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Evaluation of Wound healing activity of methanolic root extract of *Plumbago zeylanica* L. in wistar albino rats

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ABSTRACT

The entire wound healing process is a complex series of events that begins at the moment of injury and can continue for months to years. The stages of wound healing are inflammatory phase, proliferation phase, fibroblastic phase and maturation phase. Several Investigators has been found that, most of the tribal people are using Plumbago zeylanica mainly for wound healing, activity apart from in other conditions. So in the present study emphasis will be laid on the pharmacological screening of the plant with special reference to the wound healing activities. Methanolic root extract of Plumbago zeylanica are having significant wound healing activity in rats.

Keywords: *Plumbago zeylanica* L, Wound healing, Methanolic root extract**,** wistar albino rats.

INTRODUCTION

Indian medicinal plants also provide a rich source for antioxidants that are known to prevent/delay different diseased states. The antioxidant protection is observed at different levels [1]. Medicinal plants have been used since time immemorial for treatment of various ailments of skin and dermatological disorders especially cuts, wounds and burns [2]. *Plumbago zeylanica* belongs family *Plumbaginaceae.*

Several phytochemical and pharmacological investigators found that most of the tribal people are using *Plumbago zeylanica L.* mainly for wound healing, activity apart from in other conditions. It was also found that little work has been reported regarding its pharmacology and phytochemistry [3].

So in the present study emphasis will be laid on the pharmacological screening of the plant with special reference to the above mentioned activities. The present experimental investigation will be an attempt to give scientific justification to the acclaimed activities [4].

Qualitative Phytochemical Evaluation : The roots were carefully dried in shade for 15 days. To ensure complete dryness plant roots were kept in hot air oven at 45˚C for 5 minutes. Then leaves were subjected to size reduction to make powder. The crushed mass of leaves was then ready for extraction. The dried and powdered leaves were subjected to hot extraction in Soxhlet apparatus with petroleum ether, chloroform, methanol and ethanol successively. Preliminary tests were carried out for the presence or absence of phytoconstituents like Glycosides, Flavanoids, Saponins, Alkaloids, Carbohydrates, Sterols, Proteins, Phenolic compounds and Reducing compounds. A description of methods adopted for performing the tests are summarized below (5-7). Test for Alkaloids, Carbohydrates, Flavonoids, Glycosides, reducing Sugar, Saponins, Sterols, Tannins, Proteins [7].

Wound Healing Activity: A wound is a disruption in the continuity of cells—anything that causes cells that would normally be connected to become separated. It is an intricate process in which the skin repairs itself after injury. In normal skin, the epidermis and dermis exists in a steady-state equilibrium, forming a protective barrier against the external environment.

MATERIALS AND METHODS

Animals: Wistar albino rats of either sex, weighing about 150–250 each, were used for the study. They were fed with standard chow (Pranav Agro Industries Ltd., Sangli, Maharashtra) and water ad libitum. They were housed in polypropylene cages maintained under standard conditions (12 hour light - dark cycle; 25 ± 3 °C; 35–60% humidity). The experimental protocol was subjected to the scrutiny of the Institutional Animal Ethics Committee, and was cleared by same before beginning the experiment

Chemicals: Framycetin (Sanofi Aventis), Wool fat, Hard Paraffin, Cetostearyl alcohol and White Soft Paraffin.

Preparation of ointment by fusion method:
(a) Preparation of simple ointment: Wool fat - 2 gm: Hard Paraffin-2 gm; Cetostearyl alcohol -2 gm; White Soft Paraffin-34 gm. Each ingredient was mixed and heated gently with stirring then cooled. The base was then packed in a wide mouth container.

(b)Preparation of 10% ointment: 4 gm methanol root extract of *plumbago zeylanica* was added slowly to the above melted ingredients and stirred thoroughly until the mass cools down and a homogeneous product is formed. The ointment was then packed in a wide mouth container [8,9].

