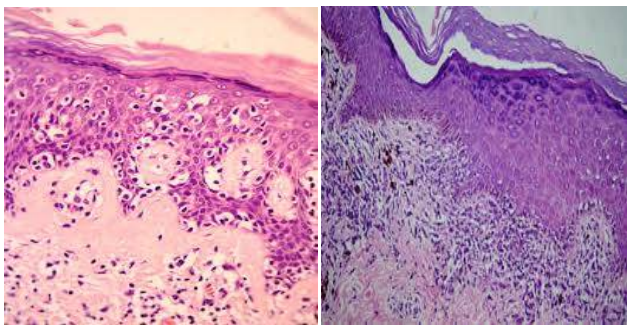
* Mean ± Standard deviation 1 – Difference statistically significant comparing the groups Noni/Saline (Student *t* test). Image Pro-plus software Media Cybernetics was used.

Interleukin-1b

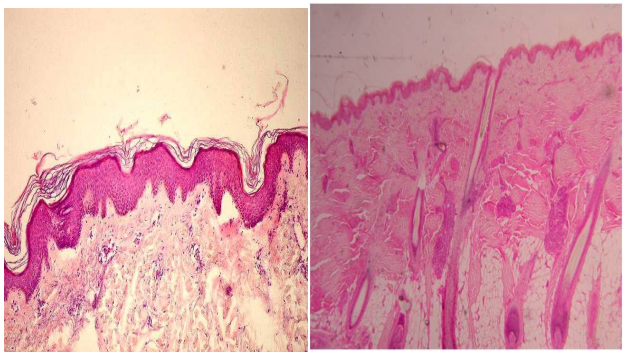
IL-1b expression was significantly enhanced in the saline (C) group than in the Noni (N) group after total epithelialization of the skin wounds. A clearly decreased immunohistochemical stainability could be noticed in the Noni group, whose data are expressed in table 1.

Histopathology

Saline rats, with edema and marked leukocyte infiltration consistent with acute injury (Figure 1). In comparison, wound tissue from (N) group showed leukocyte infiltration significantly lower than that observed in Saline group (C). These histological alterations were promoted by administration of nanostructured Noni hydroalcoholic extract in the infected wounds. The histological slides (Figures 3 and 4) suggest differences in neutrophil accumulation between the Saline (C) and Noni (N) groups.



Figures 3 Histological section of wound tissue taken from a Saline (C) group rat demonstratingsignificant neutrophil infiltration, giant cells, and follicles. HE, 100x



Figures 4 Histological section of wound tissue taken from Noni (N) group rat, demonstrating low neutrophil infiltration and good epithelial regeneration.HE, 100x.

Bacteriological examination

Culture results of the samples taken from wound fluid on third-day post treatment revealed wound infection in only the rat number 4 of group Noni (N), where Proteus mirabilis, Escherichia coli and, Enterobacter sp were isolated. In the rats whose wounds were treated with Saline (C), polymicrobial infection with more than 100,000 CFU/g was detected in all the wounds (Table 2).

Table 2 Detection of bacteria in the wound fluid of animals in groups treated with Noni (N) and treated with Saline (C).

Rat Number	Noni (N) group/bacteria	CFU/g	Saline Group (C)/ bacteria	CFU/g
1	NBG	0	Klebsiella sp; Proteus Mirabilis; Proteus vulgaris; Staphylococcus coagulase negative	> 100,000
2	NBG	0	Proteus mirabilis; Escherichia coli; Staphylococcus coagulase negative	90,000
3	NBG	0	Proteus mirabilis; Escherichia coli; Staphylococcus coagulase negative	>100,000
4	Proteus mirabilis; Escherichia coli; Enterobacter sp	40.000	Klebsiella sp; Proteus mirabilis	>100,000
5	NBG	0	Escherichia coli; Enterobacter sp; Proteus mirabilis	>100,000
6	NBG	0	Proteus mirabilis; Escherichia coli	>100,000

NBG, no bacterial growth; CFU/g, Colony forming unit per gram.

