

Wound-healing evaluation of ointment from *Stryphnodendron adstringens* (barbatimão) in rat skin

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This study evaluated the cicatrizing effect of an ointment containing 1% of the ethyl-acetate fraction extracted from the stem bark of “barbatimão” (*Stryphnodendron adstringens*), in wounds made in the skin of rats, after 4, 7 and 10 days of treatment. Control wounds were treated with a base ointment without extract. The proliferation of keratinocytes in the area of reepithelialization was evaluated by counting the number of epithelial cells that were blocked in metaphase by vincristine sulfate. The length of the reepithelialized margin and the contraction of the wound were measured. Topical application of the “barbatimão” ointment stimulated proliferation of the keratinocytes, but had no effect on the length of the epithelium or on the contraction of the wounds.

Uniterms: *Stryphnodendron adstringens*/pharmacognosy. *Stryphnodendron adstringens*/ cicatrizing effect. “Barbatimão”/pharmacognosy. Wound/healing/experimental study. Reepithelialization.

Neste estudo, avaliou-se a atividade cicatrizante de uma pomada contendo uma fração acetato de etila 1% obtida de cascas de “barbatimão” (*Stryphnodendron adstringens*) em feridas excisionais na pele de ratos após 4, 7 e 10 dias de tratamento. Feridas controle foram tratadas com pomada base, sem extrato. A proliferação dos queratinócitos na área reepitelizada foi avaliada através da contagem do número de queratinócitos bloqueados em metáfase, pelo sulfato de vincristina. O comprimento da margem reepitelizada e a contração das feridas foram mensurados. As feridas tratadas com barbatimão apresentaram um maior número de mitoses do que aquelas tratadas com a pomada base, em todos os tempos avaliados. A aplicação tópica da pomada de “barbatimão” estimulou a proliferação epitelial contudo não teve efeito sobre a migração dos queratinócitos ou sobre a contração das feridas.

Unitermos: *Stryphnodendron adstringens*/farmacognosia. *Stryphnodendron adstringens*/efeito cicatrizante. “Barbatimão”/farmacognosia. Feridas/cicatrização/estudo experimental. Reepitelização.

INTRODUCTION

In cutaneous lesions, tannins bind to the proteins of the injured tissues, precipitating them (Fernandez *et al.*, 2002) and creating a protective layer (Neto *et al.*, 1996). This layer isolates the wound site from the environment, reducing the permeability and exudation of the wound (Brown, Dattner, 1998; Bedi, Shenefelt, 2002) and promoting tissue repair. Tannins also exhibit vasoconstrictor and anti-inflammatory properties (Mota *et al.*, 1985; Kapu *et al.*, 2001) and stimulate the growth of epidermis,

aiding reepithelialization (Palermo *et al.*, 2002; Lopes *et al.*, 2005). Reepithelialization involves the proliferation and migration of cells from the edges of the wound, and is regulated by mechanisms involving genes, growth factors, integrins, extracellular matrices (ECM) and metalloproteinases (MMPs) (Santoro, Gaudino, 2005).

Stryphnodendron Martius, Leguminosae, popularly known as “barbatimão”, is a Brazilian savannah tree. Extracts of the stem bark from species of *Stryphnodendron* have several medically useful properties, such as anti-inflammatory (Melo *et al.*, 2007), antimicrobial (Lopes *et al.*, 2005; Ishida *et al.*, 2006), antiulcerogenic (Audi *et al.*, 1999), trypanocidal (Holetz *et al.*, 2005), antioxidant and cicatrizing (Lopes *et al.*, 2005) properties, probably

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because of their high tannin content (about 20%) (Audi *et al.*, 2004). The content of tannins in *Stryphnodendron adstringens* was demonstrated by Mello *et al.* (1996a; 1996b, 1999), who isolated 23 compounds from these tannins. Recently, Lopes *et al.* (2008) reported five new compounds from stem bark of *S. adstringens* and *S. polyphyllum*. Popular uses such as a cicatrizant have been evaluated by previous studies, which have demonstrated a significant cicatrizant property of the bark extract from *S. adstringens* (Neto *et al.*, 1996; Palermo *et al.*, 2002).

The aim of the present study was to evaluate the cicatrizant activity of a semipurified fraction of the bark of *S. adstringens*, on the regeneration of the epidermis in wounds to the skin of rats.