Treatment Protocols: The animals were numbered, weighed and then divided into four groups with five animals in each as follows:

Group I : Served as vehicle control and applied simple ointment.
Group II : 2%, w/w, framycetin ointment applied.
Group III : Normal ointment base
Group IV : 10%, w/w, root extract ointment is applied.

Excision wound model : Hairs were removed from the dorsal thoracic central region of anaesthetised mice. The mice were depilated on the back. One excision wound was inflicted by cutting away a 300 mm^2 full thickness of skin from a predetermined area; the wound was left undressed to the open environment. Then the ointments were applied (as stated above) calculated as percent reduction in wound area.

$$\% \text{ Wound contraction} = \frac{Healedarea}{totalarea} * 100$$

The progressive changes in wound area were monitored planimetrically by tracing the wound margin on graph paper every alternate day. Epithelialisation time was noted as a number of days after wounding required for the scar to fall off leaving no raw wound behind. From the healed wound, a specimen sample of tissue is isolated from each group of rats for histopathological examination [3].

Figure 1 : Photographic representation of excision wound in rat and measurement of wound area in excised rat.

Histopathological studies of excision wound

Procedure: Wound tissue specimens from control, test and standard groups were taken after complete healing of excision and that tissue fixed in neutral buffered formalin (10% formaldehyde in Phosphate buffered saline) over night. After fixation, the tissues placed in 70% isopropyl alcohol for 3 hours and then in each ascending strength (80%, 90%, 100% isopropyl alcohol) for 2 hours each. The amount of alcohol used should be 15 times of the size of the tissue.

Then the tissues were dipped in acetone for a period of 1 – 2 h with periodical shaking. After removing the acetone, Xylene was added to check for the milky appearance. If milky appearance found then repeat the dehydration procedure. The dehydrated tissue was impregnated in paraffin wax (m.p. = 56˚C) for a period of 1h at 58 – 60 ˚C. Molten paraffin poured into L-block along with the tissues and allowed it to become hard. The tissue was sectioned into very thin (2–8 or 5 – 10 micrometer) sections using a microtome. The tissue Mounted on the slides with Mayer's albumin solution (a mixture of equal parts of egg white and glycerin, beaten and filtered with the addition of 1% sodium salicylate) and incubated in warm oven for 2 h at 60ºC [10].

Slides containing paraffin sections were placed on a slide holder and deparaffini- zed with Xylene for 30 minutes and the excess Xylene blotted. The tissue was rehydrate successively with 100%, 90%, 80% isopropyl alcohol for 2 – 3 min. each and put it into water for 3 min.The excess water blotted, the tissue was kept into Hematoxylin stain for 1 – 2 min. Then again kept into tap water for 1 – 2 min. The slides containing tissue sections dipped into 1N HCl followed by Scott's water (Sodium Bicarbonate 3.5 g, Magnesium Sulfate 20 g, distilled water 1L) for 1 min each.The thin sections of the tissue were stained with Eosin I bluish solution and observed for the histological changes under Leica microscope [11].

Statistical analysis: The values were calculated as mean ± S.E.M. The significance of the difference of the mean value with respect to control group was analyzed by one way ANOVA followed by Dunnet's t-test using Statistica 8.0. $P<0.05$ or above was considered to be significant [5].

RESULTS

The results of excision wound model are shown in table 10. The MPZ extract exhibited significant wound healing activity as compared to control in excision wound model. It is observed that the wound contracting ability of the 10% (w/w) extract ointment treated groups showed significant wound healing from the sixth day onwards. The wound closure time was lesser, as well as the percentage of wound contraction was more with the 10%(w/w) extract ointment treated group. The epithelization of wound with 10%(w/w) extract ointment treated group was found to be earlier as compared to control. In the 10%(w/w) extract ointment treated rat the wounds were completely healed (epithelization period)in 16 ± 2 days whereas in the control animals it took more than 20± 2 days.

Table 1: Phytochemical Analysis of *Plumbagozeylanica*Linn Root

Type of phytoconstituent	Pet. Ether extract	Chloroform extract	Ethanol extract	Methanol extract
Alkaloids	+	++	—	++
Carbohydrates	++	+	++	++
Flavanoids	—	—	—	+
Glycosides	+	++	++	++
Saponins	—	—	—	+
Steroids	—	—	—	+
Tannin	++	++	—	++

—: Absent; +: Slightly Present; ++: Moderately Present.

