Wound healing activity of Alternanthera brasiliana Kuntze and its antioxidative profiles in experimentally induced diabetic rats

Chandana Choudhury Barua¹, Shameem Ara Begum², Debesh Chandra Pathak² and Rumi Saikia Bora³

¹Department of Pharmacology & Toxicology, ²Department of Veterinary Pathology, ³Department of Livestock Production and Management, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781 022, India.

ABSTRACT

Alternanthera brasiliana Kuntze is a neotropical native species used against inflammation, cough and diarrhea etc. Traditionally it is used as hemostatic in north east India, but its excellent wound healing activity in excision, incision, aged, burn, immunocompromised wound were reported by us. Keeping in mind the delayed healing of wound in diabetic patients and its complications, the present study was undertaken to evaluate the healing efficacy of topical application of Alternanthera brasiliana in experimentally induced diabetic wounds in Sprague Dawley rats. The animals were divided into three groups of six animals each. Group I is control, group II is treated with topical application of the extract and group III is used as standard. Prohealing activity was assessed by wound contraction, histopathological study, modulation of enzymatic and non enzymatic parameters. There was significant (P<0.05) increase in wound contraction, augmented levels of superoxide dismutase, catalase, reduced glutathione, hydroxyproline, protein and ascorbic acid level in the treated group as compared to the control group. It can be hypothesized that A. brasiliana favours wound healing in diabetic animals, due to the presence of various phytoconstituents which are known for augmenting healing and its antioxidant activity as well. The study has paved the way for more detailed study of this highly medicinal plant considered as Brazilian penicillin or Joy wood in Brazil.

INTRODUCTION

Diabetes mellitus is one of the major contributors to chronic wound healing problems. The diabetic patients with ulcer become at high risk for major complications which include infection and amputation. Due to the present fast life of the humans, a drastic increase in chronic disease conditions mainly diabetes has been determined. Most of these patients tend to face a tremendous problem when they get an infected wound. The pathophysiological relationship between diabetes and impaired healing is complex. Vascular, neuropathic, immune function and biochemical abnormalities each contribute to the altered tissue repair. Despite treatment of these chronic wounds, which involves tight glucose control and meticulous wound care, the prognosis for their healing is quite poor (Greenhalgh, 2003). Wound healing is impaired in diabetic patients with infection or hyperglycemia (McMurry, 1984). Natural products are still the primary health care system in some parts of world. The past decade has seen considerable change in opinion regarding ethnopharmacological therapeutic applications. The presence of various life sustaining constituents in plants has urged scientists to examine these plants with a view to determine potential wound healing properties (Gupta et al., 2011).

Alternanthera brasiliana Kuntze belonging to the family Amaranthaceae is a herbaceous plant commonly known in Brazil as Penicillín, Brazilian Joy Weed - is a neotropical native species, which grows easily on poor and deforested soil, is used against inflammation, cough and diarrhea in Brazilian popular medicine (Brochadoa et al., 2003). The extracts of A. brasiliana exhibited antinociceptive effects in mice (Macedo et al., 1999) antimicrobial effect (Bivatti et al., 2003) and anti-herpes simplex virus activity (Lagrotta et al., 1994). After validation of its wound healing, angiogenic and antioxidant property in normal excision and incision wound model (Barua et al., 2009), we have studied its healing...
efficacy in burn (Barua et al., 2012) and immunocompromised (Barua et al., 2012) wound models and results are very encouraging. This has prompted us to evaluate the wound healing potential along with antioxidant profiles following application of ointment of methanol extract of the leaves of this plant in excision wound model in experimentally induced diabetic rats to supplement its prohealing efficacy in impaired wound as described above.

MATERIALS AND METHODS

Plant material

Leaves of A. brasiliana were collected from the medicinal garden of the Department of Pharmacology & Toxicology, College of Veterinary Science, Khanapara during the month of Feb –June, 2010, were identified by Taxonomist, Dr. I. C. Barua, Principal Scientists, Dept of Agronomy, Assam Agricultural University, Jorhat, Assam. A voucher specimen was deposited in the Herbarium of Botanical Survey of India (Specimen No: AAU/CVSC/PHT/02).

Preparation of Methanol Extract

The leaves were washed with water, air-dried and powdered in an electric blender. About 250g of powdered leaves was soaked in 1000 ml methanol for 72 h in a beaker and the mixture was stirred every 18 h using a sterile glass rod. Filtrate was obtained after passing through a fine muslin cloth and then by filter paper (What man No 1), three times. It was then concentrated in Rotary evaporator (Equitron, Roteva) at 50°C–60°C under reduced pressure. A dark brown methanol extract of A. brasiliana (MEAB) obtained was stored in air tight container at 4°C till further use. Yield was 6.12% (w/w) in terms of dry leaves.

