Healing activity evaluation of the galactomannan film obtained from *Cassia grandis* seeds with immobilized *Cratylia mollis* seed lectin


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**Abstract**

Galactomannan films from *Cassia grandis* seeds, associated or not with Cramoll 1.4, were used on topical wounds of rats for the evaluation of the healing process during 14 days. All of the films were evaluated by cytotoxic assay, FTIR and lectin hemagglutinating activity (HA). Forty-five male rats were submitted to aseptic dermal wounds (Ø=0.8 cm) and divided in groups (n=15): control, test 1, and test 2, treated respectively with saline, galactomannan film and film with immobilized Cramoll 1.4. Macroscopic evaluations were performed by clinical observations and area measurements, and microscopic analysis by histological criteria. Epithelial cell proliferation and differentiation was immunohistochemically assessed using CK14 and PCNA. The presence of CO peaks in the FTIR spectrum confirmed the immobilization of Cramoll 1.4 in the film, while the residual HA confirmed the stability of the lectin after immobilization with 90.94% of the initial HA. The films presented non-cytotoxicity and cell viability exceeding 80%. All of the animals presented re-epithelialization around 10 days, furthermore test 2 group showed a diffuse response at the stromal tissue and the basal layer associated with wounds completely closed with 11 days of experiment. The results suggest a promising use of the films as topical wound curatives.

**Keywords:**
Natural polymers
Topical wound curatives
Wound healing

**1. Introduction**

The wound healing is defined as a rehabilitation process with an ordered sequence of physical, chemical and biological reactions. This dynamic event comprises overlapped phases including coagulation, inflammation, proliferation and remodelling, which involves cell wall components, adhesion and activation of platelets, chemical mediators, inflammatory cells (such as polymorphonuclear leukocytes, macrophages and lymphocytes) and fibronectin; all of them acting in consonance in order to reconstruct the tissue integrity which was previously interrupted by an injury. The process initiates by extravasation of blood constituents, platelet aggregation and migration of inflammatory cells to the site of the wound; the transition from the inflammatory to the proliferative phase is characterized by migration and proliferation of fibroblasts, keratinocytes and endothelial cells, followed by angiogenesis, leading to re-epithelialisation and granulation tissue formation. Finally, the recovery of the normal tissue occurs by reduction in cellularity, vascularization, and collagen deposition, consisting in the remodelling phase [1,2].

Natural polymers are biodegradable products with potential interesting for biomedical engineering due to two major
advantages over non-biodegradable polymers: they are gradually absorbed by the human body, and some of them are able to regenerate tissues through the interaction of their biodegradation with immunologic cells [3]. In what concerns the development of different dressings for damaged tissues, natural polymers have been well studied due to their inherent characteristics, including biodegradability, biocompatibility, absence of toxicity, and some structural similarities with human tissues, as well as their implication in the wound repair [4].

The most used wound dressings are biopolymers such as chitin, chitosan and derivatives [5,6], hyaluronic acid [7], cellulose and derivatives [8], alginate [9], collagen [10], fibrin [11] or silk fibroin [12]. In general terms and according to their applications, wound dressings can be classified in different types; the film-type wound dressings are normally adhesive, with a whiteness tendency, durable, conformable, easy to manipulate, cheap, semi-permeable to oxygen and water vapour, and often impermeable to liquid and to bacterial contamination [13]. Polysaccharides are largely used as film-type wound dressings due to their ability to mimic the structure and composition of the skin. When in contact with wound exudate, these systems efficiently work on the absorption of wound fluids, thus creating a moist environment [14].

Galactomannans are polysaccharides widespread in nature. They are mostly obtained from leguminous plants and their purification process generally yields heterogenous polymers with (1→4)-linked β-mannopyranose main chains to which (1→6)-linked α-D-galactopyranosyl units are attached [15]. Scientific publications about the characterization and application of galactomannan films are still scarce when compared to other polysaccharides. In addition, the main works reported films’ physicochemical properties rather than their biotechnological applications [16,17].

Albuquerque et al. [18] extracted the galactomannan from Cassia grandis seeds, a native plant in Brazil northeast. An extensive experimental rheological characterization showed that the galactomannan presents flow behavior, exhibiting shear-thinning zones at low concentrations, and a gel-like state above the transition liquid–solid point. The ability to provide both liquid and solid features suggests the use of low concentrations of galactomannan as films with potential application in pharmaceutical and biomedical industries.

