Galactomannan of Delonix Regia Seeds Modulates Cytokine Expression and Oxidative Stress Eliciting Anti-Inflammatory and Healing Effects in Mice Cutaneous Wound

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Research Article

Keywords: Plant polysaccharides, Galactomannan, Delonix regia, Inflammation, Wound healing

Posted Date: July 19th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-697652/v1>

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Version of Record: A version of this preprint was published at International Journal of Biological Macromolecules on January 1st, 2022. See the published version at <https://doi.org/10.1016/j.ijbiomac.2022.01.144>.

Abstract

Objective and design

To investigate the healing mechanism of *Delonix regia* galactomannan (GM-DR) in a mice model of excisional cutaneous wound.

Materials and subjects

Female Swiss mice were used in all treatments.

Treatment

GM-DR (% 0.01-1) was topically applied to the wounds during 14 days.

Methods

The wound healing effect of GM-DR was evaluated by the following parameters: wound closure, clinical signs (hyperemia, edema, exsudate, nociception), oxidative stress markers (malondialdehyde – MDA, reduced glutathione - GSH), histopathological and histomorphometric analysis (collagenesis, blood vessels, polymorphonuclear, mononuclear, fibroblast/myofibroblast cells) and immunohistochemical (inflammatory growth factor mediators).

Results

GM-DR reduced wound area (7 - 14th day) and hypernociception (6 h - 5th day), leukocyte infiltration (2 - 7th day), expression and levels of IL-1 β (2th day), IL-6 (2th day), MDA (44% - 2th day), and increased fibroblast/myofibroblast, granulation tissue, collagen deposition, GSH (25 - 50%, 2-5th day), Transforming Growth Factor Beta (TGF- β) expression (7-10th day) and Smooth Muscle Alpha Actin (α -SMA) (7-14th day).

Conclusions

GM-DR accelerates the mice healing process acting both in the inflammatory and proliferative phases.

Introduction

Healing of wounds constitutes a complex process involving both molecular and cellular events that ultimately results in scar formation [1, 2]. The high cost of health services and the impact on the life quality of individuals with disabilities in the healing process motivate the development of effective products presenting favorable cost/benefit ratio [3, 4]. In this context, effects of plant polysaccharides have been demonstrated on the healing process of cutaneous wounds, along with its immunomodulatory properties and low toxicity [5–7].

Galactomannans are neutral polysaccharides found in the endosperm of leguminous seeds, are composed by (1→4) linked β -D-mannopyranosil partially substituted at O-6 with α -D-galactopyranosil groups [8]. These polysaccharides attracted the scientific and industrial interest for the properties of forming viscous solutions in aqueous medium [9]. Reports regarding its biological activities include analgesic [10, 11] and immunomodulatory effects [12].

Delonix regia (flamboyant) is a Fabaceae tree used for urbanism around the world, and its seeds contain an easily extractable galactomannan possessing molar mass in the magnitude order of 10^5 g mol^{-1} , and mannose:galactose in the range from 2:1 to 3.9:1 [13–16]. Hydrogels from *D. regia* galactomannan had been developed for usage and drug delivery system as scaffold for cell culture and soft tissue engineering in osteoarthritis [16–18]. For our knowledge there is a single report demonstrating the use of the Fabaceae galactomannan from *Caesalpinia pulcherrima* formulated for cutaneous wounds [18], however, the healing properties of *D. regia* galactomannan has not yet been investigated.

In view of the high prevalence of wounds in clinical practice and the problems associated to its treatment, added to the beneficial effects of polysaccharides on the healing process, the aim of this study was to investigate the healing effect of the seed galactomannan of *Delonix regia* in a mice model of excisional cutaneous wound.

Materials And Methods

Plant material

Seeds of *Delonix regia* (Bojer ex Hook.) Raf. were collected in the municipality of Limoeiro do Norte, Ceará, Brazil, and identified in the Laboratory of Taxonomy of Angiospermae (Department of Biology/Federal University of Ceará). A voucher specimen (No. 57951) was deposited at the Prisco Bezerra Herbarium/Federal University of Ceará. In accordance to the Brazilian Federal Law No. 13123/2015, the access activity was registered at the National System for the Management of the Genetic Heritage and the Associated Traditional Knowledge (SISGEN, code A54ED41).

