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Wound Healing Potential of Methanolic Extract of *Calophyllum inophyllum* Linn. Bark

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ABSTRACT
The present study provides a scientific assessment for the wound healing potential of methanolic extract of Calophyllum inophyllum Linn. bark (MECI). Excision, incision and dead space wound model were inflicted upon four groups of six rats each. In excision and incision wound model, Group I was assigned as control (ointment base). Group II and III were treated with 5% and 10% MECI topical ointment respectively. Group IV was treated with standard nitrofurazone ointment (0.2%). In dead space wound model Group I was assigned as control (1% w/v CMC). Group II and III were treated with 200 and 400 mg/kg b.w., p.o. MECI, respectively. Group IV was treated with standard nitrofurazone ointment (0.2%). The parameters observed were percentage of wound contraction, epithelialization period, tensile strength and hydroxyproline content. Treatment with 10% MECI topical ointment exhibited significant (P < 0.0001) wound healing activity in excision and incision wound model, whereas 400 mg/kg b.w., p.o. MECI extract exhibited significant (P < 0.0001) wound healing activity in dead space wound model. MECI stimulates wound contraction, decreases epithelialization period, and increases tensile strength and hydroxyproline content.
KEY WORDS: Excision wound model, incision wound model, dead space wound model, *Calophyllum inophyllum* Linn.

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INTRODUCTION

Healing is a multifaceted and intricate process initiated in response to injury that restores the integrity and function of damaged tissues. Healing process can be broadly categorized into three stages; inflammatory phase consisting the establishment of homeostasis and inflammation; proliferate phase (consisting of granulation, contraction and epithelialization) and finally the remodeling phase, which ultimately determines the appearance and strength of the healed area [1]. Several plant products, natural products [2], which are composed of active principles, such as flavonoids [3] and alkaloids and biomolecules [4] have been reported which promote the process of wound healing.

Calophyllum inophyllum Linn. is medicinally and economically important plant belonging to the family Guttiferae (Clusiaceae). The genus comprises of about 180-200 species widespread in humid tropics. Although a handful species have been identified in the new world, the genus is primarily found in the Indo-Pacific region, particularly Malaysia [5]. Different parts of the plant, such as seeds, leaves, nuts and bark possess therapeutic value, and can be used as antiseptic, astringent, purgative, diuretic and expectorant. Oil from the nuts traditionally has been used as a cosmetic and medicine [6]. *C. inophyllum* is reported to contain various compounds like xanthones, coumarins, steroids, flavonoids and triterpenoids [7]. Few of these compounds are reported to have diverse pharmacological activities, such as anti-HIV, anticoagulant, anti-cancer, anti-inflammatory, antiplatelet aggregation activity etc. [8-13].

However, despite the various bioactive phytochemical and diverse medicinal activities attributed to this plant, no biochemical studies have been carried out to shed light on the role of this plant in wound healing. In the light of the above, the current study was undertaken to investigate its role on percentage of wound contraction, epithelialization period, tensile strength and hydroxyproline content in excision, incision and dead space wound model.

MATERIALS AND METHODS

Plant Material

Calophyllum inophyllum Linn. bark was identified and collected from the Lalbagh Botanical Garden, Bengaluru, and was authenticated at National Botanical Research Institute (NBRI), Lucknow, India (Ref. No: NBRI/CIF/276/2012). The barks were washed, shade dried, pulverized into moderately coarse powder, passed through a 40 mesh sieve and stored in an air tight container for further use.

Extraction

The powder was loaded into Soxhlet extractor and was subjected to extraction with methanol to get methanolic extract. The methanolic extract was concentrated to dryness using rotary evaporator, giving yield as 4.10% w/v and preserved in a refrigerator. The extract were stored in airtight vials and kept refrigerated (2-8 °C) until further analysis [14]. Crude extract were weighed and suspended in CMC (1% w/v) for oral use.

Preliminary Phytochemical Studies

Phytochemical screening was carried out to identify the presence of alkaloids, glycosides, flavonoids, triterpenoids, proteins, saponins, steroids and tannins in MECI [15].

