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Wound tissue remodeling by latex exudate of *Himatanthus drasticus*: A plant species used in Brazilian folk medicine☆

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ABSTRACT

This work investigated the healing properties of proteins extracted of latex (HdLP) on excisional wounds. Cell toxicity of HdLP was investigated carried out in murine fibroblasts after incubation with HdLP (12.5–100 μg/ml). The dermal irritability test was performed to evaluate dermal reactions. The wounds were performed and treated with vehicle or HdLP (0.5 %, 1.0 %, and 2.0 %). The macroscopic parameters, histological analysis and measurement of inflammatory markers and mediators were evaluated. HdLP did not exhibit cytotoxicity and did not induce skin irritation. HdLP stimulated the release of IL-1β at the beginning of the inflammatory phase. This effect probably favored the earlier release of IL-10 by macrophages, during the proliferative phase. The shortening and completeness of healing were characterized by fibroblast proliferation and the presence of newly synthesized collagen fibers. This was accompanied by well-organized re-epithelialization. The involvement of latex proteins in this activity is reported for the first time.

1. Introduction

Latex is composed of soluble compounds, ions, subcellular structures, proteins, complex molecular assemblages and all other typical cellular structures that are constitutive or are de novo synthesized by highly differentiated cellular structures named laticifers

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[1]. Laticifers are not part of ordinary plant tissues, nor are they present in specific organs. They grow by elongating cells and form complex channel-like networks through the plant body, across all tissues and almost all organs [2]. The laticifers accumulate latex that is released when the plant is injured. Almost all scientific evidence supports the diversified roles played by latex in plant defense [3].

Many lactescent plants are exploited in folk medicine and are described in well-established pharmacopoeias such as Ayurveda [4, 5]. The latex extracted from these species is reported to possess many pharmacological, toxicological, and other biological properties [6–8]. Some well-defined latex compounds have been investigated for antimetabolic activities, making them potentially useful for cancer therapies [9–11]. The recent literature has highlighted substantial potentials of latex fluids and latex compounds to prevent or treat adverse clinical conditions in humans [12,13].

Himathanthus drasticus (Mart.) Plumel - Apocynaceae is a tree and the latex produced is most easily collected from the bark. The latex obtained from this plant is widely used in folk medicine in Brazil's Northeast region, where it can be bought in local street markets. Sellers recommend the latex, locally called "Janaguba", to prevent or treat many kinds of inflammatory and other disorders, including cancer, gastritis, ulcers, diabetes, hepatitis and parasitosis, among others [14,15]. The potential antidiabetic capacity of this latex has been experimentally supported [16]. Among the ethnopharmacological usages of the latex is the capacity to treat gastric ulcers [17]. Earlier evidence suggested a gastro protector effect of the latex [18]. In the extensive ethnopharmacological survey performed by Souza et al. [19], the use of *H. drasticus* latex to treat gastric ulcers was implicated. In the present study, these questions were approached by testing the latex in skin wounds since it would be an easier and clearer model to follow the time-course of the healing process and then answer whether the latex helps healing processes. Therefore, the prospective wound healing activity of latex compounds still needs further investigation. Nevertheless, the components of the *Himathantus drasticus* latex responsible for this effect are unknown. In the present study, these questions were approached by testing the latex in skin wounds since it would be a more reliable model to follow the process.

Wound healing is boosted by integrated physiological events in order to restore damaged tissue. This process is divided into at least three detectable stages: inflammation, proliferation, and remodeling. Inflammation is the shorter and acute phase of the healing process. In the first 48 h, inflammatory cells infiltrate the injured tissue and release cytokines such as IL-1β, TNF-α, and IFN-γ. These events result in edema and hyperemia, macroscopically documented on day 2. The proliferation stage initiates at day 2 and progresses until day 10, after tissue injury. The cellular proliferation and vascular events, observed in this phase, induce formation of new collagen fibers, fibroplasia, angiogenesis, and re-epithelialization. The result of this internal work is macroscopically seen by the contraction of the lesion. These events together are responsible for the restoration of the barrier function in the injured area. During the extracellular matrix repair, the basement membrane containing keratinocytes is differentiated in the stratified epidermis. Tissue remodeling, the later and longer phase of wound healing, starts two weeks after the injury and progresses for months and even years, in order to increase the tensile strength in the scar [20]. At the end of this phase, the architecture of the injured tissue will be completely restored.

In the present study, the latex of *Himathantus* was collected from taxonomically identified native plants. The latex was processed to obtain its soluble protein fraction, here called HdLP, and was tested topically to treat excisional wounds surgically induced in experimental mice. The results suggested that the topical application of HdLP induces promising organized tissue remodeling and faster healing, as revealed by histological analysis.

2. Material and methods

Ketamine chlorhydrate and xylazine chlorhydrate were purchased from Vetbrands (São Paulo, Brazil); iodopovidone was purchased from ADV (São Paulo, Brazil); and hematoxylin–eosin was purchased from Merck Millipore, São Paulo, Brazil. All other chemicals were of analytical grade unless otherwise specified.

Himatanthus drasticus (Mart.) Plumel (Apocynaceae) specimens used to extract latex were located at the following geographic position (3°46′58.81″S/38°27′59.42″W) and constituted a unique community of individuals. The plant name was checked (April 2020) at "The Plant List" (www.theplantlist.org). The local name given to the plant is "janaguba" and is the same term used in English literature. Vouchers were prepared and examined at the Institutional Herbarium of the Federal University of Ceará, and a reference material was registered under the code 40408. The legal permission to access and study *H. drasticus* was formally requested to SisGen (Genetic Heritage and Associated Traditional Knowledge System) according to the current Brazilian legislation. The access code for this study is A689147. The samples used in this study were the same used in the recent study of Morais et al. [16], and further phytochemical information is available there.

2.1. Himatanthus drasticus latex proteins (HdLP)

The latex proteins used in this study were obtained using the methods described by Refs. [9,16]. The latex (500 ml) was extracted from the trunk of the trees after removing the external layer through mechanical injury. The latex released was collected in distilled water to perform homogeneous mixing 1:1 (v/v) at a final volume of 1 L. The volume was gently shaken to homogeneity and stored at 8 °C until processing in the laboratory. The water-latex mixture was centrifuged for 10 min at 4 °C at 10.000×*g*. The precipitated matter was removed. The liquid phase was dialyzed against distilled water for 72 h at 8 °C. Dialysis membranes with a retention capacity of compounds over 8.000 Da were used. The dialysis water was renewed three times daily in order to remove all dialyzed substances of low molecular mass. The final volume was again centrifuged as previously, and the precipitated material was removed. This procedure separates insoluble matter (centrifugation), water-insoluble compounds (i.e. secondary metabolites, rubber particles, polyisoprenes; starch) and water-soluble compounds of small size (salts, carbohydrates, free amino acids, peptides). The water-soluble

proteins remain soluble and retained by the dialysis membrane. This fraction is further freeze dried and stored for use. Soluble proteins (HdLP) were used for the production of an ointment that was topically applied to treat excisional wounds, as described later. Preliminary biochemical and pharmacological characterization of HdLP has already been reported [15]. HdLP represents 3.1 % of the dry matter obtained when the latex is fractionated.

