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Pharmacological Evaluation of the Wound Healing Activity of Leaves of Erythrina Variegata on Experimental Animals

Pravin P. Paikrao*, Anjali M. Wankhade, Vivek V. Paithankar, Jugalkishor V. Vyas, Shubhangi A. Bhagat

Department of Pharmacology

Vidyabharati College of Pharmacy, C K Naidu Road, Camp, Amravati, Maharashtra, India- 444602

Abstract:

***Background*:** Medicinal plants play important roles to treat various ailments. Erythrina variegata is one of the folklore plants frequently used for wound treatments. Nevertheless, pharmacological investigations have not been performed to substantiate activity of the plant extract in wound healing. Hence, this study provides Pharmacological evaluation of the wound healing activity of leaves of Erythrina variegata on experimental animals.

Materials And Methods: The leaves extraction was carried out using 80% ethanol. The leaves extract was prepared in 5% (w/w) and 10% (w/w) extract ointments. An acute dermal toxicity study of the extract was conducted in female mice by observing the signs of toxicity. Then 5% and 10% (w/w) ointments of the extract were applied topically to investigate their wound healing activity using excision and incision wound models in Swiss albino mice. Parameters such as wound contraction, Period of epithelialisation, and tensile strength were determined.

***Results*:** In acute dermal toxicity test the application of 10% (w/w) ointment formulation the site of application no signs of dermal toxicity were observed in mice. Both 5% and 10% (w/w) ointments significantly reduced period of epithelialization and increased wound contraction rate and tensile strength.

Conclusion: The results of this study showed that both 5% w/w and 10% w/w of 80% ethanolic leaves extract of E. variegata possesses wound healing activities.

***Keywords*:** Erythrina variegata, Wound healing activity, Excision wound model, Incision wound model.

I. INTRODUCTION

Wound is defined as the disruption of the anatomic and cellular continuity of tissue caused by chemical, physical, thermal, microbial, or immunological injury to the tissue. Wound healing processes consist of integrated cellular and biochemical cascades leading to re-establishment of structural and functional integrity of the damaged tissue [1]. Various growth factors such as transforming growth factor beta (TGF-β), platelet activation factor (PAF), epidermal growth factor (EGF), and platelet-derived growth factors (PDGF) seem to be necessary for the initiation and promotion of wound healing [2].Various treatment options are available likes analgesics, antibiotics, and nonsteroidal anti-inflammatory drugs for the wound management but these therapies produces unwanted side effects [3, 4].

In recent years, several studies have been carried out on herbal drugs to explicate their potential in wound management and these natural remedies proved their effectiveness as an alternative treatment to available synthetic drugs for the treatment of wound [5]. Many natural herbs have been pharmacologically reported possessing potent wound healing activity [6].

Erythrina is a genus of flowering plants belonging to the family Fabaceae. The Erythrina genus has between 100 and 10 different tree species. The term "coral tree" is used here to refer to all of these species. Erythrina variegata is a medium-sized deciduous little tree with thorny stems and branches, triangular leaves and enormous coral red blooms. The majority of the growth occurs in Bangladesh, but it has spread across much of Asia. It's been used as a people's treatment in tropical and sub-tropical countries and it's well-known for its medical specialties. The varicolored leaves and stunning red blossoms of this delectable type of plant are the main reasons for its full growth. This is a fast-growing plant that may reach 50-60 feet tall and has green and yellow leaves that are about a six inches long. In the spring, this tree is adorned with magnificent red blooms that are 2.5 inches long and organised in dense, six inches long racemes. These blooms are followed by red/brown seed pods with nephrotoxic seeds that are twelve inches long. Erythrina is usually derived from the Greek word "Eruthros," which means "red," and it depicts crimson species [7,8]. The juice from the leaves is mixed with honey and ingested to treat tapeworm, roundworm, and threadworm

in India; women take this juice to stimulate lactation and menstruation; it is commonly mixed with castor oil to treat dysentery; a warm poultice of the leaves is applied externally to relieve rheumatic joints.

