ORIGINAL ARTICLE

Wound healing activity of the methanol extracts of *Clematis* species indigenous to Ethiopia

Segewkal Hawaze, Habtewold Deti, Sultan Suleman
Department of Pharmaceutical Chemistry, School of Pharmacy, College of Public Health and Medical Sciences, Jimma University, Jimma, Ethiopia

Objective: To investigate the vulnerary activity of the methanol extracts of *Clematis longicauda* steud ex A. Rich, and *Clematis burgensis* Engl leaves. **Materials and Methods:** *In vivo* wound healing activity of methanol extracts of *C. longicauda* and *C. burgensis* were studied using excision wound model, re-sutured incision wound model, histopathological analysis and anti-inflammatory activity test. The variables studied were percentage of wound contraction and epithelialisation period in excision wound model; tensile strength in incision wound model; and percentage of acetic-acid-induced capillary permeability inhibition in the anti-inflammatory activity test. The histopathological study qualitatively assessed presence of inflammatory cells, fibroblast proliferation, collagen formation and angiogenesis. Differences between experimental groups were compared by one-way analysis of variance, followed by Dunnett's test. $P < 0.0001$ was considered statistically significant. **Results:** *C. longicauda* and *C. burgensis* extracts treated animals showed significant reduction in wound area and faster rate of epithelialisation, $P < 0.0001$. Extracts of these plants also demonstrated statistically significant wound breaking strength and inhibition of vascular permeability induced by acetic acid. Histological studies on granulation tissue sections showed formation of collagen bundles in the *C. longicauda* and *C. burgensis* extracts treated and standard drug-treated groups while inflammatory cells were present in control. **Conclusion:** Methanol extracts of *C. longicauda* and *C. burgensis* had comparable wound healing activity to Madecassol, containing 1% of *Centalla asiatica* extracts.

Key words: Anti-inflammatory activity, *Clematis burgensis* Engl, *Clematis longicauda* steud ex A. Rich, wound healing activity

INTRODUCTION

A wound is a break in the epithelial integrity of the skin, causing loss of cellular and anatomical or functional continuity of living tissues.[1] Proper restoration of disrupted anatomical continuity and disturbed functional status is achieved through three overlapping stages namely; inflammation, cellular proliferation and remodelling; but the healing process is complete upon knitting of disrupted surfaces with collagen.[2-4] Moreover, in order to avoid tissue damage tissue perfusion and oxygenation, along with nutrition and moist wound healing environment are also required.[5] Therefore, the aim of wound healing studies is to shed light on factors influencing healing process, so that they could be either included or excluded in the clinical practice.[6]

Only 27% of respondents from poor households in low-income countries who needed treatment for chronic conditions reported having received it.[7] Of the six million people suffering from chronic wound worldwide, Sub-Saharan African and South Asian countries have high rate of wound infections due to lack of access to essential medicines. Given this fact and dependence of 80% of the world population upon traditional medicine to treat various skin diseases, the use of medicinal plants as alternatives cannot be over emphasised; since herbal medicines are easily available and affordable.[3,8]

Furthermore, research findings elucidate that one-third of the traditional medicines in use are used for management of dermatological disorders, which is 10-fold of the modern dermatological agents available.[9] There are also several reports confirming the use of medicinal plant extracts for wound healing.[2,9-12] Except the study done on anti-microbial activity and phytochemical content of *Clematis longicauda* steud ex A. Rich and *Clematis burgensis* Engl. leaves extracts, there are no previous studies done on biological activities of these plants.[13] Thus, this investigation is undertaken with the objective to evaluate the wound healing potential of these plants and affirm traditional claims.

Access this article online	
Quick Response Code:	Website: www.greenpharmacy.info
	DOI: 10.4103/0973-8258.122058

MATERIALS AND METHODS

Plant Collection and Extraction

Leaves of *Clematis longicauda* steud ex A. Rich and *Clematis burgensis* Engl. were collected in October 2012

Address for correspondence: Segewkal Hawaze, School of Pharmacy, College of Public Health and Medical Sciences, Jimma University, P. O. Box: 378, Jimma, Ethiopia. E-mail: segewkalh@gmail.com
Received: 26-08-2013; **Accepted:** 07-10-2013

from Unquuree and Caala villages of Sekoru district, South-western Ethiopia. The areas lie at an altitudinal range of 1693-1740 m above sea level and have a dry and hot climate with a mean annual temperature of 19.2° and annual rainfall that varies from 1300 to 1800 mm. Clay soil with a thin layer of humus top is the main soil type and evergreen montane thickets and shrubs are characteristic vegetation types of the area**.** Botanical identification was performed by taxonomy and ethnobotany specialist, and voucher specimen was stored in Regional Herbarium.