Table 2. Evaluation of MPZ and framycetin ointment on wound healing by excision wound method in rat

Post wounding days	Wound area (mm^2) (mean ± SEM) and percentage of wound contraction			
	CONTROL	FRAMYCETIN	BASE	ROOT
0	511.91±0.46	510.00±1.48	515.5±10.7	512.5±8.40
3	482.7±18.08 (1.43)	443.5±6.45* (3)	485.5±13.7 (1.21)	448.01±6.7 (2.56)
6	420±19.1 (5.15)	403±6.3** (11.86)	432.5±51.6 (4.95)	410±7.2* (9.3)
8	350.2±24.5 (10.96)	311.0±5.01** (20.39)	362±40.4 (10.24)	318±6.2** (19.1)
10	263±15.895 (22.87)	259±2.9** (44.6)	280.5±33.9 (21.45)	269±6.6** (39.41)
12	189.5±26.5 (43.22)	130.25±5.5** (71.5)	198±12.5 (43.22)	145±5.8** (68.03)
14	109.7±30.0 (63.4)	73.75±5.1** (83.87)	111.5±3.69 (63.1)	75.50±5.2** (81.6)
16	43.5±18.4 (80.64)	00 (100)	65±5.8 (80.95)	00 (100)

*Each value is the mean ±S.E.M. of five rats.; *P < 0.05, **p<0.01 vs. control, One way ANOVA followed by Dunnet's t-test.; % wound contraction is given within parentheses.*

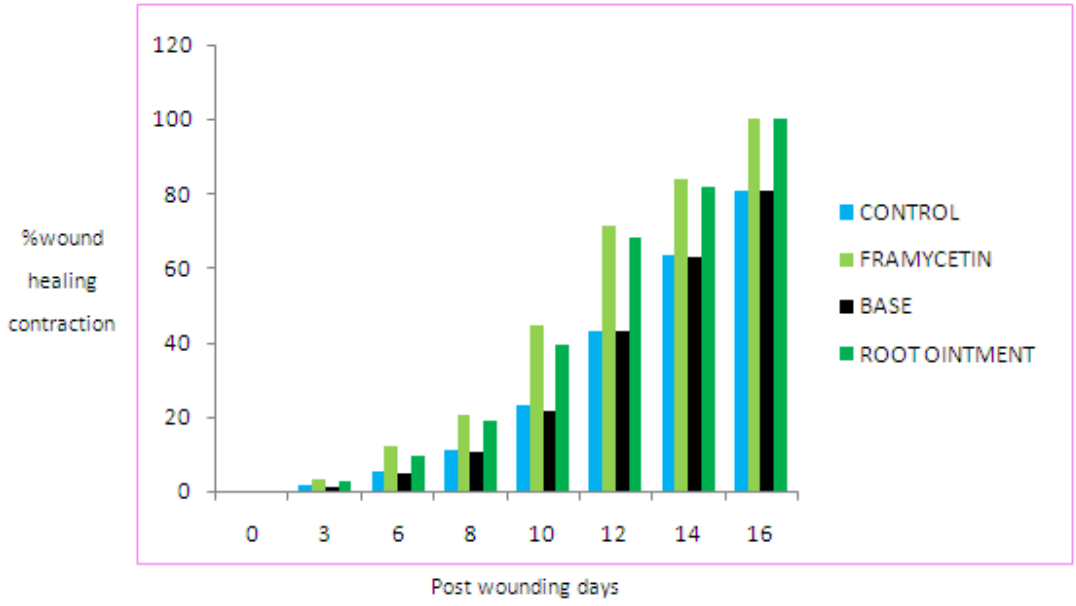
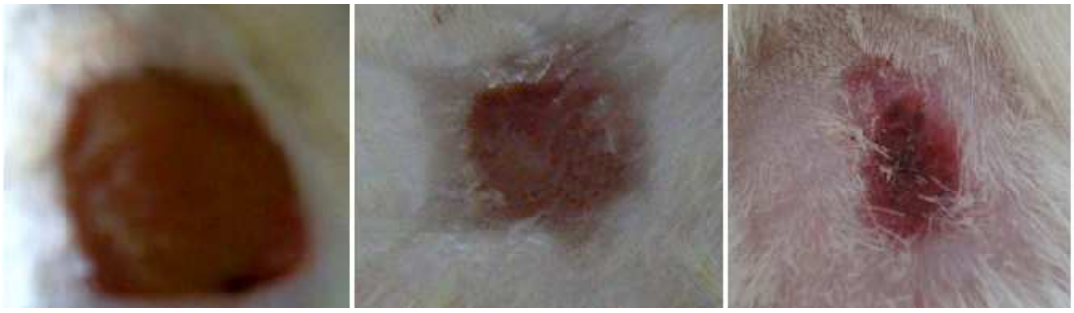
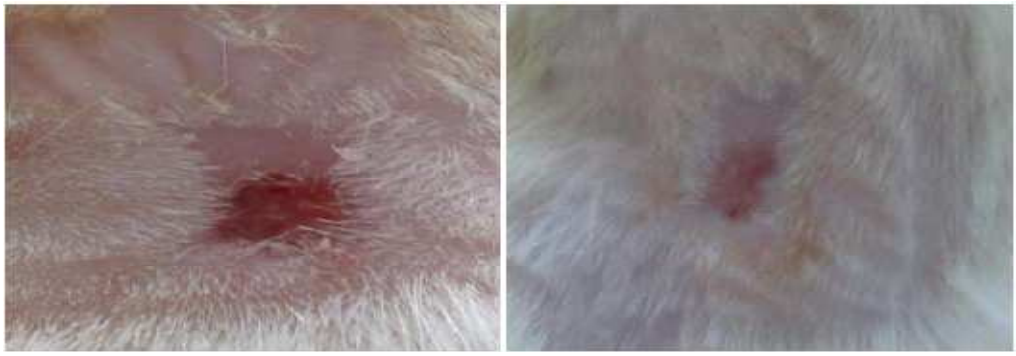


