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Copaiba oil loaded into self-nanoemulsifying drug delivery system enriched with powdered coconut water as a strategy for therapeutic enhancement of skin wound healing

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Introduction

Healing of cutaneous wounds is a multi-cellular and multi-molecular process which occurs after loss of skin integrity involving a complex signaling network, cells interactions and participation of cytokines and growth factors on functional damage reestablishment [1,2]. After injury, the skin immediately initiates complex cellular and molecular responses designed to restore the skin barrier function, homeostasis and reduce the risk of infection [3,4]. The exudative, proliferative, and extracellular matrix remodeling phases are sequential events that occur through the integration of dynamic processes involving soluble mediators, blood cells, and parenchymal cells [5].

In recent years, tremendous progress in molecular and cell biology of tissue repair have amplified the interpretation of the biological process involved in wound healing contributing to treatment and patient care [6,7]. According to a report by Market and Markets, the global wound care market is expected to reach USD 22.01 billion by 2022, up from USD 17.69 billion in 2016 [6].

Wound healing is a highly efficient biologic process in healthy individuals. However, chronic wounds can be developed when those process failed to both proceed through an orderly and timely reparative process and produce anatomic and functional integrity of the injured site [6, 8]. Chronic wounds are characterized by chronic, persistent inflammation, impaired angiogenesis/re-epithelialisation, dysregulated cytokine/growth factor networks and/or increased protease activity [8-10]. The management of chronic wound comprises a relevant aspect of medical practice and in most cases, requires specific treatment for its success such as diabetic patients that chronic wounds become a higher source of morbidity and mortality [1, 11, 12].

Although many phytomedicines, traditionally used to optimize wound healing, have already been scientifically studied, the efficacy of these substances and their mechanisms of action in the inflammatory repair process are still controversial [13,14].

Taking in account the medicinal importance of the plants used by different ethnic groups to treat diseases, specific factors need to be addressed, such as: limitations of chemical and morphological approaches for plant authentication; therapeutic efficiency along with whether a specific substance is toxic or beneficial to the human use; enable for oral and/or topic use; the ease of obtaining the drug; low cost; drug stability; whether the herbal formulation does not require feasibility damage, among others. In Brazil, this set of benefits, linked with strong scientific validations, made it possible to include some herbal medicines in the public health program of the Unified Health System (so called SUS) [15]. This is the case of the copaiba specimens (*Copaifera* genus) that in the Amazonian region of Brazil, since the 16th century, become an important folk medicinal source, and is one of the natural resource included in the SUS´s public health care program [16].

Copaiba is a common occurrence plant in South and Central America, India and West Africa, belonging to the Fabaceae family and Caesalpinioideae subfamily [15, 17]. Copaiba oil (CO) is a medicinal complex mixture composed of a majority of diterpenes (copalic acid, kaurenoic acid, alepterolic acid, and polyalthic acid) and sesquiterpenes (β-caryophyllene, α-copaene, β-elemene, α-humulene, and germacrene D) that gives the wide panel of CO biological activities [15, 17-20].

In Brazil, *in natura* CO is largely administered to treat skin lesions, infectious, urogenital, respiratory, gastrointestinal and oncologic diseases [15, 17-24]. Scientifically, it was proved that copaiba oil shows anti-inflammatory, antimicrobial, analgesic and healing proprieties, among other therapeutic benefits [15, 17, 18, 23-31].

Historically, it is worth mentioning, that controversial results for *in natura* CO applied directly on skin to treat healing process, wherein inflammatory response at early stages with significant increase in fibroblast proliferation phenomena and collagen deposition in wounds have been largely observed [32-46].

Indeed, CO presented high healing potential such as: increase wound contraction, collagen type-I and accelerates healing in rats, and also facility the regenerative process reducing the healing time when administrated for the topical treatment of cutaneous lesions in diabetic mice [47, 48].
In other study CO effect after the topical administration in the healing process of urinary bladder in rats, showed that CO improve the quantity of giant cells and vascular proliferation, but not interfere in the collagen physiology [49]. In opposite way, *in natura* CO did not accelerate the topical wound healing of traumatic ulcers after oral administration in male Wistar rats [50].
Nowadays, pharmacological CO-based formulations development still the major obstacle to overcome due the physical and chemical characteristics of the copaifera oil, specifically, due of its lower water solubility, which is the biggest challenge. In this sense, studies have been developed by using liposomes, micelles, nanoparticles, gel and emulsified systems [16, 21, 52]. The observed nanotechnologic results encourage the advances of copaiba oil biotechnological applications, such as: **i)** CO-loaded nanosystems for the treatment of pulmonary hypertension, cancer and endometriosis, besides development of new antibiotics and antifungals; **ii)** a carbomer-based hydrogel useful as topical systems for delivery of CO directly into de vaginal mucosa to treat *S. agalactiae* colonization and infection; **iii)** a hydrogel containing copaiba oil nanoemulsions was applied to anti-inflammatory activity with high edema inhibitions in mouse ear (>67%) and rat paw (>37%) models; **iv)** general formulations containing CO-loaded into nanoparticles with different physicochemical proprieties have been produced to many biological applications presenting low toxicity in some of that it is important highlight the histological analysis showing decrease of inflammatory factors, as dermis and epidermis hyperplasia and inflammatory cells infiltration [16, 21, 52-64].
Since Wound healing is a complex cellular and molecular response involving different biological messages at different healing stages of different cell types. So, adequate controlled for biosystem delivery represent a crucial step for therapeutic success in wound healing process of oral and/or topical treatments [65,66].
Concerning to oral medicines, the self-microemulsifying drug delivery system (SMEDDS) as well as SNEDDS systems (self-nanoemulsifying drug delivery system) is an innovative strategy to formulate and increase the bioavailability of lipophilic bioactive compounds with low solubility and/or high first pass metabolism. The process of formulating self-emulsifying DDS-systems (SEDDS) is quite simple, involving the mix of an oil phase, a surfactant and often a cosurfactant (co-solvent), diluted in water, affording a single-phase and transparent system as a final product. SMEDDS systems are thermodynamically stable isotropic mixtures of oils and surfactants, and shows the ability to dissolve hydrophobic moiety, encapsulating in a single unit dosage for administration form. Meanwhile, SNEDDS systems are kinetically stable. Usually low-energy is need to produce SEDDS systems and involves entropy changes when emulsification occurs that is greater than the energy required to increase the surface area of dispersion droplet size which ranges from 1 nanometer to 100 nm [67-77].
Although, some studies have shown the healing effect of CO on skin lesions, controlled studies are still required in order to provide its effectiveness on oral and/or topical uses. In this present work, it was developed a copaiba oil SNEDDS system (so called CO-SNEDDS) containing lower quantities of copaiba oil dispersed in a saline aqueous medium (enriched with powdered coconut water) affording higher therapeutic result. So, the effect of the CO-SNEDDS formulation in the skin ulcers repair process was then evaluated. Histopathological analysis of angiogenesis, collagen and evaluation of the inflammatory process were performed. In addition, the effectiveness of the CO-SNEDDS system was also assessed by immunohistochemical analysis of COX-2 expression and VEGF production.