Preparation of Formulation and Standard Used

Simple ointment was prepared from the 5% and 10% methanolic extract of *Calophyllum inophyllum Linn.* bark by trituration method in a ceramic pestle and mortar using White soft paraffin obtained from S.D. Fine Chemical, India [16]. About 5 g and 10 g of semisolid extract was incorporated into the 100 g of simple ointment base B.P. Simple ointment base was used as the control group and was applied twice per day. Extract ointment was used twice per day to treat different groups of animals. Nitrofurazone (0.2%) obtained from Rexin Pharmaceutical Pvt. Ltd., Delhi was used as standard drug for comparing the wound healing potential of extract in different animal models and was applied twice per day.

Experimental Animals

The experiments were carried out with Albino wistar rats weighing 150-200 g obtained from the Laboratory Animal Services Division of Central Drug Research Institute, Lucknow, India. Research on experimental animals was conducted in accordance with the internationally accepted principles for laboratory animal use and care (1088/07/ CPCSEA). They were kept in polypropylene cages (22.5 x 37.5 cm) and were maintained under standard housing conditions (room temperature, 24-27°C and humidity, 60-65 %) with a 12-h light and dark cycle. They were allowed free access to standard pellet diet and water, *ad libitium*. The experimental protocols were approved by the Institutional Animal Ethics Committee, which follow the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and conform to the international norms of the Indian National Science Academy. Ethical norms were strictly followed during all experimental procedures. [Hygia/M.Pharm/16/2011-12].The rats were anesthetized prior to and during infliction of the experimental wounds.

The surgical interventions were carried out under sterile conditions using ketamine anesthesia (100 mg/kg b.w., *i.p.*). Animals were closely observed for any infection and those which showed signs of infection were separated and excluded from the study and were replaced.

Wound healing activity

Excision wound model

Animals were anesthetized prior to and during creation of the wounds. A full thickness excision wound was created for this study according to Morton and Malone. All the animals were shaved on the part to be exposed and ethanol (70%) was applied as antiseptic on shaved region. A round circular seal of 400 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 400 mm^2. After achieving the full haemostasis wound was blotted with cotton swab soaked in warm saline and animals were placed in their individual cages. The wound was left untreated to the open environment and no local or systemic antibacterial agents were applied. The simple ointment, extracts and standard were administered topically as per the protocol [17]. All the cages were cleaned on alternative days and the bedding material was changed daily. All the animals were inspected daily and healing was assessed based on physical parameters namely wound contraction and complete epithelization period [18]. The animals were divided into four groups of six rats each. Group I was assigned as control (ointment base). Group II and III were treated with 5% and 10% MECI topical ointment respectively. Group IV was treated with standard nitrofurazone ointment (0.2%) once daily from 0 to 21 post wound days or till complete healing, whichever earlier. The wound contraction was assessed by tracing the wound on days 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 post wounding days or till complete wound healing, whichever occurs earlier using transparency sheets and a permanent marker. These wound tracings were retraced on1 mm^2 graph paper to assess area and then wound contraction was calculated as percentage of original wound size

(400 mm^2) for each animal in all the groups. Changes in wound area were also calculated to indicate the rate of contraction.

$$\% \text{ wound contraction} = \frac{\text{wound area on day 0} - \text{wound area on day n}}{\text{wound area on day 0}} \text{x100}$$

Day n is any specific day on which % wound contraction is calculated [19].

Epithelialization period

The period of Epithelialization was calculated as the number of days required for falling of the dead tissue remnants of the wound without any residual raw wound [18].

Incision wound model

In incision wound model, 6 cm long paravertebral incision were made through the full thickness of the skin on either side of the vertebral column of the rats, after all the animals of each group were anesthetized under light ether anesthesia [20]. No systemic or local antimicrobials were used throughout the experiment. All groups were treated same as in excision model, the both edges kept together and stitched with Black silk surgical thread (no. 0000) and a curved needle (no. 9) was used for stitching. The continuous threads on both wound edges were tightened for good closure of the wound. After stitching, wound was left undressed then ointment base, standard ointment and extracts ointment were applied daily until 10 days; when wounds were cured thoroughly the sutures were removed on the day 10 and tensile strength of cured wound skin was measured using tensiometer [21].

