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EFFECT OF AQUEOUS EXTRACT OF LEAVES OF *KALANCHOE PINNATA* IN THE HEALING OF SKIN WOUNDS IN WISTAR RATS

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Abstract: *Kalanchoe pinnata* is a species widely used in folk medicine in Brazil and other parts of the world. In Brazil, it is included in the list of plants of interest to the Unified Health System (Renisus). **Goal**: To evaluate the effect of topical application of aqueous extract of *Kalanchoe pinnata* in the healing of skin wounds on the back of rats. **Methodology**: 20 mice were used (*Rattus norvergicus*), of the Wistar lineage, adults, males, acquired by the study institution (UNINOVAFAPI). The animals were submitted to the production of a cutaneous wound on the back and randomly divided into three groups: Control Group (CG), consisting of 06 animals submitted to daily cleaning of the wound with saline solution; Standard Group (GP), consisting of 07 animals treated with Trofodermin® (clostebol acetate/neomycin sulfate); and Extract Group (EG), consisting of 07 animals treated with the aqueous extract of*Kalanchoe pinnata*. Healing was evaluated by macroscopic analysis of the evolution of the appearance of the lesion and measurement of scar retraction of the wound by digital planimetry, as well as by histological study on slides stained with hematoxylin-eosin (H.E.) considering the parameters of inflammatory cellular elements, including collagenization and re-epithelialization. The project was approved by the Ethics Committee in the Use of Animals of the Centro Universitário UNINOVAFAPI (opinion nº _0002/15). **Result:** AThe mean area of the skin wound at the end of the treatment was significantly smaller in the EG when compared to the CG. The histological findings confirm predominantly complete epithelialization in the EG, with significantly lower infiltration of mononuclear cells (p=0.008) compared to the other groups, that is, in the healing process of the EG there was a predominance of the final stages of the healing process. Furthermore, reepithelialization in EG was significantly higher (p=0.016)

compared to GC. **Conclusion**: The analysis of the results obtained indicates that the aqueous extract of leaves of *Kalanchoe pinnata* favored the healing of open skin wounds in rats.
Keywords: *Kalanchoe pinnata*, Wound Healing, Phytotherapy, Medicinal Plants.

INTRODUCTION

The species: *Kalanchoe pinnata* (figure 1) belongs to the Crassulaceae family. The gender:*Kalanchoe* comprises approximately 125 species and many of them are native to Madagascar (Africa) (COSTA et.al, 2008). It is a perennial succulent plant that grows between 1-1.5 m in height and has opposite leaves with 10 to 20 cm in length, being popularly known as coirama or saião. It is widely used in traditional medicine in America, Africa, India, China and Australia due to its homeostatic and healing properties (KAMBOJ; SALUJA, 2009). In Brazil, it is included in the List of Medicinal Plants of Interest to the Unified Health System (RENISUS) (BRASIL, 2009).

Regarding the healing effect, a study carried out in rats showed that the ethanolic extract of *Kalanchoe pinnata,* in a model of wounds by skin excision in the dorsal region, showed that on the 11th day of treatment, the animals in the extract group had a reduction of 86.33% of the wound, a result similar to that found in the standard group (85.49%) treated with 2% mupirocin and better than that found in the control group (69.36%) treated with 100% vaseline. According to the authors, this healing-promoting activity could be explained by the antioxidant action of the polyphenolic compounds present in this species (NAYAK; MARSALL; ISITOR, 2010).

In an experimental model of gastric ulcers, a healing and gastroprotective effect of *Kalanchoe pinnata* in ethyl acetate fraction extract, which has a higher concentration of flavanoids and phenolic compounds than the crude extract and aqueous fraction. of gastric ulcers (SOBREIRA, 2013).

In view of the above, and considering the wide popular use of this species, as well as the existence of a limited number of scientific studies that evaluated its effectiveness in wound healing, the present study aimed to evaluate the effect of topical application of aqueous extract of leaves of *Kalanchoe pinnata* in the healing of skin wounds on the back of rats.

METHODS

This study was carried out at the Experimental Surgery Laboratory of the UNINOVAFAPI University Center and the ethical principles of animal experimentation defined by the National Council for the Control of Animal Experimentation (CONCEA) were observed. The project was approved by the Ethics Committee in the Use of Animals of the Centro Universitário UNINOVAFAPI, opinion nº 0002/15.

ANIMALS AND EXPERIMENTAL GROUPS

A total of 20 mice were used (*Rattus norvergicus*), of the Wistar lineage, adults, males, acquired by the study institution (UNINOVAFAPI). The animals were kept throughout the experiment in cages (50x36x17cm) lined with pine sawdust, with standard food (Labina-Purina diet) for rodents and water ad libitum in an acclimatized room at 25ºC.

