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Aloe vera and copaiba oleoresin-loaded chitosan films for wound dressings: microbial permeation, cytotoxicity, and *in vivo* proof of concept

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ABSTRACT

Chitosan films are commonly used for wound dressing, provided that this polymer has healing, mucoadhesiveness and antimicrobial properties. These properties can be further reinforced by the combination of chitosan with polysaccharides and glycoproteins present in aloe vera, together with copaiba oleoresin's pharmacological activity attributed to sesquiterpenes. In this work, we developed chitosan films containing either aloe vera, copaiba oil or both, by casting technique, and evaluated their microbial permeation, antimicrobial activity, cytotoxicity, and *in vivo* healing potential in female adult rats. None of the developed chitosan films promoted microbial permeation, while the cytotoxicity in Balb/c 3 T3 clone A31 cell line revealed no toxicity of films produced with 2 % of chitosan and up to 1 % of aloe vera and copaiba oleoresin. Films obtained with either 0.5 % chitosan or 0.5 % copaiba oleoresin induced cell proliferation which anticipate their potential for closure of wound and for the healing process. The *in vivo* results confirmed that tested films (0.5 % copaiba-loaded chitosan film and 0.5 % aloe vera-loaded chitosan film) were superior to a commercial dressing film. For all tested groups, a fully formed epithelium was seen, while neoformation of vessels seemed to be greater in formulations-treated groups than those treated with the control. Our work confirms the added value of combining chitosan with aloe vera and copaiba oil in the healing process of wounds.

1. Introduction

Sustainable biopolymers, such as chitosan, have been used in the production of wound dressings (Yoshida et al., 2021). Some commercial chitosan dressings are available on the world market, such as the Japanese brands Beschitin and Chitopack, and also American brands, such as HemCon® Bandage, Syvek Patch, Clo-Sur PAD, Chito-Seal, M−Patch, and Trauma DEX. Usually, these dressings are recommended for bleeding control, the reason why they are also called hemostatic dressings (Singh et al., 2017).

Because of its physicochemical and biological qualities that make it suited for dressings, chitosan is one of the most studied biopolymers for this purpose. Chitosan also has a high film-forming capacity and mucoadhesive characteristics that may be used in a variety of administration routes (Andreani et al., 2015; Barbosa et al., 2016; Severino et al., 2014; Severino et al., 2013).

Chitosan aids in the histoarchitectural remodeling of the tissue (Muzzarelli, 1989), in the activation and multiplication of inflammatory cells in granular tissues (Alemdaroğlu et al., 2006) and macrophage activity, all of these promoting the healing process (Balassa and

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Prudden, 1978). In some studies, chitosan resulted in a significant reduction in healing time and little scarring (Muzzarelli et al., 1999). Compounds like copaiba oleoresin and aloe vera have been used in wound dressings because of their healing properties, that aid synergistic effects to bioactivities of chitosan.

Aloe vera (*Aloe barbadensis* Miller) is an herbaceous succulent plant, native from South Africa that grows in tropical zones. The fresh leaves of aloe vera contain two main components, a bitter yellow juice regulated by U.S. Food and Drug Administration as a laxative and cathartic agent, containing mainly anthraquinones and a clear mucilaginous gel obtained from the leaf pulp, applied in topical treatment of skin wounds and burns. The colorless mucilaginous gel obtained from the parenchymatous cells in the fresh leaves contains a liquid phase (99–99.5 %), composed by water, and a solid phase (0.5–1.0 %), containing mono- and polysaccharides (27.8 % of the dry weight of the gel, e.g., glucomannan, acetylated polymannan, acemannan, and mannose-6-phosphate), proteins (8.9 %), non-starch polysaccharides and ligin (35 %), vitamins (A, C, E, B12, thiamine, niacin, and folic acid) and minerals (sodium, potassium, calcium, magnesium, manganese, copper, zinc, chromium, and iron) (Boudreau and Beland, 2006).

The biological activity of the polysaccharides and glycoproteins present in leaf pulp of aloe vera has been attracting the healing and anti-inflammatory properties. The healing effect is mainly associated to glucomannan presence that acts in the fibroblast growth and stimulates the proliferation and activity of fibroblasts, improving the collagen production and secretion by these cells (Choi and Chung, 2003). Also, aloe vera extract contains anti-septic and antimicrobial properties related with the presence of lupeol, salicyclic acid, urea nitrogen, cinnamonic acid, phenols and sulfur, which present inhibitory activity against fungi, bacteria and viruses (Joseph and Raj, 2010; Pandey and Mishra, 2010). The enzymes (such as amylase and lipase) present in aloe vera extract can promote digestion by breaking down fats and sugars, and the carbohypeptidase inactivates bradykinins and produces and anti-inflammatory effects (Shelton, 1991).

Copaiba (*Copaifera* L.) is a Leguminosae tree native to Central and South America, and Africa (Veiga Junior, 2002). Several studies have reported on the pharmacological effects of products derived from the hydroalcoholic extract of copaiba leaf (Alves et al., 2013; Brancalion et al., 2012; Senedese et al., 2013), particularly copaiba oleoresin (Veiga Junior, 2002).

The presence of sesquiterpenes, which make up more than 90 % of copaiba oleoresin's makeup, is responsible for its pharmacological effect (Leandro et al., 2012). The pharmacological activity, on the other hand, cannot be assigned to a single component since its biological activity is aided by the interaction of multiple others. The biological activity of copaiba oil has been investigated for antibacterial (Pieri et al., 2012; Santos et al., 2012), anti-inflammatory (Gelmini et al., 2013; Kobayashi et al., 2011), and anti-psoriatic effects (Gelmini et al., 2013) *Copaifera langsdorffii* oleoresin showed no cytotoxic effects on fibroblasts at 100 μg/mL and at appropriate doses enhanced wound healing in rabbits (Masson-Meyers et al., 2013). Beneficial effects of copaiba oleoresin were also observed on wound healing in rats (Paiva et al., 2002).

Our research group previously published two papers, in which the physicochemical properties of chitosan films containing different ratios of aloe vera extract (Yoshida et al., 2021) and copaiba oleoresin (Debone et al., 2019) were evaluated. As previously shown by us, the presence of aloe vera in the composition of chitosan films resulted in superior barrier properties when compared to commercial dressings (Yoshida et al., 2021). The absorption capacity of the chitosan films decreased as aloe vera content increased, indicating a crosslinking or complexing behavior. This behavior was also demonstrated by elongation at break, which decreased by adding aloe vera to the films. Thermal analysis revealed that, regardless of aloe vera concentration, aloe vera made films more stable below 200 °C. The aloe vera slightly altered the films' physicochemical properties, and the active concentration did not affect the final properties. Our findings suggested that the films have appropriate properties for wound dressing application (Yoshida et al., 2021). Chitosan films containing 1.0 % (wt/wt) Copaiba oil demonstrated appropriate fluid handling properties for wound dressing applications. Copaiba oil was evenly dispersed throughout the chitosan matrix film. The barrier properties of chitosan improved as the oil was loaded into the chitosan network, reducing the material's water affinity. Copaiba oil was found to reduce elongation at break, resulting in a more resistant film (higher tensile strength) (Debone et al., 2019).

Based on the results of these two studies, the developed chitosan films containing aloe vera, copaiba oil and both were selected for the evaluation of their biological activities. In the present work, we characterize the microbial permeation, antimicrobial activity, cytotoxicity, and *in vivo* healing potential of the most promising chitosan films.

Table 1
Composition of all samples of Chitosan films.

