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Casearia sylvestris Improved Cutaneous Burn Repair in Diabetic Rats

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Abstract: Objectives: The burn repair associated with diabetes complications showed considerable changes in the healing process and the development of alternative therapies to favor the repair is important. This study investigated the efficacy of the *Casearia sylvestris* in the burn repair in diabetic and non-diabetic rats. Methods: The animals were divided into four groups (n = 20): (C) non-diabetics treated with carbopol gel; (G) non-diabetic treated with *C. sylvestris* extract in carbopol gel; (DM-C) diabetics treated with carbopol gel; (DM-G) diabetics treated with *C. sylvestris* extract in carbopol gel. Burn was induced using a metal plate (2 cm diameter/120 °C/20 sec). Samples were collected on the 3rd, 7th, 14th and 21st days after the injury for histomorphometric analysis (inflammatory infiltrate, fibroblasts, blood vessels and collagen), MPO (Myeloperoxidase) and NAG (N-Acetilglicosaminidase) dosage, hydroxyproline, glycosaminoglycans, TGF-β1, VEGF, collagen I and III. Key findings: *C. sylvestris* decreased the inflammatory process during the experimental period in diabetic and non-diabetic animals which has been demonstrated by MPO, NAG quantification, and TGF-β1 expression. *C. sylvestris* also increased blood vessels and decreased VEGF expression during the studied period in non-diabetic animals. Fibroplasia and collagenase were increased in all experimental periods in the DM-G group by means of de hydroxyproline quantification, collagen I, collagen III and glycosaminoglycans. Conclusions: *C. sylvestris* application can modulate favorably the time of inflammation and collagenous process in the repair of burn injuries in diabetic rats.

Key words: Burn, healing, herbal medicine, diabetes.

1. Introduction

Studies involving human and animal models showed considerable changes in the healing process when associated with diabetes complications, such as the reduction of the migratory capacity of fibroblasts in collagen synthesis, whereas keratinocytes show reduced proliferation, differentiation, and alterations in their morphology. These complications could result in delayed tissue repair with reduced re-epithelization, neovascularization and ECM (extracellular matrix) density. The increase of oxidative stress seems to inhibit cellular activation, migration and chemotaxis which prolongs inflammatory response and delays the subsequent stages of the healing process [1-4].

The repair of burned tissues involves complex biological and molecular events mediated by cytokines and growth factors, with interaction and proliferation of different cell types as well as collagen deposition and ECM [5]. Burn in patients with diabetes is a significant clinical problem, being a major challenge for professionals and the healthcare system [6]. These injuries promote several systemic alterations such as increased metabolism, loss of fluid volume, high infection risk and disturbances during wound healing [7].

The development of new therapies that favor the repair of burn is important for better results regarding speed and quality of healing [8, 9]. The use of medicinal plants as a treatment for wound healing has long been used [10, 11]. There are many studies about the effect of herbal medicines in the treatment of burn [12-14]. Plants represent important sources of bioactive substances that can be used in therapeutic proposals due to the great diversity of metabolites produced, as well as serving as models for synthetic

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chemicals [15]. Among the varieties of medicinal plants suggested for tissue repair, *Casearia sylvestris* Swartz (Salicaceae), also known as "guaçatonga", native in Mexico, Central America and South America, has ecological, pharmacological, and commercial importance [16]. The species is popularly used in the Brazilian herbal medicine due to its anti-inflammatory, antiviral drugs, antirheumatic, antiophidical [17, 18] in the wound healing and gastritis [19]. Most of its pharmacological properties are attributed to clerodic diterpenes present in different parts of the plant, such as casearins, casearvestrins and caseargrevins [20]. Studies with casearvestrins demonstrated that these presented promising bioactivity in citotoxicity and cancer cell assays, as well as in antifungal assays [21, 22]. The *C. sylvestris* responded immediately to incisional wounds repair in mice, contributing to a continuous reduction of the wound [23] and inflammatory process, also increased fibroblast proliferation and local angiogenesis [24].

In view of the above, the study investigated the efficacy of the *Casearia sylvestris* extract in the repair of 2nd-degree burn in diabetic and non-diabetic rats.

2. Material and Methods

2.1 Plant Material

The leaves of *C. sylvestris* Swartz, Salicaceae (ex-Flacourtiaceae) were collected in the morning of November/2015, after the flowering period of the species that occurred between June and August, in the Campus of High School of Agriculture Luiz de Queiroz-ESA/ESALQ, located in the city of Piracicaba, State of São Paulo, Brazil.

2.2 Preparation of the Hydroalcoholic Extract of the Leaves and Gel Formulation

The hydroalcoholic extract was obtained by dynamic maceration from 50 g dry *C. sylvestris* leaves, plus 300 mL of 70% v/v hydroalcoholic solution. This process was performed at room temperature and away from light. Three extractive periods were performed and, after filtration, the solvent was evaporated in vacuo on a rotary evaporator at 40 °C. This solution was called stock solution.

