EVALUATION OF WOUND HEALING ACTIVITY OF FLAVONOIDS FROM *IPOMOEA CARNEA Jacq.*

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Received : 10.04.07 *Accepted : 05.05.07*

ABSTRACT

Natural products have numerous medicinal applications and play important roles in the biology of the organisms that accumulate them. Flavonoids are one large group of natural products with a diverse number of functions in plants and in human health. The isolates of the flowers of *Ipomoea carnea* (Family: Convolvulaceae) was screened for wound-healing activity on the male wistar rats by Excision wound model and Incision wound model respectively. The studies on excision wound model reveals significant wound healing activity of the extract, which is comparable with the reference control sulphathiazole. The isolates of *Ipomoea carnea* show significant activity on all wound models.

KEY WORDS : *Ipomoea carnea*, Wound-healing activity, Kaempferol, Kaempferol-3-O-â-D-glucoside.

INTRODUCTION

One of the largest classes of naturally occurring polyphenolic compounds are the flavonoids[1]. Over 4000 structurally unique flavonoids have been identified in plant source[2]. Primarily recognized as the pigments responsible for the actumnal burst of hues and the many shades of yellow, orange and red in flowers, fruits, vegetables, nuts, seeds, herbs, species, stems, as well as tea and red wine[3-4]. The carbon skeletons of the flavonoids compounds are made up of two distinct units C6-C3-C6 compounds in which the two C6 groups are substituted benzene ring, and the C3 is an aliphatic chain which contain a pyran ring glycosides or in the free state as aglycones with hydroxyl or methoxyl groups present on the aglycone. The average daily intake of flavonoids in the United States is between 150 and 200 mg[5]. Numerous medicinal plants contain therapeutic amounts of flavonoids, which are used to treat disorders of the aquaresis and as anti-inflammatory, antispasmodic and anti-allergic agents. The many pharmacological effects of flavonoids are linked to their ability to act as strong

antioxidants and free radical scavengers, to chelate metals, and to interact with enzymes adenosine receptors and biomembranes. Some flavonoids also possess wound-healing activity[6].

Ipomoea carnea L. belonging to the family Convolvulaceae and a native of South America is cultivated in India as hedge. It is a large diffuse (or) straggling shrub with milky juice. The flowers are pale rose (or) light violet. The leaves have been recorded to contain a polysaccharide Ipomose and saponins. The water-soluble toxic present in the plant has been reported to cause haemolysis and reduce blood pressure. The ether soluble toxin affected the CNS. When administered orally it acted as a mild purgative[7].

Several members of the species Ipomoea (Convolvulaceae) are being used traditionally for the treatment of a large number of disease conditions. Among them, anticancer activity of *Ipomoea bahiensis* has been reported[8]. The present paper describes the wound healing activity of different extracts from *Ipomoea carnea* flower. It also described the wound healing activity of isolated flavonoids, such as Kaempferol, Kampferol-3-O-â-D-glucoside from *Ipomoea carnea* flowers.

EXPERIMENTAL

Extraction and Isolation: Fresh flowers of *Ipomoea carnea* collected in January 2006. Fresh flowers of *I. carnea* (1 kg) were extracted with 95% ethanol (5L) under reflux. The extract was concentrated *in vacuo* and the aqueous concentrate, subjected to successive fraction with various solvents *viz.,* diethyl ether, chloroform and ethyl acetate. The ethyl acetate extract was chromatographed over 40 g Si gel H in a vacuum liquid chromatography column (VLC) (13 x 4 cm). Gradient elution was carried out using chloroform and increasing the polarity with ethyl acetate in 5% stepwise elutions to 100% ethyl acetate (400 x 100 ml) and then with ethyl acetate and increases the polarity with methanol in 5% stepwise increments to 75% ethyl acetate 25% methanol (15 x 100 ml). Fractions 5 and 6 (150 mg) were combined and purified on a sephadex LH-20 column, (40 x 2 cm) using methanol as eluent to give compound 2 (Kaempferol-3-O-â-D-glucoside) ^{MeOH}

