

# Wound-healing Activity of *Clerodendrum infortunatum* L. Root Extracts

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## ABSTRACT

Validation of the ethnotherapeutic claim of *Clerodendrum infortunatum* L. was investigated to evaluate its wound-healing potency in experimental rats. For topical application, 4% (w/w) ointment cream bases of petroleum ether, chloroform and ethanol extracts were prepared and assessed for their effect on excision, incision and dead space wound models. Significant wound healing was observed in animals treated with chloroform and ethanol extracts, similar to the reference standard drug Nitrofurazone. It was evaluated by increased area of epithelialization, followed by an increase in wound contraction, skin breaking strength and tissue granulation dry weight. Histopathological studies of the granulation tissue also indicated that there was an increase in collagen formation in those rats treated with chloroform and ethanol extract compared to the control and petroleum ether extract treated animals. The chloroform and ethanol extract showed significant ( $P < 0.01$ ) results compared with the control. The presence of bioactive constituents, including flavonoids, is thought to promote the wound-healing process due to their antioxidant and antimicrobial activities. Further studies are in progress to isolate the active compounds.

**Keywords:** granulation tissue, skin breaking strength, *Verbenaceae*, wound contraction

## INTRODUCTION

Wound healing consists of a systematic progression of events that establish the integrity of damaged tissue. The process of wound healing is essential to prevent the invasion of damaged tissue by pathogens and to partially or completely reform the damaged tissue. Healing involves different phases, including inflammation, granulation, fibrogenesis, neo-vascularization, wound contraction and epithelialization (Clark and Henson 1996). The process of wound healing is promoted by several natural plant products, which are composed of active principles like flavonoids, triterpenes, alkaloids, tannins and other biomolecules. These agents usually influence one or more phases of the healing process. The wound healing properties of *Aloe vera* (Chitra *et al.* 1998), *Tridax procumbens* (Udupa *et al.* 1995) and curcumin (Sidhu *et al.* 1998), have been reported and experimentally studied on various animal models. Recently, Kartick *et al.* (2008) reported that *Pluchea indica* also has wound-healing properties.

The plant *Clerodendrum infortunatum* Linn. is an indigenous medicinal plant that belongs to the *Verbenaceae* family, and is widely distributed in various parts of India, Ceylon, Malaysia and Bangladesh. Traditionally, the plant is used as an aphrodisiac, antipyretic, and antihelminthic. The plant is useful in relieving thirst and burning sensation, foul odours, and blood diseases (Khatry *et al.* 2005). The root of the plant is prescribed for tumors, certain skin diseases and scorpion bites (Kirthikar and Basu 1987). Roots and fresh juice of leaves of *C. infortunatum* were used in eliminating ascarids and tumors and also as a laxative (Anonymous 1992). Two flavonoids from the roots of *C. infortunatum*, cabruvin and quercetin, showed strong antifungal activity (Roy *et al.* 1996). The phytochemical investigations on leaf of *C. infortunatum* have revealed the presence of flavonoids (Sankara and Ramachandran Nair 1973). The ethanol extracts *C. infortunatum* possess potential antibacte-

rial activity (Nishanta Rajakaruna 2002).

Flavonoids are one of the important biologically active substances present in *C. infortunatum* leaves and roots. These polyphenolic compounds display a remarkable spectrum of biological activities. However, the medicinal value of *C. infortunatum* root extracts pertaining to wound healing has not yet been reported, and is the focus of this study.

## MATERIALS AND METHODS

### Plant material collection and extraction

Roots of *C. infortunatum* were collected from Lakkinakoppa range forest of Bhadra Wild life Sanctuary, Karnataka, India. The plant was authenticated by Dr. V. Krishna (one of the authors) who is also a taxonomist and the same was confirmed from the 'Flora of Davanagere District' (Manjunath *et al.* 2004). The specimen is deposited at Kuvempu University, Shankaraghatta, Karnataka, India. The material was air dried in shade for two weeks, powdered mechanically and stored in airtight containers for further investigations. One kilogram of the powdered material was refluxed with 1/10 (w/v) petroleum ether, chloroform and ethanol in a Soxhlet apparatus for 48 h in batches of 250 g each. Every time, before extracting with the next solvent the mark was dried. The yield of petroleum ether, chloroform and methanol extracts was 4.8, 2.2 and 16.5 g, respectively.

### Chemicals

Solvents petroleum ether, chloroform and ethanol were obtained from Merck India Pvt. Ltd., (Mumbai, India). Folin-Ciocalteu reagent, sodium carbonate, Gallic acid was purchased from Himedia Pvt. Labs., (Mumbai, India). White Bees wax, Hard paraffin, Cetyl alcohol and White soft paraffin were purchased from SD Fine Chem. Ltd, (Biosar, India). All other chemicals and reagents used were of the analytical grade.

