Wound healing activity of *Celtis timorensis* Span. (Cannabaceae) leaf extract in Wistar albino rats

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*Celtis timorensis* Span. (Cannabaceae), commonly known as stink or stinking wood, has been traditionally used in the treatment of wounds and cuts by the Badagas living in Nilgiri hills of Tamil Nadu. However, effects of this plant on wound healing have not yet been clearly elucidated. Here, we evaluated wound healing activity of ethanolic extract of *C. timorensis* leaves on rats using excision, incision and dead space wound models. The topical application was made in the case of excision and incision wound model whereas oral treatment was done with dead space wound model. Soframycin skin ointment and Vitamin E were used as standard drugs. The following differences were noted in the group of experimental animals which were treated with an extract of *C. timorensis* (5 and 10% topically, 200 and 400 mg/kg orally) when compared with the control: a high rate of wound contraction ($P <0.01$), high skin breaking strength ($P <0.01$), a significant increase in the granulation tissue weight ($P <0.01$) and hydroxyproline content ($P <0.01$). On day 16, the extract-treated animals (10% ointment) showed 95.76% reduction in the wound area compared to the controls which was 74.08%. Histological studies of granulation tissue obtained from the extract treated groups showed increased well organized bands of collagen, more fibroblasts and blood vessels when compared with the controls which showed inflammatory cells and decreased collagen fibres and fibroblasts. Enhanced wound contraction, increased skin breaking strength, hydroxyproline and histological findings suggest the use of *C. timorensis* for the management of wound healing.

Keywords: Badagas Dead space, Epithelization, Excision wound, Granuloma tissue, Incision wound, Nilgiri tribes, Skin breaking strength, Stinking wood, Tribal medicine, Wound models

Wounds are physical injuries that result in an opening or breaking of the skin. Wound healing of the skin is a complex biological process involving temporal interactions between numerous types of cells, extracellular matrix molecules and soluble factors. The wounds commonly heal in an orderly and systematic manner and repair of injured tissues occurs as a sequence of events, which includes homeostasis, inflammation, proliferation and remodelling. The complex and dynamic process of wound healing involves a specialized cells such as macrophages, platelets, fibroblasts, epithelial and endothelial cells. These wound cells interact with each other and also with the extracellular matrix. The actions of the wound cells are regulated by various proteins and glycoproteins, such as cytokines, chemokines, growth factors, inhibitors and their receptors. Each stage of wound healing has certain milestones that must occur for normal healing to progress. Disruption of one or more of these interactions can significantly interfere with the wound repair process. Several drugs obtained from the natural sources are known to increase the healing and repair process of different types of infected wounds. Some of these natural drugs have been screened scientifically for their therapeutic efficacy to repair wounds in different pharmacological models. However, many of the traditionally used herbs and herbal formulations remain unexplored for their usefulness against infections and wounds.

*Celtis timorensis* Span., commonly called stink or stinking wood, is a species of flowering plant belonging to Cannabaceae family. Traditionally, the plant used in the treatment of liver complaints, jaundice, urinary tract stone, pilesworm, bronchitis, hypertension, whole plant used as nervine, antidepressant, anticonvulsant, cutaneous eruptions and as mosquito repellent. The leaves are used for backache and paste of root bark applied on cuts and wounds. The methanol extract of leaves of this plant demonstrated hepatoprotective and...
antioxidant activities. C. timorensis has been reported for its antidepressant activity, antioxidant activity and antidiarrheal activity. In view of these cited activities, observations and traditional uses of plant, the present study was undertaken to explore the wound healing potential of ethanolic extract of leaves of C. timorensis using excision, incision and dead space wound models.

Materials and Methods

Collection of plant materials

Fresh leaves of C. timorensis were collected from Chittor district of Andhra Pradesh, India. The plant was identified and authenticated taxonomically by Dr K Madhava Chetty, SV University, Tirupathi. A voucher specimen of the collected sample was deposited in the herbarium of the institute for future reference. Freshly collected plant material was cleaned and dried under shade. The dried sample was powdered and used for further studies.

Extraction of plant materials

The shade dried leaves were made into coarse powder and extracted with 70% ethanol by cold maceration method for 72 h with intermittent shaking. The extract was filtered and concentrated at high vacuum, and stored in the refrigerator till further use.

Chemicals

Soframycin ointment was obtained from Aventis Pharma Limited, Chloramine T from Sigma-Aldrich, hydroxyproline and propranolol from SD Fine Chem Ltd.

