

Pullulan film incorporated with nanocapsules improves pomegranate seed oil anti-inflammatory and antioxidant effects in the treatment of atopic dermatitis in mice

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ABSTRACT

This study aimed to prepare pullulan films containing pomegranate seeds oil (PSO) based nanocapsules, and evaluate the formulation efficacy in the treatment of atopic dermatitis (AD)-like lesions induced by 2,4-dinitrochlorobenzene (DNCB). The Eudragit RS 100® nanocapsules (PSONC) were prepared by the interfacial precipitation of preformed polymer, whereas the films were produced by the solvent casting method. Pomegranate seed oil nanoemulsions (PSONE) were prepared by the spontaneous emulsification method for comparative reasons. Both nanosystems presented adequate mean diameter (248 ± 16 nm for PSONE and 181 ± 6 nm for PSONC), polydispersity index (below 0.2), zeta potential (-25.63 ± 1.1 mV for PSONE and $+43.13 \pm 0.7$ mV for PSONC) and pH in the acid range (6.77 ± 0.27 and 5.31 ± 0.17 , PSONE and PSONC). By a pre-formulation study, sorbitol (6.5%) and PEG 400 (1.5%) were considered the most suitable plasticizers for developing pullulan films (6%) intending topical application. In general, pullulan films were classified as flexible and hydrophilic, with high occlusive properties, $57.6 \pm 0.8\%$, $64.6 \pm 0.8\%$ for vehicle, PSONCF (pullulan film containing PSONC), respectively. All formulations (films and nanocarriers) presented no irritant potential in the chorioallantoic membrane test. In the *in vivo* model, the treatments with free PSO and PSONCF attenuated the skin injury as well as the mechanical hypernociceptive behavioral induced by DNCB exposure to mice. Importantly, the biochemical analyses provided evidence that only the treatment with PSONCF modulated the inflammatory and the oxidative stress parameters evaluated in this study. In conclusion, these data lead us to believe that PSONC incorporation into a pullulan film matrix improved the biological properties of the PSO in this AD-model.

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disorder that commonly affects children (30%) and adults (10%) worldwide. It is the primary sign of a triad condition known as atopy, which is the inherited tendency to produce immune responses to ordinary environmental elements such as dust, pollen and allergens, resulting in AD, asthma and allergic rhinitis (Kapur et al., 2018; Maliyar et al., 2018; Thomsen, 2014). AD is characterized by highly pruritic eczemas associated with

excoriation, redness and serous exudates. The first line prescription therapy consists of topical corticosteroids to treat the inflammatory symptoms including flares and pruritus. However, several adverse effects are related to the long-term use of these drugs, such as skin atrophy, adrenal suppression and high risk of bacterial infection, which highlight the importance of developing new pharmaceutical approaches (Maliyar et al., 2018).

The interest of the pharmaceutical and cosmetic industry in nanotechnology has been growing in recent years, and systems such as

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nanoemulsions (NE) and nanocapsules (NC) are now widely exploited for pharmacological applications. NC is a vesicle system in which an oily core is surrounded by a polymeric shell (Roberts et al., 2017). Such nanosystem can present advantages over conventional formulations, such as increased stability (de Mattos et al., 2015), controlled release (Minai et al., 2019), decrease toxicity of active compounds (Rigon et al., 2019), and so on. Interestingly, regarding cutaneous application, due to characteristics such as shape, size and surface charge, NC can facilitate an intimate contact of substances with the *stratum corneum* and promote the deposition of particles in hair follicles, appendages and deeper layers of the tissue, enhancing the pharmacological activity (Roberts et al., 2017).

For the manufacturing of the NC, a suitable oil is required for the core. Because they can be used as a structural component of the nano-carrier as well as an active pharmacological substance, vegetable oils have been explored in this context as double agents (Marchiori et al., 2017; Mota Ferreira et al., 2016; Rigon et al., 2019). The pomegranate seed oil (PSO) is a vegetable-derived oil obtained from the seeds of the *Punica Granatum* fruit and is widely studied for its interesting pharmacological characteristics. The literature reports PSO antioxidant, anti-inflammatory (Ferreira et al., 2016; Marchiori et al., 2017) and neuro-protective actions (Al-Sabahi et al., 2017; Amri et al., 2017), as well as its use in the treatment of diabetes (Anusree et al., 2018), multiple sclerosis (Binyamin et al., 2015), ischemia and several types of cancers (Bassiri-Jahromi, 2018; Costantini et al., 2014; Yayla et al., 2018). The PSO possesses high concentration of polyunsaturated fatty acids such as α -linoleic acid, in addition to vitamin E and phytosterols, but its pharmacological activity is mainly attributed to the presence of punicic acid, its major compound (Asli Bedel et al., 2017; Khajebishak et al., 2019; Shabbir et al., 2017). Its anti-inflammatory action occurs by the inhibition of the cyclooxygenase enzymes (COX) 1 and 2, while the antioxidant property is based on the scavenger of free radicals, which promotes attenuation of oxidative stress (Lansky and Newman, 2007). In this sense, our research group has already demonstrated the potential applications of PSO as the oil core for preparing NE and NC (Ferreira et al., 2015; Marchiori et al., 2017; Rigon et al., 2019). The improvements achieved by encapsulating PSO alone or in combination with active ingredients were showed using *in vitro* and *in vivo* approaches regarding the antioxidant, antitumor and anti-inflammatory properties (Ferreira et al., 2015; 2016; Mota Ferreira et al., 2016). However, NE and NC are liquid formulations that do not possess an adequate consistency for cutaneous application and therefore, the development of a different dosage form is required.

Polymeric films are flexible dosage forms from a stable nature, easy to produce, transport and very cost-effective (Karki et al., 2016). They are well accepted by patients, being promising candidates for a site-specific application, promoting a longer residence time at the action site, and being suitable for the incorporation of aqueous-based formulations (Morales and McConville, 2011). In contrast with other cutaneous formulations, polymeric films are less associated with skin irritation and sticky sensation during administration (Karki et al., 2016). Due to its versatility, the application of these dosage forms through the cutaneous route is well reported in wound healing (Alves et al., 2016), burns (Baoyong et al., 2010; Pereira et al., 2014), diabetic ulcers (Hussain et al., 2018) and other skin injuries where reepithelization, reconstruction and angiogenesis are involved (Fajardo et al., 2013; Mofazzal Jahromi et al., 2018; Zhang et al., 2015). They can be composed by natural or synthetic polymers, where the natural polysaccharides are highlighted for their interesting characteristics such as low toxicity, biocompatibility and biodegradability (Pérez-Gago and Rhim, 2014). Pullulan is a carbohydrate obtained from the fermentation of *Aureobasidium Pullulan* fungus. It is a non-ionic and linear polysaccharide composed of D-glucose repeated units connected by a periodic sequence of glycoside bonds. Because of its molecular structure, pullulan is hydrophilic, bioadhesive and widely studied as stabilizer, gel and film-forming agent (de Lima et al., 2017; Ferreira et al., 2015;

Horinaka et al., 2018; Li et al., 2018; Prajapati et al., 2018; Wu et al., 2013). In this study, pullulan was the polymer of choice for the development of the films not only because of its interesting characteristics, but also for a matter of compatibility with the nanostructured systems, as both of them have pullulan in its composition as a stabilizer agent.

Therefore, due to: (1) the limitations of the treatment of AD, (2) the relevance of the pharmacological properties of PSO; (3) the promising results subsequently obtained in previous works of our research group through PSO incorporation in nanostructured systems and (4) the interesting characteristics of polymeric films as a platform for the therapeutic management of different clinical conditions, this study aimed to incorporate nanostructured systems (NC) containing PSO in polymeric films and evaluate its pharmacological performance against the injuries caused by an *in vivo* model of AD.

2. Materials and methods

2.1. Materials

Pullulan was donated by Hayashibara. PSO was purchased from Florien (São Paulo, Brazil). Span 80® (sorbitan monooleate) was acquired from Sigma Aldrich (São Paulo, Brazil). Eudragit RS100 was kindly donated by Evonik (São Paulo, Brazil). Sorbitol was obtained from Delaware® (São Paulo, Brazil) and PEG 400 was acquired from Labimpex (São Paulo, Brazil). DNCB was purchased from Sigma (St. Louis, MO, USA) and it was used as an inductor of AD-like lesions. The hydrocortisone (HC) was obtained commercially and it was applied as a comparative substance clinically used in the treatment of AD. All other solvents and reagents were from analytical grade and obtained from standard commercial suppliers.

2.2. Pre formulation studies

Polymeric films were prepared by the solvent casting method (Li et al., 2018; Siemann, 2005). Pullulan (6 w/v %) was dispersed into 25 mL of water and kept at 50 °C under magnetic stirring for 1 h. After, different concentrations of Glycerol (0.5, 1, 1.5, 2, 3, 4 and 5%) and PEG 400 (0.5, 1.5, 2, 5 and 10%) were tested separately in order to optimize the film's composition. The plasticizers were added to the mixture and kept under magnetic stirring until solubilization. The final dispersions were layered in Petri dishes and taken to the oven at 50 °C for 24 h. After the exclusion of glycerol as a plasticizer, sorbitol was tested at 2, 4, 6 and 8%, concomitantly with PEG 400 1.5 %. In order to evaluate which plasticizer type and concentration were more suitable for developing Pullulan films, semi quantitative evaluations were performed such as folding endurance, thumb tack test and apparent elasticity and flexibility. A score was developed (from 0 to 5) based on Notario-Pérez and collaborators study (Notario-Pérez et al., 2019) pliability table, to rate the thumb tack test, apparent elasticity and flexibility, while folding endurance was estimated up to 300.

2.2.1. Thumb tack test

The thumb tack test consisted of putting the thumb into contact with the sample surface and withdrawn it. Upon these circumstances, it is possible to observe the difficulty of pulling the thumb from the film surface and therefore, imply the strength of adhesion between the sample and the skin (Minghetti et al., 2004). The test was performed at standard conditions according to the ASTM D882, 25 °C and relative humidity of 62%.