Material and Methods

Material

Copaiba oil (CO) was purchased in a traditional market specialized in natural products (CEASA-RN, Brazil). Powdered coconut water was purchased from ACP Biotecnologia (Fortaleza city, Ceará state of Brazil). All reagents used were acquired from Sigma-Aldrich (Saint-Quentin Fallavier, France).

Copaiba oil chemical approach

The authenticity of the commercial copaiba oil sample was verified by High-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS). Specifically, copaiba oil was analyzed in a gas chromatography (Thermo Scientific - Trace 1310) equipped with a flame ionization detector (GC-FID). The sample (5.0 mg) was derivatized *in situ* by using the reagent TMSD (trimethylsilildiazomethane), affording methyl esther as derived compounds of the diterpene carboxylic acids, present in the copaiba oil sample.
Split injections (1:20) were performed in a DB-1 dimethylpolisiloxane column (25 m x 0.25 mm x 0.25 µm), using He as carrier gas at 2 $mL.min^{-1}$. Oven temperature was programmed from 120 ^{o}C to 150 ^{o}C (at 3 $^{o}C.min^{-1}$), followed by another heating ramp until 290 ^{o}C, at 15 $^{o}C.min^{-1}$. Detector and injector temperatures were set at 300 ^{o}C and 270 ^{o}C, respectively.
Two standard mixtures were injected at this same condition: a homologous series of linear hydrocarbons from tridecane to heptadecane (C13 to C17), and a mixture containing the sesquiterpenes caryophyllene, humulene and caryophyllene oxide. The mixture of this three sesquiterpenes, very common constituents from copaiba oils, were used to correct the LRI obtained and compare them with literature data.
The homologous series of hydrocarbons were applied to obtain the Linear Retention Index (LRI) of the copaiba oil constituents, and then compared with literature.
The derivatized copaiba oil samples were injected into a gas chromatography (Trace CG Ultra, Thermo Scientific), coupled with mass spectrometry detector (DSQ II, Thermo Scientific) with quadrupolanalyser and auto-injetor (AI 3000, Thermo Scientific).
Indeed, mass spectra were obtained by electron impact (70 eV), from 40 to 400 u.m.a. and using a similar oven program from GC-FID: 120 ^{o}C to 150 ^{o}C, at 2^{o} $C.min^{-1}$, followed by 150 ^{o}C to 260 ^{o}C, at 15 $^{o}C.min^{-1}$, and finally from 260 ^{o}C to 290 ^{o}C, at 14 $^{o}C.min^{-1}$. Samples were injected (split 1:40), using He as carrier gas at 2 $mL.min^{-1}$ in a zebron ZB-5ms (Phenomenex-20 m x 0.18 mm x 0.18µm) column. Mass spectrometry experiments were useful to confirm the sesquiterpene compounds identification by comparing their mass spectra with automatic database (NIST) and also to obtain the mass spectra of the derivative methyl esthers diterpenes. All results were compared with mass spectra data from previously copaiba oils isolated substances which were stored to compose a personal data library.
The applied phytochemical methodology is in accordance with our previous reported studies [35, 56, 67-79].

Copaiba oil SNEDDS-type colloidal formulation

The SNEDDS cosurfactant-free formulation based on copaiba oil (CO-SNEDDS) were obtained by using phases diagram performed on different concentration of constituents (oil, surfactant and an aqueous saline medium). Ternary phases diagram was constructed using the surfactant mass

titration methodology into the aqueous and oily phases in order to obtain a polar o/w microemulsion region. The phases diagram was prepared with the vegetable oil phases comprising copaiba oil mixed with the surfactant (Tween 80®) with the following weight ratios 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9 of surfactant and oil phases (respectively). The surfactant and oil mixed amounts were stirring and then diluted dropwise with double-distilled aqueous phase (doubly distilled water add with powdered coconut water, affording a specified saline medium). Hence, the ternary phase diagram regions of the CO-SNEDDS system was produced by using the mechanical stirring Vortex (IKA, Staufen, Germany) finding separately the optimized homogenization under moderate temperature (50 °C to 65 °C), for 5 minutes. Aiming at to confirm the colloidal system homogenization, approximately 5 mL was charged to the centrifugation tube under accelerated centrifugation (20 minutes) at rang 3500 rpm for (Centribio 80-2B, Teknika, Brasil).
The CO-SNEDDS formulation was chosen from the ternary phase diagram by fixing the emulsifier and aqueous phase amounts. So, the CO-SNEDDS based colloidal system is composed by 20% of Tween 80®, 1% of copaiba oil and 79% of the aqueous phase [doubly distilled water add with powdered coconut water at the ratio (9:1)] and it is in accordance with our previous reported study [67]. After physicochemical characterization the obtained formulation, was classified as nanoemulsion SNEDDS-type system (so called CO-SNEDDS system).

Physicochemical characterization of copaiba oil SNEDDS colloidal formulation

The applied physicochemical protocol for the characterization of the CO-SNEDDS colloidal system is describe below and is in accordance with our huge previous reported studies on physicochemical protocols for nanostructured systems development and their characterizations [67, 80-89].

Determination of the refractive index

A drop of the CO-SNEDDS formulation was analyzed on the Analog Abbe Benchtop RI Refractometer (ABBE, Model: 2WAJ; BioBrix, Breda, Nederland), previously calibrated with Milli-Q® ultrapure water. The refractive index was obtained at 25 ± 0.5 ° C, in triplicate.

Determination of pH

The pH measurement was performed by using a bench microprocessor pHmeter (Tecnopon, Model MPA-210, Piracicaba, Brazil), previously calibrated with pH 4.0 and 7.0 buffer solutions at 25 ± 0.5 ° C, in triplicate.

Surface tension assessment

The study of surface tension was performed using the SensaDyne Tensiometer equipment (Florida, USA), using the maximum bubble pressure measurement method. Two capillaries with different diameters were used, pumping nitrogen (N_2) at a constant pressure (200 kPa). The capillaries were immersed in the sample, at constant temperature (30 ° C ± 2) and with control of the gas bubbling frequency.

Droplet diameter determination

The microemulsion droplet diameter was determined using the dynamic light scattering technique (Nanotrac Particle Size Analyzer, Microtac Incorporation, USA). The measurement was performed with a refractive index of 1.4635, in triplicate.

Rheology

The viscosity assessment was performed using Haake Mars rheometer (Thermo Scientific, Brazil). The analyzes were performed at 25 °C with a shear rate variation from 1 to 370 s^{-1}.

Thermodynamic stability

The temperature resistance evaluation was performed in the temperature range between 0 and 100 °C. The dynamic stability test was performed by centrifugation (Centribio, 80-2B-5ML-110) at 4000 rpm for 60 minutes.