For the preparation of the gel plus the hydroalcoholic extract of *C. sylvestris*, the gel formulation of carbopol 940 (carbopol 2%, methylparaben 0.15%, propylene glycol 8%, aminomethylpropanol q.s. and distilled water q.s.p.) was used. Twenty mL of the extract was added to 80 g of gel (w/v). The carbopol 940 gel was chosen because of better rheological characteristics and because it increases the contact time of the extract with the wound and consequently its time of possible action in the healing.

2.3 Animals

All surgical and experimental procedures used in this study were conducted according to the experimental requirements and biodiversity rights of the National Institutes of Health for the Care and Use of Laboratory Animals (NIH 80-23, revised 1996) and also with the standards established by the Arouca Law, approved by the ethical principles in animal research adopted by COBEA and approved by the Ethics Commission on Animal Use of the University Center Hermínio Ometto-UNIARARAS (023/2015).

One hundred and sixty male Wistar rats (*Rattus novergicus albinus*) were used obtained from the Animal Experimentation Center of the Hermínio Ometto University Center-UNIARARAS, with age of approximately 120 days and the average weight of 300 g. The animals were housed in individual polycarbonate cages under constant conditions of temperature (23 ± 2 °C) and humidity (55%), and a cycle of 12:12 hours light/dark with free access to standard commercial feed and drinking water.

2.4 Induction of Experimental Diabetes

After 24 hours of fasting, the animals received intravenous administration (penile vein) of Alloxan solution [(Sigma®, Co., USA) (2,4,5,6 tetraoxohexahydropyrimidine)]. Subsequently, glycosylated solution (80%) was administered orally

for 24 hours to avoid the complications of alloxan hypoglycemia. The previous feeding has significant importance as regards host sensitivity and the performance of the diabetogenic agente, since fasting promotes greater senditivity and interferes with the animals response to aloxane [25]. Some studies point out that drug sensitivity increases with increasing fasting time [26, 27], however, fasting over 24 hours is not recommended for ethical reasons [28]. The characterization of the diabetic animal model was performed by measuring the peripheral blood glucose from tails of rats after 7 and 30 days of diabetes induction, using reagent strips for readings in portable blood glucose meter (Accu-Chek Active®, AM Roche Diagnostics, EUA). The 40 animals considered to the protocol had blood glucose ≥ 200 mg/dL [29]. Before the euthanasia of rats (3, 7, 14 and 21 days post-burned) a glucose measurement was performed to check the stability of induce diabetes.

2.5 Experimental Procedures

After anesthesia by an intraperitoneal administration with the combination of ketamine hydrochloride (3.0 mL/kg) and xylazine hydrochloride (1.0 mL/kg), the dorsal region trichotomy was performed in all animals. The burn (2nd degree) was performed in their dorsum skin by applying an aluminum metal plate (2.0 cm in diameter), adapted to an apparatus that maintains the constant temperature of 120 ºC, for 20 seconds [5]. To ensure the same pattern of burns in all animals, a support was used to hold the aluminum plate with the same pressure on the skin of the animals' backs. Subsequently, the animals were housed in individual cages and received oral analgesia: dipyrone sodium 500 mg/mL, one drop postoperatively, 12 and 24 hours later.

The four experimental groups randomly formed by 20 animals were: (C) non-diabetic treated with carbopol gel; (G) non-diabetic treated with extract of *C. sylvestris* incorporated into carbopol gel; (DM-C) diabetic treated with carbopol gel, and (DM-G) diabetic treated with the extract of *C. sylvestris* incorporated into carbopol gel.

Topical treatment, initiated shortly after the experimental injury, it occurred daily and at the same time for 21 days. Gel application (± 1.0 mg) occurred with a sterile swab, and the animals were immobilized without sedation.

2.5.1 Collection and Preparation of Tissue for Histomorphometric Analysis

After the 3rd, 7th, 14th and 21st days of injury and treatment, the samples (n = 5/experimental time) were collected after euthanasia with anesthetic deepening intraperitoneal administration with the association of ketamine hydrochloride (6.0 mL/kg) and xylazine hydrochloride (2.0 mL/kg). For this purpose, the 25 mm diameter area was delimited at the center of the injury to obtain standardized samples.