Isolation of *Delonix regia* galactomannan

Delonix regia seeds (50 g) were swelled under water vapor (1 atm, 30 min) and its isolation was performed as previously described (da Silva-Nascimento *et al.*, 2020). Chemical characterization data also estimative of included weight average molar mass by high-performance size-exclusion chromatography, determination of mannose:galactose ratio by gas after acid hydrolysis and derivatization ($5.8 \times 10^5 \text{ g mol}^{-1}$ and 2.39:1, respectively). Such analysis was performed similarly to those for *Caesalpinia pulcherrima* galactomannan [19].

Animals

Female Swiss mice (25–35 g), maintained at 26 ± 1 °C under 12/12h light/dark cycle, receiving food and water *ad libitum*, were brought to the laboratory at least 1 h before the experiments. The protocol was approved by the local ethics committee (CEUA/UECE No. 01724279/2019), which followed the guidelines of the Brazilian Council of Control in Animal Experimentation (CONCEA).

Experimental protocol

Animals had their dorsal regions previously shaved and disinfected with chlorhexidine 2% gluconate. Two circular, full-thickness wounds were induced using a biopsy punch (8 mm diameter), until exposition of the panniculus carnosus. After the procedure, the animals were kept in individual cages.

Daily topical treatment was performed by direct application (100 µL) of vehicle (0.9% saline) or GM-DR (0.01 to 1% w/v) 24 h after excision until day 14. Six animals were allocated for each experimental group.

Evaluation of wound closure and hypernociception

Wounds were photographed using Sony DSC W110 digital camera, and the areas was measured using the ImageJ 1.52a software (<http://imagej.nih.gov/ij>). Percentual wound closure was calculated according to the Eq. 1:

$$\text{Eq. (1) \% closure} = 100 \times (A_0 - A) / A_0,$$

being A_0 the wound area at day 0, and A the wound area at the endpoint of interest.

Signs of hyperemia, edema and exudate were evaluated in the wound region according to the following scores: (0) absent; (1) mild; (2) moderate; (3) intense. Crust detachment (fissures, fragility) and scar tissue were reported as relative frequency (f%) of the signal appearance.

Hypernociception was evaluated using digital algesimeter (Insight equipamentos, Brazil) equipped with a polypropylene tip (4.1 mm²), which was applied to the wound edges in order to evoke behavioral responses (winches and/or writhing) [20].

Histopathological analysis and oxidative stress markers quantification

Skin samples were fixed in 10% v/v formaldehyde and embedded in paraffin for preparation of H&E stained slides (3 µm thickness). Blind evaluation was performed according to the stages of the wound inflammation from 1 to 6: (1) intense acute process, (2) mild to moderate acute process, (3) mixed process, (4) intense chronic process, (5) mild to moderate chronic process and (6) fibrosis/absence of inflammatory process. Ulcer was reported as (0) absent or (1) present [adapted from 21].

For quantification of polymorphonuclear, mononuclear and fibroblast/myofibroblast cells and blood vessels the samples immediately below the ulcer/reepithelization of each animal (5 fields per slide) were

photographed (40x magnification; Nikon Eclipse H550S microscope; Japan) and analyzed (Plugin “Cell Counter” ImageJ’s software - National Institutes of Health, EUA) [21].

Since collagen plays the major role in wound contraction during healing, collagen deposition was evaluated by examining sections (3 μm thickness) of wounds stained using Picrosirius Red (Scytech®, Tokyo, Japan). The same methodology described for image capture of HE staining was used, and the photomicrographs (conventional and polarized light) were quantitatively analyzed using the ImageJ® software. After calibration using the color threshold command, the RGB function was adjusted in images using conventional light microscopy (red = minimum 71, maximum 255; green = minimum 0, maximum 69; blue = minimum 0, maximum 92), and polarized light microscopy (red = minimum 0, maximum 255; green = minimum 0, maximum 255; blue = minimum 0, maximum 32) to determine the percentage of the total collagen area and the types I (yellow fibers) and III (greenish fibers) [22].

Homogenates of skin samples were used to determine the tissue levels of oxidative stress markers (MDA,GSH) by ELISA [21, 23].

