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Effect of *Plantago major* on Wound Healing in Hyperglycemic Mice

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Abstract

Background: *Plantago major* is used as a wound-healing, anti-inflammatory, analgesic and antioxidant agent. **Objective:** The aim of this study was to investigate the effect of topical treatment of cream containing *P. major* extract on the healing process in hyperglycemic mice. **Methods:** The control group (CG) received topical treatment with the cream base, and the intervention group (IG) received topical treatment with the cream base containing *P. major* extract. The healing process was examined on days 0, 3, 7 and 14 post-injury. Specifically, leukocytes, blood vessels and fibroblasts were examined using hematoxylin and eosin staining, and cytokines (interleukin [IL-1β], IL-10 and tumour necrosis factor [TNF-α] and growth factors (transforming growth factor [TGF-β1] and vascular endothelial growth factor [VEGF]) were examined with western blotting. **Results:** Compared with the GC, the IG showed more inflammatory cells on day 3 post-injury, more fibroblasts on days 3 and 7 post-injury, and more blood vessels on day 7 post-injury. Western blotting revealed a higher VEGF expression on day 7 post-injury in the IG compared with the CG. **Conclusion:** *P. major* increased in inflammatory cells, but without increasing the protein expression of pro- and anti-inflammatory cytokines, and increased in fibroblasts, blood vessels and VEGF protein expression.

Keywords

Wound Healing, Diabetes Mellitus, *Plantago major*, Cytokines

1. Introduction

Diabetes mellitus (DM) is one of the most prevalent diseases in the global popu-

lation. It is estimated that by the year 2045, the number of people living with DM will increase by 46%, and 1 in every 8 adults will have this disease [1]. The lack of treatment for DM can lead to complications in various organs, such as the heart, eyes, kidneys, nerves and circulatory system [2]. Of these, lower limb damage is the complication that leads to the most hospitalizations [3], specialist care and medication [4].

Diabetic foot ulcers (DFUs) are a major cause of morbidity and mortality from DM throughout the world. It is estimated that a person with DM has a 34% chance of developing a DFU during their lifetime. The likelihood of a DFU recurring is also high—around 40% in the first year after the lesion closes and 65% over the next 5 years [5]. Among people with DFUs, 20% are likely to experience lower limb amputation and hospitalization during their lifetime [1]. The most common causes for the development of DFUs are diabetic neuropathy, which can affect up to 50% of people with DM, and peripheral vascular disease, which causes areas of ischemia in the lower limbs due to poor blood flow, resulting in an inadequate healing process [6].

All phases of the tissue re-epithelialization process are altered in the presence of DM. At the onset of the inflammatory phase, M1 macrophages cannot undergo conversion to M2 macrophages [7]. This inability leads to chronic inflammation and negatively affects granulation tissue, angiogenesis and wound epithelialization [8]. In the proliferative phase, there is abnormal angiogenesis, a reduction in extracellular matrix activity and wound contraction [7] [9]. When M1 macrophages do not differentiate into M2 macrophages, there is poor vascularization and the inability of keratinocytes to migrate and epithelialising wounds, a phenomenon that can delay healing or even promote non-healing [9]. Tissue remodelling involves an increase in matrix metalloproteinase levels and a decrease in tissue inhibitor of metalloproteinase levels, resulting in abnormal extracellular matrix and chronification of lesions [10] [11].

Researchers from throughout the world are seeking innovative topical treatments for skin wounds. Among the various approaches is the use of medicinal plants, which the World Health Organization [12] recognises as extremely beneficial in preventing disease, maintaining health, and improving quality of life, especially in chronic disease situations [13]. *Plantago major* L. is a medicinal plant that is currently being studied extensively for its contribution to tissue repair, although its mechanism of action has not yet been fully elucidated. *P. major* is a member of the Plantaginaceae family. Studies indicate that it has numerous biological effects, including the ability to promote wound healing and treat infectious diseases, as well as anti-inflammatory, analgesic, antioxidant, weak antibiotic, immunomodulatory, and antiulcerogenic activities [14]. *P. major* is mentioned in the 1st edition of the Brazilian Pharmacopoeia Herbal Medicine Form, published in 2011, as an anti-inflammatory and antiseptic for the oral cavity [15]. It is also included in the American Homeopathic Pharmacopoeia, where its morphological characteristics are described and aucubin is listed as a reference constituent [16]. This iridoid

glycoside is found in many plants.