2.2. Cell culture

The murine fibroblast L929 cell line (NCTC clone L929; ATCC) was obtained from Rio de Janeiro Cell Bank (Brazil). Cells were grown in Dulbecco's modified Eagle's medium (DMEM- Gibco®) supplemented with 10 % fetal bovine serum (FBS - Gibco®) and antibiotics (100 units/ml of penicillin and 100 μg/ml of streptomycin - Gibco®) at 37 °C in humidified air containing 5 % CO_2.

2.3. Effect of HdLP on cell viability

Cell viability was evaluated by the 3-(4,5-dimethyl thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) method [21]. Cells (4×10^3 cells/200 μl/well) were seeded in a 96 well plate in DMEM containing 2.5 % FBS (5 % CO_2, 37 °C) for 24 h. The stock solution of HdLP (1 mg/ml in sterile water) was filtered through a 0.22 μm membrane filter (Millipore®). The cells were incubated with concentrations of HdLP ranging from 12.5 to 100 μg/ml or with sterile water (control group). Doxorubicin (5 μM) was used as a positive control. After 72 h, 20 μl of the MTT (5.0 mg/ml, w/v) was added and the enzymatic reduction of MTT to formazan, indicating viable cells, was evaluated after 3 h (5 % CO_2, 37 °C). The cultured medium was removed and the formazan was dissolved in 150 μl of dimethyl sulfoxide (DMSO). The absorbance was determined by a microplate reader (Biochrom Elisa Asys Expert Plus) at a wavelength of 540 nm. Cell viability was calculated as a percentage of optical densities of the samples divided by the control group density multiplied 100.

2.4. Production of ointment containing HdLP

A water-in-oil (W/O) pharmaceutical ointment formulation was prepared considering the characteristics of the active principle (protein) and hydrophilic origin and used to treat open cutaneous lesions in an excision model (greater need for occlusion). Initially, HdLP (1 g) was dissolved in 3 ml of distilled water (aqueous phase). Then, to emulsify the dissolved proteins, anhydrous lanolin was used in a proportion of up to 10 % (w/v). The aqueous suspension was slowly added to the lanolin under constant homogenization. After emulsification, the final volume was made up by adding polyethylene petrolatum to obtain a homogeneous semi-solid preparation. Ointment samples were produced to contain HdLP at 0.5, 1.0 and 2.0 % (w/w). The preparations were stored at 8 °C until use.

2.4.1. Animals

Adult male Swiss mice, 12 weeks old and weighing 25 ± 3.0 g, were obtained from the Central Animal House of Federal University of Ceará, Brazil. They were kept under standard conditions of temperature (25 °C ± 3), humidity (55 ± 10 %) and light/dark cycle (12h/12h), with solid chow and water *ad libitum*. One hundred sixty-eight animals were used in this study.

All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Before handling the animals, the experimental procedures were approved by the university's institutional committee for animal use and care and registered under approval number 39/2014.

Stratified randomization was performed and the animals were allocated in the following groups: sham group (without treatment, only surgery and daily handling); regederm® group (commercial product composed of latex proteins isolated from *Hevea brasiliensis*); vehicle group (topical treatment with ointment without HdLP); and HdLP group (topical treatment with *Himatanthus drasticus* latex proteins ointments – concentrations). The vehicle consisted of the same components as the ointment without the HdLP.

2.4.2. Skin irritation model

Skin irritation tests were performed as the previously described procedure [22]. Swiss mice (19 ± 1g) were shaved on the dorsal surface (4 cm^2) and then were left under observation for 24 h. One region (1 cm^2) of the shaved area was submitted to scarification with a scalpel blade until the presence of noticeable tissue fluid, but not blood. The shaved animals were randomly allocated into three groups: sham (did not receive treatments), vehicle (ointment without HdLP) or 2.0 % HdLP, ointment with HdLP at the higher concentration using on wound healing assay (n = 6/group). The scarified region received approximately 50 mg of vehicle or 2.0 % HdLP (1 mg) ointments and was applied once a day for six consecutive days. A non-scarified area (1 cm^2) on shaved animals also received vehicle or 2.0 % HdLP. The thickness of non-scarified and scarified regions was measured with the aid of a digital caliper (mm), after 1, 2, 4, and 6 h after treatments, and once a day for six consecutive days. The dermal reactions were documented in non-scarified and scarified regions, including the presence of edema and erythema after the first and second day of treatment. The results were submitted to statistical analysis (n = 6 animals/group; ANOVA–Tukey test).

2.4.3. Wound healing assays

The excisional model is the most commonly used in evaluating the wound healing process. It allows the analysis of different wound progression parameters, among them: macroscopic aspects that trace a profile of the temporal evolution of the wound, inflammatory response, reepithelization, biochemical and molecular markers [23]. The first assay was performed to evaluate the macroscopic parameters of wound healing i.e., edema, hyperemia and the reduction of wound area. Mice (n = 10 per group) were anesthetized by

intramuscular injection with 10 % ketamine chlorhydrate (100 mg/kg) and 2.0 % xylazine chlorhydrate (10 mg/kg) before the surgical procedure. After shaving the dorsal surface skin, the region was prepared for aseptic surgery using 1 % iodopovidone followed by 70 % ethanol. A single circular excisional wound (1.0 cm^2) was made on the dorsal surface of the animals with a tissue punch, reaching the dermo-epidermal region. At the end of the surgical procedure, the animals received subcutaneously 1 ml of 0.9 % sterile saline solution for fluid replacement and were kept in a warm environment until complete anesthesia recovery [24]. After the surgery, the animals were kept in individual cages on a ventilated shelf used exclusively for this experiment, throughout the evaluation period of 14 days. The cages were sanitized daily for maintenance of a clean environment during the trial period. This experimental was performed similar to that reported in our previous studies that can be consulted to other details [22,24,25].

2.4.4. Macroscopic analysis

Macroscopic analysis (n = 10 per group) of the wound areas to evaluate edema, hyperemia and contraction were carried out at 2, 9 and 14 days after surgery [26]. Edema and hyperemia were scored as (0): absent; (1): mild; (2): moderate and (3): intense at day 2. Areas of excisional wounds were measured using a caliper and were calculated as follows: A = π x R x r, where A, R and r are area, large radius and small radius, respectively [25]. After the macroscopic study, animals were sacrificed with an anesthetic overdose and biopsies of excisional wounds with adjacent normal skin were removed for histological study and for evaluations of mediators of inflammation.

