EVALUATION OF WOUND-HEALING ACTIVITY OF LEAVES OF *URTICA PARVIFLORA* ROXB AND *CALLICARPA ARBOREA* ROXB IN RATS

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Summary

Leaves of *Urtica parviflora* Roxb and *Callicarpa arborea* Roxb were chosen to investigate wound healing property on rats. Wound healing study was performed by excision, incision and dead space wound models by administering the methanolic extracts of the two plants at the dose of 300mgkg $^{-1}$day^{-1} p.o and topically applying alcoholic extracts (5% w/w) formulated as an ointment prepared by Indian Pharmacopoeia method. Healing was assessed by the rate of wound contraction, time until complete epithelialization, granulation tissue weight, breaking strength, estimation of hydroxyproline and histopathological parameters. Complete wound contraction was shown by both the plants in the study period. In excision, incision and dead space wound models, all the test drugs showed significant ($P<0.0001$) wound healing activities compared to the control. Moreover the ointment formulation of *Callicarpa arborea* Roxb had been observed to have equipotent wound healing activity as of the standard drug Framycetin. Histological examination of granulation tissue showed the enhanced wound healing property of the extracts by decrease number of macrophages and increased deposition of fibroblasts and collagen.

Key words: *Urtica parviflora, Callicarpa arborea,* Excision, Incision and Dead space wound models.

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Introduction

Wounds are referred to as disruption of the normal anatomic structure and function in a living body (1). It is a very complex, multifactor sequence of events involving several cellular and biochemical processes. The objectives in these processes are to regenerate and reconstruct the disrupted anatomical continuity and functional status of the skin arises due to injury. These processes are initiated immediately after wounding and occur in four stages. The first phase is coagulation, which controls excessive blood loss from the damaged vessels. The next stage of the healing process is inflammation and debridgement of the wound followed by re-epithelialization, which includes proliferation, migration and differentiation of squamous epithelial cells of the epidermis. In the final stage of the healing process collagen deposition and remodeling occurs within the dermis (2, 3). Many traditional remedies are based on systematic observations, methodologies and have been time-tested but for many of them, scientific evidences are lacking.

The plants, *Urtica parviflora* Roxb (Urticaceae), commonly called as Stinging Nettle (English), is a monoecious, perennial herb consisting of long stoloniferous rhizomes (4) and *Callicarpa arborea* Roxb (Verbenaceae), commonly known as Beauty Berry (English), is a small, moderate sized tree about 12m in height found in deciduous or mixed evergreen and deciduous forests on mountain slopes (5,6).The leaves and fresh roots of *Urtica parviflora* are used for the treatment of fracture, dislocation of bones, boils, and decoction of herb is used as a febrifuge (7). Leaves and barks of *Callicarpa arborea* are used for the treatment of rheumatism, cutaneous diseases and juice of fruit relieves fever (8, 9). The literature revealed that *Urtica parviflora* contains acetylcholine, histamine, 5-Hydroxytryptamine, malic acid, aspartic acid, serine, tyrosine and tryptophan. (10). *Callicarpa arborea* has been reported to contain β-sitosterol, β-amyrin, lupeol, epilupeol, ursolic acid, oleanolic acid, L (+)-α-amino-β-(p-methoxyphenyl)-propionic acid, masnilic acid, betulinic acid and baurerol (11). Literature revealed no research works on wound healing activity was performed in these plants. In this context the study was undertaken to explore the wound healing activity of *Urtica parviflora* and *Callicarpa arborea* in experimental animal models.

Methods

Plant material

The fresh leaves of *Urtica parviflora (U. parviflora)* and *Callicarpa arborea (C. arborea)* were collected at Majhitar, East Sikkim, India and were authenticated by Botanical Survey of India (BSI), Gangtok, Sikkim and the herbaria were preserved in the institutional museum (HPI / PK/ No. 131and132).

Extraction

The leaves of *U. parviflora* and *C. arborea,* free from dirt were separated and shade dried for ten days and made to powder by a mechanical grinder. The powdered drugs (500g) were extracted with methanol by continuous hot extraction process (soxhelation). The solvent was recovered and the extracts were concentrated under reduced pressure. The extract yield was found to be 5% for *U. parviflora,* 7.5% for *C. arborea*.