Based on literature reviews, there are numerous therapeutic possibilities for the use of *P. major*, including as a wound-healing agent [17]. A recent scoping review suggests the use of *P. major* as a facilitator of the healing process of pressure injuries [18]. These effects may be related to the presence of phenolic compounds in these plants. In a pioneering clinical study, the use of 10% *P. major* daily for 2 weeks to treat pressure sores and DFUs reduced the wound area and erythema and promoted a higher healing rate compared with the control group [19]. Although *in vitro*, *in vivo*, clinical and ethnopharmacological studies indicate that *P. major* promotes tissue re-epithelialization, there is a lack of understanding of the molecular mechanisms in hyperglycemic animals. Hence, there is a need for preclinical studies investigating the molecular mechanisms in the presence of *P. major*, understanding that this is a natural agent that can be used in the healing process, collaborating in difficult healing such as in hyperglycemic patients. In addition, it is an easily accessible plant found in several regions of the world, and there are few reports of adverse reactions [20]. Thus, the aim of this study was to investigate the effect of topical treatment with a cream containing *P. major* extract on the healing process in hyperglycemic mice.

2. Methods

2.1. 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) Cell Viability Assay

The MTT assay was performed to determine the appropriate *P. major* extract concentration to use to treat hyperglycemic mice. For the assay, 4×10^4 HaCaT cells (a human keratinocyte cell line) were seeded in 24-well plates. When the cells reached 70% - 80% confluence, they were treated with 1%, 3%, or 6% *P. major* extract (from a 10% mother tincture in 65% alcohol) or the assay control (65% alcohol) for 24 or 48 h. MTT was prepared fresh in a buffer containing NaCl (144 mM), KCl (5.9 mM), $MgCl_2$ (1.2 mM), HEPES (10 mM), $CaCl_2$ (2 mM) and glucose (1.58 mM). The HaCaT cells were washed with phosphate-buffered saline (PBS). Then, 500 µL of MTT was added to each well (0.5 mg/mL), and the plate was incubated for 2 h. After this incubation, the MTT was removed, and 300 µL of dimethyl sulfoxide (DMSO) was added. The plate was placed on a plate shaker for 15 min to dissolve the MTT crystals. Absorbance was measured at 490 and 560 nm with a spectrophotometer (FlexStation 3, Molecular Devices, San Jose, CA, USA).

2.2. Mice and Diabetes Induction

This study was approved by the Ethics Committee for the Use of Animals of the State University of Campinas (UNICAMP), under protocol no. 5251-1/2019. Isogenic male C57BL/6JUnib mice were obtained from the Multidisciplinary Center for Biological Research in Laboratory Animal Science at UNICAMP and housed for 5 - 8 weeks at 20˚C and a 12-h photoperiod. They were provided with standard fee and water *ad libitum*. During the experiment, the mice weighed 19.5 - 26.5 g.

DM was induced in mice by intraperitoneal streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO, USA) injections (50 mg/kg) for 5 consecutive days [21] [22]. After 14 days, the glycemic level of each mouse was assessed after an 8-h fast. Blood samples were taken from the tail vein and blood glucose was measured using a glucose meter Accu-Chek Active (Roche, Basel, Switzerland). Mice with a glycemic index ≥ 250 mg/dL were considered to be hyperglycemic and included in the study. The mice were divided into the control group (CG, n = 17) and the intervention group (IG, n = 19).

2.3. Wound Model

The mice were anesthetized with 15 mg/kg ketamine hydrochloride and 30 mg/kg xylazine hydrochloride administered intraperitoneally. The anesthetics and dosages were chosen based on previously established safe analgesia protocols for mice. It is important to note that the protocol used did not show any effect of the anesthetics on tissue re-epithelialization [23]. Two circular wounds in the dorsal midline region were made with a 6.0-mm dermatological punch (Miltex® Inc., Princeton, NJ, USA) until the muscle fascia was exposed. To control pain after the procedure, the mice received intraperitoneal tramadol hydrochloride (Eurofarma Laboratórios SA, São Paulo, Brazil) 20 mg/kg [22].

