

## Healing Effect of the Ointment Made of *Equisetum pyramidale* in the Treatment of Cutaneous Lesions in Diabetic Rats

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### ABSTRACT

*This study aimed to evaluate the anti-inflammatory and repair effects of the ethanolic extract and ointment of Equisetum pyramidale on the skin lesions of rats induced to diabetes. After the induction of diabetes with alloxan, a surgical procedure was performed on the back of each rat. Then, they were separated into treatment groups: G1- ethanolic extract of E. pyramidale; G2- ointment (extract of E. pyramidale 80g + 20g of vaseline:lanolin 1:1); G3- control (vehicle vaseline:lanolin 1:1); and G4- no treatment during 3, 7 and 14 days. The samples were embedded in paraffin and stained with hematoxylin-eosin for histological analysis. The findings showed that the use of ethanolic extract as well as the ointment decreased the inflammatory cells at the site of inflammation, resulting a faster healing, with less crusting and lower amount of secretion in comparison to the control group. Therapy with topical herbal was an effective method in the inflammatory process of tissue repair, contributing to a faster and more organized tissue re-epithelialization.*

**Key words:** Diabetes, skin lesions, medicinal plants, anti-inflammatory activity, reepithelialization tissue

### INTRODUCTION

Diabetes Mellitus is currently considered a public health issue worldwide, both in terms of the number of people affected and in relation to the costs involved in controlling and treating its complications (Spadella et al. 2005). The patients with diabetes mellitus are characterized by presenting a relative, or absolute deficiency of insulin, hyperglycemia, glycosuria, tendency to develop atherosclerosis, microangiopathy, nephropathy and neuropathy. Also, the condition may trigger the infections, such as chronic

vascular and neurological complications, and changes in the immune response characterized by reduced chemotaxis and phagocytosis of neutrophils (Foss et al. 2005).

Faced with this harsh reality, it is essential to better understand the pathophysiology of diabetes and its complications, the search for a treatment capable of minimizing the endocrine-metabolic changes caused by the disease, and especially the chronic lesions on the diverse organs (Lerco et al. 2003). In view of the aggravating factors listed on the consequences of diabetes mellitus, especially in regard to healing, new therapeutic methods have been sought that can solve, or even minimize, the

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flaws diabetics present in the process of tissue repair (Dealey et al. 2008).

Thus, currently, the use of plant species with anti-inflammatory, antimicrobial and antioxidant properties in the treatment of skin wounds has intensified. The main goal of such studies remains to intervene in the healing process of the wounds caused accidentally, or resulted by the surgeries, with lessened side effects, at low cost and easily accessible to the population, especially the poorest.

Considering the vulnerability of diabetics to infections, especially skin wounds, and on the grounds of the positive results obtained with the aqueous ethanol extract and ointment from *Equisetum pyramidale* (Equisetaceae), successfully employed in the treatment of 2nd intention wounds in experimental models (Oliskovicz et al. 2006; Matias 2010), this study aimed to evaluate the healing action of *E. pyramidale* extract and ointment in the treatment of skin wounds in diabetic rats.

## MATERIALS AND METHODS

### Collection of plant material and preparation of ointment:

The upper parts of *Equisetum pyramidale* were collected from the Garden of Medicinal and Aromatic Plants (Horto de Plantas Medicinai e Aromáticas) in Campo Grande - MS - in January 2008. For the preparation of the ethanolic extract, 500 g of the plant were used, dried in air circulating kiln at 40°C (Marconi ® Model MA35) and ground in a stainless steel knife mill (Marconi ®, Model MA048). The extraction was carried out in ultrasonic bath for 60 minutes (UNIQUE ®, Model 1450), followed by maceration at room temperature. This procedure was repeated for 15 days. The solvent was evaporated under vacuum in a rotary evaporator (Tecnal Model MA120), yielding 22 g of crude ethanol extract. This extract was incorporated into the lanolin / vaseline-base vehicle (1:1). The concentration established was 20% crude extract and 80% vehicle. The drying method, the preparation of the crude ethanol extract of *E. pyramidale* and ointment at 20% followed the procedures described by Matias (2010).