Preliminary Phytochemical Test

The preliminary phytochemical test of the leaf extracts for the presence of alkaloids, flavanoids, terpenoids, glycosides, saponins and tannins was performed by the standard methods (12-14).

Animals

Healthy male albino rats weighing between 160-220gm were used in the study. They were individually housed in aseptic condition and maintained on normal diet and water. They were kept in plastic cages at 23 ± 1^0C in 12:12 hr dark: light cycle. All experiments were carried out between 10:00 and 16:00 hrs. The animal experiments were conducted as per protocol approved by the Institutional Animal Ethics Committee (IAEC) No. HPI/07/60/IAEC/0005.

Wound Models

Excision wound model

For excision wound study, the male albino rats were divided into six groups, each comprising six animals. They were starved for 12 hrs prior to wounding. Under light ether anaesthesia, wounding was performed aseptically. A circular wound of about 2.5 cm diameter was made on depilated dorsal thoracic region of each animal, washed with normal saline and observed during the study. Wounds were traced on 1mm^2 graph paper on the day of wounding and subsequently on alternative days until healing processes were complete. Changes in wound area were calculated, giving an indication of the rate of wound contraction. The alcoholic extracts (5% w/w) were formulated as an ointment prepared by Indian Pharmacopoeia method. The prepared ointments (500 mg) were applied on the wound, once daily for 18 days, starting from the day of wounding in groups V and VI. The extracts were orally fed to the animals in the dose of 300 mg/kg in a form of slurry with the help of oral feeders in group III and IV. No medication other than the extracts was given to the test groups. The standard group was treated with Framycetin (1%) ointment (Soframycin skin ointment, Aventis). While the control group only received the vehicle (2% gum acacia) orally. The percentage of wound closure was observed on 2^{nd} to 18^{th} post wounding days. The period of epithelialization was calculated as the number of days required for falling of the dead tissue without any residual raw wound (15).

Incision wound model

Two paravertebral incisions of 6 cm lengths were made in the skin on either sides of the vertebral column with the help of a sharp sterile blade. The liner wounds are at least 1cm away from the vertebral column. The wounds were sutured using 4-0 number silk thread using a (No. 11) bend needle. The sutures are spaced 5 mm apart. On 8^{th} day the sutures were removed and breaking strength was determined on 10^{th} post wounding day. The breaking strength was measured with a manually operated instrument in terms of weight (16). The animals were treated with drugs as in excision wound model except that the treatment was given up to 9^{th} day.

Dead space wounds
The wounds were made in the region of axilla and groin under light ether anaesthesia where sterilized grass piths of 2.5 cm length and 0.3 cm diameter were introduced in each side to induce granuloma formation. The wounds were sutured and mopped with a saline swab. The animals were treated with drugs except the control group for 9 days from the day of wounding. The granuloma tissues formed on implanted piths were dissected out on the 10^{th} post wounding day. One of the pith was used to determine the tensile strength by the manually operated instrument in terms of weight, while the other pith containing the granuloma tissue was used for estimation of hydroxyproline content by Woessner method (17).

Determination of wound breaking strength
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. Weights ware added slowly and the gradual increase in weight pulled 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, and the procedure was repeated on the contralateral wound (18). 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.

Histopathology Study
The histopathology study was carried on the section of granuloma tissue to observe the stages of keratinization, fibrosis, collagenation, epithelization and neovascularisation. The tissues were fixed in 10% formalin and dehydrated with 90% ethanol, embedded in paraffin, made in to sections of 7µm thickness, stained with haematoxyline-eosin dye and subjected to microscopy. The results are evaluated by numbering 1 to 5. '1' indicates least and '5' indicates maximum similarity with normal tissue in all the groups.

Statistical Analysis:
Values are expressed as Mean ± SEM. Statistical analysis (Graph Pad Prism Software) was made by using Tukey-Kramer Comparisons ANOVA test at different time intervals. $P<0.001$ was taken as significant compared to control.

Results

Preliminary Phytochemical Test
The preliminary phytochemical analysis of the methanolic extracts of *Urtica parviflora* and *Callicarpa arborea* showed the presence of the major phytoconstituents like terpenoids, flavanoids and proteins.