2.4. Treatment

The topical treatments used in this study were produced by a private handling pharmacy. The base cream was prepared from the aqueous phase containing distilled water, Amigel, Aristoflex AVC, octyl stearate, cetyl alcohol, ethylenediaminetetraacetic acid, glycerine, Emulium Delta, Silicone DC 9040, Silicone DC 245 and phenoxyethanol. Studies have used *P. major* in wounds in varying concentrations [24] [25]. Based on the cell viability assay, 3% *P. major* extract was introduced into base the cream. To obtain the cream formulation, a mother tincture of *P. major* (Laboratório Schraibmann Ltda, São Paulo, Brazil) was used. The final appearance of both the base cream formulation and the cream containing the plant active ingredient resulted in a product with a creamy texture and a light yellow colour. The wounds were treated topically once a day with 0.1 mL of the base cream (without *P. major*) for the CG or with the cream containing the *P. major* extract for the IG until the end of the experiment. The wounds were not sutured or covered—they were allowed to heal by secondary intention. There were no signs of infection. The wounds were observed every day for clinical signs of superficial infection such as purulent drainage, abnormal granulation tissue, abnormal foul odor, edema, induration, and erythema. After the injury, each mouse was housed individually to prevent traumatic damage to the wounds by other mice.

2.5. Macroscopic Evaluation of the Lesions

The wound area was measured on days 0, 3, 7 and 14 post-injury using digital images obtained with a PowerShot camera (SX400 IS, Canon, Tóquio, Japan) and

the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The same examiner took all of the images. The percentage of the wound area was estimated as: [(total lesion area immediately after wounding at day 0 − wound area that was still not covered with epidermis)/lesion area at day 0] × 100.

2.6. Tissue Extraction

Prior to tissue extraction, each mouse was anesthetized with 15 mg/kg ketamine hydrochloride and 30 mg/kg xylazine hydrochloride administered intraperitoneally. Tissue was extracted on days 3, 7 and 14 post-injury. As the wounds were made with a dermatological punch, two wounds were produced on each mouse. The wound on the right side was used for western blotting and the wound on the left side for histological analysis. On day 14 (the end of the experience), the mice were euthanized by cervical dislocation.

2.7. Histological Analyses

The wounds were pinned on cork to preserve the tissue structure. They were incubated individually in containers containing a 3.7% formaldehyde solution (pH 7.2) for 8 h. The fragments were placed in individual cassettes and incubated in 70% alcohol overnight, followed by incubation in different concentrations of alcohol for 30 min each and then two incubations in xylene (20 min each). At the end, the samples were bathed and embedded in paraffin (3 × 40 min) at 60˚C. Finally, 5 µm-thick sections were cut on a microtome (Zeiss, Oberkochen, Alemanha) and mounted on slides.

The slides were stained with hematoxylin and eosin and examined under a light microscope with a 40× objective lens (model DM 2000, Leica. Leica Microsystems, Heerbrugg, Switzerland). Images were taken with the LAS V 4.7 software (Leica). Using ImageJ v1.49, leukocytes and fibroblasts were counted based on the number of nuclei per field, and the number of blood vessels per field was counted.

2.8. Western Blotting

Tissue samples from days 3, 7 and 14 post-injury (n = 10, 14 and 10, respectively) were homogenised in solubilisation buffer (100 mM tris-hydroxymethyl-aminomethane [pH 7.4], 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM ethylenediaminetetraacetic acid, 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, 0.1 mg/mL aprotinin and 10% Triton-X-100) using a polytron PTA 20S generator (model PT 10/35, Brinkmann Instruments, Flórida, USA) at maximum speed. The homogenate was centrifuged at 14,881 *g* for 40 min at 4˚C. The supernatant was removed, and the protein concentration was determined using the Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich) and a microplate reader (measured at 562 nm; FlexStation 3, Molecular Devices, USA). The samples were mixed with a Laemmli buffer containing 100 mM dithiothreitol and heated at 95˚C for 5 min. Each sample (120 µg of protein) was subjected to gel electrophoresis in a mini-gel apparatus (Mini-Protean, Bio-Rad, Hercules, CA,

USA). The separated proteins were electro-transferred from the gel to the nitrocellulose membranes at 120 V for 90 min.