The anesthetized animal was secured to the table, and a line was drawn on either side of the wound 3 mm away from the line. This line was gripped using forceps one at each end opposed to each other. One of the forceps was supported firmly, whereas the other was connected to a freely suspended light-weight metal plate. Weight was added slowly and the gradual increase in weight, pulling apart the wound edges. As the wound just opened up, addition of weight was stopped and the weights added was noted as a measure of breaking strength in grams. Three readings were recorded for a given incision wound. The mean reading for the group was taken as an individual value of breaking strength. The mean value gives the breaking strength for a given group [18].

$$\text{Tensile strength} = \frac{\text{breaking strenght (g)}}{\text{cross section area of skin (mm2)}}$$

Dead space wound model

The animals were divided into four groups of six rats each. Group I was assigned as control. Group II and III were treated with MECI 200 mg/kg and 400 mg/kg b.w., *p.o.* (suspended in 1% w/v CMC) respectively. Group IV was treated with standard nitrofurazone ointment (0.2%) topically. Dead space wound was created by implanting polypropylene tubes (2.5 cm × 0.5 cm) subcutaneously in the lumbar region on dorsal side. Animals were received MECI from 0 day to 9th post-wounding day. On 10th post-wounding day, granuloma tissue formed on implanted tube was dissected carefully and employed for determination of breaking strength and estimation of hydroxyproline content [22].

Hydroxyproline Estimation

Estimation of hydroxyproline involves the determination of the amino acid present in the collagen fibers of granulation tissue which helps clinically to understand progress rate at which the healing process is going on in the connective tissue of wound. The amount of hydroxyproline in granulation tissue was estimated [23].

Statistical analysis

All treated groups were compared with the control groups. The results were analyzed statistically using one-way analysis of variance (ANOVA). All tests were conducted using GraphPad Prism Software, Inc.

RESULTS

Excision wound study

The significant increase in the wound-healing activity was observed in the animals treated with MECI compared with those who received the placebo control treatments. In the excision wound model, 10% MECI topical ointment treated animals showed a significant reduction ($P < 0.0001$) in the wound area [Table 1; Fig. 1; Fig. 2] and epithelialization period [Table 2].

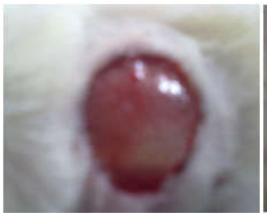
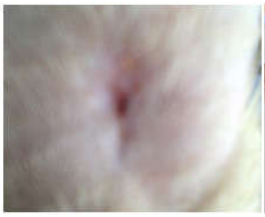


Fig 1 **Fig 2** **Fig 3**

Fig. 1 A circular excision wound on day 0.
Fig. 2 A completely healed excision wound after 21 days treatment.
Fig. 3 Tensiometer**:** for the measurement of tensile strength of skin.

Table 1: The effect of MECI on wound contraction in excision wound model.

