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Healing Activity of *Ilex paraguariensis* in an Experimental Cicatrization Model.

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ABSTRACT

The present study aimed to verify the effects of the use of a gel containing *Ilex paraguariensis* on the cicatrization process of tissue lesions in an experimental model. The *in vivo* evaluation was performed using a dorsal punch model. The sample consisted of 80 male *Rattus Norvegicus* rats, of *Wistar* lineage, divided into Control Group and Gel *Ilex* Group for 3, 7, 14, and 21 days of treatment. The macroscopic analysis of wound contraction revealed that the *Ilex* provided an epidermal regeneration after 21 days of treatment. The histological analysis showed, in the intermediate and final inflammatory phases, better delimitation of the epidermis, and proliferation and organization of collagen. The polarized light analysis showed that the groups treated with *Ilex* presented an accelerated process of tissue regeneration and mature collagen proliferation in all analyzed periods. The immunohistochemistry analysis showed increased expression of Transforming Growth Factor Beta (TGF-β1) and Vascular Endothelial Cell Growth Factor (VEGF-α). The *Ilex paraguariensis* aqueous extract was effective in healing epidermal wounds in an animal model. It is a promising therapy for wound healing, and so, future studies evaluating adjusted concentrations of extract are suggested.

Keywords: Ilex; Wound Healing; Collagen; Vascular Endothelial Cell Growth Factor; Transforming Growth Factor Beta.

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INTRODUCTION

Healing is a complex biological process that involves a series of cellular, extracellular and molecular events. It mainly comprises four interdependent and well-coordinated stages, including hemostasis, inflammation, proliferation and remodeling [1-2].

The use of topical administration drugs, such as gel formulations, is excellent for healing processes, because the polymers contribute to hydration and maintenance of a humid wound environment (2), and drugs with healing effects can be incorporated into those pharmaceutical forms, besides presenting a more affordable cost [1-3].

The *Ilex paraguariensis* A. St. Hil. (Aquifoliaceae), popularly known as *erva-mate*, is a plant native to South American countries (Brazil, Paraguay, Uruguay and Argentina) (4), which has great economic and social importance [5-6]. It is widely used in the preparation of stimulating beverages, and the most consumed forms are: the *chimarrão* (or *mate*), which is the hot water extract form dried green leaves; the *tererê*, cold water extract from dried green leaves; and *mate tea*, hot water extract from toasted leaves [4].

Besides being used for stimulating effects for being a source of caffeine, it is also used as a therapeutic agent due to its antioxidant, anti-inflammatory, hypocholesterolemic and antimicrobial activities [7-8], being used in the treatment of arthritis [6], obesity [9-10], liver and intestinal disorders [11].

The pharmacological activities of *Ilex paraguariensis* are related to its phytochemical composition, with phenolic compounds and methylxanthines being the main constituents (mainly present in leaves), mostly phenolic compounds [12-13]. Among these compounds, there is a higher concentration of chlorogenic acid, followed by quercetin and rutin in small quantities [13].

In addition, *erva-mate* is also popularly used in the form of poultice in the treatment of wounds and ulcers [14]. Martelli [15], in his study that reports the perspectives on the use of herbal medicines in tissue healing, shows that *Ilex paraguariensis* is among the several medicinal plants native to the Brazilian flora that present antimicrobial properties and meet criteria of environmental preservation and self-sustainable management, proving to be interesting in this context.

The search for new compounds with healing properties is still a challenge for both the pharmaceutical industry and academy [16]. Seeking more effective and affordable alternatives for the treatment of wounds is important, by the fact that it is a universal problem in the field of medical care, and generates great expenses for health systems [17].

In this context, the present study aimed to verify the effects of the gel containing *Ilex paraguariensis* in the healing process of tissue lesions in an experimental model.

MATERIALS AND METHODS

Plant material and extract

Ilex paraguariensis A. St. Hil. leaves were harvested in Erechim (27º38'03" S and 52º16'26" W), in the State of Rio Grande do Sul, Brazil. A voucher specimen was identified by Dr. Branca Maria Severo and deposited in the Herbarium of the University of Passo Fundo (RSPF 11074).

Extract preparation

The *Ilex paraguariensis* extract was prepared by turbo-extraction according to Luz [3]. Briefly, 200g of dried leaves were extracted for five minutes, with 1000mL of ethanol 20º GL (Gay-Lussac) as the liquid extractor (1:5 m/v). After filtration, this solution was evaporated under reduced pressure yielding the crude hydroethanolic extract. As previously described [12], in the *Ilex paraguariensis* extract, nine major compounds were identified by liquid chromatography coupled to photodiode-array detection (LC-PDA) and liquid chromatography-mass spectrometry (LC-MS) analysis: theobromine, chlorogenic acid, caffeine, 4-O-

caffeoylquinic acid, and rutin. The identification was based in the retention time, UV absorption spectra, mass spectra, and by the comparison with authentic samples.