Surgical procedures

The *in vivo* experimental study was approved by the Ethics Committee on the Use of Animals (CEUA) at Universidade Potiguar (CEUA / UnP, 0019/240712). Twenty-four male Wistar rats, 120 days old, weighing about 300g, were randomly divided into group 1 or control group (G1-7 and G1-14, according to the date of euthanasia, n = 6), and group 2 or treated group (G2-7 and G2-14, n = 6). The number of animals per group (n = 6) was based on reported studies [90-98]. The rats were housed in individual cages lined with wood shavings; with free access to water and diet; in an environment with controlled temperature (22 ºC ± 2 ºC) by automatic adjustment; noise below 60 dB; humidity around 45% ± 15%; with a 12-hour light-dark cycle [92-94].
The animals were anesthetized by intramuscular injection of Zoletil® 50 (0.1 mL/100 g), in the medial region of the right thigh. Under anesthesia, rats were positioned and immobilized in the prone position on the operating table submitted to trichotomy in the dorsal region (approximately 4 cm^2). Then, skin antisepsis was performed with chlorhexidine digluconate spray. An incision of total skin fragment (1 cm^2) was made on the animal's back [95]. Hemostasis occurred by compression with sterile gauze for 2 minutes [96, 97].
The animals postoperative pain was treated 50 mg/kg of dipyrone, single dose intraperitoneal. The random experimental groups received a daily topical application of 1 mL of the CO-SNEDDS formulation, and the control groups 1 mL of 0.9% NaCl solution (topically), during the predetermined period of the study. On the euthanasia day, all animals were weighed and anesthetized, and an excisional biopsy was performed including intact perilesional tissue.

Hematoxylin and eosin stain and cell count

Collected wound tissues were processed for histological and immunohistochemical examinations. The tissues were fixed in 10% neutral formalin and paraffin-embedded tissues were serially sectioned at a thickness of 5 µm and stained with hematoxylin-eosin and Masson's Trichrome. The cuts were analyzed microscopically by two pathologists without prior knowledge of the identification of the groups. The slides were examined under light microscopy (Olympus CX31,

Hamburg, Germany). Total number of neutrophils and mononuclear lymphocytes was counted in ten High Power Fields (HPF), ×400 magnification, based in their morphology.

Masson's trichrome staining and aniline blue quantification

Masson's trichrome staining was performed utilizing a staining kit (#EP-11-20013, EasyPath, Indaiatuba, Brazil) according to the manufacturer's instructions. The area stained by the aniline blue was divided by the total area analyzed. Percentage of collagen fibers (aniline blue stained area) was calculated and tabled.

Immunohistochemical (IHC) procedures

The immunohistochemical reactions was performed on sections (3 μm) of paraffin-embedded tissue. Then, the sections were washed in PBS, treated with heat-induced antigen retrieval using with Citrate buffer (pH 6.0) in a microwave (24 minutes), followed by endogenous peroxidase activity with 10% H_2O_2 using single cycle of 25 min. and incubated with the primary antibodies. The following antibodies utilized in this study are described in the Table 1.1. The immunohistochemical staining was performed with Envision purchased to DakoCytomation (Glostrup, Denmark), and used according to the manufacturer's instructions. Then, the slides were exposed to diaminobenzidine tetrahydrochloride (DAB) and counterstained with Carazzi hematoxylin. Breast cancer sections was used as control for all immunohistochemical reactions in this study, whereas the negative control was obtained by omitting the specific primary antibody. For IHC analysis, was obtained microphotographs of the wound area. Ten HPF (200×) were analyzed for each sample. Percentage of COX-2 or VEGF-positive cells was performed considering the number of positive cells in each field. A single research fellow manually counted all of the positive cells; the percentage was calculated and tabled. To determine the micro vessel density (MVD) we performed a quantitative analysis, considering $CD31^+$ vessels in wound area. For each sample, all vessels were counted (that had the greatest density of positively stained vessels). MVD was defined as the number of positively stained vessels per area (in mm^2).

TABLE 1.1
Overview of utilized antibodies.

Analyses	Antigen retrieval	Clone	Company	Dilution	Positive control
COX-2	Citrate/MW	CX-294	Dako	1:200	Breast carcinoma
VEGF	Citrate/MW	VG1	Dako	1:40	Pyogenic granuloma
CD31	Citrate/MW	JC70A	Dako	1:100	Pyogenic granuloma

Statistics Analysis

The data of histological and IHC analysis were presented as mean ± SD and statistical evaluation was performed by test t student. Values lower than $P<0.05$ were considered significant. Analysis were performed by using the SPSS.

Results

Chromatographic and spectrometric analysis allowed to identify sesquiterpene (about 82.35%) and diterpene (about 5.31%) constituents which were analyzed as their methyl esters derivative compounds. Specifically, the major identified terpenes comprise: β-caryophyllene (32.84%), germacrene D (18.78%), α-copaene (5,96%), δ-cadinene (5.47%), α-humulene (4.47%), copalic acid (4.44%). γ-muurulene 4.19%), and bicycle-germacrene (3.36%).

The composition of the commercial powdered coconut water (commercially sample so called ACP) showed several main components per 100 g, and comprises about: **i)** lauric acid (1.41 g), linoleic acid (0.54 g), myristic acid (0.60 g), palmitic acid (0.42 g), oleic acid (0.12 g), and caprylic acid (0.02 g), as the main amount of lipid portion; **ii)** fructose (7.80 g) and glucose (6.20 g), as the main content of carbohydrate components:); **iii)** potassium (5.17 g); sodium (2.24 g), magnesium (0.51 g), calcium (0.49 g), phosphor (0.43 g), iron (0.008 g), zinc (0.003 g), manganese (0.002 g), as the main mineral components, added to a traces of and other mineral, including copper and selenium; **iv)** vitamin C (0.016 g), B1 (0.004 g), B3 (0.003 g) for the vitamin components portion, with along traces of vitamin B6 and B2; **v)** glutamic acid (0.012 g), alanine (0.007 g), aspartic acid (0.005 g), leucine (0.005 g), valine (0.005 g), proline (0.005 g), phenylalanine (0.005 g), glycine (0.004 g), arginine (0.002 g), histidine (0.002 g), isoleucine (0.002 g), lysine (0.002 g), serine (0.002 g), methionine (0.001 g), threonine (0.001 g), as the major amino acid compounds, and also traces of others, including tyrosine; **vi)** 12.00 g of protein portion.

With regard to the ACP (powdered coconut water) preparation its industrial-scale production has a patent request [99]. Shortly, the fruit (green coconut) is selected and through a strict processing control affording the ACP product which is patent protected [99]. In that process a non-thermal sterilization method, using filter sterilization process by 0.22 and 0.80 µm Millipore® membrane is applied aiming at to determine the best relationship between the maintenance of sterility and less retention of the nutritional products. According to the ACP Biotecnologia who commercialize this product, this material has shelf stability and longevity, maintaining the inherent properties of the original product. As part of the ACP analysis quality control, sample groups were inoculated in the broths thioglycolate, BHI and liquid sabouraud methods for evaluation of the microorganism growth (anaerobic and aerobic bacteria and fungi). After incubation for 14 days at 37 ºC (aerobic and anaerobic bacteria) and at 22 °C (fungus), the test material containing the culture media inoculated with aliquots of ACP filtered by Millipore® of 0.22 and 0.80 µm, shows no evidence of bacterial and fungal contamination. The results obtained in the Biuret's proof suggest the preservation of the total protein's parameters of the product ($P>0.05$) in all evaluated methods [99].