2.2.2. Apparent elasticity and flexibility

The elasticity and flexibility were first performed manually, and a score was developed modifying the pliability table of Notario-Pérez and co-workers (Notario-Pérez et al., 2019). This assessment is considered qualitative and, detailed as well. In our work, we based the score rating films as:

- (0) **Very Fragile:** breaks easily, even during unmolding.
 (1) **Fragile:** breaks at any attempt to fold.
 (2) **Rigid:** it does not break unless great force is applied.
 (3) **Slightly flexible:** can fold to a certain point before breaking.
 (4) **Flexible:** can be folded effortlessly without breaking, recovering its initial shape.
 (5) **Too flexible:** can be folded in any way without breaking, but sticks to itself and shows problems to recover its initial shape.

2.2.3. Folding endurance

The number of times the film samples could be folded repeatedly at 180° angle in the same place until it breaks is the value of the folding endurance. The test was performed by repeatedly folding the film at the same place up to 300 times, which corresponds to excellent flexibility (Karki et al., 2016; Mukherjee and Bharath, 2013).

2.3. Preparation of PSO nanocapsules

A PSO-based nanocapsule stabilized with pullulan (PSONC) was prepared by the interfacial deposition of preformed polymer method (Marchiori et al., 2017). An organic phase containing PSO (3.0 w/v%), Span 80® (0.1925 g) and Eudragit RS 100® (0.25 g) was solubilized in acetone (125 mL) and mixed with a pullulan (0.1925 g) aqueous phase (125 mL), stirring for 10 min. The formulation was concentrated at 25 mL of final volume. For comparative reasons, a PSO nanoemulsion (PSONE) was also prepared by the spontaneous emulsification solvent diffusion method (Ferreira et al., 2015) in the same conditions of PSONC, as the only difference between them was the absence of a polymeric shell.

2.4. PSO nanocapsules characterization

The following basic characteristics were evaluated on the nanocapsules systems: average diameter and polydispersity index (PDI) in ultrapure water after dilution (1:500) via photon correlation spectroscopy and zeta potential in NaCl 10 mM (1:500) via electrophoretic mobility (Zetasizer Nanoseries, Malvern Instruments, UK). The pH was evaluated by directly immersing the electrode of a calibrated potentiometer (Model pH 21, Hanna Instruments, Brazil) in the formulations. Measurements were performed at room temperature (25 ± 2 °C) in triplicate.

2.5. Preparation of nano-based polymeric films

The polymeric films were prepared with the same method described in section 2.2, incorporating pullulan (6 w/v %) to 25 mL of the nanocapsules (PSONCF) and adding PEG 400 (1.5 w/v %) and Sorbitol (6, 6.5 or 7 % w/v). The final solutions were taken to the oven for 24 h at 50 °C in Petri dishes. For comparative purposes, a nanoemulsion based film (PSONEF) and a water-based film with no nanostructures (VEHICLE) were also prepared on the same conditions.

2.6. Films characterization

Films were assessed in means of weight, thickness and mechanical properties (tensile strength, Young's modulus and percent elongation). The chosen formulation with a final sorbitol concentration was then assessed by recovery size, scanning electronic microscopy (SEM), wettability test, *in vitro* occlusion and formulation safety evaluations and *in vivo* experiments.

2.6.1. Weight variation

Films were weighted directly from the Petri's dish in order to calculate the yield. After, it was cut to 19 cm² strip, placed in aluminum sachets and stored at room temperature (25 ± 2 °C).

2.6.2. Thickness

The thickness of the films was measured using a manual digital micrometer (Kingtools, BR) with an accuracy of 0.01 mm. Measurements were taken at least from five random positions for each film and values were calculated and averaged.

2.6.3. Mechanical properties of the films

Tensile strength, percent elongation and Young's modulus were assessed using a universal testing machine (EMIC DL1000 – Equipment and Systems Ltda – São José dos Pinhais, PR, Brazil), according to ASTM ID: D882-18 (ASTM D 882-02, 2002), at room temperature (25 ± 2 °C). The load cell used in the assessment was 100 N. Films strips measuring 6 × 3.0 cm² were fixed between two grips with an initial separation of 4 cm and 50 mm/min of the cross-head speed. The tensile strength was determined by the force needed to rupture the film divided by the cross-sectional area of the strip, whereas the percent elongation was determined by the percent change in the length of the sample from its original size. The Young's modulus was calculated using the stress and strain values to predict the performance of the material (Equation (1)). The stress and strain values were also calculated from the mechanical properties results (Equation (2) and Equation (3)). The mechanical properties were used to determine the optimum sorbitol concentration. All tests were performed in triplicate. The results were expressed in MPa (tensile strength and Young's modulus) and percentage (elongation).

$$E = \sigma / \varepsilon \quad (1)$$

where: E = Young's modulus; σ = Stress; ε = Strain.

$$\sigma = F / A \quad (2)$$

where: F = Force applied (N); A = Area force applied to (m²).

$$\varepsilon = \Delta l / l \quad (3)$$

where: Δl = Change in length (m); l = length (m).

2.6.4. Average recovery of nanostructures size from the films

From this point on, only the films containing 6,5% sorbitol and 1,5% PEG 400 were analyzed. An amount of PSONCF, PSONEF and VEHICLE (0.1 g) was dispersed in 50.0 mL of distilled water and kept under magnetic stirring until complete solubilization. These dispersions were filtered in a cellulose acetate filter (10 µm) and analyzed via photon correlation spectroscopy (Zetasizer Nanoseries, Malvern Instruments, UK) for PDI and diameter maintenance.

2.7. SEM analysis

The distribution of the nanoparticles in the final film composition as well as its morphology was performed using scanning electron microscopy (JEOL JSM 6360, Japan). A small piece of film was lyophilized and cryofractured in order to analyze the sides sections after fracture. The samples were placed on a double-sided adhesive carbon tape, mounted on the SEM sample slab and coated with gold (Denton Vacuum II, 100 Å) under reduced pressure (30 mtorr) for 40 s to increase conductivity.

2.8. Wettability test

In order to determine the wettability, the contact angle was evaluated in the fresh films by sessile drop method, which is based on the deposition of a drop of distilled water (11 µL) onto the film surface while the contact angle was measured in five seconds, before the swelling started using a goniometer (Drop Shape analysis, model DSA 30S, Krüss; Hamburg, Germany) connected to a computer with dedicated software to assess the contact angles. The evaluation was performed at room temperature (25 ± 2 °C) with relative humidity of 62%, where the under

and upper layer of the films were analyzed ($n = 3$) (ASTM D 882-02, 2002). The results were expressed in medium angle of measurements (θ).

2.9. *In vitro* occlusion test

The *in vitro* occlusion was evaluated using a modified method (De Vringer and De Ronde, 1995), where 50 mL beakers were filled with 25 mL of distilled water and covered with cellulose acetate membrane filter paper (90 mm, Sterlitech, USA). Filter papers were attached to the beakers using an elastic rubber band. PSONCF, PSONEF and VEHICLE films were cut in circles (16 cm²), with an average weight of 1.15 g, in order to completely cover the top of the beakers. The same amount of petroleum jelly (1.15 g) was used as a positive control, and a beaker without any formulation was used as a negative control. The experiment was conducted in an oven at 32 °C for 48 h, and beakers were weighed at specific time intervals (6, 24 and 48 h). The results were expressed in percentage of the occlusion factor, calculated by the following equation:

$$F = (A - B/A) \times 100$$

where F is the occlusion factor, A is the water loss from the negative control beaker, and B is the water loss from the sample beaker.

2.10. *In vitro* safety evaluation

The irritation potential of nanostructure aqueous dispersions and films was evaluated by the Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM). The experimental study was approved by the Ethical Research Committee of the Federal University of Santa Maria, Rio Grande do Sul, Brazil and registered under the number CEUA N° 5428271020. Fertilized chicken eggs incubated at 37 ± 0.5 °C with a relative humidity of $65 \pm 2\%$ for 10 days were donated by Languiru (Teutônia, Brazil). The eggshell was removed, exposing the chorioallantoic membrane (CAM). Saline solution (0.9% NaCl w/v) and 0.1 M sodium hydroxide (NaOH) were used as negative and positive control, respectively. Polymeric films were cut into 2×1 cm stripes and placed on the CAM surface. For the nanoemulsion and nanocapsule dispersions, 500 µL were placed in contact with the CAM and after 20 s, the formulations were removed with saline solution. Pure PSO was also evaluated (10 µL). The CAM was observed for 5 min for all samples to determine the occurrence of any irritation effects in the CAM blood vessels (coagulation, lysis or hemorrhage). The assay was carried out in

quintuplicate. The results were photo documented.

2.11. Animals

Female BALB-c mice (6–8 weeks old) were kept in a separate animal room, under a 12/12-h light/dark cycle (the lights were turned on at 07:00 AM), in a controlled temperature environment (22 ± 2 °C), with standard diet and water *ad libitum*. The experimental study was approved by the Ethical Research Committee of the Federal University of Pelotas, Rio Grande do Sul, Brazil and registered under the number CEEA 23357-2018. All procedures were performed according to National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize both the suffering and the number of the animals used, taking into consideration the consistent effects of the treatments.

2.12. Experimental design

The experimental design of this study is depicted in Fig. 1. As previously described by Chan et al., allergen sensitization and challenge induced by DNCB lead to the development of the skin lesions similar to those of AD (Chan et al., 2013). For the sensitization, the dorsal skin of each mouse was shaved and 200 µL of 0.5 v/v-% DNCB in acetone/olive oil (3:1 ratio) was applied on the shaved area on experimental days 1–3. These animals were challenged by applying 200 µL of 1.0 v/v-% DNCB on the dorsal skin on days 14, 17, 20, 23, 26 and 29.