2.5.2 Structural and Morphometric Analysis

After removal, the tissue fragments were immersed in a fixative solution containing 10% formaldehyde in Millonig 7.4 pH buffer for 24 hours at room temperature. The pieces were then washed in buffer and subjected to the standard procedures for soaking in Paraplast (Histosec®-Merck). Longitudinal section cuts of 5.0 μm thickness were stained with: HE (hematoxylin and eosin) for quantification of the inflammatory number of infiltrates and fibroblasts, in addition to Gomori trichrome, for percentage quantification of collagen and number of blood vessels. The section images were captured through the Leica Microscope®DM2000. Six images of the injury area were captured from every five animals in the group at the 3rd, 7th, 14th and 21st days of follow-up, which were analyzed using ImageJ software. In the quantification of inflammatory infiltrate and fibroblasts 400× of magnification were used and for quantification of blood vessels and percentage of the collagen area 200× [30]. The counts were performed in double-blind, considering the means of the counts of the six images/animal.

2.5.3 MPO (Quantification of Myeloperoxidase)

The intensity of the neutrophil accumulation in the

injuries of the animals was measured by the dosage of the enzyme myeloperoxidase. Samples (*n* = 5/experimental time) were homogenized in POLYTRON® PT 3100 at 13,000 rpm with 200 µL of 0.1 M NaCl buffer, 0.02 M $NaPO_4$, 0.015 M NaEDTA (buffer 1). After centrifugation it was resuspended in $NaPO_4$ buffer (pH 5.4) containing 0.5% HTAB (hexadecyltrimethylammonium bromide) (buffer 2). Then, 5 µL of the supernatant from the samples was placed in a 96-well plate for the assay. And 30 µL of TMB ("3, 3 ', 5, 5'-tetramethylbenzidine") and then 100 µL of H_2O_2 was added to each well of the plate. The reaction was then quenched with 4.0 M sulfuric acid and read on a plate reader at 450 nm. The results were expressed in D.O. MPO/mg tissue [30].

2.5.4 Quantification of NAG (N-Acetilglicosaminidase)

The NAG dosage was determined for the determination of the macrophagic infiltrate by the macerated supernatant of the samples. For NAG determination, 3.0 µL of the supernatant from the macerated samples (*n* = 5/experimental time) from rats of different experimental periods was placed in duplicate in a 96-well plate with 22 µL of HTAB 0.5% (buffer 2 MPO). Thirty µL of the substrate 4-nitrophenyl N-acetyl-β-D-glucosaminide 2,24 mM (Sigma-Aldrich), diluted in 50 mM citrate buffer pH 4.5 was added to these wells. They were then incubated at 37 °C for 60 minutes. Finally, 50 µL of 200 mM glycine buffer, pH = 10.4 was added. The absorbance was measured by spectrophotometry on an ELISA reader with a wavelength of 450 nm. The results were expressed in D.O. NAG/g of tissue [31].

2.5.5 Quantification of GAGs (glycosaminoglycans)

Tissue samples were dehydrated in acetone for 24 hours, oven dried at 60 ºC for 2 h and weighed. The determination of the total GAGs content (mg/g dry tissue) of the samples (*n* = 5/experimental time) was determined from the release of the polysaccharides by digestion with papain (10 mg/g tissue in buffer 30 mM sodium citrate pH 3.5 containing 40 mM EDTA and 80 mM 2-Me) at 50 °C for 24 h. After digestion, the samples were centrifuged and to the supernatant, two volumes of methanol were added to precipitate the GAGs for 24 h at 4 °C. After centrifugation, 40 µL of water was added to each sample, followed by dosing. The quantification of the sulfated GAG contents was done by the method using DMMB [32] with adaptations. The reading was made in spectrophotometer visible light at 540 nm.

2.5.6 Quantification of HO-Pro (hydroxyproline)

Fragments of tissue (*n* = 5/experimental time) were immersed in acetone and then in chloroform: ethanol (2:1) for 48 h. The samples were hydrolyzed (HCl 6 N, 1 mL for each 10 mg of tissue, 16 hours, 110 °C) and neutralized (NaOH, 6 N). The hydroxyproline quantification was performed according to the method of Stegemann, & Stalder [33] with some modifications [34]. Hydroxyproline concentrations of 0.2-6 µg/mL were used for the standard curve and the reading was made at 540 nm.

2.5.7 Western Blotting Analysis

The densitometry values of TGF-β1, VEGF, Collagen type I and type III signals were developed according to a protocol developed by Ni et al. [35] and expressed relative to proteins stained with β-actina, which were taken as 100%.

2.6 Statistical Analysis

Data were expressed as the mean ± standard error of the mean. All data were tested by the Kolmogorov-Smirnov normality test. For the data that fit the normality curve, the statistical test chosen was two-way ANOVA and Tukey's post-test. For the data that did not fit the normality curve, the statistical test used was Kruskal-Wallis and post-test of Dunn and Mann-Whitney, all performed in Statistic software and GraphPad Prism® version 5.0 [36].

