RESEARCH ARTICLE

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Corresponding Author
Vivek P. Kahale
Ph.D. Research Scholar
University Department of Pharmaceutical Sciences
Rashtrasant Tukadoji Maharaj Nagpur University
Mahatma Jyotiba Fuley Shaikshanik Parisar, Futala Square,
Amravati Road Nagpur – 440 033
Phone: +919890181872, 0712-2500324 fax: 07122500355
Email: vivekkahale@gmail.com

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To Develop and Standardize the Animal Model for Diabetic Foot Ulcer Using Alloxan: Attenuate the Complication by Combination of Extracted Aloe Vera

Vivek.P.Kahale [1*], Naresh .J.Gaikwad [1]
Department of Pharmaceutical Scinces , Rashtrasant Tukadoji Maharaj Nagpur University,Nagpur M.S. India

ABSTRACT

The core finding of the present preclinical study is to develop and standardize the animal model of diabetic foot ulcers by using varying doses of Alloxan by using excision and incision wound ulcer technique. Optimization with subcutaneous administration of the chemical inducer (Alloxan) through blood glucose and body weight monitoring of rat. Administration of prescribed oral and topical doses of Aloe Vera is remarkably protective effects in diabetic and its complication using male wistar rats against alloxan induced Diabetic Foot Ulcers (DFU). Diabetes mellitus is an endocrine disorder. Diabetes mellitus is now defined as a metabolic disorder in which the body's capacity to utilize glucose, fat and protein is disturbed due to insulin deficiency, insulin resistance or both. Diabetes is a condition which is known to be associated with a variety of connective tissue abnormalities. The collagen content of the skin is decreased as a result of reduced biosynthesis and or accelerated degradation of newly synthesized collagen and epithelisation. These qualitative and quantitative abnormalities contribute to the impaired wound healing observed in diabetes. Aqueous extract of Aloe Vera (AGE, Oral) with ethanolic extract of Aloe Vera (AG, Topical) act as DFU protective agent. Relevant doses of Aloe Vera treatment significantly attenuated damage of rat against Alloxan induced connective tissue abnormality in diabetic wound ulcer. The histopathological examination and wound ulcer contraction results show that Aloe Vera treatment is effective in DFU models; it could be used as an effective therapeutic agent in the management of Diabetic foot ulcers and related conditions.

Keywords: Alloxan, Diabetic Foot Ulcers, Optimization, extracted Aloe Vera.

Introduction

Diabetes mellitus is now defined as a metabolic disorder in which the body's capacity to utilize glucose, fat and protein is disturbed due to insulin deficiency, insulin resistance or both. According to the information of the International Diabetes Federation, there are currently at least 382 million diabetics in the world[10] and the World Health Organization estimates that there are 347 million people with diabetes mellitus.[11]

The combination of peripheral neuropathy, peripheral arterial disease and infection would result in unhealing ulcers, gangrene and amputation. Amputation leads to significant morbidity and mortality[1-5].The lifetime incidence of the diabetic developing a foot ulcer has been estimated at 15 – 25% and even with aggressive care approximately 14-24% of diabetics with a foot ulcer ultimately require an amputation.[6-9]

Diabetes mellitus is a condition which is known to be associated with a variety of connective tissue abnormalities. The collagen content of the skin is decreased as a result of reduced biosynthesis and or accelerated degradation of newly synthesized collagen. However, medical therapies for wound care are currently limited with a focus on minimizing weight bearing, antibiotics, and diligent dressing changes. Development of novel therapies to improve wound healing in diabetics is therefore an essential and emerging field of investigation. These qualitative and quantitative[12] abnormalities contribute to the impaired wound healing observed in diabetes[13]

Over the years, several animal models have been developed for studying diabetes mellitus or testing anti-diabetic agents. These models include chemical, surgical (pancreatectomy) and genetic manipulations in several animal species to induce diabetes mellitus. The diabetogenic drugs used include: alloxan monohydrate, streptozotocin with or without nicotinamide, ferric nitrilotriacetate, ditizona and antiinsulin serum. The aim of the present review is to piece together all the various experimental models developed for studying diabetes hyperglycaemia when compare to alloxan induction method. It was noted that, although chemical induction of diabetes mellitus with streptozotocin was the most widely used procedure, alloxan induced – diabetes model was the known drug induced diabetes. The existence of experimental animal model of a disease aids not only the understanding of the pathophysiology of such disease, but also the development of drugs for its treatment.[14]