Immunohistochemistry for cytokines and α -SMA expression

The area of ulcer/scar was marked on a slide for the tissue microarray procedure. The slide was paired with a paraffin block and a tissue microarrayer (Quick-Ray Unitma Co. Ltd., Seoul, Korea) was used to punch a sample (2-mm diameter) from the paraffin block. The sample was transferred to receptor paraffin blocks with capacity for 36 samples each [adapted from 24]. Sections (2.5 μm thick) were immunoreacted with the primary antibodies anti-TNF- α (1:100), anti-IL-6 (1:300), anti-IL-1 β (1:150) and anti-TGF- β (1:400) (Abcam™) and α -SMA (1:400) (Dako™). Slides were incubated with biotinylated ready-to-use, monoclonal anti-rabbit IgG secondary antibody, at room temperature for 30 min (K4065, Dako™). Paired sections were treated with the control IgG in substitution to the primary antibody. Staining intensity was measured according to the following scores: (0) no cells; (1) mild, 1–33% cells; (2) moderate, 34–66% cells; (3) 67–100% cells [adapted from 22].

Statistical analysis

Parametrical data was expressed as mean $\pm$ SEM, and analyzed by t test or one-/two-way ANOVA, followed by the Bonferroni’s test. Clinical signs, histopathological and immunohistochemical data were expressed as Median (maximum and minimum) and analyzed by the Mann-Whitney’s, or Kruskal-Wallis’s test, followed by the Dunn’s test. Categorical data (absent/present) was expressed as relative frequency (%) and analyzed by the Chi-Square test. $P < 0.05$ was considered significant.

Results

GM-DR reduces inflammatory signs and accelerates crust detachment and scar formation in excisional wounds

Table 1 summarizes the clinical signs displayed by the experimental groups. Intense edema was found in the wounds of the vehicle-treated animals, but the animals treated with GM-DR (0.01%, 0.1% and 1%) showed significant reduction, compared to the edema present from days 2 to 7 post-ulceration. Surrounding hyperemia was significantly reduced in the animals treated with 0.1% GM-DR during the first week. Exudate was not observed at any group, which suggests the absence of microbial growth.

From day 5, crust detachment was observed in all groups. At day 7, crust was present by 83% in the vehicle-treated animals, but was reduced to 33% by GM-DR at 0.01 and 0.1%, being absent at 1% GM-DR.

Crust was completely detached from the wound bed in most animals. All groups showed significant scar tissue formation, which was more evidenced (17%) after the galactomannan treatment, independent on the concentration tested.

GM-DR promotes wound closure, and reduces surrounding hypernociception in excisional wounds

Along the first week after ulceration the wound area was reduced by 40% in the vehicle-treated animals but was not altered by GM-DR at 0.01% and 0.1% w/v. However, at 1% GM-DR significantly increased either the wound area by 61% and the wound closure index by 24% from day 7 ($82.31 \pm 1.70\%$ vs. control: $66.27\% \pm 4.57$, $p < 0.05$) to day 14 ($94.4\% \pm 2.54\%$ vs. control: $82.7\% \pm 1.9$) (Table 2).

The mechanical threshold, determined by the pressure applied in the tissue surrounding the excisions, was reduced in comparison to the intact-skin animals. This reduction was partially reverted by the treatment with 1% GM-DR at hour 6 (54.26 ± 3.73 g vs. control: 29.01 ± 1.66 g; $p < 0.05$), being persistent until day 7 (82.84 ± 5.33 g vs. control: 63.76 ± 6.74 g; $p < 0.05$). GM-DR at 0.01% and 0.1% reduced the nociceptive response only at day 5. Thus, 1% GM-DR was chosen to be used in the following experiments (Table 2).

GM-DR reduces inflammatory cell infiltrate, and increases fibroplasia and collagenesis in excisional wounds

This semi-quantitative analysis of the healing process revealed that although GM-DR had promoted histological improvement in the inflammatory parameters, there was no statistical difference compared to controls (Table 3; Fig. 1). At the 2nd day post-ulceration, the wound tissues treated or non-treated with GM-DR presented similar profile, showing acute inflammatory infiltrate that ranged from mild to moderate to mixed inflammatory infiltrate in the saline, with predominance of mononuclear cells in the GM-DR treated group. At the 5th day after ulceration, the ulcers were still present in the GM-DR treated group, but showing predominant chronic inflammatory infiltrate (Table 3; Fig. 1).

