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EXCISION AND INCISION WOUND HEALING ACTIVITY OF STEM BARK AQUEOUS EXTRACT OF *BAUHINIA VARIEGATA* LINN. IN DIABETIC ALBINO RATS

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ABSTRACT

The basic objective of the present work was to assess the wound healing activity of aqueous extract of *Bauhinia variegata* stem bark by providing better tissue formation and protection against microbial invasion. The stem bark of *Bauhinia variegate* were subjected for extraction with aqueous. Various ointments of extracts in various proportions were prepared and subjected for assessment of wound healing activity in diabetic albino rats using four parameters i.e., Wound contraction studies (Excision Wound), Tensile strength measurement (Incision Wound), Hydroxyproline content determination (Incision Wound) and Histopathological studies (Incision Wound).Based on the comparison of wound healing activity of various formulations, the formulation comprising of 4% (w/w) aqueous extract of stem bark of *Bauhinia variegata* found to be superior to that of control and standard formulation (0.3% w/w neomycin ointment). The present studies evidenced the significant wound healing activity of *Bauhinia variegata* by increasing cellular proliferation, formation of granular tissue, synthesis of collagen and by increase in the rate of wound contraction.

KEY WORDS: Excision, Incision, *Bauhinia variegata*, wound contraction, cellular proliferation, collagen, granular tissue.

INTRODUCTION

Despite the advances made in orthodox medicine, there has been a global resurgence of interest in traditional systems of medicine over past few years and used in medical practice since antiquity[1]. *Bauhinia variegata* L (Synonyms: *Phanera variegata* Benth**),** which commonly known as mountain ebony, orchid-tree, poor-man's orchid, camel's foot and Napoleon's hat[2-3], belongs to the family Leguminosae. It was planted in garden, park and roadsides as ornamental plant in many warm temperate and subtropical regions. It was native to Southeast Asia and grows in tropical and subtropical climate[4-6]. All parts of the plant (leaves, flower buds, flower, stem, stem bark, seeds and roots) were used in traditional medicine. It was traditionally used in the treatment of bronchitis, leprosy, and tumors. The stem bark was used as astringent, tonic, anthelmintic and antidiabetic. Infusion of the leaves was used as a laxative and for piles. Dried buds were used in the treatment of worm infestations, tumors, diarrhea, and piles[7-12]. The phytochemical screening revealed that *Bauhinia variegata* contained terpenoids, flavonoids, tannins, saponins, reducing sugars, steroids and cardiac glycosides. Pharmacological studies showed that *Bauhinia variegate* exerted anticancer, antioxidant, hypolipidemic, antimicrobial, anti-inflammatory, nephroprotective, hepatoprotective, antiulcer, immunomodulating, molluscicidal and wound healing effects. The objective of the present review is to highlight the chemical constituents and the pharmacological and therapeutic effects of *Bauhinia variegate*[13]. Wound may be defined as loss or breaking of cellular and anatomic or functional continuity of living tissues. Normal wound healing response begins the moment the tissue is injured. The fibroblast is the connective tissue cell responsible for collagen deposition that is needed to repair the tissue injury. Collagen is the most abundant protein in the animal kingdom, accounting for 30 % of the total protein in the human body.[14] The alcoholic extract of the plant is traditionally important and have been used by traditional practioners in Bundelkhand region of India for its healing property. Keeping this in view it was worthwhile considered to investigate further for its traditionally claimed wound healing property.

MATERIAL AND METHOD

Collection & identification

Bauhinia variegata stem bark were collected from K.C. Jain traders, Lalitpur and identified by Dr. H. B. Singh, Head, RHMD, NISCAIR, New Delhi.

Preparation of extract

The bark was shade dried, powdered mechanically, and sieved by using a mesh (size no. 10/44). It was extracted with distill water in a soxhlet extractor. The concentrated material was reduced to a thick mass at room temperature and water was removed by placing it in a desiccators. The weight of the dried mass was recorded and used for experimental studies [15].