Aloe Vera (L.) Burm. f., (Liliaceae) has been used in the traditional medicinal practices of many cultures for a host of curative purposes. It has been used in the treatment of a variety of disorders including wounds and burns[15]. The fresh gel, juice or for modulated products have been used for general health. In addition to its wound healing property, *Aloe Vera* has also been shown to have antidiabetic and hypoglycemic properties[16-17]. In spite of its wide use as folk remedy over a long period of time, the biochemical details of its action on physiological/pathophysiological functions have not yet been worked out.

METHODS

Subjects:

Adult male Wistar rats born and reared in the Animal House of the Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur (India) was used in the present study. Young healthy male rats (250–300 g) were group housed (Six per cage) and maintained at 23±2 ◦C under 12:12 hrs light (08:00–20:00 h)/dark cycle with free access to rodent chow and tap water. The animal studies were approved by the Institutional Animal Ethics Committee, (sanction letter No. IAEC/UDPS -2014/39) constituted for the purpose of control and supervision of experimental animals by Ministry of Environment and Forests, Government of India, New Delhi, India. The proposal for experimentation has been approved by IAEC of the Department (sanction letter No. IAEC/UDPS -2014/39).Animals were naive to drug treatments and experimentation at the beginning of all studies. All tests were conducted between 08:00 and13:00 h.

Drugs and solutions:

- Alloxan Monohydrate (Sigma-Aldrich Labs, Bangalore, India). Alloxan dissolved in double saline water. Drug solutions were prepared fresh and their doses are expressed in terms of free bases. Other chemicals used in the present investigation were of analytical grade.
- Aloe Vera gel was prepared according to method described (Chithra et.al.1996). In brief, mature, healthy, fresh leaves of Aloe Vera were washed with water. The leaves were cut transversely into small pieces. The thick epidermis was selectively removed and the solid gel present in center of leaf grounded in blender, homogenized and centrifuged at 10,000 Xg for 30 min at 4° C. Fibers were removed by filtration and supernatant was lyophilized and stored at room temperature until use

Treatment schedule:

To develop and standerdise the animal model of dibetic foot ulcer, the rats were divided in to 5 Groups and each group in the study consists of 6 rats. Fallowing groups are for the optimization of inducer to develop the experimental model.

Group I: Untreated Control

Group II: Rats injected with inducer of dose 120 mg/kg

Group III: Rats injected with inducer of dose 140 mg/kg

Group IV: Rats injected with inducer of dose 160 mg/kg

Group V: Rats injected with inducer of dose 180 mg/kg

After the optimization fixed the dose of inducer and "Diabetes Foot Ulcer" model was induced by injecting alloxan monohydrate used as a 2% solution in physiological saline subcutaneously at various dose level of body weight to overnight fasted animals. Control animal were injected with saline physiological solution. Diabetic foot ulcer treated with Aqueous extract of Aloe Vera (300 mg/kg, p.o.) and topical Aloe

Vera gel (30 mg/kg) twice daily and comparison with marketed product containing Aloe Vera.

Surgical Wounding Procedures:

Only those rats with severe diabetes (fasting blood glucose >300 mg/dl) were selected for ulcer induction.[2] One week after glucose estimation, the ulcer induction was carried out by two methods i.e. excision and incision wound model. The day of wound induction was considered as day 0.

Diabetic Wound Ulcer Models:

A. *Excision wound ulcer:*

Excision wound ulcer was produced by method described earlier.[2] In brief, on day 0, rats were anesthetized with ketamine (100 mg/kg, i.m.) and xylazine (5 mg/kg, i.m.) combination, [34] and rectangle was marked on dorsal surface of the foot using a signet and then a layer of skin in full thickness (standard area 2 X 5 mm) was removed. One day after wound induction (day 1), the wound became slightly larger.