Fresh coconut (*Cocos nucifera* L.) water is a clear, sterile, colorless, slightly acidic and naturally favored drink, mostly consumed in tropical areas. It is a rich source of nutrients and has been used for medical purposes. A study designed to assessing the possibility of measuring the growth of bacterial in fresh, stored or sterilized coconut water was performed using turbidity measurements (at wavelengths between 600 nm and 800 nm) or by dry biomass determinations. The findings showed significant differences in the UV spectra before and after sterilization procedure and during the storage of the coconut water. Although changes in total carbohydrates were observed, they were not significant ($p > 0.05$). The enormous differences in the characteristics before and after storage suggests that the use of turbidity and dry biomass measurements for measuring the growth of bacteria in fresh, autoclaved and gamma irradiated coconut water before storage is practicable without any possibility of interference by the innate turbidity, color and dry matter of the coconut water. However, this is not practicable after storing the coconut waters at 4°C, since there were increases in the turbidity and dry matter of the coconut water to levels that will mask the turbidity

of a growing bacteria culture [100, 101]. Hence, this result strongly justifies the research applications of *Cocos nucifera* L. water in its powdered form, such it was done in the present work.
Once the colloidal SNEDDS-type system (self-nanoemulsifying drug delivery system), composed by CO load on lower concentration (1%) and Tween 80® as surfactant (20%) dispersed in an aqueous saline medium (79%), containing double-distilled water add with powdered coconut water (9:1), an expanded physicochemical study was performed. Initially, the preparation of the CO-SNEDDS system characterized by the high water/surfactant concentrations, involved: **i)** weighing and mixing the constituents of the composition, on a precision scale; **ii)** heating under a moderate temperature (below 70 °C, for 5 min.); **iii)** centrifugation at a constant speed (3500 rpm, for 15 min.). The final product was homogeneous and visually transparent or slightly with Tyndall effect [67, 102]. The obtained CO-SNEDDS nanosystem was then, characterized by refractive index, pH, surface tension, viscosity analysis and droplet diameter which formulations and its data are already patent protected [103].
Figure 1.1 shows the phases diagram focusing on Winsor IV and I regions. The surfactant content of the CO-SNEDDS formulation was effective to lower the interfacial tension and effectively afford the formation of interfaces. Hence, the emulsifier stabilizes the droplets by forming a membrane at the interface which prevent coalescence causing a strong and elastic physical barrier enough to prevent the coalescence of dispersed droplets and also by reduction of the interfacial tension. When the interface is saturated with the surfactant molecules self-assembling are formed and the surface tension reached its minimum value at the critical micellar concentration (c.m.c.) and remains constant above it. Figure 1.2 shows the whole description of droplet diameter analysis of the CO-SNEDDS nanosystem colloidal formulation.

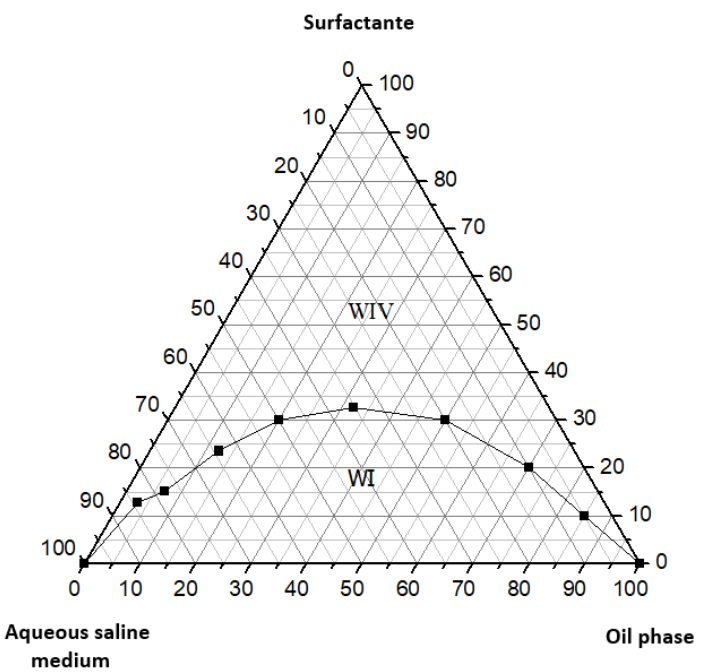

FIGURE 1.1
Ternary phases diagram of the CO-SNEDDS nanosystem colloidal formulation.

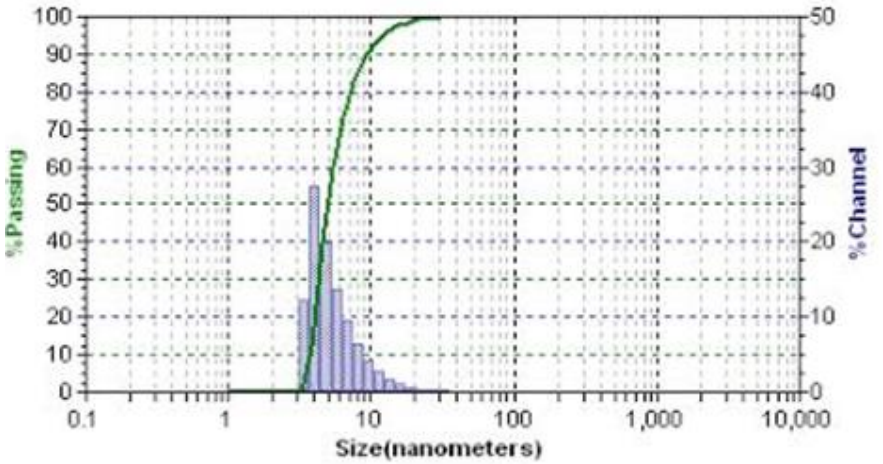

FIGURE 1.2
Description of droplet diameter analysis of the CO-SNEDDS nanosystem colloidal formulation.

CO-SNEDDS treatment reduces the inflammatory response early after wound induction

The infiltration of inflammatory cells into wound healing tissues has been associated with prolongation of healing time [104]. To quantify the number of lymphocytes and global neutrophils in the cutaneous tissue a morphological analysis was performed. Interestingly, it was found that CO-SNEDDS *in vivo* treatment significantly reduces the number of neutrophils at 7 (13.17±2.317 vs. 7.333±2.582 cells, control and CO-SNEDDS treated animals, p=0.0021, respectively) and 14 days (9.167 ± 1.424 vs. 1.000 ± 0.3651 cells, control and CO-SNEDDS treated animals, p=0.0002, respectively) compared with untreated wounds (Figures 1.3E and 1.4E).