### Animal study

Twenty four adult male Wistar rats (*Rattus norvegicus albinos*), weighing 250 to 300 g were used. The rats were obtained from the UFMS vivarium. During the experiment, the rats were kept in individual polypropylene cages equipped with the beds of selected wood shavings, maintained at 22°C by natural ventilation and provided with food and water *ad libitum*.

The rats were randomly divided into four groups (N = 2, each group): Group 1 (treated with the extract), Group 2 (treated with the ointment), Group 3 - control (treated with the sterile vaseline), and Group 4 (with no treatment) for 3, 7 and 14 days post-operative. All the studies were conducted in accordance with the standards and approval of the COMMISSION ON ETHICS IN THE USE OF ANIMALS / UFMS n. 162/2007.

For the induction of diabetes, the rats were anesthetized with an anesthetic solution containing ketamine + xylazine (100 +10 mg mL.Kg, respectively) via intraperitoneally and kept in supine position to receive the intravenous injection (dorsal penile vein) of Alloxan prepared at the time of use. The solution used was obtained from a stock of 50 mg of Alloxan and 0.8 mL of citric acid at a ratio of 0.1 mL for each 100 g of body weight, resulting in a final dose of 62.5 mg of Alloxan/kg body weight.

The rats were treated with glucose solution (10%) during the first six hours post-injection to prevent the seizures and death, common in the hypoglycemic phase. After 24 h, glucose was removed from the water. Glucose monitoring was performed after the induction to confirm whether diabetes was present. The blood glucose level was measured before induction of diabetes and 72 h later. The rats whose values were not higher than, or equal to 250 mg per deciliter of blood (mg/dL) were disposed (Carvalho, 2002). Glycemia levels were measured again on Day 5 post-treatment to confirm whether the rats remained diabetic. Finally, the blood glucose level was measured on the day of sacrifice to determine whether any reversal of diabetes occurred. The measurements were made by removing the blood from the tail vein and placing a drop on Advantage II® reactive tape. The blood glucose levels were read using the glucometer® device, which showed an average blood glucose level of  $315.2 \pm 70$  mg/dL.

For inducing the skin lesions, the dorsal region of the animals was shaved, cleaned and numbed with an anesthetic solution containing ketamine + xylazine (100 +10 mg.kgmL, respectively) by the

intraperitoneal route. With a scalpel, an incision (1 cm wide x 2 cm long) was performed on all the experimental rats. The induced lesions on each rat were treated on a daily basis in the following proportions: extract group (0.1 g of ethanol extract on the lesion); ointment group (0.1 g on the lesion); control group (0.1 g of sterile vaseline on the lesion), and untreated group (no treatment on the injury). At the end of the study periods, the rats were sacrificed in CO<sub>2</sub> chamber, as recommended by COBEA.

