Effect of Ethyl Acetate Fraction of Flowers of *Pterospermum Acerifolium* (L) Willd. on Wound Healing in Streptozotocin Induced Diabetes

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Abstract

Pterospermum acerifolium (L.) Wild. has been reported to possess both wound healing as well as hypoglycemic properties. Hence, an effort has been made to further explore the plant's efficacy in wound healing in diabetic rodent model. The collected aerial parts of the plant were extracted with methanol. Gels of different concentrations were prepared with ethyl acetate fraction of the methanolic extract of the plant (PAFEF) along with methyl paraben, propyl paraben, carbopol and triethanolamine. The diabetes induced rodents were subjected to various excision (wound contraction, period of epithelization and hydroxyproline content) and incision (tensile strength) wound healing tests. The parameters were measured using framycetin sulfate cream (1% w/w) as standard. Formulations containing various concentrations of PAFEF were found to increase wound healing on the diabetes induced rodents compared to control. Formulations containing highest concentration of PAFEF showed increased rate of wound contraction demonstrated by enhanced epithelization.

Keywords: ethyl acetate fraction of *Pterospermum acerifolium* (L.) Willd., wound healing, hydroxyproline content, streptozotocin-induced diabetic rats

INTRODUCTION

Wound is a disturbed state of tissue caused by physical, chemical, microbial and immunological insults typically associated with loss of function. Wound healing is a complex process that involves interaction of complex cascade of cellular and bio-chemical actions to restore the structural and functional integrity with regain of strength of injured tissues and involves continuous cell – cell interaction and cell matrix interactions that allow the process to proceed in different phases including inflammation, wound contraction, re-epithelialization of tissue, re-modeling and formation of granulation tissue with angiogenesis. Wound healing is impaired in diabetic patients because of hyperglycemia. Several other factors which delay or reduce the wound healing process includes bacterial infection, necrotic tissue and interference with blood supply, lymphatic blockage, if the above factors could be altered by any agent, an increased healing rate could be achieved [1]. In diabetic patient wound heals slowly and can worsen rapidly, so it requires close monitoring. The factors influencing impairment of wound healing in diabetic patients are, increased blood glucose level, poor circulation, diabetic neuropathy, immune system impairment, infection.

The prevalence of diabetes is increasing worldwide and has been estimated to be double in the next 20 years. The major increase in morbidity and mortality of diabetes is due to the development of complications mainly due to impairment of wound healing process. Foot ulcers occur in 25% of all patients with diabetes and failure of healing eventually leads to infection and amputation. Impaired wound healing is therefore responsible for most of the morbidity (and mortality) in diabetes [2].

The wound healing activities of plants have been explored ever since ancient times. *Pterospermum acerifolium* (L.) Willd. (Family- Sterculiaceae) is one such plant reported to possess wound healing properties in methanolic extract. *Pterospermum acerifolium* commonly known as Kanakchampa, is an evergreen large tree up to 24m in height and 2.5 m in girth. It is found in the sub-Himalayan tract and outer valleys from Yamuna eastwards to West Bengal, Assam and Manipur, up to an altitude of 1200 m [3]. The plant has been used pharmacologically as an anti-inflammatory, anti-diabetic, wound healer, analgesic, anti-oxidant, anti-ulcer and anti-cancer [4].

The plant is rich in various chemical constituent such as amino acids, sugars, flavonoids and glycosides. It has been reported that flowers are rich in carbohydrates, also containing bitter principles of kaempferol-3-O-glucoside as the major component and glycosides of luteolin and quercetin as minor components 3, 7-diethyl-7methyl-1, 5-pentacosanolide, n-hexacosan-1, 26-diol and kaempferol, kaempferide, β-sitosterol, β-amyrin, friedelin which is useful in leucorrhoea, inflammation, ulcer, and leprosy [5].

Pterospermum acerifolium (L.) Willd. is reported to possess wound healing properties in methanolic extract [6]. In the present study an effort has been made to show the reduced wound healing activity in diabetes and further evaluating healing property of ethyl acetate fraction of methanolic extract of *P. acerifolium* flowers in diabetic rats.

MATERIALS AND METHODS

Plant Collection

The flowers of *Pterospermum acerifolium* were collected from the campus of BIT Mesra, Ranchi in the month of March - April 2013. It was authenticated from taxonomy department of National Botanical Research Institute (NBRI), Lucknow. The voucher specimens (NBRI/CIF/247/2011) were retained in the Department of Pharm. Sciences and Technology, BIT-Mesra, Ranchi for future reference.

