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Healing and local anti-inflammatory activity of the aqueous extract of *Leonurus sibiricus* L. leaves in an animal model

Atividade cicatrizante e anti-inflamatória local do extrato aquoso das folhas de *Leonurus sibiricus* L. em modelo animal

Actividad cicatrizante local y antiinflamatoria del extracto acuoso de hojas de *Leonusus sibicirus* L. en un modelo animal

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Abstract
This study analyzed the healing and local anti-inflammatory activity of compounds from the aqueous extract of leaves of the plant *Leonurus sibiricus* L. (rubim) by evaluating the resolution of cutaneous wounds made in Wistar rats. The wounds were treated daily with the extract of *L. sibiricus* 10% (m/v) in sodium alginate 5% (m/v) to obtain a gel that facilitated its application; sodium alginate 5% (m/v) and Dexpanthenol 42 mg/g were used as negative and positive controls, respectively. The wounds were evaluated daily for appearance (crusting, swelling) and reduction during 21 days. The data were statistically analyzed and the results indicated that the extract of *L. sibiricus* favored a better healing evolution of the wounds when compared to the positive control, besides presenting less inflammatory exudate. Regarding the time of resolution (closure) of the wounds, no significant difference was observed between the animals treated with the extract of *L. sibiricus* and the negative control, however, the extract of *L. sibiricus* favored a more homogeneous healing giving the wound a better visual appearance, which was confirmed by microscopic analysis. These results corroborate the common sense knowledge of the population that uses this plant in relation to the indicated therapy.
Keywords: Cicatrization; Phytotherapy; Wounds; Traditional medicine.

Resumo
Este estudo analisou a atividade cicatrizante e anti-inflamatória local de compostos do extrato aquoso de folhas da planta *Leonurus sibiricus* L. (rubim) por meio da avaliação da resolução de feridas cutâneas confeccionadas em ratos

Wistar. As feridas foram tratadas diariamente com o extrato de *L. sibiricus* 10% (m/v) em Alginato de sódio 5% (m/v) para obtenção de um gel que facilitou a sua aplicação; Alginato de sódio 5% (m/v) e Dexpantenol 42 mg/g foram usados como controle negativo e positivo, respectivamente. As feridas foram avaliadas diariamente em relação ao aspecto (crosta, edema) e à redução durante 21 dias. Os dados foram analisados estatisticamente e os resultados indicaram que o extrato de *L. sibiricus* favoreceu uma melhor evolução cicatricial das feridas quando comparados ao controle positivo, além de apresentar menor exsudato inflamatório. Em relação ao tempo de resolução (fechamento) das feridas, não foi observada diferença significativa entre os animais tratados com o extrato de *L. sibiricus* e o controle negativo, entretanto o extrato de *L. sibiricus* favoreceu uma cicatrização mais homogênea conferindo à ferida um melhor aspecto visual, o que foi confirmado por análises microscópicas. Esses resultados corroboram o conhecimento de senso comum da população usuária dessa planta em relação à terapêutica indicada.
Palavras-chave: Cicatrização; Fitoterapia; Ferida; Medicina tradicional.

Resumen
Este estudio analizó la actividad cicatrizante y antiinflamatoria local de compuestos del extracto acuoso de hojas de la planta Leonurus sibiricus L. (rubim) mediante la evaluación de la resolución de heridas cutáneas realizadas en ratas Wistar. Las heridas fueron tratadas diariamente con extracto de L. sibiricus al 10% (m/v) en alginato de sodio al 5% (m/v) para obtener un gel que facilitó su aplicación; Se utilizaron alginato de sodio al 5% (m/v) y dexpantenol 42 mg/g como controles negativos y positivos, respectivamente. Las heridas fueron evaluadas diariamente en relación a su apariencia (costra, edema) y reducción durante 21 días. Los datos fueron analizados estadísticamente y los resultados indicaron que el extracto de L. sibiricus favoreció una mejor evolución de la cicatrización de las heridas en comparación con el control positivo, además de presentar menor exudado inflamatorio. En cuanto al tiempo de resolución (cierre) de la herida, no se observó diferencia significativa entre los animales tratados con el extracto de L. sibiricus y el control negativo, sin embargo el extracto de L. sibiricus favoreció una cicatrización más homogénea, dándole una mejor apariencia visual a la herida, lo cual fue confirmado por análisis microscópicos. Estos resultados corroboran el conocimiento de sentido común de la población que utiliza esta planta en relación a la terapia recomendada.
Palabras clave: Cicatrización de heridas; Fitoterapia; Heridas; Medicina tradicional.

