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Wound dressings loaded with an anti-inflammatory jucá (*Libidibia ferrea*) extract using supercritical carbon dioxide technology

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ABSTRACT

N-Carboxybutyl chitosan (CBC), collagen/cellulose (Promogran®) and hyaluronic acid-based (Hyalofill®) polymeric matrices/dressings were loaded with an extract obtained from jucá (*Libidibia ferrea*) and in order to develop wound dressings endowed with anti-inflammatory activity. Jucá fruits were subjected to supercritical fluid extraction (SFE) using CO_2 at 25 MPa and 50 °C and the resulting extract was later incorporated into the above referred wound dressings by a supercritical fluid impregnation/deposition method (SSI). GC analysis revealed that the obtained SFE extract is particularly rich in unsaturated (52%) and saturated (26%) fatty acids as well as in terpenoids (13%) such as lupenone and gamma-sitosterol. Extract loading yields depended on the affinity of the hydrophobic extract for the specifically employed wound dressing material and was almost 2-fold greater for CBC than for the other two commercial wound dressings. The prepared extract-loaded dressings were cytocompatible with RAW 264.7 macrophages (viability > 85% at 24 h) and down-regulated the expression of TNF-α and IL-1α pro-inflammatory cytokines as well as the production of nitric oxide, which confirms the anti-inflammatory capacity of the employed jucá extract. Nevertheless, such effect was somehow counteracted by a pro-inflammatory activity that was exhibited by CBC. Prepared dressings presented a wide range of water vapor ($(2.9–14.7) \times 10^{14}$ kg/(s m Pa)) and oxygen permeability (150 up to 830 barrer) which make them potentially suitable for the management of various wound types at different healing stages.

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1. Introduction

The wound healing process may be compromised/delayed by many local and systemic factors that lead to the need of specialized care and treatment [1]. Moreover, each of the wound stages involved in the process is usually characterized by the generation of some characteristic tissues and/or secretions which may as well require some specific treatment. The use of wound dressings may enhance the body's own healing mechanism by creating a proper physiologic wound environment (moisture and permeability to oxygen, water vapor and carbon dioxide), by acting as a barrier for microorganisms and/or by releasing bioactive compounds to the wound site [2–4].

The choice of the dressing material is critical since its interaction with the wound may significantly influence the healing process. Natural-based biodegradable and biocompatible materials are gaining increasing attention [5]. *N*-Carboxybutyl chitosan (CBC) is a highly water soluble chitosan derivative that preserves the original chitosan bactericide/fungicide activity and that also leads to limited scar formation [6]. CBC can also be processed by applying different methods to tune the permeability to water vapor and oxygen, the swelling capacity and the ability to control drug release [7].

Wound dressings made from natural body components may be advantageous in order to improve biocompatibility and to avoid inflammation and/or rejection. Collagen is present in the extracellular matrix (ECM) and acts as a natural substrate for cellular attachment, growth and differentiation [8,9]. Collagen-based materials are successfully used worldwide to prevent wound infections in burns and to treat chronic wounds, serving both as dermis substitutes and as platforms for drug release. Promogran® by Johnson & Johnson Medical is a commercially available wound dressing composed of 55% collagen and 45% oxidized regenerated cellulose (ORC), which is intended to absorb fluids/exudates and to form a soft gel that physically binds and inactivates those proteases that can degrade ECM components and which are excessively active in acute wounds [10,11]. It also binds naturally occurring growth factors and protects them from proteases [12]. On the other

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hand, hyaluronic acid (HA), which is present in connective tissues, actuates in the early response to injury together with fibrin and to support fibroblasts and endothelial cells that will originate granulation tissue [13]. HA also presents anti-oxidant and anti-inflammatory activity [14]. Hyaluronan and hyaluronan-binding proteins regulate inflammation, tissue injury, and repair by regulating inflammatory cell recruitment, release of inflammatory cytokines, and cell migration [15,16]. High molecular weight HA dressings enhance angiogenesis and are appropriate in the management of chronic wounds and diabetic foot ulcers [17,18]. Hyalofill® from ConvaTec, Ltd. is an ester of HA with benzyl alcohol, which facilitates sterilization and handling, and that forms soft, cohesive gels in contact with wounds.

Natural antimicrobial, antioxidant and anti-inflammatory substances obtained from vegetal raw materials can be used as coadjuvants that can stimulate the healing processes and protect wounds against external aggressions [19–24]. However, the therapeutic activity of these extracts is strongly dependent on the extraction methodology, which can notably affect the chemical nature, composition, amounts and stability of the extracted compounds [25–27]. Organic solvents and/or high extraction/loading temperatures can induce degradation. Once the extract is ready, it is necessary to implement adequate procedures to load into wound dressings the appropriate amounts of bioactive compound(s), in order to be efficient without causing toxicity. Supercritical fluid methodologies, namely supercritical extraction (SFE) and supercritical impregnation/deposition (SSI) using supercritical carbon dioxide ($scCO_2$), present several remarkable advantages when compared to conventional solid–liquid extraction/loading methodologies, namely a density-dependent solvent power and excellent transport properties which will control the ability to dissolve solutes and to extract them from solid matrices at relatively low temperatures [28]. Moreover, SFE is an effective method to obtain bioactive products from plant materials in a selective way and without organic solvent contaminants [29–31]. On the other hand, SSI allows the homogeneous impregnation/deposition of polymeric matrices with bioactive substances in a relatively short time and, when properly employed, it does not alter and/or damage their physical, chemical, and mechanical properties. Loading yields and loading depths can be easily controlled by tuning some of the operational conditions [7,32–35]. In pharmaceutical processing, $scCO_2$ presents an additional advantage since it may as well temporarily plasticize and swell most polymeric matrices at relatively low temperatures, which will help and enhance the loading process [36]. Upon depressurization, the CO_2-soluble compounds precipitate and become entrapped within polymer matrices, while the dense CO_2 returns to its gaseous state originating a final material that is free of any residual solvent and that may be sterilized, depending on the process conditions used [36,37]. In general terms, the higher and the most favorable the interaction of bioactive molecules with the polymer functional groups, the higher the achieved loaded amounts [33,34,38,39]. When dealing with multicomponent systems (such as natural extracts), the involved interactions are much more complex and the chemical nature of the involved polymeric matrices should be taken in consideration in order to maximize the affinity for some specific compounds.

This work aims to extract bioactive substances from jucá fruits (*Libidibia ferrea* or *Caesalpinia ferrea*, widely known as Brazilian ironwood) using a SFE methodology and later, to load those extracts (using a SSI method) into various polymeric matrices, namely into synthesized CBC foams and into commercially available Promogran® and Hyalofill® dressings, and in order to improve their final performances as wound dressings. Jucá fruits extracts have been reported to present relevant healing, antifungal, antimicrobial, anti-inflammatory, analgesic, antiulcer and cancer chemopreventive properties [26,40–44]. Other relevant objectives of this work are the evaluation and elucidation of the relationships between extract compositions, extract loading yields, extract release behaviors from employed wound dressing materials, cytocompatibility with fibroblasts and macrophages, and anti-inflammatory ability against LPS-stimulated macrophages. Other functional relevant properties for the envisaged wound dressing application, such as hydrophilicity, oxygen permeability and water vapor transmission were also evaluated.

2. Materials and methods

2.1. Materials and chemicals

Employed materials and chemicals were: carbon dioxide (99.5%) from Praxair Spain, ethanol (99.5%, p.a.) from Panreac Quimica SA Spain, ethyl acetate (99.9%) from Chromasolv Plus, Sigma–Aldrich, Inc., USA, *n*-alkanes C8-C40 GC standards (aliphatic hydrocarbons kit) from Supelco, Sigma–Aldrich, USA, and potassium sulfate (99%) and lithium chloride (99%) from Fluka. Water was purified by reverse osmosis (Milli-Q water, Millipore). Chitosan (mean molecular weight and 73% deacetylation degree), levulinic acid (98%) and sodium borohydride (99.5%) from Sigma–Aldrich were used to prepare CBC foams. Promogran® and Hyalofill® were obtained from Johnson & Johnson Medical and ConvaTec, Ltd., respectively. Jucá (*Libidibia ferrea*) fruits were harvested in Belém do Pará, Brazil. A voucher specimen (MG200833) was deposited at the Emílio Goeldi Paraense Museum, Belém, Brazil.

2.2. Synthesis of N-carboxybutyl chitosan (CBC) foams

CBC was prepared from chitosan using levulinic acid and sodium borohydride and according to a previously described method [45]. The final solution was sequentially washed with ethanol and water to remove non-reacted compounds and lower molecular weight polymeric chains. The solution was dialyzed into water for 3 days and then concentrated in a rotary evaporator (100 mbar, 40 °C). Finally, 1 ml aliquots of the concentrated solution were poured in cylindrical wells (1 cm in diameter), frozen with liquid nitrogen, and freeze-dried for 2 days. The average thickness of the prepared disc-shaped foamed structures was 0.60 ± 0.05 mm. Samples were later cut into a quadrangular shape (1 cm × 1 cm). Dried samples were stored in a desiccator (at ambient temperature) until further use.

2.3. SFE from jucá fruits

Particle size distribution of the comminuted raw fruits was analyzed using a sieve series (120–18 mesh), under mechanical stirring (Retsch, Germany) and the mean geometric diameter of the particles (calculated according to the American Society of Agricultural Engineers ASAE, S319.2 method) was found to be 0.93 mm. Raw material was placed inside the high pressure extraction cell (~17.8 cm^3) which was thermostatized at 50 ± 0.1 °C and pressurized at 25 ± 0.5 MPa. The employed SFE apparatus was previously described [46] and high pressure liquid pumps were used to deliver the liquefied CO_2. The outlet CO_2 mass flow rate ($(3.50 \pm 0.15) \times 10^{-3}$ kg min^{-1}) was measured using a mass flow meter (Series GFM, Dwyer). After a 30 min static period to allow an initial contact between raw material and $scCO_2$, continuous SFE conditions were maintained until the complete raw material exhaustion (less than 3 h). Extracts were recovered into refrigerated flasks (to improve the precipitation of volatile CO_2 soluble compounds) and were stored at approximately −18 °C until further analysis. All these SFE conditions were selected taking into account a preliminary study that confirmed the anti-inflammatory activity

of extracts obtained at these experimental conditions against raw 364.7 macrophages cell line [26].

Obtained extract was solubilized in ethyl acetate (at a concentration of 30 mg/ml) and analyzed by gas chromatography (GC-FID): GC chromatograph (Finnigan-Tremetrics, model 9001, Austin, USA); split injector (523 K, split ratio 1:10); FID detector at 523 K; DB-5 (J&W Scientific) cross-linked fused-silica capillary column (60 m × 0.32 mm i.d., 3 μm). Sample injection volume was 5.0 μl. Oven temperature was programmed from 50 °C (5 min hold) up to 250 °C, at a 2.5 °C min^{-1} heating rate, and maintained for 50 min [47].