Different parts of the plant have been used in traditional medicine as nervine sedative, collyrium in ophthalmia, antiasthmatic, antiepileptic, antiseptic, and as an astringent. In previous study the alkaloids extracted from the leaves of E. variegata are reported to have anti-inflammatory, analgesic antimicrobial and antioxidant activity [9,10,11]. The purpose of this study was to evaluates the wound healing activity of leaves extract of erythrina variegata linn.

II. MATERIALS AND METHODS

2.1 Collection and Authentification of Plant Material

The leaves of Erythrina variegata were collected from local area of Washim district (Maharashtra) and plant was identified and authenticated by Assistant Professor N. N. Kakpure, Department Of Botany, Vidyabharati Mahavidyalaya Camp Amravati.

2.2 Extraction method

The leaves of Erythrina variegata Linn. were dried under shade and then made in to a coarse powder with a mechanical grinder, then the coarse powder was subjected to Soxhlet extraction with 80% ethanol for 8 hours. The extract was collected, filtered through Whatman filter paper and the total alcoholic extract was concentrated using rotary evaporator and the percentage yield of the extract was calculated. Finally, the dried extract was packed in a closed vessel and stored in deep freezer until being required for the experiment.

2.3 Calculation of Percentage Yield

The percentage yield of extract was calculated by using following formula.

$$\text{Percentage yield} = \frac{\text{Weight of extract}}{\text{weight of powdered drug taken initially}} \times 100$$

Preliminary Phytochemical Screening: Ethanolic extract of Erythrina variegata leaves (EEEV) was subjected to various phytochemical screening tests for the identification of the phytoconstituents present in Erythrina variegata using standard procedures [12].

2.4 Ointment Formulation

Simple ointment B.P. was prepared using hard paraffin, cetostearyl alcohol, white soft paraffin and wool fat. The master formula used for the preparation of ointment was taken from British Pharmacopoeia [13]. M.F is Master Formula; R.F is Reduced Formula. The 200 g of simple ointment base was prepared by placing hard paraffin (10 g) in a beaker and melted over water bath. The other ingredients such as cetostearyl alcohol (10 g), white soft paraffin (170 g), and wool fat (10 g) were added in descending order of melting point, respectively, after removing from melting.

All the ingredients were melted over a water bath with constant stirring until they became homogeneous. The mixture was removed from the heat and stirred until cold. To prepare hydroalcoholic extract ointment, 10 g and 20 g of the powdered extract were incorporated into portion of simple ointment base to prepare 5% and 10% (w/w) ointment, respectively, by levigation. The remainder of simple ointment base was gradually added and mixed thoroughly. Finally, the extract ointment was transferred to a clean container for topical application during the experiment [14].

Table 1: Master formula and Reduced Formula used for simple ointment preparation

Sr. No	Ingredients	Master formula	Reducing formula
1	Wool fat	50gm	10gm
2	Hard paraffin	50gm	10gm
3	White soft paraffin	850gm	170gm
4	Cetostearyl alcohol	50gm	10gm
		1000gm	**200gm**

2.5 Acute Dermal Toxicity

Acute dermal toxicity was done according to OECD draft guideline 434 and Mulisa et al. with some modifications. Two groups consisting of 5 female albino mice each and showing normal skin texture were housed individually in a cage. Around 10% of the body surface area fur was then shaved from the dorsal part 24 h before the study. Extract ointments (5% and 10%) were applied on the shaved area. At the end of the exposure period (24 h), the residual test substance was removed and the animals

were observed for 24 h and for the next 14 days for development of any adverse skin reactions like inflammation, irritation or redness [15, 16].