In order to evaluate the treatment of the PSO nanostructured films on the AD-like skin lesions, mice were randomly divided into seven experimental groups ($n = 7$ animals/group): normal control mice were sensitized and challenged with acetone/olive oil (3:1) and AD-induced mice were sensitized and challenged with DNCB. All others experimental groups were sensitized and challenged with DNCB, as well as received the respective treatments: the free PSO (250 µL); the vehicle film ($2.5H \times 2.5 L$); the PSONCF (15.0 v/w-% of in $2.5H \times 2.5 L$); the PSONEF (15.0 v/w-% of PSO in $2.5H \times 2.5 L$) and 1% of hydrocortisone (0.5 g), as a comparative substance clinically used in the treatment of AD. The experimental groups were designed as control; DNCB; DNCB + free PSO; DNCB + vehicle; DNCB + PSONCF; DNCB + PSONEF and DNCB + HC, respectively. All those treatments mentioned above were applied on the dorsal skin of mice at the same days of DNCB treatment (14, 17, 20, 23, 26 and 29). The treatment schedule was based on previous studies that applied the same animal model and assessed the

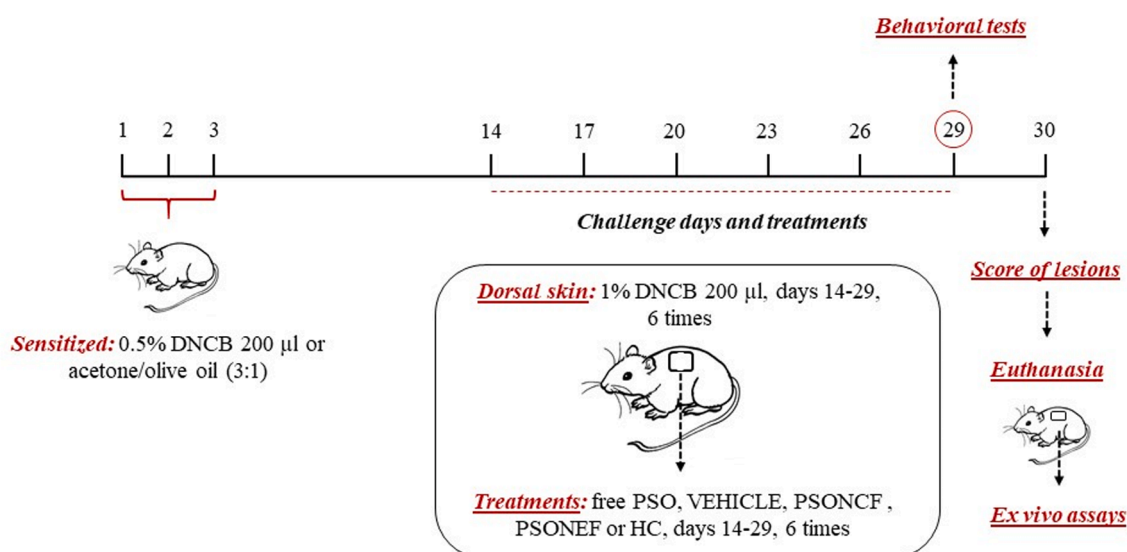


Fig. 1. Schematic representation of the experimental design. Abbreviations: DNCB – 2,4-dinitrochlorobenzene; free PSO – free pomegranate seed oil; PSONCF – pomegranate seed oil nanocapsule film; PSONEF – pomegranate seed oil nanoemulsion film; HC – hydrocortisone.

pharmacological action of films formulations (Alves et al., 2016; Voss et al., 2018). Specifically, at the moment of the application, the films were lightly moistened with water to favor the adherence of the formulation onto skin of mice. The animals were monitored in order to ensure that the films were not removed from the application site. Additionally, it was possible to observe that its adherence was efficient, and the film samples lasted in the animal's dorsal skin until next change.

Furthermore, it was of our interest to investigate the effect of the PSO nano-based polymeric films not only on the skins lesion induced by DNCB, but also on the comorbidities related to the AD-signs. Then, in order to investigate the development of the nociceptive behavior as a comorbidity of the AD-like signs, the animals were submitted to the open field test (OFT) and the von-Frey test on day 29 of the experimental protocol. Twenty-four hours later, on the last day of the protocol (day 30), the skin severity scores were determined to assess the manifestations of the AD-like signs in mice. Following, the animals were euthanized by inhalation of isoflurane anesthetic, the dorsal skin of each mouse was collected and the samples were quickly frozen at -20°C to further biochemical analyses: reactive species (RS) levels, superoxide dismutase (SOD) and myeloperoxidase (MPO) activities.

2.13. *In vivo tests*

2.13.1. *OFT*

This test evaluates the exploratory behavior and the general locomotor activity of mice. The apparatus was made of plywood ($30\text{H} \times 45\text{L} \times 45\text{W}$) in which the floor was divided into 9 squares (3 rows of 3) by masking tape markers. Each animal was placed at the center of the apparatus and observed for 4 min. The locomotor (number of segments crossed with the four paws) and exploratory (number of rearing) activities were recorded during 4 min (Walsh and Cummins, 1976) and expressed as the number of crossings and rearings, respectively. The arena was cleaned with 30% ethanol after each session.

2.13.2. *Von-frey test*

As described by Vivancos et al. (Cunha et al., 2004), the mechanical hypersensitivity in mice was estimated using an electronic anesthesiometer (Insight, Ribeirão Preto, SP, Brazil). This apparatus consists in a device connected to a specific paw pressure transducer. First, the animals were acclimatized during 30 min in an individual clear plastic chambers on an elevated wire mesh platform, which allowed to access the plantar surface of the paws. Then, a stimulus was applied on the mouse hind paw using a blunt tipped probe connected to a transducer. The paw withdrawal threshold, defined as a measure of mechanical hypersensitivity, was evaluated on day 29 of the experimental protocol. The results were expressed as mechanical threshold (g) to cause the paw withdrawal.

2.13.3. *Skin severity scores*

The dorsal skin of the animals was photographed, and severity scores were assessed as previously described to Park et al (Park and Oh, 2014). The characteristic signs of skin lesions were classified as: (1) pruritus/itching, (2) erythema/hemorrhage, (3) edema, (4) excoriation/erosion, and (5) scaling/dryness. Finally, the score was ranked as: 0 (no signs), 1 (mild), 2 (moderate), 3 (severe).

2.14. *Ex vivo assays*

2.14.1. *Tissue preparation*

For the MPO assay, the dorsal skin fraction was homogenized in PBS (20 mmol/L, pH 7.4) containing ethylenediaminetetraacetic acid (0.1 mmol/L). Then, the homogenates were sonicated for 2 min and finally they were frozen and thawed three times. Right after the homogenization step, the samples were centrifuged at 2000 rpm during 15 min in which the supernatant (S_1) was collected. Next, the S_1 fraction was also centrifuged at 14,000 rpm for 30 min at 4°C to yield a final pellet (P_2)

that it was resuspended in a medium containing potassium phosphate buffer (50 mmol/L, pH 6.0) and hexadecyltrimethyl ammonium bromide (0.5 wt/v-%) prior to performed the enzymatic assay.

To elucidate the involvement of oxidative stress, the samples of dorsal skin were homogenized (1:10, w/v) in 50 mM of Tris-HCl at pH 7.4. The homogenates were then centrifuged at 2,500 rpm for 10 min and the low-speed supernatant (S_1) was used for RS levels and SOD activity assays.

2.14.2. *Protein concentration*

The protein concentration was estimated according to the method described by Bradford, using a bovine serum albumin (1 mg/mL) as a standard (Suzuki et al., 1983). The color was measured spectrophotometrically at 595 nm.

2.14.3. *MPO activity*

MPO activity was estimated in accordance to the method previously described (K et al., 1983). In this assay, an aliquot of resuspended P_2 was added to a medium containing the resuspension buffer as well as N,N,N',N' -tetramethylbenzidine (1.5 mmol/L). The kinetic activity of MPO was started after the addition of H_2O_2 (0.01 v/v %), and the color reaction was spectrophotometrically measured at 655 nm at 37°C . The results were expressed as optic density (OD)/mg protein/min.

2.14.4. *RS levels*

An aliquot of 1 mM 2',7'-dichlorofluorescein diacetate (DCHF-DA) in ethanol was incubated with the S_1 and 10 mM Tris-HCl buffer at pH 7.4 for 1 h at room temperature in the dark. DCHF-DA, easily oxidized to DCF, is used as a fluorescent probe to detect intracellular RS levels. Thus, the oxidation of DCHF-DA into DCF was determined at 488 nm for excitation and 525 nm for emission. DCF fluorescence intensity was expressed as arbitrary fluorescence units (Hempel et al., 1999).

2.14.5. *SOD activity*

This method is based on the capacity of SOD in inhibiting the epinephrine autoxidation in adenochrome. The SOD activity was spectrophotometrically determined as described by Misra and Fridovich (Misra and Fridovich, 1972) and the color reaction was measured at 480 nm. The S_1 (10, 20 or 30 μL) was added in a 50 mM Na_2CO_3 buffer pH 10.3. Enzymatic reaction was started by adding an aliquot of epinephrine. One unit of the enzyme was defined as the amount of SOD required to inhibit the rate of epinephrine autoxidation by 50%. The enzymatic activity was expressed as U/mg protein.