2.4. SSI extract-loading into CBC, Promogran® and Hyalofill® dressings

2.4.1. SSI extract-loading procedure

The employed supercritical impregnation/deposition apparatus was already described in literature [32–34] and, in general terms, consists in a compressed air-operated CO_2 liquid pump, a visual high-pressure stainless steel impregnation/deposition cell, a thermostatic controlled water bath, and a magnetic stirring plate as an auxiliary tool to dissolve and to homogenize the high pressure mixture (jucá extract + scCO_2). Dried and previously weighed (2–8 mg) CBC, Promogran® and Hyalofill® quadrangular samples (1 cm × 1 cm), were fitted into stainless steel supports and then placed into the high-pressure cell that already contained a known amount of jucá extract (~40 mg). The system was then pressurized up to 27.0 MPa and the temperature was set to 50 °C. Notice that the employed operational pressure in the SSI process was slightly higher than that used in SFE experiments (to enhance the extract solubility into the solvent mobile phase). Magnetic stirring (900 rpm) was always employed in order to solubilize and homogenize the supercritical fluid phase mixture. All SSI experiments were carried out for 15 h and replicated. Two depressurization rates (3 and 10 MPa min^{-1}) were assayed in order to verify the influence of this variable on the loaded extract amounts.

2.4.2. Quantification of the loaded/released extract amounts

The loaded amounts of jucá extracts (in loaded dressings) were quantified gravimetrically after the immersion/leaching in ethyl acetate (which is a good solvent for the substances present in extract and do not significantly degrade/dissolve any of the employed dressings). Solvent was renewed three times in order to guarantee that all extract was removed. Non-loaded specimens were also washed to confirm that employed dressing materials did not dissolve in ethyl acetate. The final dressing weights (after extraction with ethyl acetate and the subsequent drying) were almost equal to their weights before the SSI loading experiments (mass differences below 6%).

The release of extract compounds (from loaded dressings) was performed in phosphate buffer saline (PBS) at 37 °C and 100 rpm. After 24 h, dressings were removed from PBS and dichloromethane was added (equal volume of both phases) in order to perform a liquid–liquid extraction of those extract compounds released into PBS. Procedure was repeated 3 times in order to achieve a complete extract removal from the aqueous phase. After dichloromethane evaporation, the solid residue was solubilized in ethyl acetate (at a concentration of 30 mg/ml) and analyzed by GC (as previously described in Section 2.3). Additionally, the composition of released extract compounds was identified by GC–MS (at the same conditions as those employed in GC-FID experiments) and using the NIST database and Kovat's indexes – determined relatively to the retention time (RT) of a series of *n*-alkanes and according to literature [47]. All samples were analyzed in duplicate.

2.5. Physical characterization of non-processed and loaded/processed wound dressings

2.5.1. Surface morphology

Scanning electron microscopy (SEM) (JEOL, model JSM-5310, Japan) micrographs of non-processed and loaded/processed dressing materials were obtained at 5 kV and at different magnifications. Samples were coated with gold (approximately 300 Å) in argon atmosphere.

2.5.2. Fourier transform infrared (FTIR) attenuated total reflection (ATR) spectroscopy

FTIR spectroscopy (Jasco, model 4200, UK) was performed at 128 scans with a 4 cm^{-1} resolution, between 500 and 4000 cm^{-1}, and using a Golden Gate Single Reflection Diamond ATR accessory. Samples were analyzed before and after the extract loading experiments.

2.5.3. Porosity

The porosity of the different wound dressing materials was determined as:

$$P = 1 - \frac{\rho_{\text{apparent}}}{\rho_{\text{solid}}} \quad (1)$$

where ρ_{apparent} (g/cm^3) is the apparent density (calculated as the ratio between samples' mass and volume of each dressing material), and ρ_{solid} (g/cm^3) is the true density measured by helium pycnometry (Accupyc 1330 Micromeritics, Micromeritics Instrument Corporation, USA).

2.6. Fluid handling properties

2.6.1. Oxygen permeability

Oxygen permeability of non-processed wound dressings was measured (in triplicate) using a Createch permeometer (model 210T, Rehder Development Co., Castro Valley, CA), at room temperature and at a 100% relative humidity (RH).

2.6.2. Water vapor sorption (WVS)

Dressings were cut into quadrangular 1 cm × 1 cm samples and dried at 40 °C until constant weight was achieved. Dried samples were then exposed to a 95% RH atmosphere (at 32 °C) in a desiccator containing a potassium sulfate saturated solution. Samples were weighed at pre-determined time intervals and the water vapor sorption capacity was calculated as:

$$\text{WVS} = \left(\frac{W_t - W_0}{W_0}\right) \times 100 \quad (2)$$

where W_0 and W_t, are the sample weight at the beginning of the experiment (dried) and at time t, respectively. Experiments were carried out in triplicate. The WVS kinetic curve was modeled using the following second-order adsorption model [48]:

$$\frac{1}{M_t} = \frac{1}{k_2\,\text{WVS}_e^2} + \frac{1}{\text{WVS}_e}t \quad (3)$$

where M_t is the water vapour adsorbed at time t, WVS_e is the water adsorbed at equilibrium and k_2 (h^{-1}) is a second-order adsorption rate constant.

2.6.3. Water vapor transmission rate (WVTR)

Measurements were performed according to the ASTM standard [7]. Permeability cells were filled with 1 g of Milli-Q water and test-sample dressings were fixed with a transmission exposed area of 0.636 cm^2. Cells were weighed and placed into desiccators at 32 °C and 20% RH (atmosphere created with a saturated lithium chloride solution). The slope of the water loss *vs.* time (t), normalized to

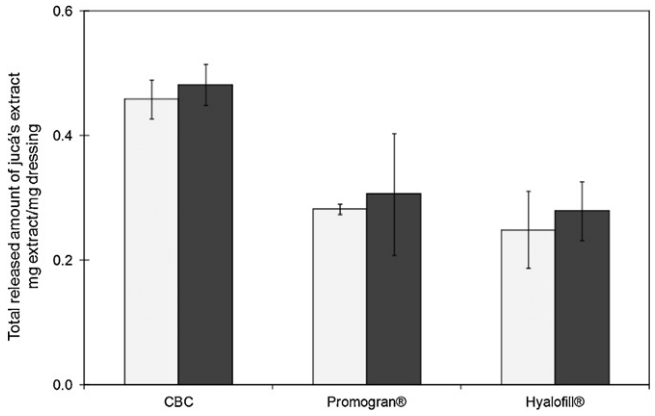

Fig. 1. Total amounts of jucá extract loaded into tested dressings (at 27 MPa and 50 °C). Depressurization rate was: 3 MPa min^{-1} (■) and 10 MPa min^{-1} (■).

the testing area (A), was employed for the calculation of the water vapor transmission rate (Eq. (4)):

$$\mathrm{WVTR} = \frac{\text{water mass lost}}{t \times A} \tag{4}$$

The water vapor permeability was then calculated as:

$$P = \mathrm{WVTR}\frac{l}{P_0(\mathrm{RH_{in}} - \mathrm{RH_{out}})} \tag{5}$$

In this equation, l is the dressing thickness (mm), P_0 is the water vapor pressure at 32 °C, and $\mathrm{RH_{in}}$ and $\mathrm{RH_{out}}$ are the relative humidity of the air inside the permeability cell (assumed to be equal to 100%) and in the desiccator (20%), respectively.

2.6.4. Water contact angle measurements

Milli-Q water contact angles measurements of non-processed dressings were performed using the sessile drop (6–7 μl) method and an OCA20 contact angle apparatus (Dataphysics Instruments GmbH, Germany). Foams were previously compressed in order to create more homogeneous surfaces. An average of 5 drops was applied in 3 different samples of each dressing material.

2.7. Cytocompatibility tests

Tests were carried out with RAW 264.7 macrophages and Balb/3T3 clone A31 fibroblasts (ATTC, Manassas) maintained in DMEM-F12 HAM with phenol red medium (Biochron AG, Germany) supplemented with 10% (v/v) heat inactivated fetal bovine serum (BioWhittaker® Lonza, Belgium) and gentamicine (B. Braun, Spain) (130 μl/100 ml) and kept in a humidified incubator at 5% CO_2, 90% air and 37 °C. The RAW 264.7 macrophages were seeded in 24 well-plates (1×10^5/2 ml in DMEM-F12 HAM without phenol red; Sigma–Aldrich, USA) and pre-incubated with samples (approx. 1 cm^2) of each dressing (non-loaded or loaded with extract) previously sterilized by UV radiation for 2 h. After that time, 20 μl of lipopolysaccharide (LPS; Sigma–Aldrich, USA) solution was added to the culture medium (final concentration 1 μg/ml). Negative (cells in culture medium without sample or LPS) and positive (cells in culture medium with 1 μg/ml LPS) controls were processed concurrently. Aliquots (500 μl) from the culture medium were collected at 2, 6, 24 and 72 h and immediately frozen at −20 °C until LDH and cytokines production tests; each aliquot was replaced with 500 μl of fresh medium. Experiments were carried out in duplicate for each tested dressing material and for each assayed time interval.

2.7.1. LDH tests

The amount of lactate dehydrogenase (LDH) released was quantified using the cytotoxicity detection Kit^{PLUS} (Roche, Spain). Absorbance was measured in a plate reader (Bio-Rad 680 Microplate Reader, USA), at 490 nm, and viability was calculated according to the cytotoxicity assay kit instructions.

2.7.2. ELISA assays for detecting IL1-α and TNF-α production

The cytokine concentrations (mouse IL-1α and mouse TNF-α), in cell culture supernatants at 2, 6 and 24 h, were quantified by specific enzyme-linked immunosorbent assay (ELISA) (Bender MedSystems, Austria) following the manufacturer's test protocol. Plates were read at 450 nm in a spectrophotometer (Bio-Rad 680 Microplate Reader, USA).

2.7.3. Measurement of nitric oxide concentration

Nitric oxide (NO) production was assayed by measuring nitrate in the supernatants, at 24 and 72 h, and according to a previously reported method [49]. Aliquots (80 μl) were transferred to 96-well plates and incubated with 100 μl of Griess reagent (Cayman Chemical, USA). After 10 min of incubation at room temperature, plates were spectrophotometrically read at 550 nm. Nitric oxide concentration was quantified by a previously obtained sodium nitrate calibration curve (0–15 μM).