Figure 1. Effects of MPZ and framycetin on excision wound model in mice.

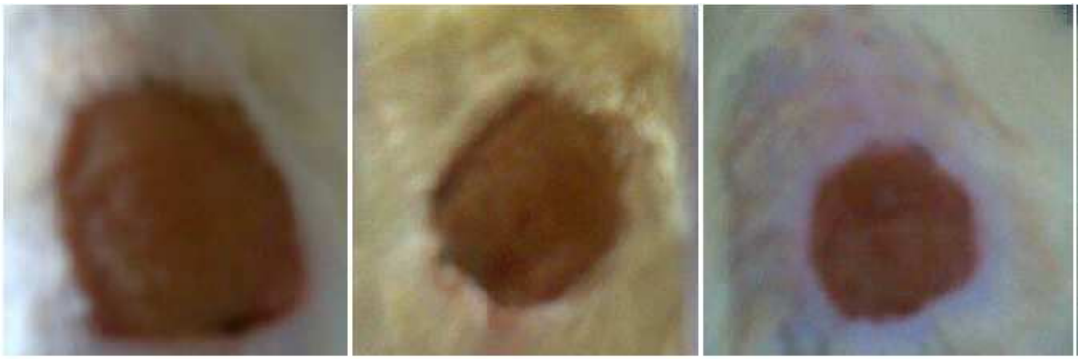


DAY 0 DAY 6 DAY 8



DAY 12 DAY 16

Figure 2:Photographical representation of contraction rate on different days in treatment group