2.15. *Statistical analysis*

The normality was assessed by D'Agostino test followed by statistical test. All data collected were expressed as mean $\pm$ S.E.M. for a minimum of three replicates ($n \geq 3$). For data considered parametric, a one-way analysis of variance (ANOVA) followed by *post-hoc* Tukey's test was performed to compare the significant difference among the experimental groups. When data were tested as nonparametric (skin severity scores), a Kruskal-Wallis analysis followed by *post-hoc* Dunn's test was used to compare different treatment effects. All statistical analyses were performed using GraphPad Prism software (version 6.0, USA). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Sorbitol (6,6.5,7%) and PEG 400 (1.5%) were considered more adequate as plasticizers for the pullulan (6%) film

In order to obtain an adequate film formulation, pullulan was tested in different concentrations (from 1 to 8), in which 6% showed to form a thin, yet strong film with a minimum amount of polymer (5% or less formed weaker films). However, pullulan alone was considered too

rigid. Therefore, two different plasticizers were tested in various concentrations separately (glycerol and PEG 400) and in association (sorbitol and PEG 400). The results showed that films with 0.5 to 2 % of glycerol were very fragile, breaking during unmolding, and from 4 to 5 % more flexible and adherent (see supplementary material – Table S1). In addition, it was observed that glycerol films are more susceptible to humidity, modifying their original characteristics. When used alone, PEG 400 produce a greasy very fragile film, displaying better characteristics only when used in combination with sorbitol, at a 1.5%. Sorbitol concentrations between 6 and 8 % associated with 1.5% PEG 400 presented an interesting flexibility and adherence characteristics, folding effortless and recovering its initial shape. Therefore, a combination of PEG 400 (1.5%) and sorbitol (6–7%) were designated for further investigation. In means of macroscopic appearance, formulations where nanostructures were incorporated (PSONCF and PSONEF) presented a milky-white color, while VEHICLE had a translucent aspect.

3.2. PSONC and PSONE presented suitable physico-chemical characteristics

After preparation, PSONC and PSONE were characterized by means of physicochemical aspects (Table 1). Both formulations presented nanometric size (248 ± 16 nm and 181 ± 6 nm, respectively) with PDI values up to 0.2 and zeta potential values of $+43.13 \pm 0.7$ mV for PSONC and -25.63 ± 1.1 mV for PSONE. The pH was at acid to neutral range (5.31 ± 0.17 and 6.77 ± 0.27 , PSONC and PSONE, respectively).

3.3. Films characterization

3.3.1. The 6.5% sorbitol concentration was considered suitable for further evaluations

The films presented a homogeneous macroscopic appearance, with white-milky color for PSONCF and PSONEF and a translucent display for VEHICLE (Fig. 2). The films weight ranged from 2.7 to 4.0 g, with approximately 82% yield and the medium thickness was 164 μ m, as showed in Table 2. SEM analysis (supplementary material – Figure S2) depicted a slightly roughened surface, without apparent difference among formulations and no presence of the nanostructures on this section cut. Mechanical properties such as tensile strength, percent elongation and Young's Modulus were assessed and showed significant difference between sorbitol concentrations in PSONEF and VEHICLE ($p > 0.05$), where higher concentrations decreased tensile strength values (Table 2). In PSONEF 6%, lower percentage elongation was observed in comparison to PSONEF 6.5% and PSONEF 7%, as well as in VEHICLE 6% and VEHICLE 7%. The Young's modulus values are also presented at Table 2, as the stress and strain curves were presented in the supplementary materials (see figure S1). As cutaneous administration is the main goal of these formulations, it cannot present neither hard and brittle characteristics that could turn application difficult, nor too flexible and elastics that would make the films fragile. For that reason, the 6.5% sorbitol concentration was chosen for further studies. After the nanostructures incorporation to the 6.5% sorbitol polymeric film,

Table 1
Nanosystems characterization.

Formulation	Size (nm)	PDI	ζ Potential (mV)	pH
PSONC	$181 \pm 6^*$	0.13 ± 0.05	$+43.13 \pm 0.7$	5.31 ± 0.17
PSONCF	$329 \pm 31^*$	$0.37 \pm 0.05^*$	–	–
PSONE	248 ± 16	0.23 ± 0.03	-25.63 ± 1.1	6.77 ± 0.27
PSONEF	334 ± 52	$0.45 \pm 0.08^*$	–	–
VEHICLE	176 ± 39	0.26 ± 0.06	–	–

Size, PDI, zeta potential and pH of PSO nanoemulsions (PSONE) and nanocapsule suspension (PSONC) as well as PSONEF, PSONCF and VEHICLE films. All data were analyzed by Student's *t* test. (*) significant difference between PSONCF and PSONC; significant difference between PDI values of PSONE and PSONEF, and between PSONC and PSONCF.

average diameter maintenance and PDI presented increased values for both PSONCF (329 ± 31 nm and 0.37 ± 0.05) and PSONEF (334 ± 52 nm and 0.45 ± 0.08) ($p < 0.05$).

3.4. All formulations were hydrophilic and PSONCF showed higher values of contact angle

Fig. 3 presents the results obtained after analyzing the under and upper side of PSONCF, PSONEF and VEHICLE films. Such evaluation revealed a significant difference between both sides, where the under-side contact angle was smaller than the upper side for all formulations ($p < 0.05$). In addition, PSONCF presented higher values of contact angle than the PSONEF and VEHICLE ($\theta 63.45 \pm 0.63$, $\theta 50.6 \pm 3.97$ and $\theta 43.63 \pm 0.60$, respectively) ($p < 0.05$), suggesting that the presence of the polymeric shell can modify the surface free energy interaction with the water drop, and therefore, increase its hydrophobicity.

3.5. PSONCF was considered occlusive

After 48 h of experiment, PSONCF showed higher occlusive properties when compared to the VEHICLE film ($57.6 \pm 0.8\%$), indicating that the nanostructures are able to modify such characteristic (Fig. 4; $p > 0.05$). PSONEF presented occlusion effects similar to PSONCF ($p < 0.05$). Regardless time, nanostructured films always showed higher occlusion factor when than pullulan VEHICLE film ($p < 0.05$). It is also important to highlight that, overall, all films formulations can be considered occlusive, displaying more than half occlusion capacity than the positive control ($93.3 \pm 0.8\%$).

3.6. All formulations were considered non irritant

In the CAM, evidence of lysis, coagulation and hemorrhage were not detected (see supplemental files – figure S3), indicating an absence of irritation potential for all formulations.

3.7. Animal model of AD-like

3.7.1. The free PSO or the PSONCF treatments attenuated the AD-like signs induced by DNCB in mice

The Fig. 5A and 5B illustrate the effect of the films containing PSO incorporated into nanosystems (PSONCF and PSONEF) on the severity of the skin lesions in mice. Our results showed that the DNCB exposure induced AD-like signs in mice, represented by an increase in the clinical skin severity score when compared with the control group ($p < 0.0001$). The free PSO or the PSONCF treatments markedly decreased the characteristics AD-like signs in the DNCB group whereas the PSONEF had no statistical difference when compared to vehicle or DNCB-treated mice. In turn, the HC and the vehicle film did not alter the severity of lesions induced by DNCB ($p > 0.05$).

3.7.2. The free PSO or the PSONCF treatments suppressed the development of a nociceptive behavior related to the AD-like signs in mice

In order to avoid any kind of unspecific results in the behavioral tests due to possible sedative effect or locomotor abnormalities related to the treatments (DNCB, free PSO, PSONCF or PSONEF and HC), the animals were submitted to the OFT. The effect of the film formulations (PSONCF and PSONEF) on parameters of the locomotor and exploratory activities in mice are shown in Table 3. The statistical analysis revealed that all the treatments performed in this study did not change the number of crossings ($p > 0.05$) or rearings ($p > 0.05$) in mice.

Fig. 6 depicts the effect of all tested formulations on the development of the mechanical hypernociception induced by DNCB in mice. The results demonstrated that the DNCB applications decreased the paw withdrawal threshold in mice when compared with the control group, indicating that the DNCB exposure triggered a mechanical hypersensitivity in mice ($p < 0.0001$). The HC, vehicle and the PSONEF

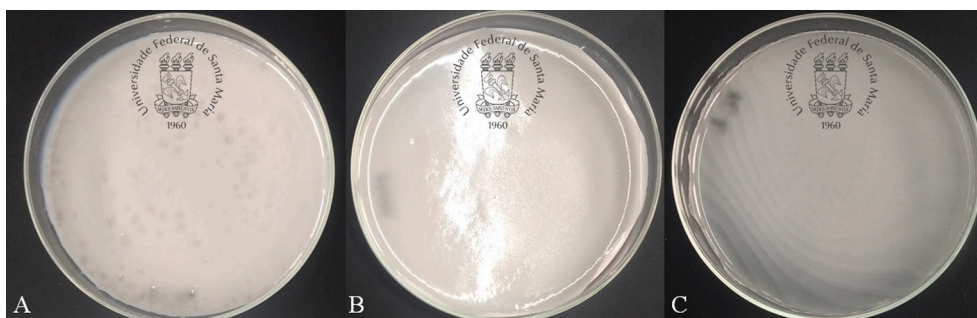


Fig. 2. Macroscopic appearance of the films. (A) PSONCF (B) PSONEF (C) VEHICLE.

Table 2

Films characterization. Influence of different concentrations of Sorbitol on films physical characteristics.

Formulation	Weight (g)	Thickness (μm)	Tensile Strength (MPa)	% Elongation	Young's Modulus (MPa)
PSONCF 6%	3.81 ± 0.00	154.7 ± 32.0	1.60 ± 0.16	205.0 ± 26.54	13071.2
PSONCF 6.5%	3.96 ± 0.03	154.0 ± 16.6	1.43 ± 0.16	215.0 ± 6.80	12450.5
PSONCF 7%	4.10 ± 0.00	160.7 ± 22.2	1.07 ± 0.22	235.0 ± 1.35	11399.7
PSONEF 6%	3.73 ± 0.02	174.7 ± 21.5	2.15 ± 0.06 [#]	90.80 ± 3.06 [*]	29518.3
PSONEF 6.5%	3.85 ± 0.05	157.3 ± 8.05	1.13 ± 0.09	214.1 ± 0.38	12520.4
PSONEF 7%	3.96 ± 0.00	173.8 ± 23.5	0.33 ± 0.09	232.2 ± 13.75	11547.3
VEHICLE 6%	2.73 ± 0.03	171.3 ± 26.3	1.20 ± 0.17 ^{\$}	271.7 ± 28.29 ^{&}	9883.1
VEHICLE 6.5%	2.83 ± 0.02	165.3 ± 12.0	0.60 ± 0.04	325.0 ± 16.51	8238.9
VEHICLE 7%	2.90 ± 0.00	167.3 ± 31.7	0.54 ± 0.10	402.8 ± 30.23	6642.7

The weight values increase according to higher concentrations of plasticizer, as well as the presence of nanocarriers or not. Thickness of films varies with no significant difference. All data were analyzed by One-way ANOVA, followed by *post-hoc* Tukey's test. (#) significant difference between all PSONEF tensile strength ($p < 0.0001$). (\$) 6% VEHICLE has significantly higher tensile strength than 6.5% and 7% ($p < 0.01$). (*) PSONEF 6% presented lower percent elongation than 6.5% and 7% ($p < 0.0001$). (&) VEHICLE 6% is significantly different than 7% ($p < 0.05$).

treatments were ineffective to increase the paw withdrawal threshold in DNCB-treated mice. In contrast, the treatments with free PSO and PSONCF improved the mechanical stimulus response in mice exposure to the DNCB, suggesting that both exerted antinociceptive effect.