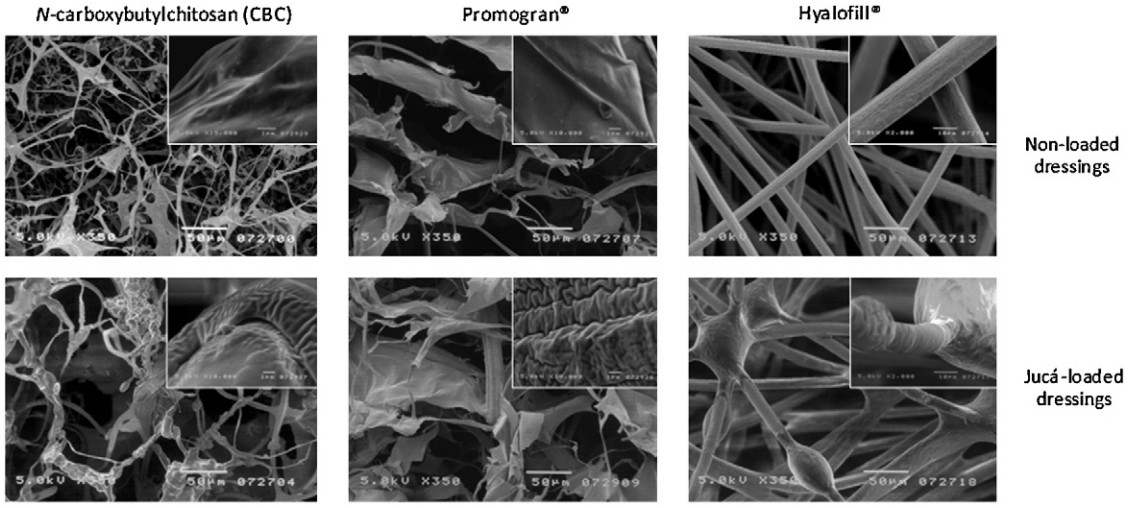

Fig. 2. SEM micrographs for non-loaded (top) and jucá extract-loaded dressings at 27 MPa and 50 °C (bottom). The images show twisted interlaced wires for *N*-carboxybutyl chitosan, CBC (on the left), layered flexible sheets for Promogran® (in the middle), and fiber-like structures for Hyalofill® (on the right).

2.7.4. Fibroblasts viability

A calcein/propidium iodide staining method was performed in order to monitor the viability of fibroblasts cultured on dressing materials. Balb/3T3 cells were seeded onto the dressings (1×10^5/2 ml) and incubated during 24 h at 37 °C (and with 5% CO_2). Subsequently, adherent cells were stained with 1 mg/ml calcein–AM (Sigma–Aldrich, Spain) and 1 mg/ml propidium iodide (Molecular Probes, USA) and observed under confocal microscope (Confocal Leica TCS-SP2, Leica Microsystems, Germany) in order to differentiate live and dead cells.

3. Results and discussion

3.1. Extract characterization and SSI extract-loading on dressings

Jucá fruits were extracted by SFE and extract GC analyses (Fig. S1 supporting information) permitted to identify six main compounds presenting relatively high abundances, namely linoleic acid (42.38%) > palmitic acid (19.28%) > elaidic acid (9.17%) > gamma-sitosterol (8.39%) > stearic acid (7.16%) and lupenone (4.67%). This corresponds to an averaged composition of 52% in non-saturated fatty acids, 26% in saturated fatty acids and 13% in terpenoids. This composition profile was somehow expected taking into account the known solubilities of the above indicated fatty acids in $scCO_2$ which follow the same trend [50,51]. Such an ability of $scCO_2$ to selectively extract oils containing high molecular mass acids (having 10 or more carbon atoms), their esters and/or their triglycerides [52,53], can be considered as an additional advantage to its non-toxic and non-flammable character, to the required low processing temperatures and to the negligible amounts of solvent residues present in the final product.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.supflu.2012.12.007.

SSI loading of the CBC foams and of the collagen and hyaluronic acid commercial dressings (thereinafter, all materials are referred as dressings) with the obtained jucá extract led to quite different loading yields which strongly depended on the employed dressing (Fig. 1). Dressings were washed/leached with ethyl acetate to completely remove the loaded extract and that removed amount was assumed to be equal to the total loaded amount. CBC dressing incorporated higher amounts of extract (~0.46–0.48 mg/mg) than the commercial dressings: ~0.28–0.31 mg/mg (Promogran®) and ~0.25–0.28 mg/mg (Hyalofill®). These results can derive from the greater hydrophobicity of the CBC matrix. Moreover, specific favorable interactions could be established between CBC and some of the compounds present in jucá extract thus enhancing extract/polymer partition coefficient and overcoming other possible diffusional constraints originated by the bulkier nature of CBC samples (according to porosity results that will be discussed latter). As previously reported, these results confirm that the SSI yields depend not only on the solubility of the solute in the supercritical solvent phase but also on the interactions that can be established between all the involved substances in the process: bioactive compounds, biopolymers and $scCO_2$ [32–34,39]. The depressurization rate effect (3 and 10 MPa min^{-1}) was minor on loading yields for all tested dressings and the observed slightly greater amounts of loaded extract at the higher depressurization rate (10 MPa min^{-1}) could be due to the fast dressing structure shrinkage during the CO_2 venting (returning to the original state after being swelled with $scCO_2$ during the loading process).

SEM micrographs (Fig. 2) indicate that the extract is mostly deposited onto the surface of the twisted and interlaced wires of the foam-like structure of CBC, onto the layered flexible sheets of the foam-like structures of Promogran® and onto the characteristic fibers of Hyalofill® (as noticed in the augmented images in

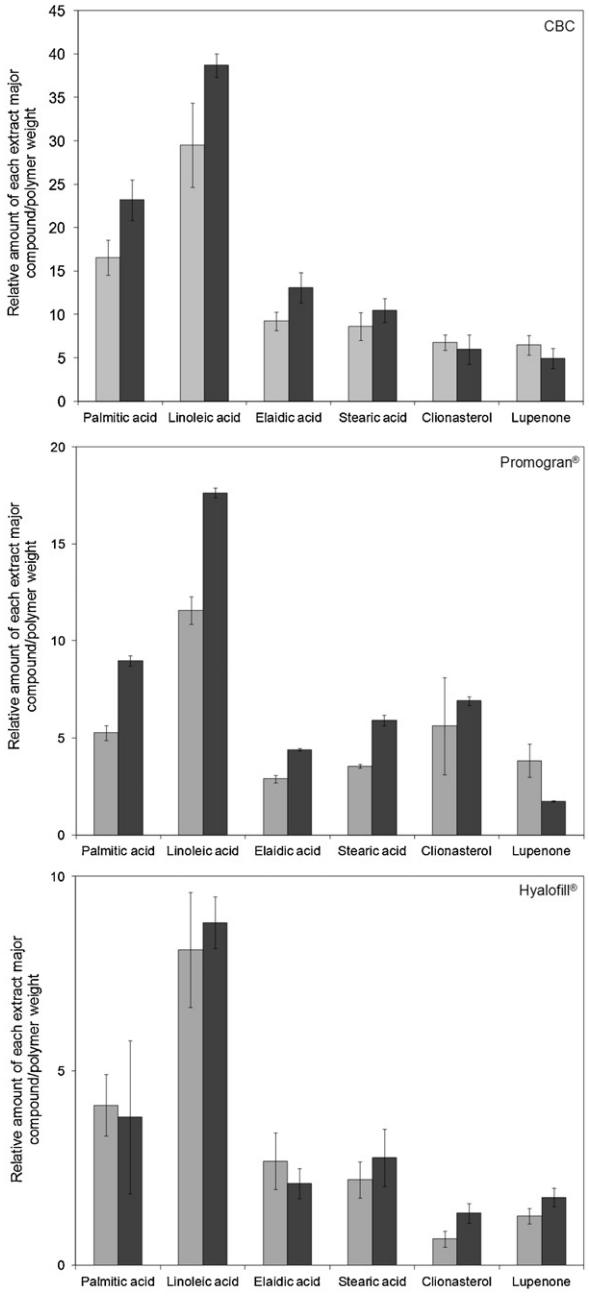

Fig. 3. Relative amounts of the six major compounds identified in jucá extract loaded into CBC, Promogran® and Hyalofill® dressings at 27 MPa and 50 °C. Depressurization rate: 3 MPa min^{-1} (■) and 10 MPa min^{-1} (■).

Fig. 2). The SSI process does not seem to affect the morphology of tested polymeric dressings. However, SEM also show that, in the case of CBC and Promogran®, extract was deposited as thin films while in the case of Hyalofill® fibers, it was also accumulated at specific points of the fibers. This occurrence may be due to some specific and different interactions that may be established between the benzyl groups present in Hyalofill® dressings and the aromatic rings of extract terpenoids, the vinyl groups and/or the carboxylic groups of those fatty acids present in jucá extract.

GC chromatogram profiles of the extract removed (with ethyl acetate) from each loaded dressing were similar to that of the raw extract (Fig. S1 supporting information), which indicates that the extract was efficiently and not selectively loaded into the dressings and also that the interactions between the employed polymer matrices and the main extract compounds are not too

Table 1
Fluid-handling capacities of CBC, Promogran® and Hyalofill® dressings.

	CBC	Promogran®	Hyalofill®
Foam thickness (mm)	0.61 ± 0.05	2.95 ± 0.09	1.98 ± 0.04
Porosity (%)	40 ± 2	83 ± 8	72 ± 6
Equilibrium WVS_e (%)	28.85 ± 0.58	56.51 ± 0.49	35.72 ± 0.67
Adsorption rate constant, k_2 (s^{-1})	120.67 ± 0.55	41.00 ± 0.46	78.81 ± 10.80
WVTR ($g\,m^{-2}\,d^{-1}$)	1623 ± 65	1549 ± 15	1635 ± 56
Water vapor permeability × 10^{14} (kg/(s m Pa)) (×10^{-14})	14.67 ± 0.58	2.89 ± 0.028	9.91 ± 0.34
Oxygen permeability (barrer)	165.41 ± 40.19	831.99 ± 76.27	152.53 ± 11.50

strong to prevent its removal by washing with ethyl acetate. Some additional peaks that were detected on the washed extracts GC chromatograms were identified as 3,4-dimethylbenzadehyde (at 20.8 min retention time), as di-2-ethylhexylphthalate (66 min) and as two non-identified compounds (33.7 and 74.1 min), and may be attributed to the presence of some impurities in CBC and in commercial dressings [54]. The relative amounts of each of the six major compounds identified in jucá extract were also quantified by GC (Fig. 3). Results show that the amount of each compound loaded into the dressings is proportional to their original composition in the raw extract and that fatty acids (mainly linoleic acid) were loaded in larger amounts.