B. *Incision wound ulcer*:

Excision wound ulcer was produced with slight modification of earlier described method [37] for incision wound. In brief, on day 0, rats were anesthetized with ketamine (100 mg/kg, i.m.) and xylazine (5 mg/kg, i.m.) combination and rectangle was marked on dorsal surface of the foot using a signet and then a layer of skin in full thickness (standard area 2 X 5 mm) was removed. One day after wound induction (day 1), the wound became slightly larger, which was parted together by stitching the skin with sutures (non-absorbent) 1mm apart, continuous threads on both wound edges were tightened for good adaptation of wound.

Histopathological Examination:

Pancreas was subjected for routine histopathological examination and fixed in 10% formal saline. Tissues were processed and embedded in paraffin wax. Sections were cut at 5µm thickness and stained with haematoxylin and eosin.[18]

The animal groups were euthanized 0, 3, 6, 9, days after wounding respectively by intravenous injections of pentobarbital (50 mg/kg). The wounded tissues with 0.5 cm of peripheral unwounded skin were harvested, pinned on a plastic plate (to keep the tissue fl at) and fixed in 10% buffered formalin. After fixation, each sample was cut into 3 pieces. All pieces were embedded together into a paraffin block, and 5 µm-thick histological sections were obtained. The tissues were stained with haematoxylin and eosin which were examined by light microscope. Ulceration necrosis and epithelization were evaluated in the skin tissue

After foot ulceration, application and administration of aqueous (oral) extract of herbal sample (AGE) with ethanol (Topical) extract of herbal sample (AG) on the wounded tissue of both excision and incision wound ulcer group of rat. The sections were collected on 0, 3, 6, 9, days and evaluated for histopathological examination of respective days.

STATISTICAL ANALYSIS

Results were expressed as mean ± S.E.M. The data were analyzed by two-way or one-way analysis of variance (ANOVA) followed by Bonferroni and Tukey's multiple comparison test respectively. Statistical significance was considered at $P<0.05$ in all the cases.

RESULT

Results of optimization of inducer:

Table 1. Variation in Blood Glucose level (mg/dl) of experimental animal with increasing dose of alloxan

Day	Normal	120 mg/kg	140mg/kg	160mg/kg	180mg/kg
0	96.25±2..24	104.93±8.23	156.65±42.21	254.32±29.13	417.56±6.26
3	107.31 ± 8.97	115.83±7.20	201.95±12.21	337.75±71.83	459.55±36.52
6	97.30 ± 21.86	104.63±26.23	195.34±21.33	351.58±48.23	539.55±54.28
9	93.80 ± 19.34	115.22±9.26	219.11±11.71	388.02±31.23	457.28±51.71
12	94.07 ± 13.04	114.52±20.48	219.96±16.43	384.87±31.83	532.85±68.70

Table 2. Variation in body weight of experimental animal with increasing dose of alloxan

Day	Normal	120 mg/kg	140mg/kg	160mg/kg	180mg/kg
0	226.73±2.88	230.52±1.89	231.14±1.32	228.42±1.32	226.97±0.96
3	228.56±3.44	229.91±2.95	231.06±0.32	223.10±3.24	217.63±7.25
6	226.95±2.82	229.23±2.13	229.92±0.83	216.56±5.92	201.24±7.21
9	230.91±3.36	228.75±1.28	230.00±5.52	212.78±10.32	189.45±8.46
12	227.59±1.28	227.42±1.27	226.20±3.05	210.00±0.85	163.67±11.82

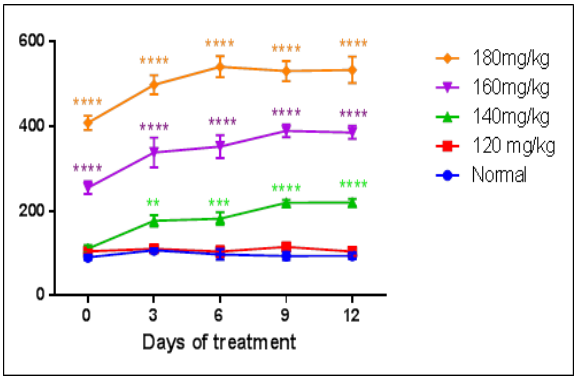


Fig.1. Influence of varying doses of alloxan in blood glucose level of experimental animal on day 0, 3, 6, 9, 12. Data are given as mean ± SEM of six rats in each group. *** P<0.001 compared to control. Significant compare to normal (**, ***, ****)