Non-ulcerated tissue was predominant in both vehicle-treated and galactomannan-treated groups at day 7. This last has also displayed discrete fibrosis, granulation tissue and mild chronic inflammatory process, as well re-epithelialized tissues in some specimens. Saline treated groups showed intense chronic inflammatory infiltrate. At the 10th day, both are re-epithelialized, the saline group had moderate

chronic inflammatory infiltrate and few signs of fibrosis while GM-DR exhibited signs of fibrosis and mild chronic inflammatory infiltrate. At the 14th day, both groups were re-epithelialized, the saline group showed chronic inflammatory infiltrate and fibrosis. Furthermore, GM-DR present few mononuclear inflammatory cells, and organized collagen fibers with remodeling signs (Table 3; Fig. 1).

The treatment with GM-DR presented decreased the leukocyte count in H&E slices. Polymorphonuclears were reduced by 65%, 68% and 80%, at days 2, 5 and 7, respectively, whereas mononuclears were reduced only at day 5 (161 ± 11 vs. saline: 228 ± 7 , $p < 0.05$) (Table 3; Fig. 1). GM-DR increased the number of fibroblasts/myofibroblasts at day 5 by 50% (341 ± 43 vs. saline 172 ± 121) and at day 7 by 43% (512 ± 48 vs. saline 290 ± 19). However, GM-DR did not change the number of blood vessels at any day of treatment when compared to the control.

Although there were no differences in total collagen deposition after galactomannan treatment, the deposition of type I collagen (yellow-reddish fibers) was increased by GM-DR from day 5 (68.05 ± 4.04 vs. saline 37.36 ± 2.4) until day 14 (37.41 ± 3.46 vs. saline 15.54 ± 2.8), whereas the deposition of immature type III collagen (whitish-green collagen) was reduced by GM-DR, from day 7 (32.33 ± 4.3 vs. saline 52.43 ± 6.37) until day 14 (34.93 ± 3.63 vs. saline 45.51 ± 3.10) (Table 3; Fig. 2).

GM-DR modulates cytokine expression and oxidative stress markers in excisional wounds

As measured by immunohistochemical staining, GM-DR reduced the expression of IL-1 β (1 [1, 1] vs. saline: 3 [2, 3]) and IL-6 (1 [1, 1] vs. saline: 2 [1, 3]) at day 2. In addition, GM-DR increased TGF- β expression at day 7 (2 [1, 3] vs. saline: 1 [1, 1]) and day 10 (2 [1, 3] vs. saline: 1 [0,1]), and that of α -SMA from day 7 (2 [1, 3] vs. saline: 1 [0,1]) to day 14 (3 [2, 3] vs. saline: 1 [1, 2]). However, TNF- α expression was unaltered by the treatment with GM-DR (Fig. 3; Table 4).

The levels of reduced glutathione were increased during the course of treatment with GM-DR by 25% at day 2 (325.2 ± 80.26 vs. saline: 244.6 ± 20.48 μ M/g tissue) and by 50% at day 5 (531.9 ± 114.9 vs. saline: 268.3 ± 109.6 μ M/g tissue) but was unaltered by GM-DR at day 7. In addition, the treatment with the galactomannan GM-DR reduced the levels of malondialdehyde (MDA) by 44% at day 2 (1038 ± 456.3 vs. saline: 256.4 ± 148.4 μ M/g tissue) (Fig. 4).

Discussion

The present study demonstrates that topical administration of *Delonix regia* galactomannan in solution enhances the healing of excisional cutaneous wounds in mice, accompanied by the following events: 1) reduction of edema, hyperemia, nociception and inflammatory cell infiltrate; 2) increased number of fibroblasts/myofibroblasts; 3) increased deposition of type I collagen; 4) modulation of oxidative stress markers and cytokines expression.

Healing wounds occurs as overlapping phases including homeostasis, inflammation, proliferation and remodeling [1], but an imbalance during the inflammatory phase may cause either prolonged healing or excessive scar formation [25, 26]. Phlogistic signs of edema and hyperemia are found early after the induction, and are decurrent from pro-inflammatory cytokines released by infiltrating leukocytes, mainly neutrophils and macrophages, in the wound bed which also produce reactive species in order to prevent bacterial infection [26], being such signs reduced by GM-DR. Additionally, *D. regia* galactomannan inhibited both IL-1 β and IL-6 expression, and TNF- α immunostaining also seemed to be diminished, although without reaching statistical significance. In view of this inhibition, it is suggested that GM-DR would be responsible for reducing leukocyte infiltrate and inflammatory signs of inflammation by the modulation of pro-inflammatory cytokines such as IL-1 β and IL-6.