The rats were submitted to the production of a cutaneous wound on the back, under anesthesia, and randomly divided into three groups: Control Group (CG), consisting of 06 animals submitted to daily cleaning of the wound with 0.9% saline solution; Standard Group (GP), consisting of 07 animals treated with Trofodermin® (clostebol acetate/ neomycin sulfate); and Extract Group (EG), consisting of 07 animals treated with the

aqueous extract of *Kalanchoe pinnata*.

Healing was analyzed by macroscopic analysis of the evolution of the appearance of the lesion and measurement of scar retraction of the wound, as well as by histological study on slides stained with hematoxylin-eosin (H.E.) considering the parameters of inflammatory cellular elements including collagenization and re-epithelialization.

PREPARATION OF AQUEOUS EXTRACT OF *KALANCHOE PINNATA*

The aqueous extract was prepared from fresh leaves of *Kalanchoe pinnata*, using dynamic maceration as an extraction method. For this, the leaves were cut into small pieces of approximately 1 cm2 and then placed in a crucible and maceration was carried out with the pistil. After the extract was prepared, filtration was carried out to remove the remaining solid part, thus obtaining the aqueous crude extract, which was stored in a refrigerator at 4°C throughout the study.

ANESTHESIA AND PRODUCTION OF THE SKIN WOUND

Rats were subjected to anesthesia with Ketamine 40 mg/kg and Xylazine 5 mg/kg. Then, local trichotomy was performed by manual traction in the dorsocostalis region in an area of approximately 6 cm2, proceeding with local antisepsis with topical Povidine.

The procedure for producing the skin wound is shown in Figure 2. Briefly, after placing the surgical field, the skin was demarcated with the aid of a ballpoint pen and a 1 cm2 hollow plastic mold specially made for this purpose. Then, surgical procedures were performed under aseptic conditions, removing the demarcated area of skin until the dorsal muscular fascia was exposed. To produce hemostasis, digital compression was performed using sterile gauze.

POSTOPERATIVE

Treatment was started immediately after surgery and was repeated daily at the same time for 14 days. No animal received dressing at the surgical wound site. The GE animals received treatment with 3 mL of crude extract of *Kalanchoe pinnata* applied directly to the wound, with the aid of a plastic syringe. The GP rats were treated with Trofodermin® ointment (clostebol acetate/neomycin sulfate) applied over the entire wound extension with the aid of a plastic spatula. The CG's wounds were only cleaned with 3 mL of 0.9% saline solution, applied with the aid of a plastic syringe. In the extract and standard groups, with each new application of the designated products, the wound was cleaned with 0.9% saline solution.

After treatment, the animals were returned to their respective cages for anesthetic recovery, with food and water available. Daily, for 14 days, all animals (GE, GP and GC) were subjected to anesthesia for the application of their respective study products or just cleaning in the case of GC.

MACROSCOPIC EVALUATION

Lesions were evaluated daily at the time of application of treatments for 14 days. Clinical evaluation was performed based on the analysis of the following characteristics: lesion measurement, presence or absence of secretion, re-epithelialization, formation of granulation tissue, presence of fibrin, occurrence of hemorrhage, presence of edema, necrosis or crust. The findings were recorded and photographic documentation of the wounds was performed.

EUTHANASIA AND OBTAINING MATERIAL FOR HISTOLOGICAL ANALYSIS

After fourteen days of specific treatment for each group (EG, GP and CG), the animals

Figure 1- Kalanchoe Pinnata leaves.

Source: Prepared by the author.

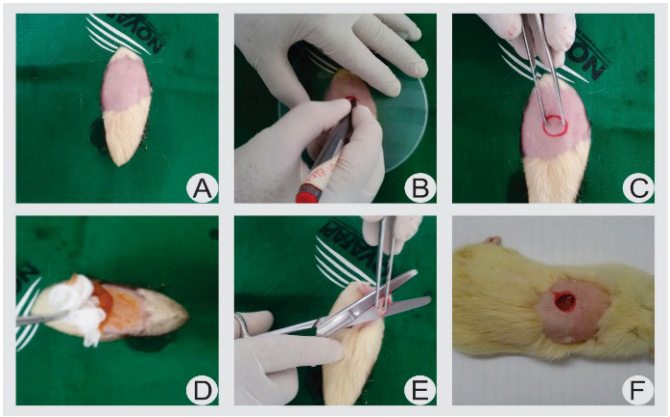


Figure 2 – (A) placement of the surgical field; (B) demarcation of the area; (C) exposure of the demarcated area; (D) antisepsis; (E) surgical procedure; (F) exposure of the surgical wound.