Parameter	**Wound area (mm²) and Percentage wound contraction**			
Post wounding days	**Control**	**5% MECI**	**10% MECI**	**Standard**
Day 2	399.56±2.30 (0.11±0.57)	395.83±1.85 (372.5±2.53)	372.5±2.53 (6.87±1.26)	326.15±1.16 (18.45±0.58)
Day 4	374.5±5.19 (6.37±1.29)	359±3.82 (10.25±0.95)	332.73±3.0 (16.81±0.76)	295.59±2.40 (26.1±0.59)
Day 6	287.5±4.93 (28.12±1.23)	282.48±3.76 (29.55±0.96)	280.31±2.9 (29.92±0.74)	202.70±2.69 (49.32±0.67)
Day 8	204.61±6.05 (48.87±1.48)	149.21±4.2^{a2} **(**63.44±1.01**)**	128.96±2.9^{b} (67.73±0.73)	121.29±2.71^{b} (69.81±0.70)
Day 10	140.14±7.01 (64.96±1.75)	100.00±4.7^{a2} 74.99±1.19**)**	89.87±2.16^{b} (77.53±0.54)	77.50±2.03^{b} (80.62±0.50)
Day 12	85.66±0.76 (78.57± 1.55)	69.55±3.04^{a2,b2} (82.60±0.76)	57.48±2.72^{b2} **(**85.66±0.74**)**	43.50±2.17^{b} (89.12±0.54)
Day 14	69.67±6.05 (82.5±1.51)	43.63±3.07^{a2} (89.08±0.76)	35.42±1.86^{b} (91.14±0.46)	26.45±3.17^{b} (93.39±0.79)
Day 16	32.53±3.42 (91.83±0.87)	29.37±3.77^{a} (92.65±0.94)	23.25±1.93^{b} (94.18±0.48)	7.60±2.74^{b} (98.09±0.68)
Day 18	18.78±2.73 (95.30±0.68)	9.09±1.31^{a2} (97.72±0.32)	3.99±2.42^{b} (98.99±0.60)	2.86±1.42^{b} (99.28±0.35)
Day 20	10.88±2.07 (97.27±0.51)	3.22±1.77^{a} (99.19±0.44)	1.69±1.54^{b} (99.57±0.38)	0.00±0.00 (100.00±0.00)

Values were expressed as mean ± S.D, (Figures in the parenthesis indicate % wound contraction). $^{a}P < 0.0001$, $^{a2}P < 0.001$, $^{a3}P < 0.01$ when compared to control group. $^{b}P < 0.0001$, $^{b2}P < 0.001$, $^{b3}P < 0.01$ when compared to standard group.

Table 2 : The effects of MECI on period of Epithelialization in excision wound model.

Group	**Treatment**	**Epithelialization Period (mean time in days)**
I	Control	27.0±1.82
II	5% MECI	22.5±1.29**^{a}
III	10% MECI	20.25±1.50***^{b}
IV	Standard	18.75±0.95***

Values were expressed as mean ± S.D. *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$, a- indicates comparison with control and b- indicates comparison with standard group.

Incision wound study

Table 3 shows the effects of 5% and 10% MECI topical ointment on wound-healing activity in rats inflicted with incision wound. In the incision wound model, a significant increase ($P < 0.0001$) in the wound-breaking strength was observed with the 10% MECI topical ointment when compared with the control.

Table 3: The effect of MECI on wound tensile strength in incision wound model.

Group	Treatment	Tensile strength in (g/mm^2) 10th day
I	Control	32.70±3.26
II	5% MECI	38.05±3.57**a
III	10% MECI	60.24±4.25***b
IV	Standard	69.91±4.02***

Values were expressed as mean ± S.D. *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$, a- indicates comparison with control and b- indicates comparison with standard group.

Dead space wound study

200 and 400 mg/kg b.w., *p.o.* MECI treated group showed significantly increased ($P <$ 0.0001) hydroxyproline level when compared with the control group [Table 4].

Table 4 : The effect of MECI on wound hydroxyproline content in dead space wound model.

Groups	Treatment	Hydroxyproline (mg/gm tissue) 10th day
I	Control	68.55±5.85
II	5% MECI	138.115±5.14**a
III	10% MECI	253.0625±6.44***b
IV	Standard	305.61±12.36***

Values were expressed as mean ± S.D. *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$, a- indicates comparison with control and b- indicates comparison with standard group.

DISCUSSION

The present study was carried out to investigate the effect of MECI on the healing of experimentally induced wounds in rats. Collagenation, wound contraction and epithelization are fundamental phases of wound healing. The phases of inflammation, macrophagia, fibroblasia and collagenation are closely interlinked. Thus, intervention at any one of these phases using drugs could eventually either promote or inhibit one or all phases of healing [24].

Herbal drugs have become increasingly used worldwide because of their safety and effectiveness.

Preliminary phytochemical screening revealed that MECI showed positive response to flavonoids, tannins, saponins, steroids and triterpenes.

The wound healing property of *Calophyllum inophyllum* Linn. may be attributed to the phytoconstituents present in the plant, and the faster process of wound healing could be a function of either the individual or the synergistic effects of the phytoconstituents.

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