MATERIAL AND METHODS

Plant material and preparation of extracts

The stem bark of *S. adstringens* (Mart.) Coville was collected in São Jerônimo da Serra, Paraná State, Brazil (S23°43'7.8", W50°45'23.5"; altitude 926 m), in November 1999. The species was identified by Prof. Dr. Cássia Mônica Sakuragui (UEM). A voucher specimen was deposited at the Herbarium of the Biology Department of the Universidade Estadual de Maringá (UEM) under number HUEM 3800.

Air-dried stem bark was extracted with Me₂CO-H₂O (7:3) (F1) according to Mello *et al.* (1999). The combined extracts were filtered and evaporated under reduced pressure, and lyophilized. This crude extract (50 g) was redissolved in H₂O and extracted with ethyl acetate (EtOAc). After evaporation, the EtOAc fraction (F3; 7.8 g) and the remaining H₂O phase (F2; 32 g) produced dark-brown solids, and both fractions were lyophilized.

Ointment preparation and animals

Tests were done using the EtOAc fraction (F3) incorporated into an ointment base (Beeler base), with a final concentration of 1%. Fifteen male Wistar[®] rats weighing from 180 to 200 g were used for the study. The animals were maintained in individual cages, in a 12-h light/dark cycle, temperature of 22 °C, with a standard laboratory diet (Nuvital[®]) and water *ad libitum*. The protocol for these experiments was accepted and approved by the UEM Animal Ethics Committee (Protocol Number 040/2003).

Cicatrizant assay

After manual depilation and asepsis, two round

excisional wounds about 7 mm in diameter were made on the back of each animal, close to the cervical area, after demarcation with a stainless steel delimitor. After the wounds were washed and their longest transverse and longitudinal axes measured, the experimental wounds (on the right side) were treated daily with an ointment containing 1% of the F3 fraction. The control wounds (left side) were treated only with the ointment base without the extract (Lopes *et al.*, 2005).

The rats were sacrificed after 4, 7 or 10 days. Two hours before sacrifice, each rat was injected with 0.5 mg/kg body weight of vincristine sulfate (Oncovin, Eli Lilly), a metaphase-arrest agent that blocks mitosis primarily by inhibiting the dynamics of spindle microtubules (Jordan *et al.*, 1992). The wounds were measured again.

The rats were anesthetized by inhalation with ethyl ether, and the wounded skin was removed. The samples were spread on cards, fixed in Bouin's solution for 6 hours, and embedded in paraffin. Blocks were cut into 4 µm-thick sections. The slides were then stained with haematoxylin and eosin. The samples taken from the skin were used to evaluate cicatrization.

Cell proliferation

Cell proliferation in the epidermis was evaluated by counting the epithelial cells arrested in metaphase, in the basal and supra-basal layers on the reepithelialization surface. In both wounds of each animal, the number of blocked metaphases was counted in 100 microscope fields, with the aid of a 105 µm ocular ruler fitted to an Olympus BX41 microscope, and a 40X objective. The results were expressed as number of metaphases/mm.

Morphometry of the wounds

The maximum length and width of each wound were measured with calipers on the day the wound was made and again on the day the animal was sacrificed; the wound area was calculated from these measurements. The degree of contraction of the wound was determined from the difference between the initial and final areas. The means of the differences between the experimental wounds and the controls were compared.

Morphometric analysis of the reepithelialization surface

Measurements of epithelium length were made in 10 sections/wound/animal from the reepithelialization surface, with the aid of a 1030 µm ocular ruler fitted to an Olym-

pus BX41 microscope, and a 10X objective. In wounds 4 and 7 days old, two margins of reepithelialization were measured, and the final measurement calculated as the sum of both. The results were expressed in micrometers.

Statistical analysis

The results were analyzed by the unpaired Student's *t*-test. The significance level was $P < 0.05$.

RESULTS AND DISCUSSION

The cicatrizant effect of an ointment containing 1% of the ethyl-acetate fraction (F3) of barbatimão (*S. adstringens*) on wounds excised in the skin of rats was assessed. Topical application of the ointment exerted a trophic effect on the keratinocytes, stimulating a proliferative response of these cells along the margin of the reepithelialization. This response was detected after 4, 7 and 10 days of treatment (Figure 1). The mechanism by which *S. adstringens* stimulates cellular proliferation remains unknown, but is believed to be related to the tannin content of its bark (Lopes *et al.*, 2005): the astringent property of tannin favors faster closure of lesions (Favaretto *et al.*, 1985).