3. Results

3.1 Confirmation of Diabetes

After the 15th day of diabetes induction using

intravenous alloxan, 40 animals had a fasting glycemic mean of 441.22 mg/dL ± 69.95 (363 mg/dL at 600 mg/dL) which was maintained for 30 days in an average weight of 318.90g ± 39.13. In daily monitoring, diabetic animals showed clinical signs of diabetes such as polydipsia and polyuria.

3.2 Analysis of Inflammatory Phase

Regarding the recruitment of inflammatory cells to the injury site, the DM-G group presented greater quantification of the inflammatory infiltrate on the 3rd, 7th and 14th days in relation to the other groups. On the 21st day, there was no difference in this parameter among the studied groups (Figs. 1A and 1B).

In the expression of TGF-β1, the G group on the 3rd day showed to be increased in relation to the other groups. On the 7th day, the DM-G group was superior to the C, G and DM-C groups. Regarding days of follow-up, it was observed a gradual reduction of this pro-inflammatory protein in the later periods (Fig. 1C).

In the quantification of MPO there was an increase in the DM-G compared to other groups on the 3rd day. On the 7th day, DM-G and DM-C were superior to groups C and G; on day 14th, DM-G and DM-C were superior to C and on the 21st day, the groups C and DM-C were superior to G and DM-G groups (Fig. 1D).

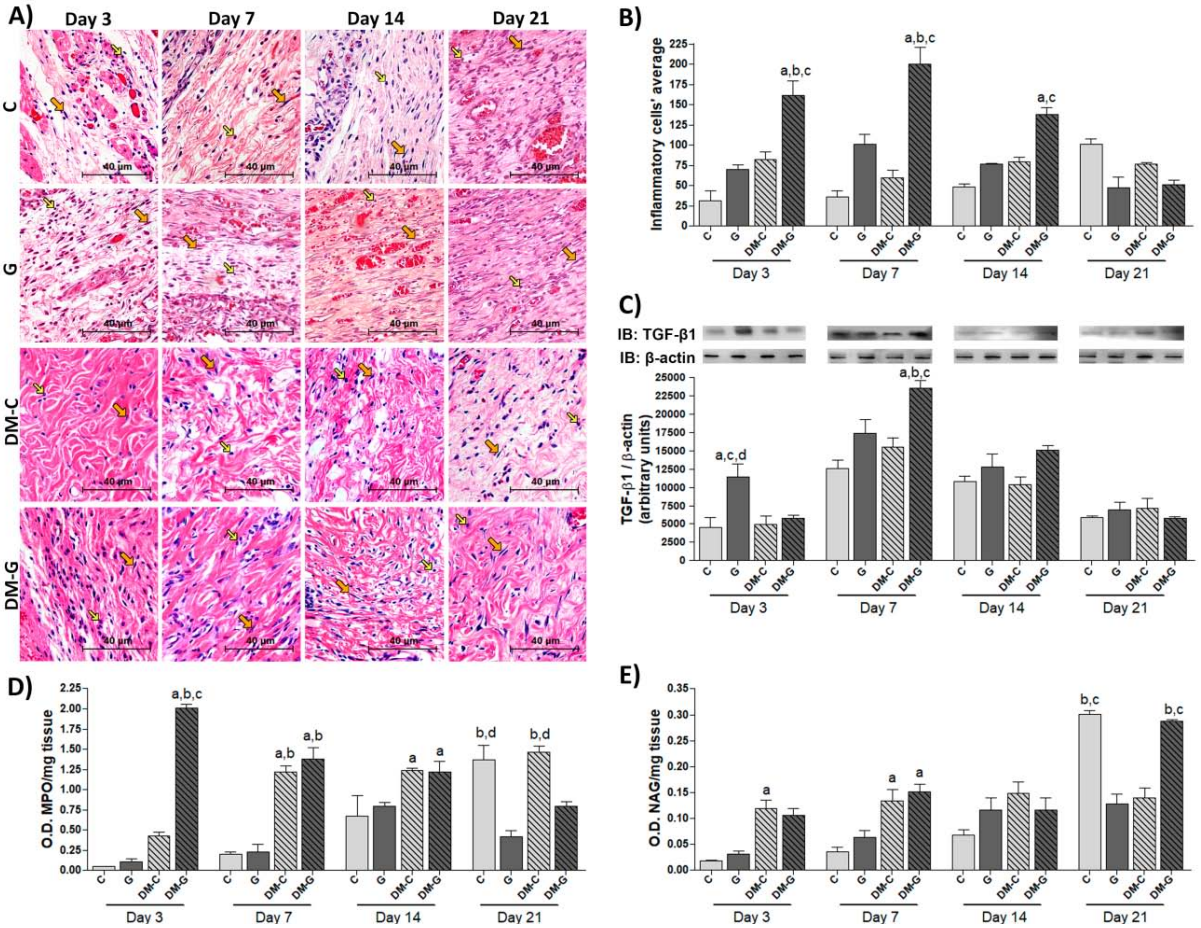

Fig. 1 Representative photomicrographs of 2nd-degree burn injuries (A) stained with Hematoxylin and Eosin from inflammatory cells (yellow arrow) and fibroblasts (orange arrow); Inflammatory cell histomorphometry (B); Immunoblotting for TGF-β1 (C); Quantification of myeloperoxidase (MPO-neutrophils) (D) and N-acetylglucosaminidase (NAG-macrophage) (E) in the groups: C, G, DM-C and DM-G on the 3rd, 7th, 14th and 21st experimental days.