Animals

A total of 80 male *Rattus norvegicus* rats, of *Wistar* strain, weighing between 200 and 250 grams were obtained from the State ;University of Londrina (Londrina, Paraná, Brazil). Three animals housed in each acrylic cage with free access to water and food, settle in a room at 23 ± 1ºC and 12-hour light/dark period. The *in vivo* evaluation of the anti-inflammatory activity was performed using the tissue lesion method with an 8mm punch. This is an induced, descriptive and observational experimental study, which followed all the guidelines of Federal Law No. 6638 and the recommendations of the Brazilian College of Animal Experimentation, with approval and register in the approval of the Animal Ethics Committee by the protocol 015/2016.

Preparation of *Ilex paraguariensis* gel

An amount of the extract was weighted, diluted in water and then incorporated into a carboxymethyl cellulose gel 3%(w/v), resulting in a formulation containing 5%(w/v) of *Ilex paraguariensis* extract.

To characterize the obtained gel, rheological measurements were made in a Brookfield's Viscometer (BROOKFIELD, model LVDVII+, USA) and the tests were conducted in a 50 mL beaker using spindle number 4. The temperature effect on the rheological behaviors of carboxymethylcellulose (CMC) base gel and Ilex gel was studied at temperatures of 25, 30 and 35ºC. The run time for each assay was set to six minutes. In the initial three minutes, the speed varied in an increasing manner to the maximum value of 3rpm. Then, the speed varied decreasing for three minutes until the minimum value close to 0rpm. (0,3; 0,5; 0,5). Both in the upstream and downstream runs it was obtained from 15 data of apparent viscosity (mPas) *versus* speed (rpm), resulting in a total of 30 data, which has been taken the average apparent viscosity for each one.

***In vivo* wound heling experiments**

The animals were divided into two groups, with 40 rats in each group: 1) Control Group (CG) 3, 7, 14, and 21 days with tissue damage and without treatment with *Ilex paraguariensis*, only CMC gel. Each group with 10 animals. 2) Ilex Group (IG) 3, 7, 14, and 21 days with tissue injury and Ilex gel treatment. Each group with 10 animals.

The animals were previously anesthetized with 80mg/kg of ketamine hydrochloride and 15mg/kg of xylazine hydrochloride. A dorsal region trichotomy approximately 4cm wide by 6cm long was performed between the fourth and twelfth vertebra after checking the anesthetic status of the animal by manual compression of the lower third of the tail. Immediately after, an 8mm punch was used to produce the lesion.

The gel was applied using the tip of a stainless-steel spatula containing approximately 24 mg of gel, which was layered on the lesion. The CG received only the gel base (blank) used in the Ilex gel formulation. The IG received only the Ilex gel.

Animals were euthanized at the end of each treatment period. They were previously anesthetized before receiving a lethal dose of 175mg/kg of intraperitoneal Thiopental [18]. Death was confirmed by the whitish coloration of eyes, lack of spontaneous breathing, and lack of reflax to the pain. The skin, where the healing process was found, was removed using surgical scissors, and samples were included in 10% formaldehyde to maintain morphological characteristics.

Histology Analysis

After euthanasia, the skin was removed, placed in 10% formalin, and taken to the Laboratory of Experimental Pathology at the Pontifical Catholic University of Paraná (Curitiba, Paraná, Brazil). Samples were identified using codes with specific numbers in each flask. These samples were sent to the Histocenter Laboratory (Guarapuava, Paraná, Brazil) without identification, only with markers provided by the researcher (ex: X1, X2). 2µm thick histological sections from the medial region to the edges were embedded in paraffin.

Three histological sections were cut from each piece. These were stained with Hematoxylin-Eosin (HE) for cellular and pathological analyses.

Immunohistochemistry (IHC) assay

To perform the IHC technique, slides were made as described for histological analysis, containing two sections per slide, per experimental group. Thermal dewaxing was performed, through 02 immersion in Xylol for 10 minutes each, and a 70º alcohol immersion bath for another 10 minutes. After a water bath, the slides were immersed in a solution of Sodium Citrate in a closed container and placed in a water bath for 30 minutes for Antigenic recovery.

Field marking was performed as described by Panis [19]. The sections were delimited with a Dako Pen® hydrophobic pen and endogenous peroxidases were blocked in a 10% hydrogen peroxide solution for 30 minutes, followed by nonspecific bond blocking by incubation in fetal serum 0.1% for 1 hour.

