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Wound Healing Activity of Aqueous Extract of *Leucas urticifolia* Leaves in Experimental Animals

Suthar Sushil*[1], Patel Ritesh[2], Kumar Vikram[3], Sharma Vikram[3]

[1]CMJ University, Shillong, Meghalya-793003, India.
[2]Department of Pharmaceutics, S. K. Patel College of Pharmaceutical Education & Research, Ganpat University, Mehsana, Gujrat, India
[3]Department of Pharmacology, Sri Balaji College of Pharmacy, Jaipur, Rajasthan, India

ABSTRACT

The present study was planned to investigate the effect of aqueous extract of *Leucas Urticifolia* on resutured incision, excision and burn wounds in Wistar rats. Resutured incision, excision and dead space wounds were inflicted under light ether anaesthesia aseptically. In incision wound model the control animals receive only distilled water and all test animals were treated with three different doses of *Lucas Urticifolia* aqueous extract (100, 200 and 400 mg/kg), orally for a period of 10 days. On the day 11, wound breaking strength of the resutured incision wound was estimated. In excision and burn wound models the animals were treated topically daily with two different concentrations (5% and 10% w/w) of extracts and the rats of standard groups were treated with 5% povidone iodine ointment topically till the complete closure. The percentage wound contraction and epithelization period were studied from day of creating wound till complete closure of the wound. The aqueous extract of *Leucas Urticifolia* show significant wound healing activity against all wound models studies. High wound breaking strength, high rate of wound contraction and decrease in period of epithelialisation were observed in treated animals when compared to control group of animals. From the results obtained it can be concluded that aqueous extract of *Leucas Urticifolia* has significant wound healing activity. The enhanced wound healing activity of aqueous extract may be due to free radical scavenging action and the antibacterial property of the phytoconstituents (flavonoids) present in it which either due to their individual or additive effect fastens the process of wound healing.

KEY WORDS: *Leucas Urticifolia*, Wound Healing activity, Incision wound, Excision wound and Burn wound.

Address for correspondence:
Sushil Suthar S/O Sh. Ram Chand Suthar
22-Gandhi Nagar, Sri Ganganagar, Rajasthan, India
Mobile No. +919799440718
E-mail: sushilsuthar05@rediffmail.com

INTRODUCTION

Wound may be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissue[1]. Wound healing is a process by which a damaged tissue is restored as closely as possible to its normal state and wound contraction is the process of shrinkage of area of the wound[2]. Three different phases constitute the physiologic process of wound-healing; (i) substrate phase, (ii) proliferative phase and (iii) remodeling phase. All these steps are orchestrated in controlled manner by a variety of cytokines including growth factors. Some of these growth factors like platelet derived growth factor, transforming growth factor B, fibroblast growth factor and epidermal growth factor etc. have been identified in self healing wounds. In chronic wounds the application of some growth promoting agents or some compounds which can enhance the in situ generation of these growth factors is required to augment the healing process[3]. Today wound healing abnormalities are among the greatest causes of disability and deformity. "I dressed the wound, God healed it" wound healing involves multiple complicated events[4]. The understanding of the mechanism of wound healing has increased dramatically during last few years. Herbs have been used as a source of drugs to combat diseases since time immemorial. The effectiveness, easy availability, low cost and non-toxic nature of herbal remedies are main reasons for its popularization. Ayurveda describes several drugs of plant, mineral, and animal origin for their wound healing properties under the term Vranaropaka. Most of these drugs are derived from plant origin[5].

Leucas Urticifolia belong to family Lamiaceae is an annual herb distributed in the Rajasthan, Punjab, Baluchistan, Sindh and Rajputana desert of Pakistan[6,7]. It is commonly known as kubo in gujrati[8], darkan in rajasthani[6], it is also known as Goma or Guldora[7]. The plant is tarditionaly also used for the treatment of diarrhea, dysentery, uterine haemorrhages, dropsy, gravel, cystitis, calculus, bronchial catarrh, skin diseases, fever and various types of mental disorders. The decoction of the leaves and apical shoots with gur is used locally as an abortifacient up to 3 months of pregnancy[9].

Leucas Urticifolia is reported to have Triterpenes like: Leucisterol, β-sitosterol, and ursolic acid[10], Diterpene: Momilactone-A[11], Flavonoids: Leufolins A, Leufolins B[12], Acids and esters: Urticic acid, Methoxybenzyl benzoate, 4-hydroxy benzoic acid[10]. The flavonoidal glucosides leufolins A and B of Leucas Urticaefolia exhiited significant inhibitory potential against the enzyme butyrylcholinesterase[12]. A survey of literature revealed that no systematic approach has been made to study wound healing activity of this plant and revealed the presence of flavonoids, which have potent antioxidant activity and helpful in wound healing. Hence the present study was undertaken to evaluate wound healing potency of aqueous extract of *Leucas Urticifolia* on various animal wound models in Wistar rats.