n = 5 rats/group (a ≠ C, b ≠ G, c ≠ DM-C, d ≠ DM-G $p < 0.05$) (Two-Way ANOVA and Tukey post-hoc).

Regarding NAG, it was observed that the DM-C on 3th day and DM-G and DM-C on the 7th day were superior to C. On the 21st day the groups C and DM-G were superior to G and DM-C (Fig. 1E).

3.2 Analysis of Angiogenesis

The G group presented a superior number of blood vessels in relation to the DM-C group in all experimental periods. Moreover, the group C was superior to DM-G on the 21st day (Figs. 2A and 2B).

Regarding expression of VEGF, the groups C, G and DM-C were a superior in relation to the DM-G on the 7th day. On the 14th day, groups G and DM-G were higher than C and; on the 21st day, group G was higher than C and the DM-C higher than C and DM-G (Fig. 2C).

3.3 Analysis of Fibroplasia and Collagenesis

In fibroplasia, the DM-G group was superior in relation to C in all experimental periods and, on the 14th day, the relation to the DM-C group also got increased (Figs. 1A and 3A).

Regarding collagenesis, the DM-G group was superior in relation to the other groups in all the experimental periods. Similarly, the DM-C had greater collagenesis compared to C in all experimental periods. In addition, group G presented superior collagenesis in relation to C on the 3rd day (Figs. 2A and 3B).

In the expression of collagen III, on the 3rd and 14th days, group G was superior than the others groups; moreover, on the 3rd day the DM-G was superior to the DM-C group and on 14th day DM-G was inferior to C. On the 7th day, the groups G and DM-C were superior to group C. About the 21st day, G was superior to DM-C and DM-G and DM-G was inferior to C (Fig. 3C).

About the expression of collagen I, there was a gradual increase in the G and DM-G during the experimental periods. On the 14th day, the DM-G group was superior in relation to C and G; on the 21st day the DM-G was superior to the other groups and G was superior to C and DM-C (Fig. 3D).

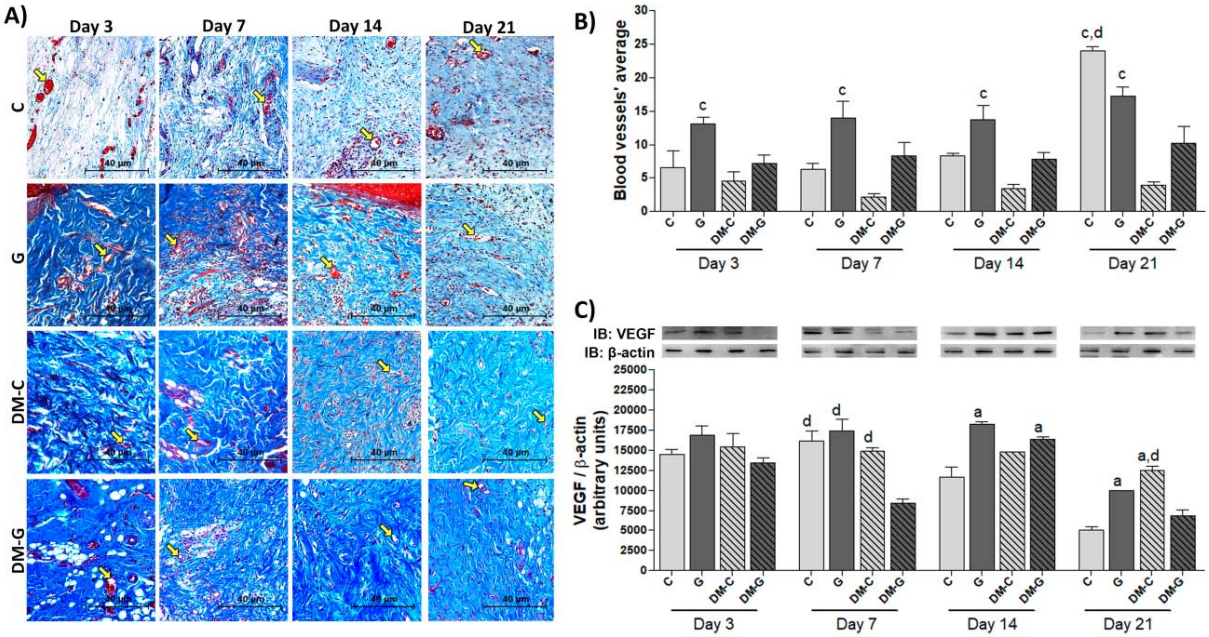

Fig. 2 Representative photomicrographs of 2nd-degree burn l injuries (A) stained with Gomori trichrome (yellow arrows): blood vessels; Blood vessel histomorphometry (B) and Immunoblotting for VEGF (C) in the groups: C, G, DM-C and DM-G on the 3rd, 7th, 14th and 21st experimental days.