The present results were similar to previous obtained with different polysaccharides, such as those extracted from *Caesalpinia ferrea* barks in the same murine model [21], but are in apparent contradiction with those obtained from mice wounds after topical administration of a galactomannan extracted from *Caesalpinia pulcherrima*, which promoted IL-1 β and IL-6 release [18]. It is known that proteins dragged during the galactomannan extraction may induce local inflammation [10]. The extractive procedure of *C. pulcherrima* is similar to ours, except for an alkaline hydrolysis performed before the final precipitation. Such step was added to our procedure in order to remove remaining proteins without rupture of glycosidic bonding. Although we have used a different approach to determine % protein, its detection limit is similar to their colorimetric procedure (0.5%), as inferred from the original report of this technique [27]. Even considering the structural differences between these galactomannans, as molar mass and distribution profile of side galactosyl groups, it is not to be excluded that low levels of remaining proteins would actually favored inflammation evoked by *C. pulcherrima* galactomannan on excisional wounds.

In our study, the reduction in the mechanical threshold applied on the wound edges was reversed by *D. regia* galactomannan as early as 6 hours after treatment. The inhibition of pro-inflammatory cytokines such as IL-1 β and IL-6 and, consequently, the reduction of migration of these inflammatory cells may justify the antinociceptive effect of GM-DR. Our group had already demonstrated the antinociceptive effect of the polysaccharide extract of *Caesalpinia ferrea* stem barks topically applied to cutaneous wounds in mice probably by the reduced expression of IL-1 β [21], and that polysaccharides extracted from *Ximenia americana* barks reduced peripheral hypernociception induced by carragennan in mice [28].

Crust detachment and scar formation were anticipated in the animals treated with *D. regia* galactomannan. GM-DR promoted healing by second intention and retraction of the cutaneous wound, being verified by the reduction of the area and increase of the wound index in the proliferative phase. It is documented that the wound contraction, a feature of the proliferative phase of the physiological healing process, involves the participation of differentiated myofibroblasts [29]. Myofibroblasts are characterized by the expression of a particular integrin smooth muscle alpha actin (α -SMA), which determines its contractility, and the increased synthesis of MEC proteins, such as collagen types I and III [30]. The treatment with GM-DR evoked the expression of α -SMA. Thus, it is suggested that this galactomannan may act in the proliferative phase inducing the differentiation of fibroblasts into myofibroblasts. In

agreement with our study, the polysaccharides extracted from the leaves of *Plantago australis* at 500 and 1000 mg/kg increased the wound healing index seven days after the excision, as well the proliferation of keratinocytes in a horizontal migration model, modulating the levels of TNF- α [31]. In addition, galactomannans extracted from *Cydonia oblonga* increased tissue elasticity and the healing rate at higher concentrations (10–20%) [32] and that from *C. pulcherrima* increased the wound healing rate in the later stage of the healing process, at the day 10 [18]. The galactomannans of *Cassia grandis* seeds also increased the retraction of the wound and reduced the infiltration of inflammatory cells from the 3rd day of treatment [6].

The most important feature of the proliferative phase is the formation of granulation tissue (due to the granular appearance generated by the newly formed capillaries) proliferation and migration of fibroblasts to the lesion (fibroplasia) [33] under stimulation of TGF- β , fibroblasts differentiate in myofibroblasts [29], contributing to wound retraction. GM-DR increased the number of fibroblasts/myofibroblasts, which was accompanied by greater expression of α -SMA from the 7th to the 14th day, reinforcing the regulator mechanism of this growth factor, in order to accelerate the formation and maturation of the granulation tissue in the proliferative phase and an effective and coordinated tissue repair.

The profuse degradation of extracellular matrix (MEC) and type III collagen, along with formation of mature type I collagen are critical in this phase, which lasts some months and years until the formation of a paucicellular scar [30, 34]. Although total collagens had not been altered by GM-DR, the deposition was increased for type I collagen, but it was decreased for type III, which indicates an effect in the proliferative phase. GM-DR may also accelerate the production of granulation tissue, as this is correlated with the maturation of the newly formed tissue, as well as with the increase in the healing rate observed since the 7th day of treatment.

However, if the inflammatory infiltrate persists, it will hamper the proliferation of fibroblasts and vascular neoformation, contributing to the deficiency in the formation of granulation tissue [25]. Thus, the inhibitory effect of GM-DR on the inflammatory leukocytes infiltrate was accompanied by proliferation and migration of fibroblasts. According *Mimosa tenuiflora* polysaccharide promoted *in vitro* fibroblast stimulation [35] and *C. pulcherrima* galactomannan increased fibroplasia associated with collagen deposition at the 14th day post-ulceration [18].