1. Introduction

The use of medicinal plants is linked to the history of mankind, becoming part of the culture of human beings in such a way that has made it very complex to date the beginning of its use (Badek *et al*., 2019); there are records of the use of ginseng herb by Chinese emperors, and this is one of the first forms that has been documented (Braga, 2011). Furthermore, in 1991, a 5,300-year-old mummy was discovered in the Italian Alps by a German mountaineering couple, and among its belongings were found mushrooms with antibacterial and anti-inflammatory activities (Peintner *et al*., 1998), showing that since the most distant times, not only the use of plants, but also of resources from nature, have been linked to the power of healing through empirical observations of the populations (Rocha *et al*., 2015; Neca *et al*., 2022e).

Brazil, with its enormous biodiversity, becomes a fortunate field of research, being considered one of the richest countries in the world in terms of plant species with therapeutic potential (Silva *et al*., 2017), it is estimated that at least half of the species used contain bioactive substances with curative and preventive properties (Lorenzi & Matos, 2002), emphasizing the need for the scientific study of medicinal plants for the research of new drugs.

Currently, medicines directly or indirectly derived from plants consist of 30% of the marketed drugs (Devienne *et al*., 2004). Due to the many people living with difficult access to health care and because synthetic medicines are expensive, the use of natural products becomes the only resource for many communities (Soares, 2007).

In Brazil, wounds are a serious public health problem due to the large number of patients with alterations in the integrity of the skin, although there are few records of these cases (Waidman *et al*., 2011; Cardinelli *et al*., 2021). Wounds are responsible for significant rates of mortality and morbidity, generating physical and emotional distress to patients (Oliveira *et al*., 2016; Tolfo *et al*., 2020), and the treatment of wounds can often be expensive for the population, leaving as the only alternative the treatment with alternative medicine.

Leonurus sibiricus L., popularly known as rubim or macaé, belongs to the Lamiaceae family and its distribution is mainly in the south and southeast of Brazil, where it grows spontaneously, being considered an invasive species (Duarte & Lopes, 2005). This plant is consumed by the population in the form of tea or topically on wounds and insect bites, assuming it has anti-inflammatory and healing activities (Wadt, 2008).

Despite the use of *L. sibiricus* by the population, no scientific studies were found confirming neither its efficacy in the described therapy nor possible adverse effects. Thereby, this study aimed to investigate the local anti-inflammatory and healing activity of the aqueous extract of the leaves of this plant in open skin wounds experimentally made in rats, through topical use.

2. Methodology

Animals

This study was approved by the Ethics Committee on Animal Use (CEUA) of the Universidade Federal do Paraná - Setor Palotina (certificate number 34/2020) and it was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen - number A58CA5E).

In the experiment, Wistar rats from 90 to 120 days old were used, regardless of sex, obeying the ethical principles of animal experimentation recommended by the Brazilian College of Animal Experimentation (COBEA). AROUCA (Brasil, 2009). The animals were kept under controlled conditions of temperature (22 ± 2.0°C), 12-hour light/dark cycle, and received water and pelleted feed for rodents *ad libitum*.

Preparation of the aqueous extract of *L. sibiricus*

Specimens of *L. sibiricus* were collected in the western region of the state of Paraná/Brazil, at coordinates: 24°49'11.7 "S 53°44'12.1 "W, in March 2020. The leaves of *L. sibiricus* were manually isolated and washed in running drinking water, then disinfection was performed in 0.1% (v/v) sodium hypochlorite solution for 20 minutes and rinsed in distilled water. The leaves were dried for 72 h at 40 °C. The dried material was ground in a knife mill until fine-grained (Pereira *et al*, 2009) material was obtained.

The ground material was used to prepare a mixture at 10% (w/v) in distilled water at 70°C; the plant cells were ruptured by sonication in an ultrasonic bath, using 5 cycles of 5 minutes on/ 5 minutes off. The mixture was filtered and used to prepare a gel with sodium alginate 5% (w/v).

Experimental wound

On experimental day zero (D0) the animals underwent surgical wounding. Each animal was anesthetized with Isoflurane 3 - 4% diluted in 100% oxygen and an area of the dorsal region below the base of the neck was trichitomized. Antisepsis of the region was done with topical solution of Chlorhexidine digliconate 10 mg/mL and analgesia was provided by Lidocaine (5mg/Kg sc). Subsequently, a 2x2 cm wound was made. The skin was removed, the wound was cleaned with saline and the animals received topical treatment according to the experimental group. For analgesia after surgery, each animal received 2.5 mg Tramadol hydrochloride (100 mg/mL) orally. Analgesia was performed immediately after surgery, 6 and 24 hours after surgery.