Sample	Chitosan (%, w/w)	Aloe Vera (%, w/w)	Glycerol (%, w/w)	Copaiba oil (%, w/w)	AgSD (%, w/w)
Chitosan film 1 %	1.0	–		–	–
Chitosan film 1 % w/ AgSD	1.0	–		–	1.0
Chitosan film 2 %	2.0	–		–	–
Chitosan film 2 % w/ AgSD	2.0	–		–	1.0
Chitosan film 1 % w/ 0.5 *A. vera*	1.0	0.5	1.0	–	–
Chitosan film 1 % w/ 1.0 *A. vera*	1.0	1.0	1.0	–	–
Chitosan film 1 % w/ 2.0 *A. vera*	1.0	2.0	1.0	–	–
Chitosan film 2 % w/ 0.5 *A. vera*	2.0	0.5	1.0	–	–
Chitosan film 2 % w/ 1.0 *A. vera*	2.0	1.0	1.0	–	–
Chitosan film 2 % w/ 2.0 *A. vera*	2.0	2.0	1.0	–	–
Chitosan film 1 % w/ 0.1 *Copaiba oil*	1.0	–		0.1	–
Chitosan film 1 % w/ 0.5 *Copaiba oil*	1.0	–		0.5	–
Chitosan film 1 % w/ 1.0 *Copaiba oil*	1.0	–		1.0	–
Chitosan film 2 % w/ 0.5 *Copaiba oil*	2.0	–		0.1	–
Chitosan film 2 % w/ 0.1 *Copaiba oil*	2.0	–		0.5	–
Chitosan film 2 % w/ 1.5 *Copaiba oil*	2.0	–		1.0	–
Chitosan film 2 % w/ 0.5 % *A. vera* 0.5 % *Copaiba oil*	2.0	0.5		0.5	–

2. Materials and methods

2.1. Materials

Aloe vera extract, copaiba oleoresin and commercial chitosan (deacetylation of approximately 82 % and molar mass of about 1.47×10^5 g/mol) were provided, respectively, by Jung consult do Brasil Produtos Naturais Ltda (Florianópolis, Brazil), Polymar (Fortaleza, Brazil) and Viafarma Ltda (São Paulo, Brazil). Components were used as received. Chitosan was used without prior purification. Acetic acid and glycerol were obtained from Sigma-Aldrich (St. Louis, MO, USA) and

used as acidic medium and plasticizer, respectively. Silver Sulfadiazine (AgSD) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The Membracel® and Curatec™ films were purchased from Vuelo Pharma (Curitiba, Brazil) and LM Farma indústria e comercio Ltda (São José dos Campos, Brazil), respectively.

2.2. Preparation of chitosan films

The films were prepared by the casting technique according to our previously described works and following the composition shown in Table 1 (Debone et al., 2019; Yoshida et al., 2021). Briefly, chitosan was solubilized in acetic acid for two hours before preparing the films. To prepare Aloe vera-loaded chitosan films, the aloe vera extract was previously solubilized in water before adding to the chitosan solution and glycerol was used as a plasticizer, while the copaiba oleoresin was directly added to the chitosan solution for homogenization without a plasticizer adding. After homogenization (24,000 rpm/min for 10 min), the filmogenic solutions were poured on Petri dishes. (0.21 g/cm^2) and dried in a forced-air oven (Tecnal TE-394/2-MP, Piracicaba, São Paulo, Brazil) at 40 °C 24 h. Two chitosan concentration were evaluated, 1 and 2 % m/m, while three aloe vera concentrations (0.5 %, 1.0 % and 2.0 % w/w) and three copaiba oleoresin concentration (0.1 %, 0.5 % and 1.0 % w/w) were assessed. For the production of AgSD-loaded chitosan film (at 1 % or 2 % of chitosan with 1 % of AgSD) the same methodology used for aloe vera-loaded chitosan films was followed, however without glycerol.

2.3. Assessment of microbial permeation

The microbial permeation was assessed according to Wittaya-Areekul and Prahsarn procedure (Wittaya-areekul and Prahsarn, 2006), after few method modifications. Fifty milliliter of nutrient broth (TSB, tryptic soy broth) was sterilized in 100 mL penicillin glass flasks (vials). Films samples were held in the mouth flasks using a wieldable PVC union. The sealing was guaranteed with parafilm all around the PVC union. The negative control was done with the use parafilm as film sample while the positive control consisted of the system (penicillin flask + PVC union) without a film sample, that is, open and exposed to air contamination. The films were previously sterilized by exposure to UV light for 5 min on each side, while the wieldable PVC unions were sterilized by gamma radiation. The flasks were exposed for 10 days in ambient conditions, and turbidity and microorganism growth were evaluated macroscopically on days 0, 5 and 10 of the experiment. Five repetitions were made for each sample.

2.4. Antimicrobial activity in agar medium

In Petri dishes containing approximately 15 mL of TSA medium, a concentration of 10^3 CFU of each tested microorganism *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538) was inoculated with the aid of sterile inoculation loops. The films used in this specific experiment were dried together with bond paper. Samples with a diameter of 9 mm from these films were sterilized in UV light for 5 min each side and placed on the inoculated plates and incubated for 12 h at 35 °C. After this period, the inhibition halos formed for each sample were read. As positive controls, AgSD films were used, while for the negative control, samples of sulfite paper of the same size as the films were used.

2.5. Antimicrobial activity in broth medium

The antimicrobial activity in liquid medium was carried out in triplicate for each sample, for the repetitions. 10^3 CFU.mL^{-1} of each tested microorganism *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538) were incubated in individual test tubes that contained 10 mL of TSB medium (tryptic soy broth, Acumedia, Michigan, USA). The film samples with a size of 10 mm in diameter were sterilized by UV radiation for 5 min on each side and then incubated in the test tubes. The positive control was set up with only the incubation of the microorganisms in the test tubes individually, while as the negative control, the test tubes contained only TSB culture medium were used. The test tubes were incubated at 30 °C, shaking for 12 h in a thermostatic bath equipment (SL 155/22, Sotelab). The results were read macroscopically, observing the turbidity.

2.6. Cytotoxicity

The cytotoxicity was evaluated following two approaches, namely, the assessment of the indirect cytotoxicity for which the extracts were prepared with culture medium; and the assessment of the direct cytotoxicity for which the cells were directly in contact with the films. This latter test was performed to compare the *in vitro* results with the *in vivo* ones. Adhesion tests were also carried out.

2.6.1. Indirect cytotoxicity

Indirect cytotoxicity was performed following too distinctive extraction procedures: the first one, for selection of non-cytotoxic films, used culture medium without serum to extract possible polar substances, and the second one used culture medium with serum for extraction of possible nonpolar substances. Balb/c 3 T3 clone A31 (ATCC® CCL¬163™) were cultured with DMEM (Gibco®, EUA) supplemented with 10 % (v/v) FBS (Vitrocell, Brazil), 1 % L-Glutamine, and 1 % antibiotic solution (10,000 UI.mL^{-1} penicillin, 10 mg.mL^{-1} streptomycin and 1 mg.mL^{-1} amphotericin B). The cells were incubated at 37 °C in a 5 % CO_2 atmosphere with controlled humidity in passages 17 to 21. When they reached 80 % confluence, they were detached from the bottles using trypsin (0.05 % trypsin solution and EDTA 0.02 % in phosphate buffer pH 7.2) and plated in 96-well plates (P96w), with 12,000 cells per well. Each film was cut and decontaminated by exposure to the laminar flow UV lamp for 15 min each side. After decontamination procedure, the films were placed separately in sterile tubes containing culture medium without serum to the proportion of 6 cm^2. mL^{-1} for 72 h at 37 °C. The extracts were filtered using 0.45 μm pore membranes, and serial culture medium dilutions (1:2) were made until the concentration of 6.25 % of the extract. The plate's cell culture medium was removed after 24 h and the extracts were added. Each dilution was performed in quadruplicate. The plates were placed in a humid incubator with 5 % CO_2 and 37 °C for 24 h. After incubation, the extracts were removed, the wells washed twice with PBS pH 7.4 and a new culture medium with MTS was added for color development for 3 h. The plates were read at a wavelength of 490 nm (Synergy HTX; Biotek). Cell viability was calculated. Culture medium was used as cell viability control and sulfadiazine and Curatec™ films were chosen as a positive control of the assay.

2.6.2. Direct cytotoxicity

In direct cytotoxicity test it was used 12-well, for each one, an insert was placed at the 1 cm diameter film previously sterilized by exposure to the UV lamp and 50,000 cells were seeded and incubated for 24 h in the humid incubator with 5 % CO_2 at 37 °C. After 24 h of incubation, cell viability was determined by using MTS as previously described.