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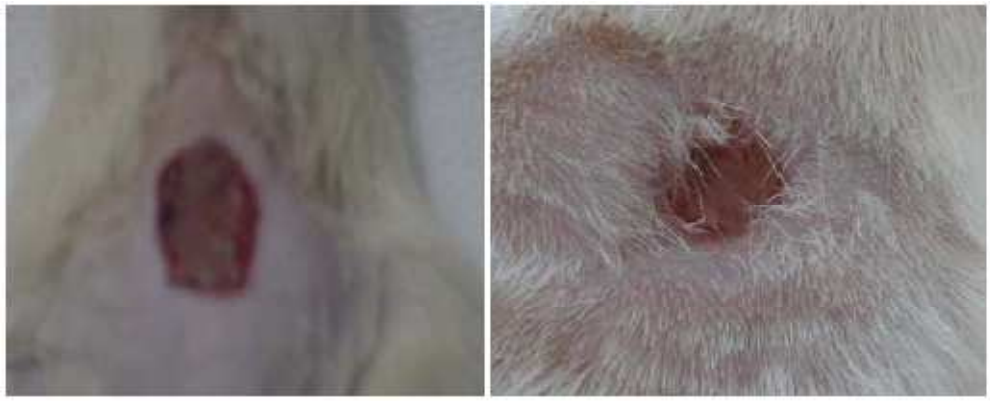

DAY 12 DAY 16

Figure 3: Photographical representation of contraction rate on different days in control group.

The multiple sections studied in histopathological examination of the tissues of the wound area treated with extract ointment (10% w/w), 2% w/w framycetin ointment and simple ointment (control) treated groups. The histological examination showed that the original tissue regeneration was much greater in the skin wound treated with extract ointments and framycetin ointment treated group without any edema, congestion, or inflammatory changes. In the control group it was partially reepithelialised and necrotic tissues were not completely replaced by granulation tissues. Collagen fibers were abnormally thickened and disordered in the wound [12].