Preparation of ointments

Three types of ointment formulations were prepared from the extract: 2.5, 5 and 10% (w/w) where 2.5, 5 and 10 g of the extract was incorporated into 100 g of simple ointment base Indian Pharmacopeia (I.P), respectively. Soframycin ointment was used as standard reference drug for comparing the wound-healing potential of the extract. For oral administration in dead space wound model, 100, 200 and 400 mg/kg suspensions of the extract were prepared in Tween 80.

Experimental animals

Albino Wistar rats (weighing between 150-200 g) of both sexes were selected for the experiment. They had free access to food and water and were maintained under standard laboratory conditions which included 12 h light-dark cycle and temperature of 28-30°C. Animals were allowed for a one week of acclimatization period prior to the study. The experimental protocol was approved by the IAEC (Institutional Animal Ethical Committee) and care of the experimental animals was taken according to the CPCSEA guidelines.

Skin irritation studies

The ointment was applied on shaven skin of the rat. Skin irritation potential of 5 and 10% ointment of C. timorensis was assessed by carrying out patch skin irritation test on albino rats (150-200 g). Rats were acclimatized for 7 days before the study. Twenty-four hours prior to the experiment, the fur from the dorsal surface of rats was removed. Animals were divided into three groups of three rats in each group. Group I, kept as control, was applied topically ointment base only, animals of Group II and III were treated with 5% and 10% of ethanolic extract of C. timorensis. The formulations were applied topically to approximately 1 cm² in area of the skin. The animals were then returned to their cages and were examined at 24, 48, and 72 h after the application of the formulation. The sites were inspected for dermal reactions such as erythema and edema. The mean erythemat and edematous scores were recorded on the basis of degree of severity: No erythema/edema = 0, slight erythema/edema = 1, moderate erythema/edema = 2, and severe erythema/edema = 3.

Evaluation of wound healing activity

Excision wound model

The rats were inflicted with excision wounds under light ether anesthesia. One excision wound was made by cutting away a 500 mm² full thickness of skin from the depilated area, the wound was left undressed to open environment. The animals were divided into five groups of six each. The animals of group I were left untreated and considered as the control, group II, III and IV were treated with 2.5, 5 and 10% (w/w) of ethanolic extract of C. timorensis whereas group V served as reference standard and treated with soframycin ointment. The ointment was topically applied once a day. The wound closure rate was assessed by tracing the wound on days 1, 4, 8, 12 and 16 post-wounding using transparency paper and permanent marker. In this model, wound contraction and epithelization period were monitored. Number of days required for falling of Eschar without any residual raw wound gave the period of epithelization. The % wound contraction was measured by using the formulation formula:

\[
\% \text{Wound contraction} = \frac{\text{Healed Area}}{\text{Total Wound Area}} \times 100
\]
Incision wound model

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 rats. The wounds were closed with interrupted sutures of 1 cm apart. The animals were divided into five groups of six animals each. The animals of group I were left untreated and considered as the control, the groups II, III and IV received 2.5, 5 and 10% ethanolic extract of *C. timorensis*, respectively. Group V received standard Soframycin ointment. The ointment was topically applied once in a day. The sutures were removed on the 8th post wound day. The skin breaking strength of the wounds was measured on the 10th day in anaesthetized rats.

Dead space wound model

The animals were divided into five groups of 6 rats in each group. Group I served as the control, group II, III and IV received oral suspension of ethanolic extract of *C. timorensis* (100, 200 and 400 mg/kg) and group V received standard drug Vitamin E (200 mg/kg) for 10 days. Under light ether anesthesia, dead space wounds were created by subcutaneous implantation of sterilized cylindrical grass liths (2.5×0.3 cm), one on either side of the dorsal paravertebral surface of the rats. On the 11th post-operative day, the granulation tissues formed on the implanted tubes were carefully detached from surfaces of the tubes and the dead space wound was excised. Wet weight of granuloma was recorded and dried in an oven at 60°C for 24 h and the dry weight noted. Wound tissues were analyzed for hydroxyproline content, a basic constituent of collagen. Tissues were dried in a hot air oven at 60-70°C to constant weight and hydrolyzed in 6 N HCl at 130°C for 4 h in sealed tubes. The hydrolysate was neutralized to pH 7 and then subjected to chloramine-T oxidation for 20 min. The reaction was terminated by adding 0.4 M perchloric acid and developed colour with Ehrlich reagent at 60°C was read spectrophotometrically at 557 nm.

Histopathology

A portion of granulation tissue in dead space wound model was taken from control, test and standard groups and after usual processing 5 μ thick sections were cut and stained with hematoxylin and eosin. The sections were qualitatively observed under the light microscope and observed in respect of fibroblast proliferation, collagen formation, and angiogenesis.

Statistical analysis

Data are expressed as mean±SEM of 6 animals in each group. To determine the statistical significance, one way ANOVA followed by Dunnett’s t test was performed. Values of *P* <0.05 were considered statistically significant.