Preparation of ointments

Various ointments of different extracts were prepared using water-soluble polyethylene glycol base as per the formulas given in table no.1. The general method of preparation was as follows: Dried extract was taken in glass mortar and triturated first. Then small parts of PEG-400 were added with triturating to dissolve or to suspend the drugs. Portions of PEG-6000 (melted at 70^0C) were added to above dispersion with triturating to form a homogenous mass of desired consistency[16].

Evaluation of wound healing activity of various prepared formulation

Experimental animals

Male Albino rats of wistar strain (150-250 g) were housed under standard conditions of temperature, 12 h light / dark and fed with standard pellet diet and water *ad libitum*. Animals were acclimatized to laboratory conditions at least 24 hours before conducting the experiments (CPCSEA Registration No. - 915/ac/05/CPCSEA).

Alloxan induced Diabetes

Diabetes was induced by a single IP injection of 120mg/kg of alloxan monohydrate in a sterile saline. After 72 h of alloxan injection the diabetic rats (blood glucose level> 250mg/dl) were separated and used for study [17].

Wound models

Excision wound model

A circular piece (300 mm^2 in area) of full thickness skin was excised from the dorsal interscapular region. Wound contraction was monitored by measuring wound area, on alternate days till the wound were completely healed. To have uniform parameters for comparison of the effects of different drugs, the wound half closure time WC-50, was calculated by Litchfield and Wileoxon method[18]. The times taken for epithelializations were measured in days required for full epithelialization were indicated by fall of scale leaving no raw wound behind. The progressive changes in wound area are monitored planimetrically by

tracing the wound margin on graph. To determine the changes in healing of wound measurement of wound area on graph paper is expressed as unit (mm^2).

Resutured incisional wound model

Incision wound were inflicted by the method of Ehrlich and Hunt[19]. Groups of animals containing six in each groups are anaesthetized and two paravertebral long incisions of 2.5 cm length are made through the skin and cutaneous muscles at a distance of about 1.5 cm from midline on each side of the depilated back of rat. After mopping the wound dry, intermittent sutures were applied by surgical nylon thread and curved needle No.11, 0.5 cm apart. On the 8^{th} day sutures were removed and on 10^{th} day, the tensile strength was measured by the method of Lee. The average of six readings per animal of a group was taken as mean and SE was calculated.

Treatments

Rats was divided into five groups, of six rats each. First group (Group I) was topically treated with Neomycin ointment (SF), Second group (Group II) remained untreated that acted as control (F); third group was treated with 2% aqueous extract (F_1), fourth group was treated with 4% aqueous extract (F_2) and Fifth group was treated with 6 % aqueous extract extract (F_3).

Methods

Tensile strength measurement

Tensile strength of wound represents the promotion of wound healing. Tensile strength (the force required to open the healing skin) was used to measure the extent of healing. The model used for this purpose consists of wooden board with a pulley that was fixed in one side of edge of board. Two Allis forceps, one is fixed to the opposite side of pulley edge and another is tied with and hanged with rope that is attached to the pan through pulley on which the weights are placed. The weights are increased slowly till it breaks the healed wound. One day before performing this experiment the sutures are removed from the stitched wound of rats after recovery[20].

Histopathological studies[21]

Five groups with six rats in each were used. Incision wounds were inflicted in rats under light ether anesthesia. A 2.5 cm long incision was made through the entire thickness of skin in each rat on its depilated back after mopping the wound dry; they were closed with interrupted

sutures, which were removed on 8th post-wounding day. On the 10th post wounding day, small pieces of skin were excised from the rats under light ether anesthesia in such a way that each piece represented the skin surrounding the incision originally made.

The sections of the skin were stained with eosin and hemotoxylin and were examined microscopically for keratinization, epithelisation, fibrosis, collagenation and neovascularization.

