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# Wound healing potential of ethanolic extract of *Kalanchoe pinnata* Lam. leaf—A preliminary study

B Shivananda Nayak*, Julien R Marshall & Godwin Isitor

Department of Preclinical Sciences, Faculty of Medical Sciences. The University of the West Indies, St. Augustine, Trinidad

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The extract of *K. pinnata* was evaluated for its wound healing activity by using excision wound model in rats. On day 11, animals treated with the ethanolic leaf extract exhibited 86.33 % reduction in the wound area, compared to petroleum jelly treated control (69.36%) and the mupirocin treated standard (85.49%). The hydroxyproline content of extract treated animals was higher, as compared to control and the standard groups. Histological analysis was also consistent with the proposal that *K. pinnata* leaf extract exhibits significant wound healing potential. The increased rate of wound contraction and hydroxyproline content in the extract treated animals supports the claims made by traditional healers of the benefits obtained from the medicinal use of *K. pinnata*.

**Keywords:** Excision wound, Hydroxyproline, *Kalanchoe pinnata*

*Kalanchoe pinnata* (Lam.) is a succulent plant native to Madagascar. In traditional medicine, *Kalanchoe* species have been used to treat ailments such as infections, rheumatism and inflammation[1]. *Kalanchoe* extract has immunosuppressive effect as well. *Kalanchoe pinnata* has been recorded in Trinidad and Tobago as being used as a traditional treatment for hypertension and for the treatment of kidney stones. *Kalanchoe pinnata* is common known as a "Master Herb" or a "cure for all" by a large community of herbal practitioners in the Caribbean region. Bufadienolide compounds isolated from *K. pinnata* include bryophillin A which showed strong anti-tumor promoting activity, as compared to bryophillin C which was less active. However, bryophillin C showed significant insecticidal properties[2-6].

Wound healing is the body's natural process of regenerating dermal and epidermal tissue. When an individual is wounded, a set of events takes place in a predictable fashion to repair the damage. Following injury, an inflammatory response occurs and the cells below the dermis begin to increase collagen production. Later, the epithelial tissue is regenerated[7]. There are three stages to the process of wound healing: inflammation, proliferation, and remodelling. Management of chronic wounds may involve the use of aggressive antibiotic therapy, anti-inflammatory therapy or a combination of both. These drug classes exhibit their therapeutic effect by modulating the duration of the inflammatory phase[8].

Current methods used to treat wounds include debridement, irrigation, antibiotics, tissue grafts, proteolytic enzymes, and corticosteroids, which possess major drawbacks and unwanted side effects. In villages people use plant extract to treat their wounds without any experimental knowledge. There is no experimental basis to prove the wound healing activity of *K. Pinnata.* Therefore, this study has been undertaken to assess the wound healing activity of *K. pinnata in* rats.

## Materials and Methods

*Plant material and extract preparation*—Leaves of *K. pinnata* were collected from a local producer. They were fresh and free from fungal infestation. The leaves were identified by Mrs. Yasmin S, Plant taxonomist at the National Herbarium, University of the West Indies, St. Augustine, Trinidad and Tobago. A voucher specimen was deposited for future use. (Specimen Number: TRIN 36533).

Leaves (400 g) were washed thoroughly with tap water followed by deionised water, and dried in a shade in a room away from direct sunlight. A fine powder was prepared by grinding the dry leaves in a blender. Fine powder (300 g) was dissolved in 600 ml ethanol and kept for 24 h at room temperature

*Correspondent author
Tel/Fax: 1-868-662-1873
E-mail : shivananda.nayak@sta.uwi.edu

(28°C). The ethanolic extract was filtered and kept for drying in a water bath maintained at 40°C. Clear ethanolic extract (15g) was obtained, and used for the studies.