Phytochemical screening

Preliminary qualitative phytochemical screening of the plant extract was done for presence of various active principles (Harbone., 1973)

Drugs and chemicals

Three different concentrations (2.5, 5 and 7.5%) of ointment of MEAB was prepared using white soft petroleum jelly (S. D. Fine Chemicals, India) as vehicle. Himax (Indian Herbs Research & Supply Co. Ltd. Darra Shivpuri, Saharanpur) was used as standard drug. Its constituents are Indradaru and Somvalka.

Experimental animals

Healthy adult Swiss albino mice of either sex, approximately of same age, weighing between 25–30 g and adult Sprague Dawley rats of either sex weighing between 180–200 g were used for the study. They were housed under controlled conditions of temperature (25 ± 3°C), humidity (50 ± 5%) and 12 hours light–dark cycles with food and water ad lib. Animals were housed individually in polypropylene cages containing sterile paddy husk bedding. The experiments were performed as per guidelines of the Institutional Animal Ethical Committee (770/03/ac/CPCSEA/FVScAAU/ IAEC/06/21) and conform to the national guidelines on the care and use of laboratory animals, India. Animals were periodically weighed before and after experiments. All the animals were closely observed for any infection and those which showed signs of infection were separated and excluded from the study.

Determination of LD₅₀ and acute toxicity

The LD₅₀ of MEAB was estimated by following up-and-down stair case method in mice using OECD TG- 425 guidelines. Animals were observed hourly for 6 hours and again after 24 hours. The parameters for motor activity and gross effect were determined after administration of MEAB orally at a dose of 2.0 g/kg body weight.

Induction of Diabetic wound

The rats were weighed and their basal blood glucose level was measured using glucometer (Ames, Bayer Diagnostic, India). Next day, after overnight fasting, the animals were injected with single dose of streptozotocin (STZ, 40 mg kg⁻¹, i.p.) in 0.1 M citrate buffer, pH 4.5 to produce diabetes (Gupta et al., 2005). The following day, the blood glucose levels of all animals in the diabetic groups were recorded and animals showing blood glucose level nearly three-folds higher as compared to control value were considered diabetic. Wounds were created on the 7th day after induction of diabetes. The animals were anesthetized by intraperitoneal (i.p.) injection of thiopentone (25 mg/ kg). The dorsal surface of the rat was shaved and the underlying skin was cleaned with 70% ethanol. The excision wounds (Morton et al., 1972) were made by excising the full thickness circular skin (approximately 250 mm²) on the preshaved dorsal surface of the rats. Animals were allowed to recover from anesthesia and housed individually in sterile cages.

Experimental design

A preliminary study was conducted for selection of the most effective concentration of MEAB ointment by using 2.5, 5.0 and 7.5% (w/w) ointment for topical application. As 5% (w/w) ointment showed optimum wound healing activity, it was selected for further detail study.

The experimental animals (rats) were randomly allocated into three groups of 6 animals each. Group I served as control and the rats received topical application of the vehicle, i.e. soft white petroleum jelly, twice daily for 7 days. Animals of group II and III received topical application of 5% (w/w) ointment of MEAB and positive control drug, i.e. Himax ointment, respectively, twice daily for 7 days.

Wound healing potential

The wound surface area was measured by tracing its contour using a transparent paper on 8th day post wounding before wound excision to determine wound contraction. The area (mm²)
within boundary was measured planimetrically (Upadhyay et al., 2009). The percent wound contraction was calculated using the following formula:

\[ \text{Percent wound contraction} = \left( \frac{\text{Initial wound size} - \text{Specific day wound size}}{\text{Initial wound size}} \right) \times 100 \]

The granulation tissue was excised on 8th post wounding day to analyze pro-healing biochemical parameters viz. hydroxyproline (Woessner, 1961) and total protein contents (Lowry et al., 1951). A 10% homogenate of granulation tissue was prepared in 0.15 M KCl containing 5 mM EDTA. After homogenization, samples were sonicated (Labsonic P, Germany) ten bursts of 5 sec each at 5 sec intervals and an aliquot was withdrawn for estimation of reduced glutathione (GSH) (Beutler et al., 1963). In the remaining homogenate, triton X-100 was added at 0.1% (v/v). Then the samples were incubated at 4°C for 2.5 hours and centrifuged at 4226 g. The supernatant was used for estimation of superoxide dismutase (SOD) (Marklund et al., 1974) catalase (CAT) (Aebi., 1984) and vitamin C content (Rae., 1984).