Extraction

The air-dried powdered plant material was extracted with petroleum ether for 48 hrs and then with methanol in a Soxhlet apparatus for 72 hours. After extraction the solvent was filtered and then evaporated in rotary evaporator to obtain the crude methanolic extract of *Pterospermum acerifolium* flowers. The methanolic crude extract was fractionated with ethyl acetate to form ethyl acetate fraction of *Pterospermum acerifolium* flowers (PAFEF).

Gel Preparation

5 ml of distilled water was taken in which methyl paraben (0.2ml) and propyl paraben (0.1ml) was dissolved by heating on water bath. Propylene glycol 400 (5ml) was added to the cooled solution. Further, required quantity of PAFEF was mixed to the above mixture and volume was made up to 100 ml. 1 g of Carbopol 934 was dispersed in 50 ml of distilled water with continuous stirring. Finally, the mixture prepared previously was mixed properly to the Carbopol 934 gel with continuous stirring to form the gel. Triethanolamine was added drop wise to the formulation for adjustment of pH (6.8-7). The same method was followed for preparation of control sample without adding any *P. acerifolium flower* extract [7]

Animals

Albino Wistar rats of both sex in the weight range of 150g to 250 g were used which were procured from Institutional Animal House of Birla Institute of Technology, Mesra. Protocol No: BIT/PH/IAEC/19/2013 dated 07.09.13. They were housed and maintained in clean polypropylene cages where food was provided in the form of dry pellets (FDA) and water *ad-libitum.*

Induction Of Diabetes

Diabetes was induced in rats by a single *i.p.* injection of Streptozotocin (50 mg/kg) in 0.1 M citrate buffer, pH 4.0. The fasting glucose levels were recorded for days 0, 3, 7 days. Animals with fasting blood glucose levels greater than 200 mg/dL are used in the study [8].

Study Design

Incision and excision wound models were used to assess the wound healing profile of *Pterospermum acerifolium.* Animals were divided into 5 groups of 6 animals each as follows:

Group I: Normal control, animals without any treatment

Group II: Diabetic control, diabetic animals treated with vehicle (gel base)

Group III: Diabetic animals treated with Framycetin sulfate cream (1% w/w)

Group IV: Diabetic animals treated with extract gel (2% w/w PAFEF)

Group V: Diabetic animals treated with extract gel (5% w/w PAFEF)

Excision Wound Model

Post anaesthetization of the animals with diethyl ether, the dorsal fur area of each animal was shaved and a circular 400 mm^2 full thickness open excision wound was made by removing a patch of skin. The groups were applied topically with their respective standard (framycetin sulfate cream 1% w/w), gel base and gel extract (2% and 5%) for 16 consecutive days. The wound area was measured on alternate days. The granulation tissues formed were removed on 16^{th} post-wounding day was used for analysis. Reduction in the wound area was expressed as percentage of the original size [9]

Measurement Of Wound Contraction

The areas on wounds were measured on days 4, 8, 12, 16 post-wounding days and the mean percentage wound contraction was calculated as follows;

$$\%\ \text{wound contraction} = \frac{\text{Healed area (wound area on day 0} - \text{wound area on day n)}}{\text{wound area on day 0}} x100$$

Determination Of Period Of Epithelization

When no raw wound was left behind it was taken as end point of complete epithelization and the days required for this was taken as the period of epithelization.

Estimation Of Collagen (Hydroxyproline) Content

Wound tissues were analyzed for hydroxyproline content, which is basic constituent of collagen. Measurement of hydroxyproline hence can be used as a biochemical marker for tissue collagen and an index for collagen turnover [9].

For preparation of protein hydrolysate, 50 mg of tissue sample, 1.0 ml of 6.0N HCl was weighed and sealed in screw-capped glass tube. The tubes were autoclaved at 151.056 kg/ cm^2 for 3 h. The hydrolysate was neutralized to pH 7.0 and brought to the appropriate volume. 1 ml of test sample was taken as test, 1.0 ml of DM water as the blank and 1.0 ml standard solution as standard. 1ml of 0.01M copper sulphate solution was added to all followed by the addition of 1.0 ml of 2.5N sodium hydroxide and 1.0 ml of 6% hydrogen peroxide. The solutions were occasionally mixed for 5 min and then kept for 5 min in a water bath at 80°C. They were chilled in ice-cold water bath and 4.0 ml of 3.0N sulphuric acid was added with agitation. 2 ml of *p*-(dimethylamino) benzaldehyde was then added and heated in water bath at temperature 70°C for 15 min. The absorbance was measured at 540 nm. The concentration of the sample was calculated by using the following formula:

$$\text{Concentration of the sample} = {}^{\text{OD of the sample}}/_{\text{OD of standard}} x \text{Concentration of standard}$$