2.6 Wound Healing Activity Testing

2.6.1 Grouping and dosing of animals

Healthy, adult white albino mice of either sex (25–35 g and 6–8 weeks of age) were used. Four groups of mice, each containing six mice, were used for excision model. Animals in group I were treated with simple ointment (as control group) and group II was treated with 0.2 % Nitrofurazone ointment (as a standard drug) whereas III and IV group was treated with 5% (w/w) and 10% (w/w) extract ointments respectively. Four groups of mice, each containing six mice, were used for incision wound model. The animals of groups I-IV were treated in a similar fashion with excision wound model. All the experiments were conducted in accordance with the internationally accepted guideline for laboratory animal use and care [17].

2.6.2 Excision Wound Model

On wounding day, animals were anesthetized under anesthetic ether using (Open Mask Method) After wound area preparation with 70% alcohol, the dorsal fur of the animals was shaved with shaving machine and the anticipated area of the wound to be created was outlined on the back of the animals on the dorsal thoracic region 1 cm away from vertebral column on the anesthetized mouse. Full thickness circular excision wounds sized about 300 mm^2 were created along the markings using toothed forceps, scalpel, and scissors. Hemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. The entire wound was left open. The mice were divided into four groups (6 mice per group) randomly and each mouse was placed in a separated cage. The treatment was done once daily topically in all the cases. The wounding day was considered as day 0. The standard drug, extract, and simple ointment were applied topically to the respective groups till the wound was completely healed [18, 19, 20].

2.6.3 Measurement of Wound Contraction

The wound closure rate was assessed by tracing the wound on days 2, 4, 6, 8, 10, 12, 14 and 16 using transparent paper and a permanent marker. The wound areas recorded were measured using 1 mm^2 scale of graph paper. Changes in wound area were evaluated, giving an indication of the rate of wound contraction and epithelialization period. The evaluated surface area was used to calculate the percentage of wound contraction, taking initial size of the wound as 100% [19] as shown below:

Percentage Wound closure = Ao – Ad / Ao × 100

Where,

Ao = Wound area on zero day

Ad = wound area on corresponding days like (2^{th} 4^{th} 6^{th} 8^{th} 10^{th} days etc..)

2.6.4 Epithelialization Period Measurement

Falling of scab leaving no raw wound behind was taken as end point of complete epithelialization and the days required for this were taken as period of epithelialisation [19].

2.6.5 Incision Wound Model

Animals were anesthetized in the same manner described for excision wound model. The dorsal fur of each mice was then shaved and a 3 cm long longitudinal Paravertebral incision 1 cm away from vertebral column was made through the skin and subcutaneous tissue. The parted skin was then sutured 1 cm apart using a surgical thread (silk no.00 round) as described by Ehrlich and Hunt with slight modification [21]. After 24 h of wound creation (on 1^{st} day), animals were treated as described under grouping section, with topical formulation of non-medicated simple ointment, extract, and standard drug once daily for nine days. The suture was removed on day 8 after incision and tensile strength was measured on the 10^{th} day after wounding using continuous water flow technique [22, 23].

2.6.6 Measurement of Tensile Strength

Tensile strength (the force required to open the healing skin) was used to measure the extent of wound healing. The model used for this purpose consists of fixed shelves with a table. There are two Allis forceps, one is fixed to the opposite side of shelve and another is tied with rope that was attached to the empty IV bag on which the weights are placed. On the 10^{th} day after wounding, each mouse was anesthetized using diethyl ether to secure animal to the table. The two forceps were firmly applied 1 cm away from healed tissue on the incised part of the skin onto the line facing each other. Water is allowed to flow into bag from tap water through IV line. A gradual increase in weight was transmitted to the wound site pulling apart the wound edges. As soon as wound gaping appeared, water flow was stopped, and the volume of water collected in the container was determined and noted as an indirect measure of breaking strength in grams [22].

2.7 Statistical Analysis

The results are expressed as mean ± standard error of mean (SEM). The statistical significance was analysed using one-way analysis of variance (ANOVA) followed by Post hoc Tukys - test was employed and p value <0.05 was considered statistically significant.