n = 5 rats/group. Representative bands are shown above the graph (C) (a ≠ C, c ≠ DM-C, d ≠ DM-G $p < 0.05$) (ANOVA Two-Way e Tukey post-hoc).

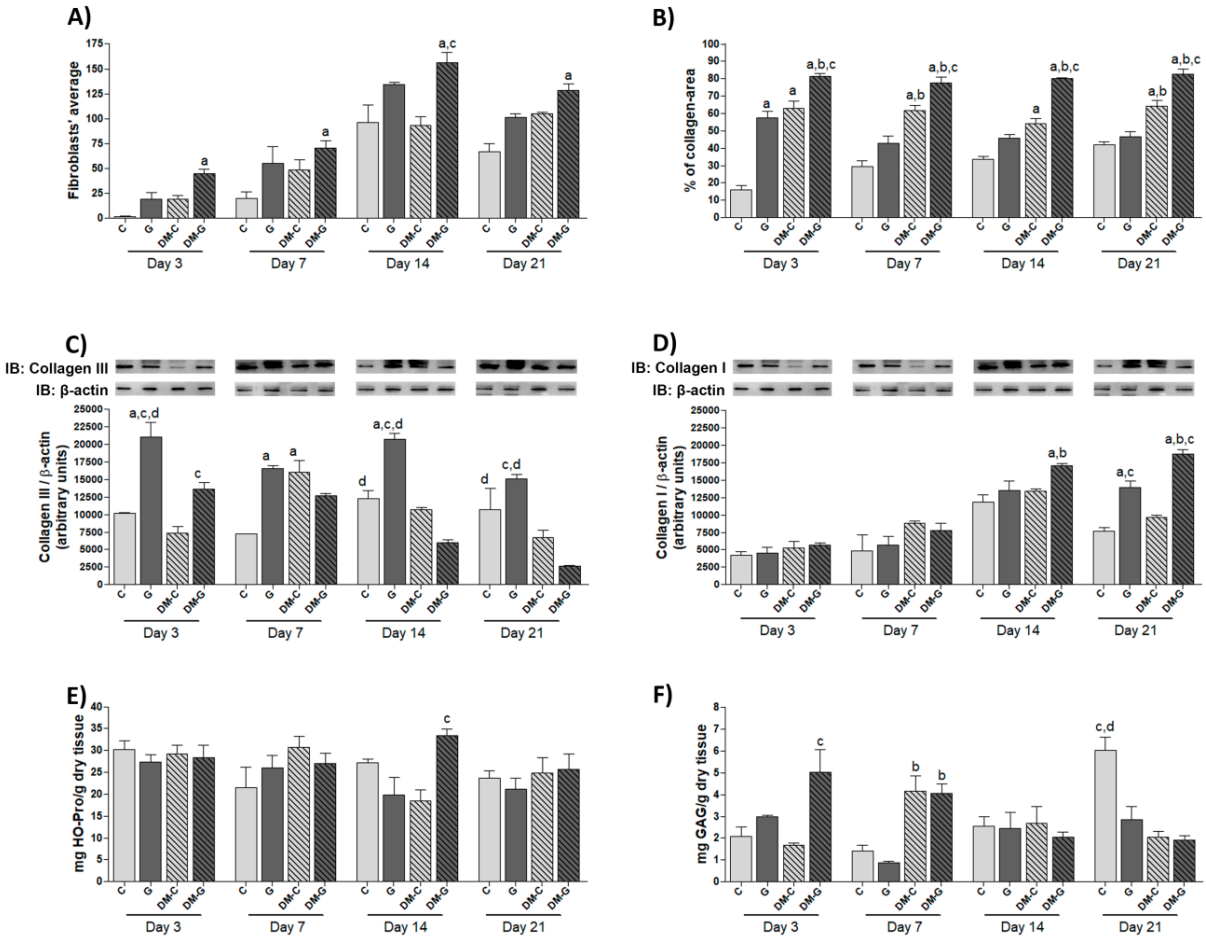

Fig. 3 Fibroblast histomorphometry (A); Determination of the percentage of collagen area (B); Immunoblotting for Collagen III (C) and Collagen I (D); Quantification of Hydroxyproline (E); Quantification of sulfated glycosaminoglycans (GAGs) (F) by means of the supernatant of the sample of l injuries by 2nd-degree burns in the groups: C, G, DM-C and DM-G on the 3rd, 7th, 14th and 21st experimental days.

n = 5 rats/group. Representative bands are shown above the graph (C and D) (a ≠ C, b ≠ G, c ≠ DM-C, d≠ DM -G $p < 0.05$) (Two-Way ANOVA and Tukey post-hoc).