## Total phenolic assay

The concentration of total phenolics in the petroleum ether, chloroform and ethanol extracts were determined according to the protocol described by Chandler and Dodds (1993). 1 mL of each root extract was mixed in a test tube containing 1 mL of 95% ethanol, 5 mL of distilled water and 0.5 mL of 50% Folin-Ciocalteu reagent. The resultant mixture was allowed to react for 5 min and 1 mL of 5% sodium carbonate was added. It was mixed thoroughly and placed in the dark for 1 h, and absorbance was recorded at 725 nm using a UV-VIS spectrophotometer (Shimadzu UV-240 Spectrophotometer, Japan). The total phenolic contents in root extracts of *C. infortunatum* were expressed as gallic acid equivalents in mg/g of the extract.

## Evaluation of wound-healing activity

### 1. Experimental animals

Wistar albino rats of either sex, weighing about 240-250 g were used for the study. The animals were obtained from National College of Pharmacy, Shimoga, Karnataka, India. Animals were housed six per polypropylene cage and given free access to standard laboratory diet (Hindustan Lever Ltd., Bangalore, India) and water during the experiment. The Institutional Ethical Committee (Registration Number 144/1999/CPCSEA/SMG) permitted the study under the certification Ref No NCP/IAEC/CLEAR/06/2007-08.

### 2. Acute toxicity studies

The acute toxicity study for all the three extracts of *C. infortunatum* root was performed using the Wistar albino rats of either sex. In the pilot toxicity experiments the three extracts were administered orally in increasing doses starting from 500, 1000, 1500, 2000 and 2500 mg/kg b.w. The signs of toxicity were observed up to 14 days after the oral administration. No symptoms of toxicity and mortality were observed up to the dose of 2000 mg/kg.bw for all the extracts.

### 3. Drug formulation

To study the incision and excision wound model a cream base was used for topical application. 4% (w/w) ointment cream (cream base + extract) of petroleum ether, or chloroform or ethanol extracts was prepared by the fusion method (melting ingredients method) as described by Bharath (1996). Briefly, for the preparation of 100 g of cream base 2 g of white Bee wax, 3 g of hard paraffin, 90 g of white soft paraffin and 5 g of cetyl alcohol were mixed and warmed to get a homogenous cream base. To study the dead space model animals were administered orally with the extracts. To dissolve the extracts 1% gum tragacanth was used.

### 4. Excision wound model

A circular wound of about 500 mm<sup>2</sup> was made on the depilated, ethanol-sterilized dorsal thoracic region of rats under light ether anaesthesia (Leite *et al.* 2002). The animals were divided into five groups of six animals. Group I was untreated (control); Group II was treated with 1% (w/w) nitrofurazone (Ranbaxy, Mumbai, India) ointment and served as a reference standard (positive control); Groups III, IV and V were treated topically with the cream base prepared from petroleum ether, chloroform and ethanol extract root of *C. infortunatum*, respectively, on a wound created on the dorsal back of rats daily until the wounds healed completely (Chatterjee and Chakravorty 1993). The percentage of wound closure was recorded on day 4, 8, 12 and 16 and the wound area was traced and measured planometrically. The actual value was converted into a percent value taking the size of the wound at the time of wounding as 100%.

### 5. Incision wound model

As explained above, rats were anaesthetized prior to and during creation of the wound. The dorsal fur of the animals was shaved

with a clipper. A longitudinal paravertebral incision, 6 cm in length was made through the skin and cutaneous muscle on either side of the vertebral column of the rat as described by Ehrlich and Hunt (1969). Care was taken to see that incision was at least 1 cm lateral to vertebral column. After the incision, surgical sutures were applied to the parted skin at intervals of one centimeter. The wounds were left undressed. The sutures were removed on the 8<sup>th</sup> post wound day and the treatment was continued. The skin-breaking strength was measured on the 10<sup>th</sup> day by the method described by Lee and Tong (1968).

### 6. Dead space wound model

For the dead space wound model the animals were divided into five groups containing six each. Group I served as the control and was orally treated with 1 ml/kg of 1% gum tragacanth readily available (Merck Pvt. Ltd., Mumbai, India). Groups II, III and IV were treated with an oral dose of petroleum ether, chloroform and ethanol extract (250 mg/kg b.w) respectively. The animals were anaesthetized with light ether anesthesia and the dead space wounds were created by subcutaneous implantation of sterilized cylindrical grass piths measuring 2.5 cm × 0.3 cm (Sisco, Bangalore, India) one on either side of the dorsal paravertebral surface of the rats. The granulation tissue formed on the grass piths were removed on 10<sup>th</sup> post wounding day and subjected to breaking strength and histological study.