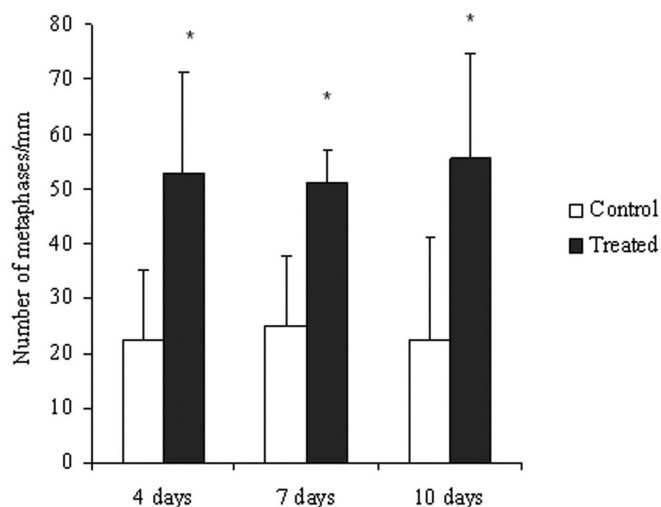


FIGURE 1 - Effect of treatment of cutaneous wounds after 4, 7 and 10 days with ointment containing 1% ethyl-acetate fraction from stem bark of *S. adstringens* on the number of metaphases/mm in the newly formed epithelium. Mean \pm S.D. ($n=5$), * $P < 0.05$ compared to the control.

Tannins are phenol compounds produced by the secondary metabolism of plants. They have an important astringent action, which increases the number of cross-links among collagen fibers in the collagen-rich matrix (tanning) (van Gulik, 1981; Haslam, 1998). In

lesions caused by burns, tannins coagulate lipid-protein complexes and accelerate the formation of a flexible scab that covers the wound (Hupkens *et al.*, 1995).

In this study, a flexible brown scab was observed after 4 days of treatment, and remained until the seventh day. On the tenth day, only a small cicatricial scar was observed.

Tannins also aid vasoconstriction, reducing vascular permeability and producing an anti-inflammatory action (Kapu *et al.*, 2001; Mota *et al.*, 1985). In addition, they have an antimicrobial effect (Schulz *et al.*, 2002; Lopes *et al.*, 2005).

Wounds treated with the ethyl-acetate fraction of two species of barbatimão were tested for proliferative activity of keratinocytes in the experimental wounds. Wounds treated with the *S. obovatum* fraction showed increases in epidermal proliferation only 4 days after treatment; whereas for *S. polyphyllum*, cell proliferation was observed after 4 and 7 days of treatment. These results suggest that the differences in the proliferative activity may be a function of the difference in the tannin content of the stem bark, which in *S. polyphyllum* is 12% and in *S. obovatum* is 19%. The extracts of *S. polyphyllum* were more effective with respect to the biological activities tested, although the extracts had a lower tannin content. This is probably a result of differences between the substances from the two species: profisetinidins were found in *S. polyphyllum*, whereas prodelphinidins were identified in *S. obovatum* (Lopes *et al.*, 2003; Sanches *et al.*, 2005; Lopes *et al.*, 2005; Lopes *et al.*, 2008).

The total tannin content of *S. adstringens* (19%) is higher than that measured for *S. polyphyllum* (12%), as shown by Audi *et al.* (2004) and Lopes *et al.* (2005). Similar results were obtained by Santos *et al.* (2002), using different analytical techniques. These authors, however, showed that *S. polyphyllum* contains a larger amount of esterified gallic acid linkage with flavan-3-ols than does *S. adstringens*, which markedly increases free-radical scavenging (Hagerman *et al.*, 1998; De Bruyne *et al.*, 1999a and 1999b), anti-glucosyltransferase, anti-viral (De Bruyne *et al.*, 1999a and 1999b), and anti-cancer activities (Dufresne, Farnworth, 2001). Nevertheless, the greater galloylation linkage with flavan-3-ols in *S. polyphyllum* was not found to be more efficacious in cicatrizant activity compared to *S. adstringens*, which in turn showed a greater mitogenic response.