2.4.5. Histological analysis

A second assay (n = 5 per group) was performed to evaluate histology and parameters associated with the proliferative phase (Table S1). HdLP (2.0 %) was used and analysis was carried out on days 9 and 14 after surgical induction of the excisional lesion. Samples of neoformed tissue were removed from wounds and were fixed for 24 h in 10 % formaldehyde dissolved in PBS 0.01 M (pH 7.4) and submitted to histopathology procedures. Sections (5 μm) were stained with hematoxylin-eosin for evaluation of fibroblasts and re-epithelialization; or Masson's trichrome for determination of collagen. Photomicrographs were taken of sections with a Leica DM microscope equipped with a Leica DFC 280 camera. The length of new epithelium (100x) in tissue samples taken on the 9th day after surgical induction of the excisional lesion was analyzed and the results were expressed as percentage of re-epithelialization (re-epithelialized area x 100/total injured area). Collagen fiber deposition was quantified in images obtained (× 200) from 15 random fields of 372 × 272 pixels. The ImageJ®1.46 software (U.S. National Institutes of Health, USA) was used to count fibroblasts and evaluate collagenesis by using the "cell counter" and "threshold color" plug-ins, respectively [27].

2.5. Markers and mediators of inflammation

The third assay, like the second (n = 6) but without the regederm® group, was carried out to evaluate inflammatory parameters (Table S1). Neutrophil infiltration in wounds was estimated indirectly by myeloperoxidase (MPO) activity, 2 and 3 days after the surgery, as previously described [28]. MPO activity, in the supernatant, was detected using 200 μl of a solution containing 2.78 mg of O-dianisidine dihydrochloride (Sigma), bi-distilled water (27 ml), potassium phosphate buffer (3 ml), and 1 % H_2O_2 (15 ml). Enzyme activity was determined by measuring the absorbance (460 nm) at 0 and 1 min. The results were reported as Neutrophils x 10^3/mg of tissue. Nitrite levels, in biopsy lysates, were determined indirectly as the total content of nitrite and nitrate (NO^{3-}/NO^{2-}) by spectrophotometry using a method reported for the Griess reaction on day 2 after the surgery [29] (Green et al., 1982). Data were expressed as micromoles of nitrite. The levels of TNF-α, IL-1β, IL-6 and IL-10 in biopsy lysates 2 days after the surgery, homogenized individually in phosphate buffered saline (pH 7.4) and processed [11], were determined using a sandwich enzyme-linked immunosorbent assay. The results were expressed as picograms/milliliter (pg/ml).

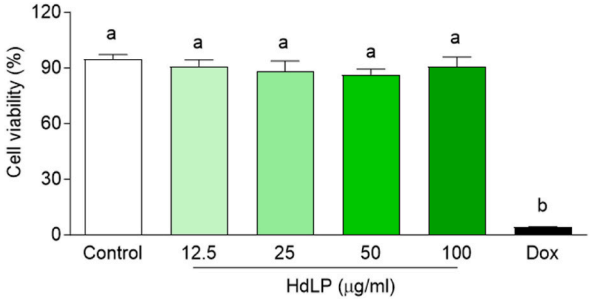

Fig. 1. Cell viability of fibroblasts (L929) by MTT expressed after 72 h of incubation with HdLP (12.5–100 μg/ml). The control group (Vehicle) was incubated with sterile water, the same used as vehicle for HdLP dissolution. The positive control used was Doxorrubicin (DOX, 5 μM). Three independent experiments (n = 4 per group/each experiment) were performed. The results were expressed as mean percentage ± standard error of the mean (S.E.M.). Different letters indicate significant differences ($P < 0.001$) between groups (Tukey test).

2.6. Statistical analyses

The results were expressed as the mean ± standard error of the mean (SEM) or in median values, depending on the type of parameter evaluated. For multiple comparisons of numerical values between groups, ANOVA was used, followed by the Tukey test using GraphPad Prism version 5.0 (USA). The medians were evaluated by the Kruskal-Wallis and Dunn's tests. The statistical significance was set at $P < 0.05$.

3. Results and discussion

HdLP did not present cytotoxicity on cultured murine fibroblasts (L929) after 72 h, compared with control, even at the highest (100 μg/ml) concentration tested (Fig. 1). There were no dermal reactions (edema and hyperemia), and no increase in the thickness of scarified and non-scarified skin in the groups treated with vehicle and with HdLP (2.0 %) compared to sham group, which did not receive any treatment (Supplementary Fig. S1). The overall macroscopic views of wound healing performance in all treatments and the control are presented in Fig. 2a. The images, representative of the time-course healing, shown in the figure, are according to the healing phases whose main characteristics were summarized in Supplementary Table S1. The images of wounds shown in Fig. 2a were taken from the same animals along the days. Day 2 is representative of edema and hyperemia, the earliest visual signs of inflammation, and these signs were prominent in all groups. The excisional wounds treated with HdLP (2.0 %) showed variations from absent to mild edema, compared to mild to moderate edema in the sham group on day 2 ($P < 0.05$). No difference in hyperemia intensity was observed between any groups (Table 1). Days 9 and 14 are representative of the proliferative and remodeling phases. The treatment with HdLP (2.0 %) accelerated the wound healing process and produced a more uniform scar on 14th day. HdLP (2.0 %) produced