Excision wound model

The result of the excision wound healing model revealed that all the four groups of animals received the extracts and ointments of the two plants showed increased wound contraction continuously from 2nd day to 18th day or the day till they healed (Table-1). The animals treated with 5% ointment of MECA shown the healing of wound completed within 14 days compared to orally treated group took 18 days. However MEUP 5% ointment group took 16 days for complete wound contraction. Hence the ointment formulation of MECA has similar potency of action like standard drug Framycetin in wound healing. The epithelization period was found to be less in ointment formulation of MECA (12.6 days) comparatively which was similar to Framycetin treated group (12.5 days).

Table 1: Wound healing effect of methanolic extracts of *Urtica parviflora* and *Callicarpa arborea* on excision wound model

Treatment Group (Gr)	Epithelization Period (days)	Wound closure (% of original area) in mm^2 on day								
		2	4	6	8	10	12	14	16	18
Control Gr-I	17.4±0.81	15.22 ±0.07	32.14 ±0.28	40.73 ±0.41	59.38 ±1.23	79.11 ±1.86	85.66 ±2.78	91.37 ±2.84	95.23 ±2.92	98.46 ±2.89
Framycetin 1%w/w Gr-II	12.5±0.43	19.28 ±0.06	39.32 ±0.19	79.16 ±0.29	86.44 ±1.01	90.21 ±2.81	98.91 ±2.68	100.00 ±2.55	–	–
MEUP 300 mg/kg p.o Gr-III	14.1±0.69	17.01 ±0.11	34.29 ±0.18	54.16 ±0.37	70.98 ±1.98	83.78 ±2.73	89.23 ±2.91	95.66 ±3.02	99.82 ±2.79	100.00 ±2.13
MECA 300 mg/kg p.o Gr-IV	13.8±0.62	16.56 ±0.08	34.97 ±0.22	56.27 ±0.46	72.14 ±2.14	84.19 ±2.05	91.17 ±2.84	94.92 ±2.93	98.71 ±2.62	100.00 ±1.97
MEUP 5%w/w Gr-V	13.2±0.48	17.91 ±0.09	36.78 ±0.26	71.23 ±0.49	78.53 ±1.69	86.74 ±1.98	95.34 ±2.32	99.33 ±2.05	100.00 ±1.93	–
MECA 5%w/w Gr-VI	12.9±0.51	18.39 ±0.09	37.81 ±0.28	74.18 ±0.47	80.35 ±1.72	88.35 ±2.00	97.62 ±1.93	100.00 ±1.86	–	–
One way ANOVA										
F	8.599	284.19	103.49	1212.1	31.304	3.000	3.000	1.838	0.7294	0.1414
df	5,30	5,30	5,30	5,30	5,30	5,30	5,30	5,30	3,12	2,6
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0259	<0.0259	=0.1355	0.5540^{NS}	0.8709^{NS}

Superscripted N= Not Significant. Other 'P' values are significant, n = 6.
MEUP = Methanolic Extract of *U. parviflora*
MECA = Methanolic Extract of *C. arborea*

Incision wound model
The breaking strength was found to be higher in MECA 5% ointment treated group 709.19g and it was equipotent to standard drug Framycetin group 712.23g (Table 6.2).In incision wound model all the test drugs shown to have significant (P<0.0001 Vs Control) wound healing activity.

Dead space wound model
The three parameters namely dry granuloma weight, breaking strength and estimation of hydroxyproline were examined in this model. Ointment formulation of both the plant extracts showed (Table-2) increased granuloma weight, breaking strength and hydroxy proline content compared to orally administered drugs. However, all the test drugs which have been investigated were significant wound healing property compared to Control (P<0.0001).

Table 2: Wound healing effect of methanolic extracts of *Urtica parviflora,* and *Callicarpa arborea* in incision and dead space wound models.