Subsequently, the sections were incubated with the primary antibodies (Santa Cruz Biotech), anti-TGF-β1 (Transforming Growth Factor Beta) (1:300) and anti-VEGF-α (Vascular Endothelial Cell Growth Factor) (1:300) in an overnight humid chamber at 4ºC for 2 hours. After incubation, the slides were submitted to 3 baths (5 minutes) in PBS and then incubated with secondary antibody and kept for 15 minutes. The slides were washed once again with PBS in a jet and three more washes with PBS drop.

Marking was revealed by incubation with 3,30-diaminobenzidine (DAB) for 15 minutes, followed by two washes in PBS, the first, jet wash and the second, drop wash. In the last stage, the sections were slightly counter-colored with Harry's hematoxylin (Merck, Darmstadt, Germany) for 30 seconds and then washed under running water. Incubated in alcohol 70º for 5 minutes in an immersion bath. Incubated in 95º alcohol for 5 minutes in an immersion bath. Incubated in Xylol for 5 minutes and incubated again in Xylol for 10 minutes. After draining all the liquid, the slides were assembled with Canadian balsam and coverslips.

For each test, negative controls were prepared in serial sections. The intensity and location of immunoreactivity with all primary antibodies used were examined throughout the content of the slide using an optical microscope. As a negative control, the primary antibody was omitted. For the image analysis study, color photomicrographs of representative areas (400x mafnification) were digitally acquired. For the semi-quantitative score, the images, in a total of 10 images for each cut of each animal were evaluated by means of the color deconvolution tool in Image J software (NIH, USA). Pixels were categorized as previously described by Chatterjee et al. [20], as strong positive (3+), positive (2+), weak positive (1+) and negative (0).

Polarized light analysis

Slides stained with Picrosirius Red were observed in an Olympus BX50 microscope (Miami, USA), and images were captured using a Dinoeye 30mm CCD, Olympus U-POT polarizer, photographed at 40X and saved in the Dinocapture software at 1024-1280 resolution. The Image-Pro Plus 4® software was used to analyze the percentage of mature and immature collagen.

Wound size assessment

All animals were photographed daily with the help of a Fujifilm Finepix Z, 10.0 Megapixels digital camera (Minato-Ku, Tokyo, Japão), without zoom approximation, kept on a tripod at a distance of 20cm. These images were transferred to the computer where the lesions were observed.

Statistical analysis

Data were analyzed in the GraphPad 5® software. The Wilcoxon test was used for the intragroup analysis; the Mann-Whitney test was used for the intergroup analysis, both with a level of statistical significance.

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RESULTS

Rheological measurements

The apparent viscosity of gels with solid dispersion in liquid phase (Fig 1A and 1B). No significant changes were observed from the bias of the flow curve from 0 to 3rpm and the downward tendency of the curve to speed from 3 to 0rpm. Thus, it was considered the average values of apparent viscosity.

The apparent viscosity decreased with the increasing of speed, which is a characteristic of pseudoplastic behavior for both gels (Figures 1A and 1B), with little variation for speeds greater than 2rpm in both gels. There is a tendency to a constant value of apparent viscosity with increasing speed that suggests asymptotic behavior typical of pseudoplastic or shear thinning fluids.

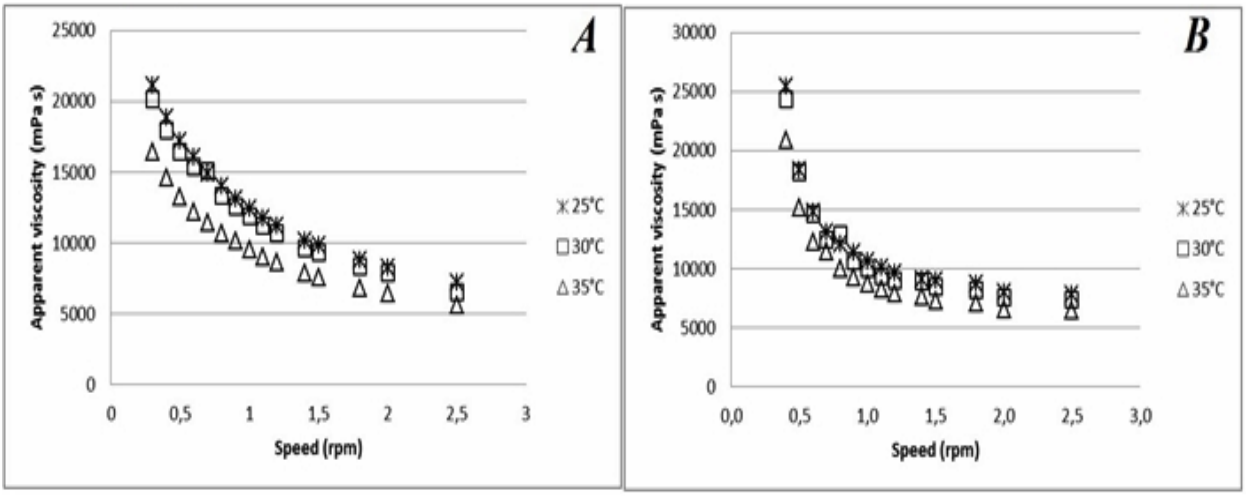

Figure 1: A) Flow curves of apparent viscosity *vs.* speed for CMC base gel at different temperatures, B) Flow curves of apparent viscosity *vs.* speed for Ilex gel at different temperatures.

