3

Development of chitosan biofilms as a biocompatible matrix for incorporation of Croton cajucara Benth loaded into SNEDDS nanoemulsion systems applied as wound dressings on in vivo experimental model and its histopathological studies

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Outline

Introduction

Croton cajucara Benth (Euphorbiaceae) occurs in the Amazonian region of Brazil and has a safe history of use in Brazilian folk medicine. This species has been frequent target of ethnopharmacological works and is well known since ancient times (about 3000 years). The set benefits of extracts and isolated compounds, linked with the strong scientific validations of the medicinal uses of *C. cajucara*, made it possible to include this plant in the Brazilian public health program so called Unified Health System, shortly named as SUS (Sistema Único de Saúde do Brasil). Both stem bark and leaves of *C. cajucara* are popularly used as tea or pill for treat several diseases including diabetes, diarrhea, stomachache, fever, hepatitis and malaria. Indeed, *C. cajucara* scientific studies proved anti-inflammatory and antinociceptive activities for the stem bark extracts and isolated phytocompounds of this plant along with other biological properties, such as: antiulcerogenic, hypoglycemic, hypolipidaemic, antioestrogen, cardioprotetor; antitumor, antigenotoxicity, antiatherogenic [1-21]. The leaves of *C. cajucara* revealed the presence of steroids and flavonoids as major compounds and it´s stem bark showed to be a rich source of bioactive terpenes (Figure 3.1), such as: *trans*-dehydrocrotonin (DCTN) and *trans*-crotonin (CTN) both are clerodane-type 19-*nor*-diterpenes and a triterpene acetyl aleuritolic acid (AAA) [3, 4, 22, 23].

The previous studies on antiparasitic activity of the crude methanol extract of the stem bark of *C. cajucara* and its isolated terpenes DCTN, CTN and AAA were investigated on *Trypanossoma cruzi.* In these assays the polar crude extract was more effective than the isolated clerodanes DCTN or CTN on trypomastigotes, and for the triterpene AAA it was observed best trypanocidal effect against epimastigotes as well as on intracellular amastigotes [24].

In this point of view, it is important to highlight that terpenes are special metabolites found in several species of plants and important reports indicated their antiparasitic activities such as antitrypanosomal, antimalarial, antileishmanial, and antifungal, among other activities [25-28].

Generally, clerodane diterpenoids are a widespread class of special phytometabolites found in a huge diversity of plant species from various families and also in organisms from other taxonomic groups. These substances have attracted interest in recent years due to their notable biological activities. The distribution, chemotaxonomic significance, chemical structures, synthesis for new derivative compounds, and biological activities of clerodane diterpenes have been shown about structure activity relationship correlations and their paharmacological importance and modern biotechnological applications [29-35].

Opening additional areas for biochemical investigation of *C. cajucara* its previous results encourage us to assay the hydroalcoholic extract obtained from the stem bark of this plant, to be applied as a nanobiotechnological approach, aiming at its effectiveness on a skin wound healing treatment.

Among the wound treatment modalities, the use of wound dressing aiming at to improve the conditions of the wound bed, protect the wound against microorganisms, absorb exudates, as well as drain the wound, where all these factors contribute to its complete healing. The rapid closure of the lesion, known as wound healing, is considered the main step within the wound management treatment. An efficient wound healing process leads to a functional and aesthetically satisfactory scar as a result of a reduced microbial contamination and accelerated healing process. For this reason, the choice of the material used to obtain the wound dressing is extremely important. In this sense, due to its biocompatibility, biodegradability, nontoxicity, antimicrobial and wound healing activities as well as the ability to form resistant and elastic films, chitosan represents an excellent alternative for wound dressings and also as a matrix for the incorporation of drugs with several therapeutic uses [36-39].

In this context, the aim of this work was to obtain a hydroalcoholic extract from the stem bark of *Croton cajucara* Benth (HAE-CC), which was incorporated (5 mg - 30 mg) into two self-nanoemulsion drug delivery systems (SNEDDS), shortly named NE1 and NE2 (nanoemulsions 1 and 2) containing the extract HAE-CC. These systems were then incorporated into a chitosan solution (CHT) affording two SNEDDS-chitosan base biofilms so called CHT-NE1 and CHT-NE2. In order to investigate the potential use of these biofilms as wound dressings, an *in vivo* experimental model with *Wistar* rats was assayed. The evolution of the healing process in the treated animals was evaluated through histopathological studies.

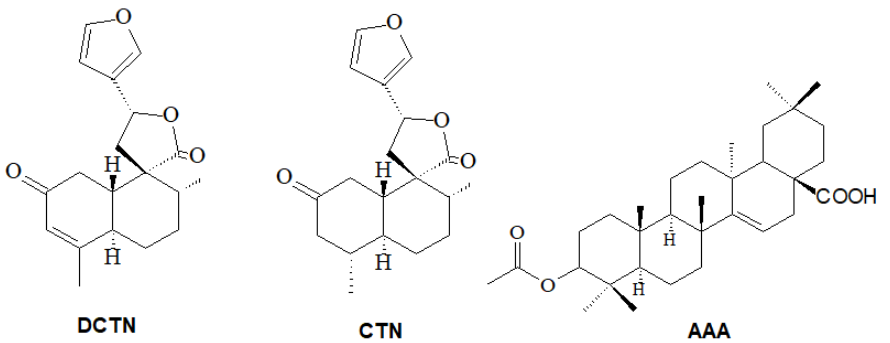

FIGURE 3.1
Chemical structures of the *Croton cajucara* Benth biomarkers *trans*-dehydrocrotonin (DCTN), *trans*-crotonin (CTN) and acetyl aleuritolic acid (AAA).