2.7. Cell adhesion assay

The cells used for this test were the same ones used in the cytotoxicity test, as well as their maintenance and number of passages. For this test, the cells were detached from the culture bottle with the aid of a cell scraper. The films were cut to a diameter of 13 mm, sterilized by exposure to the UV lamp for 15 min each side and placed in a sterile 12-well plate. Culture medium (DMEM supplemented with 10 % fetal bovine serum) was added and placed in the incubator. After 24 h the medium was removed and cell suspension with 50,000 cells was added

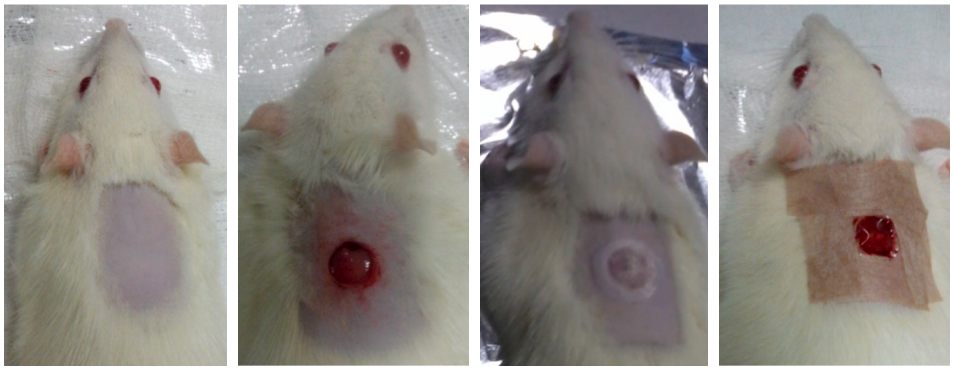


Fig. 1. Representative photos of the preparation and performance of surgery for subsequent fixation of curative membranes. (a) back after epilation; (b) skin removal; (c) dressing film placed over the wound; (d) film wrapped with Micropore® for fixation on the animal's skin.

to each film. The plates were placed in a humid incubator with 5 % CO_2 and 37 °C for 4 h. After incubation, the films were removed from the wells and transferred to another plate; were fixed with methanol for 5 min, washed with PBS pH 7.2, observed under an optical microscope with a 40X magnification objective and photographed. As a control, an empty well of the culture plate was used.

2.8. In vivo tests

The protocol implemented for the animal experimentation was approved by the Institutional Animal Care and Use Committee of Federal University of São Paulo (UNIFESP, Protocol #0777/10, 2010). Thirty-five female adult rats (*Rattus novergicus* albinus, *Rodentia mammalia*), with an average age of 45 days, weighing an average of 210 g, from the bioterium of the Nuclear and Energy Research Institute - IPEN/CNEN, were used. The animals were bred and kept in this bioterium, separated into 7 experimental treatment groups: Group I- treated with a Membracel® (commercial crystalline cellulose-based dressing); Group II - treated with chitosan film; Group III – treated with 1 % AgSD-loaded chitosan film; Group IV - treated with 0.5 % copaiba oil-loaded chitosan film; Group V - treated with 0.5 % Aloe vera-loaded chitosan; Group VI- treated with 0.5 % copaiba/0.5 % Aloe vera-loaded chitosan film; Group VII - control group (no chitosan film, dressing made with dry gauze).

In the surgical procedure, the animals were anesthetized with Ketamine (75–100 mg/kg) and Xylazine Hydrochloride (10 mg/kg), intramuscularly. Once the anesthetic plane was reached, each animal was placed on a surgical board, in the prone position. The chirurgic technique (Fig. 1), after epilation, followed the same standardization for all animals, that is, a deep circular wound (~16 mm in diameter) guided by a transparent plastic mold and removal of the *panniculus carnous*. The animals received a dressing according to the belonging group and this was fixed on the wound with adhesive tape (Micropore®) around it and the film was rehydrated with one to two drops of sterile saline solution. At the end of the procedure, the animals were placed back in their respective cages and observed until their recovery from anesthesia.

The animals received food and water, were examined daily, and the condition of the wound and its possible complications were noted in the protocol sheets and photographic records. The permanence time of each dressing was also recorded, and wound measurements were taken with a caliper. The lesions were photographed with standardized distance and conditions using a single digital camera. Euthanasia was carried out, one day after the closure of the wound in each animal, by CO_2 inhalation. The animal's dorsum skin was dissected and preserved in a 10 % formalin solution, for further histological analysis, in sections stained with hematoxylin-eosin, by optical microscopy.

2.9. Statistical analysis

Statistical analysis was performed by a BioEstat 5.3 (Instituto Mamirauá, Tefé, AM, Brazil) using Tukey's test for each time with $p < 0.05$.

3. Results and discussion

3.1. Microbial permeation

The microbial permeation test was adapted from Augustine et al.

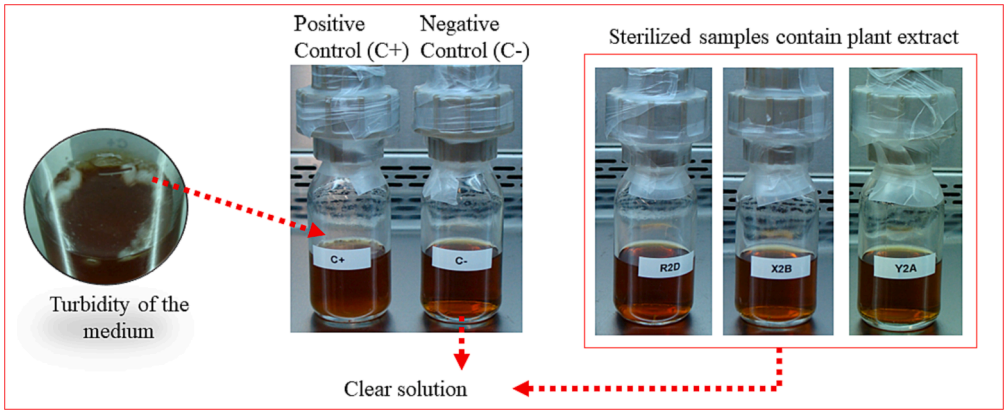


Fig. 2. Turbidity of the media of sterilized samples containing the plant extract, in comparison to the positive and negative controls.

(2015) (Augustine et al., 2015) that proposed an alternative method for skin substitute and wound dressing applications. In our work, the membranes were also evaluated *in vitro* as a barrier to microbial penetration for 10 days. The test was modified from Wittaya-Areekul and Prahsarn (2006) (Wittaya-areekul and Prahsarn, 2006) and was similar to that proposed by Augustine et al. (2015) (Augustine et al., 2015). The macroscopic evaluation of the positive control flasks ensures that the nutrient broth was suitable for microorganism growth and could represent an open wound, while the negative control flasks were tested to represent the efficiency of the sterilization process. The results showed that only the positive control presents microorganism growth, whereas all the membranes tested showed no visible microbial contamination, even after the tenth day. This indicates a good potential of the films for being used as wound dressing due to their capability to prevent the microbial permeation and consequently a secondary microorganism infection (Wittaya-areekul and Prahsarn, 2006).

For the microbial permeation tests, the 1 and 2 % chitosan films were evaluated, having different aloe vera and copaiba oleoresin concentrations, as well as the chitosan films containing AgSD. The results showed that none of the chitosan film samples allowed the microbial permeation since the turbidity of the nutrient broth was not seen for all samples, therefore, all broths were completely cleaned from the beginning to the end of the 10 days of the experiment. However, it was observed that for the positive control samples, the turbidity of the medium occurred from the second or third day of the experiment. All negative control flasks also did not show turbidity, indicating the proper choice of parafilm as an impermeable medium to microbial permeation (Fig. 2).