*Animals*—Healthy inbred male Sprague Dawley rats weighing 180-200g body weight used for the study were individually housed and maintained on normal food and water *ad libitum*. The rats were anaesthetized prior to and during infliction of the experimental wounds. The surgical interventions were carried out under sterile conditions using ketamine anaesthesia (120 mg/kg body weight). Animals were closely observed for any infection and if they showed signs of infection were separated, excluded from the study and replaced. The study was approved by the ethics committee for animal experimentation (EC-AE3-2009) by the Faculty of Medical Sciences, the University of the West Indies, St. Augustin. Excision wound model was used to evaluate the wound-healing activity of *K. pinnata* leaf extract. An acute toxicity study was done to select the dose. The male rats were applied with increasing doses (0.25, 0.5, 0.75 and 1.0 g/kg body weight) of extract for 14 days. The doses up to 1.0 g/ kg body weight did not produce any sign of toxicity and mortality. The animals were physically active.

*Excision wound model*—Animals were anaesthetized prior to and during creation of the wounds. The rats were inflicted with excision wounds as described by Morton and Malone[9]. The dorsal fur of the animals was shaved with an electric clipper and the area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision wound of circular area 250 $mm^2$ and 2 mm deep was created along the markings using toothed forceps, a surgical blade and pointed scissors. The animals were randomly divided into following three groups of 6 animals each: Group 1 (standard) animals were topically applied with mupirocin ointment (2% ointment base, manufactured by Glenmark Pharmaceuticals, India); Group 2 (control) animals were applied with petroleum jelly (100%, manufactured by Unilever pharmaceuticals, CT), and group (3) (experimental) animals were treated topically with the ethanolic extract of *K. pinnata* using petroleum jelly as a base, at a dose of 100 mg/kg body weight until day 11. This was done every day and special care was taken to avoid the variation in the dose. The wound closure rate was assessed by tracing the wound on alternate days (day 1, 3, 5, 7, 9 etc.) using transparency paper and a permanent marker. The wound areas recorded were measured using a graph paper.

*Estimation of hydroxyproline*—Dry granulation tissue from both control and treated group was used for the estimation of hydroxyproline. Hydroxyproline present in the neutralized acid hydrolysate was subsequently oxidized by sodium peroxide in presence of copper sulfate followed by complexing with para-dimethylaminobenzaldehyde to develop a pink color and that was measured at 540 nm by spectrophotometer[10].

*Histological study*—Granulation tissue was obtained on day 11 from the test and control group animals for the histological study. For the better appreciation of collagen deposition, Hemotoxylin and Eosin stains and Van Gieson stains were used. Fibers were stained red in Van Gieson's reagent and purple in Hemotoxylin and Eosin.

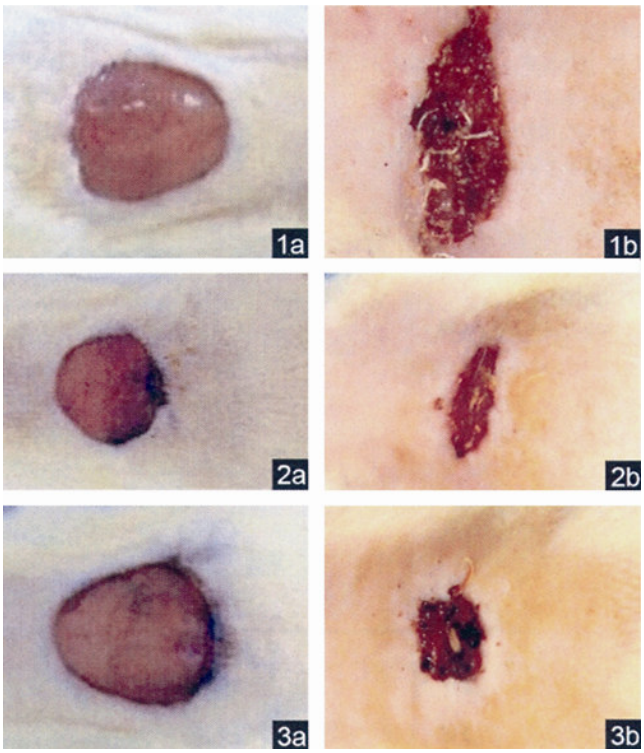
*Statistical analysis*—The means of wound area measurements between groups at different time intervals were compared using One-way ANOVA, descriptive test, followed by Tukey's post-hoc test. Data were analysed using the SPSS (Version 16.0, Chicago, USA) and *P* value was set at $<0.05$ for all analyses.