Wound contraction studies[22]

Eleven groups with six rats in each group were used. The skin of the impressed area on the depilated back of each rat was excised to the full thickness under light ether anesthesia to obtain a circular wound area about 300 mm^2. Measuring wound area that was traced on transparent polythene paper monitored wound contraction. Later the wound area was assessed using a graph paper. Wound contraction was also expressed as the percentage decrease of original wound size (about 300mm^2) on every alternate day.

Determination of Hydroxyproline content in granular tissue by Colorimetry[23]

Hydroxyproline is an amino acid present in the collagen fibers of granulation tissue. Its estimation helps clinically to understand progress rate at which the healing process is going on in the connective tissue of the wound. Firstly, prepared reagents with hydroxyproline standard and to obtained a standard curve. A sample of granulation tissues weighing around 300 mg was homogenized in glass homogenizer, 10 ml of 6N HCl was added to the homogenizes tissue in glass test tubes. The test tubes were capped and hydrolyzed for 3 hours at 130^0 C. They were then opened and contents were decanted to graduate glass cylinders, the tubes were washed thoroughly with water and the washings were combined with the hydrolysate. Further, they were processed in a manner similar to that described for obtaining the standard curve. After titration the samples were diluted to a volume of 50 ml with distilled water such that 2 ml of these diluted samples contain approximately 1-10 mcg of hydroxyproline and further experimentation was carried on with 2ml of the above solution. The hydroxyproline contents of the granulation tissue were calculated from standard curve.

Statistical analysis

The data was statistically analyzed by one-way ANOVA followed by Dunnett multiple comparison test with equal sample size. The difference was considered significant when $p<0.001$. All the values were expressed as mean ± standard deviation (S.D.)

RESULT

There is a report that *Bauhinia variegata* extracts possesses excellent wound healing property. There are reports that it also offers same degree of protection against infection of microorganism. The wound healing property of *Bauhinia variegata* extracts are presumably because of its constituents promote cell division and therefore facilitates the healing of wound. Thus process of wound healing has two components one formation of new tissue and two protections from microbial invasion during the healing process.

Preparation of ointments

Five different formulations of different concentrations of ingredients were formulated to decide the effects of the drug. Various ointment formulations of *Bauhinia variegata* extracts were prepared using polyethylene glycol ointment base (Table No.1). These formulations were prepared to study the effect of different concentration of ingredients on wound healing.

Selection of topical base was important to prepare topical formulations with optimum flow, spreadability and release properties. Gels are semisolid systems that fulfill these properties but incorporation of *Bauhinia variegata* extracts in a gel system is very difficult because the drugs are insoluble in a common polymer solvent system. Oil in water creams of drug's extracts can be prepared but generally release of drugs through cream bases is poor as drug get partitioned into the oil phase. Polyethylene glycol ointment bases shows good drug release properties than creams and other ointment bases [24]. These bases spread easily and mix readily with skin exudates and do not hydrolyses and deteriorate. They do not support mould growth and irritate the skin. They also act as percutaneous absorption enhancements.

Hence, it has been decided to prepare ointments of *Bauhinia variegata* extracts with polyethylene glycol base. All the developed ointments were stored in tightly closed containers, evaluated for physical characteristics, and wound healing activity.

Evaluation of wound healing Activity

In experimental study to evaluate, the wound healing capability of selected formulations, four parameters were taken into consideration.

Wound contraction Studies

Wound contraction indicates the rate of reduction of unhealed area during the course of treatment. Greater the reduction better is the efficacy of medication. Table no. 2. & figure no. 1 records the reduction of wound area of different groups over the period of 20 days. It was observed that fastest healing of wound took place in the group of animals treated with F_2

(Group IV) formulation and F_3 (Group V) i.e. wound were cured within 16 and 18 days. The wounds of animals treated with F_1 (Group III) formulation took time longer than 18 days to heal completely.