III. RESULTS

3.1 Yield of the Leaves Extract: The % practical yield of ethanolic leaves extract of Erythrina variegata was found to be 16% w/w.

3.2 Phytochemical Constituents of the Leaves Extract of Erythrina variegata and Solvent Fractions: According to the qualitative phytochemical screening study, the leaves extract of Erythrina variegata was found to be positive for the presence of Alkaloids, Steroids, Flavonoids, Proteins, Phenolic compounds, Glycosides and Carbohydrates whereas Tannins, Saponins and Terpenoids were absent.

3.3 Acute Dermal Toxicity: In acute dermal toxicity test, the application of 10 % w/w ointment formulation, the site of application did not show any sign of inflammation, irritation or redness. There were also no overt signs and symptoms observed when the animals were monitored for 48 h. Moreover, no other signs of toxicity or mortality were noted during the 14 days of cage side observation.

3.4 Excision Wound Model:

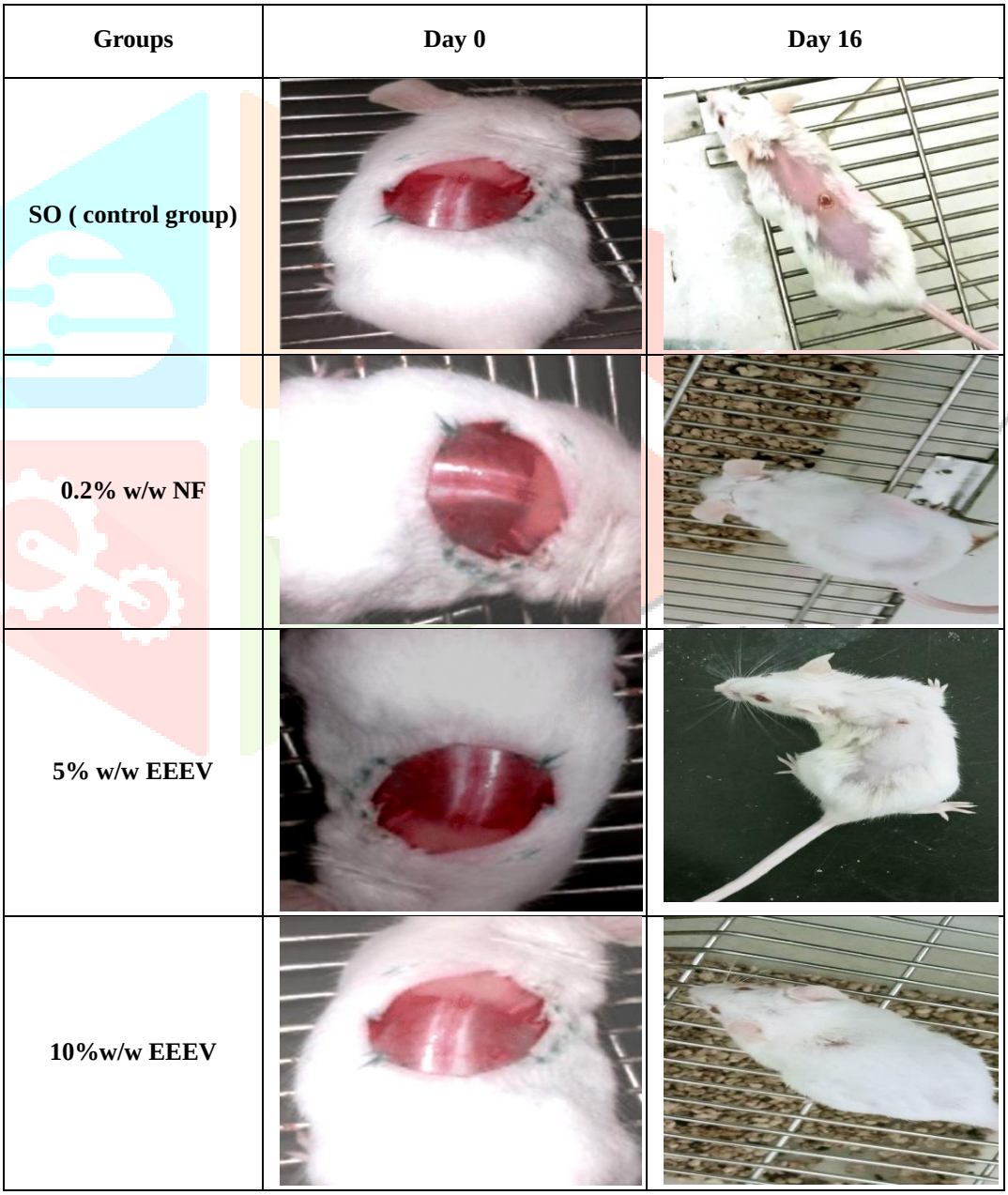

Figure 1: Wound contraction progress in simple ointment, 0.2% w/w NF, 5% w/w and 10% w/w EEEV Extract ointment treated groups in mice from day 0 to day 16 post wounding days in excision wound model