3.2. Antimicrobial activity

The 2 % chitosan films showed the best handling properties, and therefore, only these were chosen for the antimicrobial activity tests. Initially, the microbial inhibition experiment was designed to be carried out following the same methodology proposed by Lee et al. (2002) (Lee et al., 2002), in which the films after incubated in plates inoculated with bacteria and for a certain period of time were removed and placed in another plate inoculated with the same amount of bacteria previously inoculated. Such a model would allow the analysis of the time required for the formation of the inhibitory halo for the films and whether it was formed in an effective time to be used as a dressing, however the chitosan films with tested bioactive did not show a good interaction with the culture medium proposed (Mueller Hinton), when the films were directly deformed over the medium, which prevented the formation of light halos around them. As an alternative to solve this repulsive effect between the films and the culture medium, sterilized metal rings were used, placed over the films to hold them over the culture medium, this action allowed the films to stay in the required position for a longer time. for the formation of halos, however, after some time, the films detached from the rings and also deformed over the culture medium, making the formation of halos impossible again.

As a second attempt to overcome this problem, the solutions of the films were placed to dry on bond paper inside the Petri dishes normally used to make them. After drying, the film-coated papers were cut in the same way and placed on the culture medium (TSA) with the bond paper side facing up, so that it would exert weight on the film throughout its area and the film would have more difficulties to wriggle over the culture medium. Through this attempt, the results demonstrated in this experiment were obtained.

It is believed that there must be an incompatibility between the constituents of the culture medium and those present in the film, specifically chitosan, a substance in greater quantity in the formulation of the film. Due to this incompatibility, the culture medium used for the test was changed, from Mueller Hinton to TSA, due to the visualization of a better interaction of the latter with the film samples.

Ponce et al. (2003) (Ponce et al., 2003) classified the diameter of the inhibition halos as follows: not sensitive for diameters<12 mm; sensitive for diameters of 13–18 mm; very sensitive for diameters of 19–23 mm; and extremely sensitive for diameters larger than 24 mm)(Pereda et al., 2011). No halos were observed for the films contained chitosan and Aloe vera or chitosan and copaiba oil, excepted for the sulfadiazine films, used as a positive control (photographs not shown), that formed inhibition halos (9 to 12 mm), when compared with the samples, and were attributed as significant, however, according to Ponce et al. (2003) (Ponce et al., 2003) classification, they will be considered as not sensitive.

The antimicrobial activity of AgSD has already been reported in other studies, such as that of Lee et al. (2002) (Lee et al., 2002) that found inhibition's zones around films containing AgSD for the bacteria *P. aeruginosa* and *S. aureus*. No significant inhibition halo was induced by the bioactive films for any of the tested microorganisms in our experiments. One of the reasons proposed by several authors who found similar results is that there is a limitation for the diffusion of the film on the agar (Pereda et al., 2011).

Some studies have shown that only the soluble protonated fraction of chitosan, which is released from the solid particle in contact with a liquid phase, in an antimicrobial test can act as a biocide, through its interaction with negative microbial cell groups as described previously, this way it can be considered that the antimicrobial activity in insoluble chitosan films is exceedingly small (Pelissari et al., 2009).

Pranoto et al. (2005) (Pranoto et al., 2005) analyzed the antimicrobial activity of chitosan films against various microorganisms (*E. coli*, *S. aureus*, *S. typhimurium*, *L. monocytogenes* and *B. cereus)* and did not observe any inhibitory activity against any film, probably due to the fact that chitosan blocked the migration of active agents.

Since chitosan is in solid form, only microorganisms in direct contact with the active sites of the film could be inhibited. In the form of a film, chitosan is unable to diffuse through the adjacent agar media. Fernandez-Saiz et al. (2009) (Fernandez-Saiz et al., 2009) reports that the chitosan film is not an effective antimicrobial agent against *S. aureus* and that only in the form of gel or solutions it has optimal biocidal properties. Under these conditions, the amine groups of the biopolymer are protonated. López-Caballero et al. (2005) (López-Caballero et al., 2005) also investigated the antimicrobial effect of chitosan on the growth of Gram-negative bacteria and found that the addition of chitosan to the bacterial culture resulted in no effect on microbial growth. These results can be attributed to the low solubility of chitosan at neutral pH and in the presence of uncharged amine groups (Pereda et al., 2011).

The incorporation of bond paper together with the films caused, among other limitations, the lack of homogeneity in the dispersion of the film solution on this surface, which may have generated a lack of homogeneity between the concentrations of bioactive and chitosan throughout the length of the films, leading to the absence of visualization of inhibition halos. One of the ways found to reduce the issue of the low diffusion of bioactive substances present in the films to the culture medium was carried out the antimicrobial inhibition test in liquid medium.

Antimicrobial activity in liquid medium was performed using an adaptation of the broth dilution method. In this method, the relationship between the proportion of growth of the challenged microorganism in the liquid culture medium and the concentration of the substance to be tested is analyzed. The evaluation is compared against a biological reference standard and the density of turbidity caused by microbial growth is considered as indicative of antimicrobial action. The method can provide quantitative results and is not influenced by the growth speed of the microorganisms, in addition to providing an intimate contact between the culture medium and the sample allowing a greater diffusion to the medium. Its main disadvantage is the difficulty in detecting contamination in the case of testing clinical materials.

As a positive control, the broth inoculated with the microorganism was used in the same concentration used for the others, and as a negative control only the sterilized culture medium. After this test, microbial inhibition tests were performed in liquid medium, using TSB broth and

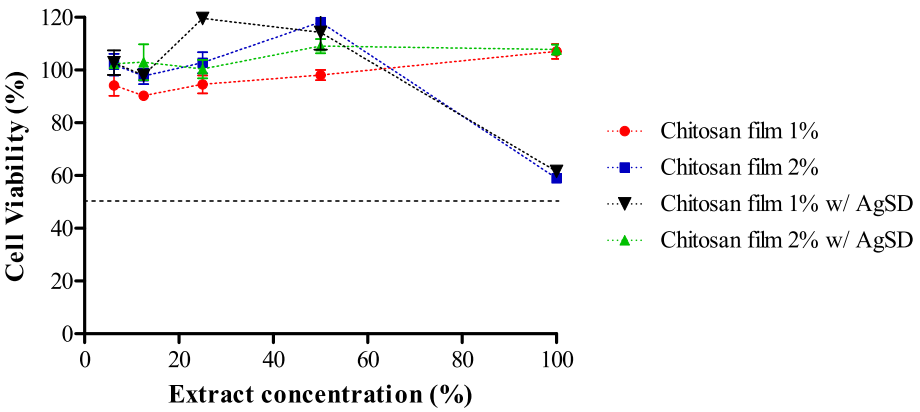


Fig. 3. Dilution curves and Balb/c 3 T3 clone A31 cells viability of the positive (chitosan film with AgSD) and negative (chitosan film) controls.