MATERIALS AND METHODS

PLANT MATERIAL

The leaves of *Leucas Urticifolia* were dried in shade and powdered coarsely. For preparation of aqueous extract, 250g of powdered leaves was macerated with 1000 ml of distilled water for seven days with intermittent stirring, filtered and concentration. The dried extract was stored at 4°C until used. The extract was subjected to preliminary phytochemical tests.

PRELIMINARY PHYTOCHEMICAL STUDIES

The aqueous extract of *Leucas Urticifolia* (LUAE) subjected to qualitative chemical investigation for the identification of the phytoconstituents- sterols, glycosides, saponins, carbohydrates, alkaloids, flavonoids, tannins, proteins[13].

DRUG FORMULATIONS

For topical application ointment of the extract was prepared using 2% sodium alginate as aqueous base containing 5% w/w and 10% w/w of drug extracts. All the preparations to be given by oral route were prepared freshly in distilled water just before dosing.

ANIMALS

Healthy Wistar albino rats of either sex and of approximately same age (12 to13 weeks), weighing between 150-200 g were used for the study. The animals were acclimatized by keeping them in animal house facility of Sri Balaji College of Pharmacy, Jaipur, Rajasthan. They were housed individually in polypropylene (32x24x16 cm) cages containing bedding material as husk and maintained under controlled conditions of temperature (23±2^{o}c), humidity (55±5%) and 12 h light and 12 h dark cycles, and were fed with commercial pellet rat chow and water ad libitum.

The norms for Good Laboratory Practice were followed for care of laboratory animals. The studies were conducted after obtaining the approval from Institutional Animal Ethical Committee clearance of Sri Balaji College of Pharmacy, Jaipur, Rajasthan. The animal house facility of this division is approved by Govt. of India under the Ministry of Environment and Forest (Reg no. 1212/ac/08/CPCSEA). The experimental protocol was approved by Institutional Animal Ethical Committee (IAEC) of Name of College and Place (Letter No. SBCP/IAEC/10-465) with CPCSEA Reg. no. 1212/ac/08/CPCSEA).

ACUTE ORAL TOXICITY STUDY

Acute oral toxicity study for the test extract (LUAE) was carried out using OCED guideline 425 (modified, adopted 23rd march 2006).[14]

SKIN IRRITATION STUDY

This study was carried out on rabbits. The skin of the animal was shaved at three different positions on the dorsal side, each about 500 mm^2. The 1st area was kept as control, to which vehicle was applied. 2nd area was applied with LUAE (5%) and the 3rd area treated with LUAE (10%). After 4 hr, the skin was observed for signs of inflammation[15].

INCISION WOUND

In incision wound model, 6 cm long paravertebral incisions were made through the full thickness of the skin on either side of the vertebral column of the rat under the ketamine anesthesia. The wounds were closed with interrupted sutures of 1 cm apart. The animals were divided into four groups of six animals each. The animals of control group left untreated and animals of other groups were treated with 100, 200 and 400 mg/kg of LUAE respectively. The sutures were removed on the 8th post wound day and wound breaking strength was measured on 11th day of post wounding by continuous water flow technique. Three readings were taken on each wound and the mean of six such readings in each animal was used for statistical analysis. Subsequently animals were sacrificed by overdose of anaesthesia[16,17].

EXCISION WOUND MODEL

The animals were randomly divided into four groups of six animals each as shown in Table 3. The animals of group I serve as a control group and left untreated, animals of other groups treated with standard 5%-povidine iodine, LUAE-5% and LUAE-10% ointment. A full thickness excision wound was created for this study according to Morton and Malone. The excision wound was created in overnight fasted animals under light ether anesthesia. A round circular seal of 500 mm^2 diameter was impressed on the dorsal thoracic central region 5 cm away from the ears of anaesthetized rats. Full thickness skin from demarked area was excised to get a wound of approximate 500 mm^2 area[18]. After achieving the full haemostasis, wound was blotted with cotton swab soaked in warm saline and animals were placed in individual cages.

Assessment of wound healing

All the animals were inspected daily and healing was assessed based on physical parameters namely wound contraction and complete epithelization period.

a. Wound contraction: It was calculated by observing progressive change in wound area planimetrically. The contraction mainly contributes for wound closure and was studied by tracing the raw wound area on butter paper on day 2, 6, 10, 14 and 18th postoperative days or till complete wound healing, whichever occurred earlier. These wound tracings were taken on 1 mm^2 graph paper to assess wound area and then wound contraction was calculated as percentage of original wound size for each animal in all the groups. Change in wound area was also calculated to indicate the rate of contraction.

$$\% \text{ of wound contraction} = \frac{\text{Initial wound size} - \text{Specific day wound size}}{\text{Initial wound size}} \times 100$$

b. Epithelization period: It was monitored by noting the number of days required for the scab to fall off from the wound surface without leaving a raw wound behind.