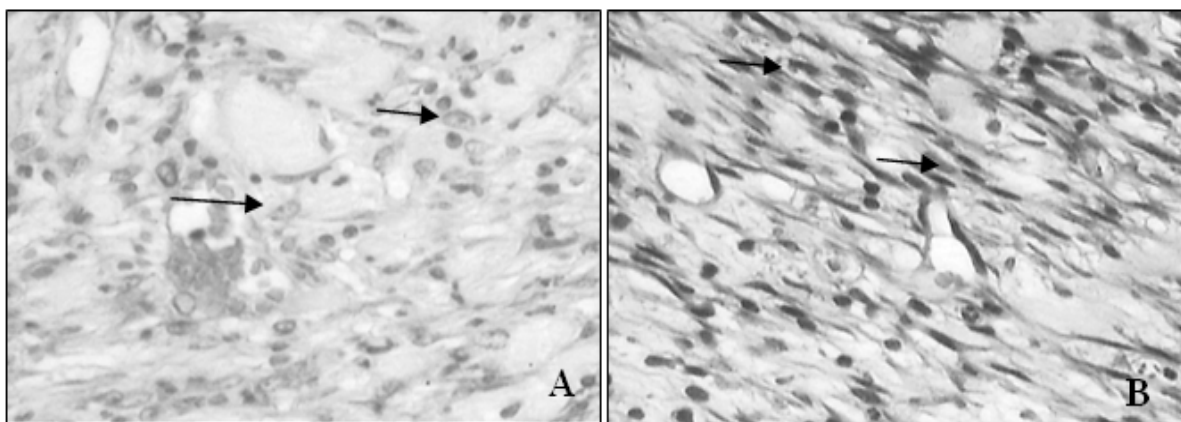
The samples of all the skin lesions were collected so as to include the part of the skin adjacent to the edges of the wound and all the scar tissue in depth. The skin lesion samples were fixed in 4% paraformaldehyde for at least 24 h. After fixation, the samples were gradually dehydrated in increasing concentrations of ethanol (70 to 100%), cleared in xylene, and embedded in paraffin according to routine histological methods. The fragments embedded in paraffin were sectioned with "820" Spence microtome, yielding the pairs of 5mm-thick sections. The histological slides were incubated for drying and the tissue sections subsequently subjected to hematoxylin eosin staining (HE) for the histological analysis.

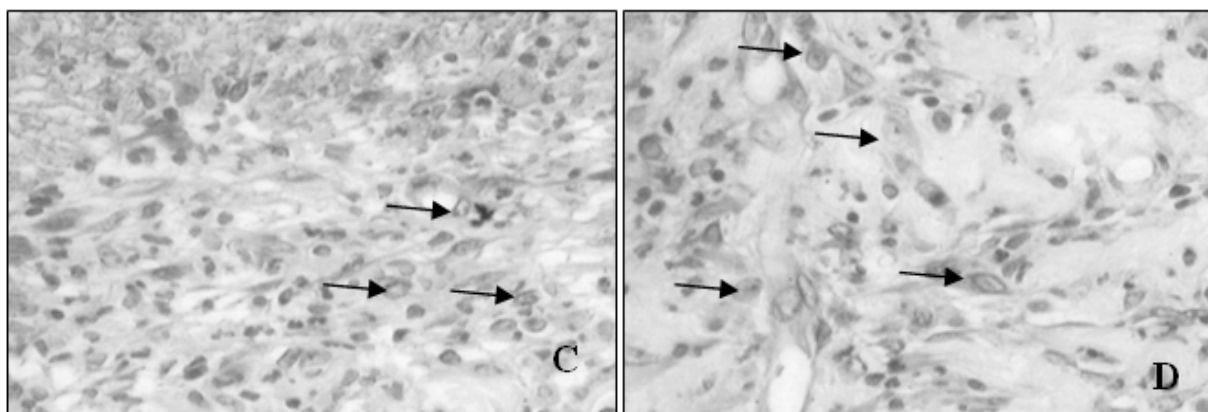
Images were captured from four microscopic fields for each histological slide with a digital camera (total magnification 100x) of AX70 Plus optical microscope (OlympusR, Tokyo, Japan). The intensity of the inflammatory infiltrate, the extent of necrotic areas and hyperemia were analyzed, yielding the classification scores: (0) absent = no infiltrate/necrosis; (1) discrete= small number of inflammatory cells/small area of necrosis and hyperemia; (2) moderate = large

number of inflammatory cells/moderate hepatic necrosis, and hyperemia; (3) intense = excessive numbers of inflammatory cells/large area of necrosis and hyperemia.