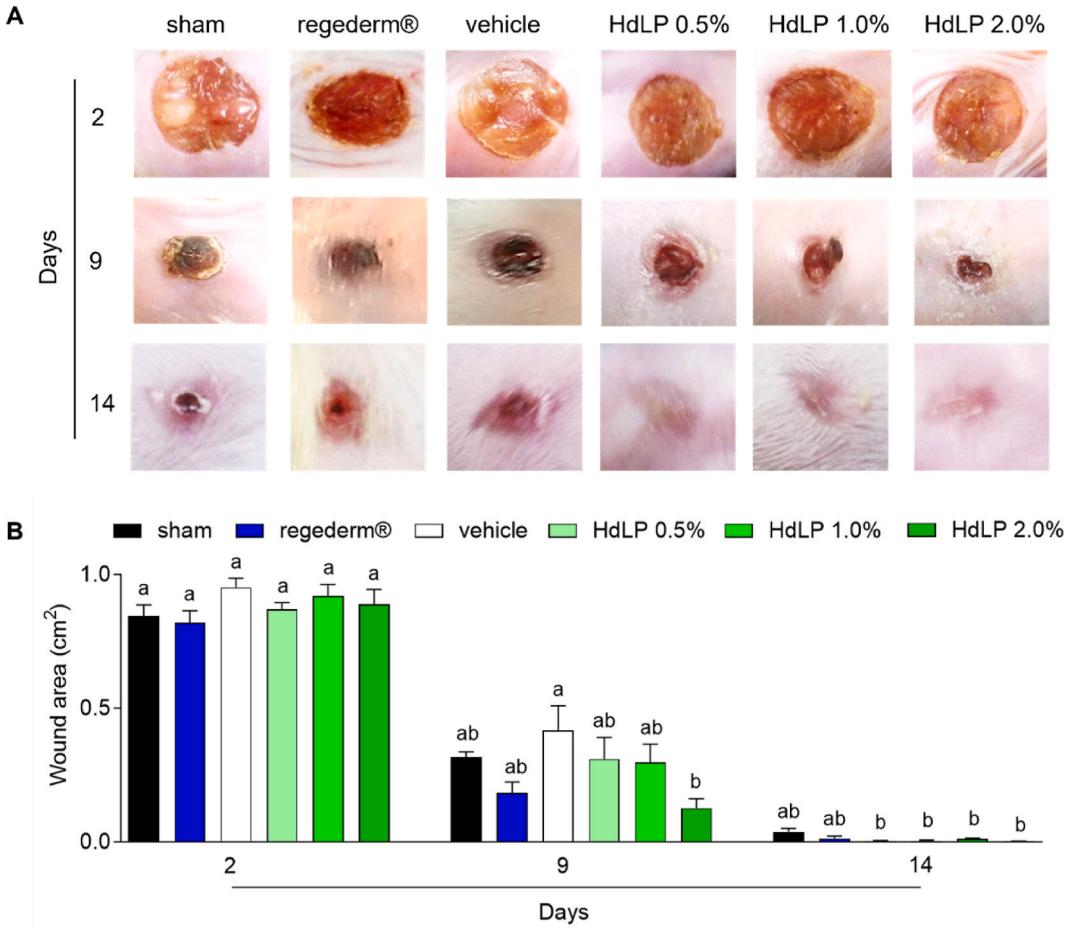

Fig. 2. Healing progress of excisional wounds: (A) macroscopic aspects, and (B) wound area (cm^2). (A) Images were selected from animals belonging to the corresponding experimental groups: sham (without topical treatment); regederm® (positive control); vehicle (ointment without HdLP); and HdLP (ointment with HdLP 0.5 %, 1.0 %, or 2.0 %). (B) Areas (cm^2) of excisional wounds were measured using a caliper at day 2, 9, and 14 days after surgery. Data are mean of three independent experiments and are expressed as mean ± standard error of mean (S.E.M.) of wound area. Equal letters indicate no significant differences ($P > 0.05$) between groups. Different letters indicate significant differences ($P < 0.05$) between groups (Tukey test; n = 10 animals/group/time).

faster ($P < 0.05$) wound contraction compared to the control group after 9 days (Fig. 2b). This advantage probably supported the complete re-epithelialization and emergence of scar as observed on day 14. The use of HdLP at 0.5 % or 1 % did not cause statistically different beneficial effects in edema, hyperemia or reduction of wound area. These groups were statistically comparable to the controls (sham, vehicle and regederm®). So only HdLP (2.0 %) was assessed in further assays.

The treatment with HdLP (2.0 %) reduced MPO activity of neutrophils on day 3, compared with the vehicle and sham groups ($P < 0.05$) (Fig. 3a). HdLP (2.0 %) attenuated the release of nitrite (corresponding to NO) on day 2, compared with the vehicle ($P < 0.01$) and sham groups ($P < 0.001$) (Fig. 3b). HdLP (2.0 %) did not alter the profile of TNF-α release (Fig. 4a). However, the treatment of wounds, with HdLP (2.0 %), increased the release of IL-1β ($P < 0.001$) and IL-10 ($P < 0.001$) on day 2, compared with the sham and vehicle groups (Fig. 4b and c).

The length of the re-epithelialized layer was greater in wounds under HdLP (2.0 %) treatment, compared ($P < 0.05$) to the sham, vehicle and regederm® groups after day 9 (Fig. 5). HdLP (2.0 %) induced higher proliferation of fibroblasts on days 9 and 14 compared with the sham ($P < 0.05$) and vehicle groups ($P < 0.001$), respectively (Fig. 6a). The collagen deposition (Fig. 6b) in remodeling tissue was increased with HdLP (2.0 %), as observed on day 14, compared to sham ($P < 0.01$) and vehicle ($P < 0.001$) groups.

At the end of the healing process (day 14), the sham and vehicle groups presented a small ulcer area and presence of residual inflammatory cells (Fig. 7a,c), while the HdLP (2.0 %) group did not, comparable to regederm® group (Fig. 7b). The treatment with HdLP (2.0 %) promoted more intense deposition of collagen, better tissue architecture organization, reduction of cellularity and greater keratinization of the epithelium compared to the controls (Fig. 7d; Fig. 8a,b,c,d).

There is no report in the literature about toxicity associated with the oral use of the latex of *H. drasticus*. In the location where this study was performed, people that believe in the preventive or curative properties of the latex, drink the latex daily, regardless the motivation. The consumed latex is a mixture of whole latex in water (1:1, v:v). This is indicative that the diluted latex drank does not induce acute toxicity. Recently, in the study of Moura et al. [7], it was reported that some latex compounds (secondary metabolites) of *H. drasticus* were toxic to mice at 2000 mg/kg. In our most recent study, we reported the chemical profile of *H. drasticus* latex and demonstrated inhibition of α-amylase and α-glucosidase by a plumieride, present in latex [16]. In the present study, all non proteinaceous molecules were eliminated of the latex.

Before starting the study about the topical effect of latex proteins on wound healing, the sample was tested for *in vitro* cytotoxicity through the MTT assay. HdLP would be completely safe for the *in vitro* assays even used at the highest tested (100 μg/ml) concentration. This result corroborates that of Mousinho et al. [9]. They did not observe cytotoxicity of HdLP against five human tumor cell lines. Instead, they found that HdLP exhibited *in vivo* antitumor effects. The reason for carrying out the cell toxicity assay is to investigate a safe concentration of the tested sample to perform, both, *in vivo* and *in vitro* assays. Thus, in all *in vitro* assays, HdLP was assayed in concentrations lower than this limit. However, *in vitro* assay gives only a picture of safeness since only one exposition of the sample is made to the cells. Under this perspective the safeness of HdLP use, observed in *in vitro* assay, should be taken with care and not broadly consolidated.