Table No.1. Formulation of ointments for standard, control, and aqueous extracts of stem bark of *Bouhinia variegata*.

Formulation ingredients (% w/w)	Standard formulation	Control formulation	Aqueous extracts of stem bark of *Bouhinia variegata*.		
	SF (0.3%)	F	F_1 (2%)	F_2 (4%)	F_3 (6%)
Neomycin	0.3	-	-	-	-
Control	-	-	-	-	-
Aqueous extracts	-	-	2	4	6
PEG- 6000	25	25	25	25	25
PEG-400 q.s. to make	100.0	100.0	100.0	100.0	100.0

Noteworthy it is the fact that treatment with F_1, F_2 & F_3 showed excellent wound healing property as compared to other formulations. The % reduction of wound in the group of animals treated with F_2, & F_3 formulation was 15 to 30% but inclusion of antimicrobial seems advisable for better results. Treatment with the standard formulation (SF) was also found satisfactory but the rate of healing was comparatively slower than the formulation of herbal extracts except F_1 – formulation.

Table No.2. Records the wound area (mm^2) of different groups over a period of 16 days.

Post Wounding Days	Standard	Control	Test		
	SF	F	F_1	F_2	F_3
	GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V
0 Day	296.28± 3.1 (O)	292.24 ± 2.4 (0)	299.42 ± 2.2 (0)	289.32 ± 2.8 (0)	294.83 ± 2.2 (0)
2 Day	288.12 ± 1.1 (2.7)	280.16 ± 4.2 (4.1)	274.32 ± 2.8* (8.3)	267.86 ± 3.9* (8.4)	273.62 ± 09* (7.8)
4 Day	271.22 ± 2.4 (8.4)	261.28 ± 3.2 (10.5)	268.62 ± 2.4* (10.2)	246.48 ± 1.2* (14.8)	258.57 ± 2.4* (12.2)
6 Day	224.08 ± 3.2* (24.4)	240.82 ± 2.8 (17.5)	218.24 ± 0.8* (27.1)	205.72 ± 2.7* (28.8)	222.36 ± 1.1* (24.5)
8 Day	167.54 ± 2.8* (43.4)	216.48 ± 2.2 (25.9)	172.28 ± 0.9* (42.4)	138.98 ± 1.6* (51.6)	169.39 ± 0.6* (42.5)
10 Day	119.82 ± 1.8* (59.5)	189.86 ± 1.2 (35.1)	128.22 ± 1.4* (57.1)	91.25 ± 0.6* (68.4)	123.73 ± 3.4* (58.0)
12 Day	78.62 ± 1.46* (73.4)	139.24 ± 1.8 (52.3)	64.84 ± 0.6* (78.3)	49.61 ± 0.9* (96.6)	75.28 ± 1.9* (74.4)

14 Day	32.18 ± 0.8* (89.1)	98.44 ± 0.4 (66.3)	36.14 ± 1.6* (87.9)	13.05 ± 0.06* (85.3)	34.94 ± 0.06* (88.1)
16 Day	08.12 ± 1.1 (97.2)	59.22 ± 1.6 (79.7)	11.86 ± 0.6* (96.0)	0.0 ± 0.0 (100.0)	09.49 ± 1.2* (96.7)
18 Day	0.0 ± 0.0 (100)	47.62 ± 0.8 (83.7)	02.12 ± 2.4 (99.2)	-	0.0 ± 0.0 (100.0)
20 Day	-	33.46 ± 2.4 (88.5)	0.0 ± 0.0 (100)	-	-

Values are Mean ± S.D. of six animals in each group. * p<0.001 as compared to control. The values shown in ( ) are the % reduction of wound area.