Experimental groups

The animals were randomly divided in three experimental groups (G1, G2 and G3) - n=8/group. All animals received a topical treatment once daily in the wound region (300 μL). The negative control group (G1) was treated with Sodium alginate 5% (w/v), the test group (G2) was treated with the extract of *L. sibiricus* leaf 10% (w/v) + sodium alginate 5% (w/v) and the positive control group (G3) was treated with Dexpanthenol 42 mg/g. Between each daily treatment, cleaning of the wound with saline (NaCl 0.9% (m/v)) was performed, and in case of necrosis (crusts), debridement was performed. Dexpanthenol was used as a positive control due to its use in research in the same scope; it helps in skin healing by stimulating the process and granulation, helping in epithelialization, since it activates fibroblasts, forming an epithelium with a high organizational level of the structures that form the epidermal layer (Faria *et al*., 2019; Ebner *et al*., 2002). The negative control, alginate, was used as a thickening and gelling agent (Garcia-Cruz *et al*., 2008), since it is an inert compound used as an excipient for controlled drug delivery (Severino *et al*., 2019).

Macroscopic wound analysis

The wounds were evaluated according to the presence or absence of bleeding, exudate, edema and crusting (necrotic tissue). For morphometric analysis the wounds were photodocumented and measured with a pachymeter on experimental days D0, D3, D7, D14 and D21. Wound area was calculated from the photos using ImageJ software (National Institutes of Health - NIH). The degree of wound area contraction was calculated using the equation *Relative wound contraction (%) = (Initial wound area - contracted area) x 100/initial wound area* (Oliveira *et al*., 2000):

Microscopic wound analysis

At D3, D7, D14 and D2, animals from G1, G2 and G3 were randomly chosen and euthanized with an overdose of Isoflurane and KCl 19.1% (w/v) intravenously (1.0 mL/350-400 g). The wound region was excised with a 0.5 cm margin of healthy skin around the lesion, deep down to the muscle fascia, and was fixed in buffered formalin 10% (v/v) for histology (Garros *et al*., 2006).

Statistical analysis

The data were analyzed using the STATISTICA 7.0 program (VinceStatSoftware). The calculation of the mean wound areas of the experimental groups and the calculation of standard deviation was performed. The Shapiro-Wilk normality assumption test and homogeneity of variance test (Levene test) were performed, and in cases where the assumptions were not met, the data were log x +1 square root transformed for normalization. After data normalization, the data were submitted to the Analysis of Variance (ANOVA) test, in addition to Tukey's Test. All analyses considered a 5% significance level ($p < 0.05$).

3. Results

Postoperative evolution

After wound preparation (Figure 1), the postoperative period of the animals occurred without complications; all recovered from anesthesia and there were no deaths. Regarding the evolution of the skin wounds, all experimental groups showed exudation, crusting and pain on handling until the D7 postoperative. From the 8th day on there was a thickening of the wound crust and the animals showed no pain on handling. From D14 it was observed an increase in wound contraction speed, and on D21, all groups already had re-epithelialization and hair growth (Figure 2).

