A Matrix Effect in Mixed Phospholipid/Fatty Acid Vesicle Formation

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Efficacy of *Ageratum conyzoides* on tissue repair and collagen formation in rats

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Summary

Background. Wound healing occurs as a fundamental response to tissue injury. Several natural products have been shown to accelerate the healing process.
Aim. To observe the efficacy of topical administration of an ethanolic extract of *Ageratum conyzoides* on cutaneous wound healing in rats.
Methods. An ethanolic extract of *A. conyzoides* was prepared, and its wound-healing efficacy on rats was studied. An open excision wound was made on the back of each rat, and 200 μL (40 mg/kg body weight) of the *A. conyzoides* extract was applied topically once daily to the treated wounds. The control wounds were treated with 200 μL of 50% ethanol. The wound tissues formed were removed at 4, 8 and 12 days after wounding, and biochemical parameters such as DNA, total protein, total collagen, hexosamine and uronic acid were estimated. The extent of epithelialization and the tensile strength of the wounded tissues were also measured.
Results. The *A. conyzoides* extract increased cellular proliferation and collagen synthesis. Wounds treated with the extract were found to heal much faster, based on the improved rates of epithelialization and wound contraction, and on the histopathological results. A 40% increase in the tensile strength of the treated tissue was seen.
Conclusions. Topical application of *A. conyzoides* accelerates the rate of wound healing.

Introduction

Healing of wounds is a chain of processes that includes removal of invading pathogens from the damaged tissue of the body, and complete or partial remodelling of the injured tissues. In general, wound healing proceeds in three interrelated, dynamic and overlapping steps: (i) coagulation and inflammation; (ii) formation of granulation tissue and matrix formation; and (iii) remodelling of connective tissue, collagenization and acquisition of wound strength.[1]

Several natural products promote the process of wound healing.[2,3] In India, several different types of medical practices, based on the different properties of herbs, have been followed for the treatment and cure of various diseases and physiological abnormalities, such as Ayurveda, Siddha and Unani. In addition, Indian folk medicine contains numerous therapies for conditions such as wounds, inflammation, skin infections, leprosy, diarrhoea, scabies, venereal disease, ulcers and snakebite. Over half of the world's population still depends upon traditional medicines for various skin diseases. Herbal medicines for wound management aim at disinfection and debridement of wounds, and at providing a moist environment to promote the natural healing process. We have already shown the

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wound-healing potential of some tropical plants such as *Centella asiatica* (Indian pennywort),[2] *Emblica officinalis* (Indian gooseberry),[3] *Terminalia chebula* (Black Myrobalan)[4] and *Butea monosperma* (Butea gum tree).[5]

Ageratum conyzoides (Family: Asteraceae) is an erect, herbaceous annual, 300–800 mm tall. Its stems are covered with fine white hairs, and its leaves are opposite, pubescent with long petioles, and include glandular trichomes. It has long been known in herbal or folk medicine in Asia and South America as a remedy for various ailments.[6] In Central Africa, it is used to treat pneumonia, but the commonest use is to cure wounds and burns.[7] Chah *et al.*[8] previously reported the antibacterial and wound-healing properties of methanolic extracts of *A. conyzoides*, and found this to be effective in inhibiting the growth of *in vitro* cultures of *Staphylococcus aureus,* a major wound pathogen. Sachin *et al.*[9] reported the wound-healing efficacy of the root extract of *A. conyzoides*. To our knowledge, there has not as yet been any report on the rationale for use of this herb in wound healing. We report on the efficacy of an ethanolic extract of leaves of *A. conyzoides* on the various phases of dermal wound healing.

Methods

The study was approved by the animal ethics committee of the Central Leather Research Institute. All procedures were carried out according to the stipulations of the Institutional Animal Care and Use Committee (IACUC).

Animals and grouping

Male Wistar rats weighing 180–200 g were used for the study. The rats were housed in wire-topped cages with sterilized rice-husk bedding under controlled conditions with a 12 h light/dark cycle and temperature of 29–31 °C, and provided with standard food pellets.