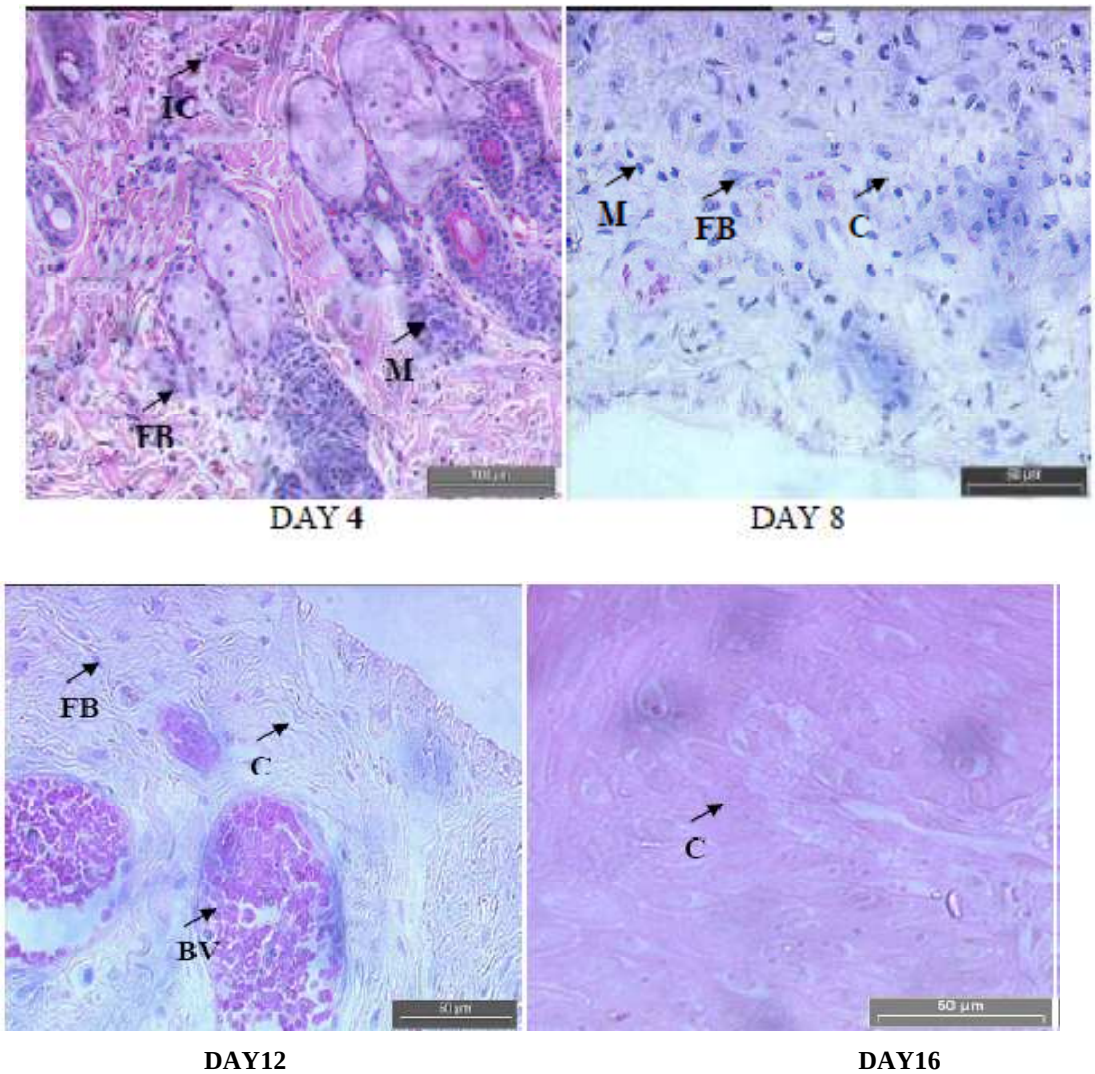

Figure 4.Hematoxylin and eosin stained sections of the granulation tissue in treated group at different time intervals. Fibroblasts (FB), macrophages (M), collagen (C) bundles, Vascularization with larger blood vessels (BV) and inflammatory cells (IC).