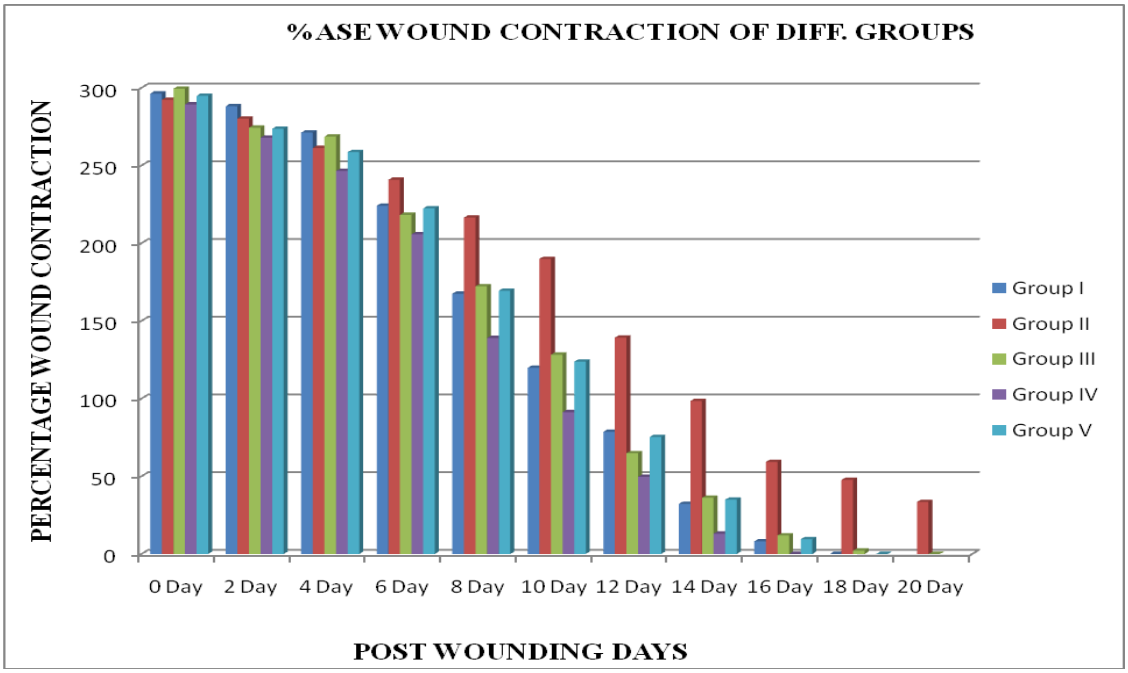

Fig. No.1. % Wound contraction of partial thickness wound of different groups of different formulations.

Tensile strength of newly formed tissue (Incision wound)

Table no.3. & figure no. 2 comprises the tensile strength of the healed skin treated with different formulation for 10 days. The wound that was untreated control had minimum tensile strength (282.8g). The tensile strength of the tissue treated with other formulation was more or less similar but comparatively greater than the untreated wound. The tensile strength of wound treated with 4% aqueous extract (F_2) was (418.8 g) but the different between this value and those treated with other formulation is not much. It may be concluded that 2% aqueous extract (F_1), 4% aqueous extract (F_2), and 6% aqueous extract (F_3) resulted in more

Table No.3. Indicate Tensile strength value in healed tissue.

S.No.	Group Models	Tensile strength of skin (g)
1	Group -I (SF)	397.4 ± 2.8*
2	Group -II (F)	282.8 ± 1.3
3	Group -III (F1)	349.6 ± 3.2*
4	Group -IV (F2)	418.8 ± 2.4*
5	Group -V (F3)	391.2 ± 4.1*

Values are Mean ± S.D. of six animals in each group. * p<0.001 as compared to control.