General comments for colloidal systems as drug carrier

Surfactants are amphiphilic molecules that possess in their structures two regions of opposing polarity: a polar (hydrophilic) and a nonpolar (hydrophobic) moieties. The presence of these two distinct regions in the same molecule renders it very prone to adsorption in the air-water and oil-water interfaces, and also on the surface of solids. The hydrophilic region is constituted by ionic or nonionic polar head groups attached to a hydrophobic tail that is constituted of one or more alkyl chains with normally eight to eighteen carbon atoms. Due to their peculiar characteristics, surfactants can act as detergents, emulsifiers, dispersants or solubilizing agents. Currently, they are largely employed in the biological field as adjuvants for the preparation of pharmaceutical nanoparticles or microemulsions, which are formed from an apparent spontaneous solubilization of two immiscible liquids (water, oil) in the presence of a surfactant, as well as a co-surfactant, if necessary. They are characteristically dispersed media (dispersed microdroplets), monophasic, thermodynamically stable, transparent or translucent systems, with lower interfacial tension and with the capacity to combine great amounts of two liquids in a single homogeneous phase. As a function of its chemical composition, the microemulsion systems (or nanoemulsions) can present a great structural diversity, being formed by dispersed and dynamic microdroplet, with a particle diameter ranging from 1 to 100 nm [40-45].
The representation of emulsified system is made by using phases diagram that are classified into ternaries, quaternaries and pseudoternaries according to the number of constituents. The ternary diagram is represented by an equilateral triangular diagram, where the microemulsion region may

vary depending on the surfactant and oil composition. The quaternary diagram consists of an extension of ternary phases diagram formed by four constituents: surfactant, co-surfactant, oil phase and aqueous phase. This type of system can be represented through a tetrahedron, where each vertex represents one of the pure components. Specifically, for microemulsified systems (or nanoemulsifiers) formed by non-ionic surfactants stands out as the main characteristic, the need for smaller amounts of surfactants for the formation of micelle aggregates (microemulsion or nanoemulsion) [46-50].

Depending on the different types of existing phases, a classification called WINSOR was postulated. It was established four types of main systems, in which Winsor IV (WIV) corresponds to a single-phase system, on a macroscopic scale, consisting of a single phase of microemulsion [41, 49-53].

Microemulsion (or nanoemulsion) present a mononuclear layer of amphiphilic molecules involving the microdroplets as a membrane, being oil-in-water polar type (o/w) when the system is water rich medium and the microdroplets are called “direct”, or water-in-oil nonpolar type (w/o), characteristic of an oil rich system with “inverse” microdroplets.

Figure 3.2 represents different types of structures found in an emulsified system, in which: **i)** region A corresponds to a microemulsion (or nanoemulsion) that is rich in water with water-like micelle (o/w); **ii)** region B: represent bicontinuous structure system (o/w and w/o); **iii)** region C: oil-rich system with water micelles in oil (w/o); **iv)** region D: system rich in surfactant, possibly presenting lamellar structures [41, 47, 51-57].

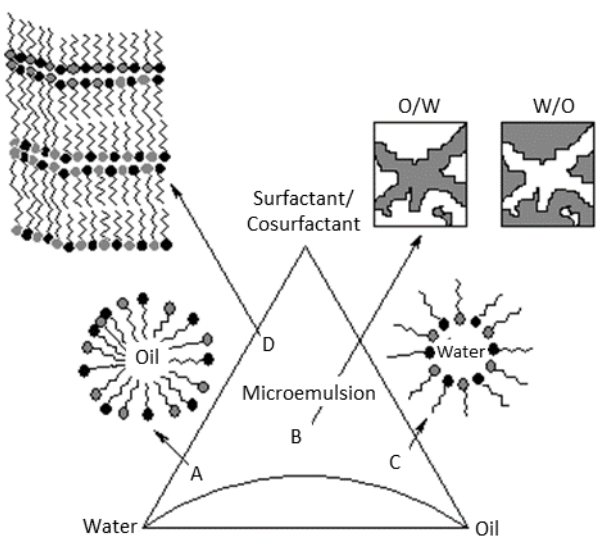

FIGURE 3.2
Typical structure of emulsified systems (adapted from Rossi et al., 2007; Friberg and Bothorel, 1988; Schulman and Roberts, 1982) [51, 57, 58, respectively].

The formation of micelle aggregates is a process that happens with decreased entropy of the system. In the critical mycelial concentration (c.m.c.), there is a transition from monomer phase from surfactants to micelles, in this concentration surfactants solutions undergo sudden changes in physical properties (electrical conductivity, surface tension, density, osmotic pressure, light scattering, among others) [48, 49, 51, 59, 60].

Infinite aqueous phase dilution tends to form direct micelles (o/w) and infinite dilution with the oily phase tends to form reverse micelles (w/o) with the aqueous phase dissolved inside. The region close to the apex of the oily phase is characterized by the presence of scattered water microdroplets in the oily phase (type w/o microemulsions), with the volume of the inner phase small. Among these two regions there may be another intermediate, where bicontinuous phases (o/w and w/o) can be observed, characterized by extensive phases of water and oil separated by an interfacial film composed of surfactant and co-surfactant. In compositions close to the water-oil binary shaft, the amount of surfactant is insufficient to facilitate the formation of microemulsions. In this region, multiple phases may exist, such as emulsions, balanced emulsions with the aqueous or oily phase, microemulsions (or nanoemulsions) in balance with the aqueous or oily phase and even three-phase systems [49, 50, 61, 62].

Surfactants can be defined taking into account the presence or absence of charges on the polar surface of their structure. Ionic surfactants present electrical charges in the hydrophilic part, and when dissociating in water, form ions (negatively or positively charged). On the other hand, non-ionic ones do not provide ions in aqueous solution and their solubility in water occurs due to the functional groups that have a strong affinity for water. This surfactant type represents an important class widely used for pharmaceutical applications with the advantage of presenting chemical stability, being resistant to variations of pH and ionic force, and can be used alone or in mixture with other surfactants. In general, it is recommended that the preparation of microemulsion (or nanoemulsion) applied to drug release have nontoxic and biodegradable surfactant [47, 49, 51, 60, 63].

The affinity between the surfactant polar headgroup and water molecules allows the solubilization of the lipophilic moiety, generally insoluble in water, through the formation of self-assembly aggregates in the bulk [40, 64, 65, 66]. These physicochemical properties grant to surfactant compounds a widely options of technological applications, such as emulsifiers, dispersants, lubricants, corrosion inhibitors, adjuvant or components in pharmaceutics, cosmetics, plastics and petrochemical industries, among others uses [65-73].