FTIR spectra (Fig. 4) of the SFE jucá extract and of the extract-loaded dressings confirmed the presence of significant amounts of free fatty acids (FFA) which are characterized by: (i) strong absorption bands between 2850 and 2920 cm^{-1}, 1374 and 1458 and 720 cm^{-1} (which are characteristic of the CH_2 and CH_3 stretching, bending and (CH_2) $n > 4$ rocking vibrations of aliphatic structures, respectively); (ii) strong absorption bands between 1690 and 1760 cm^{-1} (corresponding to the C═O stretching for acids); (iii) relatively strong bands between 1000 and 1300 cm^{-1} (characteristic of the aromatic rings of terpenoids). Those bands appearing between 900 and 1000 cm^{-1} (as well as around 3010 cm^{-1}) also confirm the presence of unsaturated C═C bounds (of FFA chains) [55]. The absence of bands at wavelengths higher than 3200 cm^{-1} (characteristic of the OH stretching vibration mode of alcohols, monomeric phenols and carboxylic acids or water in samples) confirmed the lipophilicity of the extract. FTIR spectra for $scCO_2$-processed samples (but non-loaded with jucá extract, not presented) were superimposable to those of the pristine dressings (lighter lines) proving that, and as expected, the SSI process did not chemically alter employed dressings. The higher intensity of the bands between 900 and 1100 cm^{-1}, corresponding to —C—O—C— stretching vibrations, for CBC and Hyalofill® were due to their higher content in glycoside groups when compared to Promogran®. In the latter dressing, the carboxylic and amino groups of the collagen fraction are the responsible for the relatively higher intensity bands between 1500 and 1700 cm^{-1}. In the 3200 and 3500 cm^{-1} region, associated with the presence of —OH and —NH amine and amide stretching vibration modes, CBC and Hyalofill® present a similar large and intense band resulting from the absorption of the —OH groups from the glycosidic structures, while Promogran® presents a sharper band probably resulting from hydrogen bonding interactions between the —OH groups from cellulose and the carboxylic groups from collagen.

The presence of the loaded extract in the dressings was confirmed by the bands corresponding to CH_2 and CH_3 stretching (2850–2920 cm^{-1}) and by a small band corresponding to C═O stretching for FFA (around 1700 cm^{-1}). Moreover, no important deviations in the characteristic dressing's bands were

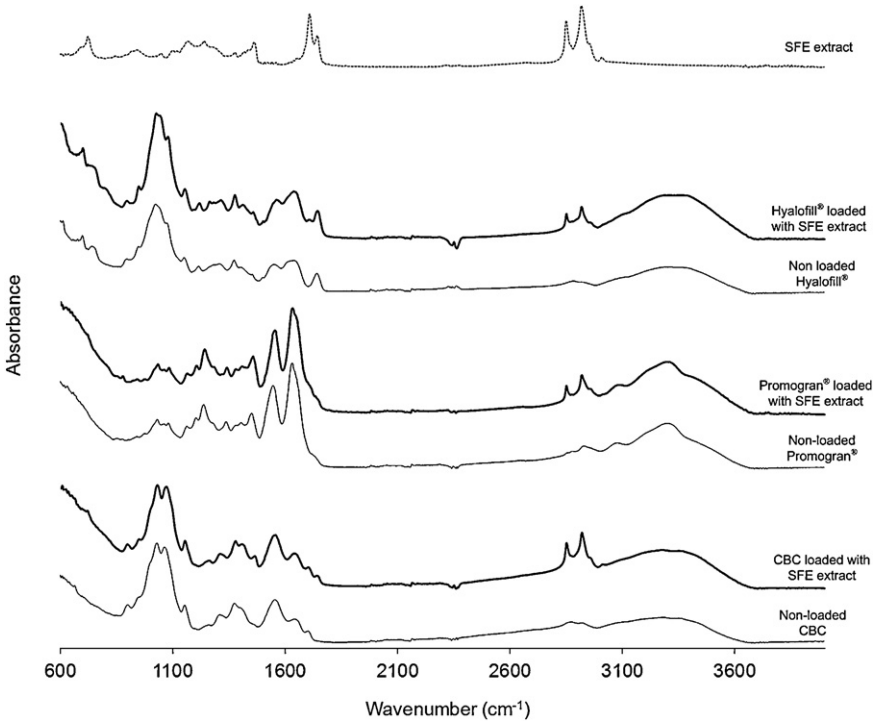

Fig. 4. FTIR-ATR spectra of the SSI-extract loaded (darker line) and of non-loaded dressings (lighter line): CBC (bottom), Promogran® (middle) and Hyalofill® (top). The dashed line on the top represents the spectra of the SFE jucá extract.

observed, which suggests weak interactions between polymers and extract.

3.2. Dressings physical characterization

Highly hydrophilic dressings may be advantageous in case of highly exudation wounds and may also enhance cell migration-proliferation and extracellular matrix deposition during the wound healing stages. However, bacterial growth may occur with excessive moisture and therefore the use of medium hydrophobic dressings enables the potential binding of the microorganisms (which can be later removed with the dressing) [56]. The equilibrium water vapour sorption (WVS_e) of Promogran® (~57%) was almost twice that attained for CBC (~29%) and Hyalofill® (~36%) (Table 1). These values were calculated from the water sorption kinetic curves (not presented), which showed a rapid initial moisture sorption followed by a slower adsorption at later stages (due to the filling of the foams' free volumes with water vapor). The CBC WVS value obtained in this work is lower than the one previously reported for similarly prepared CBC foams, however, having greater porosity (~92–93%) [7]. The amount of absorbed moisture will always depend on polymeric matrices porosity and hydrophilicity. Promogran® samples presented a high porosity (83 ± 8%), which was similar to the one obtained for Hyalofill® samples (72 ± 6%, and considering the standard deviation) and was 2-fold that obtained for CBC samples (40 ± 2%). Moreover, the water contact angle measured for CBC was equal to 99.3 ± 0.7° while that of Promogran® was equal to 90.8 ± 0.5°. The fibrous structure of Hyalofill® samples made it difficult to correctly measure the water contact angle. Thus, if compared to Promogran® and to Hyalofill® samples, CBC can be considered as having a denser structure and a lower hydrophilicity.

The rate at which the dressings absorb moisture (k_2) was inversely proportional to their water vapor absorption capacity and porosity (Table 1) which means that CBC gets saturated faster than the commercial dressings. Promogran® and Hyalofill® presented a more sustained WVS behavior (especially Promogran® with a k_2 value that is half the value measured for Hyalofill®) which is probably due to the slower diffusivity of water molecules into their structures.

An efficient dressing should maintain an optimal moisture environment in order to enhance the wound healing process [3]. Moreover, it should also balance wound occlusion, inhibit scab formation, facilitate cellular migration, accelerate re-epithelialization, diminish inflammatory response and enhance collagen biosynthesis, without diminishing its water vapor permeability (in order to avoid accumulation of large volumes of exudates which may result in tissue maceration and in the occurrence of wound infection) [57,58]. Intact human skin transpires water vapor at a rate that ranges between 240 and 1920 g m^{-2} day^{-1}. On the other hand, the WVTR of uncovered wounds can be in the order of 4800 g m^{-2} day^{-1}, and that of freshly excised wounds of approximately 10 times that of intact skin [59]. Daily WVTR (evaluated at 24 h and under static conditions) of CBC, Promogran® and Hyalofill® dressings resulted to be very similar (Table 1), and the slight lower value observed for Promogran® may be attributed to its greater thickness which in turn leads to a lower permeability. All tested dressings presented WVTR similar to those of intact skin and also to those already reported for other hydrocolloid commercial dressings such as Duoderm® (gelatin, sodium carboxymethylcellulose and pectin composite material), Biofilm® (polyester fabric sheet coated with a polyisobutylene adhesive containing hydrophilic particles of gelatin, pectin and carboxymethylcellulose) and Biobrane II® (silicone film with a nylon fabric partially imbedded in the film and to which collagen was chemically bound) [58,60]. The measured WVTR values for Hyalofill® were lower than those previously reported for two benzyl hyaluronate membranes (2157 g m^{-2} day^{-1} for Hyaloskin® and 2327 g m^{-2} day^{-1} for HYAFF®). Besides the differences in these materials composition (different composition in hyaluronic acid carboxyl groups substituted by benzyl ester groups), the different dressing thicknesses may also affect the WVTR values and thus justify these discrepancies. Therefore and to account for the distinct materials thicknesses and experimental conditions, comparisons must be also made in terms of the water vapor permeability which ranked in the following order: CBC > Hyalofill® > Promogran® (Table 1). This permeability trend is the opposite of the values obtained for the WVS equilibrium results which means that dressings which absorb lower amounts of water (lower affinity) are the ones that present the higher water vapor permeability. Under high-humidity conditions, water also act as a plasticizer that favors polymeric chain relaxation and allows

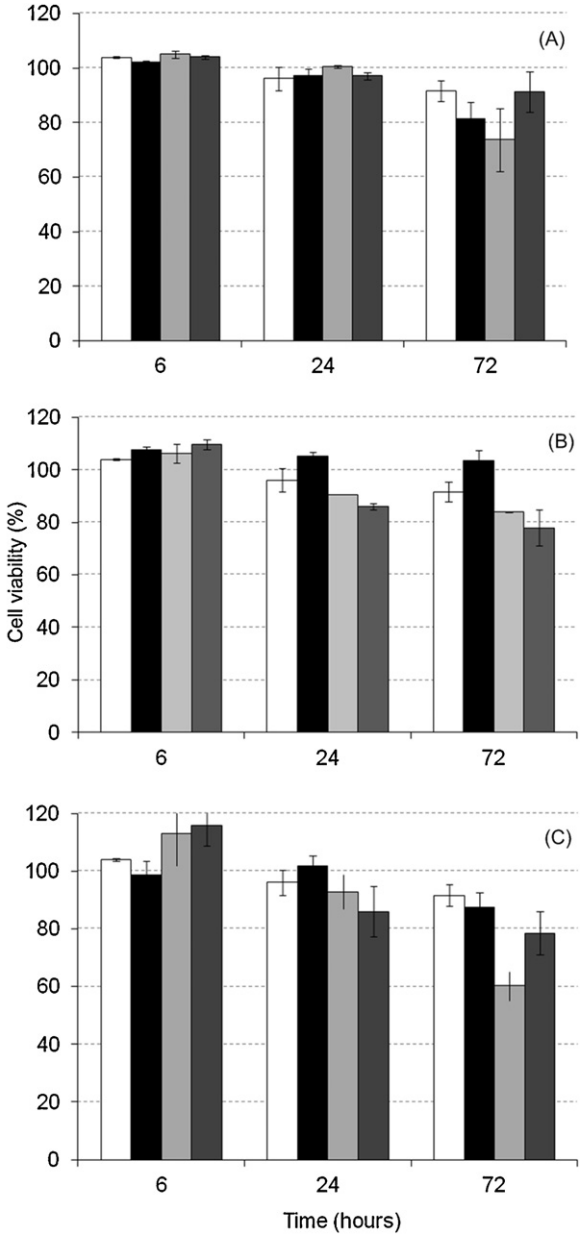

Fig. 5. Cell viability of LPS-stimulated macrophages after exposure to CBC (A), Promogran® (B) and Hyalofill® (C). Control with LPS only (□); cells in contact with non-loaded dressings (■); jucá extract-loaded dressings (depressurized at 3 MPa min^{-1}) (■); jucá extract-loaded dressings (depressurized at 10 MPa min^{-1}) (■).