The residue from the Et_2O fraction was taken up in a small quantity of Me_2CO and left in an ice-chest for a few days. An yellow solid that separated was filtered and recrystallised (MeOH) when pale yellow needles were obtained (m.p 276-78°C, yield 0.05%, tetra acetate 184 – 86°C) it had λ_{max} nm 266, 320, 370, +(NaOMe), 278, 316, 420: +($AlCl_3$) 268, 303, 350, 424; + ($AlCl_3$-HCl) 269, 302, 352, 420; +(NaOAc) 274, 386 and + (NaOAc-H_3BO_3) 267, 320, 372.

Hydrolysis of the isolated compounds: A few mg of the glycosides were reflux with 10%, HCl in 50% methanol for 3 hours. The aglycone and sugar fractions were identified by chromatography.

Wound healing activity: Incision and excision wound models were used to evaluate the wound healing activity of isolates of *Ipomoea carnea.*

Animals: Healthy, male Wistar rats (150-200g) were used in the study. They were individually housed under standard conditions of temperature (25 ± 2Ú C), 12 hr light/dark cycle, and relative humidity of 45-55% and fed standard pellet diet (Hindustan Lever rat pellets) and water *ad libitum.*

Animals were periodically weighed before and after experiments. The rats were anaesthetized prior to infliction of the experimental wounds. The surgical interventions were carried out under sterile conditions using ketamine anesthesia (10 mg/kg). Animals were closely observed for any infection; those that showed signs of infection were separated and excluded form the study. An acute toxicity study was conducted for the extracts by the stair-case method[9].

Incision wound model: The rats were anaesthetized prior to and during creation of the wounds, with 1 ml of intravenous ketamine hydrochloride (10 mg/kg body weight). The dorsal fur of the animals was shaved with an electric clipper. A longitudinal Para vertebral incision of 6 cm long was made through the skin and cutaneous tissue on the back as described by Ehrlich and Hunt[10]. After the incision, the parted skin was sutured 1 cm apart using a surgical thread and curved needle. The wounds were left undressed. Drugs were removed on 8th post wound day and continued the application of the drug. The skin-breaking strength was measured by the method of Lee[11] on the 10th day evening after the last application. Then the granulation tissue was taken on the 11th day for further studies.

Histopathological study: The healing tissue obtained on the 11th day from all four groups of animals of the incision wound model was processed for histological study. The amount of collagen was quantified.

Excision wound model: The rats were inflicted with excision wounds as described by Morton and Malon[12]. The rats were anaesthetized prior to creation of wounds, with 1 ml of intravenous ketamine hydrochloride (10 mg/kg body weight). The dorsal fur of the animal was shaved with an electric clipper and the area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision wound of 2.5 cm depth (circular area = 4.90 cm^2) and 0.2 cm depth was created along the marking using toothed fourps, a surgical blade and pointed scissors. The entire wound left open[13-14]. The animals were divided into four groups of 10 each. The group

1 animals were left untreated and considered as the control. Groups 2 animals served as reference standard and treated with sulphathiazole ointment. Animals of group

3 and 4 were treated with isolated compound 1 and 2 of *Ipomoea carnea* (200 mg/kg body weight /day) respectively for 14 days. The parameters studied were wound closure, epithelialisation time, collagen content and weight of the excision wound model were taken on 1st, 5th and 15th day following the initial wound using transparent paper and a permanent marker. The recorded wound areas were measured with graph paper. The period of epithelialisation was calculated as the number of days required for falling of the dead tissue remnants without any residual raw wound.

In the excision wound model, granulation tissue formed on the wound was excised on the 15th postoperative day and its weight recorded. The tissue was dried in an oven at 60Ú C and the dry weight was again noted. The protein content of the tissue extract was also estimated[15], Acid hydrolysate of the dry tissue was used for the determination of hydroxyproline[16].

Statistical analysis: Results, expressed as mean ± SE were evaluated using the t – test.