Colloidal systems (microemulsion or nanoemulsion) shows slow drug release favoring the prolonged effect with adequate plasma concentration of the bioactive compound [54, 60]. Some natural antioxidant agents evaluated in their none encapsulated form show low side effects and have been investigated to promote health. However, prolonged use of these free drugs may promote liver damage and trigger carcinogenesis [74-77]. In this sense, other advantage for colloidal carrier systems consist in reducing effective dose, prolonged effective action, and lower toxicity of bioactive compounds [42, 50, 54, 55, 64, 78-80].

Hence, encapsulation of natural and synthetic drugs in colloidal systems reduces toxicity and improves the therapeutic effects (enhanced efficacy by using drug lower content). Relying on colloidal systems biological finds, preparation, physicochemical characterization, stability, bioavailability and pharmacokinetics discussion of micellar solutions (microemulsion or nanoemulsion), as well as its *in vitro* and *in vivo* medicinal applications comprise a huge field of the scientific studies. In this point of view, preparation, characterization, and properties of chitosan films for delivery natural and synthetic products have been largely described, including preparations for wounds and burns: antimicrobial and wound-healing effects and antibacterial and antioxidant properties of chitosan films for potential wound healing applications [81-89].

Material and Methods

Plant material and biomarkers isolations

The stem barks of *C. cajucara* were commercially acquired from Ver-o-peso market located at Belém city of Pará state of Brazil (Amazonian region of Brazil). The authenticity of the plant was proved by applying a specific chromatography approach previously described [5, 22, 23, 90]. Specifically, the initial phytochemical procedure comprise the herbal maceration of the powder stem barks by using the polar solvent mixture ethanol:water (8:2). Then, following chromatographic fractionation of the crude hydroalcoholic extract of *C. cajucara* (HAE-CC) aiming at to isolate the plant biomarkers *trans-dehydrocrotonin* (DCTN), *trans*-crotonin (CTN) and acetyl aleuritolic acid (AAA) (Figure 3.3). The purity of the isolated compounds was proven by thin layer chromatography (TLC) using authentic samples (AAA, CTN and DCTN), as well as infrared spectroscopic analysis and NMR data. Mps: uncorr.; IR under $CHCl_3$ solution and ^1H NMR: 300 MHz.

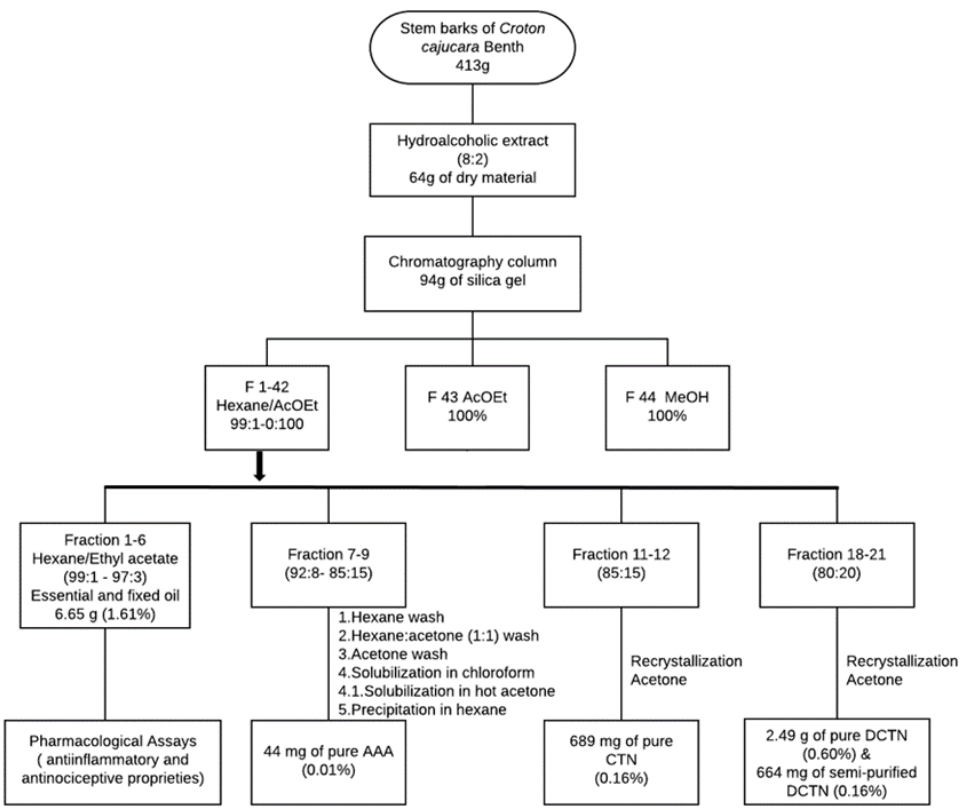

FIGURE 3.3
Chromatographic procedure applied for the isolation of terpenoids (AAA, CTN, and DCTN) from *Croton cajucara.*

Croton cajucara Benth (HAE-CC) loaded into SNEDDS colloidal system

The target SNEDDS-carrier system was prepared according to the previously described

methodology, by using as sufactant Tween 80 (preferential content ranging from 9% to 15%), and as organic phase a vegetal oil, widely used in human feed (ranging from 0,5% to 2%), under a neutral aqueous medium (double distilled water, in a preferential content ranging from 90,5% to 83,0%). The applied procedure to obtain the present SNEDDS system required a biocompatible mixture of surfactant/phase oil affording a o/w colloidal systems without co-surfactant need. Indeed, this biotechnological colloidal based formulation is already protected under a patent request (BR102018068447-7). Specifically, to prepare the SNEDDS-carrier precise amounts of oil phase and surfactant were mixed together using a magnetic bar at moderate temperature and speed (on a magnetic stirring plate), followed by (20 min.) water addition. Both phases were gradually heated (55 °C to 65 °C) and during the water addition (for each 10 mL of solution) the highest temperature was maintained [91]. The phases diagram (Figure 3.4) were obtained from the titration with double-distilled water and the oil phase of predetermined mixtures.