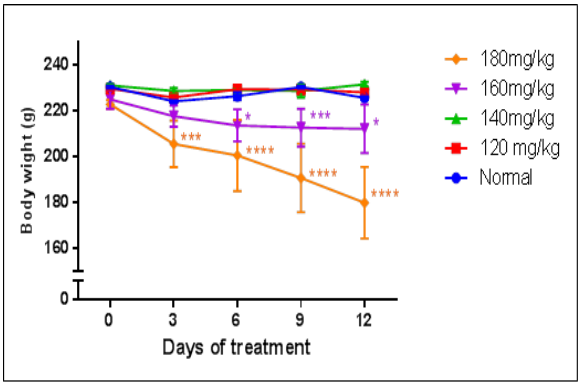


Fig.2. Influence of varying doses of alloxan in body weight (g) of experimental animal on day 0, 3, 6, 9, 12. Data are given as mean ± SEM of six rats in each group. *** P<0.001 compared to control. Significance compare with normal group (**, ***, ****)

Result of Wound Ulcer Contraction:

The result of effect of various treatments on wound area is shown in graph 7.3 on day 3, 6 and 9. On day 1, all the groups treated with Alloxan (diabetic groups) exhibited significantly increased wound size compared to control [F (4, 25) = 21.37, 86.36, 144.1, P<0.0001). There was decrease in wound size in control group (non diabetic) and showed complete healing on day 9. There was gradual increase in wound size in vehicle treated diabetic rats. The wound size was gradually decreased in diabetic animals treated with aloe vera extract and topical application of aloe vera gel on day 3, day 6 and day 9 (P<0.01). Significant reduction in the wound size was observed in diabetic rats treated with both aloe vera extract as well as topical application of aloe vera gel on day 3, day 6 and day 9 (P<0.01) compared to diabetic rats treated with vehicle.

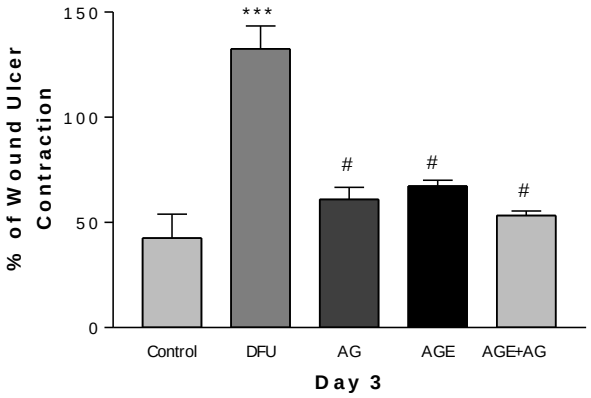


Fig.3a. Rate of wound ulcer contraction of aloe vera treated and untreated ulcers in diabetic rats on day 3. Data are given as mean ± SEM of six rats in each group. *** P<0.001 compared to control; # P<0.01 compared to DFU (one-way analysis of variance followed by Newman-Keuls test.)

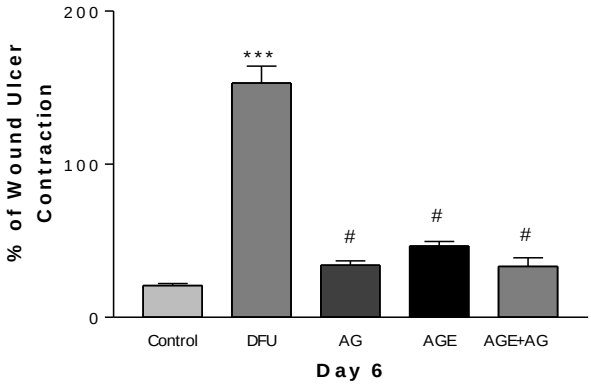


Fig.3b. Rate of wound ulcer contraction of aloe vera treated and untreated ulcers in diabetic rats on day 6. Data are given as mean ± SEM of six rats in each group. *** P<0.001 compared to control; # P<0.01 compared to DFU (one-way analysis of variance followed by Newman-Keuls test.