Recently, Albuquerque et al. [19] used the galactomannan extracted from C. grandis seeds at 0.8% (w/v) for the production of films containing different concentrations of lactoferrin, bioactive peptides, and phytosterols. The galactomannan film behaved as a promising structure for the immobilization of biomolecules foreseeing a great number of possible applications in food and pharmaceutical industries. Other galactomannans have been successfully used as matrix for immobilization of different biomolecules, such as nanoparticles [16], lipids [17], peptides [20], antioxidants [21] and lectins [22]. Therefore, galactomannan–based films with an immobilized biomolecule that improves the wound healing process may represent a good candidate for the treatment of wounds and should be exploited.

The lectin extracted from Cratylia mollis seeds, also known as Cramoll, is a biocompatible with different molecular forms that has been well studied by structural analysis [23,24] and employed in several biotechnological applications [25]. Preparations containing isoforms 1 and 4 (Cramoll 1,4) have been reported as potent healing agent in different experimental models [26,27]. In fact, Cramoll present properties that could enhance the wound healing process, e.g. immunomodulatory [28], antitumor [29], anti-inflammatory [30], and proliferative potential even in oxidative stress situation [31].

The objective of this work was the development of a potential wound dressing based on the galactomannan extracted from C. grandis seeds, with enhanced wound healing activity by Cramoll 1,4 immobilization. NaCl 0,15 M and galactomannan films with or without immobilized Cramoll 1,4 were used for evaluate the healing activity in male rats. Further, the epithelial cell proliferation and differentiation process was immunohistochemically assessed using cytookeratin 14–(CK14) and proliferation cell nuclear antigen (PCNA)-specific antibodies.

2. Material and methods

The pods of C. grandis were collected in the city of Angelim, while C. mollis seeds were collected in Ibimirim, both of cities in the State of Pernambuco (Brazil). Ethanol 99.8%, aceton PA, sodium chloride and phenol were obtained from Vetec Fine Chemicals Ltda. (Brazil). All other chemicals were of analytical grade.

2.1. Extraction of the galactomannan from C. grandis seeds and preparation of the filmogenic solution

The galactomannan from C. grandis seeds was obtained according to Albuquerque et al. [18]. Briefly, the pods of C. grandis were immersed in distilled water at room temperature (25 °C) for 18 h and then separated in a half part, revealing the seeds, which were removed and dried until reaching a constant weight. The dry seeds were boiled in distilled water 1:5 (w/v) at 100 °C for 1 h and conserved in water at 25 °C, for 18 h, to facilitate removal of the hull. The residual without hull was triturated in a blender with 0.1 M NaCl 5% (w/v) at 25 °C, filtered through a veil tissue and after using a screen printing cloth, and precipitated with 46% ethanol 1.3 (v/v) for 18 h. The white precipitate obtained was washed with 100% ethanol 1.3 (w/v) for 30 min and two times with acetone 1.3 (v/v) for 30 min, filtered on screen printing cloth between each washing and finally dried until constant weight. The dry precipitate was milled and called galactomannan.

Pilot experiment indicated that low concentrations of galactomannan [<1.0% (w/v)] were able to produce films, however filmogenic solutions containing polysaccharide concentrations below 0.8% (w/v) produced brittle films. Thus, the filmogenic solution was prepared in distilled water with galactomannan at 0.8% (w/v) and glycerol at 0.2% (v/v), under magnetic stirring (500 rpm), for 12 h, at 25 °C, to achieve thinner, more flexible films.

2.2. Extraction of the lectin from Cratylia mollis seeds and immobilization on the filmogenic solution

The lectin extracted from C. mollis seeds, known as Cramoll, containing its isoforms 1 and 4 (Cramoll 1,4), was obtained according to Correia and Coelho [23]. Briefly, the seeds were dried at 25 °C and crushed to obtain a flour, which was dissolved in 0.15 M NaCl 10% (w/v), under magnetic stirring (500 rpm), for 18 h, at 4 °C. Thus, the obtained saline extract was fractionated with ammonium sulphate (0–40% and 40–60%). The precipitate obtained from the 40–60% fraction was dialysed and purified by affinity chromatography on Sephadex G-75 column.

Cramoll 1,4 was immobilized by entrapment as follows: 0–0.5 mg/mL of Cramoll 1,4 were added to the filmogenic solution under magnetic stirring (500 rpm), for 30 min, at 25 °C, and the pH was adjusted to 5.8. The final solution was cast onto a 90 mm diameter Petri dish and kept at 30 °C until drying.

