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Wound healing activity of *Euphorbia neriifolia* leaf ethanolic extract in rats

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Abstract

Objective: *Euphorbia neriifolia* Linn. (Euphorbiaceae) is a commonly occurring plant around the dry, rocky, hilly areas of North, Central and South India. The milky latex and leaves of this plant is frequently used by the natives of Central India in case of burn, piles and to treat the deep cracks in soles of legs. The present study was designed to explore the wound healing activity of *E. neriifolia* leaf along with its effect on wound tissue growth and oxidative status. Methods: LD_{50} was determined following the OECD guideline No. 420 and 425. Wound healing activity was evaluated on excision wound and dead space wound model along with determination of hydroxyproline, protein, catalase and superoxide dismutase in wound granulation tissue. Results: *E. neriifolia* leaf extract in 200 & 400 mg/kg doses showed significantly ($p<0.05$) enhanced epithelization. *E. neriifolia* at 200 and 400 mg/kg dose significantly ($p<0.001$) increases hydroxyproline content, protein content, catalase activity and decreases superoxide dismutase activity in granulation tissue. The positive control groups were treated with vitamin C (250 mg/kg, i.p). Conclusions: *E. neriifolia* augments wound contraction and epithelisation. It also increased protein and hydroxyproline content, collagenation with added antioxidant activity on the wound site in 200 & 400 mg/kg doses. These activity may be due to presence of flavonoids and saponins which reduced oxidative stress in wound area and promote healing.

Keywords: *Euphorbia neriifolia*; wound healing; Epithelization; Hydroxyproline; Antioxidant; Flavonoid.

1. Introduction

In traditional system leaves *Euphorbia neriifolia* Linn. (Euphorbiaceae) are used as aphrodisiac, diuretic, in cough and cold, bleeding piles and ano-rectal fistula [1]. The tribal population of Chhattisgarh region uses the milky latex as an ingredient of aphrodisiac mixture. Latex is used to de-root skin warts, ear ache and in arthritis [2]. Plant is bitter, laxative, carminative, improves appetite, useful in abdominal troubles, bronchitis, tumors, leucoderma, piles, inflammation, enlargement of spleen, anemia, ulcers, fever, and in chronic respiratory troubles [3, 4]. The natives of Chhattisgarh use boiled 'thohar' milk in castor oil after adding salt externally to cure the deep cracks in soles of legs and it is also used commonly like aloe gel in case of burns. Application of lukewarm leaves reduces itching pain and swelling in piles [5]. Topically applied aqueous extract of *E. neriifolia* latex showed wound healing activity in guinea pig by increasing epithelization, angiogenesis, tensile strength and DNA content in wounds [6].

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The present study was undertaken to find out the wound healing activity of *E. neriifolia* leaf along with antioxidant activity.

2. Materials and methods

Plant material: The leaves of *E. neriifolia* were collected from Hoshangabad District of Madhya Pradesh, India, in the month of September. The plant was identified with the help of available literature and authenticated by Dr. A. P., Shrivastava, Principal, Pandit Khushilal Sharma Govt. Ayurveda College and Institute, Bhopal, India. A voucher specimen was deposited in the herbarium of department (No. 1085).

Preparation of extract: The leaves were air dried under shade and milled into coarse powder, extracted in Soxhlet extractor successively with different organic solvents such as petroleum ether (60–80°C), chloroform, acetone and 95% ethanol in increasing order of polarity [7]. The marc was dried in hot air oven below 50°C before extracting with next solvent. The extracts obtained with each solvent was distilled to remove 1/4th of solvent then the extracts were dried using a vacuum oven below 30°C and percentage weight calculated in terms of w/w. 95% ethanolic extract was dark brown in colour and extractive value was 4.85 % (w/w) of the dry weight of starting material. Presence of triterpenoidal steroids was confirmed by the Salkowski test and Noller's test [8]. Presence of saponin was confirmed by Froth test and Hemolysis test [9]. Presence of flavonoids was confirmed by Shinoda test and Alkaline reagent test [10].

Experimental Animals: Wistar albino rats (weighing 150- 200 g) of either sex bred in animal house facility of department of pharmacy, Sagar University, M.P were used. The animals housed under standard laboratory conditions maintained at 24 ± 1°C and a 12:12 hr light dark cycle. Food and water were given *ad libitum*. Ethical Committee approval was obtained before carrying out these experiments. Animals were fasted over night before the experiment and randomly divided into different groups containing six in each group. Extract was suspended in 2% (w/v) carboxy methyl cellulose (CMC) prepared in distilled water.