The collected leaves were washed in running tap water, shed dried, powdered and used for extraction. Seventy grams of powder was extracted using soxhlet apparatus with methanol (analytical reagent grade, Applichem GmbH, Germany). The extract was collected and concentrated under reduced pressure using rotary vacuum evaporator (Bibly Sterilin ltd., Staffordshire, England) at 40° and the semi-solid mass was dried in an oven (Memmer GmbH co. KG, Germany) at 40° for 10 days.

Preparation of Test Samples for Bioassay

Excision and re-sutured incision wound models were used to evaluate the wound healing activity. Test samples were prepared for the *in vivo* wound models in an ointment base (vehicle) consisting of glycol stearate, 1,2-propylene glycol and liquid paraffin (3:6:1) in 10% (w/w) concentration; 0.5 g/cm^2 of the test ointment was applied topically once daily on the wounded site immediately after each wound was created with a surgical blade. The vehicle group was treated with the ointment base only, whereas the reference drug group was treated with 0.5 g/cm^2 of Madecassol (Madecassol® Bayer, Germany), which contains 1% extracts of *Centalla asiatica*.

Animals and Grouping

Wound healing activity experiments were performed on Wistar albino rats of either sex (150-180 g) and anti-inflammatory activity experiments were performed on Swiss albino mice (20-25 g). All animals were kindly donated from Ethiopian Health and Nutrition Research Institute (EHNRI). The animals were transported following the guidelines for transportation of laboratory animals. In the laboratory, the animals were housed in polypropylene cages and maintained under environmentally controlled room provided with a 12:12 h light/dark cycle for each 24 h periods at a temperature of approximately 25°. They were fed on pellets and tap water *ad libitum*. The animals were allowed to acclimatise to the laboratory environment for 1 h before being subjected to experiments. All experiments were carried out in a quiet laboratory setting with ambient illumination and temperature close to that of the animal house. The study was carried out following the principles of laboratory animal care.[14] The rats were divided into five groups consisting of six in each group, where Group-I served as wound control, untreated; Group-II served as vehicle control, treated with ointment base; Group-III served as reference, treated with Madecassol; Group-IV served as experimental group, treated with ointment of *C. longicauda* methanol extract and Group-V served as experimental group, treated with ointment of *C. burgensis* methanol extract.

Excision Wound Model

The fur was shaved from the dorsal thoracic region of the rats and it was disinfected with 70% alcohol prepared from ethanol (analytical reagent grade, Applichem GmbH, Germany). A full thickness excision wound through a surgical seizer was formed on each animal from a circular template 500 mm^2 along the markings under light diethyl ether (ultrapure, Applichem GmbH, Germany) anaesthesia.[11] Vehicle, Madecassol, *C. longicauda* and *C. burgensis* ointments were applied on the wounded site immediately after wounding. Wounds were left undressed open to environment and animals were kept individually in separate cages. Number of days required for falling of the escher without any residual raw wound commencing from day of excision was noted as period of epithelialisation period. The area of wounds was obtained by tracing the wounds onto a transparent paper and measuring using a graph paper. Wound areas were measured on the day of wounding and subsequently on fourth, eighth, twelfth, sixteenth and twenty-second post-wounding days. The degree of wound healing was calculated as:

Degree of contraction = [1− (wound area on corresponding day/ wound area on zero day)] ×100