2.3. Cytotoxicity assay

The cytotoxic activity was determined by the bromide method [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT). Volumes of filmogenic solutions (200 μL) containing galactomannan 0.8% (w/v), added to glycerol 0.2% (v/v) and different
Cramoll 1.4 concentrations (0.1–0.5 mg/mL), were prepared under sterile conditions; then, the solutions were placed in 96-well plate and maintained at 25 °C in order to obtain the films. The dried films received a suspension of Vero cells with 10^5 cells/mL (monkey kidney fibroblasts) in RPMI medium, and the plate was incubated for 24–48 h at 37 °C. Subsequently, 5 mg/mL of the MTT stock solution was added to each well to stop the incubation time. After 3 h, the RPMI medium and the MTT excess were aspirated and 200 μL of dimethylsulfoxide (DMSO) were added to each well to dissolve the formazan crystals, whose quantity was measured at 595 nm absorbance [32]. All experiments were repeated three times with three replications per experiment.

2.4. Biological activity

The biological activity of the lectin and its stability after the immobilization in the galactomannan film were evaluated via hemagglutinating activity (HA) according to the methodology described by Correia and Coelho [23], using a suspension of rabbit erythrocytes at 2.5% (v/v) treated with glutaraldehyde. The HA was tested for a Cramoll 1.4 (0.5 mg/mL) free solution and the Cramoll 1.4 immobilized in the galactomannan filmogenic solution, being expressed as log_{10} in the results.

2.5. Immobilization of Cramoll 1.4 in the galactomannan film

The immobilization of Cramoll 1.4 (0.5 mg/mL) in the galactomannan film was confirmed by determining the composition of chemical bonds through Fourier Transformed Infrared (FTIR) spectroscopy on a VERTEX 70 (Bruker Optics, USA) spectrometer in Attenuated Total Reflectance mode (ATR). The spectra were scanned between 4000 and 500 cm⁻¹ using 16 scans at a resolution of 4 cm⁻¹.

2.6. Surgical procedure and treatment groups

All experimental procedures involving animals were approved by the Animal Ethical Committee of the Universidade Federal de Pernambuco (protocol n.² 23076.027752/2012-81). The rats were carried out in the LIKA/UFPE vivarium and kept in individual cages in a macro environment controlled with ad libitum supply of water and food, complying with the standards established by the Brazilian College of Animal Experimentation.

Forty-five male Wistar rats (90–120 day-old, weighing 250–300 g) were divided into experimental groups (n = 15) and anesthetized by intramuscular injection with xylazine hydrochloride 2% (w/v) and ketamine hydrochloride 10% (w/v) at 1:1 ratio. After the anaesthesia, each animal was shaved on the dorsal thoracic region and the clean skin was marked for the production of cutaneous wound. Aseptic dermal wound (Ø = 0.8 cm) was made by skin incision and division of epidermal layer under antisepsis with povidone-iodine 1% (w/v) and sterile solution of NaCl 0.15 M. The lesions were treated according to the group to which the animal belonged: control group, treated with NaCl 0.15 M; test 1 group, treated with galactomannan film; test 2 group, treated with the film with immobilized Cramoll 1.4 (0.5 mg/mL).

2.7. Healing activity evaluation

The healing activity was assessed according to the methodology described by Monteiro et al. [33]. After surgical procedures, the animals were clinical evaluated daily for observation of the following parameters: edema, hyperemia, exude, primary and secondary crusts, detachment and re-epithelialization. On specific days (3, 7 and 14), a visual proof of the wound healing pattern was recorded by taking digital photographs from a constant distance at the indicated time point. The time taken for full re-epithelialization of the wound biopsies was noted, the rate of contraction and surface area was measured by the standard planimetric method, by tracing the wound on transparent graph sheet. The percentage of wound contraction was calculated using the following formula:

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\% \text{ wound contraction} = \frac{[\text{wound area day } 0 - \text{wound area day } (n)]}{\text{wound area day } 0} \times 100
\]

where n = number of days (3rd, 7th, 14th day). Then, five animals from each group were sacrificed following the surgical procedure, using lethal doses of sodium thiopental (200 mg kg^{-1}). Skin fragments were collected with a wide margin (±1 cm) from the original lesion and stored in 10% (v/v) formalin, according to Aragão-Neto et al. [34].