Histological analysis

Figure 2 shows the histological sections (4x magnification) stained with hematoxylin-eosin (HE) from the CG and IG groups through 3, 7, 14, and 21 days of treatment.

The lesions are characterized by an acute to a subacute inflammatory process, and the inflammation found was the alterative-erosive type, typical of lining epithelia. The inflammation is shown in a diffuse distribution with variable intensity according to the treatment period.

In the 3-day treatment groups, the CG and the IG showed an inflammatory infiltrate with the presence of polymorphonuclear cells.

In the 7-day treatment groups, vascular, exudative, and proliferative phenomena were observed. In CG exudative predominance and angiogenesis were observed. In the IG, disorganized collagen was observed in the dermis and cell proliferation in the epidermis.

In the 14-day treatment groups, collagen proliferation was observer. The IG presented better delimitation of the epidermis in relation to the dermis compared with the other groups.

In the 21-day treatment groups, the thickening of the epidermis, collagen organization, and presence of crust were observed in the CG and IG.

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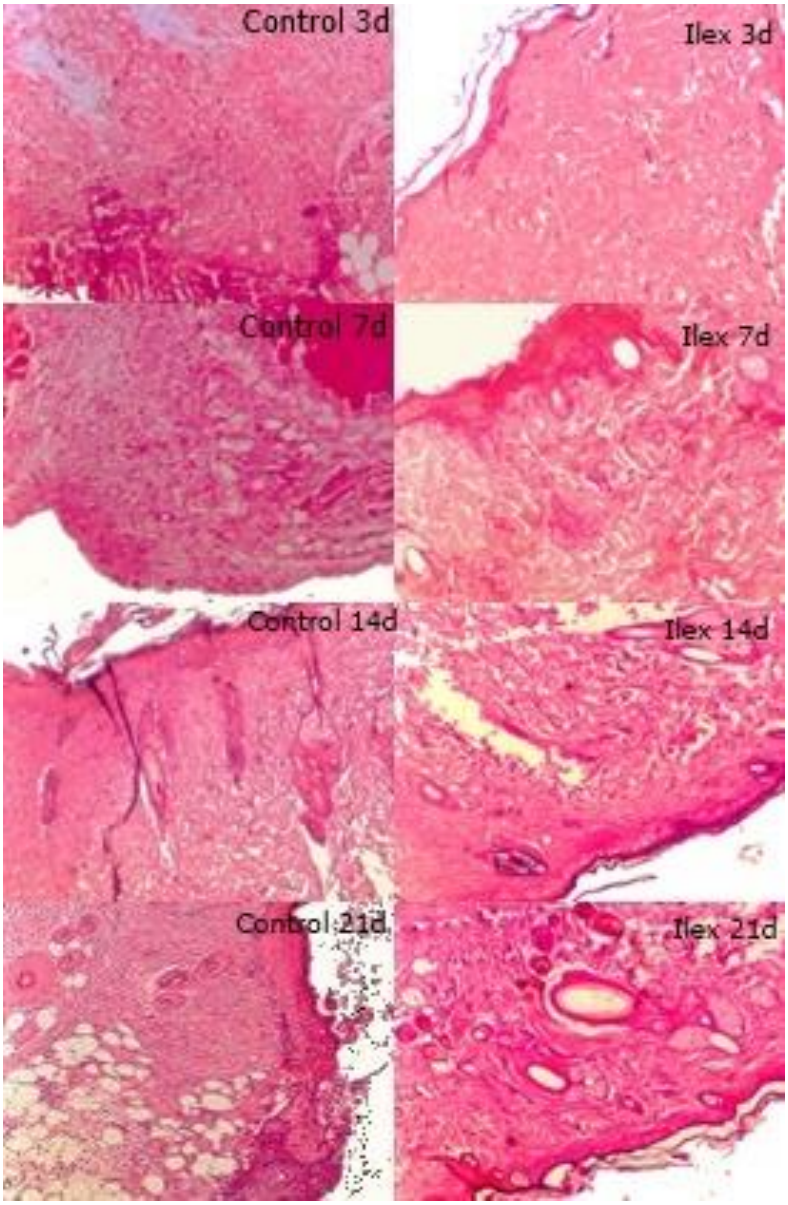

Figure 2: Histological analysis at 4 X magnification in the 3, 7, 14, and 21-day treatment groups.