In total, 48 animals were used for the study, divided into two groups (control and treated). Rats were anaesthetized with a mild dose of diethyl ether, then a full-thickness open excision wound, 2 cm^2 in size, was made on the back of each rat as reported previously.[4] The treated rats received 200 μL (40 mg/kg body weight) of the extract applied topically until the wounds healed completely, while the control rats were treated with 200 μL of 50% ethanol for the same period. Six rats from each group were killed on days 4, 8 and 12 post-wounding. The wound tissue was removed, and used for various biochemical analyses. Separate groups of animals were used to measure the rate of contraction and period of epithelialization of wounds.

For the measurement of tensile strength, incision wounds were created. The skin was shaven, and a linear full-thickness incision, 60 mm long, was made on either side of the midline. After the wounds had been mopped dry, intermittent sutures were placed 10 mm apart. Grouping and *A. conyzoides* administration was performed as described above.

Collection and extraction

A. conyzoides was collected from the field research laboratory at the Centre for Advanced Studies in Botany located at Maduravoyal Chennai, in the month of January. The identity of the plant was authenticated by P. R. Jayaraman (Madras Christian College, Chennai). The leaves were dried in the shade, then minced, weighed, powdered and homogenized in 10–20 v/w of 50% ethanol, and filtered to yield a viscous supernatant, used as the crude extract.

Chemicals

L-hydroxyproline, glucuronic acid, calf thymus DNA, chloramine-T and bovine serum albumin were from Sigma Chemical Company, St. Louis, USA. *p*-dimethyl aminobenzaldehyde and Folin's phenol reagent were from Loba Chemie, Mumbai, India. Methyl cellosolve was obtained from Merck, Darmstadt, Germany. All other reagents used were of high analytical grade.

Biochemical parameters

Nucleic acids were first extracted as described previoulsy.[10] First, 100 mg of the wound tissue was homogenized in 5 mL of ice-cold distilled water, then 5 mL of 10% trichloroacetic acid (TCA) was added, and the samples were kept in an ice bath for 30 min to precipitate the proteins and nucleic acids. The contents were separated by centrifugation, and the pellets were washed, first with 1 mL of 10% of TCA and then with 3 mL of absolute alcohol. The lipid-free sediment was resuspended in 5 mL of 5% TCA, and kept at 90°C for 15 min to separate the nucleic acids. The solution was separated by centrifugation, and the supernatant was used to estimate DNA[11] and total protein,[12] as described previously. The total collagen content in wound tissues was estimated by the hydroxyproline index.[13] Previously described methods were used to estimates levels of hexosamine[14] and uronic acid.[15]

Biophysical parameters

The rate of wound contraction was determined by tracing the wound surface on to a transparent graph sheet, and measuring the surface area by planimetry. The period of epithelialization was taken as the number of days for shedding of eschar without any raw wound left behind.

For the measurement of tensile strength, the sutures were removed on day 7 post-wounding, and the tissues were removed day 8 to determine the tensile strength of the wounds.[16]

Histopathology

After the rats were killed, the wound tissues of each animal were removed. These samples were then separately fixed in 10% formalin–saline, dehydrated through a graded alcohol series, cleared in xylene, and embedded in paraffin wax (melting point 56 °C). Serial sections of 5 μm were cut and stained with haematoxylin and eosin and with van Gieson stain. The sections were examined under a light microscope and photographs taken.

Interrupted SDS-PAGE

The subunit composition of the isolated collagen was investigated by interrupted SDS-PAGE as described previously.[17] Briefly, collagen bands were separated by SDS-PAGE using a 3% stacking gel with 5% separating gel, and stained with Coomassie Brilliant Blue.

Statistical analysis

Data are expressed as mean ± SD, and were evaluated using the Student paired and unpaired *t*-tests. $P < 0.05$ was considered significant. All statistical analyses were performed using SPSS software (version 10.0; SPSS Inc. Chicago, IL, USA) and Graph Pad Prism (version 5.0; GraphPad software Inc., San Diego, CA, USA).