Source: Laboratory of Experimental Surgery at Uninovafapi, 2015.

Figure 3 – Area of the wound healed 14 days after surgery using the polyline method.

Source: Prepared by the author.

of all groups were euthanized by application of an excessive dose of sodium thiopental (100 mg/kg) intraperitoneally and lidocaine (10 mg /kg) intraperitoneally.

After euthanasia, the animals were placed on the surgical board and the wounds were examined for crusts, secretions and hypertrophic scars. Then, each wound was photographed with the aid of a "Sony Cyber-Shot, 7.2 mega pixels" digital camera held on a tripod at a constant distance of 13 cm, for further analysis by digital planimetry.

Then, the material was collected for histological analysis. In this procedure, a fragment of skin was removed with a 1 cm margin of intact skin around the lesion, with depth to the dorsal musculature of the animal. Each piece was preserved for 48 hours in an identified plastic container containing 10% formalin, with subsequent preparation of histological slides.

DIGITAL PLANIMETRY

The measurement of the remaining area of the wounds was performed using the digital planimetry method, using the IMAGE J software, in which the periphery of the wound was delimited by the "polyline" method and its total area was calculated (Figure 3).

MICROSCOPIC EVALUATION

Histological sections were stained with hematoxylin-eosin (H.E.). Histological slides were evaluated for the presence of: vascular proliferation, polymorphonuclear cells, mononuclear cells, fibroblast proliferation, collagen fibers and re-epithelialization. The microscopic analysis was performed by recording the findings qualitatively with an ordinal variable (Chart 1), in order to allow the use of non-parametric statistical tests.

STATISTICAL ANALYSIS

Data were analyzed using statistical software: *Minitab Statistical®*. Comparison of means of skin wound area (cm2) between groups was performed by one-way ANOVA followed by Tukey's post hoc test. The histological evaluation variables were analyzed using the non-parametric Kruskal-Wallis test. The significance level used to reject the null hypothesis was set at $p<0.05$.

RESULTS

MACROSCOPIC FINDINGS2

Until the 5th day of follow-up after wound induction, there were no significant differences in the evolution of wounds between the groups; in all groups the presence of exudation and the formation of a delicate crust was observed. From the 6th day, there was a thickening of the crusts, which made it difficult for the substances under analysis to penetrate, especially in the GE and GC. Thus, debridement was performed for better penetration of these substances.

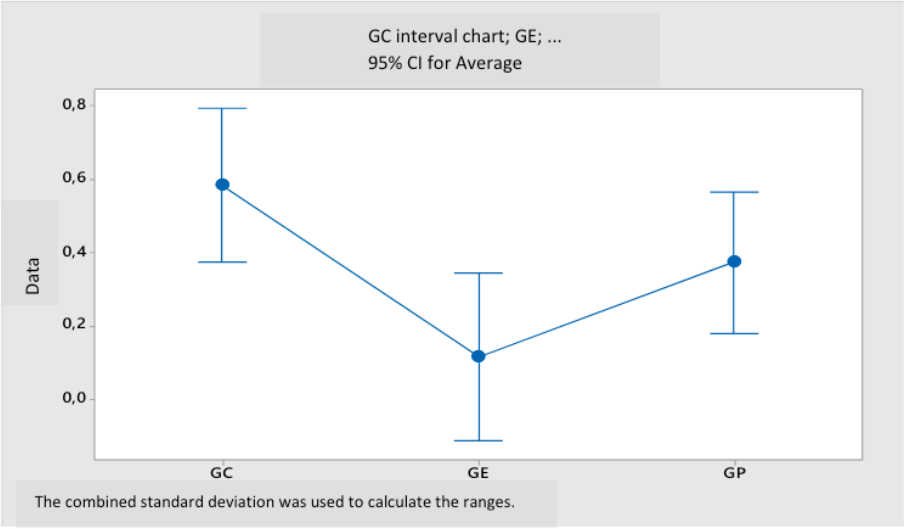
From the 9th day, an advance in the re-epithelialization process was found in GE and GP in relation to GC. On the 13th day, the progress of reepithelialization of the GP and EG was maintained, except in one of the rats in this group, Rat 5, in which a more extensive lesion was observed. On that occasion, global slowing of re-epithelialization was observed in GP, with the presence of edema and pus in the wound of one of the animals, Rat 1. On the 14th day, no significant changes were found in the evolution of the wounds in relation to the 13th day.