Regarding the quantification of hydroxyproline, the DM-G group was superior on the 14th day in relation to the DM-C group (Fig. 3E).

In the quantification of sulphated glycosaminoglycans, there was an increase in the DM-G group compared to DM-C on the 3rd day. On the 7th day, DM-C and DM-G were superior to G and, on the 21st day, C was higher than the DM-C and DM-G groups (Fig. 3F).

4. Discussion

The study to verify the efficacy of experimental Herbal medicine in the healing of injuries associated with diabetes has presented great importance and a growing interest [37]. *C. sylvestris* was incorporated into the Carbopol gel, since it presents adequate solubility, bioadhesive properties and the important characteristic of compatibility with many excipientes and does not interfere with the active of the crude extract used [38]. On the other hand it can be used as a vehicle for different herbal medicines since it does not interfere with its properties [39].

4.1 C. sylvestris and the Inflammatory Process

Tissue repair disorders are characteristic of patients with diabetes, of which the synthesis and deposition of

collagen fibers, the inflammatory response, fibroplasia, reepithelialization, and angiogenesis are compromised, as well as increased levels of proteinases and altered macrophage function [1, 40, 41]. Major problems in the wound healing in diabetes are observed in the inflammatory phase which is responsible for vascular and cellular events [42]. There is also an increase in oxidative stress that seems to inhibit cellular activation, migration, and chemotaxis, and the end result is a prolonged inflammatory response that delays the subsequent stages of the healing process [43].

In our study it was observed an increase of inflammatory infiltrate and quantification of MPO during the inflammatory, as well as a gradual decrease of TGF-β1 in the groups treated with *C. sylvestris* extract in diabetic and non-diabetics animals after the 7th day. It indicates that this phytotherapic increased the inflammatory response in the first stages of healing with subsequent reduction, highlighting its important self-control of inflammation. *C. sylvestris* has shown antioxidant and anti-inflammatory effects in experimental models in rats, suggesting that their constituents favor the reduction of cell migration and the activation of acute inflammation [18]. TGF-β1 has a proinflammatory action, released by platelets after the injury, which initiate the inflammatory phase of healing [44]. The chemotactic effects of this growth factor on neutrophils, macrophages, and fibroblasts induce an increase in their synthesis and an increase of concentration in the inflammatory phase, but also a decreasing in the reepithelization phase [45] which corroborates to this study results.

The inflammatory process modulation is attributed to clerodan diterpenes, such as casearins, casearvestrins and caseargrevins, present in different organs of *C. sylvestris* [20,46]. Second-degree scald burns treated with hydroalcoholic extract of *C. sylvestris* in non-diabetic rats showed good healing results attributed to the inhibition of the synthesis of inflammatory mediators stimulated by active extracts (clerodanic diterpenes), suggesting its therapeutic potential in the treatment of the inflammatory conditions of these injuries [47]. Therefore, the results obtained in our study also indicated that *C. sylvestris* favorably modulates the inflammation in diabetics animals, highlighting its importance in the repair of 2nd-degree burns in diabetes.

During the first healing phase it occurs hemostasis, leukocyte migration and the onset of the tissue repair cascade with the migration of neutrophils from the blood vessels to the injury site [48], with subsequent reduction of this cell type. Thus, in this experimental model, *C. sylvestris* favored the increase of MPO, a neutrophil marker, in the initial healing period especially when associated with diabetes, to a reduction in the last studied periods. Such results corroborate with the studies of Baskaran et al. [49] in rats with burns that observed that MPO levels up to the 7th day after injury reflected a continuous recruitment of neutrophils through the release of inflammatory mediators from the injured tissue. It was observed that *C. sylvestris* reduced the late inflammatory response due to its ability to inhibit cell migration and the enzymatic activity of MPO [18]. Neutrophils also favor the recruitment of macrophages to the injury site, which are present in all stages of healing, making phagocytosis of dead cells, exogenous particles (tissue debridement) and stimulating the synthesis of cytokines and chemokines [50].