Results

Wound healing

A significant reduction in wound size was seen in the animals treated with *A. conyzoides* compared with controls (Table 1, Fig. 1). Biochemical parameters such as DNA, total protein and collagen content in the wound tissues of control and treated wounds are shown in Table 2.

Biochemical results

Levels of hexosamine and uronic acid, the ground substance on which collagen is deposited, were significantly increased by almost twofold in the wound tissues of the control and treated wounds until day 8. The collagen content was increased by 54% compared with the control on day 4, and by 100% for on day 8, then it reduced to 30% on day 12. Similar trends were seen for the DNA and total protein content.

Biophysical results

There was a significant decrease of 33% in the time taken for re-epithelialization of the treated wounds (Fig. 2a) compared with the control. There was also a significant difference in tensile strength, with the treated wounds having an increase of 40% compared with the control wounds (Fig. 2b).

Histopathology

On day 7, compared with the treated group, the control wound tissue had fewer cells and lower collagen deposition, with minimal cellular infiltration. Fibroblasts and macrophages were present in the treated group, with moderate collagen deposition (Fig. 3). Hyalinized collagen, stroma and proliferating capillaries were also seen in the treated wound tissues. On day 14, the wound tissues contained keratinocytes, which were clearly differentiated from the epidermal layer and

Table 1 Effect of *Ageratum conyzoides* leaf extract on percentage wound closure in control and treated rats.

	Day			
Group	3	6	9	12
Control	11.27 ± 1.29	36.75 ± 4.08	50.44 ± 1.33	67.39 ± 2.59
Treated	17.00 ± 1.22**	52.33 ± 4.83**	82.53 ± 2.93**	93.18 ± 18**

Values are expressed as mean ± SD for six animals. **$P \leq 0.05$ compared with the corresponding control.

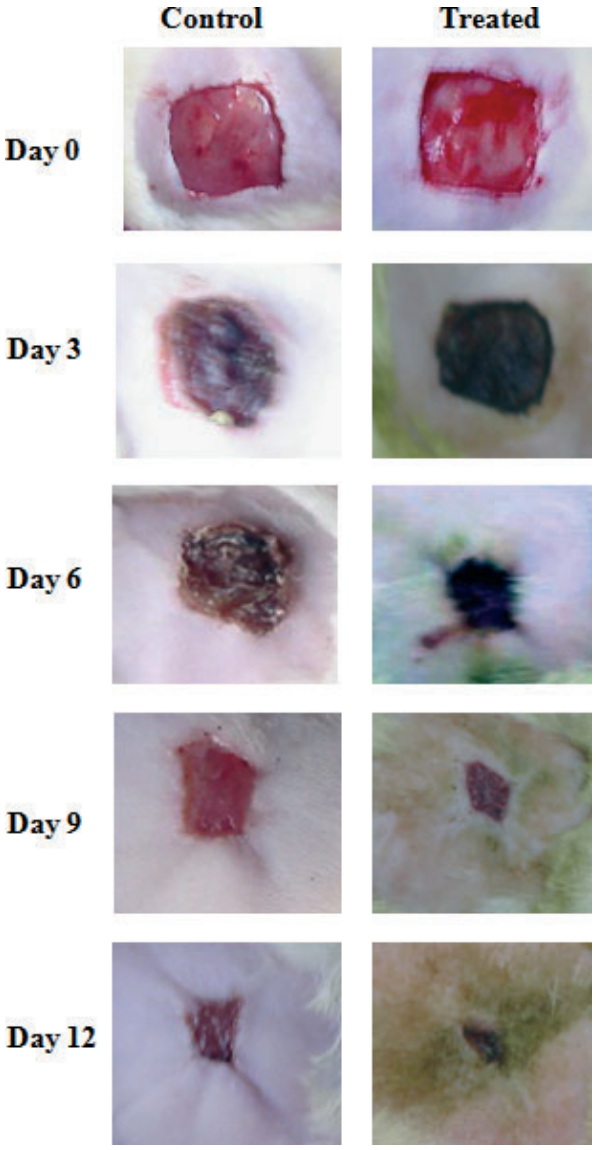

Figure 1 Contraction rate of control and treated wounds on different days (scale bar = 10 mm).