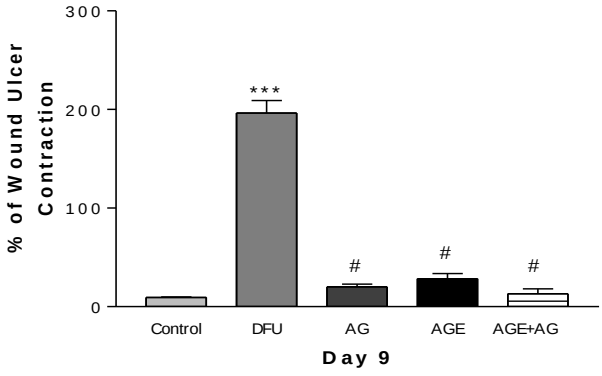


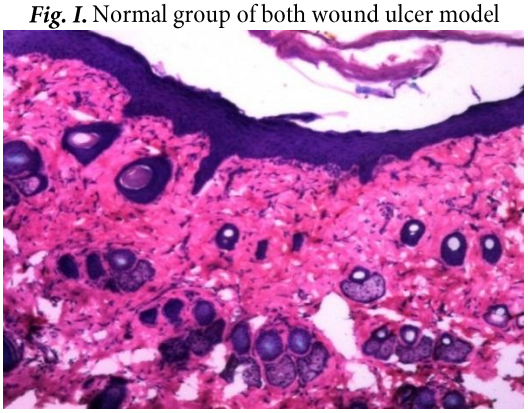
Fig.3c. Rate of wound ulcer contraction of aloe vera treated and untreated ulcers in diabetic rats on day 9. Data are given as mean ± SEM of six rats in each group. *** P<0.001 compared to control; # P<0.01 compared to DFU (one-way analysis of variance followed by Newman-Keuls test.)

Table 3. Period of epitheliazation of Aloe Vera treated and untreated ulcer wound on diabetic rats. Values are mean ± SEM of six rats in each group.

Sr.No.	Group	% of Wound Ulcer Contraction		
		Day 3	Day 6	Day 9
1	Control	42.590 ± 11.401	20.698 ± 1.364	9.257 ± 0.586
2	DFU	132.567 ± 10.884	153.26 ± 10.930	193.367 ±12.668
3	AGE	67.363 ± 2.669	46.580 ± 3.070	28.373 ± 8.287
4	AG	60.920 ± 5.814	34.030 ± 2.860	20.033 ± 2.959
5	AGE + AG	53.33± 2.108	33.33 ± 5.578	13.053 ± 5.093

Result of Histopathological Examination:

Fig. I. Normal group of both wound ulcer model



Stratum cornium, epidermis and dermis layer of skin on '0' day in both group

Fig.II. **0 Day**

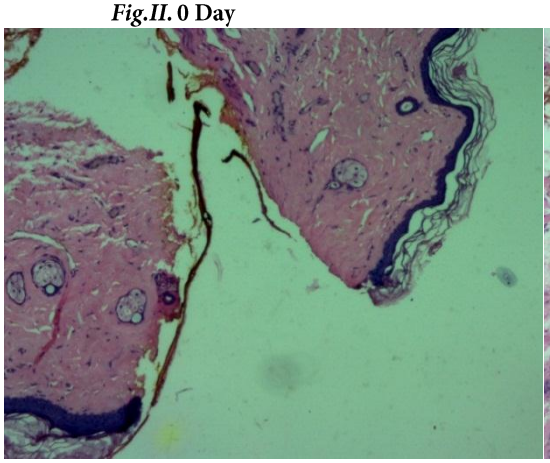
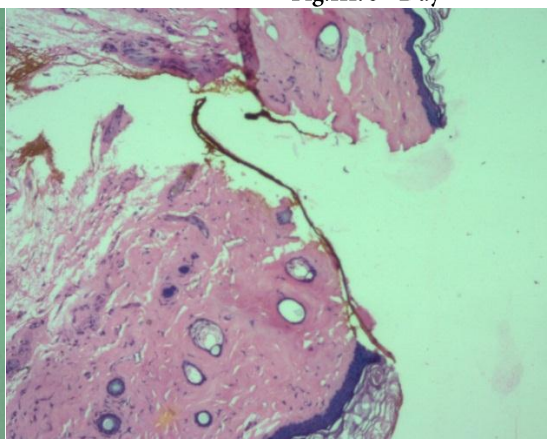


Fig.III. 3rd Day



Excision wound ulcer plate on '0' & '3' day diabetic Foot ulcer model of rat tissue