Immunohistochemistry (IHC) assay

Figure 3 and figure 4 shows, respectively, the presence of VEGF-α and TGF-β1 by brown staining. VEGF-α had the highest presence on the twenty-first day in the control group with mean and standard deviation of 2.8±0.44, while in the Ilex group that happened on the third day with mean and standard deviation of 2.0±0.70. In the statistical analysis through Kruskal-Wallis the value of p=0.0009 and in the Dunn's post-test, a value of $p<0.05$ was obtained between the control 3D/Ilex 21D and control 21D/Ilex 21D.

The presence of TGF-β1 was its greatest presence on the twenty-first day in the control group with mean and standard deviation of 2.8±0.44 and in the group treated with Ilex on the fourteenth day with mean and standard deviation of 2.6±0.54. In the statistical evaluation using the Kruskal-Wallis test, a difference was noted with a p-value of 0.0006 and in the Dunn's post-test, a difference between the 21D/Ilex 21D and Ilex 14D/Ilex 21D controls with a p-value of 0.0016 and 0.0077, respectively.

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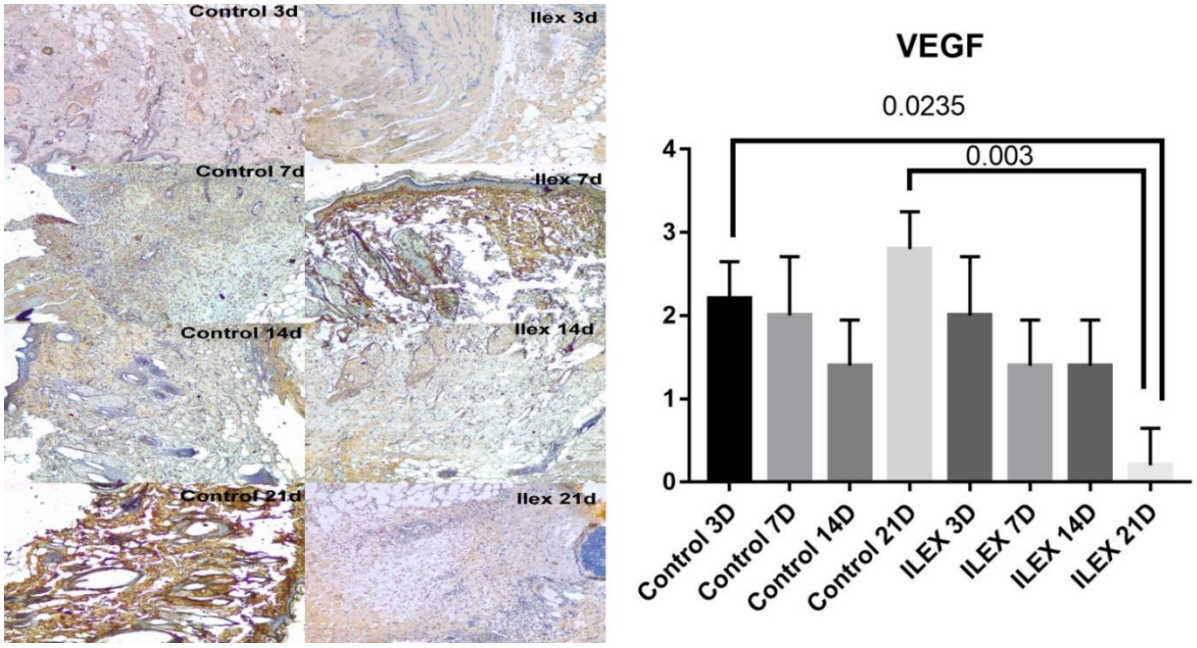

Figure 3: Expression of VEGF-α on healing using Ilex-based gel. A) IHC for VEGF-α in the control group shows that (brown) is highly present in basal lamina keratinocytes on the twenty-first day. B) IHC for VEGF-α in the control group shows that (brown) is highly present in basal lamina keratinocytes on the third day.