After transfer, the membranes were incubated in a blocking buffer (5% bovine serum albumin [BSA], 10 mM Tris, 150 mM NaCl and 0.02% Tween 20) for 1 h at room temperature to block non-specific protein binding. Then, the membranes were incubated with the appropriate primary antibody (diluted 1:1000 in 3% BSA, 10 mM Tris, 150 mM NaCl and 0.02% Tween 20), for 3 hours at room temperature on a shaking table. The primary antibodies were: interleukin 1beta (IL-1β, 25 kDa; SC1252, Santa Cruz Biotechnology, Dallas, Texas, USA); IL-10 (15 - 20 kDa; SC1783, Santa Cruz Biotechnology, Dallas, Texas, USA); tumour necrosis factor alpha (TNF-α, 20 kDa; SC8301, Santa Cruz Biotechnology, Dallas, Texas, USA); transforming growth factor beta1 (TGF-β1, 56 kDa; Ab31013, Abcam, Waltham, MA, USA); vascular endothelial growth factor (VEGF, 150 kDa; ab1316, Abcam, Waltham, MA, USA); and β-actin (42 kDa; Ab8227, Abcam, Waltham, MA, USA). After removing the primary antibody, the membranes were triple washed in basal solution for 10 minutes each. The peroxidase solution was prepared according to the primary antibody-antigen. Then, the membranes were incubated with the appropriate secondary antibody (diluted in 1% BSA, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20) for 2 hours at room temperature on a shaking table. At the end of the incubation, the membranes were washed again 3 times in basal solution for 10 minutes each wash. VEGF was normalized to β-actin expression.

2.9. Statistical Analysis

The data were analysed using GraphPad Prism version 6.01® (GraphPad Software, La Jolla-CA, USA) with analysis of variance (ANOVA) to compare the groups, and Student's t-test to compare the treatment days. The results are expressed as mean ± standard deviation. A p-value < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Cell Viability

Treatment with the *P. major* extract significantly increased HaCaT cell proliferation after 24 h ($p < 0.05$): by 22.64% (for the 1% concentration), 22.04% (for the 3% concentration) and 23.47% (for the 6% concentration). After 48 h, there was no increase in cell proliferation ($p > 0.05$; **Figure 1**). At that time, the 6% concentration decreased HaCaT viability by 11.21%. Based on these results, the cream used to treat the IG contained 3% *P. major* extract.

3.2. Morphometric Analysis

The wounds were monitored for up to 14 days post-injury. On day 3, the CG and the IG showed granulation tissue within the lesion covered by a yellowish crust and edges beginning to contract. On day 7, the lesions showed contracting edges, with granulation tissue for the CG and a yellowish crust covering the entire lesion

for the IG. On day 14, there was re-epithelialization in both groups. There was no significant difference between the groups ($p > 0.05$; Figure 2(A) and Figure 2(B)).