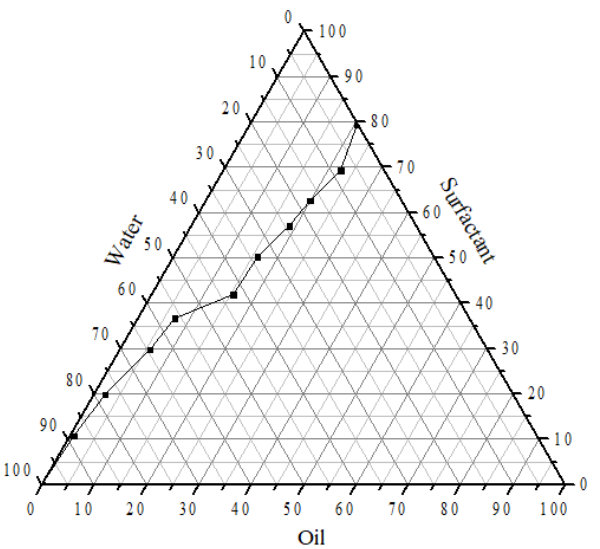

FIGURE 3.4
Ternary phase diagram of the target carrier SNEDDS-type nanoemulsion system without herbal encapsulation.

Sequentially, the *C. cajucara* hydroalcoholic extract (HAE-CC) in two different contents (5 mg/mL and 30 mg/mL) were solubilized in the target SNEDDS-carrier (shortly named NE-system) that satisfactorily load the HAE-CC extract affording two herbal formulations, designed as NE1 and NE2, which contain 5 mg of HAE-CC per 1 mL of the NE-system or 30 mg/mL, respectively. These single-phase o/w-type self-nanoemulsion (NE1 and NE2) remained isotopically stable after centrifuge procedure (3500 rpm) as well as by water dilution (upon thirty dilutions) ensuring no phase change. Physicochemical characterization of the NE-system without incorporation of the herbal extract, comprise: **i)** c.m.c. (critical micellar concentration); **ii)** self-assembling is formed when the interface is saturated with the molecules of surfactant component. Then the surface tension reached its minimum value at the c.m.c. and remains constant above it; **iii)** viscosity; **iv)** droplet size [92].

Chitosan biofilms based on nanoemulsified *Croton cajucara* extract (HAE-CC)

Chitosan (medium molecular weight) biofilms were produced dissolving 2% of chitosan (w/v) in 2%

acetic acid solution (v/v). After the complete dissolution of chitosan (CHT), the CHT-solution was mixed with equal volumes (1:1, v/v) of nanoemulsion NE1 and NE2 (5 mg/mL and 30 mg/mL, respectively), by magnetic stirring for 30 min., affording two CHT-modified solutions (CHT-MS1 and CHT-MS2). Then, 24 mL of this resulting dispersion (CHT-MS1 and 2) were poured into Petri dish, then the plates were taken to an over at 50 ^{o}C for 24h in order to evaporate the solvent used to obtaining the target chitosan solution. After that time, two chitosan biofilms containing HAE-CC load into the target NE-carrier were obtained and designed as CHT-NE1 and CHT-NE2. Posteriorly, plates were immersed in 1M NaOH solution for 30 min., and the biofilms were washed with distilled water up to neutral pH. Finally, the novel nanobiofilms (CHT-NE1 and CHT-NE2) were stretched, dried at room temperature, cut (1 cm^2) and stored in a dry place before experiment.

***In vivo* studies**

In *vivo* experimentation was conducted according to the standards of the National Council for Control of Animal Experimentation (CONCEA), using 15 rats of the *Wistar* lineage, males, two months of age, weighing between 220 g to 320 g, coming from the bioterium of Potiguar University. The research was approved by the Ethics Committee on the Use of Animals (CEUA) of Potiguar University under N^{o}. 018/2015. The animals were packed under ventilated shelving (Model: ALE02; Brand ALESCO/BRASIL 2007®) for adaptation remaining one week in acclimatization period. The room evaluated daily had controlled temperature (20 ± 2 °C), humidity of 45 ± 15%, environment noise below 60dB, and ambient lighting, with light-dark cycle of 12h. The diet offered was standardized with commercial rat food (Purina®) and filtered water *ad libitum*. The animals were randomly divided into 3 groups with 5 animals each. The experimental groups were submitted to 2 types of topical treatments: one with only chitosan biofilm and other with the nanoemulsioned systems of *Croton cajucara* Benth incorporated into a chitosan solution to afford the nanobiofilms (CHT-NE1 and CHT-NE2). The animal groups were identified as follows:

i) Group A (n=5): animals treated only with chitosan film (CHT-film), from which CHT1, CHT2, CHT3, CHT4, and CHT5, identify each animal of this group.
ii) Group B (n=5): animals treated with the formulation CHT-NE1, which contain chitosan/HAE-CC (5 mg/mL) and each animal of this group was designed as: CHT1-NE1, CHT2-NE1, CHT3-NE1, CHT4-NE1, and CHT5-NE1.
iii) Group C (n=5): animals treated with the formulation CHT-NE2, which contain chitosan/HAE-CC (30 mg/mL) and each animal of this group was designed as: CHT1-NE2, CHT2-NE2, CHT3-NE2, CHT4-NE2, and CHT5-NE2.
After the division of the groups, the animals were marked with inert ink in the syrup, receiving ordinary numbering, and their cages identified according to the experimental group to which they belonged, as well as all stages of the experiment and their variables recorded in a book agenda of minutes and computational archive (Microsoft /Windows/Excel 2013®).
For induction of anesthesia, Zoletil® 50 (50 mg/Kg) was administered intramuscularly in the region of the right quadriceps, with disposable syringes of 1mL and needle 0.45 x 13 26G 1/2" Descarpack®. After verifying total rat anesthesia by caudal pressure test, trichotomy and antisepsis of the dorsal region of the animal with 2% ® spray chlorhexidine diluconate was performed. For wound incision, digital caliper (mark "ZAAS precision" - 8/200mm/2015/Brazil®) was used to standardize the size of the lesion (1cm^2). All surgical instruments were previously sterilized in autoclave (BRASMED 21Lts,2014/Brazil®). After performing the lesions, samples described before were placed on wounds.