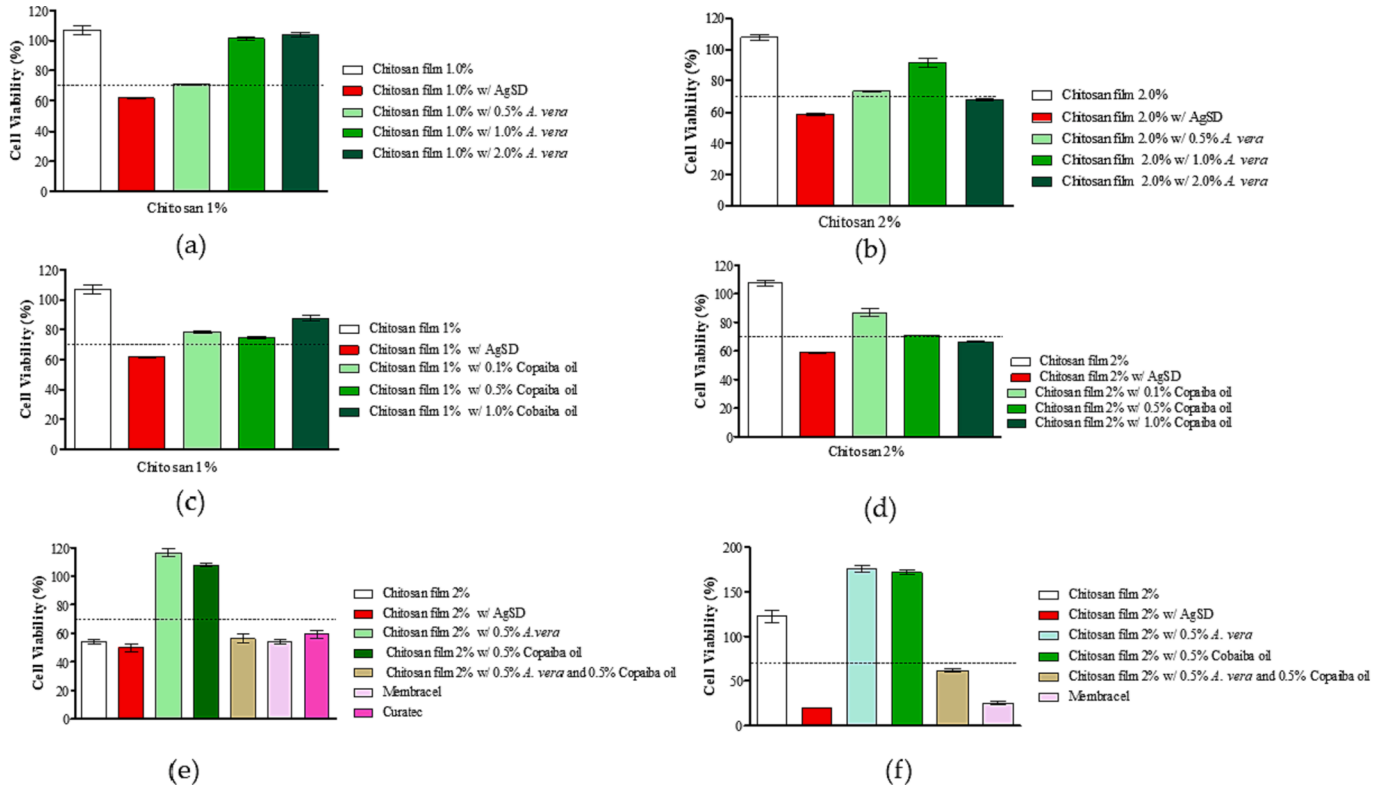


Fig. 4. Cytotoxicity results in Balb/c 3 T3 clone A31 cell lines of films produced with 1 % (a) or 2 % (b) of chitosan, combined with different concentrations of *A. vera* (0.5 %, 1.0 % and 2.0 %); films produced with 1 % (c) or 2 % (d) of chitosan, combined with different copaiba oleoresin (0.1 %, 0.5 % and 1.0 %) in comparison with chitosan film containing AgSD; Films obtained with chitosan 2 % containing different concentrations of *A. vera* and/or copaiba oil (e); and cell viability of chitosan films using the direct cytotoxicity test (f).

an inoculum of 10^3 CFU/mL for each tested microorganism. The results showed that the films do not present antimicrobial activity in the broth medium. Although similar experiments have not been found in the literature with the use of culture broth for antimicrobial activity for sulfadiazine, the results of this experiment show that diffusion of sulfadiazine in liquid medium is more effective than in culture medium. The results also suggest that even the highest concentrations of aloe vera extract and copaiba oleoresin were not sufficient to promote microbicidal activity in our films.

Three alginate-chitosan matrices using different proportions of alginate, chitosan, aloe vera and silver nanoparticles (AgNPs), which were incorporated into the polymeric system through immersion methods, were evaluated by Chabala, Cuartas & López (2017) (Gómez Chabala et al., 2017) and the results showed antibacterial activity against *S. aureus* and *P. aeruginosa*. Nevertheless, the strains used at their experiments are different from the ones used in our work and the method, although not fully described, was also different. The authors used a recharging process with aloe vera gel for 24 h and subsequently 20 μL of silver nanoparticles addition, which could have led to the antibacterial real activity.

On the other hand, Haeriah et al. (2017) (Haeriah et al., 2017) developed a patch of chitosan an Aloe vera and their results regarding the antimicrobial effect against *S. aureus* are similar of that here obtained, as the inhibition halo do not exceed 12 mm.

3.3. Cytotoxicity

Devices for use in the healthcare field must undergo biological tests

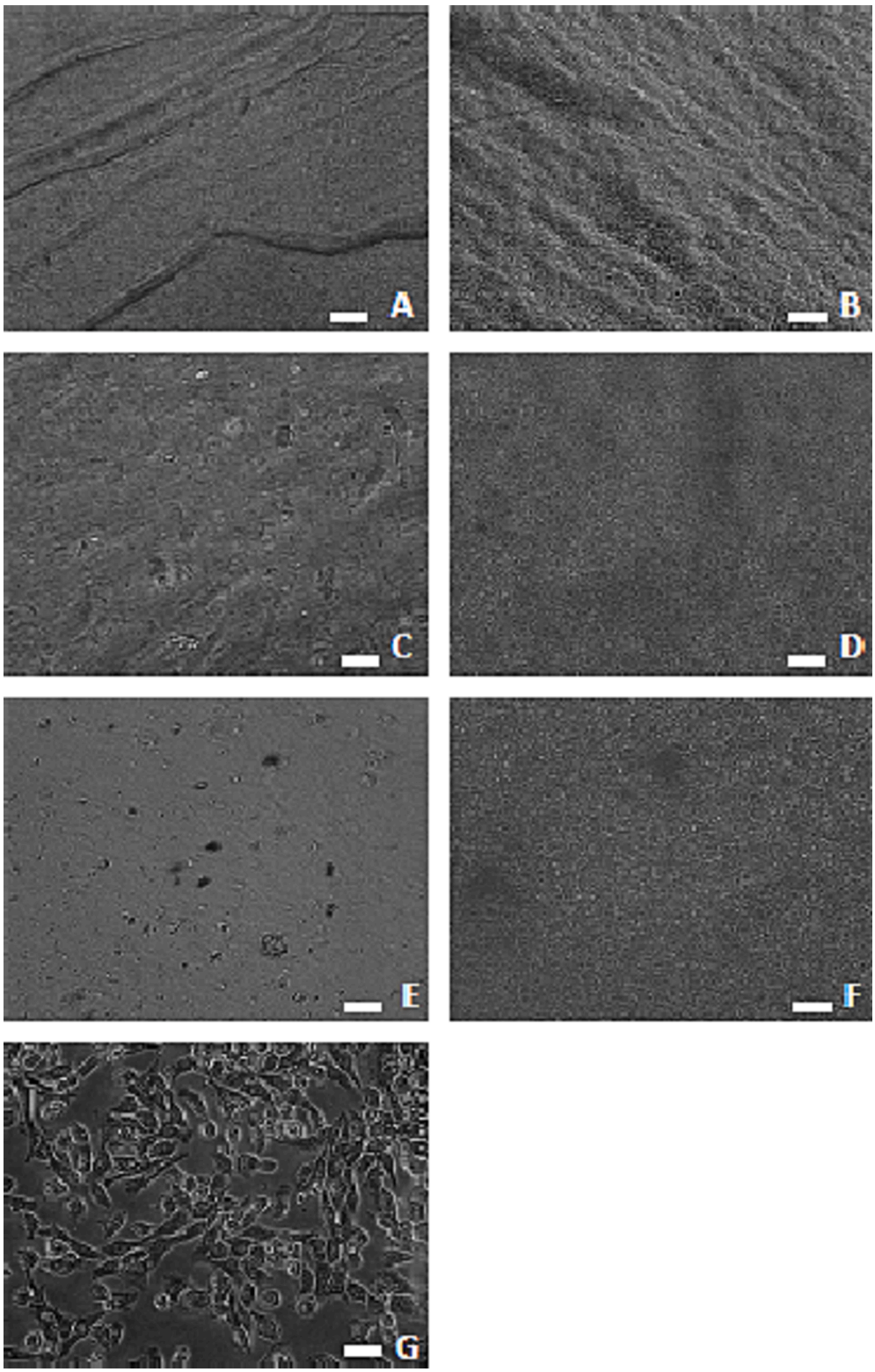


Fig. 5. Microphotographs of the chitosan films submitted to the adhesion test. (a) Group I- Membracel (commercial crystalline cellulose-based dressing); (b) Group II - chitosan film; (c) Group III – 1 % AgSD-loaded chitosan film; (d) Group IV – 0.5 % copaiba oil-loaded chitosan film; (e) Group V – 0.5 % aloe vera-loaded chitosan; (f) Group VI – 0.5 % copaiba/0.5 % aloe vera-loaded chitosan film; (g) Group VII - control group – gauze (40x magnification). Bar = 1 mm.

to assess their safety and risk. According to the ISO 10,993 - "Biological evaluation of medical devices", a material is considered cytotoxic when the viability of the cells is lower than 70 %, when compared to the control of cells without treatment. Fig. 3 shows the chitosan film extracts as a negative control, that is, the non-cytotoxic reference film; and the extract with AgSD incorporated as a positive control, that is, the cytotoxic reference film.