2.8. Histological analysis

The skin lesions induced in rats undergoing different treatments were located, sectioned, and set in 10% (v/v) buffered formalin. After setting, the samples were washed with water, immersed in 70% (v/v) ethyl alcohol for 3–4 days and embedded in paraffin. Five-μm thick paraffin sections were taken and stained with hematoxylin & eosin (HE). Histological analysis of the skin sections was carried out using a light microscopy system Zeiss (Axio Scope A1) with Axiom Vision software (40× Magnification).

2.9. Immunohistochemical analysis

Serial sections were immunohistochemically processed by the method described by Hosoya et al. [35], with some modifications. The sections were subjected to autoclaving, while immersed in citric acid buffer (pH 6.0), for 15 min at 121 °C for antigen retrieval. After that, they stained for 20 min at room temperature (25 °C) and were subsequently treated with 0.3% H₂O₂ in a solution of 0.1 M phosphate buffered saline (PBS, pH 7.4), for 15 min at room temperature in order to inactivate endogenous peroxidase. Then, the sections were pre-treated with goat serum for 30 min at room temperature and incubated with primary antibodies for 18 h, overnight, at 4 °C. Mouse monoclonal antibodies against rat Cytokeratin-14 (CK14, 1:200) and rat Proliferative Nuclear Cell Antigen (PCNA, 1:400) were employed. The sections were rinsed in PBS and consecutively reacted with biotinylated goat antibody against mouse IgG (secondary antibody). They were then allowed to react with horseradish peroxidase (HRP)-conjugated streptavidin. The immune complexes were visualized using 3,3′-diaminobenzidine tetrahydrochloride (Liquid DAB Substrate Kit). The immunostained sections were counter-stained with Mayer’s hematoxylin (Lab Vision, CA, USA), dehydrated with xylene and mounted with Entellan® (Merk). Immunohistochemical analysis was carried out using a light microscopy system Zeiss (Axio Scope A1) with Axiom Vision software.

2.10. Statistical analysis

Statistical analysis were performed using two-way method of analysis of variance (two-way ANOVA). The statistical significance was set at 5% (p < 0.05) and the software used for data entry and processing was the Graphpad Prism for Windows, version 5.0 from Graphpad Software, Inc.
3. Results and discussion

3.1. Cytotoxicity evaluation

The immune system is challenged constantly by adverse factors able to cause diseases to which the medicine still has limited tools for treatment and prevention. In this context, products from natural sources are generating renewed interest in scientific research for the development of new treatments [36].

Macrophages are immunologic cells with essential role in maintaining homeostasis regardless the variations in external conditions. When an injury occurs, macrophages become the predominant cell population before migration and proliferation of fibroblasts; they also attract additional cells related to the healing process and regulate the proliferation and chemotaxis of fibroblasts, the collagen synthesis and the migration and replication of endothelial cells [36]. Regarding the importance of the properties of immunologic cells in the wound repair, the usage of fibroblast cell lines to evaluate the toxicity of products suitable for biotechnological applications is a considerable factor in the biological characterization of potential wound dressings.

Cell viability of the galactomannan film and the film with immobilized Cramoll 1.4 in different concentrations were calculated as a percentage of viable cells that were treated and compared to the untreated cells 100% viable (positive control) for 24 and 48 h. None of the films showed significant cytotoxicity when compared to respective untreated controls (Fig. 1). These results corroborate previously reported works showing that Cramoll did not induce apoptosis or toxic effects in a range of normal cells, for instance immune and proliferative cells [28,31,37] and Vero cells [31]. Moreover, these findings encouraged us to test this new formulation (the film with immobilized Cramoll 1.4) in a wound healing model.

The galactomannan film was non-cytotoxic and presented cell viability of 89.80 ± 26.27% and 101.2 ± 16.07%, respectively after 24 and 48 h of incubation. The film with immobilized Cramoll 1.4 in different concentrations (0.1–0.5 mg/mL) presented similar behavior with cell viability continually increasing with the increase in the concentration of immobilized lectin. Our results suggest that the immunomodulatory and mitogenic properties of Cramoll 1.4 allowed fibroblast cells to exercise their activity in healing process. In addition, the cytotoxicity evaluation was performed to simulate in vivo processes that usually occur when any substance promotes wound healing and tissue regeneration. With this result, the samples were test for other assays, as one can confirm in the following results.