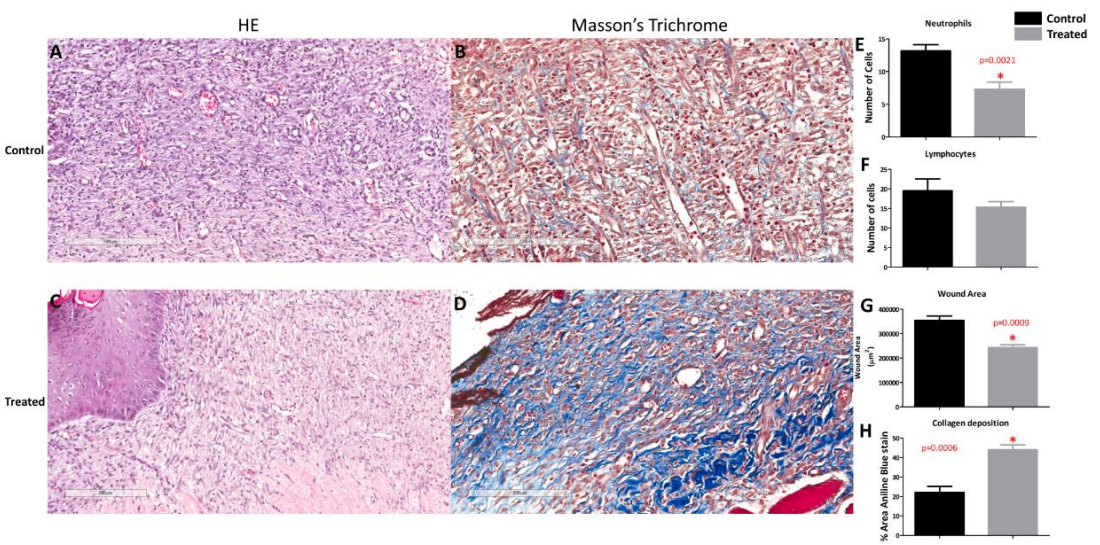

FIGURE 1.3
H/E findings and Masson's trichrome analysis on control and treated wounds at 7th day. (A) Control group showed an exuberant granulation tissue with moderate amounts of neutrophils and lymphocytes, and discrete fibroblastic activity (B). In CO-SNEDDS treated group, it was observed a higher number of fibroblasts (C) and collagen deposition (D) when compared to control group. The graphs demonstrated the number of neutrophils (E), lymphocytes (F), wound area (G) and percentage of collagen deposition (H). Mean ±SD, Student's t-test, n=6 for each group.

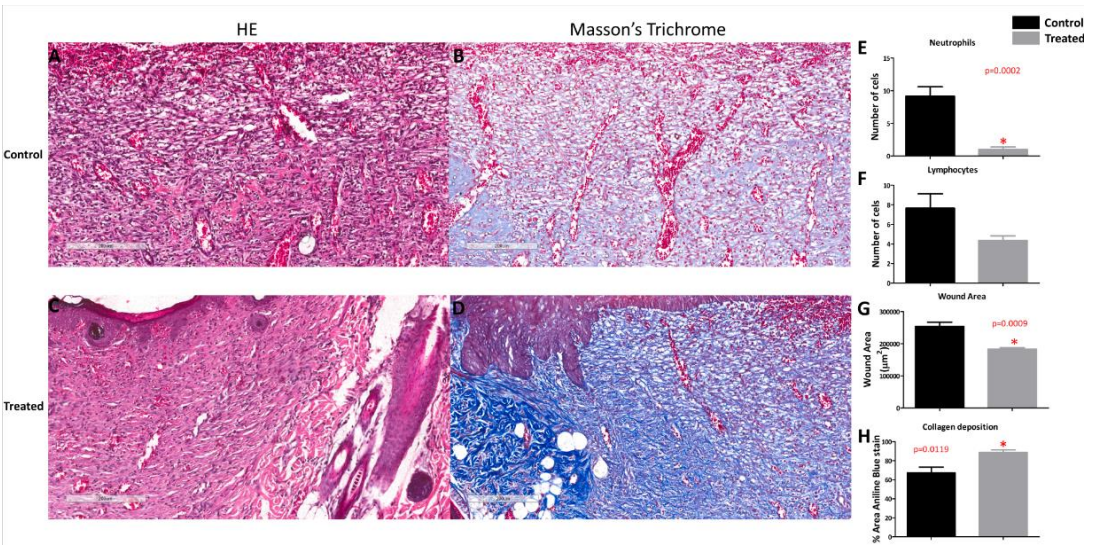

FIGURE 1.4
H/E findings and Masson's trichrome analysis on control and treated wounds at 14th day. Microphotographs of the control group wounds revealed a considerable number of fibroblasts and blood vessels, incomplete reepithelization (A) and discrete collagen deposition (B). CO-SNEDDS treated wounds demonstrated complete reepithelization (C) intense neocollagenesis, presenting thicker collagen fibers (D) compared to control group. The graphs demonstrated the number of neutrophils (E), lymphocytes (F), wound area (G) and percentage of collagen deposition (H). Mean ±SD, test t student, n=6 for each group.

The number of lymphocytes was consistently smaller in CO-SNEDDS treated wounds (15.33 ± 1.406 and 4.333 ± 0.4944 cells at 7 and 14 days, respectively, $p>0.05$) compared with control group (19.50 ± 3.041 and 7.667 ± 1.453 cells at 7 and 14 days, respectively, $p>0.05$), but did not observe significant difference (Figures 1.3F and 1.4F). At 7th day, H/E stain of cross-sections through wounds revealed an exuberant granulation tissue for both groups, however, treated wounds showed less inflammatory cells and a considerable number of fibroblasts, indicating that CO-SNEDDS treatment induces a proliferative phase early, while control group was in inflammatory stage (Figures 1.3A and 1.3B). Furthermore, the CO-SNEDDS treatment significantly reduces the wound area compared with untreated wounds, at 7th ($p=0.0009$) and 14th ($p=0.0009$) day, indicating an earlier closure of the wounds (Figures 1.3G and 1.4G).

CO-SNEDDS treatment inhibits the COX-2 expression and stimulates the VEGF production

The COX-2 is involved in angiogenesis induced by vascular endothelial growth factor (VEGF) [105, 106]. To analyze the effects of CO-SNEDDS treatment on COX-2 and VEGF expression, immunohistochemical evaluation of formalin-fixed paraffin (FFPE) embedded tissues was

performed. COX-2 showed a cytoplasmic expression in fibroblasts and inflammatory cells in the damaged area. At 7 days, overexpression was observed in untreated wounds, and the CO-SNEDDS treatment (p=0.0005) seems inhibit this expression (Figures 1.5A and 1.5B). In the wound treated with CO-SNEDDS and untreated at 14 days, the expression of COX-2 was lower and no differences were detected (p>0.05).