### Statistical analysis

The results of these experiments are expressed as mean ± S.E. of six animals in each group. The data were evaluated by one-way ezANOVA followed by Tukey's pair-wise comparison test. The values of  $P < 0.01$  were considered as statistically significant.

## RESULTS AND DISCUSSION

### Total phenolic assay

Biological activities of phenolic compounds are well known due to the presence of potential antioxidants and free radical scavengers (Rice-Evans *et al.* 1995; Marja *et al.* 1999; Sugi-hara *et al.* 1999). Therefore, many of the medicinal properties of the plants are attributed to the presence of phenolics and flavinoids. In the present study we have tried to estimate the total phenolic content of the plant *C. infortunatum* L. The total phenolic contents of extracts obtained from leaves using petroleum ether, chloroform and ethanol was found to be 66.6, 164.2 and 460.0 mg/g in terms of gallic acid equivalent respectively. Extraction with ethanol yields the highest amount of phenolic content followed by chloroform and petroleum ether extract. The estimation of phenolic content of *C. infortunatum* was done using Folin-Ciocalteu reagent that produced blue colour by reducing yellow hetero polyphosphomolybdate anions (Huang *et al.* 2005). It was observed that as the number of hydrogen donating groups increases in the phenolic compounds the intensity of blue colored complex also increases. This is a clear indication of the high phenolic contents in the extracts. The results obtained from this estimation are in agreement with the observations made by Shrivastava and Patel (2007) where the major chemical components reported from the genus *Clerodendrum* are phenolics. Thus, it can be clearly said that the phenolics constitute as the major components of this plant and therefore, this characteristic can be apparently correlated to some of the pharmacological effects.

### The percentage of wound closure

Wounds are physical injuries that result in an opening or break of the skin. Proper healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin. Healing is a complex and intricate process initiated in response to an injury that restores the function and integrity of damaged tissues. Wound healing involves continuous cell-cell and cell-mat-

**Table 1** Effect of topical application of root extracts of *C. infortunatum* on excision wound model.

Groups	4 <sup>th</sup> day	8 <sup>th</sup> day	12 <sup>th</sup> day	16 <sup>th</sup> day	Epithelialization (days)
I	24.07 ± 1.06	38.17 ± 0.63	57.31 ± 0.45	74 ± 0.94	24
II	53.16 ± 1.81*	85.22 ± 1.84*	95.99 ± 1.55*	100*	14*
III	29.10 ± 1.25	47.22 ± 1.18	60.30 ± 0.68	78.61 ± 1.01	22
IV	38.19 ± 1.52*	63.30 ± 1.38*	85.35 ± 0.91*	92.96 ± 1.57	18*
V	48 ± 1.73*	78.16 ± 1.68*	93.96 ± 1.11*	100*	16*
F	65.7	98.5	334	170	49.6

Group I – control, Group II – Nitrofurazone, Group III – Petroleum ether extract, Group IV – Chloroform extract and Group V- Ethanol extract  
Values are mean ± S.E.; n = 6 in each group. \*P < 0.01 is compared to control.

rix interactions that allow the process to proceed in three overlapping phases viz. inflammation, cellular proliferation and remodeling (Glynn 1981; Martin 1996). The healing process can be enhanced by using appropriate chemical compounds. There are several compounds isolated from plants or plant products being used as major ingredients in ointment based medicines. In this study while studying the wound healing using the excision wound repair model we have observed that the wound contraction of standard and extract ointment treated groups was found to be more significant ( $P < 0.01$ ) than the simple ointment base treated group. In brief, on day 14<sup>th</sup> the standard ointment treated wound was completely healed, where as the ethanol and chloroform extract ointment treated group healed 100% on day 16<sup>th</sup> and 18<sup>th</sup> respectively. On day 18<sup>th</sup> wound contraction in control animals was found to be 74.94%. It was also observed that epithelialization period of treated and a standard ointment group was less when compared to simple ointment base treated group (Table 1). These results of excision wound repair using ethanol extract based ointment exhibited the complete healing process for a minimum period of epithelialization (i.e. 16 days) indicating the medicinal property of the said plant. A vast majority of the medicinal plants have been proved to possess similar kind of wound healing activity at 16 days of epithelialization (Singh *et al.* 2005). The basic principle of optimal wound healing is to minimize tissue damage and provide adequate tissue perfusion and oxygenation, proper nutrition and moist wound healing environment to restore the anatomical continuity and function of the affected part (Pierce and Mustoe 1995). The wound repair can be mainly attributed to the scavenging activities of the beneficial compounds present in the extracts. We have clearly witnessed an enhanced wound contraction induced by the chloroform and ethanol extracts. This could be attributed by the enhanced contractile property of myofibroblast resulting in the increase of epithelialization. Thiem and Grooslinka (2003) reported that topical application of compounds with free radical scavenging properties in patients have shown to improve wound healing significantly and protect tissues from oxidative damage. The presence of phenolics in *C. infortunatum* also supports these results as phenolic compounds are known for free radical scavenging property.