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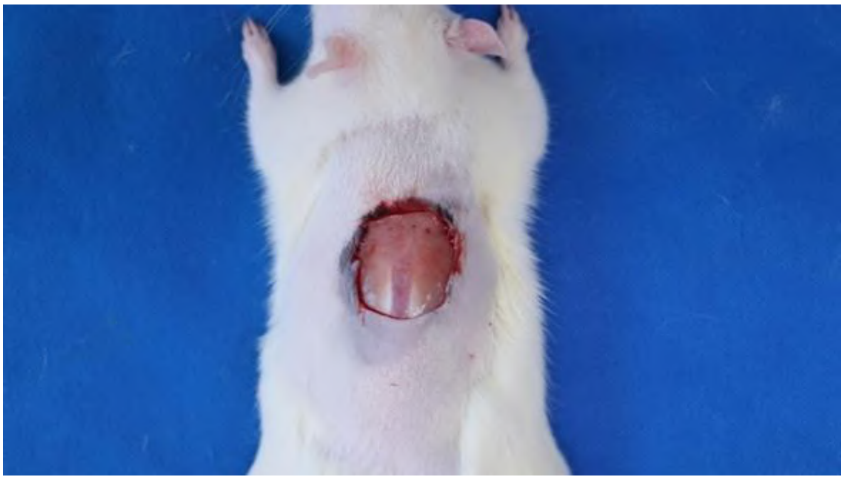
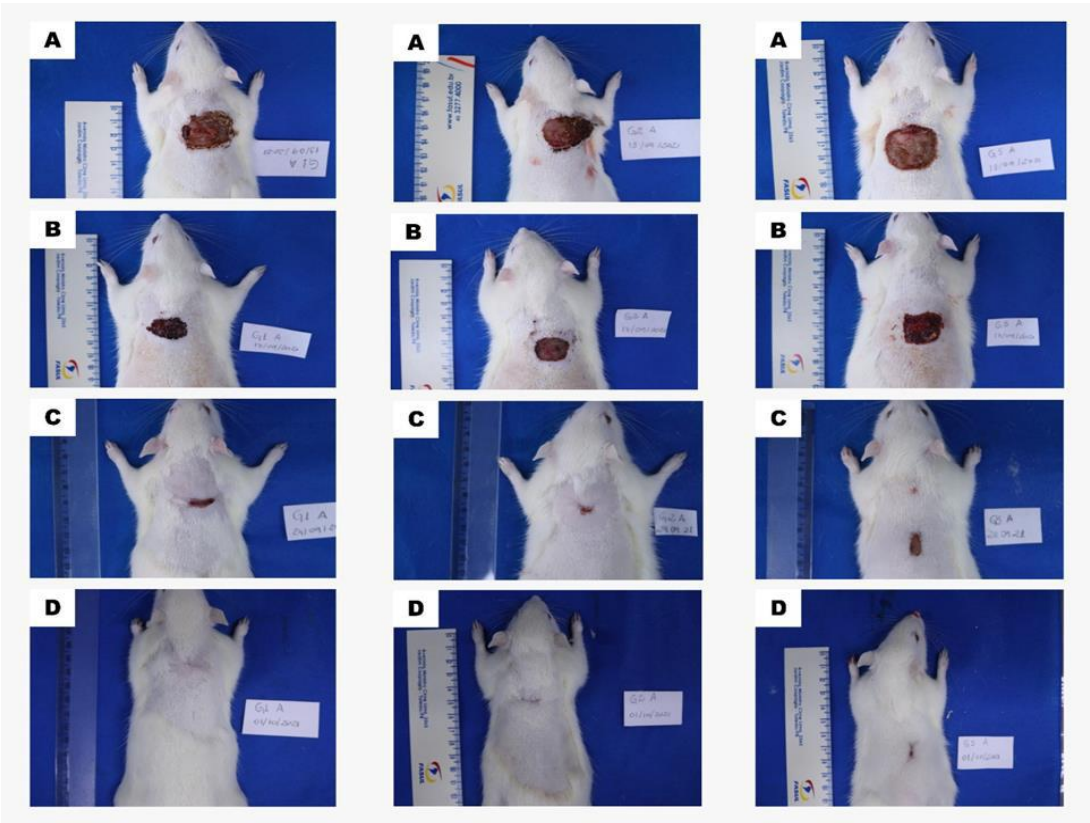
Figure 1 - Wound on the back of Wistar rats (D0).



Source: Authors (2021).

Figure 2 - Photos representing the evolution of wound healing. Columns from left to right: G1, G2 and G3. D3 (A), D7 (B), D14 (C) and D21 (D).

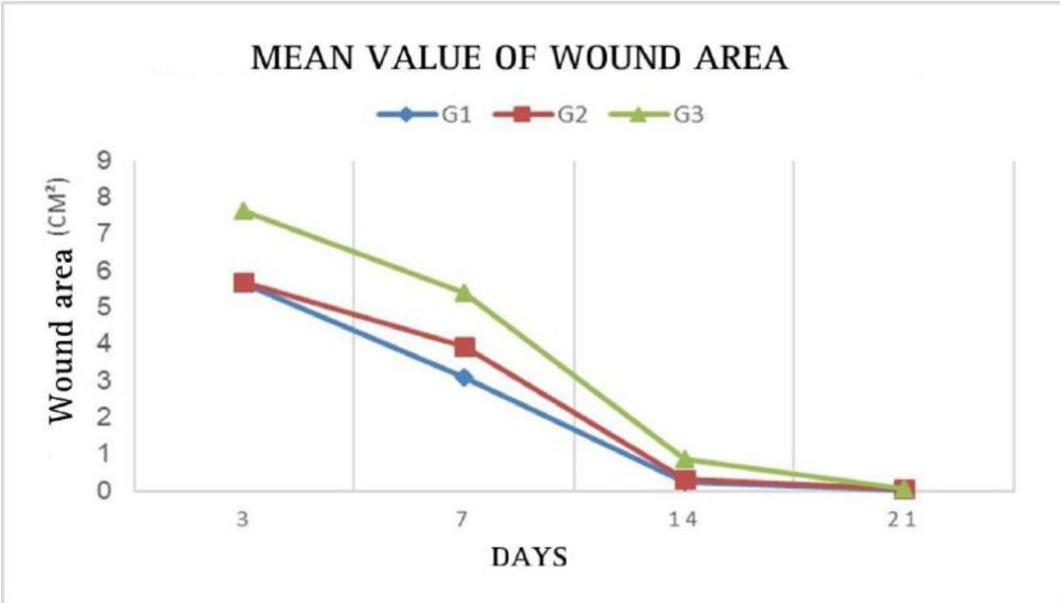


Source: Authors (2021).