**Histopathological study**

For histological studies, granulation tissues collected on eighth post treatment day was fixed in 10% neutral formalin solution and dehydrated with a sequence of ethanol-xylene series of solution. The materials were processed by conventional paraffin embedding method. Microtome sections were prepared at 6 μ thicknesses, mounted on glass slides, stained with hematoxylin and eosin and Vangeison’s stain (Lee et al., 1968) followed by observation for histopathological changes under light microscope.

**Statistical analysis**

Data were expressed as mean ± SE and statistical significance between experimental and control values were analyzed by one way ANOVA followed by Dunnett’s test using Graph Pad Prism 2.01 (Graph Pad Software Inc., La Jolla, CA, USA). P<0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

**Phytochemical Screening**

MEAB showed presence of alkaloid by Wagner’s and Dragendorff’s test, steroid by Salkowski’s and Lieberman Burchardt’s test and triterpenes by Salkowski’s and Lieberman Burchardt’s test. The constituents like triterpenoids and alkaloids present in MEAB might play a major role in the process of wound healing, however further phytochemical studies are needed to find out the active compound(s) responsible for wound healing promoting activity. Triterpenoids are known to promote the wound healing process, mainly due to their astringent and antimicrobial property (Benzie et al., 1996).

These active constituents support the process of wound healing by increasing the viability of collagen fibrils, by enhancing the strength of collagen fibres either by augmenting the circulation or by preventing the cell damage or by promoting DNA synthesis (Halliwell et al., 1999).

**LD₅₀ and acute toxicity studies**

In acute toxicity study, there was no change in motor activity and gross behaviour during 24 h of observation and MEAB was found to be safe up to 2000 mg/kg body weight, p.o. The low toxicity of the plant observed in this study suggests that MEAB is safe and did not affect any of the parameters studied.

**Wound healing potential**

Significant wound healing activity was observed in animals treated with 5% (w/w) ointment of MEAB in the diabetic wound. Percent wound contraction on day 8th in the extract treated group was 89.76% which was significantly (P<0.05) higher compared to the control (45.75%) and standard (73.38%) groups (Table 1). MEAB showed faster wound healing compared to control group and wound contraction rate was also quicker. This might be due to stimulation of interleukin-8, an inflammatory α-chemokine which affects the function and recruitment of various inflammatory cells, fibroblasts and keratinocytes. It may increase the gap junctional intracellular communication in cultured fibroblasts and induces a more rapid maturation of granulation tissue (Moyer et al., 2002).

**Table 1:** Effect of topical application of MEAB (5% w/w) on wound area contraction (mm²).

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Area of wound (mm²) (Percent wound contraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>140.93±2.50 (45.75)</td>
</tr>
<tr>
<td>A. brasiliiana</td>
<td>25.88±2.14 (89.76)</td>
</tr>
<tr>
<td>Standard (Himax)</td>
<td>67.53±2.38 (73.38)</td>
</tr>
</tbody>
</table>

Values are mean ± SE; n = 6.

Mean in a column bearing same superscript do not differ significantly (P<0.05).

**Estimation of protein, hydroxyproline content and antioxidant analysis**

MEAB increased cellular proliferation and collagen synthesis at the wound site as evidenced by increase in total protein content of granulation tissue (P < 0.05) when compared with the control group (Table 2). The hydroxyproline content of the granulation tissues following topical application of 5% (w/w) ointment of MEAB was found to be 66.17±2.70 mg/g tissue which was higher than the control group (50.83±2.66 mg/g) (Table 2). Increase level of hydroxyproline indicates enhanced collagen content in the granulation tissue. MEAB increased the gap junctional intracellular communication in cultured fibroblasts and induces a more rapid maturation of granulation tissue (Moyer et al., 2002).

Table 2 shows the antioxidant levels in the granulation tissue of control, MEAB.
extract (5.0%) and Himax treated diabetic rats. Reduced GSH concentration in granulation tissue was significantly \((P < 0.05)\) increased in treated rats \((1.67\pm0.07)\) in comparison to control \((1.54\pm0.05)\) animals. Similarly, there was significant increase in GSH level as compared to control group. GSH plays important role in many of the processes involved in wound healing, such as opposing the oxidative stress associated with inflammation and infection and participating in many of the processes associated with proliferation of the cells to form new tissue (Gupta et al., 2002).