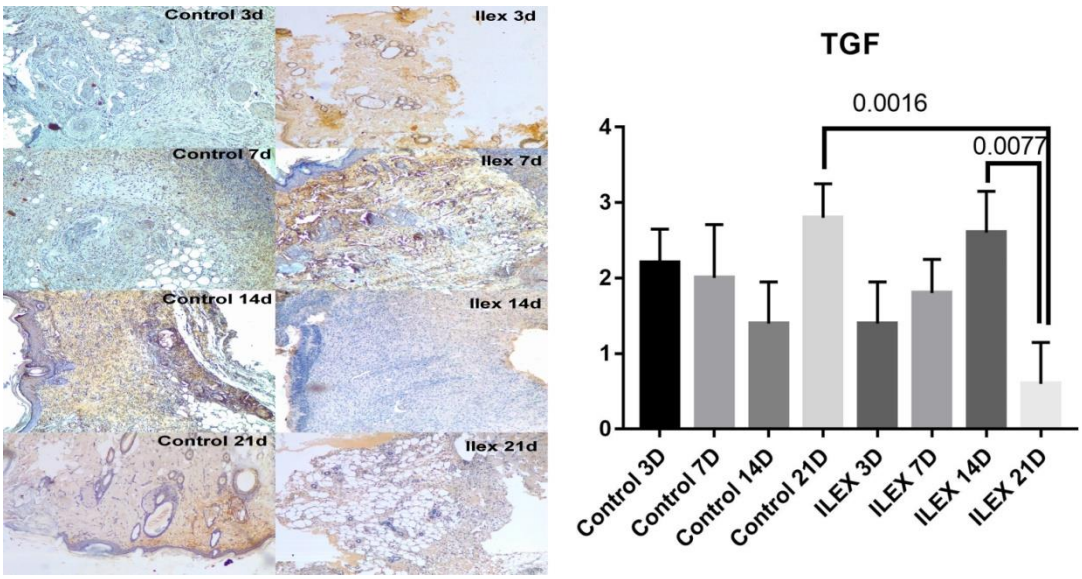

Figure 4: Expression of TGF-β1 on healing using Ilex-based gel. IHC for TGF-β1 on the fourteenth day the control group shows that (brown) is highly present in basal lamina keratinocytes on the twenty-first day. IHC for TGF-β1 on the seventh day in the control group shows that (brown) is highly present in basal lamina keratinocytes on the fourteenth day and.

Polarized light analysis

Figure 5 shows the analysis to quantify the mature collagen present in the lesion region using *Picrosirius Red*. The birefringence shows mature collagen in red coloration and immature collagen in greenish coloration. Mature collagen was observed in the IG in greater quantity in all analyzed periods compared to the observed in the other groups.

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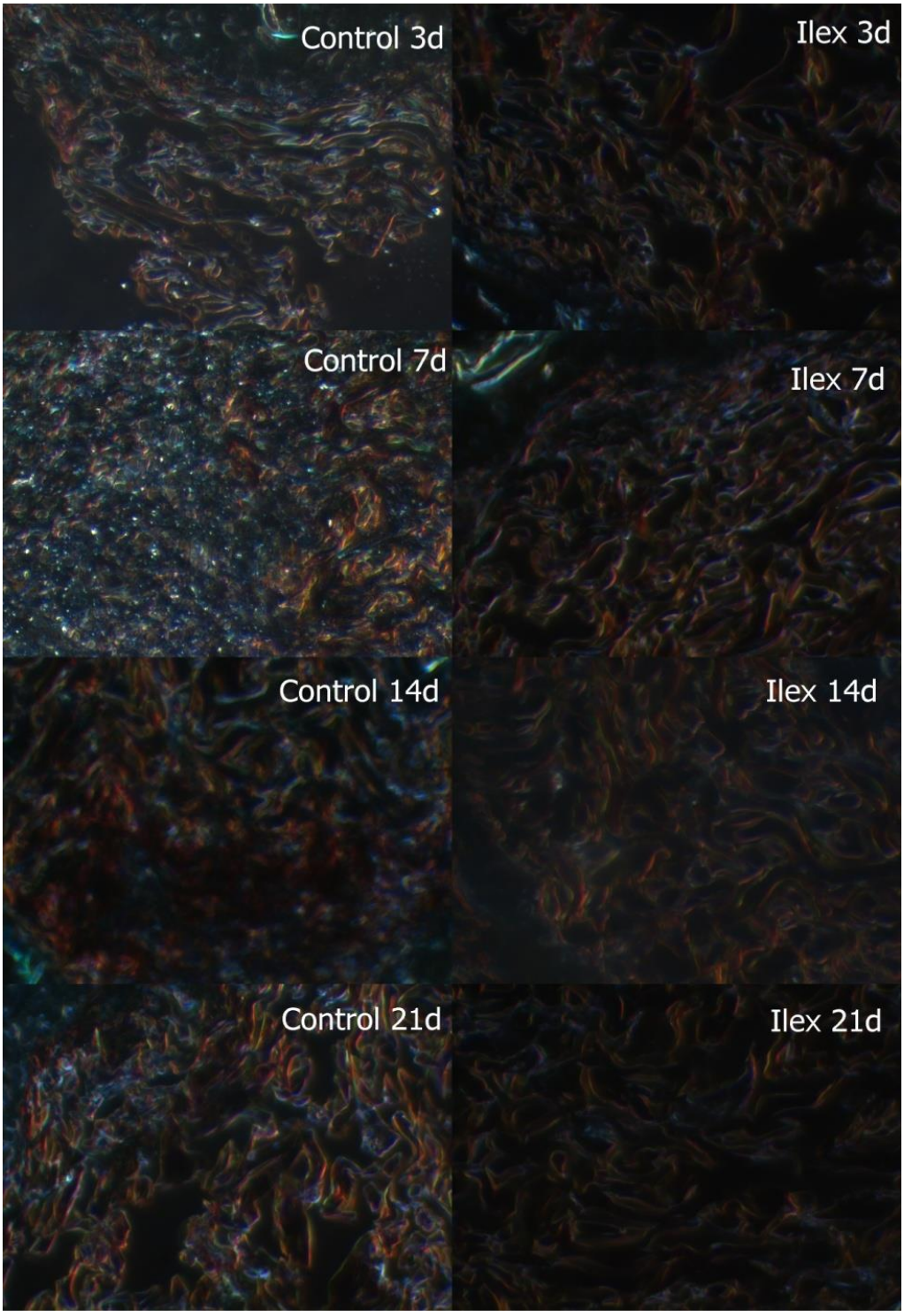