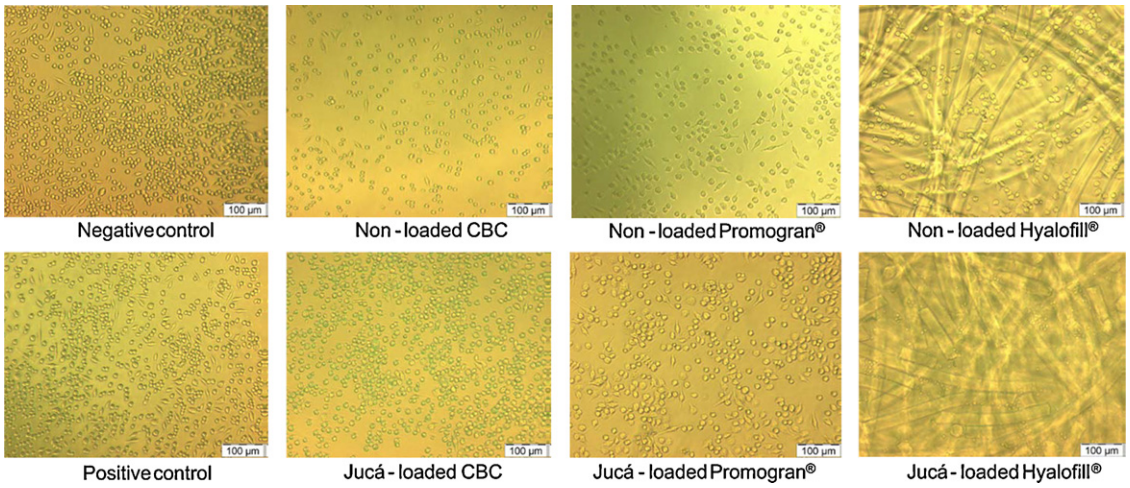

Fig. 6. Micrographs (optical microscopy) (10×) of the RAW 264.7 cell macrophages after exposure for 24 h to non-loaded and to jucá extract-loaded dressings, compared to negative control (cells in culture medium without sample or LPS) and positive control (cells in culture medium with 1 μg/ml LPS).

an increase in the penetrant water flow, thus providing flexibility and swellability to the foams (which will result in an increase in the WVP) [61,62]. These effects seem to be more pronounced for CBC and for Hyalofill® dressings which may be an indication that, after the saturation, their molecular structures become rearranged in order to facilitate water permeation. On the contrary, the higher hydrophilicity of Promogran® (higher water affinity) results in lower water vapour permeability since water molecules interact stronger with the collagen/cellulose based material.

Finally, the adequate dressing permeability to oxygen and to carbon dioxide is also a crucial factor for wound healing enhancing [63]. It is known that the rapid restoration of microcirculation occurs in an anaerobic environment but high levels of oxygen are necessary for the growth of fibroblasts and for the collagen formation [64,65]. Promogran® presented a higher permeability to oxygen (~830 barrer) than CBC and Hyalofill® (~160 barrer). These results are certainly related with their corresponding dressing hydrophilicity since an increase in the oxygen permeability is noticed as the water content increases, thus leading to the loss of barrier properties in the hydrated state. The higher the hydration state of the material the higher the water plasticization effect (that will lead to polymer chain relaxation, to free volume increase and to improved gaseous permeation). This effect is much more pronounced for relative humidities higher than 80% (as in this work) [66].

3.3. Cytocompatibility and anti-inflammatory activity

The anti-inflammatory activity of jucá extract obtained using SFE is related to its six major compounds. Terpenoids (lupenone and sitosterol) extracted from different raw materials have been shown to present anti-inflammatory activity [67–70]. However, the activity of fatty acids as anti-inflammatory agents and wound healing promoters is not so clear [71]. In the case of injury, inflammatory cells (mainly macrophages and leukocytes) release chemical mediators like cytokines (interleukins and tumoral necrosis factors) and eicosanoids (prostaglandins and leukotrienes), among others, that regulate the intensity and duration of the inflammatory phase. It has been reported that the presence of a certain level of pro-inflammatory cytokines is essential for normal wound healing, because it initiates and regulates the cascade of molecular and cellular processes during the inflammatory stage [72,73]. However, unresolved or chronic inflammation can delay wound healing and, therefore, a delicate ratio between pro-inflammatory and anti-inflammatory mediators should be attained. Polyunsaturated fatty acids, also known as PUFAs, may interfere in the evolution of the inflammatory process and alter pro-inflammatory mediators production since they (and/or their metabolites) may be converted by COX-2 and/or LOX-5 into pro-inflammatory mediators. In addition, they may also be converted by LOX-15 into compounds that are not playing a role in the arachidonic acid pathway and that work as anti-inflammatory agents [74]. In this way, PUFAs and their metabolites attenuate the generation of pro-inflammatory mediators by blocking the action of LOX-5. In turn, they inhibit the production of inflammatory eicosanoids, adhesion molecules and cytokines, interleukins (IL-1 and IL-6) and tumor necrosis factor (TNF), which can cause bone, muscle and tissue mass loss during prolonged inflammation [75,76,73]. Linoleic acid (LA), the major component in jucá extract (~42%), is the most abundant essential fatty acid (PUFA) in human skin, being a precursor of both arachidonic acid (AA) and gamma-linoleic acid (GLA) [77]. However, this conversion is quite slow and it is further restricted under inflammatory conditions. Thus and after injury, a reinforced dose of LA, provided by the application of the LA rich extract, may help to rebalance the ratio of anti-inflammatory/pro-inflammatory mediators. In fact, LA has been successfully used for the prevention and treatment of pressure ulcers [78,79]. Although PUFAs supplementation alone may be not enough to treat inflammation, their use may reduce the requirements of NSAIDs and/or improve their efficacy [79] (Fig. 5).

Taking into account this previous information, extract-loaded dressings were tested regarding cytocompatibility and the capacity to regulate inflammatory mediators. The LDH assay measured to what extent this cytosolic enzyme was released to the culture medium due to increased membrane permeability (which is indicative of cell damage or lysis) [60,80]. All non-loaded dressings led to an excellent biocompatibility against macrophages, and only minor decrease in cell viability was observed after 72 h for CBC (~80%) and Hyalofill® (~90%). This means that CBC, as its precursor quitosan [81], is highly compatible with the cells [82,83]. Jucá-loaded dressings showed lower cell viability after 72 h: roughly 80%, except for Hyalofill® depressurized at 3 MPa min^{-1} which was close to 60%. These results indicate that the extract of jucá induce some toxicity on macrophage cells when begins to be released from the dressing. Nevertheless, the visualization of the cells by microscopy after 24 h of culture in contact with non-processed and

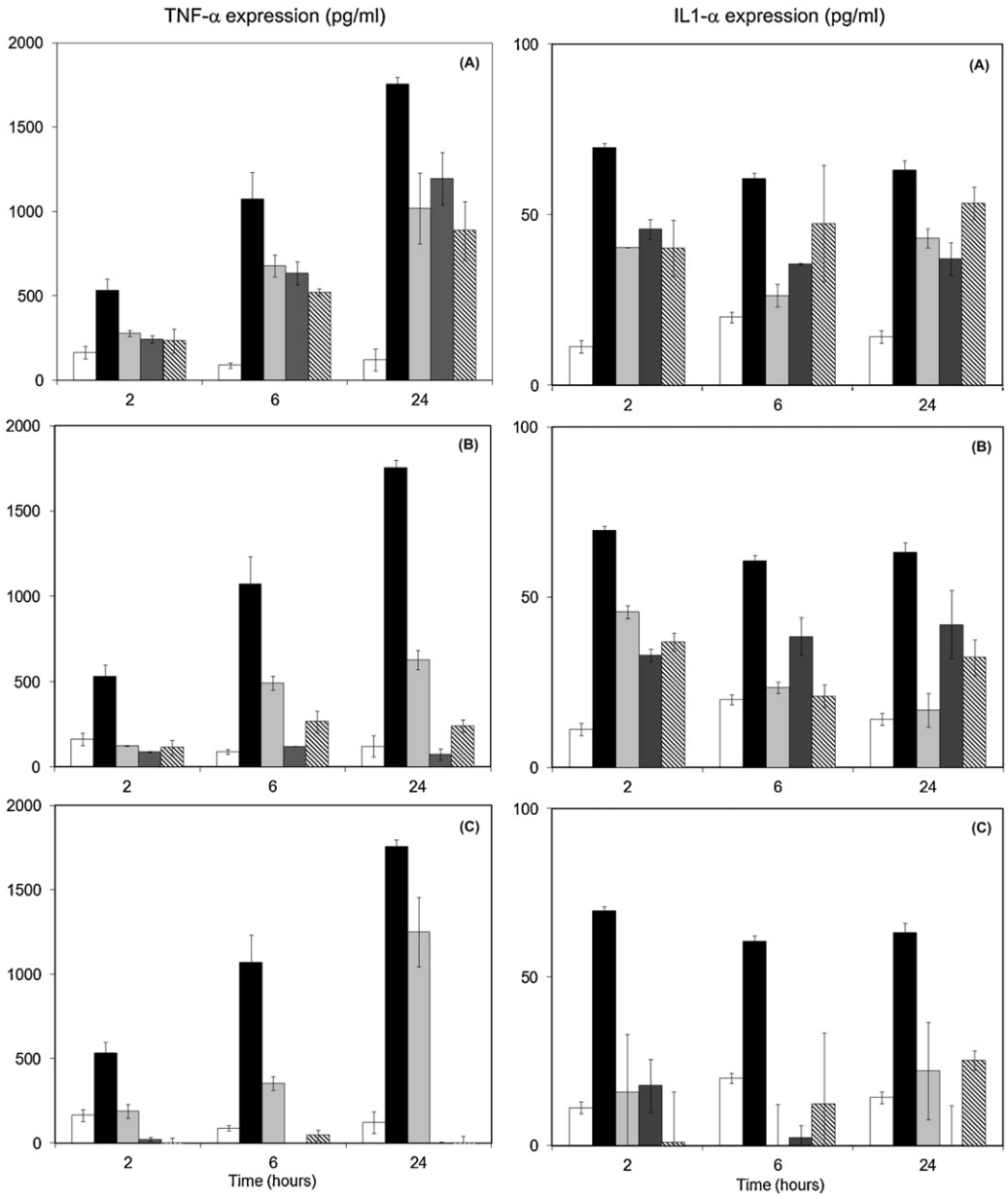

Fig. 7. TNF-α and IL-1α secretion from LPS-stimulated RAW 264.7 cells cultured in the presence of loaded and non-loaded CBC (A), Promogran® (B) and Hyalofill® (C) dressings, after incubation for 2, 6 and 24 h at 37 °C with 5% CO_2. The TNF-α and IL1-α released into incubation media were quantified by ELISA. Negative control (cells only) (□), positive control (cells with LPS) (■), non-loaded dressings (■), jucá extract-loaded dressings (depressurized at 3 MPa min^{-1}) (■), jucá extract-loaded dressings (depressurized at 10 MPa min^{-1}) (▨).

extract-loaded materials did not show significant morphological changes when compared with a negative control, confirming the minor cytotoxic effect on macrophages (Fig. 6). Additionally, adhesion of fibroblasts to the dressings was tested using confocal microscopy (colour images in Fig. S2 supporting information). The cells directly seeded on non-processed dressings exhibited adhesion ability and thus proliferated; except in the case of Hyalofill® that showed minor cell adhesion. Extract-loaded CBC and Promogran® dressings showed a dramatic decrease in fibroblast cell viability. These results suggest that the hydrophobic components of the extract avoid cells adhesion, which could be related to an increase in the inherent hydrophobicity of these dressings. Previous research has shown that materials with water contact angles higher than 90° (which is the case of the dressings studied in this work) make cell adhesion difficult [84,85]. This behavior is advantageous for clinical applications since new re-epithelialization cells are less likely to adhere to the dressing and be removed with the dressing when it is changed, minimizing trauma to the wound and allowing those cells to migrate and proliferate effectively [86].