Table 2: Effect of topical application of the 80% ethanolic extract of the leaves of Erythrina variegata on wound contraction of excision wound model in mice.

Wound Area in mm^2 on Post-Wounding Days								
Groups	**Day 2**	**Day 4**	**Day 6**	**Day 8**	**Day 10**	**Day 12**	**Day 14**	**Day 16**
SO Base	291.00 ± 0.85	250.83 ± 0.74	199.33 ± 0.49	186.33 ± 0.66	146.83 ± 0.90	102.66 ± 0.95	75.00 ± 1.21	46.00 ± 1.18
0.2% (w/w) NF	265.66 ± 0.33	171.5 ± 0.80^{a1c1}	141.66 ± 0.61^{a2c1}	93.66 ± 0.42^{a3c2}	44.66 ± 0.95^{a3c2}	13.33 ± 0.61^{a3c2}	2.3± 0.06^{a3c2}	00 ± 00^{a3c2}
5% (w/w) EEEV	282 ± 1.03	240.66 ± 0.42	174.33 ± 0.55^{a1}	141 ± 0.44^{a2}	105.5 ± 1.31^{a2}	74.66 ± 1.08^{a2}	29.5 ± 0.61^{a2}	6.66 ± 0.42^{a2}
10% (w/w) EEEV	266.33 ± 0.66	185.16 ± 1.30^{a1c1}	151.33 ± 0.66^{a2c1}	107.16 ± 1.01^{a3c2}	53.66 ± 0.55^{a3c2}	17.5 ± 0.34^{a3c2}	4.63 ± 0.05^{a3c2}	00 ± 00^{a3c2}

Notes: Values are expressed as mean ± SEM (n = 6 animals in each group) and analyzed by one-way ANOVA followed by Tukey post-hoc test; numbers from 2-16 indicate the day on which contraction rate measurement was taken, [a]compared with simple ointment (control group) and [c]compared with 5% (w/w) extract; [1] $p < 0.05$, [2] $p < 0.01$, and [3]$p < 0.001$.

Abbreviations: SO; simple ointment base, NF; nitrofurazone, EEEV; Ethanolic extract of Erythrina variegata.