Fig.IV. **6th Day**

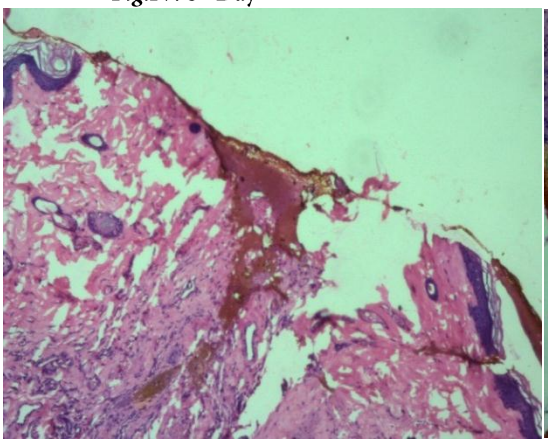
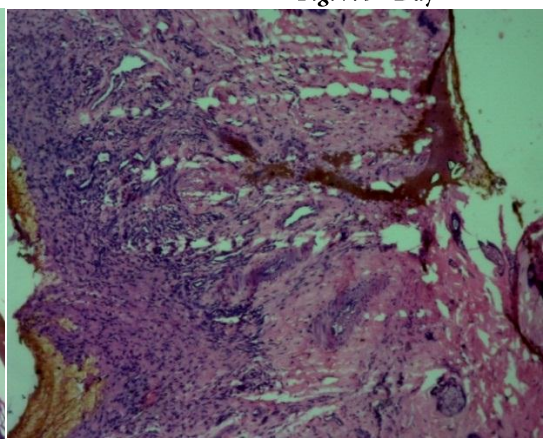
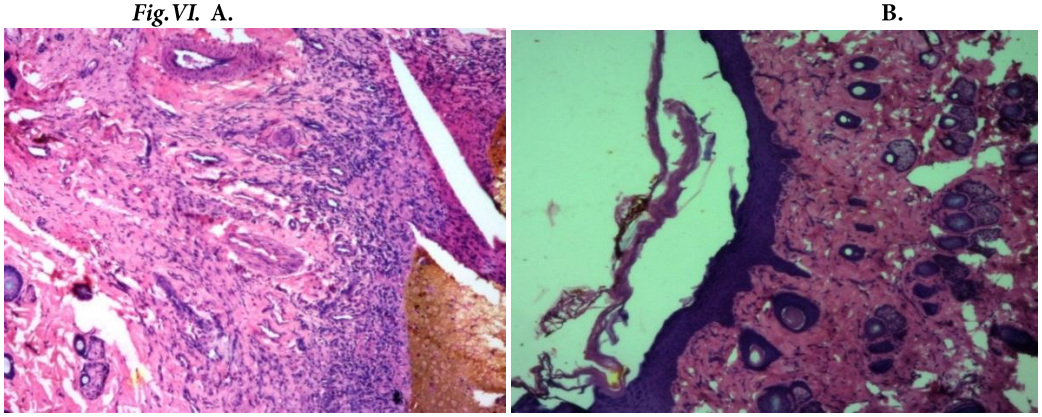


Fig.V. **9th Day**



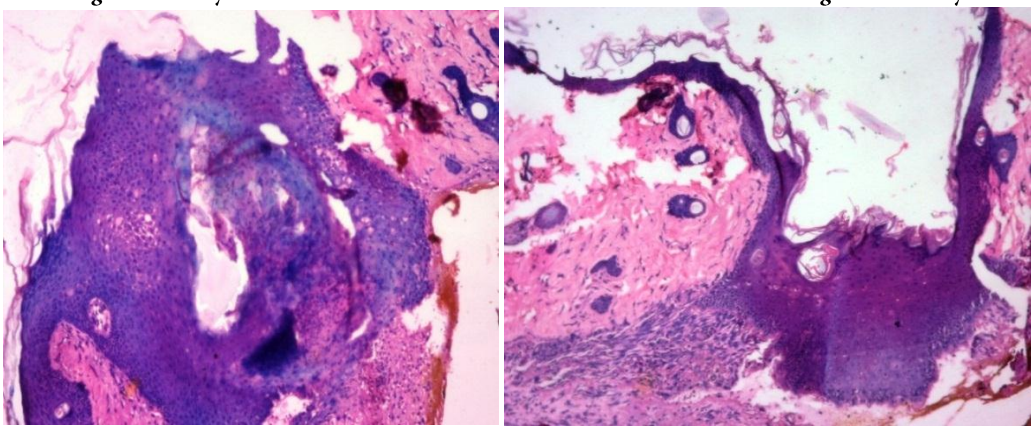
Excision wound ulcer plate on '6'&'9' day diabetic Foot ulcer model of rat tissue

Fig.VI. A. B.