In *in vivo* assays, wounds were exposed to HdLP daily, for 14 days. HdLP was incorporated in an inert pharmaceutical formula to reach a maximum concentration of 2.0 %. Approximately 1 mg of HdLP incorporated in the formula was the maximum exposure, per application, on superficial wounds in animals. The HdLP tested *in vivo* did not induce adverse effects observed, as shown in all measurements performed, including histology, compared to the respective controls. So, HdLP did not show any cytotoxic or detectable physiological adversity in all assays performed. This indicated that HdLP was not cytotoxic for topical use on excisional wounds.

The healing process of excisional wounds involves three sequential phases, each characterized by key events with overlapping periods: inflammatory, proliferative and remodeling (Table S1). The process starts with an acute inflammatory phase with signs of hyperemia and edema [30]. These signs can be macroscopically seen and evaluated in a comparative basis. On excisional wounds, HdLP (2.0 %) attenuated the edema but did not interfere with the hyperemia. The intense migration of neutrophils to the injured site is the earliest cellular event of the inflammatory phase (Table S1). The increased level of NO in the inflammatory phase, resulting from the activity of myeloperoxidase on activated neutrophils, is associated with the production of reactive oxygen species (ROS), which generate oxidative stress and results in tissue damage [27]. HdLP (2.0 %) reduced MPO activity of neutrophils; as a consequence, the nitrite levels were also attenuated at the inflammation response. This observation suggests that HdLP prevents tissue damage through the reduction of release of injurious substances in the acute inflammation phase.

Another important event on inflammatory response is the activation of macrophages, motivated by prior neutrophil activity. This occurs in the intermediate stage of the inflammatory phase of the healing process and is essential to begin the transition from the inflammatory to the proliferative phase [31]. The release of chemical mediators, by activated macrophages, stimulates angiogenesis and fibroplasia, the main processes associated with the proliferative phase [32]. HdLP (2.0 %) increased the release of IL-1β, IL-10 but not TNF-α, after 2 days of induced-wound. Therefore, it is suggested that HdLP modulates macrophage activity for tissue homeostasis. A previous healing study demonstrated that biomaterial, containing proteins isolated from the latex of *Calotropis procera,* induced the

Table 1
Semi-quantitative evaluation of edema and hyperemia signs on excisional wounds induced by HdLP.

		sham	regederm®	vehicle	HdLP 0.5 %	HdLP 1.0 %	HdLP 2.0 %
Day 2	Edema	1 (1–2)^{a}	1 (0–2) ^{ab}	1 (0–2) ^{ab}	1 (0–2) ^{ab}	1 (0–2) ^{ab}	1 (0–1)^{b}
	Hyperemia	1 (1–2)^{a}	1,5 (0–2)^{a}	1 (0–2)^{a}	1 (0–2)^{a}	1 (0–2)^{a}	1 (0–2)^{a}

Data represent the median and range of scores from two separate experiments. Scores: (0) absent, (1) mild, (2) moderate and (3) intense. Different letters indicate significant differences between groups $p < 0.05$ (n = 10 animals/group, Kruskal–Wallis test followed by Dunn's test).

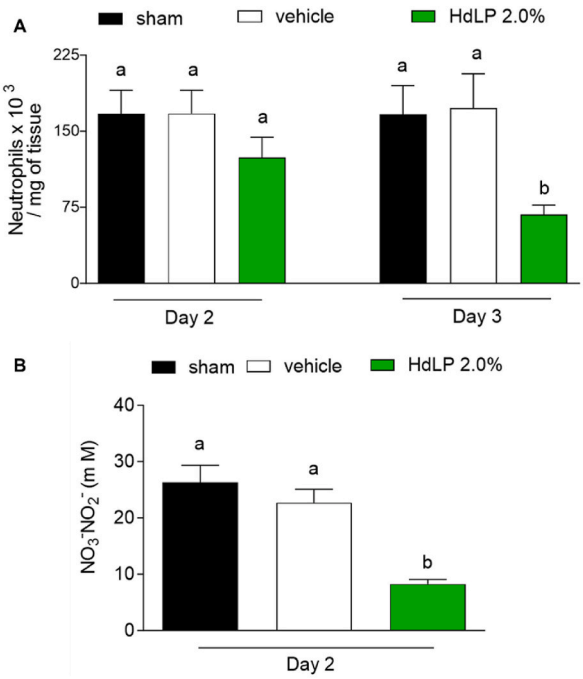

Fig. 3. Neutrophil infiltration (A) and nitrite level (B) measurement in tissue samples from 2 to 3 after surgery. Animals were sacrificed and wounds were removed to determine neutrophil infiltration and nitrite level by Griess reaction. Groups: sham (without topical treatment); vehicle (ointment without HdLP); and HdLP (ointment with HdLP 2.0 %). Data are expressed as mean ± standard error of mean (S.E.M.) of neutrophil number x 10^3/ mg of tissue (A), and nitrite (NO_3^-/NO_2^-) level (μM) (B). Equal letters indicate no significant differences ($P > 0.05$) between groups. Different letters indicate significant differences ($P < 0.05$) between groups (Tukey test; n = 6 animals/group/time).

production of IL-1β and TNF-α in skin wounds, which was shown to improve the healing performance observed in animals [24]. Although the mechanisms underlying the anti-inflammatory effects of HdLP may differ from those performed by proteins of *C. procera* latex, the results observed in both studies suggest the latex compounds were beneficial for tissue healing. The latex of different plants, even of those belonging to closely related taxa, have been shown to contain different proteomes, making it difficult, to make direct comparisons between different latex proteins and their pharmacological activities [2]. Unlike the latex of *H. drasticus*, which is orally consumed by people, the latex of *C. procera* is not ingested and toxic effects associated with it have been documented [33].

The healing process occurs to restore the integrity of the damaged tissue and it is essential for the reconstruction of the skin barrier. Re-epithelialization is the earliest event in the proliferative phase (Table S1). During this event, keratinocytes, located at the edges of the cutaneous wound, start proliferating and migrating towards the center of the wound, in order to rebuild the epidermal barrier from a new basement membrane [34]. The results showed the length of the re-epithelialized layer was greater in wounds under HdLP (2.0 %) treatment, after day 9. This result suggests that treatment of wounds with HdLP (2.0 %) afforded earlier proliferation of keratinocytes as compared to the control groups.

The proliferative phase is orchestrated by activity of keratinocytes, fibroblasts and endothelial cells and it involves synthesis of the extracellular matrix and revascularization (Table S1). In addition to restoring the physical barrier of the skin to the external environment, keratinocytes are important in regulating the activity of fibroblasts, since the keratinocytes secrete growth factors and enzymes that act in tissue remodeling. These events can be investigated with using histology. The proliferation of fibroblasts increased on day 14 in wounds treated with HdLP (2.0 %), compared to vehicle group, which suggests the topical use of HdLP stimulated the proliferative phase.