LD_{50} was determined according to the guidelines of Organization for Economic Co-operation & Development (OECD) following the Up & Down method (OECD guideline No. 425) and Fixed dose method (OECD guideline No. 420). Based on these agreements a *Limit test* was performed to categorize the toxicity class of the compound and then *Main test* was performed to estimate the exact LD_{50} [11, 12]. The limit test was started from 2000 mg/kg dose. LD_{50} was found greater then the test dose so the test substance could be classified in the hazard classification as class 5, 2000 mg/kg<LD_{50} <5000 mg/kg in the Globally Harmonized System (GSH). LD_{50} of *E. neriifolia* leaf extract was found to be 2779.71 mg/kg from main test [13,14]. A dose range of 100, 200 and 400 mg/kg was selected for *E. neriifolia* leaf extract. The doses selected for the study starts from 1/7 of LD_{50}.

Excision wounds: The albino rats were divided into five groups, each containing 6 animals. A round seal of 2.5 cm diameter was impressed on the dorsal thoracic central region 5 cm away from the ears of the anaesthetized (pentobarbitone sodium 45 mg/kg, i.p) rats. Full thickness skin from demarked area was excised to get a wound measuring 500 mm2 [15]. After achieving full haemostasis by blotting the wound with cotton swab soaked in warm saline, animals were placed in their individual cages. The animals were treated daily as follows, from 0 day to 21st post-wounding day. Group I Control (2% CMC suspension), Group II, III and IV extract 100, 200 and 400 mg/kg, p.o. and Group V, vitamin C (Ascorbic acid, 250 mg/kg, i.p).

In this wound model, two physical attributes of healing namely wound contraction and epithelization period were studied. Contraction mainly contributes for wound closure in the first two weeks was studied by tracing the raw wound area on butter paper on alternate days. These wound tracings were retraced on 1 mm^2 graph paper to assess area and then wound contraction was calculated as percentage of original wound size (500 mm^2) for each animal of group on the day of wounding and subsequently on alternate days for 21 days or till complete healing whichever was earlier. Changes in wound area were calculated, giving an indication of the rate of contraction. The number of days required for falling of eschar without any residual raw wound gave the period of epithelisation.

Dead Space Wounds: Albino rats were divided into five groups each containing 6 animals same as before. One pre-weighed (2.5 x 0.25 cm) polypropylene tube was implanted beneath the dorsal para vertebral skin of the anaesthetized rats (pentobarbitone sodium 45 mg/kg, i.p). The animals were treated daily as follows from 0 day to 9^{th} post-implantation day. On the 10^{th} day the harvested granulation tissues were carefully dissected out, along with the tubes. The pieces of granulation tissue were collected, dried at 60°C overnight and weighed. The granulation mass, which was dried at 60°C for about 24 hrs was weighed and placed in sealed tube containing 10 ml of 6N HCl. Heating the sealed tubes at 110°C for 24 hrs hydrolyzed these tissues. The hydrolysate was cooled and excess of acid was neutralized with 10N NaOH using methyl red as indicator. The volume of neutral hydrolysate was made up to 20 ml with distilled water. From this 0.1 ml was used to estimate hydroxyproline.

Hydroxyproline was determined as per the method of Neuman and Logan [16,17]. The reaction mixture contains 0.1 ml hydrolysate and 1 ml each of NaOH (2.5N), $CuSO_4$ (0.01M) and H_2O_2 (6%). Immediately test tubes were placed in a water bath at 80°C for 16 min and then cooled for 5 min. 2 ml freshly prepared para-dimethyl aminobenzaldehyde (5% in n-propanol) and 4 ml H_2SO_4 (3N) was added. Test tubes were once again placed in a hot water bath at 80°C for 15 min and then cooled for 5 min. absorbance of the pink colored samples were compared to standard hydroxyproline of known concentration at 540 nm.

The following formula was used for the calculation of hydroxyproline:

$$\text{C U } (\mu g) = \frac{\text{A U}}{\text{A Std}} \text{ X C Std } (\mu g) \text{ X } \frac{\text{H Vol}}{\text{Vol assay}}$$

Protein content was measured according to method of Lowry *et al*. [18]. The reaction mixture contains hydrolysate (0.1 ml), 4 ml of freshly prepared alkaline mixture (2% sodium carbonate in 0.1N NaOH and 1 ml 4% aqueous copper sulphate), Folin-Ciocalteau reagent (0.4 ml) and allowed to stand for 10 minutes for the reaction to complete. The absorbance was noted at 540 nm against blank.

$$\text{C P (mg/ml)} = \frac{\text{A Spl}}{\text{A Std}} \text{ X C Std (mg/ml)}$$

C U	:	Concentration of unknown (mg)
A U	:	Absorbance of unknown
A Std	:	Absorbance of standard
C Std	:	Concentration of of standard (mg)
H Vol	:	Hydrolysate volume
Vol assay	:	Volume taken for assay

C P	:	Concentration of protein (mg/ml)
A Spl	:	Absorbance of sample
A Std	:	Absorbance of standard
C Std	:	Concentration of of standard (mg/ml)

Catalase was determined as per the H_2O_2 decomposition method of Aebi [19]. The reaction mixture contains hydrolysate (20 µl) and phosphate buffer (0.1M, pH 7.0, 2 ml). Reaction was initiated by adding 1 ml of 30 mM H_2O_2. The solution was mixed well and the decrease in absorbance (initial absorbance should be approximately 0.500) was determined at 340 nm after every 15 sec for 60 sec. The results were expressed as H_2O_2 decomposed/min/mg of protein, using 71 as molar extinction coefficient of H_2O_2.