Fig. 4 **(a) - (b)** and Fig. 4 **(c) - (d)** show the decrease in cell viability of pure extracts when prepared without serum in the culture medium. Fig. 4 **(e)** shows the decrease in cell viability of pure extracts when

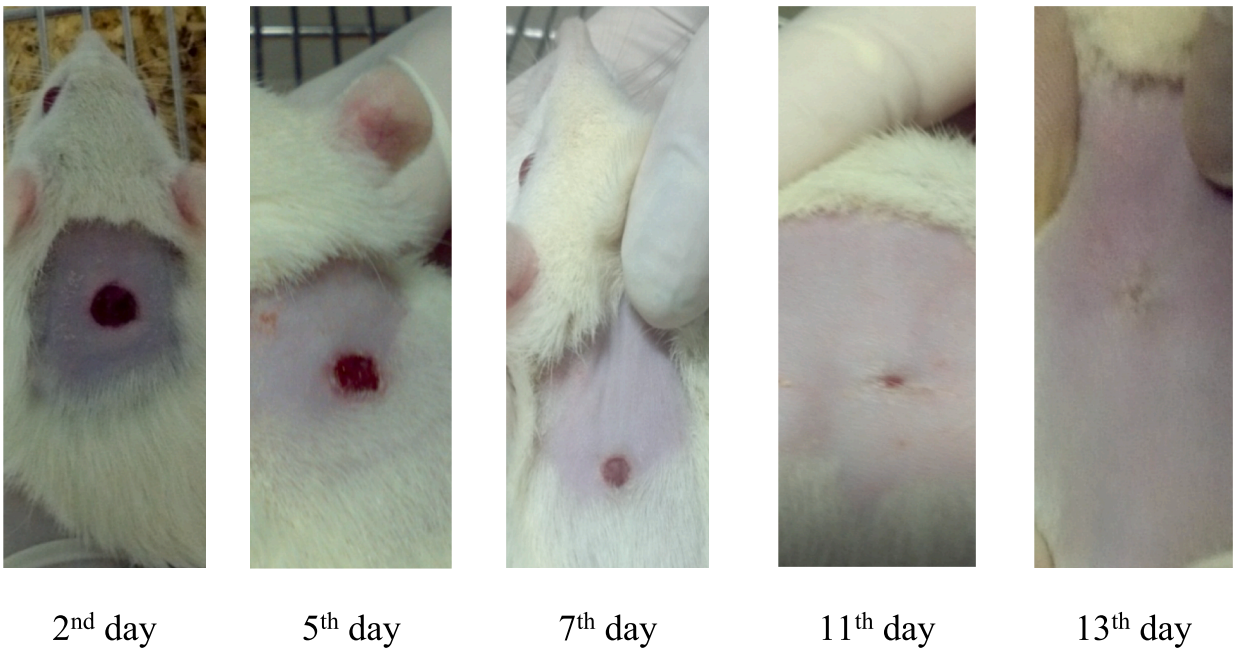


Fig. 6. Representative photos of wound healing in different time (2, 5, 7, 11 and 13 days).

prepared with the serum in the culture medium.

Chang et al. (2013) (Chang et al., 2013) studied the cytotoxicity of chitosan/2-glycerophosphate hydrogels with and without silver. The concentration of 2-glycerophosphate and the molar mass of chitosan were evaluated. They used human fibroblasts (HS68) in the presence of fetal bovine serum and verified that cytotoxicity is little affected by the molar mass of chitosan and by the concentration of 2-glycerophosphate. After 12 h, the cell viability of HS68 cells was of the order of 22.7–33.3 %, 47.7–61.3 % and 77.9–87.9 %, respectively, for hydrogels with 12 ppm, 6 ppm and 0 ppm silver. Thus, the results obtained in our study demonstrate that AgSD presented cytotoxicity lower than metallic silver, in the order of 50 %, with or without fetal bovine serum, even after 24 h.

On the other hand, Sun et al. (2012) (Sun et al., 2012) studied chitosan films containing different proportions of hydroxyapatite for use as scaffolds in bone regeneration. They used murine fibroblasts (L-929 cells) and evaluated the cytotoxicity of the composites using the same technique as this work, that is, the exposure of these cells to different concentrations of diluted extracts from the films. The cells exposed to concentrations of 6.25 to 100 % of the diluted extract of each film showed no significant reduction in viability after 24 h, which showed that the composite films were cytocompatible.

Kangjian et al. (2012) (Kangjian et al., 2012) prepared a film with the polymers chitosan, collagen and Chondroitin sulfate and incorporated microspheres containing bFGF (Recombinant Human Fibroblast Growth Factor – Recombinant Human Basic Fibroblast Growth Factor). They evaluated the cytotoxicity of the films on fibroblasts and found that the film promoted cell proliferation after 1 to 3 days. Cell proliferation has been attributed to sustained release of bFGF.

In Fig. 4 **(a) – (b)** and Fig. 4 **(c) – (d)**, where the cytotoxicity test was performed to evaluate possible polar toxic residues, it was observed that the compositions of 2 % aloe vera-loaded 2 % chitosan film (Fig. 4(b)) and 1 % copaiba-loaded 2 % chitosan film (Fig. 4 **(d)**) exceeded the limit allowed by ISO 10993–5 standard. In the other compositions, the films were considered non-cytotoxic.

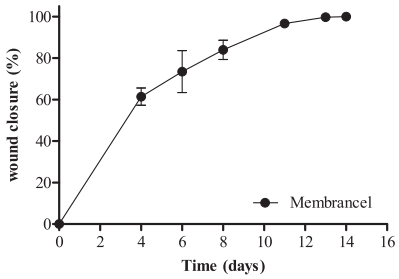
Some films were selected for *in vivo* tests, however, before these tests, cytotoxicity tests were performed in the absence of serum to check the cytotoxicity of non-polar compounds present in the films. For these tests, only films containing 2 % chitosan were selected. The film containing 0.5 % copaiba oleoresin was chosen since for 1.0 % the cytotoxicity was observed (Fig. 4 **(d)**); In addition to this, films containing 0.5 % aloe vera were also chosen, in order to compare films with the same concentration of actives, although the films with 0.5 and 1.0 % aloe vera showed acceptable cytotoxicity for use as a biomaterial (Fig. 4(b)). In addition to these two films, new films were also prepared with a mixture of both bioactive in identical concentrations (0.5 % aloe vera and 0.5 % copaiba oleoresin). All these samples were selected for *in vivo* tests with rats.

In Fig. 4 **(e)**, where the cytotoxicity test was carried out to assess possible nonpolar toxic residues, it was observed that most of the films analyzed have nonpolar cytotoxic components, including commercial dressings. Except for the compositions those containing aloe vera 0.5 % and copaiba oil 0.5 %, the figure shows an increase of approximately 20 % in cell viability, which suggests a better investigation of effects of these components on cells in culture and using molecular biology techniques to assess the proliferative effect.