The healed scar on day of epithelization was excised and used for determination of collagen content[19].

BURN WOUND MODEL

The animals were randomly divided into seven groups of six animals in each group as shown in Figure 2. Partial thickness burn wound was created on overnight starved animals under light ether anesthesia, by pouring hot molten wax at 80^0 C into a metal cylinder of 300 mm^2. The back of the animal just below the neck was shaved just before creating the wound. The molten wax in the cylinder was allowed to solidify which took about 10-12 minutes. Then the metal cylinder with wax adhered to the skin was removed which left a distinctly demarked partial thickness circular burn wound of 300 mm^2 area[19,20]. After animals recovered completely from anesthesia, they were kept in individual cages. All the animals were inspected daily and healing was assessed based on physical parameters namely wound contraction and complete epithelization period as described above.

STATISTICAL ANALYSIS

Results are expressed as mean ± S.D. Statistical differences between means were determined by One-way ANOVA followed by Tukey's post hoc test using GraphPad Prism 5. P value <0.05 was considered significant.

RESULTS

PHYTOCHEMICAL SCREENING

The phytochemical tests revealed that the aqueous extract of *Leucas Urticifolia* leaves possess alkaloids, flavonoids, tannins, glycosides, carbohydrates and proteins in aqueous extracts.

ACUTE TOXICITY STUDY

The alcoholic extract of *Leucas Urticifolia* was found to be safe upto dose of 2000 mg/kg b.w. without produce any mortality and other toxic effects. Hence the 1/20th, 1/10th, and 1/5th of doses was taken, which were found to be 100, 200 and 400 mg/kg body weight.

SKIN IRRITATION STUDY

LUAE (5%, and 10% ointment) did not show any irritation and there was no evidence of any notice able inflammation and redness.

RESUTURED INCISION WOUNDS

The results of Incision wound healing activity are shown in Table 1. In incision wound model, LUAE-200 and LUAE-400 mg/kg treated animals showed significant increase in wound breaking strength (P<0.001) respectively when compared to the control group.

Table. 1. Effect of *Leucas Urticifolia* aqueous extracts orally on incision wound healing.

Groups	Control	LUAE-100	LUAE-200	LUAE-400
Wound breaking strength (g)	197.00±9.75	207.83±11.49^{ns}	245.16±14.52***	312.16±10.47***

Values are expressed as mean ± SD on six animals in each group; 'ns' no significaant '***' P<0.001 when compared to control group.

EXCISION WOUND HEALING

The results of excision wound model are given in Table 2. In excision wound model, the mean percentage closure of wound area was calculated on the 2, 6, 10, 14 and 18 post wounding days. The rate of wound closure in LUAE treated animals is significant (P<0.001) more on 10, 14 and 18th day as compared to that of control. LUAE-5% and LUAE-10% also significantly (P<0.001) reduced the epithelization time from 21.83±0.98 to 18.33±1.50 and 17.83±1.47 days, when compared with control. Povidone iodine showed significant effect, i.e. p < 0.001 as compared with control.

The collagen content in healed scar of the control group was 41.83 mg/g of tissue. The application of povidone iodine ointment and LUAE ointment caused a significant (P<0.001) increase in collagen content of the scar tissue.

Table 2. Effect of topical application of aqueous extract of *Leucas Urticifolia* on healing of excision wound in wistar albino rats

Groups	Wound area (mm) on different days					Epithelization period (Days)	Collagen content (mg/g)
	Day-2	Day-6	Day-10	Day-14	Day-18		
Control	349.00±21.03 (32.20±4.20)	239.00±12.18 (52.20±2.43)	198.00±11.55 (60.40±2.31)	143.33±10.44 (71.30±2.08)	46.83±7.54 (90.63±1.50)	21.83±0.98	41.83±2.31
Standard	364.16±17.13 (27.16±3.42)	218.00±16.29 (56.4±3.25)	25.16±3.81*** (94.96±0.76)***	1.00±1.67*** (99.80±0.33)***	- (100±00)***	13.16±1.16***	58.33±1.87***
LUAE-5%	347.83±14.66 (30.43±2.93)	250.33±19.10 (49.93±3.82)	134.83±8.10*** (73.03±1.62)***	107.50±18.75*** (78.50±3.75)***	4.16±4.83*** (99.16±0.96)***,ns	18.33±1.50***	44.16±1.72***
LUAE-10%	365.66±24.86 (26.86±4.97)	246.33±16.18 (50.73±3.23)	124.83±10.90*** (75.03±2.18)***	73.00±18.18*** (85.40±3.63)***	3.16±3.65*** (99.36±0.73)***,ns	17.83±1.47***	52.33±1.63***

Values are expressed as mean ± SD on six animals in each group; values in paranthesis indicate percentage wound contraction; '***' P<0.001 when compared to control group; 'ns' no significant when compared to standard group.