## Results

Phytochemical analysis of *K. pinnata*, by qualitative methods, showed the presences of the large family of secondary plant metabolites known as cardiac glycosides. Differential analysis of the cardiac glycoside super family indicated the presences of the sub-groups; cardenolides and bufadienolides.

On the $11^{th}$ day post-wounding, there was a significant increase in the wound-healing activity in the animals treated with *K. pinnata* ethanolic extract (Fig. 2b) compared to animals which received the control treatment (Fig. 1b) and Standard treatment (Fig. 3b). Significant progressive reduction in the wound area of the extract treated animals was observed by day 11 (86.3%) when compared to control (68.0%) and standard (85.5%). There was significant increase in hydroxyproline content in extract treated animals (22 mg/g tissue) as compared to controls (19 mg/g tissue), however, more hydroxyproline content was observed with standard treatment (35 mg/g tissue).

Histological analysis of granulation tissue samples revealed that the control group generally exhibited




Figs 1–3—Effect of *K. pinnata* ethanolic extract on excise wound [a= day 1, b= day 11, 1: control–petroleum jelly treated, 2: extract treated, 3: Mupirocin treated. Note 70, 87 and 86 % wound closure in 1b, 2b and 3b respectively]

more sparsely and diffusely dispersed collagen fibers, as well as focal areas of vascularization with minute blood vessels (Fig. 4a). Fibroblasts, characterized by vesiculated nuclei were also evident between the collagen fibers, while a few spindle shaped fibroblasts were distributed in circular focal areas. Collagen fibers were hardly distinguishable in the Van Gieson-stained section (Fig. 4b). The standard granulation tissue had, in addition to sparse diffuse collagen fibers, few skeletal myocytes (Fig. 5a). Larger blood vessels and greater concentration of strands of collagen fibers in variable orientation was, however, observed in sections stained with Van Gieson's reagent (Fig. 5b). The experimental granulation tissue had the greatest concentration of fibrocytes and dense wavy bundles of collagen fibers (Figs 6a and b). Sections through hair follicles were also evident.

## Discussion

Animals treated with ethanolic extract of *K. pinnata* exhibited significant increases in the rate of wound contraction. There was also a significant decrease in edema at the wound site 3 days after the initial application with the extract, as compared to animals