Results

Skin irritation studies

In skin irritation studies, there is no sign of erythema and edema was found up to 72 h after application of ethanolic extract of *C. timorensis* ointment.

Excision wound model

In excision wound model, the wound area of 10% extract-treated group as measured for every four days, showed significant contraction from 35.47% on day 4, to 61.58% on day 8, 75.44% on day 12 and 95.76% on day 16 (Table 1). In comparison, vehicle group showed only 16.77% contraction on day 4, 37.05% on day 8, 51.36% on day 8 and 74.08 % on day 14. Overall, the contraction of wound was in the order of standard group>10% extract-treated>5%-extract treated>2.5%-extract treated>control group. Wounds dressed with 10% extract were found to be epithelialized faster (9.16 days), followed by standard group (10.83 days) while the 5%-extract treated group showed an average of 11 days. There were no significant differences in the mean epithelization time among wounds dressed with 2.5% extract and control group. This indicated that the healing potential of the extract was dose dependent and was effective only at 5 and 10% concentrations.

Incision wound model

The breaking strength of the incision wounds was increased in drug treated groups to significant extent i.e. 605±21.77 in control increased up to 790±18.71 with 5%-extract treated group and with 10%-extract

<table>
<thead>
<tr>
<th>Post-wound healing days</th>
<th>Control</th>
<th>Standard</th>
<th>EECT-2.5%</th>
<th>EECT-5%</th>
<th>EECT-10%</th>
<th>Epithelization in days</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>16.77±1.27</td>
<td>40.16±2.06**</td>
<td>22.70±2.71</td>
<td>23.92±3.83</td>
<td>35.47±3.20**</td>
<td>15.33±0.42 (Control)</td>
</tr>
<tr>
<td>8</td>
<td>37.05±2.65</td>
<td>56.56±2.63**</td>
<td>42.48±2.28</td>
<td>47.29±3.21*</td>
<td>61.58±2.35**</td>
<td>13.33±0.33 (2.5%)</td>
</tr>
<tr>
<td>12</td>
<td>51.36±3.96</td>
<td>79.58±1.92**</td>
<td>55.10±1.94</td>
<td>72.66±2.00**</td>
<td>75.44±2.19**</td>
<td>11.0±0.85 ** (5%)</td>
</tr>
<tr>
<td>16</td>
<td>74.08±2.26</td>
<td>94.10±2.64**</td>
<td>82.32±2.45</td>
<td>91.64±2.01**</td>
<td>95.76±2.71**</td>
<td>9.16±0.47** (10%)</td>
</tr>
</tbody>
</table>

[No. of animals n=6, EECT: Ethanolic extract of *Celtis timorensis*. *P* <0.05, **P <0.01 when compared to control group]
treated group to 818±20.58. In incision wound model, a significant increase ($P < 0.01$) in breaking strength (grams) was observed in rats treated with 5% and 10% extract ointment, respectively when compared to control group (Table 2).

**Dead space wound model**

The mean dry weight of granulation tissue in control group was 76.16±8.18 which significantly increased ($P < 0.01$) to 109±7.89, 123±8.06 and 121.33±5.76 in groups treated with 200, 400 mg/kg and standard drug, respectively. The mean wet weight of granulation tissue in control group was 274.33±14.68 which significantly increased ($P < 0.05$) to 356.66±16.21 in 200 mg/kg group and 387.83±10.45 and 440.33±18.36 ($P < 0.01$) in 400 mg/kg and standard group treatment, respectively (Table 3). Hydroxyproline is a major component in the ground substance of granulation tissue. In the present study, 200 and 400 mg/kg group had significantly high hydroxyproline level (30.49±0.53 and 34.80±1.33 μg/mL) when compared to control group ($P < 0.01$). The standard drug (Vitamin E) also showed significant increase in hydroxyproline content ($P < 0.01$) compared to the control group.

**Histopathological evaluation**

The photomicrographs of the granulation tissue obtained from the animal wound are shown in Fig. 1. The images revealed decrease in collagen bundles and few blood vessels in the control group, whereas moderate edema and few blood vessels were seen in 100 and 200 mg/kg group and thick collagen bundles, increased fibroblasts and blood vessels were seen in animals treated with 400 mg/kg group.