accumulated in the basal lamina of epidermis. Collagen fibres were densely packed and arranged in parallel. There was greater accumulation of the collagen fibres in the extracellular matrix (ECM) region, compared with controls, with prominent thick bundles of collagen fibres embedded with proliferating fibroblasts. By contrast, the collagen fibres in the control wounds were loosely packed with an irregular arrangement, and there were undifferentiated keratinocytes under the basal lamina layer. There was incomplete epithelialization, with less fibrous tissue at the wound site than seen in the treated wounds.

Table 2 Effect of *Ageratum conyzoides* leaf extract on biochemical parameters in control and treated rats.

	Day		
Group	4	8	12
DNA			
Control	2.08 ± 0.27	3.71 ± 0.13	5.11 ± 0.11
Treated	3.23 ± 0.11**	6.21 ± 0.98**	7.63 ± 0.13**
Protein			
Control	3.07 ± 0.42	5.26 ± 0.08	8.10 ± 0.12
Treated	5.71 ± 0.14**	10.25 ± 0.25**	11.60 ± 0.43**
Collagen			
Control	2.80 ± 0.10	4.78 ± 0.75	7.46 ± 0.47
Treated	4.33 ± 0.27**	9.59 ± 0.79**	9.70 ± 0.75**
Hexosamine			
Control	515 ± 34.2	590 ± 29.6	470 ± 18
Treated	970 ± 43**	1251 ± 56**	673 ± 62**
Uronic acid			
Control	150 ± 20	348 ± 24	230.5 ± 20
Treated	370.0 ± 14**	541.2 ± 23**	345.6 ± 18*

Values (mg/g of tissue dry weight for DNA, protein and collagen; μg/g of tissue dry weight for hexosamine and uronic acid) are expressed as the mean ± SD from six rats in each individual experiment. $^{*}P \leq 0.01$, $^{**}P \leq 0.05$ compared with the corresponding control.

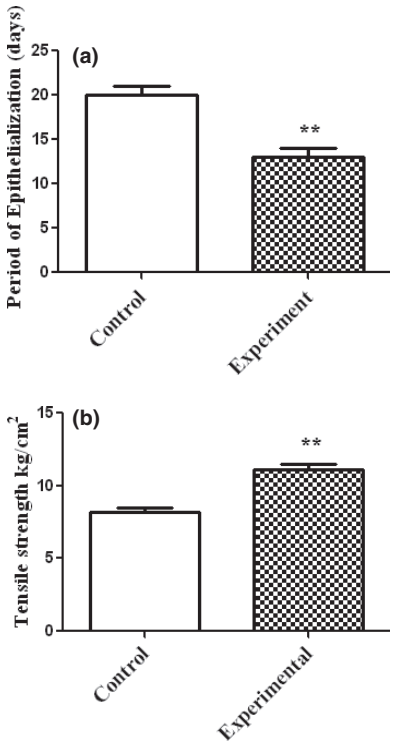

Figure 2 (a) Period of epithelialization (measured as the number of days required for shedding of eschar without leaving any raw wound and (b) tensile strength of tissues in control and treated rats. Values are expressed as mean ± SD for six animals. $^{**}P \leq 0.05$ compared with the control.