Superoxide dismutase was estimated as per the nitro-blue tetrazolium (NBT) reduction method of Misra and Fridovich [20]. The reaction mixture contains hydrolysate (100 µl), sodium carbonate (0.05M, 1 ml), NBT (0.01%, 0.4 ml) and EDTA (1 mM, 0.2 ml). Zero minute absorbance was noted at 560 nm and the reaction was initiated by addition of 0.4 ml of hydroxylamine hydrochloride (2.4 mM). Reaction mixture was then incubated at 25°C for 5 min, the reduction of NBT was noted. One enzymatic unit of SOD is the amount of protein in the form of enzyme present in 100 µl of sample required to inhibit the reduction of 24 micromolar NBT by 50% and is expressed as unit/mg of protein.

2.1 Statistical Analysis

All pharmacological observations are presented in tables as mean ± standard error. Experimental data was analyzed using one-way ANOVA followed by Turkey-Kramer multiple comparison test. P value less than 0.05 were considered statistically significant. Graph Pad Prism Version 3.02 was used for statistical calculations.

Results

Excision wounds: In the control animals, wound contracted progressively and required an average period of 22.50 ± 1.22 days for epithelization. *E. neriifolia* in 200 & 400 mg/kg doses

Fig. 1 Effect of *E. neriifolia* leaf extract on granulation tissue weight in dead space wound healing. Data expressed as mean ± SEM, n = 6 per group. ***p<0.001.

Fig. 2 Effect of *E. neriifolia* leaf extract on hydroxyproline and protein content of granulation tissue in dead space wound healing. Data expressed as mean ± SEM, n = 6 per group. **p<0.01 and ***p<0.001.

significantly ($p<0.05$) enhanced the epithelization as evidenced by the shorter period required for eschar dropping and promoted the wound contraction. Ascorbic acid reduced the period of epithelization to 16.45 ± 0.94 days, which is highly significant ($p<0.01$) compared to control animals as depicted in Table. 1.

Fig. 3 Effect of *E. neriifolia* leaf extract on catalase content of granulation tissue in dead space wound healing. Data expressed as mean ± SEM, n = 6 per group. *p<0.05 and ***p<0.001.

Fig. 4 Effect of *E. neriifolia* leaf extract on superoxide dismutase content of granulation tissue in dead space wound healing. Data expressed as mean ± SEM, n = 6 per group. ***p<0.001.

Dead Space Wounds: Increase in granulation tissue weight is highly significant in all the tested dose levels for *E. neriifolia* extract. Granulation tissue weight was 147.24 mg at 400 mg/kg extract treatment compared to 36.83 mg of control and for ascorbic acid treated group it was 165.60 mg. *E. neriifolia* extract at 200 and 400 mg/kg doses significantly ($p<0.001$) increased hydroxyproline content, protein content, catalase activity and decreased superoxide radical scavenging activity in granulation tissue. The results were summarized in Figure. 1, 2, 3 & 4. Increase in hydroxyproline content was extremely significant (26.72 mg) in ascorbic acid treated group compared to control group (2.95 mg). Ascorbic acid showed extremely significant antioxidant activity and increased protein content of granulation tissue. From the above findings it is clear that *E. neriifolia* leaf extract promotes growth of tissue by increasing wound collagen content also shows good antioxidant activity in wound tissue.

Discussion

The study was designed to investigate the influence of *E. neriifolia* on different phases of wound healing namely wound contraction, epithelisation, protein content, hydroxyproline content, collagenation and antioxidant activity on the wound site.