Macroscopic and microscopic analysis of the wounds

The wound area showed a gradual reduction in all experimental groups when the mean wound area was compared between them (Figure 3). Table 1 shows the values of the mean wound area classified according to the group and day of analysis. It was observed that until the D3 postoperative, the clinical appearance of the wounds was similar in G1, G2 and G3, with high hyperemia, elevated edges, serosanguinous exudate and pain at wound cleaning and debridement. In G1 and G2 a yellowish film was formed due to the gel-like characteristic of the sodium alginate that adhered to the skin wound. In addition, the same groups showed partial crust formation, while in G3 there was little wound contraction in the same period of time, formation of much granulation tissue and significant presence of edema.

Figure 3 - Mean value of wound area (cm2) on D3, D7, D14 and D21 (p>0.05).



Source: Authors (2021).

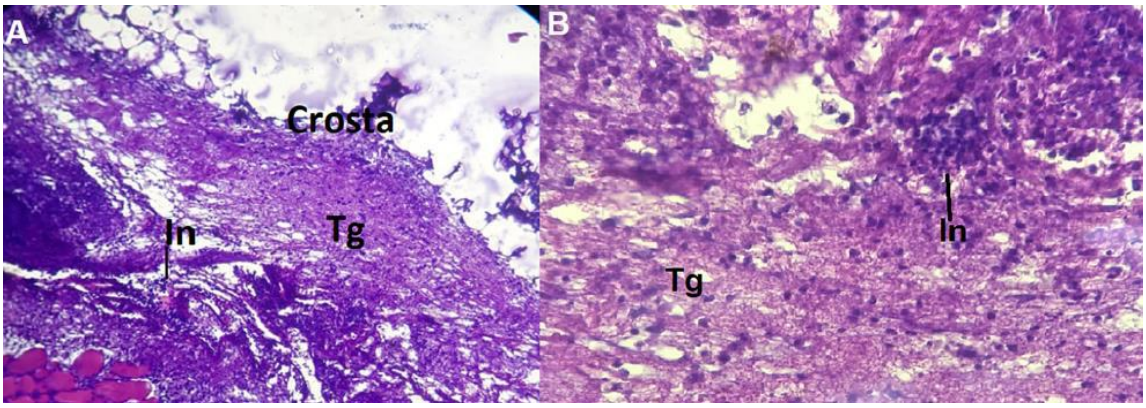
Table 1. Mean values and standard deviation of wound areas of G1, G2 and G3 on D3, D7, D14 and D21. Different lower case letters differ by Tukey's test at 5% level.

	GROUPS		
DAYS	G1	G2	G3
3	5.64 ± 0.68	5.68 ± 1.09	7.65 ± 1.02
7	3.11 ± 0.35	3.93 ± 1.21	5.40 ± 0.87
14	0.25 ± 0.10	0.32 ± 0.21	0.87 ± 0.37
21	0.01 ± 0,02	0.07 ± 0.12	0.05 ± 0.07
	a	a	b

Source: Authors (2021).

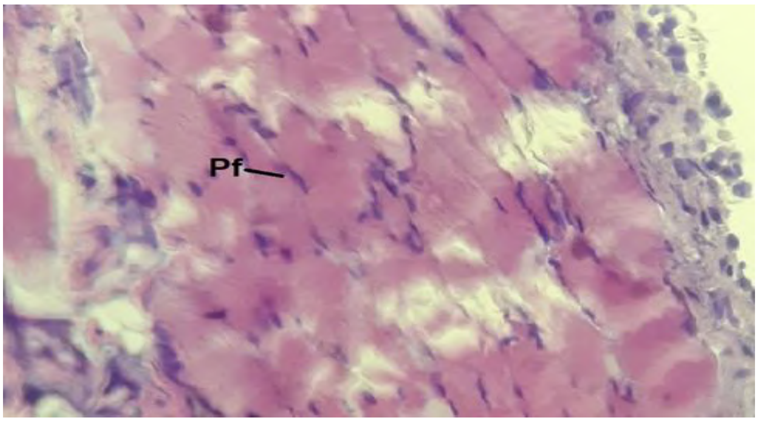
The histological analysis of G1 on D3 shows the presence of crust with thin thickness, granulation tissue just below and inflammatory infiltrate (Figure 4). G2 showed granulation tissue with greater fibroblastic proliferation (Figure 5) and G3 showed a disorganized granulation tissue with inflammatory infiltrate, without crust formation (Figure 6). From D7 all groups evolved with crust formation in the wound region, and G1 and G2 had a similar evolution, with thickening of the crust and considerable contraction of the wound area. G3 showed crusting, but with little contraction of the wound area, and some wounds were still swollen. On D14 all groups started to evolve in a similar way; the wounds were already at least 50% smaller compared to the initial size, the scabs had already disappeared, and on D21 all wounds already showed a process of re-epithelialization, and G3 still had individuals with incomplete wound closure and a worse aesthetic appearance, with the presence of a more evident and less uniform scar.