Excision wound ulcer plate on '12' day diabetic Foot ulcer model of rat tissue

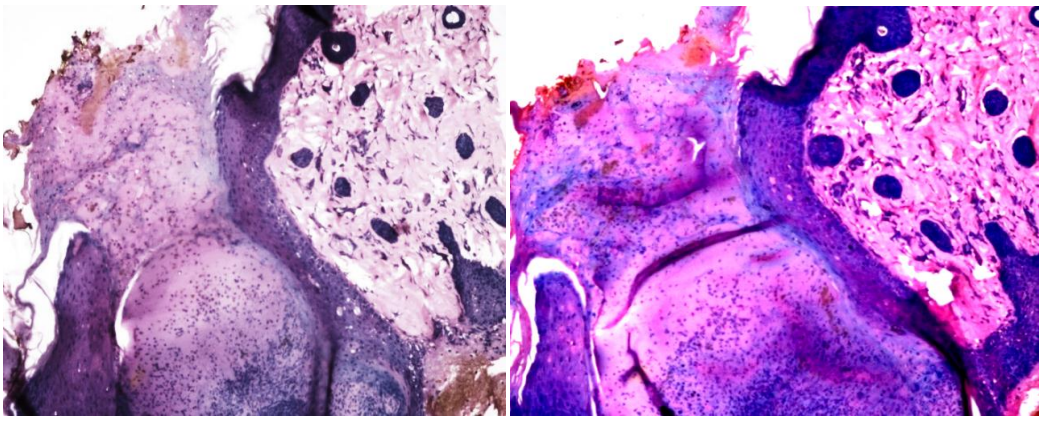
Fig.VII. 3rd Day *Fig.VIII.* 6th Day



Incision wound ulcer plate on '3' & '6' day diabetic Foot ulcer model of rat tissue

Fig.IX. 9th Day

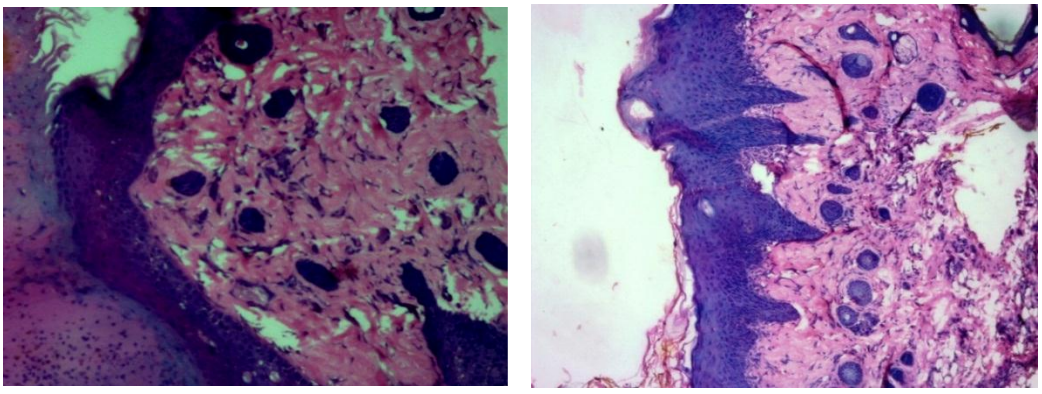
A. B.