![Fig. 1 — Histopathology of granulation tissue in dead space wound model. (A) Control animals showing decrease in collagen bundles and few blood vessels; (B) Animals treated with EECT-100 mg/kg showing decrease in fibroblasts and collagen fibres, focal aggregates of macrophages, areas of moderate edema and mild decrease in blood vessels; (C) Animals treated with EECT-200 mg/kg showing decrease in fibroblasts and thin collagen fibers, focal aggregates of macrophages, areas of moderate edema and mild decrease in blood vessels; (D) Animals treated with EECT 400 mg/kg showing thick collagen fibers, increase in fibroblasts, areas of mild edema and mild increase in blood vessels; (E) Standard group animals showing thick collagen bundles, diffusively scattered inflammatory cells comprising of lymphocytes and decrease in macrophages, areas of mild edema and increase in blood vessels]
Discussion

Topical application in the form of ointment was preferred to internal medication as this mode of application was reported to be effective in faster wound contraction due to larger availability of the drug at the wound site15. Three different models were used in our study to assess the wound healing effect of ethanolic extract on various phases of wound healing, which run concurrently, but independently of each other. The standard drugs soframycin and Vitamin E were used as reference drugs to assess the healing potency of the crude drug against the control. In the excision wound model, healing of the excision wounds can be monitored by recording wound area changes (closure rate) at conveniently fixed intervals of time. It was also monitored by recording the epithelization period. Epithelization can be directly measured in terms of days for falling of Eschar without any residual raw wound. Topical application of the extract improved wound contraction and closure, and the effects were distinctly visible starting from 4th post-wounding day. Wound contraction occurs through the centripetal movement of the tissues surrounding the wound, which is mediated by myofibroblasts16. Since the extract enhanced wound contraction, it would have either enhanced contractile property of myofibroblasts or increases the number of myofibroblasts recruited into the wound area17. Epithelization involves proliferation and migration of epithelial cells across the wound bed. Also, re-epithelization decreases the wound size. Wound re-epithelization is a hallmark of successful wound care18. In this study, the epithelization time was also found to be significantly shorter in animals treated with ointments containing the crude extract. Therefore, the shorter epithelization period in the extract treated group might be due to facilitated proliferation of epithelial cells and/or increasing the viability of epithelial cells.

Treatment with ethanolic extract of C. timorensis and soframycin exhibited significant wound healing activity in the incision wound model. This is also evident from the high breaking strength of the wounds in the treatment groups on day 10. Wounds of soframycin-treated rats had the highest breaking strength followed by those of 10, 5 and 2.5%-extract and the control group. However, the breaking strengths of 10, 5% and soframycin-treated wounds were significantly higher than those in control group. Higher tensile strength is indicative of increase in collagen and its maturation leading to the formation of inter and intra-molecular cross-links. The low breaking strength in the control wounds resulted in prolonged wound-healing time. In dead space wound model, the ethanolic extract of C. timorensis at (200 & 400 mg/kg) produced a significant increase in the wet granuloma tissue as well as in the dry weight. The increase in dry granulation tissue weight is indicative of higher protein content and collagen maturation19. Treatment with C. timorensis has demonstrated a significant increase in the hydroxyproline content and collagen distribution of the granulation tissue after 10 days of injury indicating increased collagen turnover. Collagen, the major component which strengthens and supports extra cellular tissue is composed of the amino acid, hydroxyproline, which has been used as a biochemical marker for tissue collagen20. Similar results were reported on hydroxyproline content using dead space wound model in rats treated with Ficus benghalensis root extract21 and Acacia Honey22. A close examination of granulation tissue sections revealed that tissue regeneration was much quicker in the treated group compared to that in control wounds. The well-formed collagen bundles in the extract-treated group support the efficacy of C. timorensis on fibroblast proliferation and synthesis of extracellular matrix during healing process. Treatment with the extract also resulted in increased angiogenesis and reduced edema and inflammation.

The higher level of hydroxyproline, high tensile strength together with the desired wound contraction and epithelization indicates interplay of different mechanisms leading to faster wound healing in the treated animals. Histopathological studies further support the biochemical and biophysical data of the study.

The results of this study support the notion that C. timorensis can promote wound healing by inhibiting inflammation, inducing collagen synthesis and promoting angiogenesis. Preliminary phytochemical analysis revealed the presence of alkaloids, carbohydrates, proteins, cardiac glycosides, tannins, flavonoids and phenolic compounds. Flavonoids and tannins are known to promote the wound-healing process due to their astringent and antimicrobial properties, which appear to be responsible for wound contraction and increased rate of epithelization23. Thus, wound healing property of ethanolic extract of C. timorensis may be attributed to the phytoconstituents present in it, which may be either due to their individual or additive effect that fastens the process of wound healing.

Conclusion

The ethanolic extract of Celtis timorensis leaves possess good wound healing activity when applied locally or administered orally. The higher doses of the
extract were more effective in all the three models tested. However, further studies should be carried out with isolated constituent of the extract to exactly determine the lead molecule responsible for the wound healing property.

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