### Tensile breaking strength

In another wound repair model of wound incision, the animals treated with the chloroform and ethanol extracts showed an increase in breaking strength ( $411.67 \pm 8.33$ ), ( $528.33 \pm 9.1$ ) respectively when compared with the control animals ( $390.33 \pm 8.19$ ). The mean breaking strength of the animals treated with the positive control was ( $P < 0.01$ ) significant (Table 2). The breaking strength is the ability of healing wound which is measured experimentally by the amount of force required to disrupt it. In the initial stages wound will be having little breaking strength because the clot alone will be holding the edges together. In these experiments an increased breaking strength of skin has been observed.

The data depicted in Table 3 shows effect of oral administration of the suspensions of the *C. infortunatum* extracts on dead space wound model assessed by the increase in the

**Table 2** Effect of topical application of root extracts of *C. infortunatum* on incision wound model.

Treatment	Breaking strength (g)
I	390.33 ± 8.19
II	606.67 ± 17.64*
III	411.67 ± 8.33
IV	528.33 ± 9.1*
V	583.33 ± 12.02*
F	72.0

Group I – control, Group II – Nitrofurazone, Group III – Petroleum ether extract, Group IV – Chloroform extract and Group V- Ethanol extract  
Values are mean ± S.E.; n = 6 in each group. \*P < 0.01 is compared to control

**Table 3** Effect of oral administration of root extracts of *C. infortunatum* on dead space wound model.

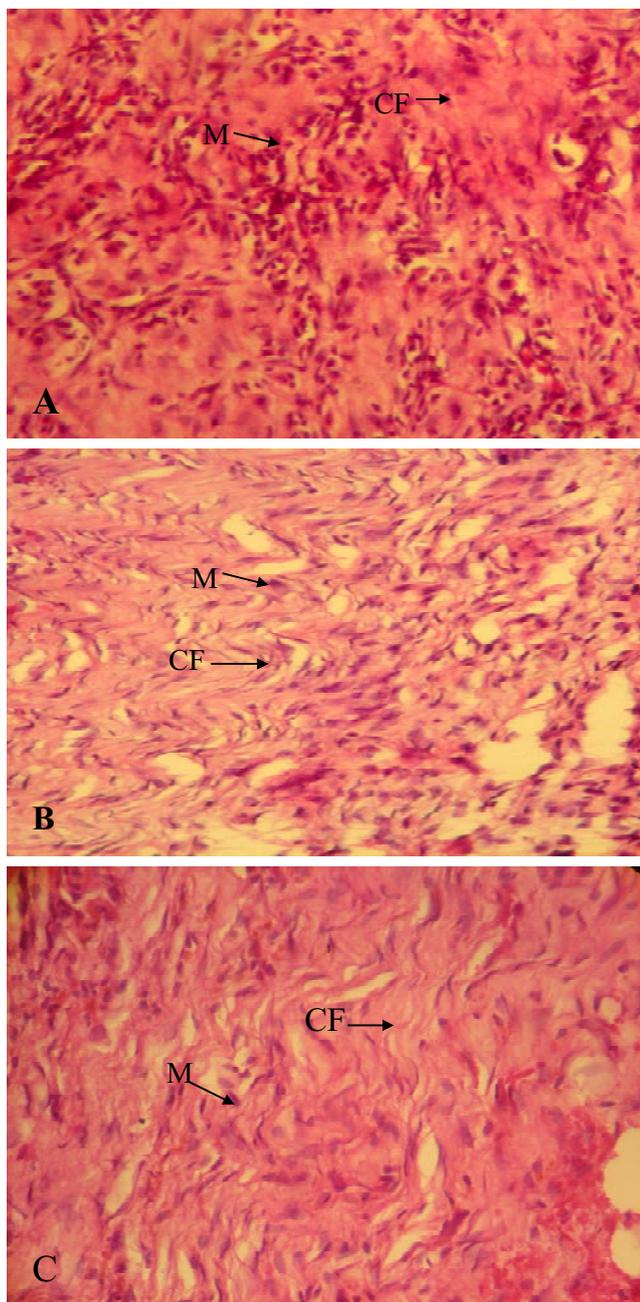
Treatment	Granulation tissues dry weight (mg/100 g)	Breaking strength (g)
I	91 ± 0.82	255.00 ± 5.00
II	105 ± 0.86	275.00 ± 6.19
III	136.43 ± 0.91*	338.33 ± 8.33*
IV	151.67 ± 1.2*	365.00 ± 8.85*
F	428	50.9