3.2. Stability and successfully immobilization of Cramoll 1.4 into galactomannan film

Classified as part of the mannose/glucose-specific binding group, Cramoll 1.4 is a lectin that binds specifically these monosaccharides and, when that occurs, deploy several biological effects [25]. HA is a test for determining the lectin presence in a sample by forming a network agglutination between lectin binding sites and carbohydrates of the erythrocytes surface [38].

Cramoll 1.4 at 0.5 mg/mL was chosen to be used in the following analyses due to its immunomodulatory and mitogenic pattern, as well as the percentage of viable cells already presented on the cytotoxic evaluation. The HA was made to a Cramoll 1.4 free solution (0.5 mg/mL) which was benchmarked to 100% HA. The HA for the lectin free solution and the galactomannan film with immobilized Cramoll 1.4 (at 0.5 mg/mL) were 3.31 and 3.01, respectively. The residual HA confirmed the stability of Cramoll 1.4 after immobilization because the lectin retained 90.94% of its initial HA. The galactomannan film without immobilized Cramoll 1.4 did not show any HA.

FTIR was used in order to evaluate possible chemical interactions between Cramoll 1.4 and the galactomannan film, in addition to modifications in their structure (Fig. 2). The FTIR spectrum for the galactomannan film is observed in the black line, which is similar to other galactomannan spectra [18]; this result confirms the nature of the polysaccharide, i.e., the galactomannan extracted from C. grandis seeds. Particular differences could be observed for the film with immobilized Cramoll 1.4 (observed in the red line of Fig. 2). According to the literature [39], characteristics amide I band corresponds to C=O stretching near to 1640 cm⁻¹ and amide II band corresponds to C–N stretching and N–H bending near to 1540 cm⁻¹ proposing the identification of proteins. The shifting observed for the peaks 1634.47 and 1535.43 cm⁻¹ can be related to the results reported for identification of proteins by FTIR, thus confirming the presence of the lectin after the immobilization process.

Cramoll 1.4 was proved to be a stable biomolecule after the immobilization process in different supports. For example, Albuquerque et al. [40] evaluated Cramoll 1.4 contained in C. grandis seeds galactomannan gel [1.7% (w/v)] by rheometry, pH, colour, microbial contamination and lectin hemagglutinating activity along time. They suggested that this gel is a promising immobilizing matrix for Cramoll 1.4 and can be further exploited for clinical and cosmetic applications. Silva et al. [41] developed a biosensor for metastatic disease diagnosis and demonstrated that Cramoll 1.4 was able to distinguish the degree of staging prostate cancer, providing the diagnostic differentiation of benign and malign hyperplasia. Avelino et al. [42] described the development of a biosensor composed by Cramoll 1.4 immobilized by electrostatic interactions on hybrid nanocomposite (gold nanoparticles and polyaniline) to distinguish abnormal glycoproteins of sera from patients infected with dengue serotypes I, II and III. Considering the broad range of Cramoll 1.4 biological activities and possible chemical interactions, it was important to guarantee the presence and stability of the lectin after the immobilization process. In our case, the successful immobilization of Cramoll 1.4 on the film matrix is an important feature to ensure the release of the lectin biological activities.

3.3. Rate of wound contracture and macroscopic evaluation

Percentages of wound contracture for control and tested groups are shown in Fig. 3A. The comparison between the wound area on the surgery day and on the sacrifice day was expressed as percentage of retraction and analysed statistically using two-way ANOVA. The results showed that the tested groups were significantly different (p < 0.05) from control on days 3 and 7. Furthermore, control group showed the slow rate of wound contraction for the entire experimental interval. There was a significant increase (p < 0.05) in the percentage of wound contraction in the test 2 group on the 7th day, however all of the groups showed same wound contraction and re-epithelialization feature at 14 days.