Re-sutured Incision Wound Model

Two paravertebral straight incisions of 6 cm length each were made through the entire thickness of the skin, on either side of the vertebral column using scalpel. After complete haemostasis wounds were closed by interrupted sutures placed 1 cm apart. 0.5 g vehicle, Madecassol, *C. longicauda* and *C. burgensis* ointments were topically applied to the wounds once daily. The sutures were removed on eighth post-wound day and application of formulations was continued. The skin breaking strength was measured on the 12th day after the last application. The animals were killed under diethyl ether anaesthesia and Allis forceps were firmly applied on either side of incision wound, three millimetres away from wound margin on adjacent normal skin. The forceps on one side was hooked to a fixed metal rod while the other forceps was attached to a thread suspended by weights running over a pulley. As soon as gapping of the wound occurred, addition of weights was stopped and simultaneously weights were lifted so as to avoid opening of the entire

wound and weights required to produce wound gapping were recorded.[15]

Acetic-Acid-Induced Increase in Capillary Permeability

Each test sample was administered orally to a group of 10 mice at 200 mg/kg body weight and Indomethacin (Indocin®, Lundbeck, Denmark) at 10 mg/kg body weight. Thirty minutes after administration 0.1 ml of 4% Evans blue in saline solution was injected intravenously through the tail of each animal. Ten minutes after Evans blue injection 0.4 ml of 0.5% (v/v) acetic acid was injected intra-peritonealy. After 20 minutes of incubation, the mice were killed by dislocation of the neck, and the viscera were exposed and irrigated with distilled water, which was then poured into 10 ml volumetric flasks through glass wool. Each flask was made up to 10 ml with distilled water, 0.1 ml of 0.1 N sodium hydroxide solution was added to the flask, and the absorption of the final solution was measured at 590 nm (CECIL instruments, Cambridge, England). Control animals were given a mixture of distilled water and 0.5% Carboxymethyl Cellulose, CMC (BDH Chemicals, UK) orally and they were treated in a similar manner.[16]

Histological Study

Twelfth post-wounding day sample granulation tissues were fixed in 10% formalin and embedded in paraffin wax. Serial sections of 5 µm thickness were cut from the paraffin-embedded tissues. The tissues were stained with haematoxylin and eosin, which were examined by light microscope (Leica Microsystems GmbH, Wetzlar, Germany). Sections were observed and qualitatively assessed in respect to presence of inflammatory cells, fibroblast proliferation, collagen formation and angiogenesis.

Statistical Analysis

The data obtained was categorised, coded and entered into SPSS (Windows v 16.0; SPSS Inc., Chicago, IL). Results were expressed as Mean ± SD. The number of observations for the excision and re-sutured incision models were each 6 ($n = 6$), while it was 10 ($n = 10$) for acetic acid induced capillary permeability in each group. The differences between experimental groups were compared by one-way analysis of variance (ANOVA) followed by Dunnett's test. $P \leq 0.0001$ was considered statistically significant.

RESULTS

There was noticeable increase in the degree of wound contraction; thus complete wound healing was achieved faster in the experimental groups when compared with control. All the five groups in excision wound model exhibited decrease in wound area from day to day [Table 1], but there was a significant decrease ($P < 0.0001$) in wound area and epithelialisation period in groups treated with Madecassol and methanol extract of *C. longicauda* and *C. burgensis*.

In excision wound model, the group of animals treated with *C. longicauda* and *C. burgensis* showed 71.97% and 67.91% contraction on 12th post-wounding day, which was close to the contraction due to Madecassol (87.77%). Likewise extracts of *C. longicauda* and *C. burgensis* demonstrated a significant increase ($P < 0.0001$) in tensile strength on 12th post-wounding day ($P < 0.0001$) comparable to Madecasol.

The effect of *C. longicauda* and *C. burgensis* extracts on the inflammatory phase of wound healing, assessed through inhibition of acetic-acid-induced capillary permeability was significant ($P < 0.0001$) comparable with that of Indomethacin [Table 2].

Haematoxylin and eosin stained 12th post-wounding day granulation tissues sections of Madecassol treated animals showed only collagen, while *C. longicauda* treated showed fibroblasts, blood vessels and collagen; *C. burgensis* treated showed fibroblasts, macrophages and collagen. Unlike the treated groups, sections from wound control animals showed inflammatory cells [Figure 1].