Oxidative stress markers is often a secondary physiological event associated to inflammation, as evidenced cell stress under persistence dense infiltrate of inflammatory cells. In contrast, the inhibition of oxidative stress by superoxide dismutase, catalase or reduced glutathione prevents cell damage [25]. GM-DR reduced the levels of MDA at day 2, as well increased that of reduced glutathione at days 2 and 5, while reduced the polymorphonuclear infiltrate. Similar effects on polymorphonuclears and MDA were obtained *C. ferrea* polysaccharides treatment [21] or with the aqueous extract of *Ocimum sanctum*, which reduced MDA concomitant to increased activity of superoxide dismutase and catalase, and increased levels of reduced glutathione [36].

Although GM-DR had stimulated fibroplasia and collagen deposition, there was no increase in the number of blood vessels. It is known that neoangiogenesis is important to supply oxygen and nutrient demand of cells throughout the healing process. However studies have already shown that mild angiogenesis, but forming structured vascular network, favors the formation of adequate scar [37]. In contrast to that observed in our study, it was demonstrated that oral administration of an ethanolic extract of polysaccharides from the root of *Sanguisorba officinalis* L. in mice resulted in the acceleration of angiogenesis, via VEGF production [38], and skin wounds treated with *C. pulcherrima* galactomannan had a more pronounced neoangiogenesis when compared to controls [18]. Further studies involving specific markers of newly formed vessels, such as CD-31 and 34, may be useful to elucidate that discrepancy.

Delonix regia galactomannan (GM-DR) promotes tissue repair in mice excisional cutaneous wounds acting as anti-inflammatory via inhibition of cytokine pro-inflammatory (IL-1 β , IL-6) and healing by stimulation fibroplasia and collagenesis via increase of TGF- β .

Abbreviations

GM-DR, *Delonix regia* galactomannan; IL-1 β , Interleukin-1 Beta; IL-6, Interleukin-6; TGF- β 1, Transforming Growth Factor Beta 1; TNF- α , Tumor Necrosis Factor Alpha; α -SMA, Smooth Muscle Alpha Actin.

Declarations

Funding

This research was supported by CAPES, CNPq and FUNCAP. Ana Maria S. Assreuy is senior investigator of CNPq (Process N $^{\circ}$. 308433/2017-3).

Conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Legal permission

Studies of *Delonix regia* galactomannan are registered (N. A54ED41) in the SisGen (Genetic Heritage and Associated Traditional Knowledge System), according to the current Brazilian regulations.

Availability of data and material

All data generated or analyzed during this study are included in this published article and are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Íasly Costa Lima, Beatriz Lima Adjafre and Skarlatt H. A. F. Sousa. The first draft of the manuscript was written by Íasly Costa Lima and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval (include appropriate approvals or waivers)

The protocol was approved by the local ethics committee (CEUA/UECE No. 01724279/2019), which followed the guidelines of the Brazilian Council of Control in Animal Experimentation (CONCEA).

Consent to participate

Not applicable.

Consent for publication (include appropriate statements)

Not applicable.

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Tables

Table 1. *Delonix regia* galactomannan reduces inflammatory signs and accelerates scarring of excisional wounds in mice

	Time course		
	Day 2	Day 5	Day 7
Edema^a			
Vehicle	2 (1-3)	1 (0-2)	1.5 (1-2)
°GM-DR 0.01%	1(1-2)	1 (1-2)	0.5 (0-1)*
GM-DR 0.1%	1.5 (1-3)	1 (0-1)	0 (0-1)*
GM-DR 1%	1 (1-2)	0.5 (0-1)	0 (0-1)*
Hyperemia^a			
Vehicle	1(0-2)	1 (0-1)	1(0-2)
GM-DR 0.01%	1.5 (0-2)	1(0-1)	0 (0-1)
GM-DR 0.1%	1(0-2)	0 (0-1)	0 (0-0)*
GM-DR 1%	1(0-2)	0 (0-1)	0 (0-1)
Exsudate^a			
Vehicle	0 (0-1)	0.5 (0-2)	0.5 (0-1)
GM-DR 0.01%	0 (0-1)	0 (0-0)	0 (0-1)
GM-DR 0.1%	0 (0-1)	0 (0-1)	0 (0-1)
GM-DR 1%	0 (0-0)	0 (0-0)	0 (0-0)
Crust detachment^b			
Vehicle	0%	83%*	83%*
GM-DR 0.01%	0%	100%*	33% [†]
GM-DR 0.1%	0%	100%*	33% [†]
GM-DR 1%	0%	100%*	100%*
Scar tissue^b			
Vehicle	0%	0%	50%*