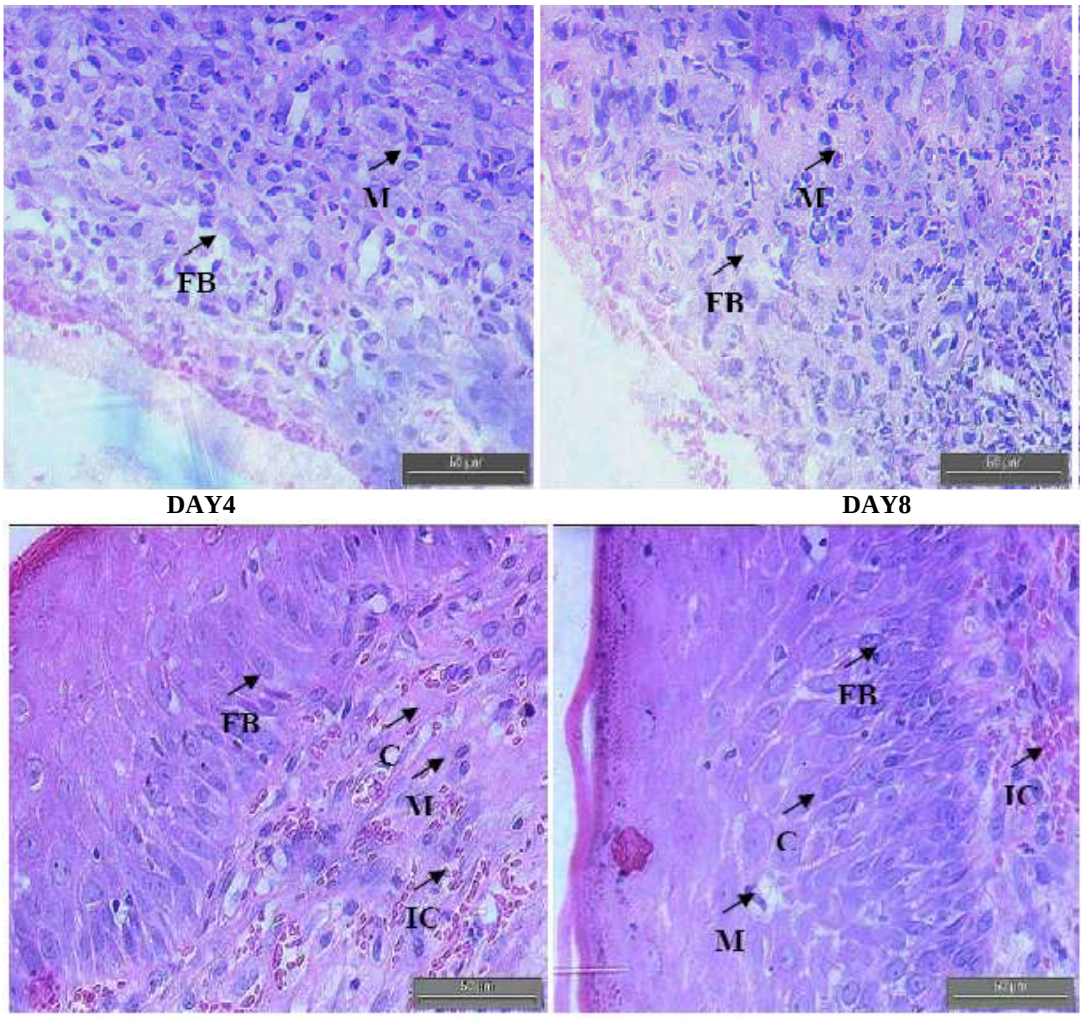

Figure 5.Hematoxylin and eosin stained sections of the granulation tissue in control group at different time intervals. Fibroblasts (FB), macrophages (M), collagen (C) bundles, vascularization with larger blood vessels (BV) and inflammatory cells (IC).

DISCUSSION

The present investigation describes some unique features of the root extract from the plant *P.zeylanica* with respect to its potential wound healing capacity in rats. Plant products are potential wound healing agents, and largely preferred because of their widespread availability, non-toxicity, absence of unwanted side effects, and effectiveness as crude preparations. Earlier it was reported that *Centellaasciatia* and *Terminaliachebula* are effective in wound healing in rats. Various activities were conducted in this study to evaluate the potential of *P.zeylanica* a wound healing agent. One such activity is the phytochemical screening test. The phytochemical results reveal the presence of tannins, alkaloids, reducing sugars and steroids in the methanolic root extract. The constituents of the root extract, such as terpenoids and alkaloids, may play a major role in the wound healing process observed in this study, however, further phytochemical studies are needed to isolate the active compound(s) responsible for these pharmacological activities.

Wound contraction, a part of the proliferative phase of wound healing, occurs through the centripetal movement of the tissues surrounding the wound, which is mediated by myofibroblasts. The increased wound contraction in the treated group may be due to the enhanced activity of fibroblasts *P.zeylanica*a root extract. A significant increase in collagen content due to enhanced migration of fibroblasts and epithelial cells to the wound site was observed during the wound healing process in the treated group. A close examination of granulation tissue sections revealed that tissue regeneration was much quicker in the treated group compared to that in control wounds (Figures 4-5). Early dermal and epidermal regeneration in the treated group confirmed that the ointment containing the *P.zeylanica* extract had a positive effect toward cellular proliferation, granulation tissue formation, and epithelialization. Incomplete epithelialization with less extracellular matrix synthesis was observed in control rats, as shown in Figure 5. Clumps of degenerating neutrophils, necrotic changes, and the persistence of inflammatory exudates in the upper dermis with loss of epidermis were also observed up to day 16. The treated rats showed marked epithelialization, a moderate amount of extracellular matrix synthesis and new blood vessel formation (13).

CONCLUSION

The results obtained in the present study clearly indicate that the Methanolic root extract of P*lumbago zeylanica* are having significant wound healing activity in rats. The wound healing effect of Methanolic root extracts of *Plumbago zeylanica* may be due to the presence of more than one active principles mentioned above. Further pharmacological and biochemical investigation will clearly elucidate the mechanism of action and will be help full in projecting this plant as an therapeutic target in wound healing and other diseases.

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