In vitro experiments have demonstrated that polymeric proanthocyanidins extracted from *Hamamelis virginiana* stimulated the growth of keratinocytes, but did not stimulate their differentiation. Its action on cell growth depended on continuous application in the culture medium, for long periods of time (Deters *et al.*, 2001).

These results suggest that the proanthocyanidins present in the F3 fraction of *S. adstringens* may be responsible for the trophic effect on the epidermis observed in this study.

Grape-seed proanthocyanidins also stimulate the expression of VEGF (Vascular Endothelial Growth Factor) by keratinocytes in culture (Khanna *et al.*, 2001). VEGF can induce the migration and proliferation of endothelial cells and increase vascular permeability (Inoue *et al.*, 1998). These events are consistent with the capacity to promote angiogenesis, which has a central role in cicatrization.

Treatment with the ointment containing 1% of the F3 fraction from *S. adstringens* did not influence the migration of keratinocytes. No difference in the length of newly formed epithelium was found between the treated and control wounds (Figure 2).

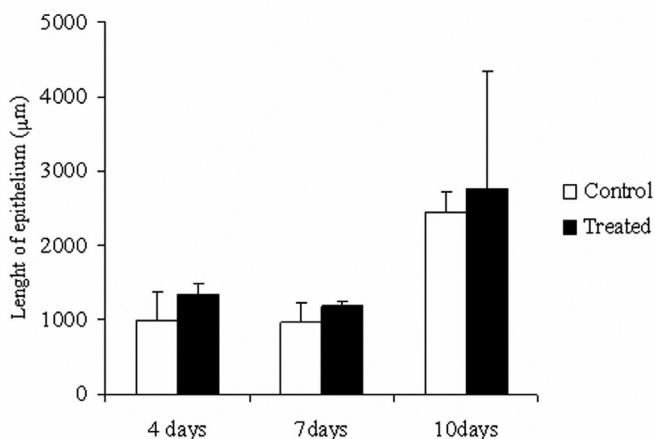


FIGURE 2 - Effect of treatment of cutaneous wounds with ointment containing 1% ethyl-acetate fraction from stem bark of *S. adstringens* on the length (μm) of the newly formed epithelium. Mean \pm S.D. (n=5) *P<0.05 compared to the control.

The phenomenon of migration is influenced by the nature of the surrounding extracellular matrix and the protein composition of the wound environment (Donaldson, Mahan, 1988; Bernstein *et al.*, 1996). Therefore, the coagulant action of the proanthocyanidins present in the barbatimão ointment may interfere with migration of the keratinocytes.

The barbatimão ointment also did not influence wound contraction (Figure 3). This suggests that the spatial arrangement of the proanthocyanidins in the fraction studied may not have been capable of causing the astringent effect that is necessary for contraction of wounds.

Therefore, the active compounds that are present in the barbatimão extract incorporated into the ointment, at

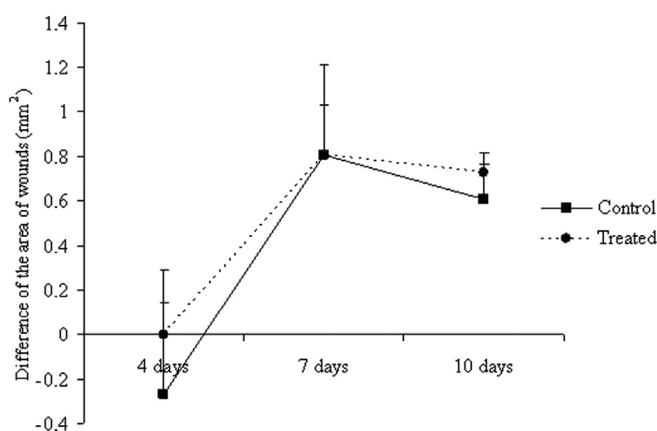


FIGURE 3 - Differences between the initial and final areas (mm^2) of cutaneous wounds treated with ointment containing 1% ethyl-acetate fraction from stem bark of *S. adstringens*. Mean \pm S.D. (n = 4), *P<0.05 compared to the control.

the dose tested, stimulated cell growth but not migration and did not influence contraction of the wounds. These results allow us to infer that the concentration and spatial arrangement of the proanthocyanidins present in the F3 fraction may be a preponderant factor in stimulating a response in the biological events that regulate the cicatricial process, being, at least in part, responsible for its medicinal use as a cicatrizant of cutaneous wounds.

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