The capacity of a wound to heal depends in part on its depth, as well as on the overall health and nutritional status of the individual. Following injury, an inflammatory response occurs and the cells below the dermis (the deepest skin layer)

Table. 1. Effect of *E. neriifolia* leaf extract of excision wound healing

Treatment, mg/ kg, p.o	% Reduction in wound area in post wounding days			Epithelization Period (days)
	6th	12th	18th	
Vehicle control	30.72	56.66	73.34	22.50 ± 1.22
E.n. extract (100)	38.02	63.15	80.19	19.97 ± 0.42^{ns}
E.n. extract (200)	36.85	65.50	85.34	19.05 ± 0.61*
E.n. extract (400)	46.66	85.17	98.82	18.15 ± 1.34*
Vitamin C ()	57.13	89.63	—	16.45 ± 0.94**

***P<0.001, **P<0.01, *P<0.05, when compared to control group. The values are expressed as mean ± SEM, n = 6 in each group.

begin to increase collagen (connective tissue) production. Later, the epithelial tissue (the outer skin layer) is regenerated. It is well known that the phases of healing namely coagulation, inflammation, microphasia, fibroplasias and collagenation are intimately interlinked. The process of wound contraction and epithelization is separate and independent. The activity of fibroblast is responsible for wound contraction and involves movement of the entire dermis. Epithelization involves the migration and proliferation of cells. It is known that stabilization of lysosomal membranes, inhibition of cellular migration and inhibition of fibroblast contraction are responsible for their antihealing effects [21]. Thus an intervention into any one of these phases by drugs could eventually lead to either promotion or depression of the collagenation, wound contraction and epithelisation.

E. neriifolia significantly enhanced the epithelization and promoted the wound contraction. Increase in granulation tissue weight and high protein content of treated group than the control group may be due to either cellular infiltration or increase in collagen synthesis. The increase hydroxyproline content in the treated group agrees with the increase in protein content, which is predominantly due to enhanced collagen synthesis in the treated group. Wound contraction is mediated by specialized fibroblast (myofibroblasts) found with in granulation tissue [22]. These cells are known to contract collagen gel, which were newly synthesized at the site of healing wound. Increased wound contraction in treated group may be due to the enhanced activity of fibroblasts [23,24].

Vitamin C is needed to make collagen (connective tissue) that strengthens skin, muscles, and blood vessels and to ensure proper wound healing. Severe injury appears to increase vitamin C requirements [25] and its deficiency causes delayed healing [26]. Ascorbic acid is required for the synthesis of collagen as it is a known stimulator of collagen production [27].

The levels of free radicals are very high in a wound due to damage to cells in wounded tissue. Immune cells migrating into the wound bed induce inflammation and release free radicals that damage nearby cells. Injuries generate superoxides and lipid peroxidation through the activation of neutrophils [28] in several types of injuries like burns [29], infected wounds, skin ulcers [30] etc. This oxidative stress may cause damage to the growing tissue (collagen the epithelium) at the reparative site. Free radicals are unstable oxygen molecules that cause damage to cellular components such as DNA, proteins or lipids and can induce cell death. The report that oxidant impairs the healing supports this assumption. Hence, a drug that inhibits oxidative stress is believed to increase the viability of cells by improving the circulation, preventing cell damage, promoting DNA synthesis etc.

Vitamin C is a highly effective antioxidant, protecting cells from damage by free radicals. It also helps prevent depletion of antioxidants stored in the skin and prevents damage by free radicals. Vitamin C is a free radical scavenger

that promotes healing [31] by accelerating differentiation or maturation of skin cells [32]. Results indicated that in the first 7 days of wound healing, high levels of ascorbic acid, hydroxyproline, and zinc accumulation occurred in the wound tissue [33]. Ascorbic acid augments wound healing in mice exposed to gamma-radiation [34], also promote fracture healing in experimental animal model [35, 36].

Earlier reports showed that when there is increase in oxidative stress, the stress itself indicates gene expression of SOD by activating the transcription factor [37, 38]. In the extract treated groups the decrease in SOD level is due to the antioxidant activity of the phytochemical constituents as the extract scavenges the superoxide radical resulting in decrease in oxidative stress. The extract also converts the superoxide radical to hydrogen peroxide as when there is increase in hydrogen peroxide it stimulates the expression of catalase [39]. This may be the reason for the enhanced activity of catalase in treated group. Thus the antioxidants present in the *E. neriifolia* like, saponin and flavonoids could be expected to promote the epithelisation by controlling the oxidative stress.

Aqueous extract of *E. neriifolia* latex showed wound healing activity by increasing epithelization, angiogenesis, tensile strength and DNA content on topical application. Present study explores the wound healing activity of *E. neriifolia* leaf ethanolic extract administered orally. In conclusion *E. neriifolia* leaf extract promoted the skin incision wound contraction as well as turnover of wound granulation tissue with added antioxidant activity. Antioxidants present in *E. neriifolia* could be expected to promote the epithelisation by controlling the oxidative stress. Therefore, wound-healing potency of *E. neriifolia* may be attributed to the phytoconstituents present in it, which may be either due to their individual or additive effect that fastened the process of wound healing. This study provides the scientific basis to the traditional uses of *E. neriifolia* leaf. This plant is easily available in a large quantity in the dry hilly areas of North and Central India. This plant can be used as a cheap source of active therapeutics by the poor and under privileged people of this region.

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