Table 1: Wound healing activity of methanol extracts of *C. longicauda* and *C. burgensis*

Groups	Wound area (mm²)±SD and degree of wound contraction (%)					Epithelialisation period (d)	Tensile strength (g)
	Post-wounding day						
	4th	8th	12th	16th	22nd		
Wound control	392.37±2.82	284.43±3.09	202.22±3.30	132.52±2.86	18.98±2.72	24.0±0.63	220±30.33
	19.34	41.53	58.43	72.56	96.10		
Vehicle control	389.48±4.39	280.45±7.85	198.13±3.85	128.47±5.77	14.57±3.4	23.5±0.55	240±35.21
	20.11	42.47	59.36	73.65	97.01		
Madecassol	336.53±2.77	222.53±2.95	60.47±2.76	0.0	0.0	13.33±0.52*	490±16.43*
	31.93*	54.99*	87.77*	100*	100*		
Methanol extract of *C. longicauda*	376.48±2.55	250.45±4.50	137.48±5.70	24.33±2.33	0.0	16.33±0.52*	390±22.80*
	23.24*	48.93*	71.97*	95.04*	100*		
Methanol extract of *C. burgensis*	359.43±1.69	265.4±3.88	157.72±2.81	53.72±2.83	0.0	17.83±0.75*	370±15.17*
	26.86*	45.99*	67.91*	89.07*	100*		

n=6, *The mean difference is significant at *P*<0.0001; SD – Standard deviation

Table 2: Inhibitory effect methanol extracts of *C. longicauda* and *C. burgensis* on acetic-acid-induced increased capillary permeability

Group	Dose (mg/kg)	Evans blue concentration (µg/mL)±SD	Inhibition (%)
Control (CMC)		10.89±1.1	
C. longicauda	200	7.12±0.71*	34.62
C. burgensis	200	7.63±0.59*	29.94
Indomethacin	10	5.24±0.38*	51.88

n=10, *The mean difference is significant at *P*<0.0001; SD – Standard deviation; CMC – Carboxymethyl Cellulose

DISCUSSION

Wound healing is the process of restoring cellular structures and functions to normal state. [1,16] The decrease in wound area, increase in tensile strength, complete wound closure was significantly higher in *C. longicauda* and *C. burgensis* extract treated groups visible on days 3, 12 and 22, respectively, which implies faster epithelialisation and collagen formation.

Qualitative phytochemical screening done on the methanol extracts of *C. longicauda* and *C. burgensis* leaves indicated presence of carbohydrates, proteins, tannins, saponins and flavonoids.[13] On top of this, there are several studies depicting the diverse biological activities of tannins, saponins and flavonoids.

Tannins are beneficial in treating dermatitis by coagulating surface proteins; reducing permeability and secretion; forming a protective layer and due to their anti-microbial properties.[17] *Vernonia scorpioides, Terminalia arjuna* and *Hibiscus rosa sinensis* posses wound healing action due to presence of tannins having effects on regeneration and organisation of the tissues.[12,18,19] Polysaccharides from *Opuntiaficus indica* also demonstrated facilitated healing of wounds.[20] Saponins are also known to promote wound healing process due to their anti-oxidant and anti-microbial activities.[21] Increase in neutrophils infiltrating granulation tissue delays the wound healing process, by meditating lipid peroxidation through production of superoxide anions.[22,23] Flavonoids detected in medicinal plants hinder lipid peroxidation. Therefore, inhibition of lipid peroxidation increases viability of collagen fibrils by facilitating vascularity and strength of collagen fibres, aiding DNA synthesis and halting cell necrosis. [24,25]

As a result it can be implied that there was rapid biosynthetic activity in groups treated with *C. longicauda* and *C. burgensis* during initial phase of granulation. In remodelling phase, maturation of collagen took place by the formation of inter- and intra-molecular cross links, as a result of which the tensile strength was increased. Increased tensile strength

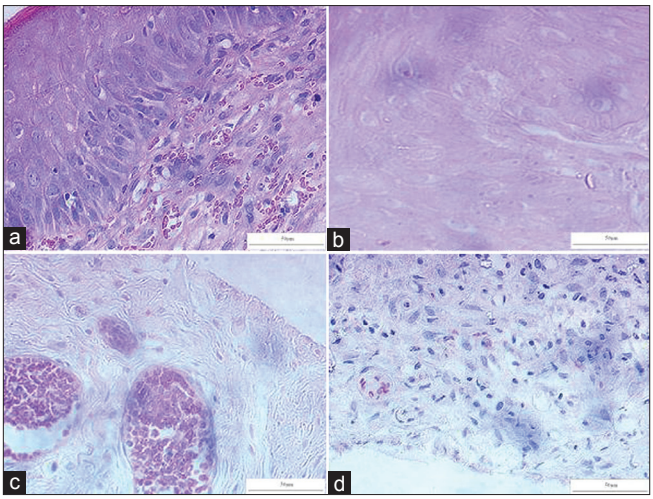

Figure 1: Photomicroscopic view (50 µm) of histopathological characteristics of haematoxylin and eosin stained 12th day granulation tissue sections (a) wound control (untreated); (b) madecassol treated; (c) *C. longicauda* treated and (d) *C. burgensis* treated