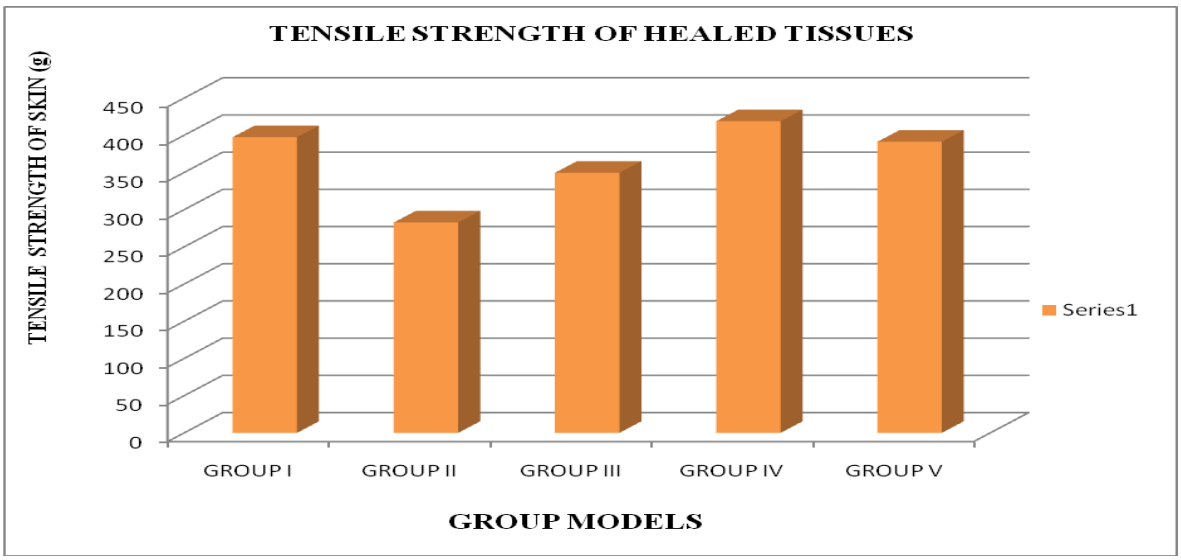

Fig. No.2. Tensile strength of different groups

Or less same tensile strength of healed tissue. It may be noted that tensile strength of tissue obtained from animal treated with F_1, F_2, and F_3 was comparable to the animals treated with standard formulation. From the results, it is observed that the wounds treated with the test formulation show increase in tensile strength compared to untreated control group thus promoting wound healing. A significant increase in tensile strength ($P < 0.001$) substantiates the tradition claim of *Bauhinia variegata*.

Determination of hydroxyproline value (Incision wound)

Table No. 4. Indicate Hydroxyproline value in healed tissue.

S.No.	Group Models	Hydroxyproline (µg/g)
1	Group -I (SF)	612.5 ± 4.4*
2	Group -II (F)	179.2 ± 3.3
3	Group -III (F1)	490.2 ± 3.1*
4	Group -IV (F2)	648.6 ± 2.9*
5	Group -IV (F3)	589.8 ± 4.8

Values are Mean ± S.D. of six animals in each group. * p<0.001 as compared to control.