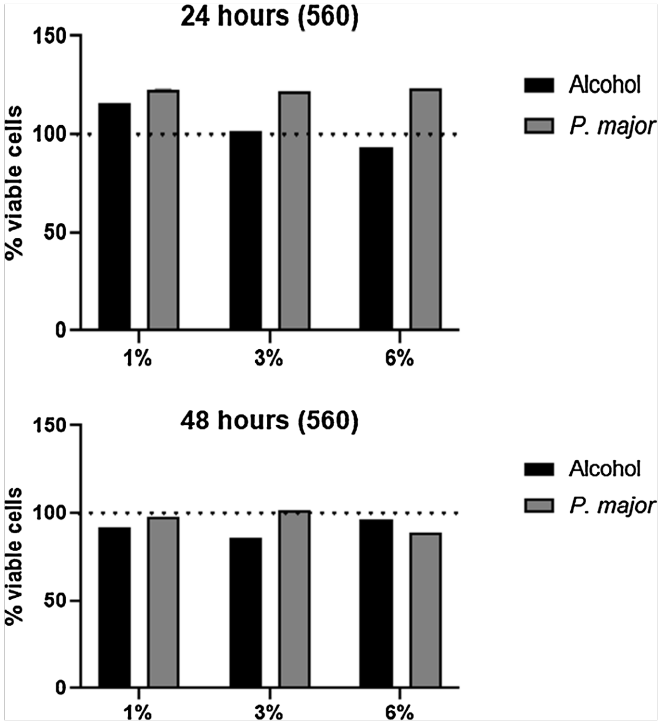

Figure 1. Viable cells and concentrations of *P. major*, in 24 and 48 hours, by cell viability studies (MTT). The results were expressed as percentages according to ANOVA (n = 4 - 6 at 24 hours and n = 3 - 8 at 48 hours).

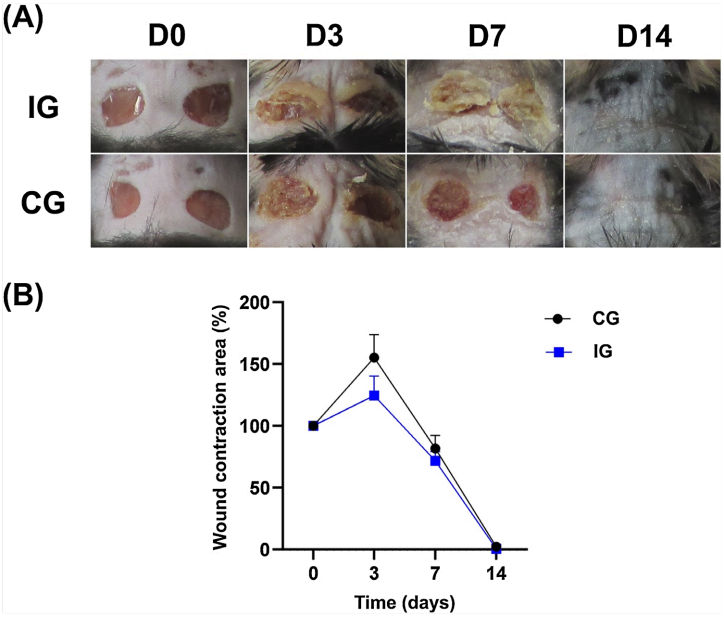

Figure 2. (A) Photos of the areas of wound contraction on the 3rd, 7th and 14th days after injury to the IG and CG. (B) Wound contraction area, in percentage, of IG and CG animals, on days 3, 7 and 14 after the injury. Values presented as mean ± SD, ANOVA, Student's T test, Bonferroni post-test.

3.3. Analysis of the Inflammatory Infiltrate, Fibroblasts and Blood Vessels

Figure 3 shows the results of the histological analysis. There was a significant increase in inflammatory cells in the IG compared with the CG on day 3 post-injury ($p < 0.05$). There was no significant difference on days 7 or 14. There was a significant increase in fibroblasts in the IG compared with the CG on days 3 and 7 post-injury ($p < 0.05$). Finally, there was a significant increase in blood vessels in the IG on day 7 post-injury ($p < 0.05$).