### Table 2: Effect of topical application of *A. brasiliana* methanol leaf extract (5.0% w/w) on 8th post wounding day on the levels of protein, hydroxyproline and antioxidants in diabetic rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Diabetic control</th>
<th>MEAB leaf extract</th>
<th>Himax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg/g tissue)</td>
<td>59.17±2.98</td>
<td>66.17±2.70</td>
<td>88.33±2.81</td>
</tr>
<tr>
<td>Hydroxyproline (mg/g tissue)</td>
<td>50.83±2.66</td>
<td>59.17±2.60</td>
<td>62.67±3.08</td>
</tr>
<tr>
<td>Glutathione (µg mg−1 protein)</td>
<td>1.54±0.05</td>
<td>1.67±0.07</td>
<td>1.77±0.06</td>
</tr>
<tr>
<td>Superoxide dismutase (U mg−1 protein)</td>
<td>7.94±0.35</td>
<td>8.13±0.30</td>
<td>9.60±0.36</td>
</tr>
<tr>
<td>Catalase (U mg−1 protein)</td>
<td>4.11±0.08</td>
<td>5.95±0.07</td>
<td>5.39±0.12</td>
</tr>
<tr>
<td>Ascorbic acid (µg mg−1 protein)</td>
<td>1.62±0.03</td>
<td>1.96±0.07</td>
<td>2.72±0.06</td>
</tr>
</tbody>
</table>

Values are mean ± SE; \(n = 6\).

Likewise SOD and catalase activity in granulation tissue was 8.13±0.30 and 5.95±0.07 respectively in MEAB treated groups which was significantly higher \((P < 0.05)\), as compared with the control groups. Results from the antioxidant enzyme revealed that, application of MEAB ointment resulted significant increase in SOD, CAT and GSH activity in the granulation tissue, thereby affording protection to the developing fibroblasts against free radicals. Wounding initiates inflammation, which in turn stimulates the production of free radicals by phagocytes. Some of the free radicals can leak into the extracellular space and inhibit myofibroblasts thereby retarding the healing process (Nayak et al., 2003).

SOD and CAT, are two powerful antioxidant enzymes of the body that are known to quench superoxide radicals and thus prevent the damage of cells caused by free radicals (Shukla et al., 1997). So scavenging effect of these two antioxidants might be one of the most important components of wound healing in MEAB treated group. It may be noted that as compared to the Himax treated standard group, there was significant increase in the level of catalase indicating promising wound healing activity of MEAB. Similarly ascorbic acid level in MEAB treated group was 1.96±0.07 which was significantly higher than the control animals \((1.62±0.03)\). Increase level of ascorbic acid in MEAB treated diabetic rat acts as a cofactor for synthesis of collagen, proteoglycans and other organic components of the intracellular matrix of tissues such as skin, capillary walls and other connective tissues, thereby promote wound healing.

The combined effect of ascorbic acid on collagen synthesis, antioxidant status and immunomodulation make it an appropriate supplement for wound repair protocols (MacKay et al., 2003).
Histopathological study

On day 7, the MEAB treated group showed more advanced layering with continuous basement membrane in addition to a better organization of the collagen bundles. MEAB and Himax treated animals showed reduced congestion and necrosis and increased angiogenesis. Control group showed less collagen fibers than MEAB and standard group. The histological studies showed a much earlier recovery and regeneration in MEAB treated group compared to the control group. Vangiones staining showed uniform, compact and regularly arranged collagen fibers in the wound tissue in MEAB treated rats, whereas standard and untreated diabetic control group had less compact and irregularly arranged collagen fibers. The histopathological study revealed increased collagen deposition in MEAB treated group as compared to vehicle treated control animals. Granulation tissue of the control group showed less collagen fibers, whereas in MEAB treated group increase in collagen deposition was evident. Since, collagen provides strength and integrity to the dermis and all other supporting tissues, synthesis, secretion and subsequent organization of collagen play an integral role in wound healing (Chithra et al., 1988).

CONCLUSION

Our data demonstrates that MEAB has promising wound-healing activity in diabetic wounds, in addition to its efficacy in burn or immunocompromised wound reported in previous studies. The healing efficacy in part might be due to its phytoconstituents and anti oxidant activity as revealed in this study. Although there are many underlying factors for its healing potential, hence this study has paved the way for delving further to unveil the mechanism of this multifarious medicinal plant.

ACKNOWLEDGEMENT

The authors extend their sincere thanks to Defense Research Development Organization (DRDO), Govt. of India, New Delhi for funding this project. The authors are also grateful to the Director of Research (Vety) and the Central Instrumentation facility (CIF), FVSc, Khanapara for providing necessary facilities to carry out the research work.

REFERENCES


How to cite this article: