

Wound healing effect of ethanolic extract from Morning Glory (*Ipomoea carnea* Jacq.) leaves by using different models in rats

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Abstract: Current study aims to evaluate the wound healing effect with apparent mechanism and determination of flavonoid (quercetin) from ethanol extract of *Ipomoea carnea* Jacq. leaves, family Convolvulaceae. The wound healing effect of ethanol extract from *I. carnea* Jacq. leaves screened by excision and incision wound methods in rats. Five groups (Negative control, vehicle control, 2.5% w/w, 5% w/w ethanol extract ointment and 5% w/w Reference Ointment Povidone-iodine group) of rats (n=6) were experimentally wounded at dorsal portion of rats. The 5% w/w ointment of ethanol extract found significant wound contraction at 18-20th days, greater tensile strength, and biochemical parameters. Ethyl acetate fraction of ethanolic extract was analysed by RP-HPLC and retention time was found 3.042 min. The percentage of quercetin was found in *I. carnea* leaves as 0.842%. The results were supported by histopathological studies which showed augment in terms of collagen fibers, fibroblast and new blood vessels. The results were evidently exhibited the traditional uses of *I. carnea* leaves for wound healing effects. The healing effect may be attributed by presence of flavonoid and other compounds present in the leaves with free radical scavenging mechanism.

Keywords: *Ipomoea carnea* Jacq, incision, excision, wound healing, histopathology.

INTRODUCTION

Wound healing is replace devitalized tissue layers and lost cellular structures. The wound healing process comprises by homeostasis, inflammation phase, proliferation and remodeling phase (Gilmore *et al.* 1991). *Ipomoea carnea* Jacq. is well-known as the Morning Glory in English belonging to the family Convolvulaceae (Anonymous, 2001; Indian Herbal Pharmacopoeia, 2002) and a inhabitant of South America. Its cultivated get around in India and mainly found in Madhya Pradesh and Chhattisgarh (Ekka and Dixit 2007). It is fast growing shrubs in all type of soil (dry and wet land, canal, lakes, and ponds) etc.

I. carnea is a folk medicine used to treat of skin disease. Leaves of *I. Carnea* posses antimicrobial and antifungal activity (Agarwal and Uppadhyay 1979; Guleria and Kumar, 2006)

Traditionally the leaves of morning glory externally applied on cuts and wounds to get quick relief (Adhikari *et al.* 2010), free radical scavenging, wound healing, (Abbasi *et al.*, 2010; Konwer *et al.*, 2007) anti-inflammatory and antioxidant activity (Sharma *et al.* 2014), antibacterial activities (Khatiwora *et al.*, 2012) and also used as laxative, mild purgative (Anonymous, 2001; Indian Herbal Pharmacopoeia, 2002). The latex of plant applied locally to relief boils and pimples in the body skin, used locally to alleviate the stomachache, nodules in breast, muscular pain, swelling (Upadhyay *et al.*, 2010).

Leafy latex of *I. carnea* are already reported the presence of flavonoid and evaluated for wound healing activity (Ambiga *et al.* 2007). Previously research reported that quercetin was present in *I. carnea* leaves (Khan *et al.* 2014). Plant flavonoids would benefit the healing process by modulating the concentrations of reactive oxygen species due to the activation of platelets, neutrophils, macrophages, lymphocytes and fibroblasts at different time points of the healing process (Ghosh and Gaba, 2013). *I. carnea* helps to compromise production and reproduction because its lead to hypothyroidism (Wilson, 1975) and leaves shows the antidiabetic effect. The leaves and flower of *I. carnea* have been recorded to contain a polysaccharide Ipomose and saponins and also contain significant amount of phenols, terpenoid and steroid (Vaishali *et al.*, 2012). In the present study ethanolic extract of *I. carnea* was evaluated for effective wound healing. Ethanolic extract was further fractionated by using distilled water and ethyl acetate and obtained a flavonoid rich fraction. A quantitative determination of quercetin was done by HPLC method.