3.7.3. The treatment with PSONCF improved the inflammatory and the oxidative stress status in the dorsal skin of mice exposed to the DNCB

The effect of the film formulations on the inflammatory and the oxidative stress parameters are depicted in Fig. 7. All the animals exposed to DNCB enhanced the MPO enzymatic activity in the dorsal skin when compared with the control group ($p < 0.0001$). In turn, the treatment with PSONCF decreased the MPO activity in the dorsal skin of mice in comparison to DNCB-treated mice (Fig. 7A).

As expected, the dorsal skin of the DNCB-treated mice had shown a significant increase in the RS levels when compared with the control group ($p < 0.0001$) whereas only the PSONCF treatment was effective in reducing the RS levels in the DNCB group (Fig. 7B). Accordingly, our data also demonstrated that the DNCB applications inhibited the SOD activity in the dorsal skin of mice when compared with the control group ($p < 0.05$). On the other hand, the treatment with PSONCF restored the activity of this enzyme (Fig. 7C).

4. Discussion

The selection of film components is majorly important and reflects directly in its performance within different administration routes. Pululan presents adequate film forming characteristics producing thin, transparent, adhesive, colorless, and odorless films and it was already reported in manufacturing of films for cutaneous administration, treatment of skin disorders and wound dressing (Li et al., 2018; Zhu et al., 2014). The use of plasticizer is equally important to provide flexibility

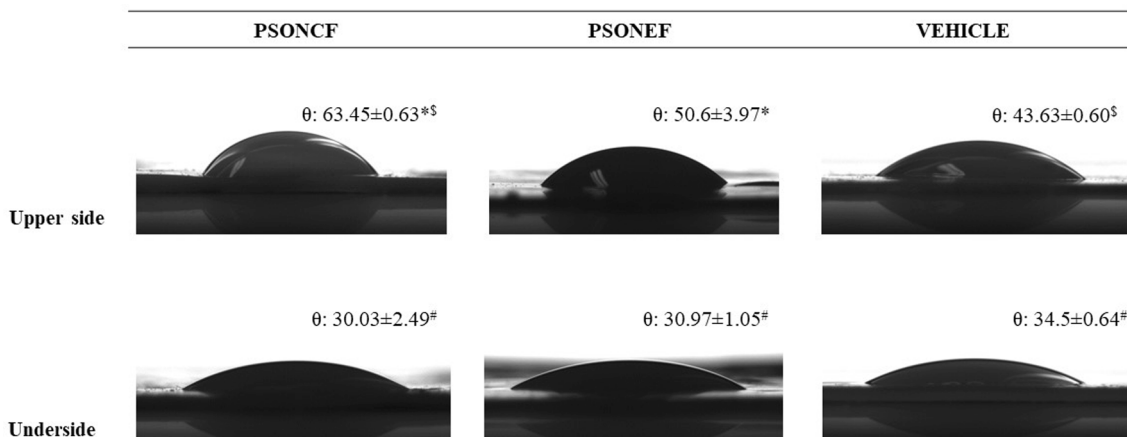


Fig. 3. Contact angle analysis of the upper and underside of all film formulations. Upper side: All data were analyzed by One-way ANOVA, followed by *post-hoc* Tukey's test. (*) significant difference between PSONCF and PSONEF and between PSONCF and VEHICLE, respectively. Underside: (#) all contact angle values were smaller than the upper side. No difference between the underside analysis.

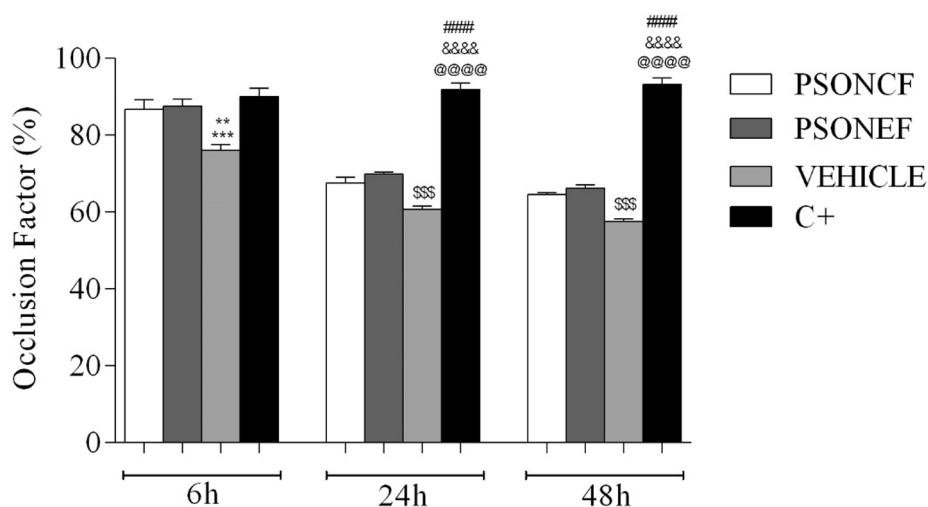
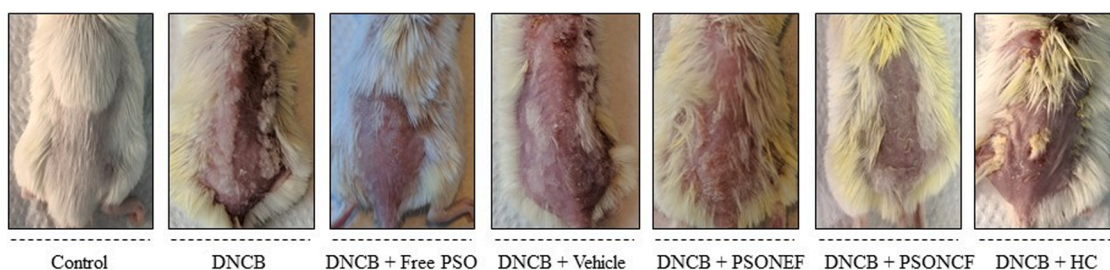


Fig. 4. Occlusion factor in 6, 24 and 48 h. All data were analyzed by One-way ANOVA, followed by *post-hoc* Tukey's test. (*) VEHICLE is significantly difference than all formulations in 6 h with (** $p < 0.01$; *** $p < 0.001$). (\$\$\$) difference among PSONEF, PSONCF and FF ($p < 0.001$), (####) difference between PSONCF and C+, (@@@@) difference between PSONEF and C+, while (&&&&) reports difference between VEHICLE and C+ ($p < 0.0001$).

A)



B)

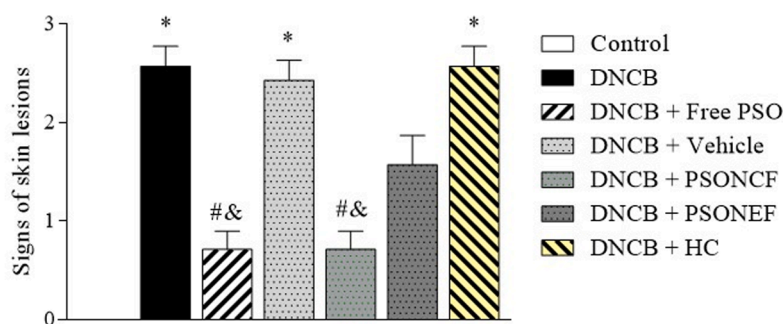


Fig. 5. Effect of the free PSO and the films formulations on the severity of the skin lesions in mice. (A) Images of the skin lesions from the groups of mice taken on the last day of the experiment (day 30). (B) Dermatitis scores. Each column represents the mean $\pm$ SEM. of 7 mice per group. (*) $p < 0.05$ compared with the control group, (#) $p < 0.05$ compared with the DNCB group, (&) $p < 0.05$ compared with the hydrocortisone (HC) group (Kruskal-Wallis followed by the Dunn's test) and the control score (0).

and improve elasticity, reducing the intermolecular forces of the film structure and increasing its softness for administration. In the pre formulation studies, apparent elasticity, flexibility, and adhesion were evaluated in different concentrations of various plasticizers. It was possible to observe that glycerol formed more elastic and flexible films, more susceptible to room humidity and, therefore, very hydrophilic. As a consequence, these formulations were considered more fragile than the sorbitol films. This take effect because as both glycerol and sorbitol are polyols, glycerol presents three linked carbons to the hydroxyl (OH) group, whereas sorbitol shows 6 carbons in its structure. Hence, sorbitol

has a higher interaction capacity with pullulan molecules, providing less mobility to their chains and lowering their interaction with water molecules. Glycerol also has a smaller molecular weight and it is known to be more hydrophilic. When accessing PEG 400, it was possible to observe that the plasticizer alone was not able to provide adequate characteristics such as flexibility or adhesion, however Antoniou and co-workers (Antoniou et al., 2014) reported that as PEG 400 has high molecular weight, it overflows the polymer matrix and is trapped in the underside of the film, which is in contact with the Petri's dish. Such phenomena modify the understructure of the film, facilitating the

Table 3

Effect of the formulations and the DNCB treatments on the locomotor and exploratory activity parameters.