## RESULTS

The histological observations of the samples of the wounds of all the groups (Day 3 post-operative) showed inflammatory reaction, hyperemia, which varied from group to group at different intensities, which induced a more accurate analysis. The skin lesions on the group treated with ethanol extract showed pattern of tissue repair in inflammatory phase (Day 3 after the surgery) with discrete inflammatory infiltrate and granulation tissue more evident than the control group (Fig. 1A). The wounds of the ointment-treated group presented thin crust with a discrete inflammatory infiltrate, showing the presence of fibroblasts (Fig. 1B). The skin lesions in the control group (treated with sterile vaseline) exhibited the pattern of tissue repair in inflammatory phase (Day 3 after the surgery), with the formation of thick crust with necrotic tissue. The inflammatory infiltrate was considered intense and inflammatory cells were predominantly lymphocytes (Fig. 1C). The lesions of the group receiving no treatment had thick crust with significant amount of necrotic tissue and granulation tissue with predominance of large quantity of neutrophils, fibroblasts and blood vessels; the vessels were present in smaller quantity than in the other groups tested. The conjunctive tissue was not very thick, loose and randomly deposited. (Fig. 1D).





**Figure 1** - Photomicrography of skin wound in diabetic rats on Day 3 post-operative . A) extract-treated group , B) ointment-treated group, C) control group treated with sterile vaseline and D) no-treatment group (arrows= inflammatory cells). (H.E). 400 X

## DISCUSSION

The present study demonstrated the effects of *E. pyramidale* in the inhibition of inflammatory process and epithelial remodeling of surgical wounds, when used in the form of extract, or ointment. The data obtained showed that both the extract and ointment reduced the inflammatory infiltrate and increased the degree of re-epithelization in the skin of the rats. However, the results also showed that the induced skin lesions presented local inflammation as a result of the production of pro-inflammatory mediators that triggered vasodilatation, PMN (polymorphonuclear) cell infiltration, and plasma extravasation, leading thus to the classic signs of inflammation (Castardo et al. 2009).

The models of skin lesions can identify the compounds with anti-inflammatory activity that may be potentially useful in the treatment of inflammatory diseases affecting the skin, as they cause conditions that resemble some types of dermatitis seen in the humans (Vane and Botting 1998; Mendonça and Coutinho-Netto 2009). These lesions stand for a useful model for screening anti-inflammatory activity of the compounds acting in the acute phase of inflammation as well as in hyper-proliferative inflammatory processes (Garros et al. 2006). In the present study, topical application of the extract and ointment on the surgical lesions induced an inflammatory response characterized by the vasodilatation and formation of erythema on Day 3, followed by the increased

thickness of crust as a result of cell extravasation, which was maximum on Day 5-6 and tended to decrease thereafter. The adherence of PMN leukocytes in the vessel wall and the degranulation of mast cells were found between Days 4 and 6. However, the maximum infiltration of PMN leukocytes in the tissue occurred only 72 h after the application of the topical treatments (Mendonça and Coutinho-Netto 2009).

The microscopic assessment of the lesions showed that the groups treated with the ethanol extract and ointment presented discrete inflammatory phase of healing that prevailed on Day 3 and 7 after the surgery, characterized by the predominance of neutrophils, a smaller amount of lymphocytes and an improved macroscopic appearance with less crusting and no exudation, up to Day 14, compared to the control and no-treatment groups, which presented with a larger quantity of inflammatory infiltrates.

During the lesion treatment, although there was no significant difference between the groups, some differences could be seen in the evolution of the lesions in the diverse types of treatment, e.g., bleeding, early infection and adhesion were only observed in the lesions that received no treatment. Therefore, the lesions treated with ethanol extract and ointment (Figures 1A and 1B) evolved better during the treatment and showed no bleeding, secondary infections, or necrotic tissues.

The species studied (*E. pyramidale*) has the triterpenes (ursolic and oleanolic acid) (Matias 2010) in its composition and, according to

Murakawa et al. (2006), this class of secondary metabolite has, as one of the mechanisms of action, effects on the process of inflammation resulting from the activation of PKC (protein kinase C) and the sequential activation of the MAP kinase pathway, phospholipase A2, activation of AA, induction of COX-2 expression and translocation/activation of LOX.