Histopathological analysis

After 28 days, animals were again anesthetized with Zoletil® 50 (50 mg/Kg, tiletamine hydrochloride and zolazepam hydrochloride at 1:1) and submitted to excisional biopsy of both scar and normal tissues. For histopathological analysis, samples were first fixed in 10% formaldehyde, dehydrated in a gradual series of ethanol, and diaphanized by automated histotechnic. Histological cuts of 0.5 µm were obtained and stained with Hematoxylin - Eosin and Masson tricheric. Histopathological evaluation of inflammatory response, type of inflammatory response, intensity of inflammation, epithelization, granulation tissue, collagen and fibroblast deposition intensities were analyzed. An experienced pathologist without prior recognition of the analyzed groups performed the analysis using a Leica optical microscope coupled with camera photomicrograph and Leica Microsistem Laz EZ software (version 3.4.0).

Results and Discussion

Herbal extraction and characterization protocols

Croton cajucara represents a medicinal source of great importance in the treatment and cure of various diseases. The commercialization of this plant occurs through leaves and stem barks, with therapeutic indication for the treatment and cure of diseases such as: diabetes, diarrhea, malaria, fever, stomach problems, inflammations of the liver, kidneys, vesicle and in the control of high cholesterol rates. This species is also marketed in handling pharmacies, in this case the shells are powdered and sold in the form of pills (250 mg in each capsule), and the leaves powder commercialized in mixture with *Peumus boldus* powder and applied for liver diseases [3, 5, 26].
The previous studies on antiparasitic activity of the crude methanol extract of the stem barks of *C. cajucara* and its isolated terpenes *trans*-dehydrocrotonin (DCTN), crotonin (CTN) and acetyl aleuritolic acid (AAA) were investigated on *Trypanossoma cruzi.* In these assays the polar crude extract was more effective than the isolated clerodanes DCTN or CTN on trypomastigotes, and for the triterpene AAA it was observed best trypanocidal effect against epimastigotes as well as on intracellular amastigotes [24].
Focusing in this promissory therapeutic effect of *C. cajucara*, in the present work 413 g of drying stem barks of this plant was used to extraction by maceration procedure from which four extractions were performed using EtOH/H_2O (8:2) until the intense brown coloration becomes light brown. After filtration, the filtrate was evaporated by reduced pressure obtaining an extract with 15.5% yield (64 g of the extracted material, named HAE-CC). Then, HAE-CC was submitted to open column chromatography of silica gel (230 - 280 Mesh) to obtain 43 fractions eluted with mixture of hexane and ethyl acetate (AcOEt) in polarity gradient. The increasing polarity approach Hex:AcOEt (100:0 - 0:100) is in accordance with our previously work [22, 23, 90].
Fractions 1-42 contained non polar components (fixed oil) in great amount (1.61%) [92], fractions group 1-42 after another chromatography process on a silica gel column eluted with mixtures of hexane-AcOEt at different ratios (Figure 3.3) afforded the triterpene AAA (0.019%), and the clerodane-type diterpenes CTN (0.16%) and DCTN (0.76%). The characterization of the AAA, CTN and DCTN, biomarkers compounds (Figure 3.1) were performed by thin layer chromatography (TLC) comparing with authentic samples and also infrared spectra (IV) and magnetic resonance NMR spectroscopy presenting good correlation with literature [22, 23, 90]. The NMR spectroscopy data sowed:

DCTN. Colourless crystals, mp 139- 140 ^{o}, $[\alpha]_D$ + 10.6^{o} ($CHCl_3$, *c* 0.6). **IR** ν_{max} cm^{-1} ($CHCl_3$): 3120, 2959, 2859, 1748, 1666, 1504, 873. **^{1}H NMR**, $CHCl_3$: 2.15 (H-1α), 2.51 (H-1β, *dd*, *J*=15.6, 2.7), 5.86 (H-3, *br s*, *J*=1.2), 3.14 (H-5, *ddd*, *J*=11, 10.5, 1), 2.24 (H-6α), 1.17 (H-6β, *dq*, *J* = 12.8, 3.4), 1.85 (H-7α), 1.60- 1.72 (H-8), 1.77 (H-10), 2.33- 2.40 (H-11), 5.40 (H-12, *dd*, *J*=8.6), 6.37 (H-14, *dd*, *J*=0.9), 7.42 (H-15, *m*), 7.42 (H-16, *m*), 1.12 (H-17, *d*, *J*=5.8) and 1.93 (H-18, *br s*, *J*=1.2).

CTN. Colourless crystals, mp 130- 132^{o}, $[\alpha]_D$ + 1.5 (CHCl3, *c* 0.8). **IR** ν max cm^{-1} ($CHCl_3$): 3139, 2965, 2921, 2883, 1756, 1704, 1505, 873. **^{1}H NMR**: 2.44 (H-1β, *tt*, *J*=12.9, 2.6), 2.36 (H-3β), 1.39 (H-4, *m*), 2.00 (H-5, *dddd*, *J*=11.0, 10.7, 10.6, 3.7), 0.97- 0.83 (H-6β), 1.76 (H-7α, *dddd*, *J*=12.7, 12.5, 12.3, 3.2), 1.57 (H-7β), 1.44 (H-8, *m*), 5.37 (H-12, *t*, *J*=8.6), 7.40 (H-15, *m*), 7.40 (H-16, *m*), 6.34 (H-14, *t*, *J*=1.38), 1.10 (H- 17, *d*, *J*=6.5) and 1.01 (H-18, *d*, *J*=6.4).