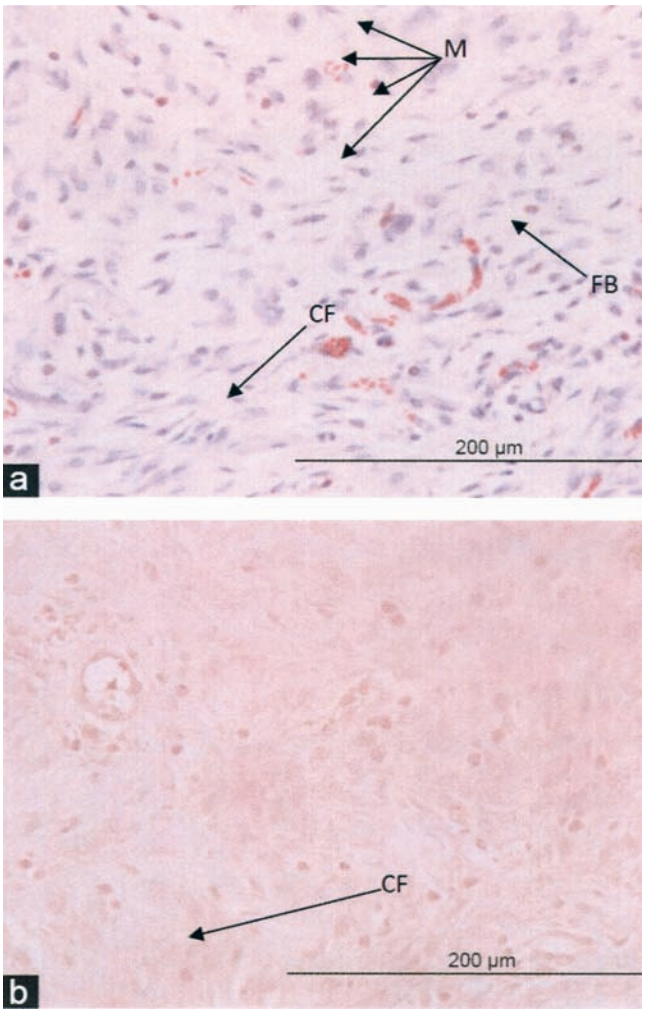



Fig. 4—(a): Section of granulation tissue of a control rat stained with Haematoxylin & Eosin (H&E) [There were numerous Fibroblast (FB), and Macrophages (M); collagen fibers (CF) were diffusely dispersed] (b): Section of granulation tissue of a control rat stained with Van Gieson [Collagen fibers (CF) are hardly noticeable within the stroma. Numerous inflammatory cells populate the area] (40X)

treated with mupirocin. Granulation tissue formed in the penultimate part of the proliferative phase is primarily composed of fibroblasts, collagen (Type III) fibres, inflammatory cells, fibronectin and new small blood vessels. Granulation tissue of animals treated with the extract exhibited a denser pattern of collagen deposition, and fewer numbers of inflammatory cells as compared to animals of the control group. Hydroxyproline content of the granulation tissue was significantly higher in animals treated with ethanolic extract as compared to controls. Increased hydroxyproline content of the granulation tissue indicates increased collagen synthesis. Collagen, the major structural component of granulation tissue,

strengthens and supports extracellular matrix tissue, and eventually replaces the temporary fibrin-fibronectin matrix. The amino acid hydroxyproline, is an integral part of the collagen fibre and is used as a biochemical marker for tissue collagen.

The wound healing, exhibited by the extract, may be attributed to the presences of steroidal glycosides. *K. pinnata* has been shown to have a significant quantity of bufadienolide; a steroidal aglycone which exists in the plant as a steroidal glycoside. Bufadienolides have been documented to exhibit anti-tumour, insecticidal and cardioprotective properties; but by virtue of the steroidal moiety of the aglycone, it is proposed that the bufadienolide aglycone can be metabolised by the cells of the wound site to produce an active therapeutic agent that may exhibit an immunosuppressive activity[11-13]. Severe oxidative stress at the wound site may lead to cytotoxicity[13]. This cytotoxicity leads to the necrosis of cells e.g. fibroblast and other cell types sensitive to reactive oxygen species. The necrotic tissue has to be cleared by debridement activity actively being accomplished by the neutrophil population in attendance. Oxidative stress at the wound site exacerbates the intrinsic process of chronic wounds by prolonging the inflammatory process[14-17]. Flavonoid biomolecules and other polyphenols have been shown to exhibit significant antioxidant activity and beneficial in reducing the concentration of ROS's at the wound site[18]. Therefore modulation of the period of the