WOUND AREA

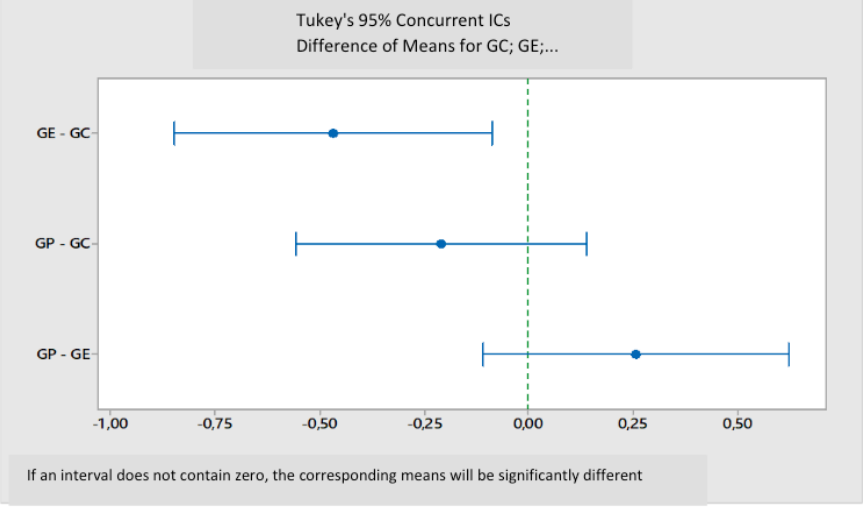
On the 14th day after the production of a cutaneous wound, a statistically significant difference was found in the average wound area (Graphs 1 and 2).

Histological Findings	Intensity of the findings			
	Absent	Discrete	Moderate	High
Vascular proliferation	1	2	2	3
Polymorphonuclear cells	0	1	2	3
Mononuclear cells	0	1	2	3
Fibroblast proliferation	0	1	2	3
Collagen fibers	0	1	2	3
Re-epithelialization	Absent = 0	Partial = 1		Complete = 2

Table 1 - Classification and attribution of indexes to the histological findings of HE (hematoxylin - Eosin).



Graph 1 – Interval graph showing that the average area of the wounds after healing is smaller in the EG compared to the GP and GC.



Graph 2 – Tukey test showing significant difference only between EG and CG.

MICROSCOPIC EVALUATION OF THE WOUND

The mean number of mononuclear cells was significantly lower in GE and GP compared to GC, while re-epithelialization was significantly higher in the GE and GP groups (Table 2).

Figure 4 shows photomicrography with the characteristic pattern of re-epithelialization observed in each group after the 14th day of surgery. In the CG there was a predominance of animals with non-epithelialized wounds. In GP, both complete epithelialization and partial or forming epithelialization were found at the same frequency. In GE, epithelialization was predominantly complete in almost all animals.

DISCUSSION

In this study, the treatment of cutaneous wound in rats with aqueous extract of leaves of *Kalanchoe pinnata* for 14 days it promoted a reduction in the wound area, an increase in re-epithelialization and a reduction in the number of mononuclear cells.

The holy leaf, as it is popularly known, is also widely used in the treatment of infections and on open wounds in several communities around the world. As an example, in Trinidad and Tobago, a follow-up study of 96 patients treated with this plant versus 382 in the control group demonstrated benefits from the topical use of *Kalanchoe pinnata* to treat infections of open wounds of the diabetic foot (BEZERRA et al, 2006).

In addition, anti-inflammatory properties of several types of extracts from this plant have been described in some studies (CHIBLI et al, 2014; FERREIRA et al, 2014). Antihypertensive, anti-visceral leishmaniasis, antidiabetic, antiviral, antiulcer and healing properties were also found, indicating the presence of bioactive compounds in this species with potential medicinal application (BOPDA et al, 2014; CAWICH et al, 2014; GOMES et al, 2009; PATIL et al, 2013; Sobreira, 2013; WANG et al, 2013).

In the present study, a median observation time (14 days) was adopted in comparison with other healing studies, as the objective was to verify the possible occurrence of faster tissue recovery resulting from the application of the extract of *Kalanchoe pinnata* compared to the healing process in GC and GP. Healing studies that assume processes and try to elucidate tissue repair mechanisms generally use follow-up periods that can vary from 7 to 21 ± 2 days. Furthermore, significant differences in findings on histological slides start from the seventh day of follow-up, so that on the 15th day, conclusive statements about healing time are already made based on macroscopic and histological analysis (GARROS et al, 2006)

Depilation of the animals' backs was performed using a manual and not a mechanical method, due to the fact that the manual traction of the hairs hardly causes skin lesions, contrary to what happens with the use of histological laminated appliances (GARROS et al, 2006).