AAA. Was characterized as the AAA derived methyl compound, obtained from methylation of the target compound AAA. Colourless crystals, mp 144- 145^{o}. **IR** ν_{max} cm^{-1} ($CHCl_3$): 2936, 1729, 1590, 1467, 1375, 1244, 1168, 1025, 596. **^{1}H NMR**, $CHCl_3$: 4.44 (H-3, *dd*, *J*=9.0, 6.6), 1.02 (H-5, *dd*, *J*=13.0, 3.1), 1.93 (H-7β, *ddd*, *J* = 12.0, 6.8, 3.7), 5.48 (H-15, *dd*, *J*=8.0, 3.4), 1.91 (H-16β, *dd*, *J*=14.2, 3.4), 3.57 (-$COOCH_3$, *d*, *J*=8.2) and 2.04 (CH_3COO-, *d*, *J*=8.2).

Focusing on the functional groups of these compounds IR spectra showed carbonyls, lactones and furan subunit regions (Table 3.1). Hence, the authenticity of commercially acquired plant material was proven by the isolation and spectroscopic characterizations of the isolated bioactive components DCTN, CTN and AAA.

Our previously pharmacological studies performed with the isolated terpenoids, e.g., *trans*-dehydrocrotonin (DCTN), *trans*-crotonin (CTN) and acetyl aleuritolic acid (AAA) showed striking correlation among these compounds with the folk traditional therapeutic use of *Croton cajucara*, being DCTN the lead compound showing remarkable biological properties. Indeed, aiming at to validate the traditional use of *Croton cajucara* as a therapeutic safe plant and also to improve its scientific studies, we have been undertaking an extensive phytopharmacological research oriented by its traditional medicine. Specifically, DCTN showed anti-inflammatory, antinociceptive, antifungic, hypolipidaemic, hypoglycemic antiatherogenic effects, among others [1-24, 26]. Advances in our research with DCTN proved that this phytocompound was nor genotoxicity or cytotoxicity to bone marrow cells (*i.p*. treated mice) [20, 21].

TABLE 3.1
IR data correlation for the terpenoids DCTN, CTN and AAA.

Isolated Constituents	Literature* (ν_{max}, cm^{-1})	Experimental (ν_{max}, cm^{-1})
DCTN	1748 (C=O lactone stretch) 1666 (α,β-unsaturated carbonyl stretch) 1504; 873 (C=C furan ring)	1755 (C=O lactone stretch) 1653 (α,β-unsaturated carbonyl stretch) 1501; 868 (C=C furan ring)
CTN	3139 (CH- aromatic stretch) 2965; 2883 (CH_2 and CH_3 stretches) 1756 (C=O lactone stretch) 1704 (C=O ketone stretch) 1505; 873 (C=C furan ring)	3129 (CH- aromatic) 2955; 2874 (CH_2 and CH_3 stretches) 1748 (C=O lactone stretch) 1705 (C=O ketone stretch) 1500; 864 (C=C furan ring)
AAA	3448 (hydroxyl stretch) 2940 (CH_2 and CH_3 stretch) 1734 (C= ester stretch) 1688 (C= acid stretch) 1467 (CH_2 plane deformation) 1370 (CH_3 plane deformation) 1298 (C-O stretch)	2936 (hydroxyl) 2936 (CH_2 and CH_3 stretches) 1733 (C=O ester stretch) 1687 (C=O acid stretch) 1450 (CH_2 plane deformation) 1373 (CH_3 plane deformation) 1297 (C-O stretch)

*Maciel et al., 1998 [23].

Preparation of the biofilms containing the nanoemulsionated systems NE1 and NE2

The target nanobiosystems (SNEDDS-type carrier system shortly named NE-carrier) was obtained with a mixture of non-ionic surfactants (Tweens class), a vegetable oil (a common food use oil) and double distilled water. The ternary phase diagram proved the formation of o/w emulsion region (WIV) (Figure 3.4).

The preparation of the target NE-carrier was performed using a nontoxic and biodegradable surfactant (co-surfactant free) for topical application on wound healing.

The ternary phase diagram of the NE-carrier system (Figure 3.4) formed by a non-ionic surfactant constituent, vegetable oil and double distilled water afforded a polar type o/w structure with wide resistance to water dilutions. Shortly, the physicochemical characterization of the target system NE-carrier without incorporation of the herbal hydroalcoholic extract, showed: **i)** c.m.c. (critical micellar concentration, with experimental value ranging from 6,0 x 10^{-3} g/mL to 9,0 x 10^{-3} g/mL); **ii)** when the interface is saturated with the surfactant molecules self-assembling are formed and the surface tension (ranging from 4 x 10^{-2} N/m to 6 x 10^{-2} N/m) reached its minimum value at the c.m.c. and remains constant above it; **iii)** viscosity with experimental value ranging from 2 x 10^{-3} N·s/m² to 4 x 10^{-3} N·s/m²); **iv)** reached strongly small droplet size (ranging from 5 nm - 18 nm). So, the biotechnological *Croton cajucara* based SNEDDS colloidal based formulation (NE1 and NE2) is already protected under a patent request [92].

Finally, the incorporation of HAE-CC into the target NE-carrier system and misted with chitosan (CHT) solution (2%, w/v) afforded the novel formulations CHT-NE1 and CHT-NE2 which is already protected under a patent request [91].

Histopathological analysis of animals treated with CHT-NE1 and CHT-NE2 nanobiofilms

Histopathological analysis showed an atrophic epidermis, with no cutaneous attachments in the dermal healing area for the group treated with chitosan film (Figure 3.5) and the inflammatory response of chronic inflammation was observed at moderate intensity. Fibroblasts were young with collagen deposition in moderate amounts. Granulation tissue is present in this group and shows a late pattern with few vessels, mild edema and extravasated red blood cells. Based on these results, it is possible to conclude that the group treated with a CHT-film without the *C. cajucara* extract (HAE-CC) incorporation, had a lower scar response to the healings of the groups treated with *C. cajucara* incorporated into biofilms. The CHT-film was obtained by dissolving 2% of chitosan (w/v) in 2% acetic acid solution (v/v). After the complete dissolution of chitosan 24 mL of this solution were poured into Petri dish, then the plates were taken to an over at 50 ^{o}C for 24h in order to evaporate the solvent used to obtaining the blank chitosan film (CHT-film). Posteriorly, plates were immersed in 1M NaOH solution for 30 min., and the CHT-film was washed with distilled water up to neutral pH. Finally, the CHT-film was stretched, dried at room temperature, cut (1 cm^2) and stored in a dry place before the *in vivo* experiment, such as: **i)** Group A animals, treated only with CHT-film, from which CHT1, CHT2, CHT3, CHT4, and CHT5, identify each animal of this group); **ii)** Group B and C animals, treated with the CHT-NE1 and CHT-NE2 formulations, 5 mg/mL and 30 mg/mL of herbal extract, respectively. Each group comprise: CHT1-NE1, CHT2-NE1, CHT3-NE1, CHT4-NE1, and CHT5-NE1 (Group B-animals, n=5) and CHT1-NE2, CHT2-NE2, CHT3-NE2, CHT4-NE2, and CHT5-NE2 (Group C-animals, n=5).