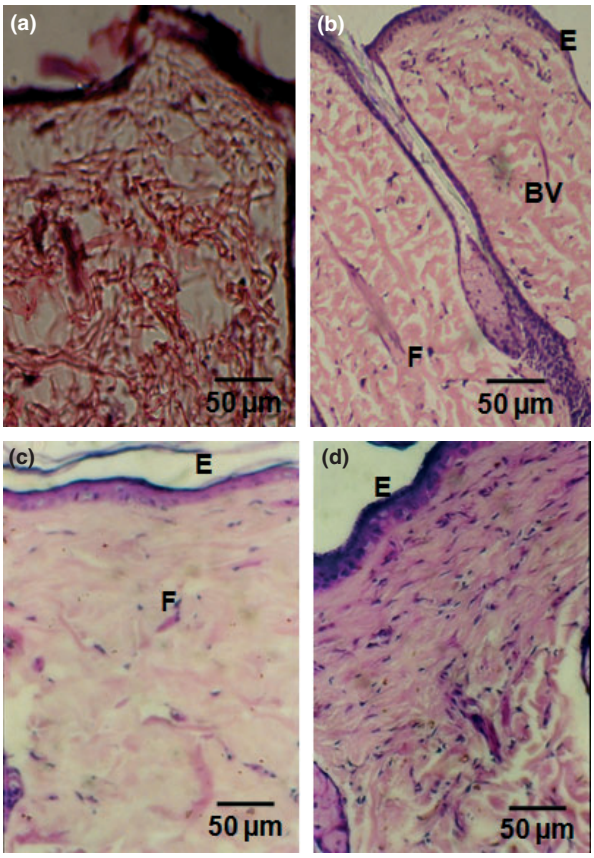

Figure 3 (a,b) On day 7, the *Ageratum conyzoides*-treated tissue has new blood-vessel formation with dense collagen deposition, whereas there was loosely packed collagen with irregular epithelialization in the control tissue. (c,d) On day 14, there was complete, epithelialization of the treated tissue with regularly arranged collagen, whereas the control tissue had a thinner epithelial layer with less collagen. BV, blood vessels; E, epithelialization; F, fibroblast. Haematoxylin and eosin with van Gieson, original magnification ×80. Scale bar = 50 µm.

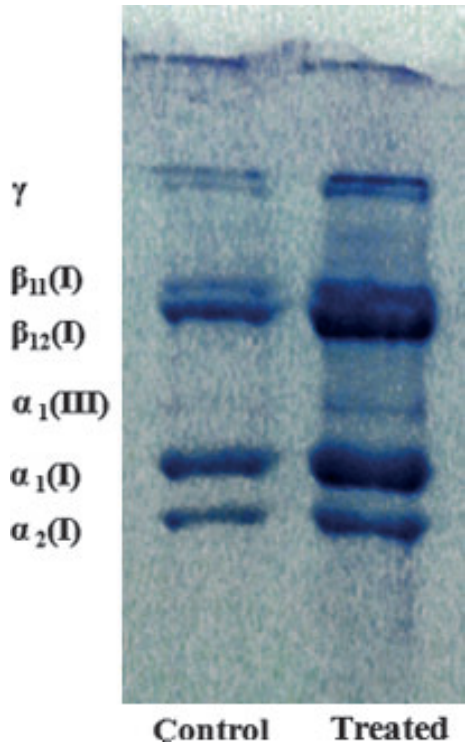

Figure 4 Interrupted SDS-PAGE showing the distribution of type I and III collagen in the acid-soluble fraction of day 8 from control and treated wound tissue. All lanes were loaded with equal amounts of collagen (25 µg).

Table 3 Results of gel electrophoresis (see also Fig. 4).

	Peak areas for collagen chains, %		
Group	Type I	Type III	Type I/III
Control	85.13 ± 0.74	14.98 ± 0.52	5.68
Treated	73.04 ± 0.25	32.38 ± 0.28	2.25

SDS-PAGE

In the SDS-PAGE pattern of acid-soluble collagen of wound tissues, a significant increase in the α1 chains of type III collagen could be seen in the treated wounds compared with the control wounds (Fig. 4, Table 3).

Discussion

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.[1] This complex cascade of event starts from the moment of injury, and the time taken can vary, depending on the extent of wounding.