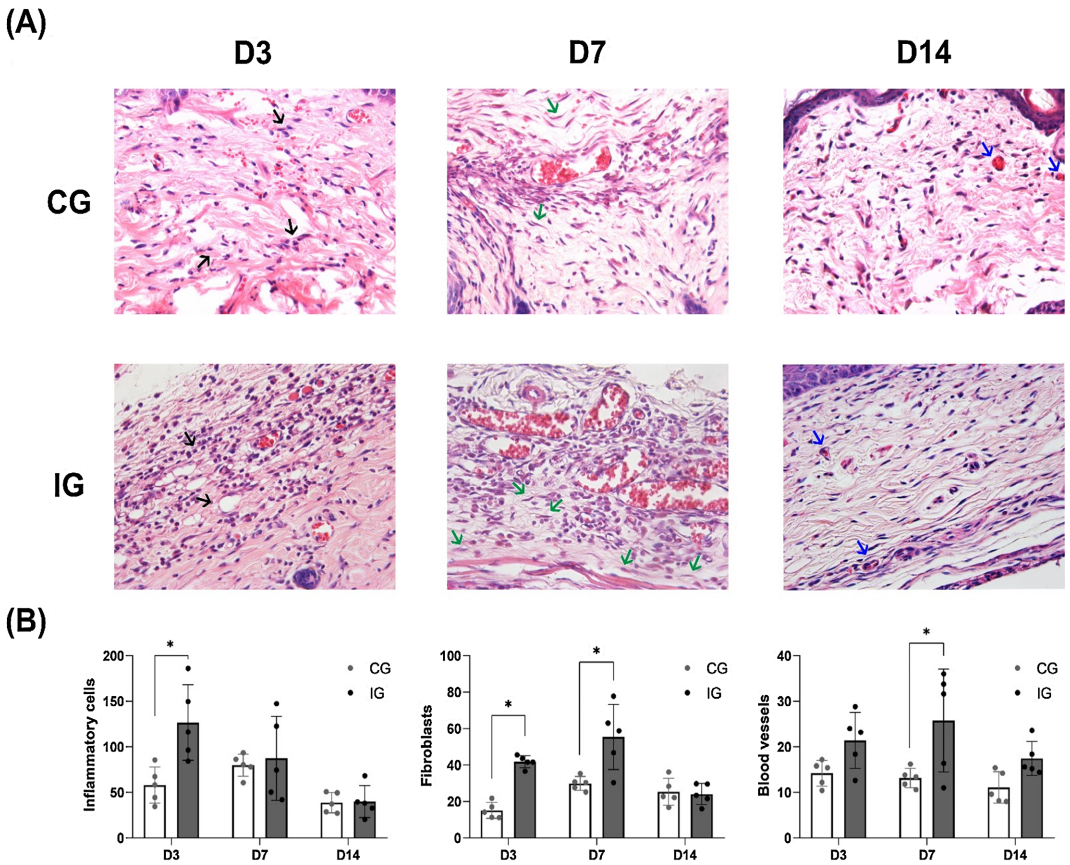

Figure 3. (A) Photomicrographs of slides stained with H&E from IG and CG, on days 3, 7 and 14 after injury. (B) Quantitative analysis of the number of inflammatory infiltrates, fibroblasts and blood vessels in the IG and CG, on days 3, 7 and 14 after injury. ($p < 0.05$ on 3rd for inflammatory infiltrate, $p < 0.05$ on 3rd and 7th for fibroblasts and $p < 0.05$ on 7th days of treatment for the number of blood vessels). Data presented as mean ± SD, ANOVA and Student's T test ($n = 5$).

3.4. Western Blotting

As presented in Figure 4, western blotting showed no difference between the CG and the IG in the levels of the pro-inflammatory cytokines IL-1β and TNF-α, the anti-inflammatory cytokine IL-10 as well as the growth factor TGF-β1 on day 3, 7 or 14 post-injury. On day 14, there was no IL-10 protein expression. On the other hand, VEGF protein expression was significantly higher in the IG compared with the CG on day 7 post-injury ($p < 0.05$).