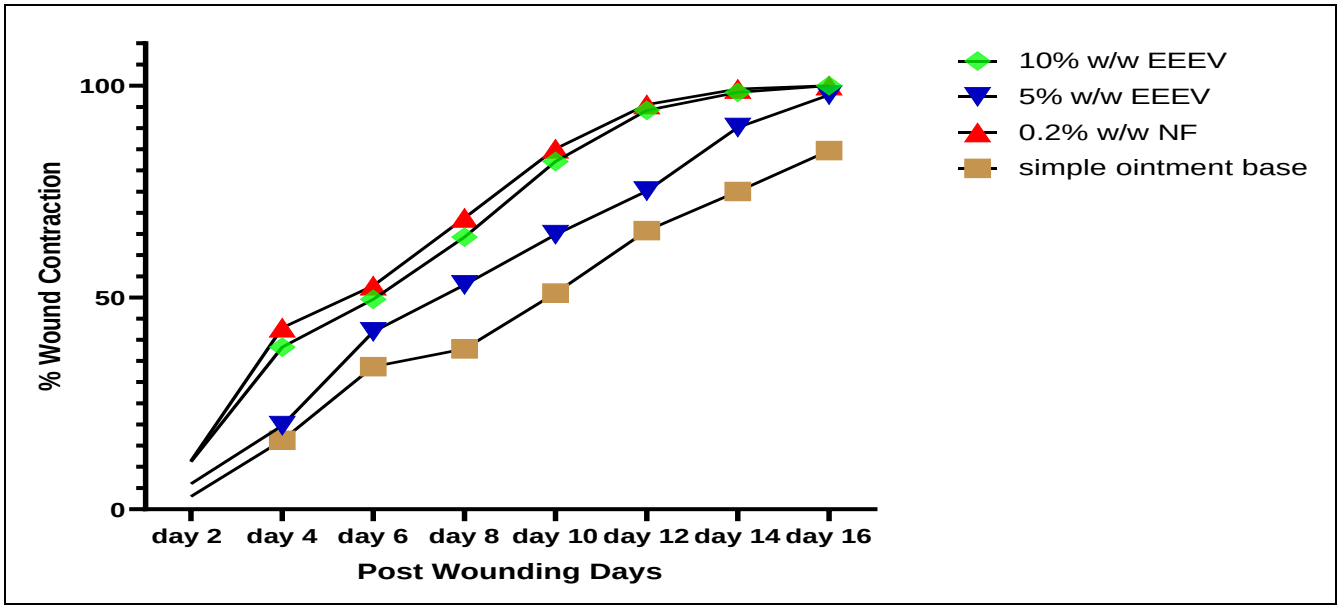

Figure 2: Percentage of wound area contraction effects of simple ointment, 0.2% w/w NF , 5% w/w and 10% w/w EEEV extract ointments treatment in mice in excision wound model.

3.5 Wound Contraction

Topical application of the ointments of 80% ethanolic extracts of E. variegata leaves showed significant effect on wound healing process in mice. The progress of wound contraction induced by treatment of simple ointment base, nitrofurazone 0.2% (w/w) ointment, 5% (w/w) and 10% (w/w) ointment of 80% ethanolic extract is shown in (**Table 2 and Figure 1)**. The plant extracts facilitated wound contraction significantly at both dose levels from 6th day to 16th day as compared to control group. The 10 % (w/w) crude extract ointment treated group showed significant ($p< 0.05$) wound contraction starting from day 4. This effect was highly significant ($p< 0.001$) from 8th day onward in comparison with the control group (simple ointment). There was ($p< 0.05$) significant difference in wound healing activity between the 10% (w/w) and 5% (w/w) extract and higher rate of wound closure was observed with 10% (w/w) EEEV ointment. The maximum percentages (rate) of wound contraction were observed in animals treated with 0.2% w/w NF extract ointment from the 12th to 16th days, which were 95.55, 99.23 and 100 % respectively. Similar percentages of wound contraction (94.16, 98.45 and 100%) were observed in animals treated with the 10% (w/w) EEEV ointment from the 12th to 16th day. The 10% extract ointment revealed better observable effect compared to the standard drug;

however, it failed to reach statistical significance. The animals treated with 5% (w/w) ethanolic extract ointment showed significant wound contraction from 6th day onward as compared to control group ($p< 0.05$). Significant wound contraction was also observed for nitrofurazone 0.2% (w/w) ointment treated group from 4th day onward as compared to control group ($p< 0.05$). The maximum percentages of wound contraction for nitrofurazone 0.2% (w/w) ointment were seen in the 12th, 14th and 16th days, which were 95.55, 99.23, and 100%, respectively. However, there was no significant difference in wound healing activity between 0.2% w/w NF and 10% w/w extracts. Furthermore, complete wound closure was observed in 10% (w/w) extract and standard ointment treated groups within 14 to 16 days, respectively **(Figure 2)**.