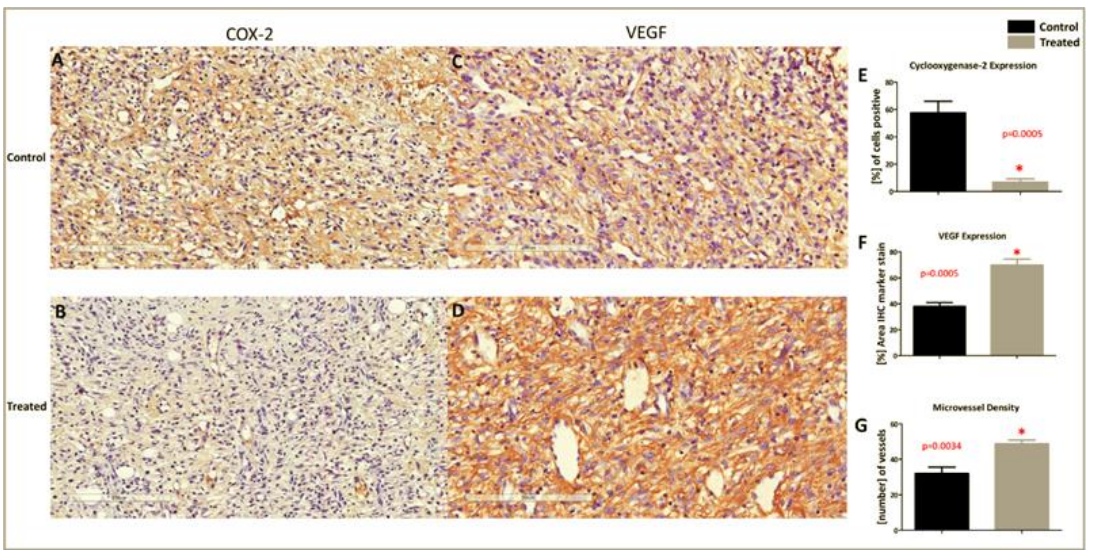

FIGURE 1.5
IHC (COX-2 and VEGF) findings on control and treated wounds at 7th day. COX-2 demonstrated a high and diffuse expression in fibroblasts and inflammatory cells in the control group (A), and discrete in NEOCACP-treated group (B) (p<0.05). The expression of VEGF was weak in the control group (C) and significantly up-regulated in CO-SNEDDS treated wounds on day 7 (D). The graphs demonstrated the percentage of positivity of COX-2 (E), VEGF (F) and MVD (G). Mean ±SD, test t student, n=6 for each group.

VEGF was expressed in fibroblasts, inflammatory cells and blood vessels. We found a higher expression of VEGF especially during early time point (7 days) in CO-SNEDDS treated wounds (p=0.0005) compared with untreated wounds (Figures 1.5C and 1.5D). Control wounds also presented VEGF expression, however, was predominantly found at 14 days (p=0.0002), compared with CO-SNEDDS treated wounds (Figures 1.5C and 1.5D). Treatment with CO-SNEDDS seems to inhibit the expression of COX-2 and increase VEGF during the inflammatory phase of the cutaneous healing process.

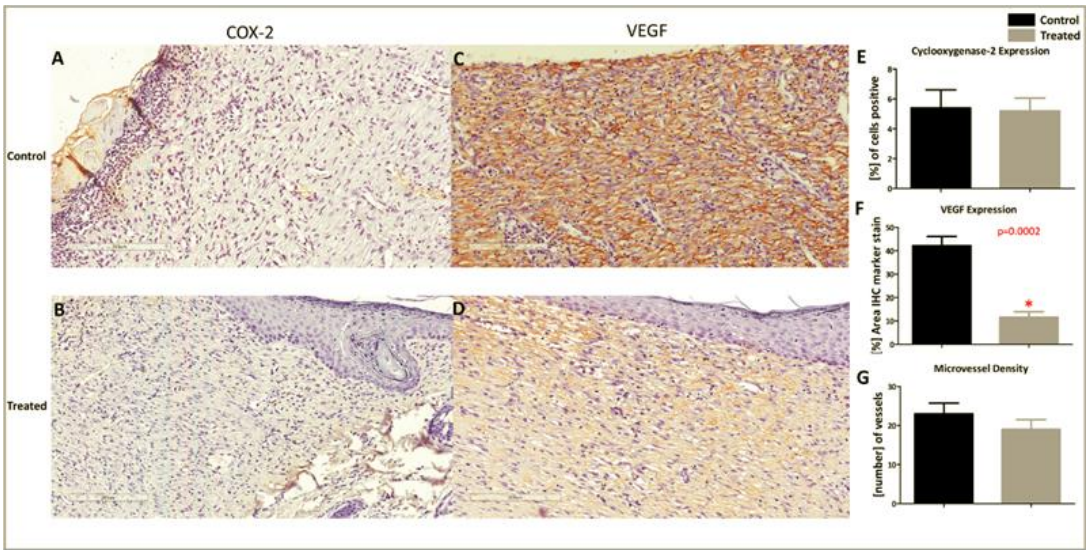

FIGURE 1.6
IHC (COX-2 and VEGF) findings on control and treated wounds at 14th day. COX-2 demonstrated a weak expression in inflammatory cells in the control (A) and CO-SNEDDS treated group (B) ($p>0.05$). Positive VEGF staining was observed in mesenchymal and in endothelial cells, and was considered intense in control group (C) and weak in CO-SNEDDS treated group (D) ($p<0.05$). The graphs demonstrated the percentage of positivity of COX-2 (E), VEGF (F) and MVD (G). Mean ±SD, test t student, n=6 for each group.

CO-SNEDDS treatment accelerated early wound healing by enhanced in neocollagenesis and angiogenesis

The angiogenesis and fibroplasia are co-dependent processes and early start of this phase has been associated with a shorter healing time [105, 106]. In order, aniline blue staining (to evaluate the neocollagenesis) and CD31 expression (to evaluate the angiogenesis) were utilized to confirm the effect of CO-SNEDDS treatment in skin wound repair. On day 7 and 14, the collagen fraction was significantly higher in CO-SNEDDS treated wounds, as compared to control group (Figures 1.3C and 1.3D; Figures 1.4C and 1.4D). The percentage of the collagen fibers stained with aniline blue was also significantly higher from day 7 onwards in CO-SNEDDS treated wounds compared to control group ($p=0.0006$), evidencing an early increase of neocollagenesis (Figures 1.3H and 1.4H).
Micro vessel density (MVD) was increased in CO-SNEDDS treated group (mean value of 32.00 vessels CD31+/mm²) compared to control group (mean value of 48.80 vessels CD31+/mm²) ($p=0.0034$) at 7 days, demonstrating that CO-SNEDDS treatment accelerated angiogenesis (Figure 5G). As expected, on 14 days (Figure 1.6G), MVD was lower in the treated group (mean value of 23.00 control and 19.00 CO-SNEDDS treated group), more no differences were detected ($p>0.05$). Treatment with CO-SNEDDS seems to modulate the inflammatory response and induce neocollagenesis and angiogenesis, contributing to the early closure of the cutaneous lesion.
Figure 1.7 shows some photographs such as: 1.7A photograph of the surgical wound and cutaneous flap performed on the back of the animals; 1.7B photograph of surgical wound immediately after surgery; 1.7C biopsy photograph of cutaneous tissue after CO-SNEDDS treatment period; 1.7D photograph of the surgical wound of an animal belonging of the CO-SNEDDS treated group, after 7 days of treatment, evidencing the formation of granulation tissue and the absence of crusts; 1.7E

photograph of the surgical wound of the control group after 7 days of the surgical procedure, evidencing the formation of coarse crust and delay of the tissue repair process.