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.supflu.2012.12.007.

To gain an insight into the anti-inflammatory features, both non-processed and extract-loaded dressings were tested in macrophage cultures to which LPS had been added. LPS is known to be a potent inductor of inflammation that induces the production of pro-inflammatory cytokines in macrophages, fibroblasts and monocytes [60,87]. Raw 264.7 cell lines exposed to LPS have demonstrated to be an adequate *in vitro* model for the evaluation of novel anti-inflammatory agents [88].

LPS-stimulated macrophages (positive control) showed a remarkable increase in TNF-α and IL-1α expression compared to

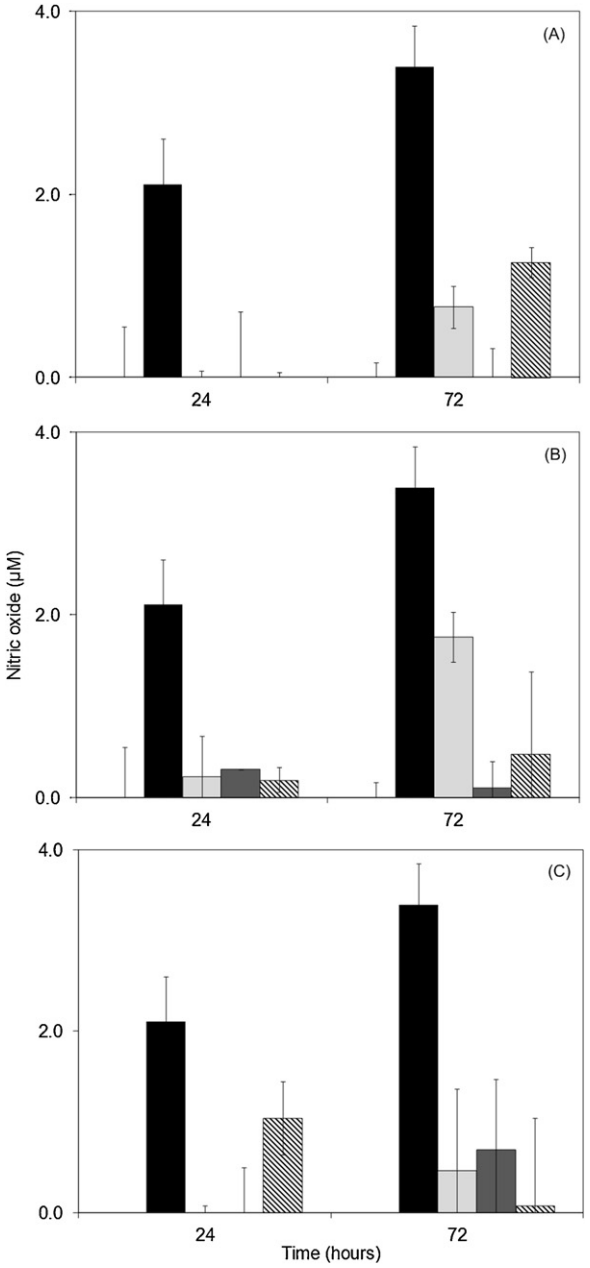

Fig. 8. Amounts of nitric oxide (NO) released from LPS-stimulated RAW 264.7 cells after exposure to CBC (A), Promogran® (B) and Hyalofill® (C) dressings. Negative control (cells only) (□), positive control (cells with LPS) (■), non-loaded dressings (▒), jucá extract-loaded dressings (depressurized at 3 MPa min^{-1}) (▓), jucá extract-loaded dressings (depressurized at 10 MPa min^{-1}) (▨).

control macrophages (negative control). Cytokines secretion was found to be time dependent; namely, TNF-α level progressively increased as a function of time from 2 to 24 h, while IL-1α level showed a brusque increase already at 2 h (Fig. 7). By contrast, when the LPS-stimulated macrophages were cultured in the presence of the dressings, lower secretion of cytokines was observed even for dressings that were not loaded. The effect was less marked for CBC dressings, in the presence of which the concentration of TNF-α was ~670 pg/ml at 6 h. This value is in agreement with those previously reported by Ma et al. [89], but significantly higher than those reported by Yoon et al. [49] when the effect of chitosan oligosaccharides on the cytokine expression levels by LPS-stimulated macrophages was evaluated. This discrepancy may be related with differences in the molecular weight and the degree of deacetylation of the tested oligosaccharides, which in turn affect the solubility in water and the easiness at which the polymer is hydrolyzed by lysozyme and *N*-acetyl-*B*-D-glucosaminidase. The slower the biodegradation, the lower is the amount of pro-inflammatory low molecular weight fragments [81]. The relatively higher values observed for CBC when compared to commercial dressings may be related to the effect of the material bioconversion products, but also to a certain pro-inflammatory activity of CBC [90]. Bianco et al. [91] reported that chitosan could mediate macrophage activation through the release of arachidonic acid (AA) and even found higher levels of cytokines released from LPS-stimulated cells in the presence of chitosan. Moreover, it was found that chitosan amino groups are recognized by the immune system and that macrophages are activated to various extents by chitin derivatives. The results here reported seem to indicate that CBC may regulate inflammation in a similar way as chitosan [92,93].

The down-regulating capacity of the jucá extract is clearly inferred from the significant reduction of the amount of TNF-α released from macrophages exposed to extract-loaded Promogran® and Hyalofill® dressings (Fig. 7). This effect was even more pronounced for Hyalofill®, probably because its fibber structure permits a faster release of the SSI loaded extract to the culture medium. It should be noticed that Hyalofill® showed the lowest extract loading yield (~0.25–0.28 mg/mg) which confirms the activity of the extract even when applied at low concentrations. Oppositely, jucá-loaded CBC dressings maintained a level of TNF-α secretion similar to that of the non-loaded CBC, probably because the pro-inflammatory activity of the CBC matrix and the slower release of the extract components to the medium, due to above commented stronger CBC-extract affinity. In fact, the CBC dressing released only 10% of the loaded extract in PBS after 24 h, while commercial dressings released almost twice this value (data not shown). Considering the chemical structure of CBC and of the fatty acids present in the extract, and the possible electrostatic interactions among them, it could be hypothesized that this complex might be recognized by the cells as a LPS derivative leading to the release of chemical mediators. However, this hypothesis was discarded since macrophages without LPS exposed to the extract-loaded CBC dressings did not induce the production of TNF-α nor IL-1α.

Finally, all the non-loaded and loaded materials, including CBC based dressings, significantly decreased the nitric oxide production after 24 h of cells exposure (Fig. 8). The slight increase in the NO produced after 72 h may be due to the stress to which cells are subjected after large periods of exposition (Fig. 6).

4. Conclusions

Bioactive wound dressings with anti-inflammatory capacity can be prepared applying supercritical fluid technology as a viable alternative to conventional methods for the extraction of natural compounds from jucá fruits and to their subsequent loading into biopolymeric materials. SFE allowed obtaining a jucá extract rich in unsaturated and saturated fatty acids, while SSI enabled a homogeneous deposition and dispersion of this extract into distinct dressings. Loading yields mainly depended on specific extract-polymer affinity and, as a consequence, the *N*-carboxybutyl chitosan (CBC) dressing achieved the highest extract loading levels. Cytocompatibility tests showed that the loaded dressings are non-toxic against macrophages but they inhibit the adhesion of fibroblasts. This may be advantageous in the case of wound dressing applications since it avoids new re-epithelialization cells to adhere to the dressing and to be removed with it without compromising the healing process. Jucá extract proved to present

anti-inflammatory capacity since it down regulated the expression of TNF-α and IL-1α mediators as well as the production of nitric oxide from macrophages and after exposure of LPS-stimulated cells to the jucá loaded dressings. This effect was more pronounced for Promogran® and Hyalofill®, probably due to the lower affinity of the extract to these materials, which lead to faster release rates, and also to the fact that CBC can have itself a pro-inflammatory activity. The present work shows that, by the combination of natural biocompatible polymers and of jucá fruit extracts, it is possible to prepare medicated wound dressings with various fluid-handling capacities and extract release profiles and within the desired ranges for skin applications. Moreover, a careful selection of the raw material and of the SFE process conditions makes it possible to obtain selective extracts, with different bioactivities, that would permit to cover a wide range of wound healing applications.