MATERIALS AND METHODS

Plant material

I. carnea leaves were collected from local area of village Kukrikheda, Barela, Jabalpur (India) in the month of September, 2012. The plant was recognized by Department of Botany, Jawaharlal Nehru Krishi Viswavidyalaya, Jabalpur, M.P. and a voucher specimen was submitted. The powdered leaves (100g) were extracted with petroleum ether for defating in soxhlet

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apparatus and extracted with ethanol (95%) for 6 hrs. The extract was concentrated under reduced pressure and weighed for determination of yield.

Chemicals and reagents

Copper sulphate, sodium tartrate, sodium carbonate, chloroform, ethyl ether, ethyl acetate of HiMedia Lab, Mumbai and Petroleum ether, methanol (HPLC grade), acetonitrile (HPLC grade), HCl (Hydrochloric acid) of Merck Ltd, New Delhi, India. All chemicals and reagents were used of analytical grade for study.

Extraction and fractionation

The dried powdered leaves (1.0Kg) of *I. carnea* was defatted with petroleum ether and extracted with ethyl alcohol for 72 hrs. Solvent recovered by distillation and remaining solvent was removed under vacuum. The semisolid ethanol extract was suspended in distilled water and extracted with ethyl acetate in a separating funnel repeatedly. Ethyl acetate fraction of *I. carnea* was dried under vacuum and produced (2.6% w/w) a brown powdered product that gave positive test for presence of flavonoids.

Chromatographic conditions

The HPLC system (Waters) consisted of a pump, UV Visible detector, Thermo C18 (250 X 4.6mm, 5 μ m) column and Data Ace software. The chromatographic study was performed at ambient temperature on a RP-HPLC with C18 analytical column through a mobile phase, acetonitrile: methanol (50:50v/v) isocratically eluted at a flow rate of 1 ml/min. The sample was injected using a 20 μ l fixed loop, and the run time was 10min. The chromatogram was observed with UV at a wavelength of 256 nm.

Preparation of standard stock solution

Standard quercetin (10 mg) was weighed accurately and makes a stock solution of 1000 ppm to a 10 ml volumetric flask with the methanol. Mobile phase was filtered through Whatmann filter paper and degassed.

Sample Preparation

Ethyl acetate fraction of *I. carnea* leaves (10 mg) was taken in 10 ml of volumetric flask and dilute with methanol to obtain concentration of 1000 μ g/ml. The resulting solution was filtered using 0.45 μ m membrane filter paper from Millipore (Milford, USA) and used for injection. The sample solution was chromatographed for the determination of quercetin concentration in fraction samples and found out percentage using regression equation.

Preparation of formulation

The semisolid ethanolic extract (brown color) was formulated as 2.5% w/w and 5% w/w ointment with simple ointment base B.P. by using fusion method (Anonymous, 1953).

Animals

Inbred house wistar rats (200–250 g) of either sex were used in the present study. The animals were acclimatized for 10 days early of the experiment to laboratory hygienic circumstances. The animals were fed pellet foods (Hindustan Lever Pvt, Bangalore, India), and water ad libitum. Animal experiments were done in Guru Ramdas Khalsa Institute of Science and Technology (Pharmacy) Barela, Jabalpur (MP) with prior approval from Institutional Animal Ethical Committee (Registration No. 1471/PO/a/11/CPCSEA, India).

Wound healing study

Grouping of animals

Animals were divided into five groups consisting of six animals in each group as following below:

Group I-Negative control

Group II-Vehicle control

Group III-2.5% w/w ethanol extract ointment of *I. carnea*

Group IV-5% w/w ethanol extract ointment of *I. carnea*

Group V-5% w/w Reference Ointment Povidone-iodine USP (Win-Medicare Pvt. Ltd., New Delhi, India)

Circular excision wound creation

An excision wound was inflicted by cutting away a 500 mm² full thickness of skin from a traced area and at the open atmosphere, left undressed (Lodhi *et al.*, 2006). Wound contraction was measured as percent contraction in each two days after wound formation. The wounds were left undressed to the environment and observed daily. The treatments were applied topically twice a day, from the wound induction time to complete healing.