Experimental Group	OFT	
	Crossings	Rearings
Control	76.3 ± 4.9	31.7 ± 2.8
DNCB	76.7 ± 6.3	23.1 ± 2.3
DNCB + free PSO	74.7 ± 4.9	26.3 ± 1.6
DNCB + VEHICLE	82.4 ± 7.6	30.3 ± 2.3
DNCB + PSONCF	77.1 ± 6.2	29.7 ± 1.9
DNCB + PSONEF	73.6 ± 6.1	27.1 ± 1.5
DNCB + HC	74.4 ± 6.8	23.7 ± 2.0

Data are reported as mean ± SEM for 7 animals per group. Statistical analyses were performed by one-way ANOVA followed by the Tukey's test when appropriate.

removal of the film from the dish. The 1.5% PEG 400 concentration was considered ideal as it is the minimum amount needed for this performance. As for sorbitol, from 6 to 8% concentrations, adhesion, flexibility and elasticity were considered adequate, but further studies were necessary to be able to differentiate it.

Regarding nanostructures characterization, PSONC presented values of mean diameter, polydispersity index and pH similar to those reported by Marchiori and co-workers (2017) (Marchiori et al., 2017). After the nanostructure incorporation to the film matrix, PDI and diameter maintenance increased. Chandra and co-workers (Chandra et al., 2018) used a similar method to evaluate lacidipine nanoparticles recovery after incorporation in a fast dissolving polymeric film. As a result, all formulations showed an increased particle size after re-dispersion, with a difference of results up to 200 nm. The author suggested that the particles may be embedded with the polymeric matrix. Also, the presence of the polysaccharide forms a colloidal dispersion with particles ranging in the nanometric scale, which can be detected by the photon correlation spectroscopy method. A previous study of our research group concluded that the increase observed in particle size after re-dispersion should not cause prejudice to potential pharmacological effects (Ferreira et al., 2020).

Physical properties of the films were considered consistent with the intended cutaneous application. The measurement of thickness is important as directly corresponds to a comfortable administration of the formulations. Zhu and co-workers developed carboxymethyl, gellan and pullulan blend edible films (Zhu et al., 2014). One of their formulations were a 4% pullulan film that showed thickness around $170 \pm 0.04 \mu\text{m}$, similar to the values obtained in this study ($164 \mu\text{m}$ average) with 6% pullulan. Although it is a different pullulan concentration, such parameter variation can be due to distinctive preparation methods. Surface morphology assessment is necessary to indicate the

homogeneity of the film. SEM analysis showed a slightly roughened surface and yet very homogeneous appearance without nanostructures presence. Machado and co-workers tailored a tenofovir-loaded nanoparticle film with poly (lactic-co-glycolic acid) and sterylamine for vaginal drug delivery (Machado et al., 2016). They suggest that the absence of the nanoparticles in the SEM analysis of the side sections after fracture is due to its intimate and uniform insertion in the polymeric film network.

Mechanical and physical properties are important assessments given the fact that polymeric films should present adequate tension so it can be pulled out of the casting mold and peeled easier from its packaging without tearing. Likewise, they cannot be too flexible as to modify its shape or structure and impair its homogeneity during administration (Karki et al., 2016). For all formulations, the tensile strength decreased as the percent elongation increased with the raise of plasticizer concentration. Tensile strength corresponds to the maximum amount of force needed to tear the film during a constant rate of deformation, as percent elongation correlates to the comparison of the length before and after deformation under stress. Lower concentrations of sorbitol shaped a harder more brittle film with high values of Young's modulus, whereas higher concentrations formed a flexible more elastic product with smaller Young's modulus. The Young's modulus or elastic modulus reflects the stiffness of the films. The influence of the nanostructures in this test were not assessed in means of statistical analysis because the aim of the test was to select one plasticizer concentration to carry out the study.

The wettability test is evaluated by the contact angle method, which is defined as the angle between the baseline of the water drop and the tangent line at the contact point of the water droplet with the flat surface. This technique, so-called the sessile drop method, can be used to learn more about a material surface free energy and its hydrophobicity. Such parameters are considered important when it comes to skin administration of a pharmaceutical formulation because it helps determine the product behavior in contact with the skin in means of adhesion, hydrophilicity, permeability and so on (Eudier et al., 2019). As described by Antoniou and co-workers, the solvent casting method can provide different textures to both sides of the polymeric film (Antoniou et al., 2014). As a result of the contact with the casting mold, the underside is usually smoother than the upper side, which is exposed to the air. Because of this difference, both sides of the films were evaluated in this test. As mentioned before in the pre formulation studies, PEG 400 overflows the polymeric matrix and its deposited in the underside of the film. Thus, the underside of all formulations presented lower values of contact angle than the upper side, suggesting this side is more hydrophilic and adherent. In the upper side, PSONCF showed a greater contact angle value. The presence of Eudragit RS100 is the only composition difference between nanoemulsions and nanocapsules, which can explain PSONCF hydrophobic nature. Overall, because of its layout, the films

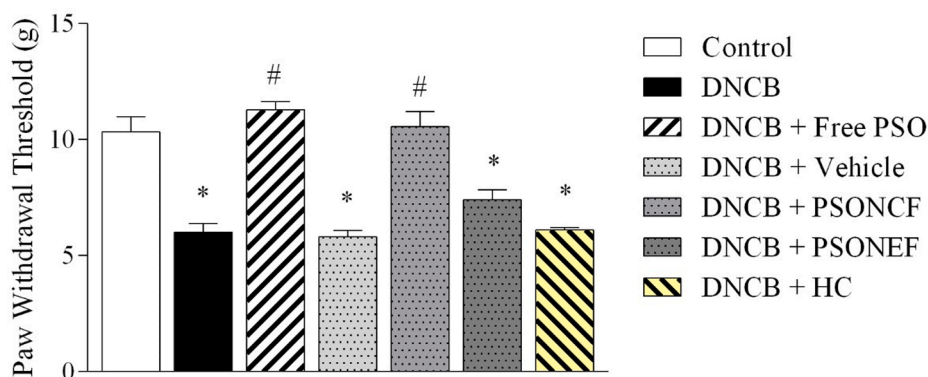


Fig. 6. Effect of the free PSO and the film formulations on the development of a mechanical hyper sensitivity induced by DNCB in mice. Each column represents the mean ± SEM, of 7 mice per group. (*) $p < 0.05$ compared with the control group, (#) $p < 0.05$ compared with the DNCB group (One-way ANOVA followed by the Tukey's test).

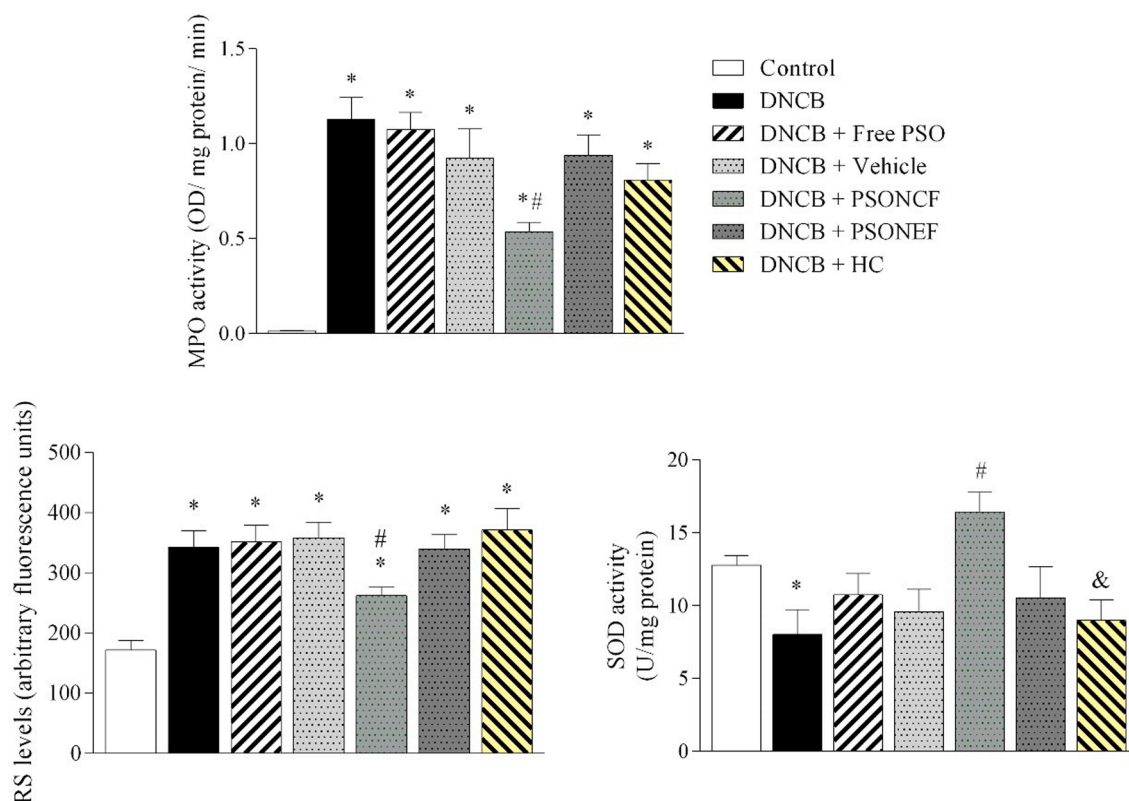


Fig. 7. Effect of the free PSO and film formulations on the MPO activity (A), RS levels (B) and SOD activity (C) in the dorsal skin of mice. Each column represents the mean $\pm$ SEM. of 7 mice per group. (*) $p < 0.05$ compared with the control group, (#) $p < 0.05$ compared with the DNCB group, (&) $p < 0.05$ compared with the hydrocortisone (HC) group (One-way ANOVA followed by the Tukey's test).

presented high wettability potential and therefore, a very hydrophilic nature as pullulan is a freely soluble and rapidly dispersible polymer. Aiming at cutaneous administration for the treatment of skin injuries such as AD, pullulan hydrophilic character can come to use if the lesion presents moisture due to exudates, as reported by Cordenonsi and co-workers (Malgari Cordenonsi et al., 2019). Dermatitis lesions can peel off to abrasions and exudate, and with pullulan fast dissolving quality the nanostructures can be rapidly available to interact locally with the injury, improving its efficacy during treatment.