Incision Wound Model

All the animals were anaesthetized with diethyl ether and the back hair of the rats were shaved, 1 linear-paravertebral incision was made with a sterile surgical blade through the full thickness of the skin at the distance of 1.5 cm from the midline of the vertebral column. The wounds were closed with 2 surgical interrupted sutures of 1 cm apart. The study comprises of 5 groups of 6 animals each and the gel was topically applied once in a day. The sutures were removed on 8th post wound day. The skin breaking strength of the wound was measured on 10th day [10,11]

Measurement Of Tensile Strength

The different formulations along with the standard ointment (framycetin sulphate cream) were applied throughout the experiment, once daily for 9 days. The sutures were removed on the tenth day and the animals were anesthetized. Small piece of healed wound was cut out such that the healed incision wound comes exactly in the middle. Four small curved needles (No: 14) were pierced through the healed skin, two on either side. To one side two needles were tied to a rod and on the other side two needles were tied to a plastic bottle, which hang freely in the air (the either side of needles were placed equidistant from the healed incision wound). Then slowly water was added to a bottle until the wound began to open. The amount of water in the bottle was weighed and considered as an indirect measure of tensile strength of the wound. The mean determinations of tensile strength on the two paravertebral incisions on both sides of the animals are taken as the measures of the tensile strength of the wound for an individual animal. The tensile strength of the extract-treated wounds was compared with control [6].

Statistical Analysis

The results were expressed as mean ± standard error of the mean (SEM). Statistical analysis of all the data was evaluated according to one-way analysis of variance (ANOVA) using statistical software Graphpad Prism version 6. The significance of difference was evaluated using one-way ANOVA followed by Dunnett's multiple comparison test. Probability values of normal control was compared with diabetic control whereas probability values of ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 were compared with diabetic control.

RESULTS

Excision Model

From the percentage wound contraction data (Fig. 1, Table 1 and Fig. 2), it is concluded that the diabetic control group showed decreased wound contraction compared to normal control. Formulation Gel2 produced greater wound contraction as compared to the other tested formulations and wound contraction increased as the day passed. The gel formulations have significant ($P < 0.0001$) wound healing activity compared to diabetic control group. The rate of wound contraction was found to reach a maximum on the 20th day in the group treated with Gel2 (5% extract) while in case of Gel1 (2% extract) it was 22nd day. It indicates that the wound healing is delayed in diabetic condition compared to normal and also that the contraction of wound increases in diabetic group as the concentration of extract increased from 2% in Gel1 to 5% in Gel2. On the 16th day of the treatment, wound contraction was 97% with reference ointment, whereas it was (75.30±2.13), (87.61±3.17) and (66±0.63) respectively for groups treated with Gel1, Gel2 and the untreated group. On day 18 no scars were observed in animal treated with Gel2 and reference ointment, which was an indication for complete wound healing or epithelization (Table 2).

Biochemical Evaluation

Hydroxyproline Content

Extract-treated groups showed significant increase in hydroxyproline content in a dose dependent manner when compared to control animals over the 16 day treatment period (Table 3, Fig. 3, 4). The hydroxyproline content in animals treated with Gel1, Gel2 and reference ointment was found significantly greater than control group of animals.

Incision Model

Tensile Strength

The tensile strength of the tissue after wound healing in different groups were determined and the amount of weight required for breaking of the tissue was observed. Diabetic groups applied with Gel1 and Gel2 showed a significant increase in tensile strength of the healed tissue in a dose dependent manner when compared to the diabetic control group (Table 4, Fig. 5).

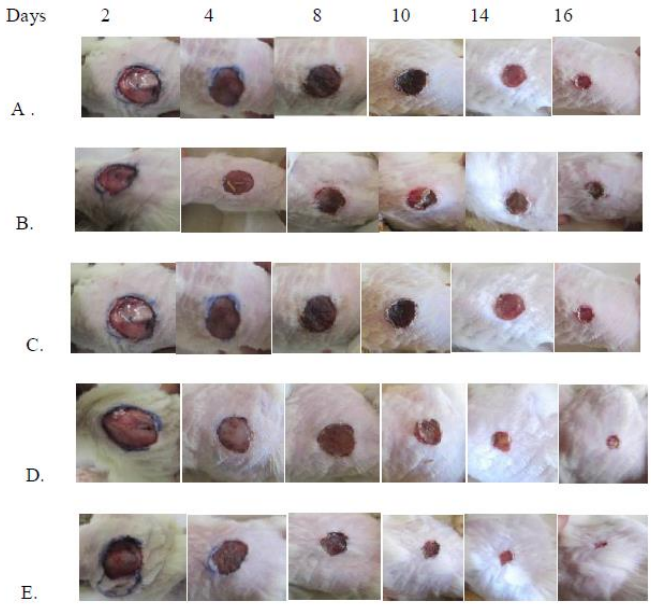

Figure 1. Wound contraction in different groups for 16 days treatment (Group I. normal control group treated with gel base; Group II. Diabetic control group treated with gel base; Group III. Diabetic group treated with gel1; Group IV. Diabetic group treated with gel2; Group V. Diabetic group treated with standard)