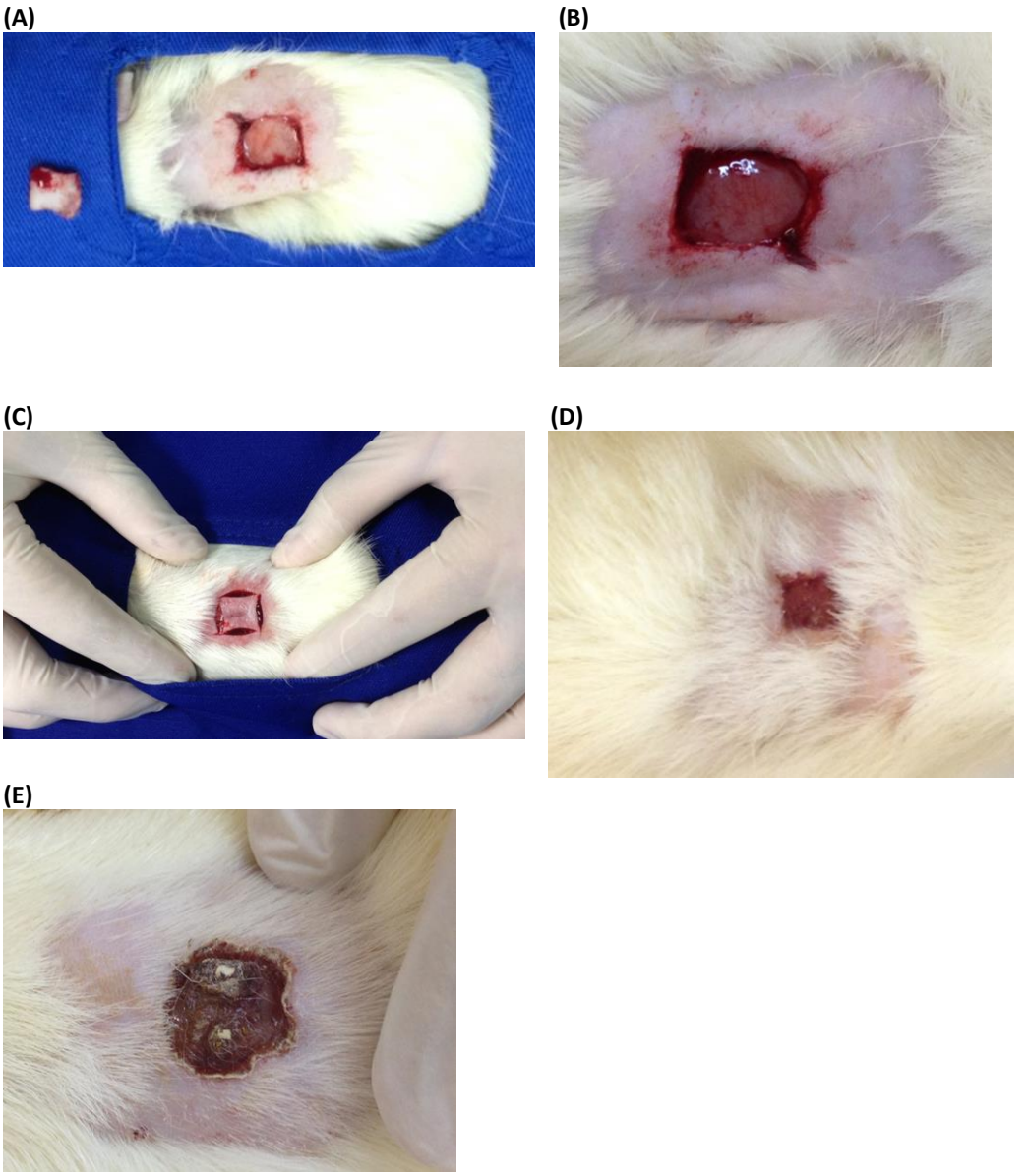

FIGURE 1.7
Photographs of the surgical wound and cutaneous flap performed on the back of the animals as well as the CO-SNEDDS treatment on the therapeutic enhancement of animal skin wound healing.

Discussion

Even in the face of scientific and technological advances, the treatment of skin ulcers is still a challenge for health professionals. Although traditionally recognized as a healing agent, previous studies with copaiba oil in its *in natura* form have not proven its benefits in the skin tissue [32, 33, 37]. However, healing was favored when the concentration of copaiba oil was reduced to 10% [36, 42]. In this sense, the purpose of this study was to develop a new copaiba oil-based nanoformulation for skin repair process. In addition to the successful delivery, the obtained self-nanoemulsifying drug delivery system (so called CO-SNEDDS system), was well tolerated by the animals, no adverse effects or deaths were observed proving to be effective both in modulating the inflammatory response, as well as in inducing neocollagenesis and angiogenesis in the treated animals.

The difference in terms of copaiba oil composition has already been described [16, 18, 42], highlighting an example, samples of copaiba resin oil, extracted from 22 species of *C. multijuga* Hayne, were analyzed by chromatography-flame ionization detection (GC-FID) and gas chromatography mass spectrometry (GC-MS), presenting 7 main constituents in all samples: α-copaene, α-humulene, α-amorphene, β-caryophyllene, caryophyllene oxide, copalic acid and pinifolic acid.

In the present study, the compounds identified in copaiba oil sample comprises 82.35% of sesquiterpenes and 5.31% of diterpenes. Hence, the chromatographic and spectrometric analysis allowed to identify the herbal CO commercial samples as *Copaifera multijuga* Hayne, by comparing its CGMS result to an authentic samples data.

The composition of the commercial powdered coconut water sample showed, per 100 g amount, carbohydrate components (75.5 g), lipids (3.4 g), mineral (8.9 g), vitamin (0,024 g), amino acid (0,096), and protein (12 g) components.

In this present study, the CO-SNEDDS nanosystem containing copaiba oil load in a saline medium enriched with powdered coconut oil, differs from the others by reducing the percentages of bioactive compound and, mainly, by the absence of co-surfactant and also to be a SNEDDS carrier which is resistant to dilutions (water or other biosolvents) and possess a rich nutritional water colloidal phase. For the thermodynamic (or kinetic) stability of the CO-SNEDDS formulation it was submitted to centrifugation resistance and temperature variations analysis in order to evaluate the stability of the CO-SNEDDS system by comparing to the original sample characteristics. The temperature resistance evaluation was performed in the extreme range of temperature (0 and 100 °C). The dynamic stability test was performed by centrifugation at 4000 rpm for 60 minutes and the acceptability criterion was the non-occurrence of phase separation. In these conditions, CO-SNEDDS showed to be stable.

In a general view, nanoemulsions favored the delivery of drugs that are poorly soluble in water improving pharmacokinetics and pharmacodynamics through different routes of administration. Other advantages including: the droplet on a nanoscale, which easily cross cell membranes; the controlled release of drugs, with a consequent improvement in prolonged efficacy; high stability; low rates of adverse and side effects; among others [65, 107, 108]. Alencar et al. [57] developed a nanoemulsion containing copaiba oil (5%), Tween 20® (1.56%), Span 80® (0.44%) and water (93%) presenting *in vitro* antimicrobial activity.