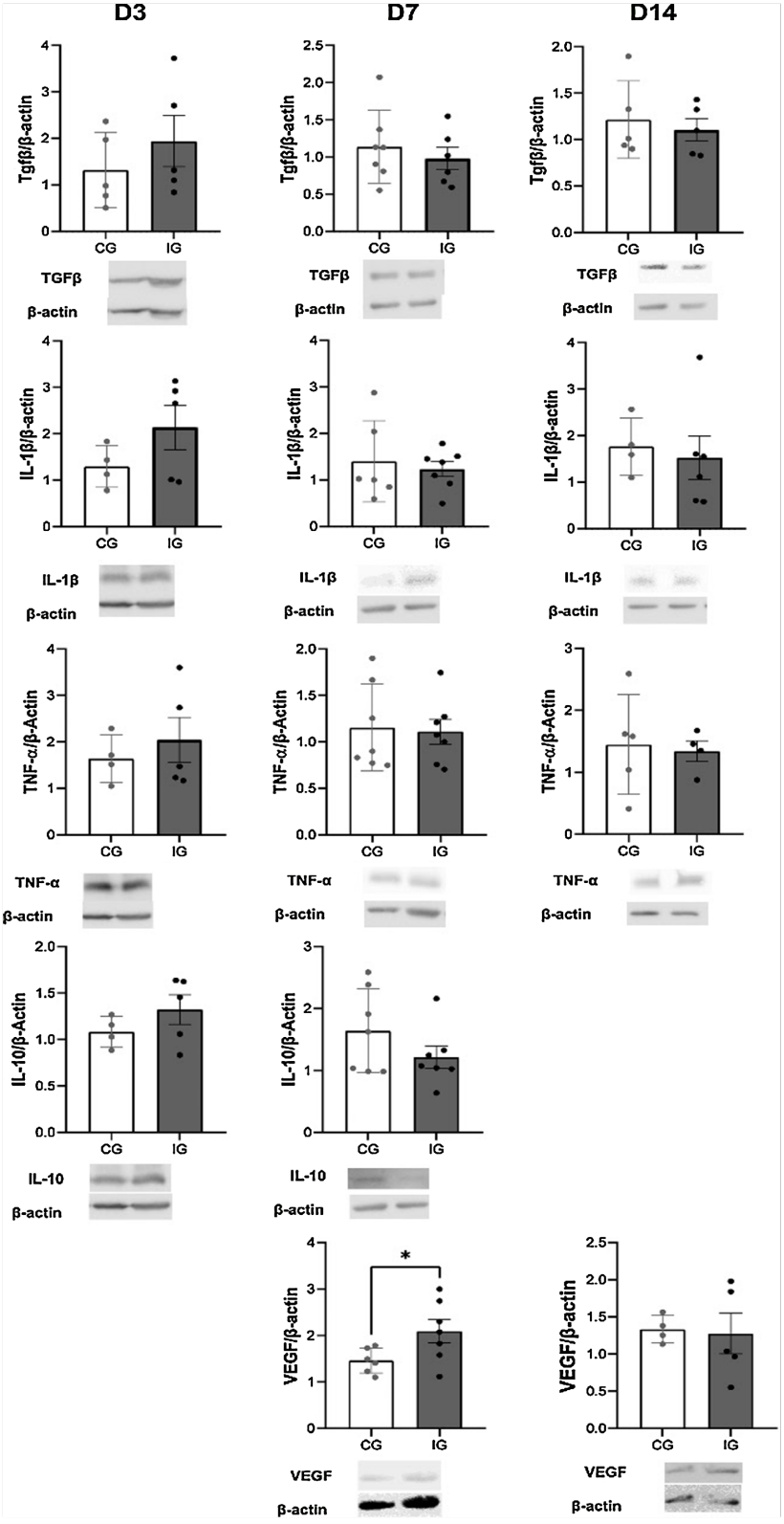

Figure 4. Levels of TGFβ-1, IL-1β, TNF-α, IL-10 and VEGF in IG and CG, on days 3, 7 and 14 after injury, by Western Blotting technique. Data were expressed as mean ± SD and Student's T-test (n = 5/7/5 for TGFβ-1, n = 5/7/5 for IL-1β and TNF-α, n = 5/7 for IL-10 and n = 7/5 for VEGF).