In the NAG quantification of macrophages, it was observed a high level of it on the 21st day in diabetics treated with *C. sylvestris*. One of the functions of macrophages is the synthesis of the EGF (epidermal growth factor) and FGF (fibroblast growth factor), which favor the chemotaxis and proliferation of fibroblasts and keratinocytes, and PDGF (platelet-derived growth factor) that, in addition to these functions, is involved in the turnover of collagen, with degradation of type III collagen and synthesis of collagen I [48, 51]. Thus, *C. sylvestris* benefited the presence of macrophages in the last experimental period promoted at the chemotaxis of fibroblasts,

which in turn, synthesized collagen by reestablishing the injured ECM, making up the remodeling phase. Baum and Arpey [52] reported that macrophages remain in the injured area to favor subsequent wound healing phases, including collagen formation, angiogenesis, and reepithelialization. Polubinska et al. [53] performed tests on human skin fibroblasts cultured *in vitro* with the supplementation of the culture medium with NAG and observed a higher concentration of collagen in wells containing NAG-treated cells; concluded that this molecule has multidirectional actions in the fibroblasts of the skin. Therefore, the results obtained in this study indicated the effects of *C. sylvestris* on the inflammatory phase of healing, highlighting its importance in the repair of 2nd-degree burns in diabetes.

4.2 C. sylvestris and Angiogenesis

The extract from leaves of *C. sylvestris* also presents angiogenic effects in the repair of skin burns [47] and in this study, blood vessels increase and a gradual decrease of VEGF was observed in the groups treated with this extract, showing its efficacy in the modulation of this pro-angiogenic factor. Ferrara [54] reports that VEGF has a fundamental action for adequate tissue repair and it promotes the growth of endothelial cells derived from arteries and veins, contributing to the formation of new vessels with increased vascular permeability. Hypoxia is one of the most potent stimulatory factors of VEGF [55] which explains its diminished expression at the end of the study period.

4.3 C. sylvestris and Collagenesis

The results of this study demonstrated that *C. sylvestris* favored fibroplasia and collagen synthesis in diabetic animals, a pathology in which the fibroblasts have diminished migratory capacity, whereas the keratinocytes present reduced proliferation, differentiation and alterations in their morphology, resulting in delayed tissue repair with decreased reepithelialization, angiogenesis, collagen synthesis, ECM density, and the release of growth factors [41]. Fibroblasts, after induction of growth factors, are activated and initiate the synthesis and secretion of ECM components, such as glycosaminoglycans and collagen fibers types I and III [56] contributing to the maintenance of the structural integrity of connective tissues, reestablishing the injured area [57] and favoring dynamic interactions with the cells, essential for cell adhesion, motility, growth, differentiation, synthesis of the ECM itself, providing support for cells and other structures [7]. In the repair process, the synthesis of molecules that make up the ECM as collagen, GAGs, adhesion glycoproteins, among others, is essential for the reestablishment of the morphology of the injured skin and its biomechanical properties constituting a method of evaluation of the medicine curing activity or plant extracts [58].

In this sense, *C. sylvestris* benefited GAGs increasing at the beginning of the healing process in diabetic animals. GAGs present important actions in tissue repair, such as transport of molecules, adhesion, recognition, growth and cellular proliferation in the initial healing phase [59]. These molecules participate in the regulation of collagen synthesis stimulating cell migration, differentiation, and proliferation in all phases of wound healing [60].

Hydroxyproline is one of the main amino acids present in the composition of collagen and indispensable for the stability of its triple helix, being used for an indirect dosage of the total concentration of collagen. Our study demonstrated an improve of collagenesis in the diabetic animals treated with *C. sylvestris* through hydroxyproline quantification which was increased in the proliferative phase and by the higher amount of collagen I and was decreased in collagen III throughout the experimental period. It is important to note that *C sylvestris* altered the proportion of collagen I and III without raising the total collagen concentration, except in the DM-G group. The expression of collagen plays an important role in the healing of injuries and in the physiological

processes of tissue repair. However, in chronic and uncontrolled inflammation, characteristic of diabetic healing, it provides an interrupted homeostasis, resulting in excessive collagen deposition, producing fibrosis [3]. Consequently, the little variation in total collagen between the groups seems to have being beneficial. It has been shown that diabetes interferes with the fibroblast behavior, that is, its proliferation and activities, for example, in the synthesis of collagen, promoting complex effects on cellular mechanisms involved in healing [1]. So, the analysis related to fibroplasia, collagen deposition and other components of ECM in this study evidenced the iimportance of *C. sylvestris* action in the 2nd-degree burns in animals induced to diabetes.

5. Conclusions

It is concluded that the hydroalcoholic extract of *C. sylvestris* leaves has modulated the inflammatory process in the 2nd-degree burn repair in diabetic rats and this benefited the fibroplasia and collagenesis, consisting in a relevant therapeutic alternative in burned in diabetes. Future studies with the fractionation of the crude extract will be relevant, as well as the action of these actives in the different cellular and molecular elements involved in the healing process in diabetics in order to establish an effective clinical practice. The study of herbal medicines in diabetes in the animal model has presented excellent results with positive future prospects for human application.

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

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