Table 3.2 shows the scores attributed to histopathological parameters related to animals treated with CHT-film and the colloidal based nanobiofilms CHT-NE1 and CHT-NE2. It was possible to evidence that for the groups treated with CHT-NE1 or CHT-NE2, the reepithelization of the epidermis was more complete than the group treated with the CHT-film control group, showing a pattern of epithelization close to the normal epithelium pattern, without atrophy. The inflammatory response of the chronic inflammation ranged from mild to absent. Fibroblasts showed a pattern of mature cells with distribution within normal limits or slightly increased in number. Collagen had a normal distribution with a well formed and refurbished tissue pattern. Granulation tissue was absent in most of the animals studied.

In general, animals treated with CHT-NE1 demonstrated better inflammatory response than those ones treated with CHT-NE2. On the other hand, this group presented highest density of fibroblasts and collagen (Figure 3.5).

Figure 3.5 (A, C and E) shows greater presence of collagen to the groups treated with CHT-NE1 or CHT-NE2 comparing with the group treated with chitosan biofilms. The highest collagen density and its highest degree of organization indicate a greater evolution of the scar process evidencing almost completed tissue repair. In addition, histograms stained with *Masson's trichrome* (Figure 3.5, B, D and F) show considerably higher blood vessel densities in groups treated with CHT-NE1 or CHT-NE2 indicating neovascularization in the experimental groups (D and F).

TABLE 3.2
Scores attributed to histopathological parameters of the inflammation and healing to each individualized animal treated with CHT-film, CHT-NE1 and CHT-NE2.

Animals	Inflammatory response	Type of response	Intensity	Fibroblasts	Collagen	Epithelialization	Granulation tissue
CHT1	+	Chronic	+	++	++	+	+
CHT2	+	Chronic/ granulomatous	++	++	+	+	++
CHT3	+	Chronic	++	++	++	+	++
CHT4	+	Chronic	+	++	++	+	+
CHT5	+	Chronic	++	++	++	+	++
CHT1-NE1	-	-	-	+	+++*	+	-
CHT2-NE1	+	Chronic	+	++	++	+	+
CHT3-NE1	+	Chronic/ granulomatous	++	++	++	+	++
CHT4-NE1	+	Chronic/ Acute	+	+	+++	+	+
CHT5-NE1	+	Chronic/ acute	++	++	++	+	++
CHT1-NE2	+	Chronic/ acute	+++	+++	++	+	++
CHT2-NE2	+	Chronic	++	++	++	+	+
CHT3-NE2	+	Chronic	++	+++	++	+	+
CHT4-NE2	-	-	-	+	+++*	+	-
CHT5-NE2	+	Chronic	+	+++	+++	+	+

CHT1/CHT5= animal number 1 to animal number 5 of the Group A (n=5) treated with CHT-film;
CHT1-NE1/CHT5-NE1 = animal number 1 to animal number 5 of the Group B (n=5) treated with CHT-NE1;
CHT1-NE2/CHT5-NE2 = animal number 1 to animal number 5 of the Group C (n=5) treated with CHT-NE2.
+: present, -: absent (Inflammatory response);
+: mild, ++: moderate, +++: intense (intensity of inflammatory response);
+: mild, ++: moderate, +++: intense (fibroblast);
+: mild, ++: moderate, +++: intense (collagen);
+: present, -: absent (epithelialization);
+: light, ++: moderate, +++: intense (granulation tissue).