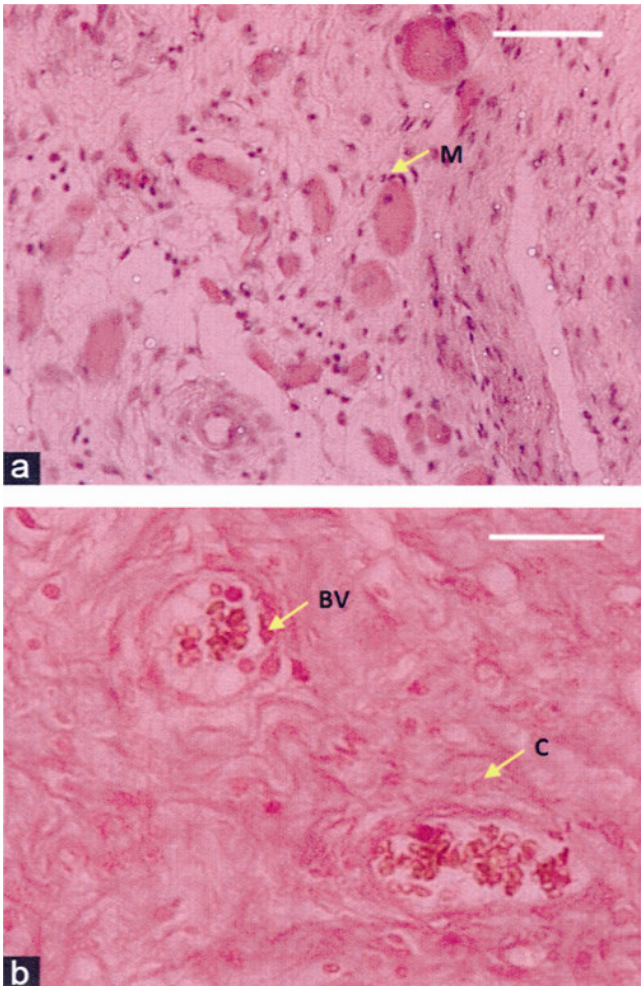



Fig. 5—(a): Section of standard granulation tissue of a rat stained with H & E [A few skeletal myocytes (M) are evident] (b): Section of standard granulation tissue of a rat stained with Van Gieson [Note the greater concentration of strands of collagen (C) fibers in different orientations. Vascularization with larger blood vessels (BV) is also becoming evident] (40X)

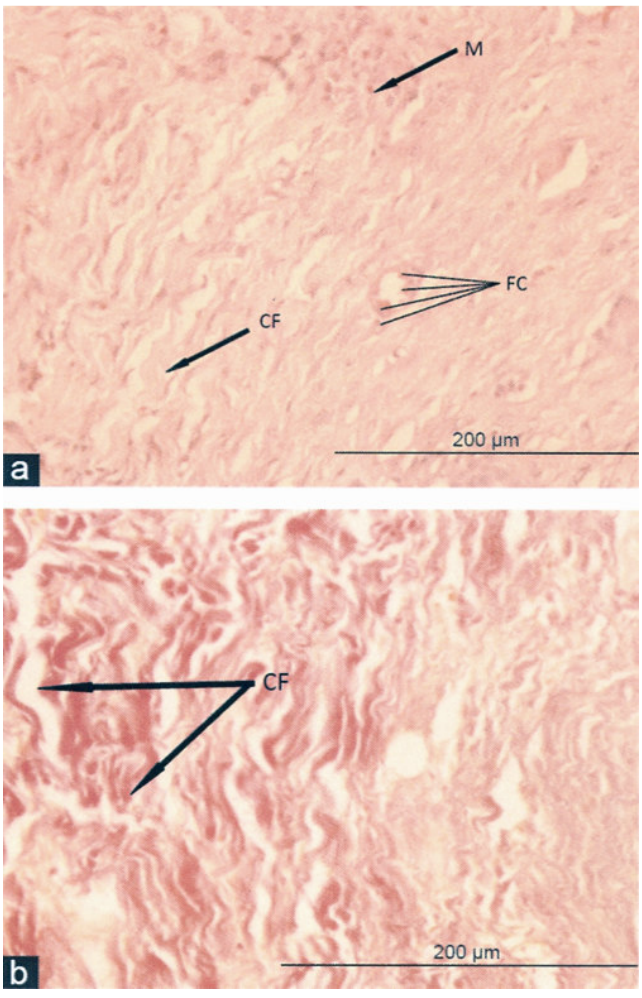



Fig. 6—(a): Section of experimental granulation tissue of a rat stained with H&E [More fibrocytes (FC) and dense collagen (CF) bundles are evident. Few Macrophages (M) are present. Population of macrophages is significantly low] (b): Section of experimental granulation tissue of a rat stained with Van Gieson[Dense wavy collagen Fibres (CF) are quite evident] (40X)

inflammatory phase by the combined effect of immunosuppressive and anti-oxidant activity may represent the basic therapeutic mode of action of the ethanolic *K. pinnata* extract.

The data of the present study demonstrates that the ethanolic extract of *Kalanchoe pinnata* facilitates significant wound healing, based on the parameters examined. Its wound healing promotion activity could be due to a potential antioxidant activity provided by the polyphenolic derivatives that were qualitatively identified. The findings at present are preliminary and warrant an indepth study. Further investigation is required to determine if the bufadienolides, isolated from *K. pinnata* can indeed exhibit the activity suggested by the results observed herein.

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