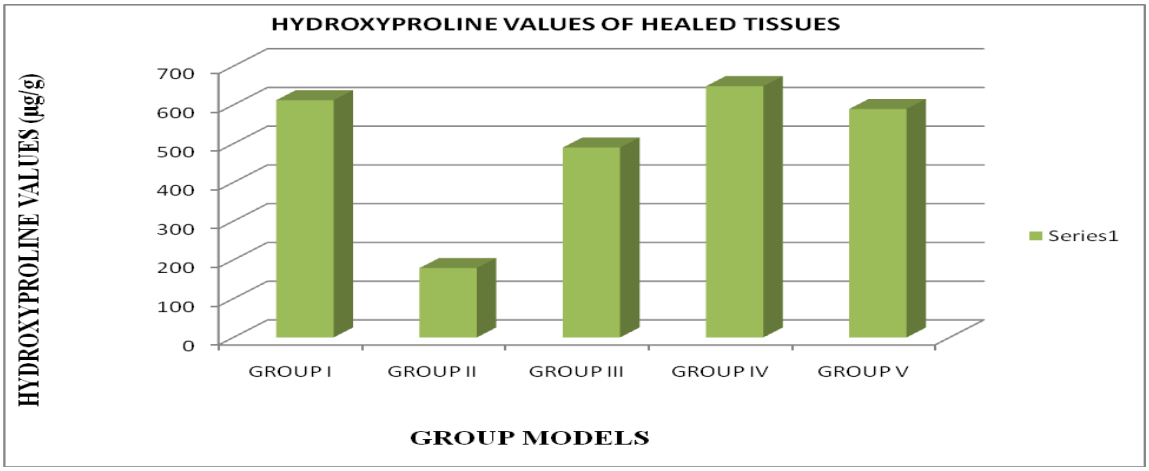

Fig. No. 3. Hydroxyproline values of different groups of healed tissues

During the healing of wound, collagen is synthesized which is one of the constituents of growing cell. Constituents of hydroxyproline are a measure of concentration of collagen. Higher concentration of hydroxyproline indicates faster rate of wound healing. Table no. 4 & figure no. 3 records the concentration of hydroxyproline in the tissue of animals, which were treated with different formulation up to 10 days. Highest concentration of hydroxyproline (648.6 µg / g) was observed in the group of animals treated with F_2. All other group of animals treated with formulation showed more or less same hydroxyproline value thus promoting wound healing.

Histopathological Studies of healed tissue (Incision wound)

In the histopathological study, healed tissues were observed for the healing markers like neovascularization, keratinization, collagenation, epithelization, and fibrosis. The test formulation showed better keratinization, epithelization, collagenation and fibrosis. However, neovascularizaion was not very prominent when compared with untreated control. The results are shown in table no. 5 & figure no. 4.

Table No. 5. Histopathological evaluation of wounds treated with different formulation.

Parameters	I	II	III	IV	V
Keratinization	4.68 ± 0.3	2.89 ± 1.1	4.54 ± 0.7	4.92 ± 1.3	4.53 ± 0.9
Epithelization	4.48 ± 0.8	2.15 ± 1.8	4.25 ± 0.9	4.73 ± 1.4	4.12± 0.3
Fibrosis	4.42 ± 0.5	2.92 ± 0.6	4.32 ± 0.3	4.84 ± 0.5	4.08 ± 0.8
Collagenation	4.72 ± 0.4	3.46 ± 0.4	4.61 ± 0.8	4.90 ± 1.7	4.26 ± 0.5
Neovascularization	4.49 ± 0.6	1.56 ± 0.9	4.29 ± 0.9	4.62 ± 1.2	4.37 ± 0.7

Values are mean ± SD from 6 readings each. A value 5 refers to maximum similarity and 0 refers for least similarity of wound from the normal tissue. All values are significant at $P<0.001$.