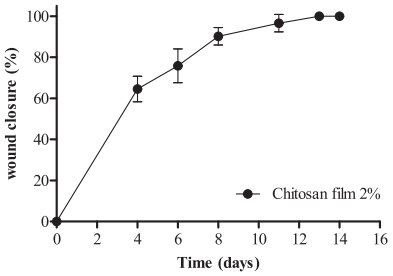
Fig. 4 **(f)** shows that 0.5 % copaiba oleoresin/0.5 % aloe vera-loaded chitosan films, AgSD-loaded chitosan film and the commercial bandage Membracel showed cytotoxicity. Membracel is a commercial and approved wound cellulose dressing typically characterized and known for its antibacterial, biocompatibility, inert, and adequate resistance dressing properties that can temporarily replace the skin (Coelho et al., 2020).

Regarding films composed only of 2 % Chitosan and separate addition of 0.5 % copaiba oil and 0.5 % aloe vera, there seems to be an increase in cell viability suggesting an increase in cell proliferation, but complementary methodologies should be used for confirmation of this phenomenon such as, for example, total cell count. With this test, we postulate that films that showed an increase in cell viability should favor the closure of wounds in a shorter time, a fact that will be discussed in the *in vivo* test.

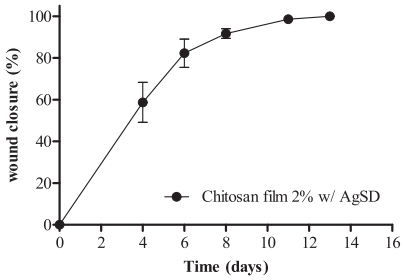
Cellular adhesion is linked to interactions between substrate components and cell surface molecules, however, the mechanisms that govern these events are not yet fully understood. That occurs because adhesion is a complex process, which involves several steps, including immediate adhesion, cytoskeleton mobilization and subsequent spreading. These steps are mediated by different sets of molecules and signals, in addition to different cell types reacting to a particular substrate in different ways (Rocha et al., 2001).



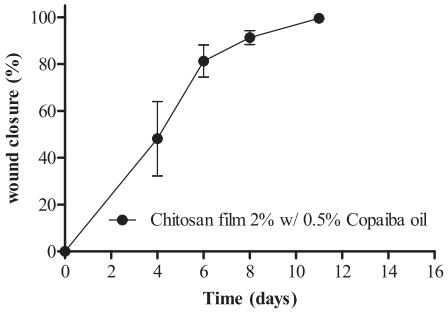
(a) Group I



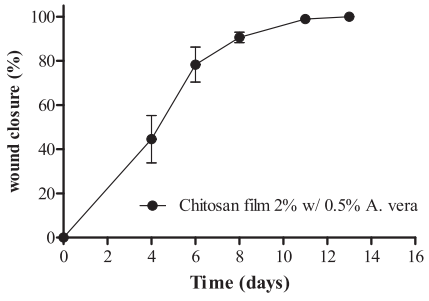
(b) Group II



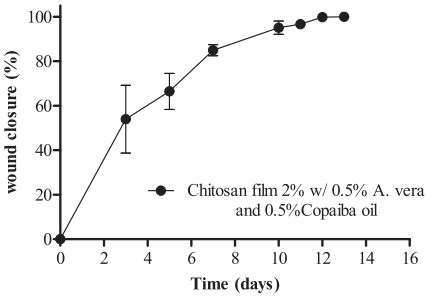
(c) Group III



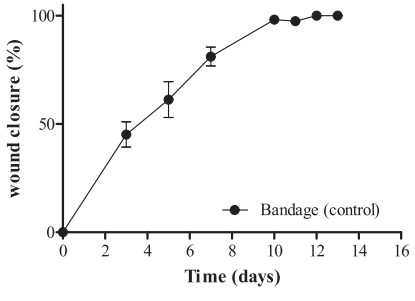
(d) Group IV



(e) Group V



(f) Group VI



(g) Group VII

Fig. 7. Representative wound closure graph analyzed for 14 days. The graphs present the results for Membracel (a), Chitosan film 2 % (b), Chitosan film 2 % with AgSD (c), Chitosan film 2 % with Copaiba oil (d), Chitosan film 2 % with Aloe vera (e), Chitosan film 2 % with 0.5 % Aloe vera and 0.5 % Copaiba oil (f), and bandage (control) (g).

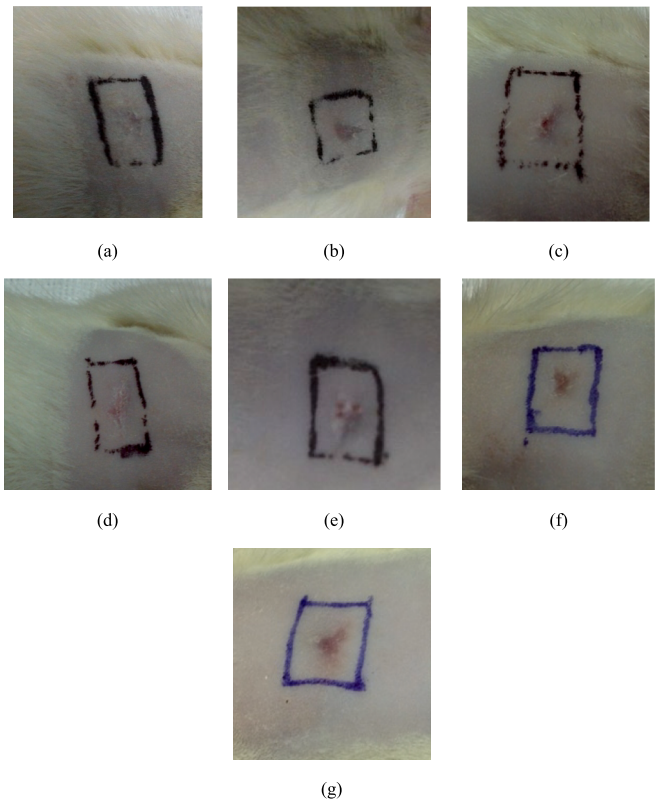


Fig. 8. Photos of the appearance of the animal's back after 1 day of wound closure. (a) Group I- Membracel (commercial crystalline cellulose-based dressing); (b) Group II - chitosan film; (c) Group III – 1 % AgSD-loaded chitosan film; (d) Group IV – 0.5 % copaiba oil-loaded chitosan film; (e) Group V – 0.5 % aloe vera-loaded chitosan; (f) Group VI – 0.5 % copaiba/0.5 % aloe vera-loaded chitosan film; (g) Group VII - control group – gauze (40x magnification).