Figure 4 - G1 - D3. Skin - HE. A) 100x. Thin crust and partial coverage of the injured area. B) 400x. Area with inflammatory infiltrate and disorganized granulation tissue. Tg - Granulation tissue. In - Inflammatory infiltrate.



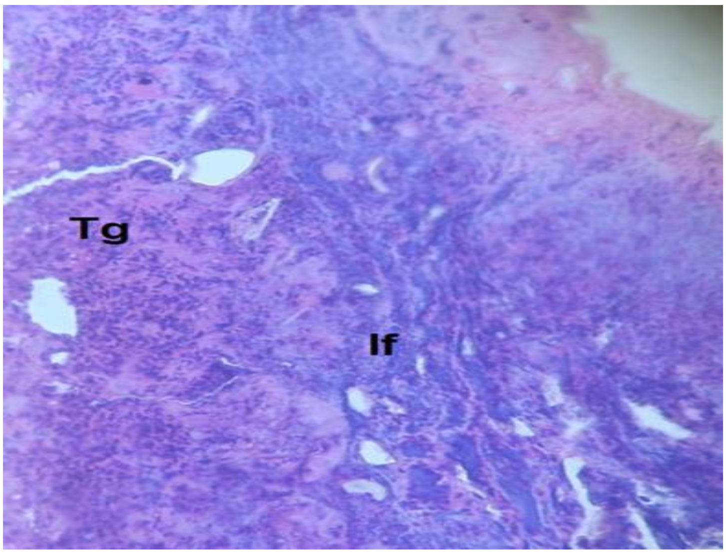
Source: Authors (2021).

Figure 5 - G2 - D3. Skin - HE. 400x. Presence of fibroblasts for collagen synthesis. Pf - Fibroblastic proliferation.



Source: Authors (2021).

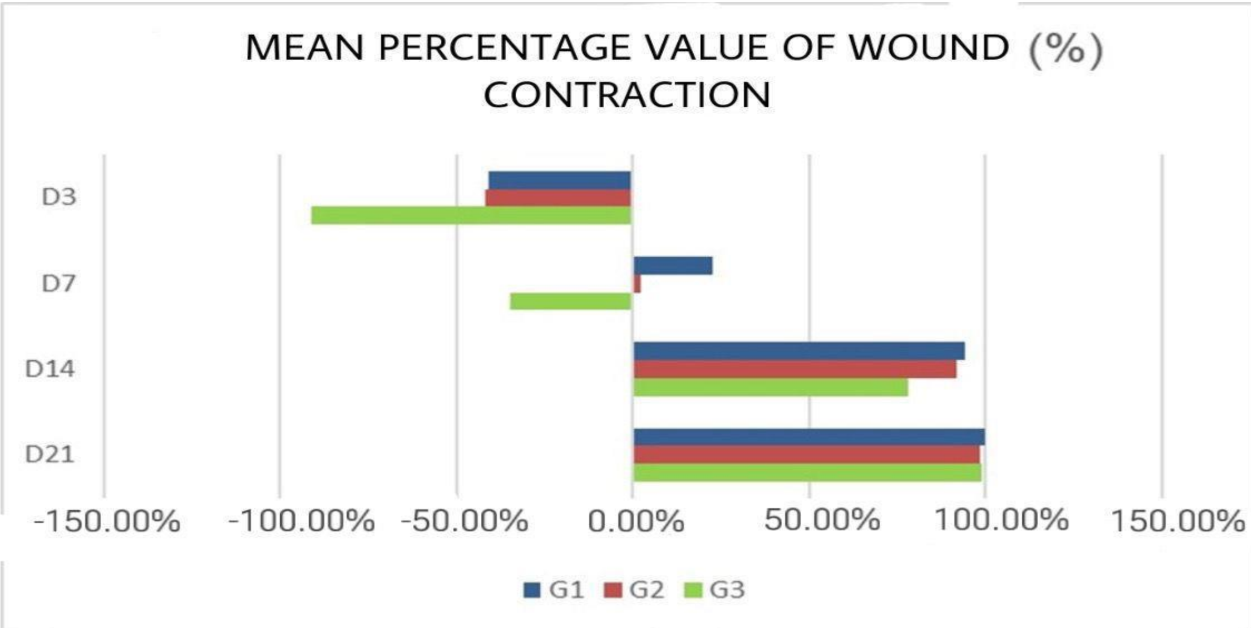
Figure 6 - G3 - D3. Skin - HE. 400x. Inflammatory infiltrate and disorganized granulation tissue. If - Inflammatory infiltrate. Tg - Granulation tissue.