Treatment Group (Gr)	Incision breaking strength(g)	Dead space		
		Dry granuloma weight(mg/100g)	Breaking strength(g)	Hydroxyproline (µg/100g)
Control Gr-I	389.87±3.86	26.32±0.41	380.44±1.12	1401.22±0.98
Framycetin 1%w/w Gr-II	712.23±2.84	62.12±0.38	612.13±2.31	2439.61±0.87
MEUP 300 mg/kg p.o Gr-III	634.47±3.91	44.39±0.53	481.37±3.02	1958.12±1.09
MECA 300 mg/kg p.o Gr-IV	656.81±3.89	49.53±0.49	498.11±3.57	1979.26±1.03
MEUP 5%w/w Gr-V	700.31±3.67	62.12±0.42	587.19±3.46	2198.78±1.20
MECA 5%w/w Gr-VI	709.19±3.54	68.16±0.44	592.49±3.08	2310.16±1.19
One way ANOVA F df P	 1153.2 5,30;35 < 0.0001	 1185.9 5,30;35 < 0.0001	 950.55 5,30;35 < 0.0001	 118893 5,30;35 < 0.0001

Values are mean ±SE of 6 replicates
P values are extremely significant.
MEUP = Methanolic Extract of *U. parviflora*
MECA = Methanolic Extract of *C. arborea*

Histopathology

The results of the histopathological examinations were recorded in five parameters and were presented in Table-3**.** The ointment form of MECA treated group showed similar stage of keratinization (4.1 ± 0.09) which is comparable to the effect of the standard drug Framycetin (4.2±0.05).The stages of epithelization, fibrosis and Neovascularization were also found to have significantly (P<0.0001) higher in test drug treated group compared to control.

Table 3: Histopathological examinations of methanolic extracts of *Urtica parviflora* and *Callicarpa arborea*

Treatment Group (Gr)	Parameters				
	Keratinization	Epithelization	Fibrosis	Collagen	Neovascularisation
Control Gr-I	0.4±0.09	1.6±0.15	2.5±0.17	2.6±0.17	0.6±0.07
Framycetin 1%w/w Gr-II	4.2±0.05	4.3±0.14	4.2±0.15	4.5±0.17	4.4±0.09
MEUP 300 mg/kg p.o Gr-III	3.6±0.16	3.9±0.17	3.7±0.08	4.0±0.15	3.8±0.10
MECA 300 mg/kg p.o Gr-IV	3.8±0.13	3.9±0.18	3.8±0.09	4.1±0.14	3.9±0.09
MEUP 5%w/w Gr-V	4.0±0.08	4.0±0.09	4.0±0.13	4.3±0.14	4.3±0.08
MECA 5%w/w Gr-VI	4.1±0.09	4.1±0.08	4.0±0.12	4.4±0.16	4.4±0.07
One way ANOVA F df P	 189.50 5,30;35 < 0.0001	 51.637 5,30;35 < 0.0001	 23.210 5,30;35 < 0.0001	 20.414 5,30;35 < 0.0001	 308.30 5,30;35 < 0.0001

Values are expressed ± SEM, n = 6.
Tukey-Kramer Multiple Comparisons Test
P values are considered very significant.
MEUP = Methanolic Extract of *U. parviflora*
MECA = Methanolic Extract of *C. arborea*

Discussion

Wound may be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissue (19). Traditional medicines are always a better choice for the wound healing purpose. Approximately one-third of all traditional medicines in use are for the treatment of wounds and skin disorders, compared to only 1–3% of modern drugs (20). Reports about medicinal plants affecting various phases of the wound healing process, such as coagulation, inflammation, fibroplasia, collagenation, epithelization and wound contraction are abundant in the scientific literature (21-23). In this study we have evaluated the wound healing activities of the methanolic extracts leaves of *U.parviflora* and *C.arborea* by orally and topically in rats.

Here, both the plants shown to have significant wound healing activities in excision, incision and dead space models. The ointment formulation of *C.arborea* showed to have equipotent wound healing activities of the standard drug Framycetin ointment in the studies. Histopathological examination confirmed the mechanism of wound healing by increased deposition of collagen, fibroblast on the granulation tissue and neovascularization. The preliminary phytochemical investigation had showed the presence of flavanoids and terpenoids in the methanolic extracts of these plants. Moreover there are plenty of research studies proved the potent wound healing activities of flavanoids and terpenoids (24). In conclusion the present study explored the wound healing properties of leaves *Urtica parviflora* Roxb and *Callicarpa arborea* Roxb in experimental models. However there is a need for evaluation of phytochemicals responsible for this activity, which may be proved a better drug candidate in future.

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