3.6 Period of Epithelization

Table 3: Effect of topical application of the 80% ethanolic extract of the leaves of Erythrina variegata on period of epithelialisation (number of days).

Treatment groups	Period of epithelialisation (days) Mean ± SEM
Simple ointment base	20.18±0.38
0.2% (w/w) NF	12.00 ± 0.28^{a3c1}
5% (w/w) EEEV	15.41 ± 0.23^{a3}
10% (w/w) EEEV	14.20 ± 0.2^{a3}

Notes: Values are expressed as mean ± SEM (n = 6), one-way ANOVA. [a]Compared to simple ointment(control group); [c]compared to 5% ointment; [1]$p < 0.05$, [2]$p < 0.01$, [3]$p < 0.001$

The time for complete epithelialization was short in extract ointment and nitrofurazone treated groups as compared to control (simple ointment treated group). On average, the period of epithelialization was 20.18, 12, 15.41, and 14.20 days for control group, standard drug, and 5% (w/w) and 10% (w/w) extract ointment, respectively. The 0.2% NF ointment treated group showed faster rate of epithelialization ($p<0.001$) compared to control group and ($p<0.05$) compared to 5% w/w extract. Similarly, 10% (w/w) extract ointment showed significant ($p<0.001$) difference of epithelialization period as compared to simple ointment. The 5% (w/w) extract also showed significant ($p<0.001$) difference of epithelialization period as compared to simple ointment treated group (Table 3).

3.7 Incision Wound Model

3.7.1 Wound Breaking Strength (Tensile Strength)

Figure 3 : Photograph of incision wound on day 0 and measurement of tensile strength on 10th day using water flow technique [24].

Table 4 : Effect of ethanolic extract of the leaves of Erythrina variegata on tensile strength of wound in incision wound model.

Groups	Wound breaking strength (gm)
Simple ointment base	135±0.28
0.2%w/w NF	309±0.96 [a3]
5%(w/w) EEEV	282±0.73 [a3]
10%(w/w) EEEV	291.5±0.61 [a3]

Notes: Data are expressed as Mean ± SEM; (n=6) and analyzed by one way ANOVA followed by Tukey post-hoc test; [a]compared with simple ointment (control group); [1]$p < 0.05$, [2]$p < 0.01$, [3]$p < 0.001$.

On the incision wound model, the extract ointments applied topically were found to be effective in increasing the tensile strength of the healing wound (Table 4). Both 5% and 10% extract ointments produced significantly (p<0.001) higher tensile strength of the healing wound in comparison with the simple ointment base treated groups. Treatment with the standard drug 0.2% (w/w) Nitrofurazone ointment also exhibited significantly (p<0.001) increased tensile strength compared to simple ointment base treated groups. The highest percent tensile strength was shown in the 0.2% (w/w) Nitrofurazone ointment treated group.

IV. DISCUSSION

In world, medicinal plants have been used traditionally for several years as topical and internal preparations to promote wound repairs. They have a great potential for wound healing by promoting the speed of wound healing with lower pain, discomfort, and scarring of the patient [25]. The leaves paste of Erythrina variegata was traditionally claimed to be used for wound healing. Therefore, in the present study we scientifically explored these traditional claims [26].

The results of this study on wound healing activity revealed that the leaves extract significantly increases wound healing effects with both 5% (w/w) and 10% (w/w) extract ointment treated groups in the excision and incision wound models. This can be supported by the fact that the greater the reduction in the rate of wound contraction is the better efficacy of medication and the wound will close at faster rate if the medication is more efficient [27].In excision wound healing model the leaves extract of E. variegata showed statistically significant wound area contraction compared to the control group. The 10% (w/w) extract ointment treated group revealed faster wound area contraction from day 6 to day 16, whereas the 5% (w/w) extract ointment treated group showed statistically significant wound area contraction starting from the 8th day onwards. The higher wound contraction rate of the extract ointment may be due to either its dose-dependent antibacterial effect or induction of macrophage cell proliferation [28].