Figure 5: Polarized light analysis of groups in 3, 7, 14, and 21 days of treatments.

Figure 6 shoes the macroscopic analysis of wound contraction in the CG and IG in 3, 7, 14, and 21 days of treatment. The IG showed complete regeneration, however, with remnants of scars. The CG showed incomplete regeneration after 21 days of treatment.

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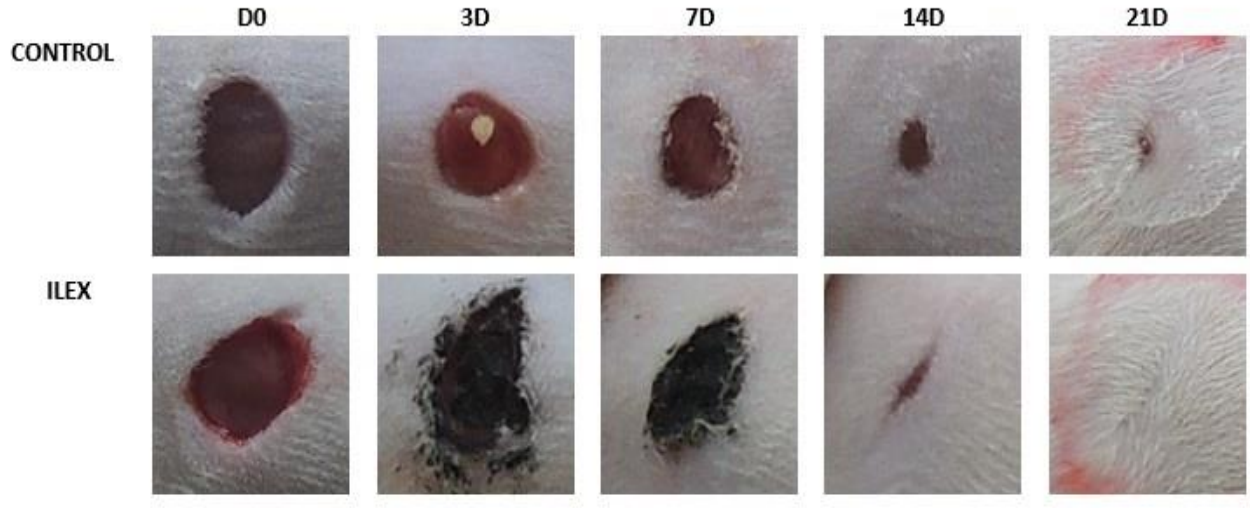

Figure 6: Macroscopic analysis of wound contraction in the CG and IG in 3, 7, 14, and 21 days of treatment.

DISCUSSION

The main objective of wound treatment is to achieve a quick repair, with satisfactory functional and aesthetic properties [21]. Thus, the treatment seeks protection against physical, mechanical and/or biological external agents, so that possible complications can be avoided. For this reason, an important factor to be considered is the pharmaceutical form in which active ingredients can be packed and how excipients can help in this process [22, 23].

Formulations with different concentrations of CMC promote a better adaptation to the wound area and can help in the healing process [22]. CMC is a great polymer for the development of formulations that make the wound treatment viable, because the polymer, by itself, presents a certain healing action.

Analyzing the application of CMC in 2% in leg ulcers for 90 days, it was observed that the polymer had effective action in reducing ulcer size, increasing granulation tissue, reducing swelling and pain, and reducing treatment costs [24].