While the signs of inflammation and associated events become residual in wounds, in the proliferative phase, of the healing process, tissue remodeling starts (Table S1). Tissue remodeling is the closing phase of the healing process and it works to produce and organize the new functional tissue, until it reaches maturity. The remodeling starts in the late stage of the proliferative phase and involves the synthesis of type III collagen (Table S1). The remodeling is marked by the replacement of type III collagen fibers with type I collagen, among other associated structural events. The provisional extracellular matrix, in which fibroblasts, myofibroblasts and macrophages are allocated, consists mainly of immature collagen (type III), proteoglycans, glycosaminoglycans, fibronectin and hyaluronic acid [35]. Thus, synthesis of new collagen is extremely important to form a new extracellular matrix and to reconstitute the structure and integrity of the skin. The higher proliferation of fibroblasts noted corroborates with the greater collagen deposition (Fig. 6b) in remodeling tissue, treated with HdLP (2.0 %), as observed on day 14. HdLP (2.0 %) promoted better tissue architecture organization, reduction of cellularity and greater keratinization of the epithelium at the same period of observation (Fig. 7d). Therefore, suggesting an improved healing process promoted. An increase in collagen deposition was observed for the topical treatment with latex proteases from *Wrightia tinctoria*, which accelerated the contraction of wounds [36]. Similarly, an ointment containing latex proteases from

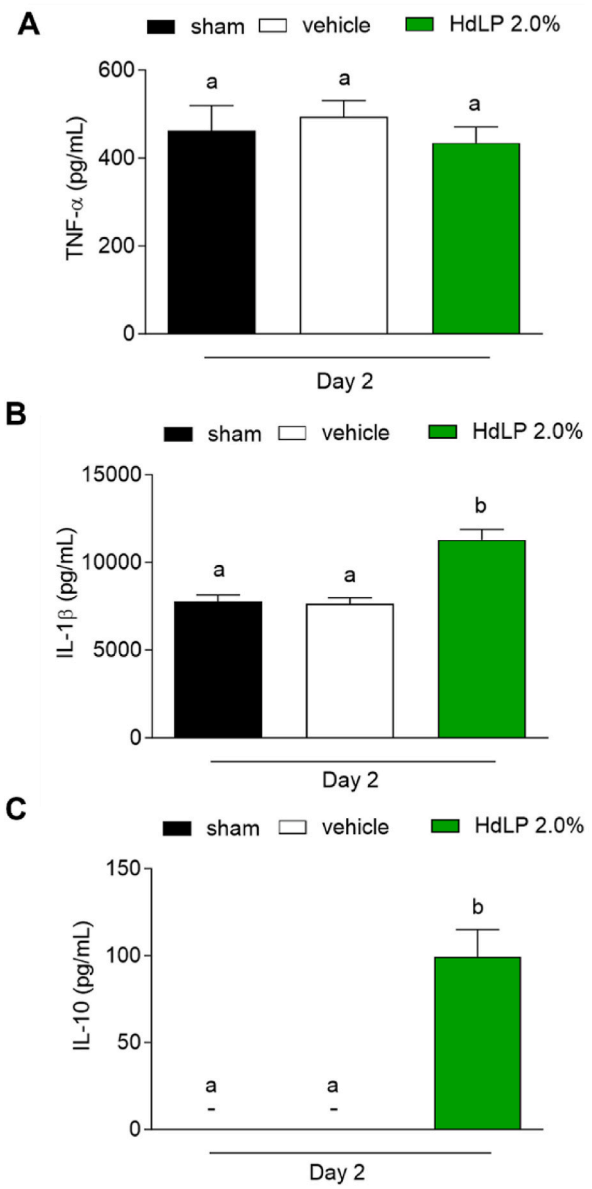

Fig. 4. HdLP effect on TNF-α (A), IL-1β (B) and IL-10 (C) levels. On day 2 after surgery, animals were sacrificed and wounds were removed to determine cytokine levels by ELISA. Groups: sham (without topical treatment); vehicle (ointment without HdLP); and HdLP (ointment with HdLP 2.0 %). Data are expressed as mean ± standard error of mean (S.E.M.) of cytokine/ml from supernatant/mg of tissue (A–C). Equal letters indicate no significant differences ($P > 0.05$) between groups. Different letters indicate significant differences ($P < 0.05$) between groups (Tukey test; n = 6 animals/group/time).

Plumeria rubra accelerated tissue repair, which was attributed to a reduction in the number of inflammatory cells as well as greater collagen deposition in the wound [37].

HdLP comprises the soluble protein fraction of the latex. This fraction represents 3.1% of the dry matter obtained when the latex is freeze dried. Therefore, when drinking the latex diluted in water, practitioners of folk medicine uptake a half of this quantity. In this study, the proteins were investigated for promoting healing because latex proteins belonging to another species (*Calotropis procera*) have been successfully demonstrated to possess this effect [22,24,25]. The results found here indicate that HdLP afforded superior performance of healing. Therefore, a common property shared with latex proteins of *C. procera*. However, as far as we are concerned, both latex exhibit very distinct proteome. While the latex of *C. procera* is diverse in proteins, with multiple isoforms of peptidases, chitinases and enzymes involved in antioxidative stress, we did not find yet counterparts in the latex of *H. drasticus*. The solely common protein confirmed in both latex fluids is osmotin. There is limited information about the proteins found in the latex fluids and there is no information whether latex proteins are constitutive or there is seasonality associated-expression or other specificities. Cho et al.

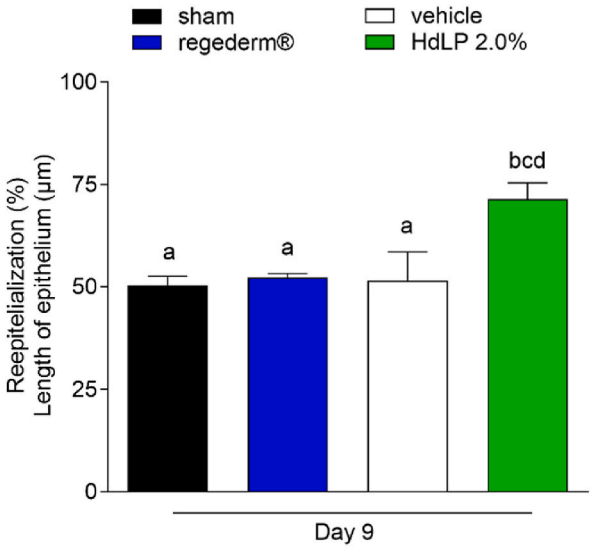

Fig. 5. Re-epithelialization induced by HdLP. On day 9 after surgery, animals were sacrificed and samples of subcutaneous tissue were removed to perform histological analyses. Groups: sham (without topical treatment); regederm® (positive control); vehicle (ointment without HdLP); and HdLP (ointment with HdLP 2.0 %). The length of the new epithelium was estimated by hematoxylin–eosin staining. Data are expressed as mean ± standard error of mean (S.E.M.) of percentage of the length of epithelium (µm). Equal letters indicate no significant differences ($P > 0.05$) between groups. Different letters indicate significant differences ($P < 0.05$) between groups (Tukey test; n = 5 animals/group/time).