Table 1. Effect of PAFEF Gel on percentage wound contraction in STZ induced Diabetic rats

Post-wounding days	Normal control	Diabetic control	Gel 1(2%)	Gel 2(5%)	Reference ointment
Day2	7.43±.1709	7.19±0.135^{ns}	8.3±0.159^{ns}	11.22±0.193**	12.33±.202**
Day4	12.68±0.086	10.42±0.256*	15.93±0.173**	20.02±.435*	23.27±1.17*
Day8	27.1±0.81	23.04±0.93*	32.1±1.71**	46.19±1.08***	48.5±2.09****
Day10	35.14±0.73	27.79±0.41	46.18±0.90	66.3±0.87	73.9±0.31
Day14	52.64±0.69	47.7±0.523*	67.08±0.99*	75.71±0.406*	85.17±1.02*
Day16	66±0.63	58.46±0.29**	75.96±1.08**	86.62±0.85***	92.9±0.73***

Values are expressed as mean± SEM (n=6). Inter group comparisons were made between Normal control Vs Diabetic control, extract treated and reference group with diabetic control group using one way ANOVA followed by Dunnett test. Symbols represent *P<0.0.5, **P<0.01, ***P<0.001, ****P<0.0001

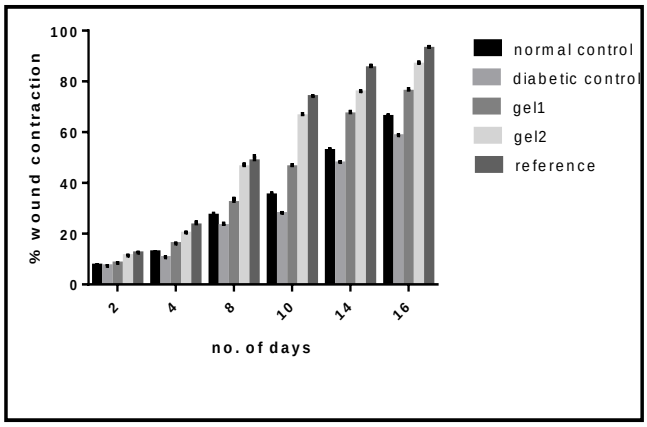

Figure 2: Percentage wound contraction with post wounding days in STZ induced diabetic rats applied topically with Gel1, Gel2 and reference ointment

Table 2. Period of epithelization of wound applied with PAFEF Gel in STZ induced diabetic rats

Period of epithelization	Normal control	Diabetic control	Gel1 (2%PAFEF extract)	Gel2 (5%PAFEF extract)	Reference ointment
	23.51±0.67	26.03±0.32**	21.64±0.38*	20.33± .44***	18.36±1.71**

Values are expressed as mean± SEM (n=6). Inter group comparisons were made between Normal control Vs Diabetic control, extract treated and reference group with diabetic control group using one way ANOVA followed by Dunnett test. Symbols represent *P<0.0.5, **P<0.01, ***P<0.001, ****P<0.0001

DISCUSSION

The observations and results obtained in present study indicated that PAFEF posses significant wound healing properties. The gel formulation prepared incorporated with the extract fraction may contribute to better healing by increasing the residence at wound area and release of active

constituents from the PAFEF. Both the formulations containing 2% and 5% PAFEF were found to increase wound healing compared to control with 5% fraction showing increased wound contraction. This is demonstrated by a significant increase in rate of wound contraction and by enhanced epithelization

Table 3. Hydroxyproline content of the granulation tissue

S.No.	Group	Hydroxyproline content
1.	Normal control(base)	97±3.45
2.	Diabetic control(base)	63±1.71**
3.	Gel1 (2%)	109.5±2.11*
4.	Gel2 (5%)	127.5±2.41***
5.	Ref. ointment (framycetin)	136.2±0.813****