CMC gel has been used with different extracts for wound contraction. Marchianti *et al* [25] evaluated the influence of different gelling agents, including CMC, added to the aqueous fraction gel of *Mammosa Merremia (Lour.)* for wound cicatrization. Formulations with CMC were preferable due to lower capacity to cause skin irritation. Also, Park *et al* [26] investigated the behaviors and characteristics of sodium carboxymethylcellulose hydrogel impregnated with chestnut honey (CH-CMC). The results indicated that the CH-CMC hydrogel optimized the healing process provided by chestnut honey when compared to the control groups (untreated group and group treated only with CMC hydrogel) [26].

Bearing it in mind, the present study used CMC as a gel-making polymer, with apparent viscosity between 5,000 and 21,000 mPas for white gel (without *Ilex paraguariensis* extract), and for *Ilex* gel, between 5,000 and 22,000 mPas. For the speeds studied, they did not present thixotropy.

Regarding rheological behavior, it was classified as pseudoplastic. The pseudoplastic character is common in topical pharmaceutical forms, such as gels and creams, and provides adequate spreadability of the product on the skin, which is directly related to the permeation of the active ingredient [24].

In the present study, better results were observed in the ilex group (IG), indicating that CMC may have optimized the activity of *Ilex paraguariensis* by the capacity to provide an environment with better humidity and absorb the exudates [27].

In relation to histological analysis, according to Figure 2, in the initial days there was intense proliferation of inflammatory cells, because after the breakdown of skin integrity, it is necessary that after the repair of local hemostasis, dead tissue removal and prevention of infections occur. Defense cells invade the injured area and assist in the elimination of bacteria, degradation of dead tissue and secretion of growth factors, cytokines and chemokines, necessary in the proliferative phase [28].

One of the factors of great importance for the healing process is TGF-β1, which is involved in almost all stages of healing, amplifying the migration of inflammatory cells, stimulating angiogenesis, the formation of granulation tissue, reepithelialization, and increasing collagen III deposition in the proliferative phase and tensor strength of the tissue through collagen deposition I [29].

In the present study, in the immunohistochemical analysis (shown in Figure 4), the presence of TGF-β1 in the IG group was observed in greater quantity on the fourteenth day, while for CG only on the twenty-first day. In addition, in IG, collagen I was observed in greater quantity in all periods analyzed compared to the control group (Figure 5).

Therefore, it is suggested that the healing process was considerably faster in IG due to the expression of TGF-β1 having occurred earlier, as well as the more efficient collagen deposition.

Furthermore, through the protein deposition that occurred in the proliferative phase, the structure formed in this stage acts as a basis for the proliferation of endothelial cells, mediated by the secretion of VEGF-α, and then there is the formation of new vessels, necessary for the synthesis, deposition and organization of granulation tissue [29]. The increased expression of VEGF-α is associated with acceleration of healing in all treated groups, as also observed by Marchianti [25], on his work using *Merremia mammosa (Lour.).*

In the present study, a higher presence of VEGF-α was observed in the group treated with *Ilex paraguariensis* on the third day, compared to the control group, which presented a higher presence only on the twenty-first day, indicating the occurrence of angiogenesis long before the control group, an essential process on this phase, corroborating what was observed by Marchianti [25] referring to the correlation of VEGF-α expression and healing time.

Chen *et al.* [30], evaluating the effect of topical application of chlorogenic acid on wound healing by excision in rats, observed that the topical application can accelerate the wound healing process and increase collagen synthesis (through positive regulation of key factors and its antioxidant potential).

Thus, since *Ilex paraguariensis* is a plant rich in chlorogenic acid [13], it can be attributed, in parts, the stimulating capacity of collagen deposition to this constituent, corroborating Chen's findings [30], because according to the analysis of polarized light (figure 5), mature collagen was observed in IG in greater quantity in all periods analyzed.

Furthermore, Thangavel [31] reports that the wound contraction occurs mainly due to increased myofibroblast activity, and that the differentiation of fibroblasts to myofibroblasts can be stimulated via increased expression of TGF-β, a process that is linked to increased collagen synthesis and complete tissue repair.

The results observed in the present study corroborate the description of Thangavel [31], because due to the increased expression of TGF-β1 and collagen deposition, a complete and effective tissue regeneration was observed at the end of the treatment.

CONCLUSION

The gel containing *Ilex paraguariensis* aqueous extract was effective in healing epidermal wounds in an animal model. It could be considered a promising therapy for wound healing (alone or as an integrative therapy). Additional studies to evaluate and adjust the concentration of the extract and formulation application are suggested.

Conflicts Of Interest

No conflicts of interest, financial, or otherwise.

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