The dorsal region of the rat, chosen to produce the lesion, is justified by the fact that it prevents the animal from reaching it, and because it is a well-known experimental model of an open wound (BOPDA et al, 2014; GOMES et al, 2009; PATIL et al, 2013). Another care used so that the animal did not remove the drug before achieving the desired effect was to use anesthesia before each application of the products, this way the rats remained anesthetized for a minimum time of 30 minutes, as a way of enabling successful treatment. topical application of the standard extract or ointment. In addition, the control group was also anesthetized to ensure the homogeneity of the study and to apply saline solution on the wound. The application of 0.9% saline solution aims to clean the wound in order to minimize the possibility of infections

	GC	GE	GP	p
vascular proliferation	13,7	8,4	8,2	0,118
mononuclear cells	14,5	7,3*	8,5	**0,008**
polymorphonuclear cells	11	9,6	9,5	0,828
fibroblast proliferation	9,5	11,1	9,5	0,85
collagen fibers	10,5	11,9	7,9	0,122
Epithelialization	8,8	14,8*	6,9	**0,016**

Table 2 – Average of the ranks of the GC, GE and GP groups of the histological variables. Kruskal-Wallis test 15 days after surgery.P – the P-value. * significant p-value.

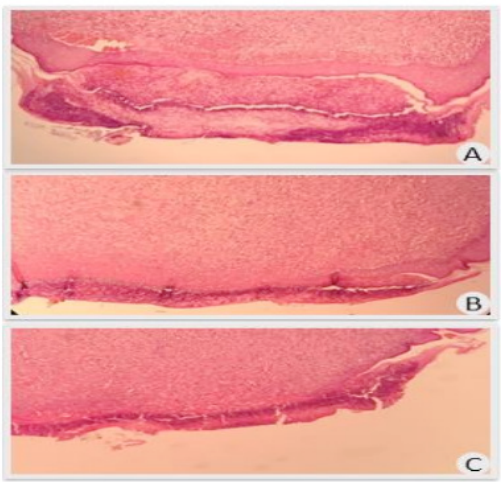


Figure 4 – (A) Complete re-epithelialization observed in GE; (B) Absent re-epithelialization under the wound in GC. (C) Partial re-epithelialization (in formation) observed in GP. H.E. stain, 40x magnification.

Source: Prepared by the author

that could compromise the study.

As for the size of the lesions, a constant measurement was used, which allowed better monitoring of healing. In this sense, standardization of wound dimensions by skin excision was previously used in other studies (GARROS et al., 2006; MARTINS et al., 2006; SILVA et al., 2006).

In the healing evolution, the retraction of the edges of the lesion deformed the original appearance, but the morphometric method used in this study, digital planimetry, with the tool: *poliline* from the IMAGE J software can be used to format any geometric area to be defined and obtain its value. Regarding the wound area, its decrease was observed over time and the smallest wound areas were observed in the group treated with the extract of *Kalanchoe pinnata*, that is, topical application of aqueous extract of leaves of this species accelerated the healing process in relation to the control group, with an effect similar to that produced by the reference treatment used, which consisted of application of an ointment based on clostebol acetate and neomycin sulfate.

In the histological analysis performed with samples obtained on the 14th day after the production of a skin lesion, a decrease in mononuclear cells was found. These findings can be justified by the fact that, in the healing process, neutrophils and monocytes are the first elements to reach the wound area with the function of debridement and phagocytosis (TAZIMA; VICENTE; MORIYA, 2008). In this study, the GC had a higher amount of mononuclear cells compared to the EG, indicating that the skin wounds of this group are still in the inflammatory phase of the healing process, while in the EG the lesions have already advanced to a slightly more advanced phase of the healing process. healing.

Another histological parameter that shows improvement in healing in EG was the greater reepithelialization found in relation to GC. Thus, the most advanced stage in the healing process in the group treated with aqueous extract of *Kalanchoe pinnata* both by the results of the histological analysis and the morphometric findings.

CONCLUSION

The results of the present study allow us to conclude that the topical use of aqueous extract of *Kalanchoe pinnata* favored the healing of open skin wounds in rats. The histological observations that reinforce the beneficial effect of this species in the healing process consist of re-epithelialization and lower presence of cells related to the inflammatory process after 14 days of treatment.

The use of extract of *Kalanchoe pinnata* in the healing process of cutaneous wounds in humans remains an open field for studies. Thus, it is important to expand the experimental study in animals using different treatment times and doses, in addition to testing other extracts and also bioactive compounds isolated from leaves and other parts of the plant, in order to demonstrate the substances involved in the skin repair process.

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