Group I – control, Group II– Petroleum ether extract, Group III – Chloroform extract and Group IV – Ethanol extract  
Values are mean ± S.E.; n = 6 in each group. \*P < 0.01 is compared to control.

weight of granulation tissue and increase in its tensile strength. In dead space wound model, the presence of the foreign body in the subcutaneous area initiates the formation of granulation tissue around the wound, which has the appearance of pink granules protruding from the floor of the wound. Microscopically these granules possess newly formed capillaries, fibroblasts and leucocytes. When more and more collagen fibers are laid down, vascularization of tissue decreases. The breaking strength of the granulation tissue increases proportionately with the collagen deposition. The reports of Azad (2002) indicated that the increase in weight of the granulation tissue is due to the presence of higher content of protein. Between the three tested groups the wound healing activity of the chloroform and ethanol extract is comparatively more than the petroleum ether extract. In the chloroform and ethanol extracts the significant wound healing activity observed could be due to the presence of bioactive chemical constituents.

Histology of the wound tissue of the control animals showed the presence of acute inflammatory cells, fibroblastic connective tissue and very little number of blood vessels (Fig. 1A). The lesser epithelialization and lesser collagen formation indicated incomplete healing of the wound in control animals. The sections of the granuloma tissue of the animals treated with chloroform extract showed moderate epithelialization, fibrosis and collagen formation (Fig. 1B). In ethanol extract treated animals the histology of granulation tissue showed complete healing with more of fibroblasts within marked increase of collagen tissue and increased number of blood vessels and lesser macrophages (Fig. 1C).

Thus, the results of this study showed that the chloroform and ethanol extracts ointment of *C. infortunatum* effectively stimulates wound contraction; increase tensile strength of incision and dead space wounds as compared to control



**Fig. 1** (A) Section of the granulation tissue of the control animal showing more number of macrophages (M) and lesser collagen fibers (CF) (Hematoxylin-eosin, 100X). (B) Section of the granulation tissue of the animal treated with chloroform extract of *C. infortunatum* showing moderate deposition of CF and moderate number of M (Hematoxylin-eosin, 40X). (C) Section of the granulation tissue of the animal treated with ethanol extract of *C. infortunatum* showing complete epithelialization. Arrow indicates the deposition of CF, lesser M (Hematoxylin-eosin, 40X).

group. The histological work indicates that the compounds in *C. infortunatum* stimulates and enhances the faster lay-down of collagen fibers.

Triterpenoids and flavanoids are known to promote the wound-healing process mainly due to their antimicrobial and antioxidant properties. In recent years, oxidative stress has been implicated in a variety of degenerative processes and diseases. These include acute and chronic inflammatory condition such as wound healing (Maier 2002). In our studies we have observed high phenolic contents. Phytochemical screening by other laboratories has also revealed the presence of flavonoids in *C. infortunatum* (Roy *et al.* 1996). The flavonoids which are responsible for the free radical scavenging activity are believed to be one of the important

components in wound healing process (Devipriya and Shyamaladevi 1999). Pankaj *et al.* (2007) have shown that *C. infortunatum* possess anti-oxidant property using few *in vitro* parameters. Similar results have been obtained in our laboratory with both *in vitro* and *in vivo* antioxidant protocols. Therefore, the results of the present investigation and the reports from other laboratories certainly indicate the presence of prohealing activity of *C. infortunatum*. Several reports are available suggesting similar type of wound healing activities of several plants which are mainly attributed to the presence of bioactive compounds (Kumar *et al.* 2007). Thus, the results of this pharmacological investigation of the plant *C. infortunatum* provides a clear pharmacological evidence of wound healing activity and hence this plant may be used as a better source of therapeutic compounds useful in wound healing processes.

## ACKNOWLEDGEMENTS

We acknowledge the encouragement of Prof. B. Abdul Rahiman, Dept of Biotechnology and Bioinformatics. We also acknowledge the help rendered by The Principal, National College of Pharmacy, Shimoga, Dr. Sanjay, and Adithya Pathological Laboratory. One of the authors G.K. is highly indebted to the SC/ST Cell, Kuvempu University for awarding Junior Research Fellowship (JRF).

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