Regarding the clinical observations (Fig. 3B), from the first to the third day after surgery, all of the studied groups presented crust with edema and hyperemia. Further, control group presented exudate throughout the first two days of the healing process, which was not observed in animals of the tested groups. The presence of exudate indicates an intense inflammatory response, which means more pain and confirms the difficulty to eat and drink of these animals. The galactomannan film and the film with immobilized Cramoll 1.4 worked as hemostatic tampons, protecting the wound from fluid loss and microbial contamination [43]. Comparing the tested groups, test 2 group showed less intense edema and hyperemia and an accelerated crust detachment. This fact could be related to the anti-inflammatory and wound healing activities of Cramoll 1.4. These characteristics result in significant reduction of pain and edema, as well as a better circulation to the injured site.
It is possible to observe in Fig. 3B that wound closing in tested groups happened faster than control wounds after the same time points. According to Ranjar-Mohammadi et al. [44], the quicker wound closure facilitates the biological event of healing by joining the wound edges. Due to the inherent characteristics of a polysaccharide, seems that the galactomannan contributed to the healing effects and promoted a faster wound healing. About 10 days, all animals presented re-epithelization, however it is important to point that Cramoll 1,4 improved significantly the wound healing when compared to the control group, and the wounds of test 2 group were completely closed with 11 days of the experiment. Finally, the scar tissue formed after the surgical procedure was much reduced in test 1 and test 2 groups when compared to the control, and its coloration was paler and more similar to mature surrounding tissue.

3.4. Histological observations

Cellular observation of wound tissues was possible by HE staining for control and the tested groups. The formation of epithelium, connective tissue, inflammatory response, fibroblast proliferation and collagen deposition could be seen in Fig. 4. On day 3, HE stained on tissue sections of control group showed inflammatory infiltrate, few fibroblasts and a dysmorphic protein layer directly in contact with the wound area; there was no evidence of collagen and epithelium extract for this group. The tested groups showed less inflammatory response and a slight deposition of collagen, confirming the quick healing effects of galactomannan and Cramoll 1,4 on full wound compared with control in the same time. It is important to highlight that the inflammation stage in wound healing process occurs shortly after the injury, so the immune system components act by removing damaged tissue and bacteria from the wound. Galactomannan and Cramoll 1,4 had absorb the protein layer on the wounds of the tested groups, thus enhancing the healing process and confirming the behavior also observed in macroscopic evaluation (see Fig. 3B).

On the 7th day, HE stained histopathological sections of all of the groups showed moderate inflammatory infiltrate; however, some particular differences could be observed for the tested groups. The connective tissue is fibrous in nature with less inflammatory components such as lymphocytes and blood vessels, which clearly indicates that galactomannan and Cramoll 1,4 improved a faster healing by preventing the prolonged inflammatory phase. It is possible to observe the presence of collagen for all of the groups, but collagen with much fibrils and moderate epithelial layer was formed in wounds that were treated with the film with immobilized Cramoll 1,4 (test 2 group).

On day 14 after surgery, the wounds of the tested groups showed higher degree of healing compared with the control, as confirmed by macroscopic evaluation (see Fig. 3). The inflammatory components already observed for all of the groups in the 3rd and the 7th
days were substituted by a connective tissue with collagen fibers, but the tested groups showed complete epithelialization with focal acanthosis, indicating a good and complete healing, while the control group presented rare epithelialization.

Our results agree to which has been reported for other works about wound healing experiments employing polysaccharides in association with different therapies, biomolecules or cells. In general, they reported that their components accelerated the transition from the inflammation and tissue granulation phases of the wound healing process and enhanced mature scar formation and extracellular matrix remodelling, leading to a faster wound contracture and closure. For example, Ranjbar-Mohammadi et al. [45] worked with a scaffold of gum tragacanth, curcumin and cells, and observed after 15 days a wound closure with well-formed granulation tissue. Aragão-Neto et al. [34] evaluated the effect of a hydrogel based on cashew tree (Anacardium occidentale L.) gum and chitosan associated or not with low level laser therapy and concluded that the hydrogel contributed for a most effective wound healing and modulation of the inflammatory process until 14 days. The healing mechanism of the polysaccharide extracted from Caesalpinia ferrea stem barks was investigated by Pereira et al. [2], who observed the complete cutaneous healing acquired by topical application (during 21 days, twice a day) at day 10. Tabandeh et al. [45] evaluated the skin wound repair of rats using a gel containing polysaccharides of Aloe vera and observed wound closure in 15 days. In this paper, the fact that the films were only applied on the day of the surgery, in addition to the presence of collagen in the tested groups already in the 3rd day could be considered as a great advantage for this matrix. It is also important to highlight that the wound repair observed in microscopic evaluation, confirmed by the contraction observed in macroscopic results, was an important characteristic for validation of the galactomannan as a healing agent.