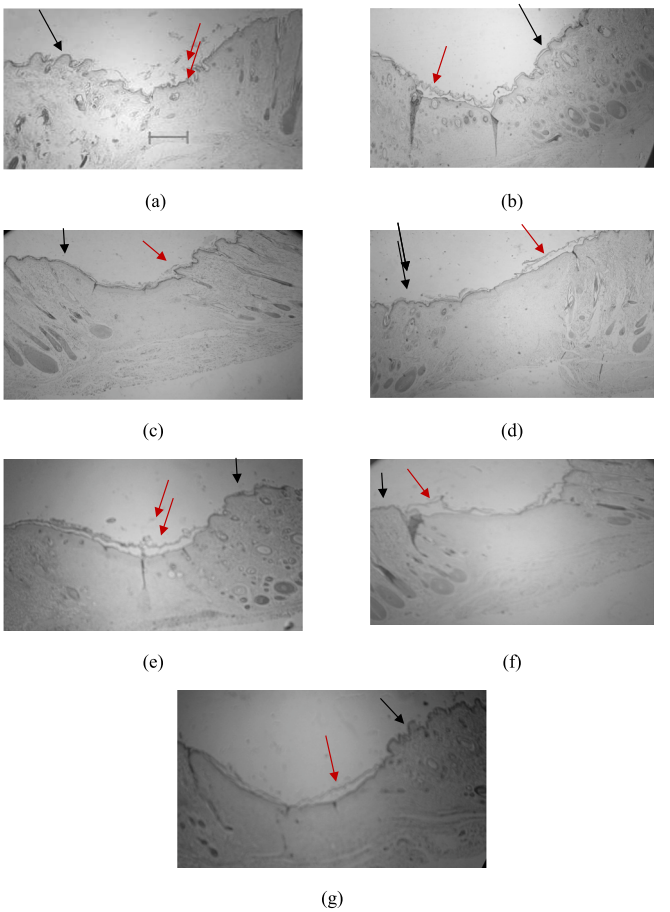


Fig. 9. Photos of the histological sections of the animal's back fragment after 1 day of wound closure (black arrow: animal's entire skin; red arrow: regenerated skin): (a) Group I- Membracel (commercial crystalline cellulose-based dressing); (b) Group II - chitosan film; (c) Group III – 1 % AgSD-loaded chitosan film; (d) Group IV – 0.5 % copaiba oil-loaded chitosan film; (e) Group V – 0.5 % aloe vera-loaded chitosan; (f) Group VI – 0.5 % copaiba/0.5 % aloe vera-loaded chitosan film; (g) Group VII - control group – gauze (40x magnification). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The cell adhesion process is a time-dependent process, also affected by the concentration of membrane receptors and ligands. In this work, cell adhesion of Balb/c 3 T3 fibroblasts was evaluated in the tested films, observing that in none of them were adhered to cells, as shown in Fig. 5. It should be noted that the incubation time of only 4 h is justified due to the knowledge that fibroblasts take 2 to 4 h to adhere to surfaces, as shown in Fig. 5G.

Hajian et al. (2017) (Hajian et al., 2017) study a thin hydrogel film composed of different ratios of PVA and aloe vera gel evaluating the ability of these hybrids to support cell growth and proliferation. Furthermore, cell spreading, and adhesion were evaluated via SEM analysis. The results demonstrated that incorporation of Aloe vera in the PVA film stimulated the proliferation of fibroblast cells, a result similar to ours. The authors postulated that this positive interaction is due to the basic fibroblast growth factor (bFGF) receptors being altered with the Aloe vera components. In addition, the authors also showed that the lack of cell adhesiveness and spreading might be considered as an advantage since it would reduce the risk of damage during membrane removal. This fact also runs with all of the samples, showing that the films do not present cell adhesive properties.

3.4. *In vivo tests*

Chitosan films with aloe vera extract (0.5 %), copaiba oleoresin (0.5 %) and aloe vera (0.5 %)/copaiba (0.5 %) presented the best physicochemical and mechanical properties according to our previous works (Debone et al., 2019; Yoshida et al., 2021). As they did not show *in vitro* cytotoxicity (except for the film containing the aloe vera and copaiba oleoresin mixture, in which cell viability was slightly below 70 %) and presented similar characteristics to commercial dressings, they were selected for an *in vivo* study. The film performance was compared to a commercial dressing (Membracel®), as well as to a pristine chitosan film and AgSD-loaded chitosan films, which is a typical drug used in the treatment of burns as ointment.

The results obtained were compared at three different levels, namely, wound closure speed, macroscopic appearance of the healed wound, and histological evaluation of healing.

To follow-up the speed of the wound closure, the animals were visually monitored and the remaining area of the wound until its closure was measured (Fig. 6)). With these measurements, the reduction of the wound area until its closure and the healing times were compared. The results were expressed as percentage of wound closure (Fig. 7).

On the sixth day, the differences between some of the groups were not significant; however, on the eighth day, Group I (Membracel®) showed a significant difference in relation to the following films (Tukey's test for each time with $p < 0.05$): Group III (AgSD-loaded chitosan film); Group IV (0.5 % copaiba-loaded chitosan film); Group V (0.5 % aloe vera-loaded chitosan film). The performance of the Membracel® dressing was inferior to these films on this day. Finally, on the eleventh day, there was a significant difference only between Group I and Group IV, and the wound closure for the latter had already occurred for all animals, which happened for Membracel® group only on the thirteenth day. The results were very similar or superior to the results

observed by most works involving chitosan films, despite having different active ingredients and different dressing addition techniques (Arockianathan et al., 2012; Hirose et al., 2007; Kofuji et al., 2010; Lu et al., 2008; Xu et al., 2007).

For this macroscopic evaluation after wound closure, the back of each animal was photographed one day after wound closure and macroscopically observed the appearance of healing. In Fig. 8 we present representative photos of this result, of one mouse for each group. Apparently, there was no difference in the result of wound closure on the back of the animals that used the test films and those that used the commercial dressing.

One day after the wound closure, immediately before the removal of the material for biopsy, the macroscopic appearance of the healing was verified, where it was not possible to observe differences between the various groups that used the test films and those that used the commercial dressing. The photos shown in Fig. 9 are illustrative of what can be seen. Apparently, in all groups, it is possible to observe a fully formed epithelium, a region of collagen that is not yet fully structured, and a neoformation of vessels that seem to be greater in some groups than in the control.

Mousavi et al. (2019) (Mousavi et al., 2019) study the healing effect of nano silver-Aloe vera composition and AgSD on burns in experimental rat models and found that the use of nano silver compounds in combination with Aloe vera compared to AgSD cream can expedite re-epithelialization and wound healing in rats.

Ranjba & Yousefi (2018) (Ranjbar and Yousefi, 2018) evaluated the effect of aloe vera with chitosan nanoparticle biofilm *in vivo*on wound healing in full thickness infected wounds with antibiotic resistant gram positive bacteria and concluded that the developed membrane presents a wound healing effects and could be used in humans.

Oryan et al (2019)(Oryan et al., 2019) investigate the effects of adipose derived stem cells (ASCs) combined with aloe vera on burn wound healing in a rat model and concluded that incorporation of Aloe vera with ASCs significantly enhanced the expression level of cytokines and growth factors and resulted in improved wound repair and regeneration.

A study to evaluate the effects of a mixture of aloe/chitosan gel in comparison to each component alone, on the healing of excisional full-thickness wounds in rats was conducted by Janahmad et al. (2019) (Janahmadi et al., 2019). The treated wounds showed healing profiles especially in combination therapy.

These recently works corroborate the results here presented showing that the films with natural products are still a very research interesting topic, and the wound healing membranes here evaluated were promising new products.

4. Conclusions

Our work corroborates the hypothesis of developing superior chitosan films combined with with *Aloe vera* or Copaiba oil for wound healing. *Aloe vera* and Copaiba did not show antimicrobial activity even at the highest concentrations of these bioactive in chitosan films; only films with silver sulfadiazine showed antimicrobial activity. The dressing containing the mixture of copaiba oil and aloe vera showed a reduction in cell viability, demonstrating the importance of studying the interaction between the two bioactive. On the other hand, dressings containing only 0.5 % copaiba oil or only 0.5 % aloe vera did not show cytotoxicity but increased cell proliferation. Additionally, cell adhesion tests showed that none of the films adhered to fibroblasts, which is an advantage for dressings that require removal, reducing tissue trauma. Healing tests demonstrated that although wound closure did not show significant differences between most samples up to the eighth day, the film containing only copaiba oil (0.5 %) showed complete closure in eleven days, i.e., two days earlier than commercial dressing and other films. In this way, the 2 % chitosan film containing 0.5 % copaiba oil is a good film for healing studies with humans in future works. Although two days may seem a short time, it can also translate two days less in the hospital and less suffering for the patient. Furthermore, the neoformation of vessels is more significant in some chitosan film groups than in the control group.

CRediT authorship contribution statement

Bianca P. Genesi: Investigation, Validation, Methodology, Writing – original draft, Data curation. **Raquel de Melo Barbosa:** Investigation, Validation, Methodology, Writing – original draft, Data curation. **Patricia Severino:** Investigation, Validation, Methodology, Writing – original draft, Data curation. **Andrea C.D. Rodas:** Investigation, Validation, Methodology, Writing – original draft, Data curation. **Cristiana M.P. Yoshida:** Investigation, Validation, Methodology, Writing – original draft, Data curation. **Mônica B. Mathor:** Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Patrícia S. Lopes:** Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **César Viseras:** Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Eliana B. Souto:** Investigation, Validation, Methodology, Writing – original draft, Data curation, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Classius Ferreira da Silva:** Investigation, Validation, Methodology, Writing – original draft, Data curation, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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