Longitudinal incision wound creation

Animals were anaesthetized by light ether before wound creation. A para vertebral 1 cm long incision was made at dorsal portion of each animal. Continuous sutures were made with black silk surgical thread on both skin edges by a curved needle (No. 11). All animal groups treatment were given same as in circular excision model. All test samples were topically applied daily up to 9th days. The sutures were removed on the day 9, upon complete healing. The tensile strength of healed tissues was measured (Increase in blood vessels and role of antioxidants were experimentally proved (Lodhi *et al.*, 2011; Chao *et al.* 2013).

Wound healing evaluation

Wound contraction measurement

Wound contraction indicates the rate of reduction of unhealed area throughout the healing development. The better efficacy of medication accelerates the rate of wound closer. An excision wound margin was traced by means of transparent paper. The percent wound contraction was determined in each 2 days interval, and showed as percentage of healed area. The epithelialization time was considered from initial day (Suntar *et al.*, 2010).

The percentage contraction was calculated using the following formula:

$$\text{Percent wound contraction} = \frac{\text{Healed area}}{\text{Total area}} \times 100$$

Wound breaking strength measurement

Breaking strength measurement indicates mechanical properties of the skin depend on the elastic fiber and collagen present in the skin. The breaking strength of the healed wound is the minimum force required to break the incision apart, which indicate the healing efficacy. On the day 9 sutures were removed and the recently formed tissue was excised. Tensile strength was measured by using a Tensiometer (Kuwano *et al.*, 1994). Usually wound healing agents promote a gain in tensile strength. It shows strength of repaired tissue under tension and quality of healed tissue.

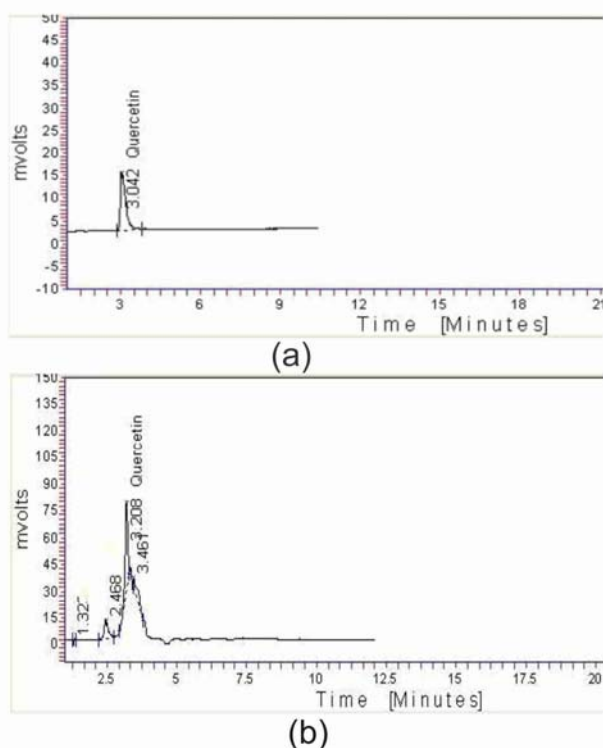


Fig. 1: HPLC Chromatogram of (a) Quercetin (Standard); (b) Ethyl acetate fraction of *I. carnea* leaves

Collagen content measurement

Wound tissues were analyzed for hydroxyproline content that is a basic constituent of collagen on 18th day. Tissues were dried at 60-70°C up to constant weight and samples were hydrolyzed with 6N HCl for 4h at 130°C. The hydrolysate subjected to Chloramine-T oxidation for 20 minutes after neutralization (pH 7). By using Ehrlich reagent (60°C) colored adduct formed (Shukla *et al.* 1999) was read at 557nm. Reference hydroxyproline was also run and values reported as µg/mg dry weight of tissue.

Protein estimation and granuloma weight

On the post wounding days 18th the protein content of skin tissues were determined by method of Lowry (Lowry *et al.*, 1951). With a mixture of copper sulphate, sodium tartrate and sodium carbonate, the tissue lysate was treated. Mixture was treated with Folin-Ciocalteu reagent after 10 minute which provides a bluish color in 20-30 minutes. By the using spectrophotometer, absorbance was taken at 650 nm.

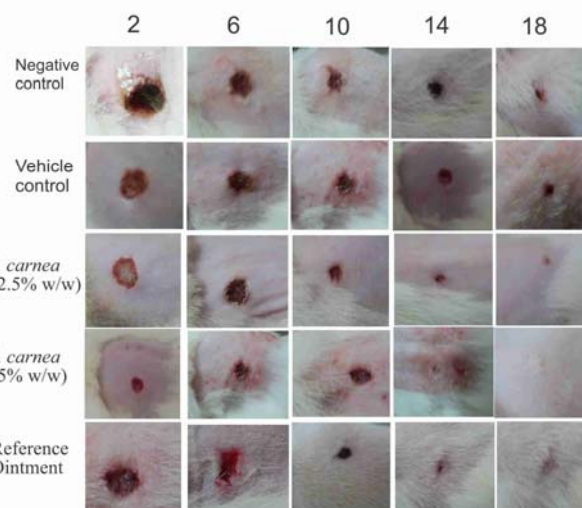


Fig. 2: Wound areas of different groups in excision wound model

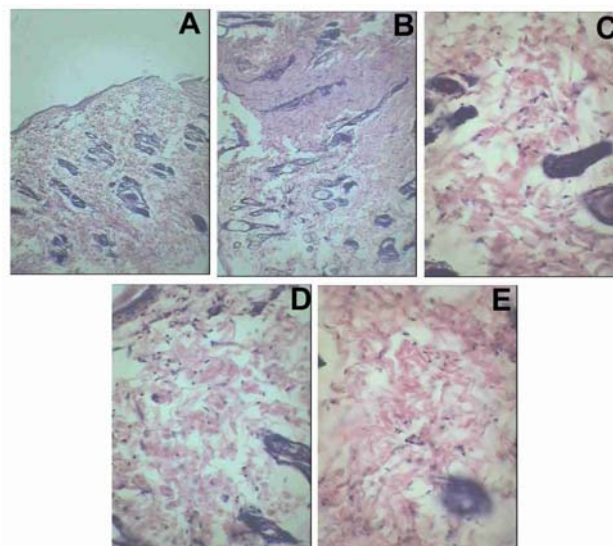


Fig. 3: Photomicrograph of rat skin tissues after post-wounding days for different treatment groups in excision wound model. Hematoxylin and eosin, ×100; (A) Negative Control; (B) Control (Vehicle); (C) *I. carnea* (2.5%w/w); (D) *I. carnea* (5%w/w); (E) Reference Ointment

Histopathological studies

The animals were anaesthetized by diethyl ether and skin sample was taken. On 18th day wound tissue sample from

each group were collected. Tissue samples were processed in 10% buffered formalin, and blocked with paraffin. The sections were cut into 6 μm thickness and stained with hematoxylin and eosin (Varshney *et al.*, 1997). The tissues were examined by light microscope.

STATISTICAL ANALYSIS

Data are presented as the mean \pm standard deviation. Treated groups were compared with the standard and results were statistically analyzed for the comparison using Student's *t*-test. The data were significant at $p < 0.05$.

RESULTS

Phytochemical studies and HPLC analysis

The ethanol extract of *I. carnea* leaves was concentrated under reduced pressure and yield was found 5.7% (w/w). Ethyl acetate fraction of ethanolic extract was analysed for determination of quercetin and found a retention time of the identified quercetin as 3.042 min (fig. 1a and 1b). With the help of standard quercetin we found percentage of quercetin in *I. carnea* leaves as 0.842%.