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References

[1] S. Rajendran, Advanced textiles for wound care Woodhead Publishing in Textiles, No. 85, Woodhead Publishing/CRC Press, Oxford/Boca Raton, FL, 2009.
[2] D.M. Wiseman, D.T. Rovee, O.M. Alvarez, Wound dressings: design and use, in: I.K. Cohen, R.F. Diegelmann, W.J. Lindblad (Eds.), Wound Healing: Biochemical and Clinical Aspects, W.B Saunders Co., Philadelphia, 1992, pp. 562–580.
[3] L.G. Ovington, Wound care products: how to choose, Advances in Skin Wound Care 14 (2001) 259–264.
[4] B.S. Liu, C.H. Yao, S.S. Fang, Evaluation of a non-woven fabric coated with a chitosan Bi-layer composite for wound dressing, Macromolecular Bioscience 8 (2008) 432–440.
[5] R. Jayakumar, M. Prabaharan, K.P.T. Sudheesh, S.V. Nair, H. Tamura, Biomaterials based on chitin and chitosan in wound dressing applications, Biotechnology Advances 29 (2011) 322–337.
[6] R.R. Muzzarelli, O. Tarsi, E. Filippini, G. Giovanetti, P.E. Biagini, Antimicrobial properties of N-carboxybutyl chitosan, Antimicrobial Agents and Chemotherapy 34 (1990) 2019–2023.
[7] A.M.A. Dias, M.E.M. Braga, I.J. Seabra, P. Ferreira, M.H. Gil, H.C. de Sousa, Development of natural-based wound dressings impregnated with bioactive compounds and using supercritical carbon dioxide, International Journal of Pharmaceutics 408 (2011) 9–19.
[8] Z. Ruszczak, Effect of collagen matrices on dermal wound healing, Advanced Drug Delivery Reviews 55 (2003) 1595–1611.
[9] P.L. Dalla, E. Faglia, Treatment of diabetic foot ulcer: an overview strategies for clinical approach, Current Diabetes Reviews 2 (2006) 431–447.
[10] B. Cullen, P.W. Watt, C. Lundqvist, D. Silcock, R.J. Schmidt, D. Bogan, N.D. Light, The role of oxidised regenerated cellulose/collagen in chronic wound repair and its potential mechanism of action, The International Journal of Biochemistry and Cell Biology 34 (2002) 1544–1556.
[11] G.S. Schultz, R.G. Sibbald, V. Falanga, E.A. Ayello, C. Dowsett, K. Harding, Wound bed preparation: a systematic approach to wound management, Wound Repair and Regeneration 11 (2003) S1–S28.
[12] J.C. Karr, A.R. Taddei, S.G. Picchietti, A.M. Fausto, F.A. Giorgi, Morphological and biochemical analysis comparative study of the collagen products biopad, promogram, puracol, and colactive, Advances in Skin and Wound Care 24 (2011) 208–216.
[13] A. Chen, Functions of hyaluronan in wound repair, Wound Repair and Regeneration 7 (1999) 79–89.
[14] R. Moseley, M. Walker, R.J. Waddington, W.Y.J. Chen, Comparison of the antioxidant properties of wound dressing materials-carboxymethylcellulose, hyaluronan benzyl etser and hyaluronan, towards polymorphonuclear leukocyte-derived reactive oxygen species, Biomaterials 24 (2003) 1549–1557.
[15] D. Jiang, J. Liang, P.W. Noble, Hyaluronan in tissue injury and repair, Annual Review of Cell and Developmental Biology 23 (2007) 435–461.
[16] D. Jiang, J. Liang, P.W. Noble, Hyaluronan as an immune regulator in human diseases, Physiological Reviews 91 (2011) 221–264.
[17] J.R. Vazquez, B. Short, A.H. Findlow, B.P. Nixon, A.J. Boulton, D.G. Armstrong, Outcomes of hyaluronan therapy in diabetic foot wounds, Diabetes Research and Clinical Practice 59 (2003) 123–127.
[18] M.E. Edmonds, A.V. Foster, Diabetic foot ulcers, British Medical Journal 332 (2006) 407–410.
[19] A. Gupta, N.K. Upadhyay, R.C. Sawhney, R. Kumar, A poly-herbal formulation accelerates normal and impaired diabetic wound healing, Wound Repair and Regeneration 16 (2008) 784–790.
[20] C.O. Esimone, C.S. Nworu, C.L. Jackson, Cutaneous wound healing activity of a herbal ointment containing the leaf extract of *Jatropha curcas* L. (Euphorbiaceae), International Journal of Applied Research in Natural Products 1 (2009) 1–4.
[21] S. Gurung, N. Skalko-Basnet, Wound healing properties of Carica papaya latex: in vivo evaluation in mice burn model, J. Ethnopharmacology 121 (2009) 338–341.
[22] R. Karodi, M. Jadhav, R. Rub, A. Bafna, Evaluation of the wound healing activity of a crude extract of *Rubia cordifolia* L. (Indian madder) in mice, International Journal of Applied Research in Natural Products 2 (2009) 12–18.
[23] C. Shenoy, M.B. Patil, R. Kumar, S. Patil, Preliminary phytochemical investigation and wound healing activity of *Allium cepa* Linn (Liliaceae), International Journal of Pharmacy and Pharmaceutical Sciences 2 (2009) 167–175.
[24] I.P. Suntar, U. Koca, E.K. Akkol, D. Yilmazer, M. Alper, Assessment of wound healing activity of the aqueous extracts of *Colutea cilicica* Boiss. & Bal. fruits and leaves, Evidence-Based Complementary and Alternative Medicine (2011), Article id 758191, 7 pages.
[25] G.S. Jensen, X. Wu, K.M. Patterson, J. Barnes, S.G. Carter, L. Scherwitz, R. Beaman, J.R. Endres, A.G. Schauss, In vitro and in vivo antioxidant and anti-inflammatory capacities of an antioxidant-tich fruit and berry juice blend. Results of a pilot and randomized, double-blinded, placebo-controlled, crossover study, J. Agricultural and Food Chemistry 56 (2008) 8326–8330.
[26] M.E.M. Braga, M.S. Ribeiro, I.J. Seabra, A.M.A. Dias, P. Ferreira, J. Gaspar, M.H. Gil, F. Ambrósio, R.C. Júnior, H.C. de Sousa, Anti-inflammatory activity of Jucá (*Caesalpinia ferrea*) extracts obtained by SFE and by ESE, in: Proceedings of the II Iberoamerican Conference on Supercritical Fluids, Prosciba 2010, Brasil, 2010, p. 89.
[27] L.C. Silva, C.A. da Silva Jr., R.M. de Souza, A.J. Macedo, M.V. da Silva, M.T. dos Santos Correia, Comparative analysis of the antioxidant and DNA protection capacities of *Anadenanthera colubrina*, *Libidibia ferrea* and *Pityrocarpa moniliformis* fruits, Food and Chemical Toxicology 49 (2011) 2222–2228.
[28] S. Pereda, S.B. Bottini, E.A. Brignale, Fundamentals of supercritical fluid technology, in: J.L. Martines (Ed.), Supercritical Fluid Extraction of Nutraceuticals and Bioactive Compounds, CRC Press, New York, 2008, pp. 1–24.
[29] M. Mukhopadhyay, Natural Extracts using Supercritical Carbon Dioxide, CRC Press, Boca Raton, FL, 2000.
[30] B. Díaz-Reinoso, A. Moure, H. Domínguez, J.C. Parajó, Supercritical CO_2 extraction and purification of compounds with antioxidant activity, J. Agriculture and Food Chemistry 54 (2006) 2441–2469.
[31] E. Reverchon, I. De Marco, Supercritical fluid extraction and fractionation of natural matter, J. Supercritical Fluids 38 (2006) 146–166.
[32] M.E.M. Braga, M.T.V. Pato, H.S.R.C. Silva, E.I. Ferreira, M.H. Gil, C.M.M. Duarte, H.C. de Sousa, Supercritical solvent impregnation of ophthalmic drugs on chitosan derivatives, J. Supercritical Fluids 44 (2008) 245–257.
[33] M.V. Natu, M.H. Gil, H.C. de Sousa, Supercritical solvent impregnation of poly(E-caprolactone)/poly(oxyethylene-b-oxypropylene-b-oxyethylene) and poly(E-caprolactone)/poly(ethylene-vinyl acetate) blends for controlled release applications, J. Supercritical Fluids 47 (2008) 93–102.
[34] V.P. Costa, M.E.M. Braga, J.P. Guerra, A.R.C. Duarte, E.O.B. Leite, C.M.M. Duarte, M.H. Gil, H.C. de Sousa, Development of therapeutic contact lenses using a supercritical solvent impregnation method, J. Supercritical Fluids 52 (2010) 306–316.
[35] V.P. Costa, M.E.M. Braga, C.M.M. Duarte, C. Alvarez-Lorenzo, A. Concheiro, M.H. Gil, H.C. de Sousa, Anti-glaucoma drug-loaded contact lenses prepared using supercritical solvent impregnation, J. Supercritical Fluids 53 (2010) 165–173.
[36] S.M. Howdle, M.S. Watson, V.K. Whitaker, M.C. Davies, F.S. Mandel, J.D. Wang, K.M. Shakesheff, Supercritical fluid mixing: preparation of thermally sensitive polymer composites containing bioactive materials, J. Chemical Society, Chemical Communications (2001) 109–110.
[37] P.J. Ginty, M.J. Whitaker, K.M. Shakesheff, S.M. Howdle, Drug delivery goes supercritical, Materials Today 8 (2005) 42–48.
[38] S.G. Kazarian, G.G. Martirosyan, Spectroscopy of polymer/drug formulations processed with supercritical fluids: in situ ATR-IR and Raman study of impregnation of ibuprofen into PVP, International Journal of Pharmaceutics 232 (2002) 81–90.
[39] F. Kikic, F. Vecchione, Supercritical impregnation of polymers, Current Opinion in Solid State and Materials Science 7 (2003) 399–405.
[40] E.S. Nakamura, F. Kurosaki, M. Arisawa, T. Mukainaka, M. Okuda, H. Tokuda, H. Nishino, F. Pastore, Cancer chemopreventive effects of constituents of *Caesalpinia ferrea* and related compounds, Cancer Letters 177 (2002) 119–124.
[41] F.K. Nakamura, M. Arisawa, T. Mukainaka, J. Takayasu, M. Okuda, H. Tokuda, H. Nishino, F. Pastore, Cancer chemopreventive effects of a Brazilian folk medicine, Juca, on in vivo two-stage skin carcinogenesis, J. Ethnopharmacology 81 (2002) 135–137.
[42] F.C. Sampaio, M.S.V. Pereira, C.S. Dias, V.C.O. Costa, N.C.O. Conde, M.A.R. Buzalaf, In vitro antimicrobial activity of *Caesalpinia ferrea* Martius fruits against oral pathogens, J. Ethnopharmacolpgy 124 (2009) 289–294.