Source: Authors (2021).

After resolution of the wound, it was possible to observe that despite being fully closed, in G1 the scar tissue in the wound region was visually larger and less uniform when compared to G2. Figure 7 shows the evolution of the relative wound contraction during the experimental days.

Figure 7 - Evolution ofwound contraction area of G1, G2 and G3 on D3, D7, D14 and D21.



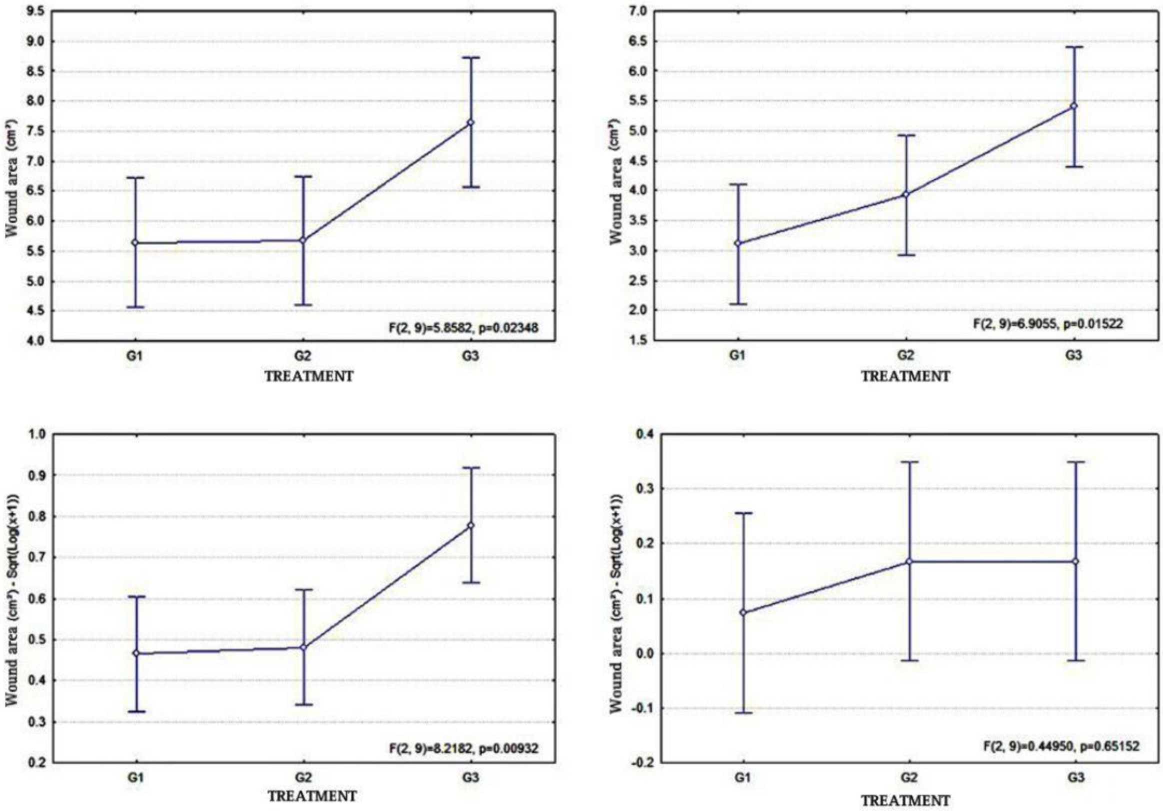
Source: Authors (2021).

Statistical Analysis

The wound area data on D3 and D7 meet the normality assumptions of the Shapiro-Wilk and Levene tests. The wound area values on D14 and D21 were normalized (square root of log + 1) to meet the assumptions of normality and homogeneity of variance.

Analysis of variance (ANOVA) revealed that there are significant differences between the mean wound areas in G1, G2 and G3 on D3, D7 and D14 with p=0.023, 0.015 and 0.009 respectively. On D21 there were no significant differences between the mean areas of the wounds in the experimental groups. When the Tukey test was performed, there was a statistical difference between G1 and G2 on none of the days evaluated in the experiment (D3, D7, D14 and D21), while G3 showed a significant difference compared to G1 and G2 on D3, D7 and D14. These analyses indicate that G3 performed worse with regard to wound reduction until experimental D14, and from this day on, all groups showed similar wound evolution (Figure 8).