GM-DR 0.01%	0%	0%	67%*
GM-DR 0.1%	0%	0%	67%*
GM-DR 1%	0%	33%	67%*

^aMedian (minimum-maximum) *p<0.05 vs. D2; †p<0.05 vs. D5 (Kruskal-Wallis's and Dunn's tests). ^b Relative frequency (f%) *p<0.05 vs. D2 . †p<0.05 vs D5 (Chi-square test). ^c *Delonix regia* galactomannan.

Table 2. 1% GM-DR promotes closure and reduces surrounding hypernociception of excisional skin wounds in mice

Parameters	Hours					Days				
	0	0.5	1	6	12	D2	D5	D7	D10	D14
Wound area (mm²) *										
Saline	-	-	-	-	-	0.0075 ± 0.00	0.0062 ± 0.00	0.0045 ± 0.00	0.0024 ± 0.00	0.0016 ± 0.00
^b GM-DR	-	-	-	-	-	0.0077 ± 0.00	0.0060 ± 0.00	0.0030 ± 0.00*	0.0010 ± 0.00*	0.0010 ± 0.00*
Wound closure index (%) *										
Saline	-	-	-	-	-	33.53 ± 8.74	51.72 ± 4.26	66.27 ± 4.57	82.13 ± 2.97	82.7 ± 1.9
GM-DR	-	-	-	-	-	39.39 ± 4.99	61.27 ± 2.33	82.31 ± 1.7*	96.94 ± 1.94*	94.45 ± 2.54*
Hypernociception (g) *										
Intact skin	82.43 ± 6.29*	124.2 ± 7.63*	105.4 ± 2.93*	111.28 ± 8.86*	116.4 ± 9.3*	118 ± 9.16*	129.78 ± 6.44*	140.2 ± 6.23*		
Saline	21.28 ± 2.12	26.7 ± 1.68	28.43 ± 1.91	29.01 ± 1.66	32.03 ± 1.66	35.15 ± 1.66	41.80 ± 4.01	63.76 ± 6.74	-	-
GM-DR	22.00 ± 1.46	39.66 ± 3.19	35.91 ± 2.75	54.26 ± 3.73*	65.6 ± 4.12*	60.22 ± 3.10*	78.36 ± 3.70*	82.84 ± 5.33*	-	-

^a Mean ± E.P.M. *p<0.05 vs. Saline (Two-way ANOVA/Bonferroni test). ^b *Delonix regia* galactomannan.

Table 3. Histopathology and histomorphometry of the inflammatory infiltrate, fibroplasia, blood vessels and collagen in cutaneous wounds treated with 1% GM-DR for 14 days

Parameters	Groups		Days				
			D2	D5	D7	D10	D14
Histological scores ^a	Saline	Epithelium	1 (1;1)	1 (1;1)	0 (0;1)	0 (0;1)	0 (0;0)
		Connective tissue	2 (1;2)	3 (3;4)	3.5 (3;5)	4 (3;4)	4.5(4;5)
	◐GM-DR	Epithelium	1 (1;1)	1 (1;1)	0 (0;1)	0 (0;1)	0 (0;0)
		Connective tissue	3 (1;3)	4 (3;4)	5 (3;6)	4.5 (4;5)	5 (4;6)
Polymorphonuclear ^b	Saline		95.5 ± 24.27	114.5 ± 18.36	22.33 ± 7.62	-	-
	GM-DR		33.5 ± 3.14*	36.33 ± 6.88*	4.33 ± 1.4*	-	-
Mononuclear ^b	Saline		197.4 ± 11.78	228.2 ± 7.12	206 ± 12.14	-	-
	GM-DR		206.5 ± 11.02	161.2 ± 11.29*	227.2 ± 21.33	-	-
Fibroblast/myofibroblast ^b	Saline		77.17 ± 17.69	172.2 ± 121.54	290.5 ± 19.16	-	-
	GM-DR		106 ± 14.63	341.3 ± 43.3*	512.2 ± 48.14*	-	-
Blood vessels ^b	Saline		-	0.16 ± 0.16	6 ± 1	-	-
	GM-DR		-	2.5 ± 1.45	5.33 ± 0.61	-	-
Total collagen (%) ^b	Saline		-	45.8 ± 4.28	59.45 ± 4.56	57.33 ± 1.26	50.7 ± 2.35
	GM-DR		-	46.89 ± 2.54	50.99 ± 2.88	53.3 ± 2.38	54.71 ± 3.30
Type I collagen deposition (%) ^b	Saline		-	37.36 ± 2.4	12.55 ± 3.44	23.4 ± 3.77	15.54 ± 2.80
	GM-DR		-	68.05 ± 4.04*	37.54 ± 3.75*	40.37 ± 2.96*	37.41 ± 3.46*
Type III collagen deposition (%) ^b	Saline		-	28.44 ± 2.52	52.43 ± 6.37	44.8 ± 3.38	45.51 ± 3.10
	GM-DR		-	20.42 ± 1.56	32.34 ± 4.3*	30.38 ± 2.43*	34.93 ± 3.63