3.5. Immunohistochemical analyses for CK14 and PCNA

The immunohistochemical analyses for CK14 and PCNA in the wound tissues were performed for all of the groups using DAB and hematoxylin staining. PCNA is able for detecting the proliferating cells, while CK14 is for epithelial differentiation. Cytokeratins are major intermediate filaments in all types of epithelia and are the most fundamental markers of epithelial differentiation. CK14 is a marker for undifferentiated keratinocytes and disappears at the onset of differentiation; it is normally expressed in several layers of the epidermis (basal and suprabasal layers), being expressed...
Fig. 4. Cellular observations of control (treated with saline 0.15 M), test 1 (treated with galactomannan film) and test 2 (treated with the film with immobilized Cramoll 1,4) at 3rd, 7th and 14th day of treatment. Dysmorphic protein layer, inflammatory response, collagen fibers, connective tissue and epithelium were indicated by blue, red, yellow, green and black arrows, respectively. Scale bars: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differently in the epidermal tissue during the re-epithelialization process [35,46]. On the 3rd and 7th days, no reactivity was observed for CK14 in all of the groups. On the 14th day, the results for immunohistochemical analysis of this antibody showed that there is a mark representative of the epithelium, i.e., CK14 was contained in the basal layer of the epithelium for all of the groups; however, this reaction revealed a more stratified tissue in the wounds of the tested groups. In addition, the regenerated epithelium of the test 2 group reacted strongly with antibodies for CK14 throughout the entire basal layer, thus the reaction was more immunopositive when compared to the test 1 group (Fig. 5). CK14 increased with the increase of cellular proliferation and tissue differentiation. This result agrees with those observed in microscopic and macroscopic evaluations until 14 days, and with Seltmann et al. [47], who reported that high levels of CK14 are known to lead to proliferation in vivo and found close relation with skin tensegrity. The same observations were reported in oral wounds during the re-epithelialization process of mucosa [35].

To explore the cellular mechanisms for the acceleration of wound healing by galactomannan film and the film with immobilized Cramoll 1,4, PCNA was used as a marker of cell proliferation by immunohistochemical analysis. As already observed for CK14 results, there was no reactivity for PCNA on days 3 and 7 for all of the groups. On the 14th day, we observed a similar behavior of the above mentioned immunohistochemical result: the control group showed a focal reaction in both stromal tissue and epithelial layer, while the tested groups showed a diffuse response. It is important to highlight that test 2 group had a stronger reaction when compared to test 1 (Fig. 5). The behavior observed for test 2 group corroborates the results already observed in the cytotoxic assay, i.e., that Cramoll 1,4 immobilized in galactomannan-based films could display efficiently its biological activities, including mitogenic pattern, immunomodulation, and wound healing agent.

Up to 14 days of experiment, all of the groups still showed a focal response at the basal layer of the re-epithelialized tissue, but intense diffuse response was observed at the stromal tissue and the basal layer of the re-epithelialized wound of test 2 group, which suggests that the healing process is almost finalized. Hence, the galactomannan film can be effectively used as wound dressing, especially as an alternative for synthetic ones; additionally, the wound repair can be stimulated and accelerated by the incorporation of Cramoll 1,4 into the matrix.

4. Conclusions

In this work, galactomannan films with or without immobilized Cramoll 1,4, proved to be an effective alternative to replace synthetic wound dressings in healing of wounds made on rat models. The lectin was successfully immobilized in the galactomannan film, producing films with 90.94% of the initial HA of Cramoll 1,4 and cell viability exceeding 80%. In what concerns the wounds of the tested groups, there were observed improved granulation, epithelium formation and collagen regeneration. These results could be associated with the innate properties of the galactomannan, which can introduce faster signalling pathway, resembling natural extracellular matrix and attracting fibroblasts to the derma layer. Moreover, the

Fig. 5. Immunohistochemical observations of CK14 and PCNA in control (treated with saline 0.15 M), test 1 (treated with galactomannan film) and test 2 (treated with the film with immobilized Cramoll 1,4) at 14th day of treatment. DAB and hematoxylin contrast. Scale bars: 100 μm.
imunomodulatory and mitogenic characteristics of Cranmol 1.4 accelerated wound healing process, as confirmed by cytotoxicity evaluation and immunohistochemical analyses.

Acknowledgments

PBSA and ACAN are recipients of a scholarship from the Fundação de Amparo à Ciência e Tecnologia do Estado do Pernambuco (FACEPE), and PAGS and GSA are recipients from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The authors express their gratitude to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for research grants and fellowships (MTSC, ILCB and MGCC).

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