Figure 8 - Mean wound area on D3, D7, D14, and D21.



Source: Authors (2021).

4. Discussion

The use of herbal medicines with the aim of accelerating the healing process and that have an anti-inflammatory effect is widely discussed in the scientific literature, with the most varied results. There are studies on the healing activity of *Caesalpinia ferrea*, popularly known as jucá, in goat skin lesions, showing a significant effect on healing (Oliveira *et al*., 2010). The effect of sugar in different formulations on healing by second intention in wounds of Wistar rats was also studied, showing that the sugar associated with chlorhexidine in the ratio 2:1 makes healing earlier (Guirro *et al*., 2019).

A review study on the healing action of medicinal plants showed that several medicinal plants used by common sense as barbatimão, mastruz, calendula and babaçu have different activities, such as anti-inflammatory, antimicrobial, stimulation of proliferation, migration and cell activity (Moreski *et al*., 2018). In this study, no significant difference was observed between the resolution of skin wounds treated with sodium alginate and sodium alginate associated with the aqueous extract of *L. sibiricus* leaves when the variable evaluated was the wound healing time. In fact, sodium alginate, despite being an inert component, has a good capacity for absorbing and retaining water, which leaves the wound initially more wet (Rodrigues, 2008), this possibly contributed to a better performance of the start of healing. However, it is important that, in addition to rapid healing, the wound presents homogeneous scar tissue, avoiding hypertrophic scars, keloids and skin imperfections that prevent the growth of hair and its complete integrity. In this context, the compounds present in the aqueous extract of *L. sibiricus* leaves seem to provide a healing process not only faster, but also more homogeneous.

Dexpanthenol is a B-complex provitamin, which when applied topically, turns into pantothenic acid - a natural constituent of the epidermis, helping to organize the forming structures of the epidermis (Idson, 1993). However, this compound performed worse in relation to wound healing during the beginning of the process, even though it helps to retain humidity in the wound, which contributes to increase the epithelialization rate; moreover, Dexpanthenol decreases the formation of crusts, which delay the healing process (Mandelbaum *et al*., 2003).

According to the results of the study and data from the literature, it can be seen that maintaining humidity in the wound is an important factor in the healing process. In this sense, sodium alginate is able to form a hydrophilic gel that holds water in the wound site and keeps it wet (Gonzalez *et al*., 2004), while Dexpanthenol is a hydrophobic compound, so it is assumed that unlike holding water in the wound, Dexpanthenol only prevents evaporation of the already existing local water over a period of time.

G1, treated with the aqueous extract of *L. sibiricus*, was the experimental group that presented the lowest edema rate, this finding corroborates other study on this plant, in which it was observed that there is a reduction of paw edema in rats induced by carrageenan with the use of a methanolic extract of *L. sibiricus* (Islam *et al*., 2005). Plants of the genus *Leonurus* contain tannins (Wadt *et al*., 1996), a polyphenol that precipitates proteins from injured tissues, favoring their repair, besides decreasing vascular permeability and wound exudation, promoting tissue repair (Passaretti *et al*., 2016), this helps to explain the lower exudation observed in G1.

Although on D3, D7 and D14, G1, G2 and G3 showed a variance between the means of wound areas with statistically significant difference, on D21 none of the three groups showed superiority regarding the variable time of wound healing. However, when we compared the quality of the healing process, it was noted that G2 presented a healing with a better clinical aspect and wound edges with better integrity, indicating that even if the variable time was not different between the groups, the macroscopic result of the final scar was better in G2.

Moreover, Dexpanthenol, despite being a drug indicated in the literature as a suitable positive control for studies of skin wounds, it has a better efficacy from D14, since it promotes an increase in collagen synthesis through the activation of fibroblasts, but the rate of synthesis and remodeling of collagen is slow (Guimarães *et al*., 2011). This helps to explain the fact that only from D14, the animals of G3 showed an evolution of healing similar to the other groups.

5. Conclusion

This study suggests that the aqueous extract of *L. sibiricus* leaves positively influences wound healing in the model evaluated, since the animals treated with the extract showed a better evolution of the lesion than Dexpanthenol, a drug already indicated for healing. However, the choice of sodium alginate as a gelling agent may have favored a better environment for the resolution of the wound, so that in relation to healing time the presence of the extract of *L. sibiricus* was not significant, unlike the homogeneity of the healing process, which was more efficient in the presence of the extract.

Therefore, it is important to expand experimental studies on animals, with different dosages, other excipients and formulations, in addition to researching the plant components responsible for positive intervention in the tissue healing process.

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