4. Discussion

The *Plantago* genus is widespread throughout the world, and its members, including *P. major*, are commonly used in folk medicine due to their wound-healing, anti-inflammatory, antidiabetic and anticancer effects [26]. Different *P. major* formulations and concentrations have been investigated and have shown promising results for the tissue repair of skin lesions. In one study, topical treatment of burns with a 50% aqueous extract of *P. major* improved re-epithelialization, reduced inflammatory cells and promoted good tissue formation on day 21 post-injury, but there was no significant difference in wound closure or microscopic findings between the studied groups [27]. The use of *P. major* in hydroalcoholic medium (10%) and *Aloe vera* improved the area of the lesion and the density of vessels, fibroblasts and collagen fibres [28]. When 10% *P. major* was incorporated into an ointment base for the treatment of skin wounds in mice, there was an early reduction in the wound area compared with the control group, but without a significant difference. Furthermore, on day 9 post-injury, there was neo-epithelialization and the presence of skin appendages [29]. An *in vitro* study conducted with dermal fibroblast cultures evaluated the biological effects of *P. major* extract encapsulated with alginate. The results encourage the use of *P. major*, especially in combination with alginate as an adjuvant in the healing process. Interestingly, *P. major* extract alone reduced fibroblast cell viability. Regarding collagen synthesis, the extract combined with alginate was more efficient when compared to *P. major* extract isolated [30].

In the present study, there were more fibroblasts and blood vessels on day 7 post-injury in the IG compared with the CG. Consistently, a study using 10% *P. major* extract on infected wounds in healthy rabbits showed an increase in the formation of new blood vessels after 3 and 7 days of treatment, as well as fibroblast proliferation, immature collagen bundles and granulation tissue organization after 7 days of treatment [31].

One of the activities of *P. major* that has been explored is its anti-inflammatory action [32] [33]. Our findings do not agree with the literature: the cream with *P. major* extract did not alter the expression of pro-inflammatory and anti-inflammatory cytokines. In a prior study that investigated the treatment of acetic-acid-induced ulcerative colitis, high doses of a *P. major* leaf extract significantly reduced the ulcerative lesion index and IL-6, TNF-α and IL1-β immunohistochemistry [34]. No previous studies have investigated tissue repair in animals with hyperglycemia and *P. major*. It is known that the healing process in the presence of DM has a prolonged inflammatory phase and lower expression of growth factors, which impairs tissue repair [35]. Measurements of macrophages and other markers may be important in understanding the healing process in hyperglycemic patients using *P. major*.

Regarding the growth factors, TGF-β1 protein expression did not differ between the IG and the CG at any time point. However, there was higher VEGF protein expression on day 7 post-injury in the IG. In a previous *in vitro* study investigating

the effect of *Plantago asiatica* extract on hair growth stimulation and the molecular mechanism of human hair follicle dermal papilla cells, the extract significantly increased cell proliferation and the expression of several hair growth factors, namely keratinocyte growth factor (KGF), VEGF and fibroblast growth factor 2 (FGF2) [33].

In summary, there was an increase in the number of fibroblasts and blood vessels as well as VEGF protein expression in the IG compared with the CG, but this treatment did not change the time it took for the lesion to close. It is known that a shorter time for the lesion to close prevents local complications, such as infection, which slows down the healing process [34] [36]. The preparation of the extract, including the part of *P. major* that is used, may lead to the presence of different bioactive compounds and influence the therapeutic effects. Bioactive compounds such as polyphenols and polysaccharides can stimulate cell proliferation and migration [29] [37], and flavonoids such as 7-luteolin, apigenin, hispidulin and baicalein [15] can contribute to the healing process [31]. Some limitations should be considered when interpreting the results of this study. First, the stability of *P. major* in the vehicle used was not investigated. Second, the bioactive compounds present in this composition were not investigated. Although *P. major* has been used in clinical practice for decades, little is known about the numerous chemical components present in each part of the plant and the most suitable ways to extract the desired bioactive compounds. Additional research is required to elucidate the molecular mechanisms of *P. major* before it can be used more widely in clinical practice.

5. Conclusion

Topical treatment of skin lesions on hyperglycemic mice with a cream containing *P. major* increased the number of inflammatory cells but did not alter the protein expression of pro- and anti-inflammatory cytokines. There were more fibroblasts and blood vessels and higher VEGF protein expression in the IG group, but these changes were not sufficient to modulate the healing time compared with the CG.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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