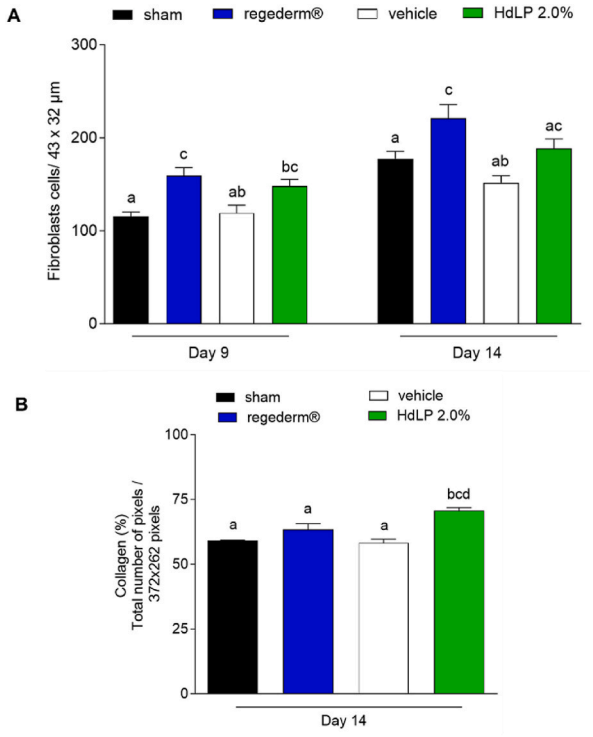

Fig. 6. Fibroplasia and collagen deposition during wound healing were improved by HdLP treatment. On day 9 and 14 after surgery, animals were sacrificed and samples of subcutaneous tissue were removed to perform histological analyses. Groups: sham (without topical treatment); regederm® (positive control); vehicle (ointment without HdLP); and HdLP (ointment with HdLP 2.0 %). Hematoxylin–eosin staining was employed to estimate fibroplasia (A) and Masson's trichrome stained sections were employed to estimate collagen deposition (B). Data are expressed as mean ± standard error of mean (S.E.M.) of the number of fibroblasts; collagen content estimated by total number of pixels in a fixed area (372 × 262 pixels) is expressed in percent. Equal letters indicate no significant differences ($P > 0.05$) between groups. Different letters indicate significant differences ($P < 0.05$) between groups (Tukey test; n = 5 animals/group/time).

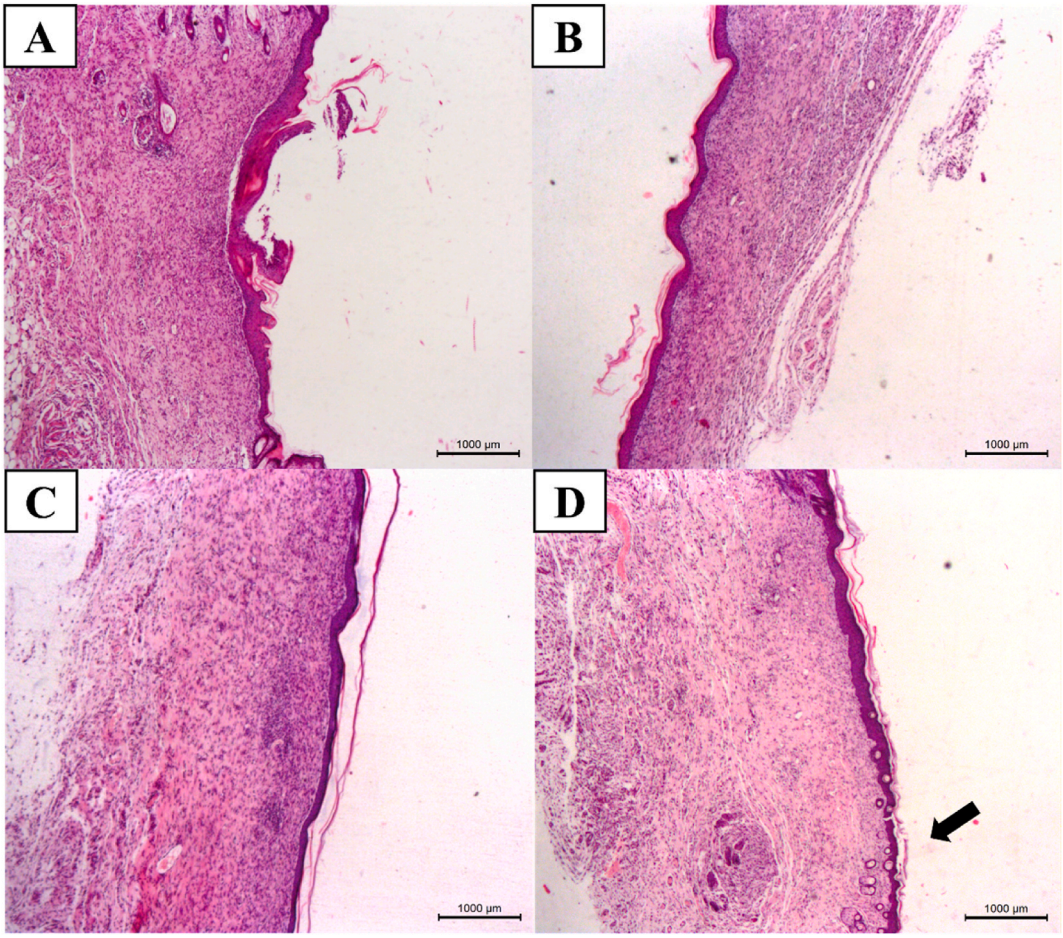

Fig. 7. Complete tissue re-epithelialization by HdLP 14 days after surgery. Photomicrographs of H&E stained histological slides, representing the new epithelium, from all groups (40× magnification). A black arrow indicates the presence of hair follicles in the HdLP (2.0 %) group. Groups: sham (without topical treatment – A); regederm® (positive control – B); vehicle (ointment without HdLP – C); and HdLP (ointment with HdLP 2.0 % – D).

[38] reported a set of common proteins annotated in latex of different plants and found some correlations with proteins found in phloem, plastids and mitochondria. The authors proposed functional correlations of these proteins with synthesis, ecology and plant defense. The latex of *H. Drasticus* is markedly poor in proteins compared to other latexes. Therefore, much more efforts will be needed to better characterize HdLP.