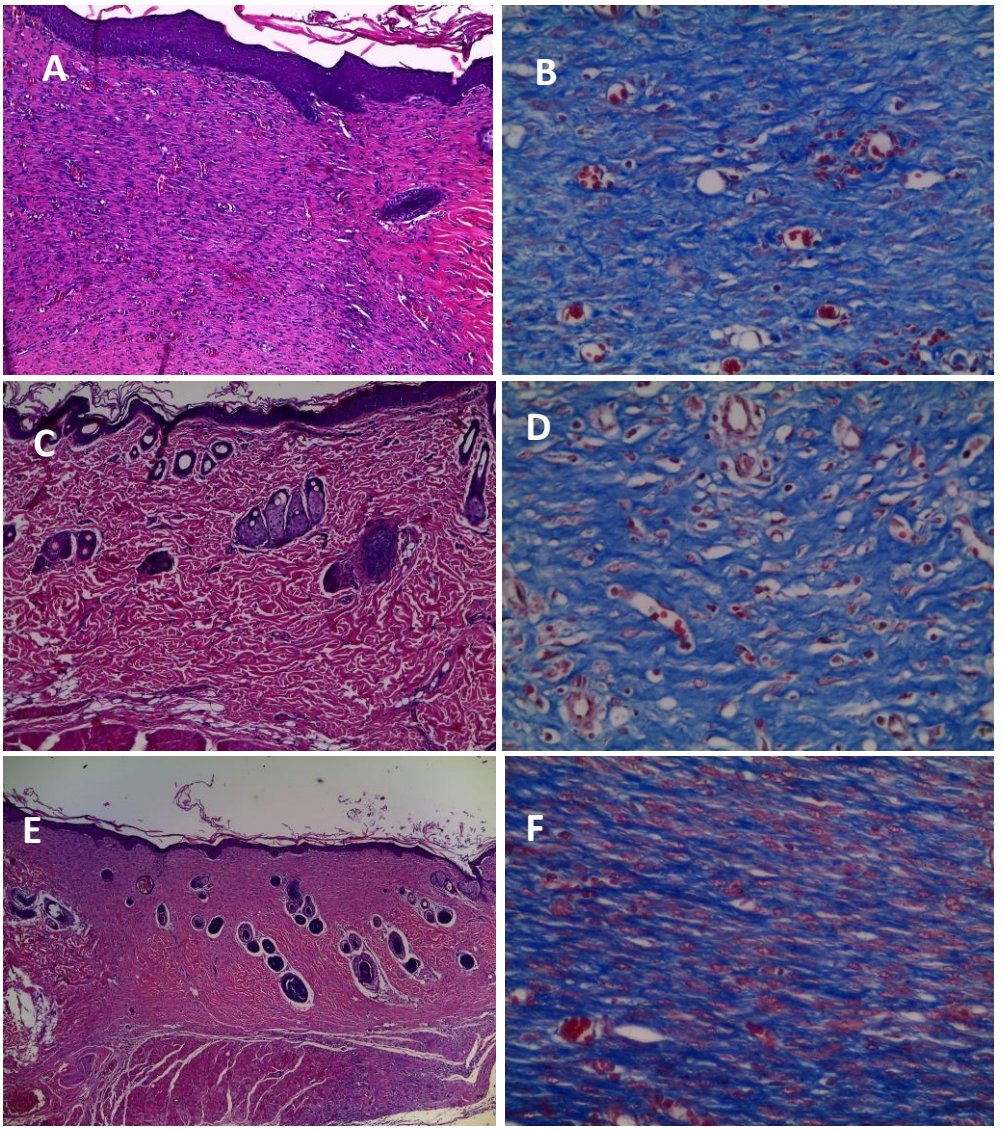

FIGURE 3.5
Histological aspect of the tissue treatment with chitosan solution (CHT-film, A and B), and with the colloidal based nanobiofilms containing the HAE-CC extract load in two different contents 5 mg (CHT-NE1, C and D) and 30 mg (CHT-NE2, E and F) after 28 days. Slides were stained with Hematoxylin-eosin (A, C and E) and *Masson's trichrome* (B, D and F).

The colloidal nanobiofilms based on chitosan/*C. cajucara* act differently from drugs administered in conventional formulations which need high drug concentration to reach the therapeutic dose in the site of action promoting adverse and toxicological effects in the normal tissue. So, the nanobiotechnological research evidenced in the present study reinforce the importance to develop interdisciplinary studies based on vegetables to produce safety formulation applied to treatment of many diseases. Indeed, compared to other studies developed with several vegetables carried in

colloidal systems, the present study contributes as follows: favors the reduction of therapeutic doses due to site-specificity and protection against side effects by reducing the frequency of drug administration; act with slow and controlled release; solubilize compounds of low and high polarity via encapsulation that maintains molecular dispersion in a biocompatible way, with gains in permeability and therapeutic functions.

Concerning to chitosan wound dressing advances it was observed healing at early phase of experimental open wound in dogs and it effectiveness as in infected and noninfected wounds in mice as well as other therapeutic applications [93-96]. In this present work, encapsulation of the hydrophobic drugs such as DCNT, CTN and AAA, present in the tested hydroalcoholic extract, into o/w micelles followed by adding a chitosan solution process, and then solvent evaporation, open additional areas for biochemical investigation of *C. cajucara*, such as skin wound healing. In this context, nanobiocarriers due to the great diversity of their properties can be used safely, applied in prolonged therapies with administration of the drug by different pathways, such as: topical, transdermal, oral, ocular, vaginal and rectal [42, 50, 54, 55, 97, 98].

Conclusion

Due to its biocompatibility, biodegradability, nontoxicity, antimicrobial and wound healing activities as well as the ability to form resistant and elastic films, chitosan represents an excellent alternative for wound dressings and also as a matrix for the incorporation of drugs with several therapeutic uses. In this context, the aim of this work was to obtain a hydroalcoholic extract from the stem bark of *Croton cajucara* Benth (EHA-CC), which was incorporated (5 mg and 30 mg) into a self-nanoemulsion drug delivery systems (SNEDDS) target carrier. The authenticity of plant material was proven by the chromatographic isolation and then spectroscopic characterizations of the biomarkers *trans*-dehydrocrotonin (DCTN, 0.76%) and *trans*-crotonin (CTN, 0.16%) which are clerodane-type 19-*nor*-diterpenes and the triterpene acetyl aleuritolic acid (AAA, 0.019%).

The SNEDDS nanoemulsions (NE1 and NE2) containing 5 mg (NE1) and 30 mg (NE2) of the extract EHA-CC were then incorporated into chitosan biofilms (CHT) affording two novel nanobiofilms so called CHT-NE1 and CHT-NE2. The applied procedure to obtain the SNEDDS-carrier system required a biocompatible mixture of surfactant and oil phase affording a polar (o/w) colloidal system without co-surfactant need. The SNEDDS-carrier system without or containing the herbal extract remained isotopically stable after centrifuge procedure (3500 rpm) as well as by water dilution ensuring no phase change.

In order to investigate the potential use of these colloidal biofilms as wound dressings, an *in vivo* experimental model with *Wistar* rats was assayed. The evolution of the healing process in the treated animals was evaluated through histopathological studies. The findings were compared between the animal group treated with CHT-film (chitosan biofilm vegetal free) and those animal groups treated with the chitosan/vegetal biofilms (CHT-NE1 and CHT-NE2, 5 mg and 30 mg, respectively). The results showed that these formulations promote more efficient healing, inducing greater neovascularization and a greater amount and organization of collagen fibers. It was also observed that the nanobiofilms were more effective than CHT-film in the healing aspect of the wounds, as evidenced by the higher (improved) neovascularization. On the other hand, the animals treated with CHT-NE1 had a better inflammatory response, whereas the group treated with CHT-NE1 showed higher densities of fibroblasts and collagen.

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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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