Concerning to the CO-SNEDDS topically therapeutic assay, animals surgical wound and cutaneous flap were performed on the back of the assayed animals and the wounds were treated with at CO-SNEDDS for 7th and 14th days. Visually at the 7th day it was possible to observe granulation tissue

formation and absence of crusts on the CO-SNEDDS treated animals (Figure 1.7), promoting an effective tissue repair process.

So, it was possible to evidence that the tissue repair process depends on a coordinated response of cellular and molecular events, which involves the release of growth factors and the recruitment of inflammatory cells that lead to complete healing of the lesion, being in accordance to literature [109]. Indeed, CO-SNEDDS modulated the inflammatory response in the treated animals, significantly reducing the number of neutrophils (Figure 1.3) in the inflammatory ($p = 0.0021$) and proliferative ($p = 0.0002$) phase, when compared with untreated wounds (Figure 1.4). It is worth mentioning that neutrophils are the first leukocytes to migrate to the injured area, followed by monocytes and macrophages, producing greater amounts of pro-inflammatory cytokines and proteases [110, 111]. Together, these data suggest that CO-SNEDDS behaved as an anti-inflammatory drug, inhibiting the migration of neutrophils, which may be associated with the improvement in healing observed in the treated group.

Cyclooxygenase (COX) is an enzyme involved in different physiological regulatory mechanisms, including the inflammatory response. Specifically, cyclooxygenase-2 (COX-2) is an inducible form of the enzyme that catalyzes the synthesis of prostaglandins (PG) from arachidonic acid, modulating inflammation and angiogenesis [96, 113, 114]. In the current study, inhibition of COX-2 expression and strong expression of VEGF at 7 days is associated with enhanced wound repair promoted by treatment with CO-SNEDDS. According to Abd-El-Aleem et al. [104] the over-regulation of COX-2 is associated with the persistence of the inflammatory phase in patients with chronic venous ulcers. Additionally, the weak expression of VEGF in 14 days may indicate that the wound treated with CO-SNEDDS was in the maturation phase, the final stage of the tissue repair process. In animals treated with bilirubin it has also been observed that the modulation of growth factors (VEGF), associated with reduced expression of cytokines and increased angiogenesis and collagen deposition, favor wound healing in rats.

The above reported results may have relevant clinical implications for the development of new skin lesion treatment protocols, in which the SNEDDS-type nanoemulsion containing copaiba oil can be used to promote healing.

Indeed, to modulating the inflammatory response, treatment with CO-SNEDDS induced neocolagenesis and angiogenesis, these effects were observed mainly in the early stages of repair, indicating its effectiveness in the treatment of wounds. Collagen deposition and blood vessel formation (BVF), as assessed by CD31 expression, were higher in rats treated with CO-SNEDDS, especially at 7 days. The early increase in neocollagenesis identified in animals treated with CO-SNEDDS was also observed in previous studies, which analyzed the effects of an ointment with copaiba oil (10%), on wounds on the dorsal skin of rats, in which an increase in angiogenesis and collagenesis in the treated group [41, 43]. Treatment with CO-SNEDDS seems to modulate the inflammatory response and induce neocollagenesis and angiogenesis, contributing to the early closure of the cutaneous lesion.

This inability to develop an effective treatment that could favor the skin repair process, combined with advances in the delivery of resinous compounds in nanoemulsions, provided the impetus to develop a product, containing copaiba oil, which could result in a new therapeutic option from local biodiversity.

In these line, with the CO-SNEDDS findings it is important to highlight that there is a lack of clarity regarding to optimal level of hydration required to support healing. Meanwhile, water is essential for the normal healthy skin function [115, 116]. Putting the theory of copaiba oil medicinal properties (focusing on its microbiologic, anti-inflammatory, analgesic and healing biological activities) [16, 18, 25-30, 32, 33, 36-38, 40, 41, 48-54] as well as the nanoemulsions therapeutic

advantages [56-77, 102] along with the benefits of hydration importance in wound healing, it is mandatory to expect from the CO-SNEDDS medicinal action: faster lower infection rates, pain reduction, collagen synthesis, less scarring, faster wound contraction and so, faster wound healing. Indeed, the CO-SNEDDS aqueous saline medium (over than 70%) provide an optimal moist healing environment and also is a rich nutritional and phytomedicinal medium to be strongly considered in the beneficial wound healing care. Hence, in theory it is also expected that the SNEDDS physicochemical characters could be effective against the prolonged exposure of tissue to the damaging components of non-healing wound exudates.
Finally, from this approach by changing the copaiba oil phase among the *copaifera* plant species (such as: *C. cearensis* Huber ex Ducke, *C. duckei* Dwyer, *C. glycycarpa* Ducke, *C. guyanensis* Desf., *C. langsdorffii* Desf., *C. lucens* Dwyer, *C. martti* Hayne, *C. officinalis* (Jacq.) L., *C. paupera* Herzog, *C. piresii* Ducke, *C. publiflora* Benth., *C. reticulata* Ducke, and *C. trapezifolia* Hayne) and also its content and/or the surfactant content and even adding a cosurfactant agent or blend surfactants option, the CO-SNEDDS may become an effective target system. So, by specific changings CO-SNEDDS may afford new biological o/w emulsion, nanoemulsion, microemulsion, SNEDDS or SMEDDS formulations based on copaiba oil which could be extensively applied on oral and topic therapeutics studies.

Conclusion

The management of chronic wound comprises an important aspect of medical practice and in most cases, requires specific treatment for its success. Historically, records revealed that copaiba oil had been used primarily to promote wound healing and treat inflammatory and infectious diseases. However, the low solubility in water has limited its pharmacological use. In this work, a self-nanoemulsifying drug delivery system containing copaiba oil (CO-SNEDDS) prepared in a salinized medium with powdered coconut water, was applied for Wistar rat skin wound healing experiment. Nowadays, self-emulsions as drug delivery systems became an attractive strategy for enhanced therapeutic profile aiming at new safety design to enhance the topic (or oral) bioavailability of a new natural product based nanomedicines for therapeutic purposes. Focusing in the CO-SNEDDS as a new modern medicinal target for wound healing propose, its histomorphometric analysis demonstrated a smaller number of neutrophils and lymphocytes in the treated group (7 days) as well as collagenesis, fibroblast proliferation and angiogenesis (14 days). Therefore, CO-SNEDDS formulation, showed to be effective in the *in vivo* applied treatment and seems to inhibit the COX-2 expression and increase VEGF during the inflammatory phase of the cutaneous healing process. Taken together, CO-SNEDDS formulation modulate the inflammatory response and induce neocollagenesis and angiogenesis contributing to the early closure of the cutaneous lesion. So, this study comprises an innovative approach for the use of copaiba oil (*Copaifera multijuga* Hayne) lower dispersed content (1%) into a SNEDDS colloidal system, co-surfactant free, and highlight the morphological and immunohistochemical characteristics of rats treated with the copaiba oil SNEDDS-type formulation.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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