^a Median (minimum-maximum) *p<0.05 vs. saline (Mann-Whitney/Dunn's test). ^b Mean ± E.P.M. *p<0.05 vs. Saline (Two-way ANOVA/Bonferroni). ^c*Delonix regia* galactomannan.

Table 4. 1% GM-DR modulates the expression of inflammatory mediators and growth factors in mice skin wounds

Antibody	Groups	Days post-ulceration				
		2°	5°	7°	10°	14°
Anti-TNF- α^a	Saline	2.5 (1.3)	2 (0.3)	2 (0.3)	2 (1.3)	1 (1.2)
	^b GM-DR	1 (0.3)	1 (1.2)	0.5 (0.1)	1 (0.1)	1 (1.2)
Anti-IL-1 β^a	Saline	3 (2.3)	2 (1.3)	2 (1.2)	2 (2.3)	2 (2.3)
	GM-DR	1 (1.2)*	1.5 (1.3)	1 (1.2)	1 (1.2)	1 (1.3)
Anti-IL-6 ^a	Saline	2 (1.3)	1 (1.2)	1 (0.2)	2 (1.3)	2 (2.3)
	GM-DR	1 (1.1)*	1 (0.2)	2 (0.3)	2 (2.2)	2 (1.2)
Anti-TGF- β^a	Saline	1 (0.2)	1 (1.2)	1 (1.1)	1 (0.1)	1 (1.2)
	GM-DR	2 (0.2)	1.5 (0.3)	2 (1.3)*	2 (1.3)*	2 (1.3)
Anti- α -SMA ^a	Saline	2 (1.3)	1 (0.1)	1 (0.1)	1 (1.2)	1 (1.2)
	GM-DR	1 (1.2)	1 (1.3)	2 (1.3)*	2 (1.3)*	3 (2.3)*

¹ Median (minimun. maximun) * p< 0.05 vs saline (Mann-Whitney/Dunn's test) ² *Delonix regia* galactomannan.

Figures

Figure 1

Photomicrographs of mice skin wounds treated with GM-DR. 1% GM-DR was topically administered once a day for 14 days. 0.9% saline was used as control. Leica microscope (200x) 5 fields - HE staining. Saline (a – e); GM-DR (g – j) (color figure online).

Figure 2

Photomicrographs of mice skin wounds treated with GM-DR. Leica microscope (200x) 5 fields in optical microscope (total collagen) and polarized light (collagen type I and III) measured as percentage of collagen area (Image J). 1% GM-DR was topically applied 1x / day for 14 days. Picrosirius staining. Saline (a – d); GM-DR (e – h) (color figure online).

Figure 3

Photomicrography of immunohistochemical for IL-1 β , IL-6, TGF- β and α -SMA in mice skin wounds. Photomicrographs of immunohistochemical slides (Leica Microscope; 400x magnification). % cytoplasmic or nuclear expression classified by scores for positive cells: (0) absence; (1 - mild) 1-33%; (2 - moderate) 34-66%; 67-100% (3 - intense) (color figure online).

Figure 4

GM-DR increases reduced-glutathione and malondialdehyde in mice skin wounds. 1% GM-DR or 0.9% saline were topically administered 1 x/day. (a) GSH (A412 nm) and (b) MDA (A535 nm) by ELISA.