Incision wound ulcer plate on '9' day diabetic Foot ulcer model of rat tissue

Fig.X. 12th Day

A. B



Excision wound ulcer plate on '12' day diabetic Foot ulcer model of rat tissue

DISCUSSION

Optimization of Inducer:

In the present study,for the development and standerdization of dibetic foot ulcer model the optimization of inducer (Alloxan) includes the blood glucose level and body weight variations were observed in various dosage. Figure 1 was observed the efficacy of alloxan in induction of diabetes on blood glucose level. In the Dosage of 180 mg/kg of body weight the blood glucose level goes above 539 ± 54.28 mg/dl that appers to cause extensive damge of beta cells.an elevation of blood glucose level to around 219.96±16.43 that is considered as diabetic by normal standards. At 120mg/kg dosage body weight we observed similar elevation but blood glucose level move towards lesser extends of 115.83±7.20. However it should be noted that these values are observed only for period of 4-5 days. In both the doses the values are seen to normalize by 9 days in case of body weight.

The dosage of 160mg/kg of body weight however appears to maintain blood glucose of 260-400mg/dl throughout the experimental period. The toxicity of alloxan was observed in the dosage of 180mg/kg, due to the toxicity the mortality also observed, while doses of 140 & 160mg/kg negligible toxicity and sometime mortality. Severe polyuria was observed (20ml Average) overnight in fasted animals treated with 180 mg/kg compare to 160 and 140 mg/kg.The weight loss is rapid in the case of animals treated with 180 mg/kg of alloxan.it is also observed that while animals treated with alloxan dose of 120 mg/kg of body weight shows initially elevation and then reduction in later stage. In the dosage of 140 and 160 mg/kg significant reduction in body weight.

Wound Ulcer Contraction:

In present study, this extract significantly reduced the wound size in excision diabetic wound ulcer model and in incision diabetic wound ulcer model. Results further revealed that topical application of aloe Vera gel also significantly reduced the wound size. The reduction in wound size was significantly higher in diabetic rats treated with both oral Aloe Vera extract and topical Aloe Vera gel in combination.

Histopathological examination:

The biopsy samples were collected from both excision and incision (Then after incision parted skin together & stitched with blue mono filament non absorbable sutures) group of all 12 cases on 0, 3, 6, 9,12th postoperative day. The tissue were collected in 10 percent formalin and processed for histopathological observations with routine H& E staining protocol for each groups.

Diabetic Foot Ulcer (DFU) on 0 day (Fig. I & II) in all the cases of both groups showed epidermis, dermis and stratum corneum with mild inflammatory changes. Few areas showed infiltration of inflammatory cells on 0 day

On the 6th post-operative day (Fig. IV & VIII) of the herbal samples treatment, hemorrhages', slight exudation, granulation tissue containing unorganized collagen fibrils, proliferation of fibroblast was seen in both group I and II. In group I, out of six cases, two cases showed mild polymorphonuclear infiltration with moderate inflammatory reaction and in case of group II no clear infiltration was seen. Odematous swelling or inflammatory reaction was not observed in group II treated with AGE and AG. It was also observed that surgical skin incision wound closed with sutures showed congestion of blood vessels and a few sites neovascularization was seen.

On 12th day, (Fig.VI & X) surgical skin incision sutured with conventional nylon showed exudation, and slight inflammatory reaction. The intensity of mild inflammation was seen, while no inflammatory reaction was seen in surgical skin incision treated with AGE and AG. Granulation tissue was observed to be dense, as compared to 6th and 9th day in both the group of surgical skin closure as well as open wound tissues of rat, congestion of blood vessel could be seen in both groups, neovascularization, newly forming blood capillaries was observed in both group after treatment with aqueous and ethanol extracted Aloe Vera. The healing tendency with granulation tissue formation and reepithelisation of skin layer was observed in group of skin incision closed with blue monofilament non absorbable sutures.

CONCLUSION

In the present study, dosage in between 140mg/kg & 160 mg/kg of body weight alloxan shows significant result as compare to normal group and it was considered to be optimize dose of alloxan for the development and standardization of "Diabetic foot ulcer" (DFU) model by using rats specifically. The use of Aloe Vera extract and Aloe Vera gel shows

excellent activity against diabetic foot ulcer in rat wound ulcer healing activity, indicate the increase contractions and reduction in wound size. The histopathological results express the confirmation in stepwise healing process that the combination of Aloe Vera extracts Provides strength and integrity to the tissue matrix to improve the wound ulcer healing.

Thus, the results of the present study provides a scientific rationale for the Aloe Vera extract and Aloe Vera gel attenuated the diabetic foot wound in rat.

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