Gastric ulcers result in loss of tissue integrity and inflammation, two important injuries, also present in the excisional wounds, experimentally performed in this study and treated with HdLP. The healing promoting of excisional wounds performed by HdLP, reported here, does not prove that HdLP could perform similarly in gastric ulcer. This hypothesis remains to be approached since to treat gastric ulcer and create a more realistic model, HdLP should be administered orally and this protocol is not comparable to that performed in this study.

Similar to the latex of *C. procera*, an osmotin protein was detected in the latex of *H. drasticus* [39]. Osmotins are typical plant defense proteins and seem to be consistently present in different lactescent plants [2]. Osmotins have been shown to mimetics the human hormone adiponectin and display a pro-inflammatory effect by stimulating IL-1β release [40]. This report is in line with the observation that HdLP stimulated the release of IL-1β at the beginning of the inflammatory phase. This is probably a good venue to initiate further studies with HdLP.

The absence of detectable toxicity or adverse effects associated to the topical use of HdLP in *in vitro* and *in vivo* assays performed in this study, is also supported by the popular knowledge amongst Janaguba users that the oral consumption of the latex is safe. The scientific validation of the medicinal properties of plants, used in traditional medicine, is the first and very important step for encouraging the protection of ethnopharmacological bioresources and promoting their rational and safer use.

At the end of the healing process, HdLP showed no difference in the contraction of the lesions compared to the healing processes observed with other treatments. This may be due to the rapid evolution of the healing process in mice that have no underlying pathological condition that could hinder or slow down the entire healing process. This is part of the limitations of the model used in this work, since the healing capacity of HdLP was investigated in a clean (non-infected) and acute wound healing model. It is worthwhile

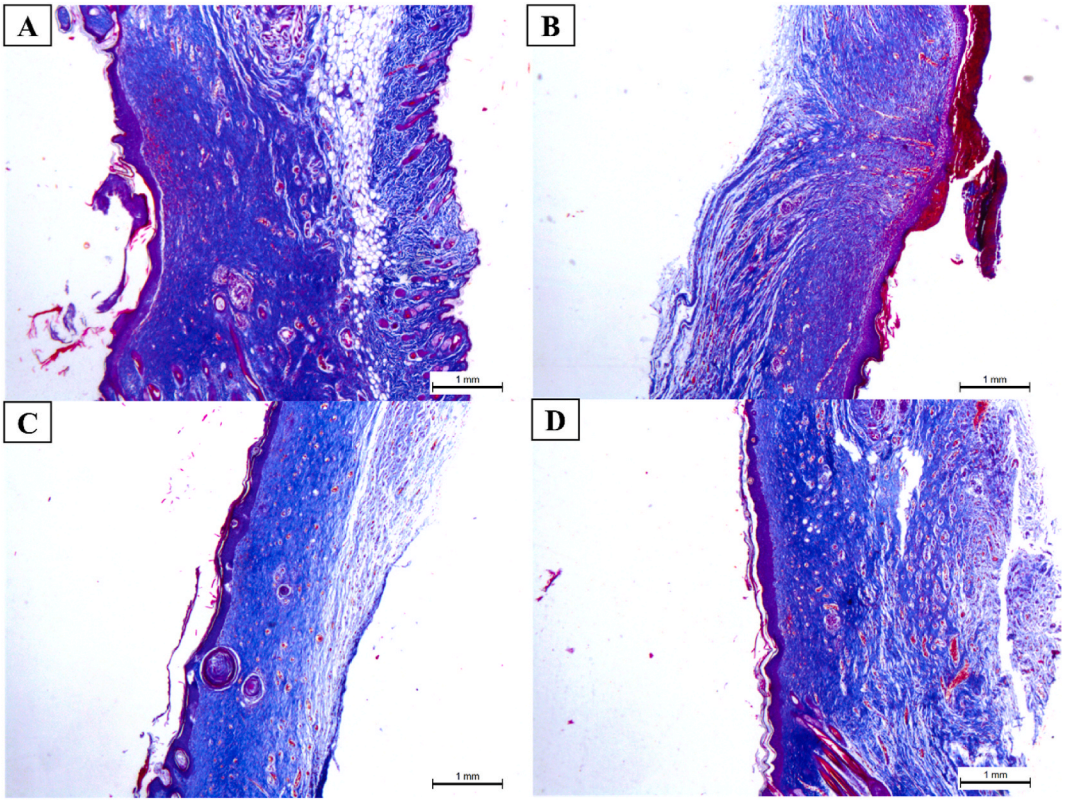

Fig. 8. Representative photomicrographs of a histological section of tissue stained with Masson's trichrome showing the deposition of collagen 14 days after surgery (40× magnification). The HdLP 2.0 % group had denser collagen fibers, more keratin filaments and thicker epidermis than the other groups (collagen stains blue; keratin stains red). Groups: sham (without topical treatment – A); regederm® (positive control – B); vehicle (ointment without HdLP – C); and HdLP (ointment with HdLP 2.0 % – D). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

investigating if HdLP is just as effective in treating other kinds of wounds, such as chronic wounds typical of diabetic or venous ulcers.

4. Conclusion

The healing process of excisional wounds that were treated with proteins isolated from the latex of *Himatanthus drasticus* was better, compared to the controls used, even compared to a commercial product, indicated for healing. The better performance of HdLP in promoting wound healing was associated with the downregulation of the inflammatory phase and a higher degree of maturity in the remodeled tissue, as revealed by measurement of inflammation markers and histological analysis. Wound healing potential is a new property attributed to the latex of *H. drasticus*, although, this work was limited to using a single cutaneous animal healing model. A more detailed investigation of the *in vitro* mechanism of action of HdLP may be useful in identifying molecular targets still not addressed.

Ethical approval

The experimental procedures were approved by the university's institutional committee (Federal University of Ceará) for animal use and care and registered under approval number 39/2014.

Consent to participate

All authors agree to participate in this study.

Consent for publication

All authors agree to publish this manuscript in its current form in this journal.

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CRediT authorship contribution statement

Tamiris F.G. Souza: Formal analysis. **Márcio V. Ramos:** Data curation, Project administration, Writing – review & editing. **Taiana M. Pierdoná:** Resources. **Liviane Maria Alves Rabelo:** Methodology. **Mirele S. Vasconcelos:** Formal analysis. **Luana D. Carmo:** Formal analysis. **Gisele F.P. Rangel:** Formal analysis. **Yuri T.C.N. Paiva:** Formal analysis. **Emilia T. Sousa:** Formal analysis. **Ingrid S. T. Figueiredo:** Formal analysis. **Nylane M.N. Alencar:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21843.

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