Wound healing activity

Wound contraction measurement

Wound area was measured in each 2 days interval by tracing on a transparent paper. By subtracting from the original wound area, the healed area was calculated. On day 6, the wound contraction of extract ointment treated groups was found to be significant ($p < 0.01$) in comparison control group. On day 18, standard ointment treated wound was completely healed while extract ointment treated group was also almost at complete healing stage. On day 18th extract ointment (5% w/w) treated group was healed 100% and base treated group showed 87.12 ± 1.24 healing (fig. 2). Epithelialization period of both treated and standard group were less in comparison to simple ointment base treated group shown in table 1.

Wound breaking strength measurement

Tensile strength in the topical application of 2.5% and 5% w/w *I. carnea* ointment groups were found 344.8 ± 28.67 gm/cm² and 366.5 ± 17.74 gm/cm², respectively. The standard group was showed 369.2 ± 23.81 gm/cm² which was significant ($p < 0.05$) than control group (209.3 ± 19.73 gm/cm²) as shown in table 2.

Collagen content, protein level and granuloma weight measurement

In case of incision wound model, the treated group with 2.5% w/w and 5% w/w of *I. carnea* ointment were found significantly ($p < 0.05$) increase in hydroxylproline level as 21.02 ± 3.04 and 25.21 ± 2.32 mg/g tissue, respectively when compared to control group (tables 3). The protein

content for wound treated with 2.5% w/w (38.90 ± 3.25), 5% w/w *I. carnea* (42.66 ± 3.27) and reference ointment (44.61 ± 2.91) group were found when compare to control group (26.40 ± 2.56) (table 3).

Histopathological study

Histopathological examination of stained sections collected from different treatment groups were exhibited different healing degrees of the wounded tissues. The results of study provide a good indication of the extract formulations in promoting wound healing (fig. 3). The observations showed that tissue regeneration was much greater in the wound treated with 2.5% w/w and 5% w/w *I. carnea* extract ointments and reference group without any edema, congestion or inflammatory changes. Both group showed proliferation of epithelial tissue covering the wound area.

DISCUSSION

In the present investigation, *Ipomoea carnea* was evaluated for the wound healing activity and determined percentage of quercetin in leaves extract. The plants of genus *Ipomoea* have long been used in folk medicine for the treatment of a wide variety of pathological conditions like inflammatory and analgesic processes, kidney disease, constipation, and digestive disorders. The abundance of latest research is being to develop more efficient systems of drug for wound healing activity. Wound repair is an integrated series of biochemical and physiological process. Wound healing process consists of different phases such as inflammation, proliferation and migration of connective tissue, production of extracellular matrix including collagen synthesis, epithelial cells migration and proliferation leading to neovascularization of wounded tissue. The remodeling phase in which cells production is balanced by cell death, collagen balanced by degradation, absorption and capillary formation by capillary obliteration (Venkat *et al.* 2010). This study, introduced potential formulation for an efficient wound healing. Angiogenesis plays an important role in wound healing and newly formed blood vessels comprise 60% of the repair tissue. Plants contain a number of free radical scavenging agents, such as flavonoids that have antioxidant property (Vaishali *et al.*, 2012). Flavonoids are also known to promote the rapid wound healing due to their antioxidant, antimicrobial and astringent properties (Tsuchiya *et al.*, 1996). Therefore, wound healing potential of *I. carnea* may be attributed due to the synergistic effect of chemical constituents present in leaves, as well as presence of quercetin in leaves that accelerates the proliferation phase of wound healing.