is indicative of increase in collagen strength and obviously facilitated wound healing.

Anti-inflammatory activity is also monumental for wound healing, since an extended inflammatory phase causes a delay in healing process; further it is also required for minimal pain and scar formation.[26] Consequently, early tissue approximation and increased tensile strength observed in the treated wounds may also be attributed to individual and/or additive, synergistic and potentiated effects of phytochemical constituents within *C. longicauda* and *C. burgensis*.

Histological examination also provided additional evidence for the wound healing study, which was based on excision and re-sutured incision model. Increase in collagen mass due to enhanced migration of fibroblasts and epithelial cells was observed in treated groups. Decreased collagen mass in wound control group might be due to prolonged inflammatory phase where degradation of collagen is greater than synthesis. Increased cellular infiltration observed from haematoxylin and eosin stained section of wound control groups may be due to presence of pathogens, but the anti-microbial property of phytochemical constituents in *C. longicauda* and *C. burgensis* could have reduced the bacterial/fungal population in treated groups, thereby indirectly reducing inflammatory cells on wound site. Early dermal and epidermal regeneration in treated groups confirmed that ointments containing *C. longicauda* and *C. burgensis* extract had a positive effect on cellular proliferation, granulation tissue formation, and epithelialisation. Well-formed collagen bundles observed on haematoxylin and eosin stained sections of treated groups may suggest efficacy of *C. longicauda* and *C. burgensis* on fibroblast proliferation and synthesis of extracellular matrix

during healing, as compared with less extracellular matrix synthesis in wound control.

Findings from this study demonstrated that the wound healing activity of *C. longicauda* and *C. burgensis* is promising and these plants could be future options of relevance if the exact mechanism of action is further researched. However, further histological, microbiological and toxicological studies are also recommended.

ACKNOWLEDGMENT

The study was financially supported by Jimma University. The authors would like to thank EHNRI for the donation of laboratory animals. We are also grateful to Laboratory of Drug Quality Assurance, Jimma University for letting us utilise the available facilities. Dr. M. Remesh (taxonomy and ethnobotany specialist), Regional Herbarium, Jimma University is acknowledged for his assistance in identification of the voucher specimens.