There is a direct correlation between *in vitro* occlusion and *in vivo* skin hydration. Formulations with occlusive components present the ability to form a barrier that can prevent transepidermal water loss, increasing water content in the *stratum corneum*. An increase of skin hydration can be useful to enhance the permeation of active compounds and therefore, useful in the treatment of skin disorders (Galvão et al., 2016). In addition, it is well stated in the literature that nanostructured systems are granted highly occlusive properties depending on their composition. Montenegro and co-workers compared the occlusion capacity of different nanocarriers (nanoemulsions, solid lipid nanoparticles and nanostructured lipid carriers) incorporated in a gel vehicle (Montenegro et al., 2017). The results showed that the content of the lipid core of the nanocarriers with similar sizes seemed to play a key role on its occlusive properties. The *in vitro* occlusion tests results of our study revealed that the nanostructures presence in the film formulation increased the occlusion factor in comparison to VEHICLE, but presented no difference between PSONCF and PSONEF. Both NC and NE formulations have similar average size and the same lipid composition, which is PSO. It is also important to highlight that all film formulations exhibited more than half occlusion capacity in comparison to petroleum jelly, a well-known occlusive substance. As a result, it is possible to imply that the occlusive properties of the films are mainly due to the PSO presence in the nanosystems. Such characteristics can improve the

pharmacological effects of the PSO providing better outcome and efficacy in the treatment of skin disorders. In this sense, reinforcing such argument, our results demonstrated that the PSONCF provided a superior pharmacological action that can be related to the formulation high occlusive properties.

The HET-CAM assay is an alternative to Draize test of ocular toxicity that can be used to assess a formulation irritation potential and therefore, its safety (Batista-Duarte et al., 2016). Various authors report the test application to formulations designed for skin administration, as well as its use to estimate nanostructured systems cutaneous tolerability (Dario et al., 2016; Yingngam et al., 2019; Yum et al., 2018). Sousa and co-workers evaluated the irritation potential of creams containing different vegetable oils (Sousa et al., 2018). All formulations were classified as nonirritant, suggesting their safety for topical application. Likewise, Contri and co-workers did not detect endpoints of irritancy when evaluating rhodamin-tagged nanocapsules incorporated in a chitosan gel vehicle (Contri et al., 2016). In our study, the positive control of NaOH presented signs of coagulation, while all developed nanostructures suspensions as well as all film formulations presented no irritation potential and are initially considered safe for cutaneous application.

AD is considered a complex disorder of high heterogeneity which can include abnormalities in the skin barrier and pathogens and allergens access to the tissue. Such factors contribute to the development of classical AD-signs, characterized by the dry and thick skin as well as the patchy eczema (Weidinger and Novak, 2016). Similarly, repeated DNCB applications, an epicutaneous sensitizer, stimulate the inflammatory cells and disrupt the skin barrier of mice. For this reason, DNCB exposure is widely employed as tool to induce a chemical animal model of allergic contact dermatitis (Jin et al., 2009). As expected, our findings showed that consecutive exposure to DNCB induced the appearance of the AD-like signs in mice, that was evidenced by an increase in the severity of

the skin lesions (dryness, erythema and erosion).

Despite that topical corticosteroid are considered the standard treatment to alleviate the severity signs associated with AD, the appearance of numerous adverse effects limits the long-term use of this therapeutic agents. Therefore, there is an urgency on the planning and developing of a new, safe and effective strategies for AD. The PSO, abundantly rich in conjugated fatty acids, polyphenols and estrogen, has been reported due to its antioxidant, anti-inflammatory and antitumoral properties (Lansky and Newman, 2007). Moreover, the nanotechnology has been widely used to improve the performance of natural products. In fact, a previous study had been showed that the PSO, used as a polymeric shell core in the NC, prolonged the anti-inflammatory effect of a topical formulation containing silibinin in an animal model of skin injury induced by UVB radiation (Marchiori et al., 2017). In this context, our results provided evidence that both free PSO and PSONCF treatments attenuated the AD-like signs in mice, represented by a decrease in the incidence of the skin lesions score.

In general, it has been proposed that the pathological characteristics of the AD-like skin lesions required immunological and inflammatory responses. More specifically, the infiltration of mast cells, neutrophils and eosinophils into the tissue, stimulated the release of T helper 2 (Th2) cytokines as well elevated the local and systemic immunoglobulin E (IgE) levels, resulting in an allergic reaction (Werfel et al., 2016). In view of the tight relationship between the inflammatory process and the pathogenesis of AD, we investigated the involvement of the MPO activity. As expected, the results of the present study indicate that consecutive exposures to DNCB increased the MPO enzymatic activity in the dorsal skin of mice. As expected, only the treatment with the PSONCF inhibited the activity of this enzyme in the dorsal skin of animals exposed to DNCB. Reinforcing the need for new therapeutic approaches to AD, our findings revealed that mice treated with HC exhibited AD-like skin lesions as well as an increase in the MPO activity on the local injury. This data agrees with the fact that the long-term steroids use lead to the development of skin thinning, cracking or bleeding (Slater and Morrell, 2015).

Furthermore, the melanocytes and keratinocytes, some types of skin cells, generate RS. It is well-known that the immune cells stimulation is associated to the RS production as well as the inflammatory mediators are activated by the oxidative molecules (Steullet et al., 2016). In this way, the RS production from the skin cells may also produce exacerbated reactive lipid species by reacting with lipid molecules or redox-sensitive lipid-metabolizing enzymes, resulting in structural and functional damages of macromolecules. In this situation, there are an enhancement on the antioxidant enzymes activity and an induction on the constitutive cytoprotective genes in order to maintain the cellular redox homeostasis (Yum et al., 2018). Thus, the RS overproduction led to an imbalance on the redox signaling and might accelerated the development of chronic inflammatory skin disease, such as AD. Indeed, our data demonstrate that repeated DNCB challenges increased the RS levels and decreased the SOD activity in the dorsal skin of mice. On the other hand, the PSONCF treatment modulated these oxidative stress markers in DNCB-treated mice.

Although both free PSO and PSONCF treatments exhibited protective effects against the AD-like skin lesions in mice, only the PSONCF restored the biochemical parameters evaluated in this study. Such nanostructured system can be retained in the *stratum corneum* or even penetrate and permeate the inner layers of the skin tissue (Guterres et al., 2007). Particularly, in the NC, the active can be entrapped in, dispersed, dissolved or adsorbed due to their core-shell supramolecular structure (Frank et al., 2015). In addition, is well established that the polymeric composition of the NCs shell developed in this study (Eudragit RS100) possesses great bioadhesive properties, which can promote an intimate contact with the skin surface. For this reason, we believe that the PSONCF increased the residence time of the PSO in the skin tissue in comparison with PSONEF and thus, improved and prolonged its anti-inflammatory and antioxidant effects. As evidenced here, we

hypothesized that the treatment with PSONCF reduced the MPO activity, indicating a decrease in the polymorphonuclear leukocytes migration to the local inflammation. The anti-inflammatory effect exerted by the PSONCF might contributed to increase the SOD activity in order to avoid the RS overproduction within the skin cells of mice exposed to DNCB. Through this study, we highlight and reinforce the role of the nanosystems, such as NC, in improving and optimizing the biological properties of the natural compounds.

Indeed, AD imposes considerable burdens upon those affected, including poor quality of life and comorbidities secondary to severe itch (Brunner et al., 2017). The presence of inflammatory mediators such as prostaglandin E₂, bradykinin and arachidonic acid might trigger the hyper sensitivity of the nociceptors at the site of damage or in an adjacent tissue (Binshtok et al., 2008). In the same way, the exacerbating production of oxidizing agents, such as RS, could sensitize the nociceptive nerve ending (Salvemini et al., 2011). Considering that both chronic inflammation and oxidative stress have been involved in the pathogenesis of AD, we investigated the development of a nociceptive behavior as a comorbidity of this disease. The findings of this study demonstrated that both free PSO and PSONCF treatments exhibited an antinociceptive effect by reducing the mechanical hyper sensibility in the DNCB-treated mice. It is in agreement with our previous findings, in which PSO-loaded NE reduced the nociceptive behavior of mice in tests of chemical and acute inflammatory pain, reinforcing the pharmacological actions of PSO and the advantages achieved by nano-encapsulation (Ferreira et al., 2016). In this context, such evidence suggested that the severe skin inflammation induced by DNCB may damage the function of the nociceptive nerve ending, resulting on the development of the mechanical hypersensitivity related to the AD-like signs.

5. Conclusion

In conclusion, this work proposed the development and characterization of a novel pullulan film associated with NC for the topical delivery of PSO in the treatment of AD-like lesions. The mechanical and physical properties of the film was suitable for cutaneous application, and all formulations were considered safe. The *in vivo* results demonstrated that both free PSO or PSONCF treatments ameliorated the severity of the skin lesions and the development of a nociceptive behavior associated to the AD-signs in mice. However, only the PSONCF treatment modulated the inflammatory and the oxidative stress markers evaluated in this study. Thus, we highlighted an improvement on the biological properties of the PSO when incorporated to NC-based pullulan film and we propose this system as a promising alternative as nanocarrier for natural compounds to AD management.