[43] L.P. Pereira, R.O. da Silva, P.H.S.F. Bringel, Polysaccharide fractions of *Caesalpinia ferrea* pods: potential anti-inflammatory usage, J. Ethnopharmacology 139 (2012) 642–648.
[44] S.M.A. Lima, L.C.C. Araujo, M.M. Sitonio, Anti-inflammatory and analgesic potential of *Caesalpinia ferrea*, Revista Brasileira de Farmacognosia – Brazilian Journal of Pharmacognosy 22 (2012) 169–175.
[45] K.S.C.R. Santos, H.S.R.C. Silva, E.I. Ferreira, R.E. Bruns, 3^2 Factorial design and response surface analysis optimization of N-carboxybutyl chitosan synthesis, Carbohydrate Polymers 59 (2005) 37–42.
[46] A.T. Serra, I.J. Seabra, M.E.M. Braga, M.R. Bronze, H.C. de Sousa, C.M.M. Duarte, Processing cherries (*Prunus avium*) using supercritical fluid technology. Part 1. Recovery of extract fractions rich in bioactive compounds, J. Supercritical Fluids 55 (2010) 184–191.
[47] R.P. Adams, Identification of Essential Oil Components by Gas Chromatography/Quadruple Mass Spectroscopy, Allured Publishing Corporation, Illinois-EUA, 2001, p. 456.
[48] Y.S. Ho, Review of second-order models for adsorption systems, J. Hazardous Materials 136 (2006) 681–689.
[49] H.J. Yoon, M.E. Moon, H.S. Park, S.Y. Im, Y.H. Kim, Chitosan oligosaccharide (COS) inhibits LPS-induced inflammatory effects in RAW 264.7 macrophage cells, Biochemical and Biophysical Research Communications 358 (2007) 954–959.
[50] O. Guçlu-Ustundag, F. Temelli, Correlating the solubility behavior of fatty acids, mono-, di-, and triglycerides, and fatty acid esters in supercritical carbon dioxide, Industrial Engineering Chemical Research 39 (2000) 4756–4766.
[51] C. Garlapati, G. Madras, Solubilities of palmitic and stearic fatty acids in supercritical carbon dioxide, J. Chemical Thermodynamics 42 (2010) 193–197.
[52] F. Temelli, Perspectives on supercritical fluid processing of fats and oils, J. Supercritical Fluids 47 (2009) 583–590.
[53] C.E. Schwarz, J.H. Knoetze, Phase equilibrium measurements of long chain acids in supercritical carbon dioxide, J. Supercritical Fluids 66 (2012) 36–48.
[54] V. Seidel, Initial and bulk extraction, in: S.D. Sarker, Z. Latif, A.I. Gray (Eds.), Methods in Biotechnology, Natural Products Isolation, 2nd ed., Humana Press, Totowa, NJ, 2006, p. 42.
[55] R.C. Sun, J. Tomkinson, Comparative study of organic solvent-soluble and water-soluble lipophilic extractives from wheat straw 2: spectroscopic and thermal analysis, J. Wood Science 48 (2002) 222–226.
[56] A. Ljungh, N. Yanagisawa, T. Wadstrom, Using the principle of hydrophobic interaction to bind and remove wound bacteria, J. Wound Care 15 (2006) 175–180.
[57] S. Thomas, Wound Management and Dressings, The Pharmaceutical Press, London, 1990, pp. 25–34.
[58] P. Wu, A.C. Fisher, P.P. Foo, D. Queen, J.D.S. Gaylor, In vitro assessment of water vapor transmission of synthetic wound dressings, Biomaterials 16 (1995) 171–175.
[59] A. Nangia, C.T. Hung, Design of a new hydrocolloid dressing, Burns 15 (1989) 385–388.
[60] L. Ruiz-Cardona, Y.D. Sanzgiri, L.M. Benedetti, V.J. Stella, E.M. Topp, Application of benzyl hyaluronate membranes as potential wound dressings: evaluation of water vapour and gas permeabilities, Biomaterials 17 (1996) 1639–1643.
[61] N.A. Peppas, L. Branon-Peppas, Water Diffusion, Sorption in amorphous macromolecular systems and foods, J. Food Engineering 22 (1994) 189–210.
[62] M. Pereda, M.I. Aranguren, N.E. Marcovich, Water vapor absorption and permeability of films based on chitosan and sodium caseinate, J. Applied Polymer Science 111 (2009) 2777–2784.
[63] L.M. Sirvio, D.M. Grussing, The effect of gas permeability of film dressings on wound environment and healing, J. Investigative Dermatology 93 (1989) 528–531.
[64] A.S. Pandit, D.S. Faldman, Effect of oxygen treatment and dressing oxygen permeability on wound healing, Wound Repair and Regeneration 2 (1994) 130–137.
[65] J.C. Phillips, Understanding hyperbaric oxygen therapy and its use in the treatment of compromised skin grafts and flaps, Plastic Surgical Nursing 25 (2005) 72–80.
[66] S. Despond, E. Espuche, A. Domard, Water sorption and permeation in chitosan films: relation between gas permeability and relative humidity, J. Polymer Science Part B: Polymer Physics 39 (2001) 3114–3127.
[67] R.D. Logia, A. Tubaro, S. Sosa, H. Becker, O. Isaac, The role of triterpenoids in the topical anti-inflammatory activity of calendula officinalis flowers, Planta Medica 60 (1994) 517–524.
[68] E.M. Giner-Larza, S. Manez, M.C. Recio, R.M. Giner, J.M. Prieto, M. Cerda-Nicolas, J.L. Ríos, Oleanonic acid, a 3-oxotriterpene from Pistacia, inhibits leukotriene synthesis and has anti-inflammatory activity, European Journal of Pharmacology 428 (2001) 137–143.
[69] M. Hamburger, S. Adler, D. Baumann, A. Forg, B. Weinreich, Preparative purification of the major anti-inflammatory triterpenoid esters from Marigold (*Calendula officinalis*), Fitoterapia 74 (2003) 328–338.
[70] S. Sosa, C.F. Morelli, A. Tubaro, P. Cairoli, G. Speranza, P. Manitto, Anti-inflammatory activity of *Maytenus senegalensis* root extracts and of maytenoic acid, Phytomedicine 14 (2007) 109–114.
[71] M. Otranto, A.P. Nascimento, A. Monte-Alto-Costa, Effects of supplementation with different edible oils on cutaneous wound healing, Wound Repair and Regeneration 18 (2010) 629–636.
[72] J.C. McDaniel, M. Belury, K. Ahijevych, W. Blakely, Omega-3 fatty acids effect on wound healing, Wound Repair and Regeneration 16 (2008) 337–345.
[73] N. Collins, C. Sulewski, Omega-3 fatty acids and wound healing, Ostomy Wound Management (2011) 10–13.
[74] W.S. Harris, D. Mozaffarian, E. Rimm, P. Kris-Etherton, L.L. Rudel, L.J. Appel, M.M. Engler, M.B. Engler, Omega-6 fatty acids and risk for cardiovascular disease: a science advisory from the American Heart Association Nutrition Subcommittee of the Council on Nutrition, Physical Activity, and Metabolism; Council on Cardiovascular Nursing; and Council on Epidemiology and Prevention, Circulation 119 (2009) 902–907.
[75] L. Ferrucci, A. Cherubini, S. Bandinelli, B. Bartali, A. Corsi, F. Lauretani, A. Martin, C. Andres-Lacueva, U. Senin, J.M. Guralnik, Relationship of plasma polyunsaturated fatty acids to circulating inflammatory markers, J. Clinical Endocrinology and Metabolism 91 (2006) 439–446.
[76] J. Ren, S.H. Chung, Anti-inflammatory effect of γ-linolenic acid and its mode of action through the inhibition of nitric oxide production and inducible nitric oxide synthase gene expression via NF-KB and mitogen-activated protein kinase pathways, J. Agriculture and Food Chemistry 55 (2007) 5073–5080.
[77] R. Kapoor, Y.S. Huang, Gamma linolenic acid: an antiinflammatory omega-6 fatty acid, Current Pharmaceutical Biotechnology 7 (2006) 1–4.
[78] V.A. Ziboh, C.C. Miller, Y. Cho, Metabolism of polyunsaturated fatty acids by skin epidermal enzymes: generation of anti-inflammatory and antiproliferative metabolites, The American Journal of Clinical Nutrition 71 (2000) 361S–366S.
[79] B. Pieper, M.H.L. Caliri, Nontraditional wound care: a review of the evidence for the use of sugar, papaya/papain, and fatty acids, J. Wound Ostomy and Continence Nursing 30 (2003) 175–183.
[80] T. Decker, M.L. Lohmann-Matthes, A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity, J. Immunological Methods 15 (1988) 61–69.
[81] G. Molinaro, J.C. Leroux, J. Damas, A. Adam, Biocompatibility of thermosensitive chitosan-based hydrogels: an in vivo experimental approach to injectable biomaterials, Biomaterials 23 (2002) 2717–2722.
[82] G. Biagini, A. Bertani, C. Zucchini, C. Rizzoli, R. Muzzarelli, A. Damadei, G. DiBenedetto, A. Belligolli, G. Riccotti, C. Zucchini, C. Rizzoli, Wound management with N-carboxybutyl chitosan, Biomaterials 12 (1991) 281–286.
[83] C. Kaiyong, L. Wenguang, L. Fang, Y. Kangde, Y. Zhiming, L. Xiuqiong, X. Huiqi, Modulation of osteoblast function using poly(D,L-lactic acid) surfaces modified with alkylation derivative of chitosan, J. Biomaterials Science, Polymer Edition 13 (2002) 53–66.
[84] E.A. Vogler, Structure and reactivity of water at biomaterial surfaces, Advances in Colloid and Interface Science 74 (1998) 69–117.
[85] W.M. Saltzman, Cell interactions with polymers, in: Tissue Engineering: Principles for the Design of Replacement Organs and Tissues, Oxford University Press, New York, 2004, pp. 348–385.
[86] C. Cochrane, M.G. Rippon, A. Rogers, R. Walmsley, D. Knottenbelt, P. Bowler, Application of an in vitro model to evaluate bioadhesion of fibroblasts and epithelial cells to two different dressings, Biomaterials 20 (1999) 1237–1244.
[87] R.R. Schumann, D. Pfeil, N. Lamping, C. Kirschning, G. Scherzinger, P. Schlag, L. Karawajew, F. Herrmann, Lipopolysacharide induces the rapid tyrosine phosphorylation of the mitogen-activated protein kinases erk-1 and p38 in cultured human vascular endothelial cells requiring the presence of soluble CD14, Blood 87 (1996) 2805–2814.
[88] D. Tweedie, W. Luo, R.G. Short, A. Brossi, H.W. Holloway, Y. Li, Q.S. Yu, N.H. Greig, A cellular model of inflammation for identifying TNF-alpha synthesis inhibitors, J. Neuroscience Methods 183 (2009) 182–187.
[89] P. Ma, H.T. Liu, P. Wei, Q.S. Xu, X.F. Bai, Y.G. Du, C. Yu, Chitosan oligosaccharides inhibit LPS-induced over-expression of IL-6 and TNF-a in RAW264.7 macrophage cells through blockade of mitogen-activated protein kinase (MAPK) and PI3K/Akt signaling pathways, Carbohydrate Polymers 84 (2011) 1391–1398.
[90] Y. Shibata, W.J. Metzger, Q.N. Myrvik, Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan: mannose receptor-mediated phagocytosis initiates IL-12 production, J. Immunology 159 (1997) 2462–2467.
[91] I.D. Bianco, J. Balsinde, D.M. Beltramo, L.F. Castagna, C.A. Landa, E.A. Dennis, Chitosan-induced phospholipase A_2 activation and arachidonic acid mobilization in $P388D_1$ macrophages, FEBS Letters 466 (2000) 292–294.
[92] J. Feng, L. Zhao, Q. Yu, Receptor-mediated stimulatory effect of oligochitosan in macrophages, Biochemical and Biophysical Research Communications 317 (2004) 414–420.
[93] T. Mori, M. Murakami, M. Okumura, T. Kadosawa, T. Uede, T. Fujinaga, Mechanism of macrophage activation by chitin derivatives, Surgery 67 (2005) 51–56.