DISCUSSION

In recent decades, popular interest in Noni has intensified due to the knowledge of the medicinal effects capable of preventing or curing cancer, diabetes, hypertension, osteoporosis, menstrual and muscular disorders, headaches, depression, stress, allergies, arthritis, asthma, bacterialinfections, obesity, gastric ulcers, insomnia, respiratory problems, multiple sclerosis and even chemical dependence [23,24] Scientific studies have identified chemical components of the various parts of Morinda citrifolia L. In the plant around 200 compounds are shown, most of which are phenolic compounds (anthraquinones) and their glycosides (damnacantal, morindone, escopein, alizarin, austrocortinin, rubiadine) [25]. Other compounds were identified as: water (90%), high amount of proteins, some amino acids (aspartic acid, glutamic acid and isoleucine)[26]. The minerals found are potassium, calcium, phosphorus and sulfur. Some vitamins such as ascorbic acid and pro-vitamin A [27]. Carbohydrates like sucrose, fructose and glucose. Some volatile compounds such as organic acids, alcohols, esters, ketones and lactones. The alkaloids xeronine and proxerone are also described [28].

In this sense, products using biotechnology in the form of nanoparticles or nanostructured compounds, can have excellent results, since, due to their reduced diameter, the substance can be used in smaller doses, avoiding the toxic effect of the plant and maintaining its phytotherapic action.
Cutaneous wound healing is a complex process involving blood clotting, inflammation, new tissue formation, and finally tissue remodeling [29]. The effects of exogenous substances on the healing process are described in this paper. However, the roles played by these exogenous treatments have remained largely unclear [30-32]. According Tintino *et* al. there do several reports that Morinda citrifolia L. has bactericidal

activity due to the phenolic compounds (anthraquinones) exist in the noni fruit. Thus, the effects of these compounds have been shown to have a direct effect in reversing the natural resistance of some bacteria to the elimination of plasmids, altering the plasma membrane, the cell wall and modifying the efflux pump [33]. Therefore, Noni has been the target of studies to prove its effectiveness as an antimicrobial action, contributing to the destruction of bacteria today multidrug resistant to traditional medicines. This study tested the antimicrobial potential modulator and fruit extracts from Morinda citrifolia L. (Noni) and noted that showed significant results for the modulator and bactericidal effect when compared to the tested drugs (imipenem, oxacillin, gentamicin, and amikacin) against *Pseudomonas aeruginosa* and *Escherichia coli* [31-33]. The present study is one of the first to demonstrate that Morinda citrifolia L inhibitors significantly improve healing of infected skin wounds in an experimental model in rats. We demonstrated that the topical application of formulated nanostructured base hydroalcoholic extract Morinda citrifolia L. (Noni) attenuated the inflammatory reaction in wound healing of infected tissues. The chemical compounds of the Noni fruit have a wide variety of beneficial health effects, including antioxidant, antimicrobial and anti-inflammatory actions [34]. These effects are mainly due to phenolic compounds such as anthraquinones, rutin, asperuloside, alizarin and escopeletin. Therefore, it was observed that these compounds inhibit the growth of various bacteria like *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus morgaii, Bacillus subtilis, Escherichia coli, Helicobacter pylori, Salmonella, Shigella, Mycobacteriumtuberculosis* and *Streptococcus pyoge nes.* [34-36].

Morinda citrifolia L. (Noni) was able to induce a marked decrease in TNFα anf IL-1β in healing tissues, as demonstrated by immunohistochemical analysis. A probable antibacterial effect was also observed, and the exact mechanism to explain this action is to be described.

CONCLUSION

In addition to its antiinflammatory properties, the protective effects of formulated nanostructured base hydroalcoholic extract Morinda citrifolia L. (Noni) in infected open skin wounds was able to reduce infection and had antibacterial action. The potential to treat these wounds with nanostructured Noni to ameliorate inflammation and infection is promising, reducing the toxicity of the plant in nature and increasing its therapeutic power using nanotechnology.

Conflict Of Interest

The author (s) declared that there are no conflicts of interest with respect to the authorship, and/or publication of this article.

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