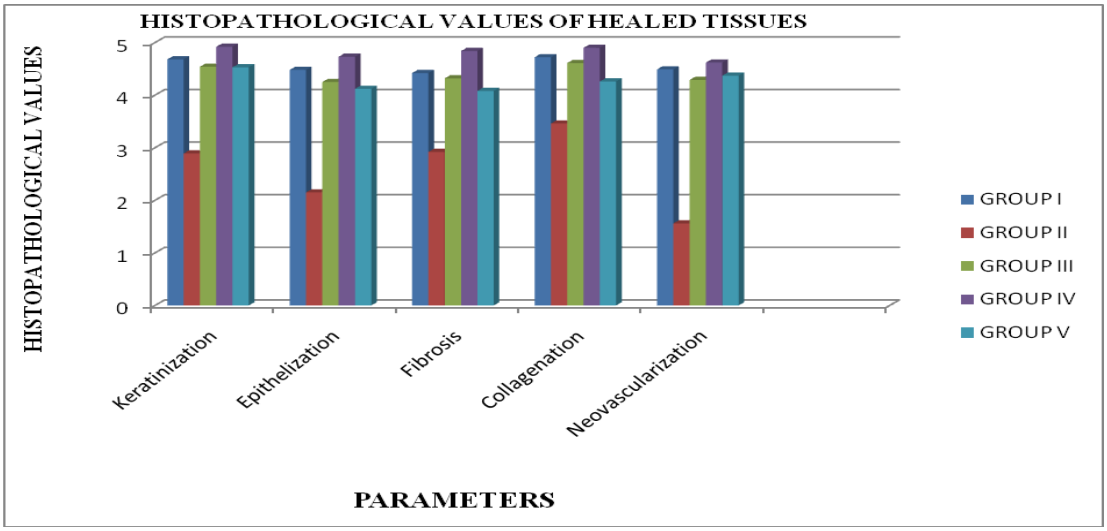

Fig No. 4. Figure of different parameters of histopathology of healed tissues.

DISCUSSION

Wound healing deficits in diabetes are diverse, multifactorial, complex and interrelated[25]. This defect is believed to be caused by impaired blood flow and oxygen release from increased blood sugar, decreased collagen and fibronectin synthesis from protein malnutrition, impaired local immune and cell defenses, and decreased anabolic activity with decreased insulin and growth hormone. In majority of patients, normal healing established tissue integrity quickly and effectively. However, at times, this healing is delayed and the ability to accelerate the wound healing becomes a highly desirable objective [26]. Wounds may be defined as loss or breaking of cellular and anatomic or functional continuity of living tissues. Wound healing involves a highly dynamic integrated series of cellular, physiological and biochemical processes, which occur in living organism. Repair through regeneration is very common in unicellular and the lower metazoan animal groups while it is highly restricted in the higher animals. Wound healing involves different phases such as contraction, epithelization, granulation, collagenation[27].

CONCLUSION

In diabetic rats, aqueous extract of *Bauhinia variegata* increased the wound contraction activity as well as epithelialization period only during the later phase of wound healing. In excision, wound study the test formulation of *Bauhinia variegata* showed better and fast healing compared to untreated control group. The wound contraction ability of *Bauhinia variegata* was so prominent initially but progressively the contraction ability of *Bauhinia variegata* was slowed. The *Bauhinia variegata* treated group showed much greater

contraction of wounds except 2% aqueous extract (F_1) than those treated with neomycin 0.3% w/w as the reference standard. The time for wound closure of *Bauhinia variegata* extract formulations (except F_1) was less than that of control group. In incision wound study, there was significant increase in tensile strength of 10-day-old wound due to treatment with *Bauhinia variegata* formulation. Increase in tensile strength is indicative of improved collagenation, which significantly contributes to better and effective healing. There was significant increase in hydroxyproline content of the 10-day-old wound due to treatment with *Bauhinia variegata* extract formulation. Increase in hydroxyproline content is indicative of improved collagen, which significantly contributes to better and effective healing. Histopathological observations of healed tissue showed incomplete healing with poor keratinization, epithelization, fibrosis and collagen formation in the untreated rats. The histopathological observation revealed better keratinization in *Bauhinia variegata* extract formulation treated animals when compared with control group. Epithelization improved with test formulation application when compared with control group that may be due to proliferation of epithelial tissue over wound area. It may be concluded that *Bauhinia variegata* extract formulation except F_1 promotes keratinization, epithelization and fibrosis comparable with neomycin treatment. Interestingly the visual examination of wounds inflected during "wound healing ability" experiments revealed that the wounds treated with *Bauhinia variegata* extracts were relatively clean and free from any inflammatory reaction like swelling and redness. Consequently, it was observed that test formulation does exert remarkable anti-inflammatory action when applied to wounds. This offers a very interesting dimension to treatment of wounds by *Bauhinia variegata* extracts except F_1 formulations. Further studies are required to isolate the compound responsible for the wound healing activity of *Bauhinia variegata* and to evaluate the mechanism of wound healing activity.

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