BURN WOUND HEALING

The results of burn wound model are given in table 3. In burn wound healing activity the period of epithelization was reduced significantly in povidone iodine, LUAE-5% and LUAE-10% treated animals (P<0.001) when compared to the control group. LUAE-5% and 10 showed significant (P<0.001) increase in percentage wound contraction on day 18th compared to control group.

Table 3. Effect of topical application of aqueous extract of *Leucas Urticifolia* on healing of burn wound in wistar albino rats

Groups	Wound area (mm) on different days					Epithelization period (days)
	Day-2	Day-6	Day-10	Day-14	Day-18	
Control	329.33±20.83 (5.97±5.94)	226.66±17.18 (35.28±4.90)	176.66±11.51 (49.56±3.28)	102.66±8.09 (70.69±2.30)	63.16±7.13 (81.96±2.03)	25.33±1.21
Standard	345.83±19.60 (1.26±5.59)	165.66±15.13*** (52.70±4.32)***	58.00±7.72*** (83.44±2.20)***	10.83±11.90*** (96.90±3.30)	(100±00)***	14.50±2.07
LUAE-5%	329.66±24.05 (5.88±6.86)	235.33±12.97 (32.81±3.70)	170.00±9.12 (51.46±2.60)	89.83±8.44 (74.35±2.41)	41.50±8.36*** (88.15±2.38)***	22.00±0.89**
LUAE-10%	329.33±17.04 (5.97±4.86)	198.16±9.64 (43.42±2.75)**	143.50±10.98*** (59.03±3.13)***	87.66±8.11 (74.97±2.31)	38.83±3.54*** (88.91±1.01)***	21.16±1.16***

Values are expressed as mean ± SD on six animals in each group; values in paranthesis indicate percentage wound contraction; '**' P<0.01, '***' P<0.001 when compared to control group.

DISCUSSION

The main objective of this study is to evaluate the influence LUAE on healing of excision, resutured incision and burn wounds in male Wistar rats. The findings of the present study clearly indicated that the LUAE treated groups significantly enhanced wound healing as assessed by wound breaking strength in resutured incision wound, wound closure rate, time taken for complete epithelisation in excision and burn wound models. The LUAE also tested for its influence on collagenation.

The LUAE ointment promote the healing in all three models by the influencing wound breaking strength, wound contraction, epithelization phase and collagenation. While the phase of collagenation give the required strength to the scars of wounds healed by primary and secondary intentions, wound contraction reduce the gap of open wound to be filled by extracellular matrix which is rich in collagen and finally covered by epithelium. Two principal component of collagenation phase are collagen synthesis and maturation. Based on the results of the study, it could be assumed that LUAE might have enhanced strength of scar by increasing the collagen levels, which could stitch the wound edge together at the repair site. However a number of phases of healing, especially coagulation, inflammation, macrophagia, fibroplasias, collagenation, wound contraction and epithelialization etc. are intimately interlinked. Therefore the treatment could influence the healing of wound by intervening in any one or more phases of healing. Thus based on present study design, it is very difficult to comment on exact location and mechanism of the prohealing action of LUAE topical applications[21].

The excision wound healing model is often used for wound healing evaluation because it represents a true wound that could be reproducibly analyzed in nonsubjective, highly controlled manner[22,23].

Phytochemical screening revealed the presence of tannins, flavonoids, alkaloids, proteins and other important constituents. Flavonoids have been documented[24] to possess potent antioxidant and free radical scavenging effect, which is believed to be one of the most important components of wound healing. Phytochemical screening revealed the presence of flavonoids in *Leucas Urticifolia.* Thus, the enhanced wound healing may be due to free radical scavenging action and the antibacterial property of the phytoconstituents present in it which either due to their individual or additive effect fastens the process of wound healing. This could be the reason for prohealing activity of *Leucas Urticifolia.* This enhanced wound contraction effect *of Leucas Urticifolia* and epithelization could possibly be made use of clinically in healing of open wounds. However confirmation of this suggestion will need well designed clinical evaluation.

In conclusion, *Leucas Urticifolia* promoted wound healing in all the three cutaneous wound models. This prohealing effect of *Leucas Urticifolia* needs to be investigated further.

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