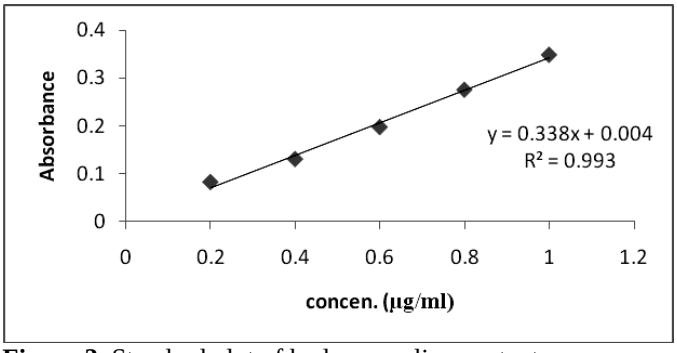

Figure 3. Standard plot of hydroxyproline content

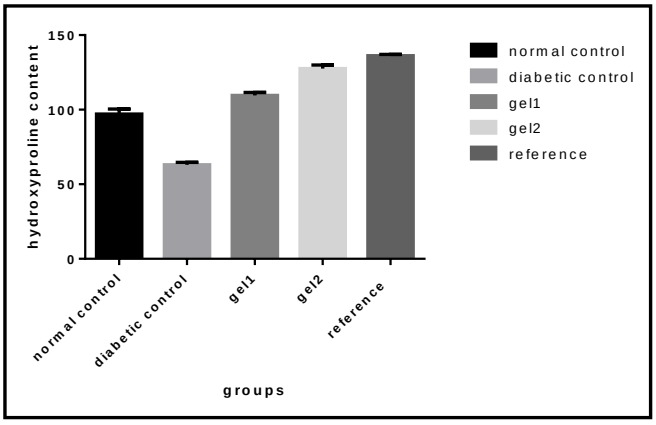

Figure 4. Effect of PAFEF gel on Hydroxyproline content

Defective collagen metabolism in diabetes may be considered as a factor in delayed wound healing in diabetic individuals. Hyperglycemia in animal studies is found to increase collagenase and protease activity in diabetic rats and impaired vascular wound healing [12]. Collagen deposition in the wound is the most important phase of wound healing [13]. Collagen is a major protein of the extracellular matrix which ultimately contributes to wound strength. Breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of this hydroxyproline therefore is used as an index of collagen turnover. The increased hydroxyproline content of the excision wound indicates faster collagen deposition leading to rapid wound healing. Significant increase in hydroxyproline content was observed in PAFEF compared to the diabetic control which is a reflection of increased collagen levels. This indicates that the healed wound has improved collagen maturation by increased cross-linking.

Table 4. Tensile strength of the tissue on 10th post wounding day

Group	Normal control	Diabetic control	Gel1	Gel2	Standard
Tensile strength	67.99±0.311	52.67±0.181*	77.63±0.518**	87.17±0.128***	92.27±0.078**

Values are expressed as mean± SEM (n=6). Inter group comparisons were made between Normal control Vs Diabetic control. extract treated and reference group with diabetic control group using one way ANOVA followed by Dunnett test. Symbols represent *P<0.0.5, **P<0.01, ***P<0.001, ****P<0.0001.

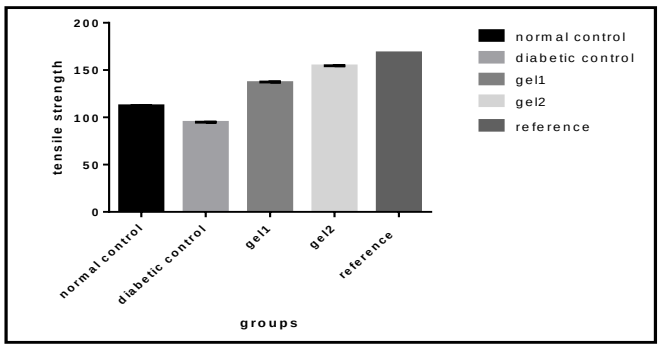

Figure 5: Effect of PAFEF gel on Tensile strength

Gain in tensile strength depends upon the increase in the collagen content [6,14]. From the incision

wound model, it can be observed that, with the increase in the dose of PAFEF the tensile strength of the incised wound increased when compared to the diabetic control.

CONCLUSION

The present study demonstrated the wound healing properties of ethyl acetate fraction of *Pterospermum acerifolium* (L.) Willd. in the diabetic condition for the first time. Further studies to isolate the bioactive constituents responsible for wound healing in diabetes and the detailed cellular assays to estimate the mechanism of action should be done. The above study also reveals the scope of *Pterospermum acerifolium* (L.) Willd. in the screening of other complications in diabetic conditions.

Acknowledgements: The authors would like to acknowledge the Department of Pharmaceutical Sciences and Technology, Birla Institute of Technology, Mesra, Ranchi, India, for providing the necessary facilities to carry out the study.

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