The involvement of each phase of wound healing largely depends on the type, location and environment influencing the wound. Epithelialization is the process by which keratinocytes migrate from the lower skin layers and divide. Contraction is the centripetal movement of the edges of a full-thickness wound to facilitate closure of the defect. The movement of fibroblasts into the the wound area facilitates ECM formation, and collagen is laid down over and throughout the amorphous material.[18]

In this study, we examined the effects of a tropical evergreen plant, *A. conyzoides*, on rat dermal wound healing. Plants can produce a number of compounds that can act as agents for wound healing. Plant-based products are preferred in many countries because of their widespread availability, ease of administration,

lack of toxicity (if used correctly), and effectiveness even as crude preparations. *A. conyzoides* is widely used in traditional medicine by various cultures worldwide, although applications vary by region.[6,7]

Several pharmacological investigations have been conducted to determine the efficacy of *A. conyzoides*. Almagboul *et al.*,[19] using a methanolic extract of whole plant, studied the inhibitory action of *A. conyzoides* in retarding the development of *S. aureus*, *Bacillus subtilis*, *Eschericichia coli* and *Pseudomonas aeruginosa*. Biokia *et al.*[20] reported that an aqueous extract of *A. conyzoides* leaves (100–400 mg/kg) had an effective analgesic action in rats. Studies in Kenya showed that an aqueous extract of the whole plant had muscle-relaxing activities, confirming its popular use as an antispasmodic.[21] These findings prompted us to carry out further investigations into this plant to examine its wound-healing efficacy.

The increase in DNA content that we found in the treated wounds indicated hyperplasia of the cells. This growth was accompanied by a concomitant increase in the total protein and collagen. Hexosamine and uronic acid are the matrix molecules that act as the ground substance for the synthesis of new ECM. The early increase in hexosamine and uronic acid shows that fibroblasts actively synthesize the ground substance on which collagen is laid down. It has been reported that there is an increase in the levels of these components during the early stages of wound healing, after which normal levels are restored.[22] We found a similar trend; compared with control, the levels of hexosamine and uronic acid increased in wounds treated with *A. conyzoides* up to day 8. Uronic acid in the wound attracts fibroblasts and stimulates collagen synthesis by providing more fluid, which facilitates greater cell mobility, early remodelling and assists the wounds to heal faster without scar formation.[23] The healing process depends to a large extent on the regulated biosynthesis and deposition of new collagens and their subsequent maturation.[24] Deposition of newly synthesized collagens at the wound site increases the collagen concentration per unit area and hence the tissue tensile strength.

Assessment of collagen content in wound tissues of control and the *A. conyzoides*-treated wounds clearly suggests that *A. conyzoides* enhances collagen synthesis and deposition. There seems to be greater and earlier maturation of collagen fibres in *A. conyzoides*-treated wounds. The significant increase in the tensile strength of wound tissues substantiates this observation.

We also found a significant increase in both collagen type I and type III in *A. conyzoides*-treated wounds, and the ratio between them was greatly reduced, showing that an excess of type III collagen was synthesized. Dermal wound tissue in the early phase of healing resembles embryonic skin, in which there is a higher proportion of type III collagen relative to type I collagen.[25] The increased type III collagen is produced by local fibroblasts, which are activated by the wound-healing process. The early appearance of type III collagen is associated with an early increase in collagen synthesis, which may function in providing wound structure and support for further wound healing. Type III collagen has a far greater platelet-aggregating activity than type I collagen, and it plays an important role in the formation of the homeostatic plug.[26]

Topical drugs are effective in stimulating faster wound contraction and healing because of their greater availability at the wound site. In our study, the rate of wound contraction was significantly higher in rats treated with *A. conyzoides* extract, and the period of epithelialization was shorter. These results support the effectiveness of *A. conyzoides* in wound healing.

Conclusion

Topical administration of an ethanolic extract of *A. conyzoides* was found to improve the various phases of wound repair, including collagen synthesis and maturation, wound contraction and epithelialization.

What's already known about this topic?

- The wound-healing potential of a root extract and of a methanolic extract of *A. conyzoides* has been reported.
- There has been no report on the biochemical significance of this plant on wound healing.
- We report the efficacy of an alcoholic extract of the leaves of *A. conyzoides* on various phases of dermal wound healing.

What does this study add?

- Topical administration of alcoholic extract of *A. conyzoides* was found to improve the different phases of wound repair, including collagen synthesis and maturation, wound contraction and epithelialization.

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