REFERENCES

1. In: Cotran RS, Kumar V, Robbins SL, Schoen FJ, editors. Inflammation and repair. In: Robbins Pathologic Basis of Disease. Philadelphia: WB Saunders Company; 1994. p. 86.
2. Babu BH, Lakshmi SM. Wound healing activity of methanolic extract of *Martynia annua* L. (Martyniaceae). Inter J Phytotherapy 2012;2:54-8.
3. Kumar B, Vijayakumar M, Govindarajan R, Pushpangadan P. Ethnopharmacological approaches to wound healing-exploring medicinal plants of India. J Ethnopharmacol 2007;114:103-13.
4. Buffoni F, Banchelli G, Cambi S, Ignesti G, Pirisino R, Raimondi L. Skin wound healing: Some biochemical parameters in guinea-pig. J Pharm Pharmacol 1993;45:784-90.
5. Pierce GF, Mustoe TA. Pharmacologic enhancement of wound healing. Annu Rev Med 1995;46:467-81.
6. Stuart E, Patricia P. Pathophysiology of Healing. London: Woodhead Publishing and CRC Press; 2004. p. 234.
7. Wagner AK, Graves AJ, Reiss SK, Lecates R, Zhang F, Ross-Degnan D. Access to care and medicines, burden of health care expenditures, and risk protection: Results from the World Health Survey. Health Policy 2011;100:151-8.
8. Annan K, Houghton PJ. Antibacterial, antioxidant and fibroblast growth stimulation of aqueous extracts of *Ficus asperifolia* Miq. and *Gossypium arboreum* L., wound-healing plants of Ghana. J Ethnopharmacol 2008;119:141-4.
9. Gavimath CC, Sudeep HV, Sujan Ganapathy PS, Padmalatha Rai S, Ramachandra YL. Evaluation of wound healing activity of *Butea monosperma* Lam. extracts on rats. Pharmacology Online 2009;2:203-16.
10. Nagappa AN, Cheriyan B. Wound healing activity of the aqueous extract of *Thespesia populnea* fruit. Fitoterapia 2001;72:503-6.
11. Suguna L, Sivakumar P, Chandrakasan G. Effects of *Centella asiatica* extract on dermal wound healing in rats. Indian J Exp Biol 1996;34:1208-11.
12. Bhaskar A, Nithya V. Evaluation of the wound-healing activity of *Hibiscus rosa sinensis* L (Malvaceae) in Wistar albino rats. Indian J Pharmacol 2012;44:694-8.
13. Hawaze S, Deti H, Suleman S. *In vitro* antimicrobial activity and phytochemical screening of *Clematis* species indigenous to Ethiopia. Indian J Pharm Sci 2012;74:29-35.
14. National Research Council (US) Committee for the update of the guide for the care and use of laboratory animals. Washington (DC): National Academies Press (US); 2011. p. 11-88.
15. Reddy JS, Rao PR, Reddy MS. Wound healing effects of *Heliotropium indicum*, *Plumbago zeylanicum* and *Acalypha indica* in rats. J Ethnopharmacol 2002;79:249-51.
16. Tumen I, Süntar I, Keleş H, Akkol EK. A therapeutic approach for wound healing by using essential oils of *Cupressus* and *Juniperus* species growing in Turkey. Evid Based Complement Alternat Med 2012:1-7.
17. Bedi MK, Shenefelt PD. Herbal therapy in dermatology. Arch Dermatol 2002;138:232-42.
18. Leite SN, Palhano G, Almeida S, Biavatti MW. Wound healing activity and systemic effects of *Vernonia scorpioides* extract in guinea pig. Fitoterapia 2002;73:496-500.
19. Chaudhari M, Mengi S. Evaluation of phytoconstituents of *Terminalia arjuna* for wound healing activity in rats. Phytother Res 2006;20:799-805.
20. Trombetta D, Puglia C, Perri D, Licata A, Pergolizzi S, Lauriano ER. Effect of polysaccharides from *Opuntia-Ficus-indica* (L.) cladodes on the healing of dermal wounds in the rat. Phytomedicine 2006;13:352-8.
21. Mukherjee PK. Quality control herbal drugs: An approach to evaluation of botanicals. New Delhi: Business Horizons; 2002. p. 546-9.
22. Shimizu N, Watanabe T, Arakawa T, Fujiwara Y, Higuchi K, Kuroki T. Pentoxifylline accelerates gastric ulcer healing in rats: Roles of tumor necrosis factor alpha and neutrophils during the early phase of ulcer healing. Digestion 2000;61:157-64.
23. Zimmerman JJ, Ciesielski W, Lewandoski J. Neutrophil-mediated phospholipid peroxidation assessed by gas chromatography-mass spectroscopy. Am J Physiol 1997;273:653-61.
24. Getie M, Gebre-Mariam T, Rietz R, Neubert RH. Evaluation of the release profiles of flavonoids from topical formulations of the crude extract of the leaves of *Dodonea viscosa* (Sapindaceae). Pharmazie 2002;57:320-2.
25. Shetty S, Udupa S, Udupa L. Evaluation of antioxidant and wound healing effects of alcoholic and aqueous extract of *Ocimum sanctum* Linn in Rats. Evid Based Complement Alternat Med 2008;5:95-101.
26. Goldsmith LA, editor. Cutaneous wound repairs. In: Physiology, Biochemistry and Molecular Biology of Skin. New York: Oxford University Press; 1991. p. 576.

How to cite this article: Hawaze S, Deti H, Suleman S. Wound healing activity of the methanol extracts of *Clematis* species indigenous to Ethiopia. Int J Green Pharm 2013;7:304-8.

Source of Support: Nil, **Conflict of Interest:** None declared.