CRedit authorship contribution statement

Verônica Ferrari Cervi: Methodology, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Conceptualization. **Camila Parcianello Sacco:** Methodology, Data curation, Investigation, Writing – review & editing. **Marcel Henrique Marcondes Sari:** Methodology, Data curation, Investigation, Visualization, Conceptualization, Writing – review & editing. **Carolina Cristóvão Martins:** Methodology, Data curation, Conceptualization, Writing – review & editing. **Lucas Saldanha da Rosa:** Methodology, Writing – review & editing. **Bruna Dias Ilha:** Methodology, Writing – review & editing. **Fábio Zovico Soares:** Methodology, Conceptualization, Writing – review & editing. **Cristiane Luchese:** Data curation, Writing – review & editing. **Ethel Antunes Wilhelm:** Data curation, Writing – review & editing. **Letícia Cruz:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpharm.2021.121144>.

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Pullulan film incorporated with nanocapsules improves pomegranate seed oil anti-inflammatory and antioxidant effects in the treatment of atopic dermatitis in mice

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Table S1. Pre formulation studies

Tests	<i>First test</i>												<i>Second test</i>			
Plasticizers	Glycerol							PEG 400					Sorbitol + PEG 400 - 1.5%			
(%)	0.5	1	1.5	2	3	4	5	0.5	1.5	2	5	10	2	4	6	8
Thumb test	0	0	0	0	1	4	5	0	0	0	1	1	1	2	3	2
Elasticity	0	0	0	0	1	4	5	0	0	0	0	1	0	0	4	4
Folding endurance	0	0	0	0	0	300	300	0	0	0	0	0	0	0	300	300

Thumb tack test, apparent elasticity and folding endurance in different concentrations of glycerol (G), sorbitol (S) and PEG 400 (P) in a 6% pullulan film. (0) to (5) corresponds to minimum and maximum values of thumb tack and apparent flexibility. NA means non applicable.

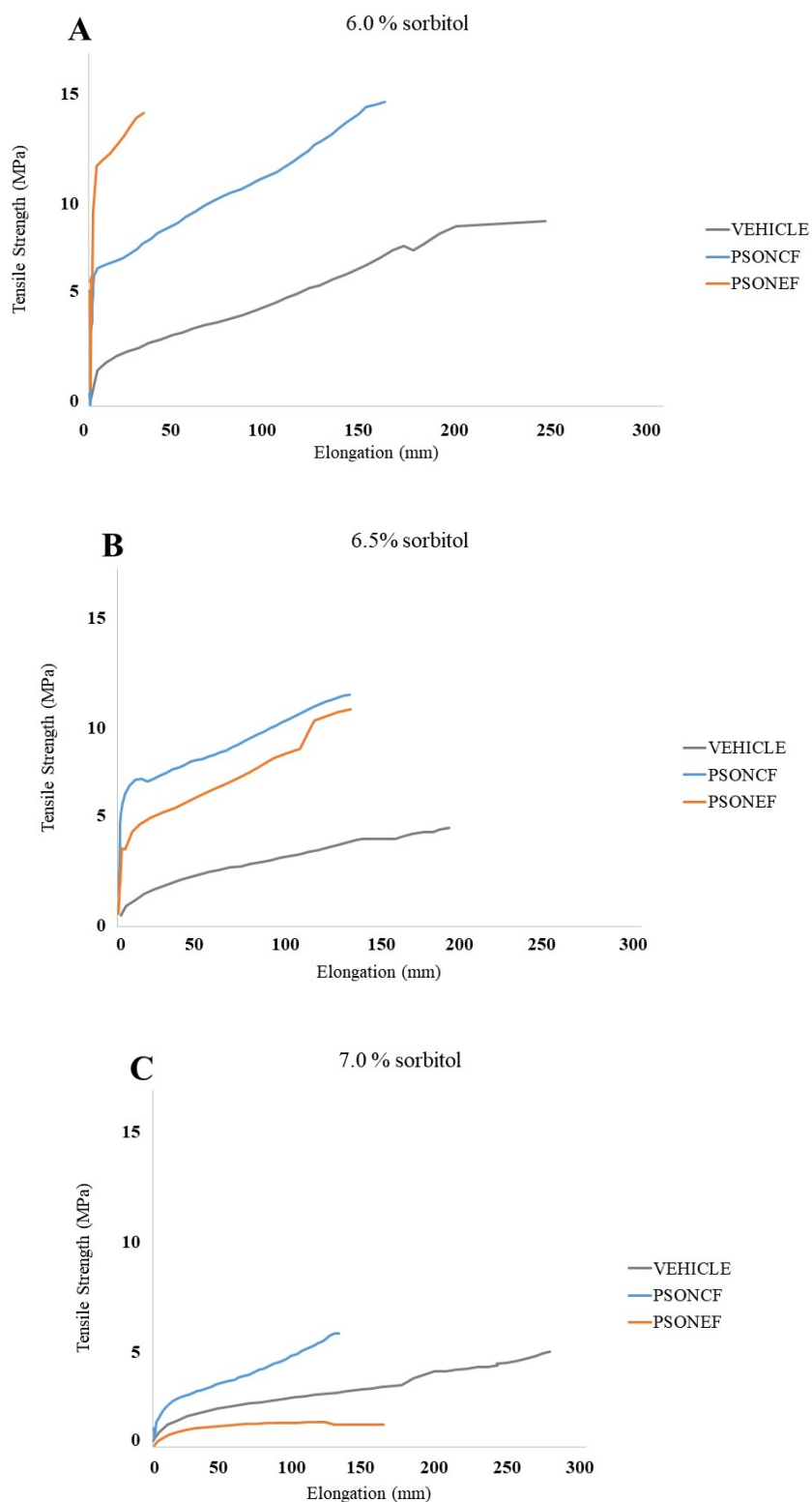


Fig. S1. Stress and strain curves representing the Young's Modulus. (A) 6.0%, (B) 6.5% and (C) 7.0% sorbitol concentration. It is possible to observe that harder and brittle films are obtained with lower plasticizer concentrations, as well as softer and weaker films are obtained with increased plasticizer concentrations. The curves were developed by plotting the tensile strength (MPa) versus the elongation (mm) values obtained during the mechanical properties evaluation using a universal testing machine

(EMIC DL1000 – Equipment and Systems Ltda – São José dos Pinhais, PR, Brazil) at room temperature ($25\pm 2^{\circ}\text{C}$).

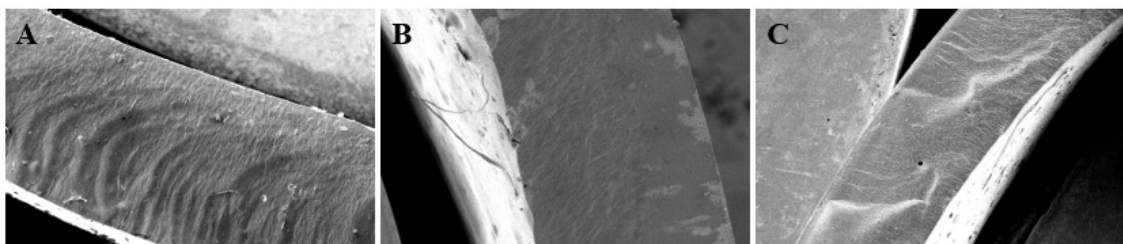


Fig. S2. SEM images of produced films. Images presents exposed side sections after cryofracture of PSONEF (A), PSONCF (B) and VEHICLE (C). The samples were placed on a double-sided adhesive carbon tape, mounted on the SEM (JEOL JSM 6360, Japan) sample slab and coated with gold (Denton Vacuum II, 100 Å) under reduced pressure (30 mtorr) for 40 s to increase conductivity.

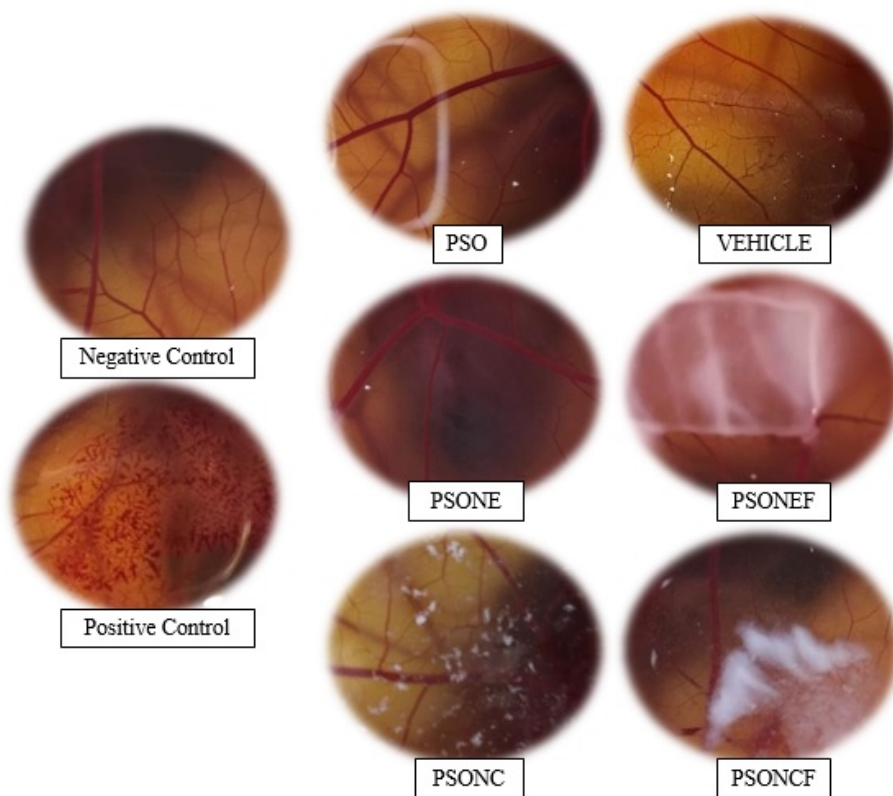


Fig. S3. Hen's Egg Test of the Chorioallantoic Membrane (HET-CAM), presents NaCl as negative control and NaOH 0.1 M as positive control. Nanostructured dispersions as well as films formulations were tested and no irritancy potential was observed.