The naturally occurring flavonoids, in the *I. carnea* leaves extract, have revealed antioxidant action in different models (Auudy *et al.*, 2003). It has been used for numerous therapeutic property such as antiinflammatory,

Table 1: Effect of prepared formulations and reference ointment on percent wound contraction area of excision wound in rats

Groups	Post wounding days (Percent wound contraction)										Epithelia-lization period
	2	4	6	8	10	12	14	16	18	20	
Negative Control	16.19 ±1.61	26.81 ±2.22	37.02 ±2.01	43.58 ±2.12	49.97 ±1.67	54.51 ±2.31	58.23 ±2.97	64.26 ±1.91	68.98 ±2.51	71.89 ±1.93	24
Control (Vehicle)	17.10 ±2.19	27.96 ±2.06	37.74 ±1.78	41.72 ±1.87	47.93 ±2.22	53.24 ±2.30	63.18 ±1.23	69.87 ±1.61	82.31 ±2.23	87.12 ±1.24	23
<i>I. carnea</i> (2.5% w/w)	16.07 ±1.83	27.49 ±2.81	38.26 ±1.16	44.97 ±2.04	54.13 ±1.43	67.27 ±2.16	77.11 ±1.85	87.21 ±1.67	98.20 ±2.19*	100.11 ±1.52	20
<i>I. carnea</i> (5% w/w)	15.26 ±1.08	28.21 ±1.93	40.21 ±2.37	49.18 ±2.45	58.98 ±1.85	70.48 ±2.45	84.21 ±1.71*	94.78 ±1.82*	100.75 ±2.23*	-	18
Reference Ointment	15.21 ±2.53	30.72 ±2.81	45.48 ±1.73	50.22 ±1.36	65.18 ±2.62	72.19 ±2.18	85.77 ±1.18*	96.02 ±2.28*	100.53 ±1.98*	-	18

Table 2: Effect of prepared formulations and reference ointment on tensile strength of tissues from incision wound model in rats

Groups	Tensile strength (gm/cm ²)
Negative Control	194.7±21.32
Control (Vehicle)	209.3±19.73
<i>I. carnea</i> (2.5% w/w)	344.8±28.67
<i>I. carnea</i> (5% w/w)	366.5±17.74
Reference Ointment	369.2±23.81

Table 3: Effect of prepared formulations on hydroxyproline and protein content of tissues from incision wound in rats

Groups	Hydroxyproline (mg/ g tissue)	Protein content (mg/g tissue)
Negative Control	12.33±2.21	25.52±2.83
Control (Vehicle)	13.48±3.05	26.40±2.56
<i>I. carnea</i> (2.5% w/w)	21.02±3.04	38.90±3.25*
<i>I. carnea</i> (5% w/w)	25.21±2.32*	42.66±3.27*
Reference Ointment	26.31±2.13*	44.61±2.91*

n = 6 albino rats per group, value represents Mean ± S.D.

*p < 0.01, when compared each treated group with control group

antifungal, antimicrobial and wound healing (Cesca et al. 2012). Flavonoids also reported to promote the chronic wound healing due to their antimicrobial and astringent properties (Ambiga et al. 2007). Breakdown of collagen produced free hydroxyproline and its peptides. The increased hydroxyproline level of the incision wound has indicated faster collagen turnover leading to rapid healing with simultaneous increase in the wound breaking strength.

The results were also confirms that ethyl acetate fraction contains quercetin which may be responsible for effective wound healing (Khan et al. 2014) and ethanolic extract produces a definite healing action. This was confirmed by a significant increase in wound contraction and by improved epithelialization. The increase in tensile strength was observed, significantly. This was further confirmed by histopathological studies and increased granuloma breaking strength.

CONCLUSION

In conclusion, the outcome of the present study showed that the ethanolic extract of *I. carnea* effectively stimulates wound contraction and significantly increased tensile strength of skin wound tissues. Furthermore the ethyl acetate fraction from ethanolic extract was confirmed the presence of quercetin which can be responsible for significant wound healing effect of *Ipomea carnea* leaves. These finding could validate the involvement of flavonoids present in *Ipomea carnea* in the management of wound healing effects.

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