Furthermore, the period of epithelialization was significantly reduced from 20.18 days (control group) to 12, 15.41, and 14.20 days for 0.2% nitrofurazone, 5% and 10% w/w extract ointment treated groups, respectively. The shorter period of epithelialization and faster wound area contraction could be due to the ability of E. variegata leaf extract to enhance collagen synthesis,induction of cell proliferation, and antimicrobial activities of bioactive constituents [29].

In incision wound model, significant increase in skin breaking strength was observed. Groups treated with 10% and 5% (w/w) extracts and standard ointments showed statistically significant increase in tensile strength as compared to simple ointment base treated group. The increase in tensile strength in the incision model may be due to the antioxidant activity of the extract, increase in collagen synthesis and maturation, formation of stable intra and intermolecular cross-link, matrix deposition, and cell migration [27,30,31].

Another possible reason for enhanced wound healing effect could be due to the crude extracts of E. variegata leaves which may possess antioxidant, free radical scavenging properties and promote cell proliferating properties. The role of antioxidant and free radical scavenging property in wound healing process is further strengthened by other studies conducted on the (Fabaceae) family which revealed that the plant possesses anti-inflammatory, antipyretic, antibacterial and antioxidant properties [30,32].

The role of phytochemicals in wound healing is also supported by different studies. For presence of alkaloids gives antibacterial activity and alkaloid extract could be attributed to the fact that extract caused an increased rate of formation of epithelial cells thus speeding up the re- epithelialization process which is critical in wound healing. flavonoids are potent antioxidants, free radical scavengers [27,31]. Polyphenols and flavonoids (prevent the synthesis of prostaglandins) possess anti-

inflammatory properties and have antimicrobial activities [33]. Glycosides isolated from the same family (Fabaceae) possess antioxidant, antimicrobial, analgesic, nervine sedative, febrifuge, anti-asthmatic, anti-epileptic and anti-inflammatory effects. Therefore, the presence of phytochemicals in the leaves extract such as alkaloids, steroids, terpenoids, flavonoids, glycosides and phenolic compounds may contribute to wound healing activities independently or synergistic effects [32]. A study revealed that phytochemical constituents identified in E.variegata plant were directly responsible for antioxidant, antimicrobial and anti-inflammatory activities through different mechanisms [34]. For example, reducing proteins due to their astringent effect, encouraging wound contraction, and increasing the formation of capillary vessels and fibroblasts [35]. On the other hand, flavonoids are responsible for reducing lipid peroxidation by preventing or slowing downcell necrosis and improving vascularity, which increases the viability of collagen fibrils by increasing circulation, preventing cell damage and promoting DNA synthesis [31]. The different phases of wound repair; wound contraction, epithelization, and tensile strength were improved by ointments prepared from 80% ethanolic leaf extract of E. variegata as compared to the simple ointment base treated group. The findings of this study therefore support the traditional claims of the plant for wound healing treatment.

V. CONCLUSION

In this study, in Excision and Incision models, the different phases of wound repair, wound contraction, epithelialization, and tensile strength, were enhanced by the 80% ethanolic extract ointment of the leaves of Erythrina variegata as compared to the simple ointment base treated group. These results collectively demonstrate that the 80% ethanolic extract possesses wound healing activity and this justifies the use of the leaves of Erythrina variegata for treatment of wounds as claimed in the folklore literature. The wound healing activity of the plant could be due to the presence of alkaloids, flavonoids, steroids, proteins, glycosides and polyphenolic compounds. Hence the present findings extend support for the traditional claims of the plant for the treatment of wound.

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