Since a significant drop in neutrophil migration were seen, it would be important to comment on the adhesion molecules that were responsible for the chemotaxis of these cells in the inflammatory response. These molecules are responsible for rolling, adhesion and transmigration of leucocytes (Rocha- Junior, 2006). The activated neutrophils present in the inflammatory focus significantly contribute to the release of free radicals, proteolytic enzymes and some inflammatory mediators (LT, PG and human leukocyte elastase) (Prieto et al. 2003). The mediators released by neutrophils are responsible for sustaining an acute inflammatory process in the psoriatic plaque, through the activation of keratinocytes and T lymphocytes (Terui 2000). Acute inflammation is a rapid response against several agents or stimuli involving recruitment and activation of neutrophils (Vergnolle 2008).

Park et al. (2004) and Murakawa et al. (2006), considering the pathways and pro-inflammatory mediators involved in the inflammatory response, observed that there were several targets on which the compounds present in plants could exert their pharmacological action. However, part of the anti-inflammatory action of these plants could also be a result of an inhibitory effect on the synthesis of arachidonic acid metabolites or blocking its actions (Park and Barbul 2004; Murakawa et al. 2006).

The present findings showed that probably the extract and ointment of the plant showed an inhibitory effect on the mediators of inflammation, with beneficial effect of anti-inflammatory activity of triterpene acids, especially ursolic and oleanolic acids, found in the plant. These effects were also reported by Matias (2010) for *E. pyramidale* extract and ointment 0,2%, used in the treatment of 2<sup>nd</sup> intention wounds in Wistar rats.

The results were also consistent with those described by Diaz et al. (2000) who found in experimental models that these acids inhibited the synthesis of prostaglandin E2, similar to indomethacin (CI = 0.95 mM). The ethanol extract, as well as the ointment, was able to reduce

leukocyte migration at the expense of neutrophils mostly, which characterized the inhibition of acute inflammatory response (Diaz et al. 2000).

Regarding the process of re-epithelization, it was noteworthy that in seven days, a tendency to greater re-epithelization was observed in the groups treated with the extract and ointment. These results could be related to the antioxidant action, justified by the presence of caffeic acid in the chemical composition of the plant (Matias, 2010). The acid was also studied by Brudzynski and Carlone (2004), who concluded that it could stimulate the proliferation of epidermic keratinocytes. The fact that there was no statistically significant difference when compared with the control group it could, in this context, reflect both the low concentration of the extract employed in this assay and the variability in the bioactive properties that characterized the plant.

Therefore, it was possible that the ethanol extract and *E. pyramidale* ointment led to a better re-epithelization by stimulating the epidermal cells on Day 14 post-operative. Another important observation was the moisturizing effect of the ointment, which prevented water loss through evaporation in the wound focus, providing a more humid micro-environment, thus promoting re-epithelization. Despite the positive results in the healing process of diabetic rats, those belonging to the groups that received no treatment showed delay in the process of tissue repair, and also evidence of infection. Loots and Santos (1999) reported that in diabetic animals, the tissue repair process was slow due to the inhibition of fibroplasia.

Therefore this study provided scientific evidence to validate the popular topical use of *E. pyramidale* as reported by Matias (2010) in the treatment of skin inflammatory processes and validated the use of ethanol extract and ointment (2.0%) to treat the 2<sup>nd</sup> intention wounds in the diabetic rats, which demonstrated the potential of the crude extract for the development of a new anti-inflammatory medicine for topical use.

## CONCLUSION

The topical use of the extract and ointment in the lesions of diabetic rats managed to regulate the effects of the inflammatory process, with a discrete condition when compared to the control group, with a better arrangement of epithelial and

hypodermic structures, accelerating the repair process. However further studies would be needed to elucidate all the pathophysiological mechanisms involved in the dynamics of the stimulation of the healing process as well as the fractionation of the compounds found in the plant studied.

## ACKNOWLEDGMENTS

The authors acknowledge the financial support of the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); Ministério de Ciência e Tecnologia (MCT); INAU (Instituto Nacional de Áreas Úmidas), Centro de Pesquisa do Pantanal (CPP); Universidade Estadual de Maringá – PR (UEM) and FUNDECT for the Master Degree grant.

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Received: October 27, 2011;  
Revised: January 18, 2012;  
Accepted: October 17, 2012.