RESULTS AND DISCUSSION

The fresh flowers of *Ipomoea carnea* have been found to contain Kaempferol and its 3-O-â-D glucoside. The UV spectrum of the flavonol aglycone obtained from the Et_2O fraction exhibited two major peaks at 367nm (band I) and 266 nm (band II), which showed a flavonol skeleton. A bathochromic shift of 49 nm on the addition NaOMe revealed the presence of a free 4'-OH group in the B-ring. A shift of +57 nm on the addition of $AlCl_3$ – HCl showed the presence of a free 5-OH in the A-ring. The $AlCl_3$ spectrum was exactly same as that of ($AlCl_3$-HCl) revealing the absence of Catechol type of substitution in B-ring. The 400 MHz ^{1}H-NMR (DMSO-d_6, TMS) spectrum of the flavonol shows a signal at the off set region around 13 ppm, revealing the presence of a free 5-OH proton. The C-6 proton occurs as a doublet at δ 6.2 ppm, higher field than that of C-8 proton, which also occurs as a doublet at which, also occurs as a doublet at δ 6.4 ppm. Protons at C-2', 3', 5'and 6' appear as two pairs of ortho coupled doublets. The H-3', 5' doublet occurs which in turn occurs at δ 8 ppm. Due to the shielding effect of the oxygen substitute. Supporting evidence for the structure of the flavonol was provided by the analysis of ^{13}C-NMR (100 MHz, DMSO-d_6, TMS) data (Table 1).

In the ^{1}H-NMR spectrum (400 MHz, DMSO-d_6, TMS) the A-ring protons at C-6 at C-8 appear separately as doublet at δ 6.2 ppm and δ 6.4 ppm respectively. The 5-OH proton resonates at α 12.6 ppm. In the bring, the protons at C-2', 3', 5' and 6' due to the free rotation of the phenyl ring appear as two pairs of ortho coupled doublets at δ 6.9 and δ 8 ppm. The H-3', 5' doublet occurs up field from the H-2', 6' doublet due to the shielding effect of oxygenation at C-4' as also due to deshielding influence of C-ring operating on H-2' and H-6'. The H-

1" signal of the glucose moiety appears at δ 5.4 ppm found downfield from the bulk of the sugar protons. The remaining glucosyl proton appear in the range δ 3.1 to 3.5 ppm. The β-linkage of the glucose to 3-OH is evident from the large coupling constant 6.3 Hz of H-1".

Comparing ^{13}C-NMR (100MHz, DMSO-d_6, TMS) spectrum of the glycoside with that of the aglycone, the carbonyl carbon at C-4 of the glycoside appears at 1-6 ppm downfield to that of the aglycone. Due to the glycosylation at C-3, its ortho carbon of the glycoside C-4 appears at 2.4 ppm downfield to that of the aglycone and due to the ortho effect C-2 appears 9.5 ppm downfield to that of the aglycone. All the other carbons of the glucoside at A and C rings appears at downfield (Fig 1 a, b).

In both the model studied, significantly improved wound-healing activity has been observed with the two fraction of *Ipomoea carnea*, compared to that of the reference standard and control group of animals. In an incision wound model compound 2 treated animals demonstrated significant skin breaking strength upto 440.0 ± 4.53 when compared to control animals (318.1 ± 3.2). Significant increase in the weight of the granulation tissue ($P < .001$) and hydroxyproline ($P < .001$) content were observed in animals treated with the compound 2 of *Ipomoea carnea* when compared to the control group of animals (Table 1). Compound 1 treated animals, showed moderate rate of wound closure, less skin breaking strength (390.0 ± 3.4), moderately increased weight of the granulation tissue and hydroxyproline content ($P < .05$).

In the excision wound model, Compound 1 treated animals showed significant reduction in the wound area faster rate of epithelialisation (10.2 ± 0.13), increased dry weight of the tissue and increased hydroxyproline content when compared with the control group of animals. But the animals treated with Compound 2 showed moderate reduction in the wound area ($P<.05$) and slower rate of epithelialisation (11.7 ± 0.15). Both hydroxyproline and granulation tissue weight were moderately ($P < .05$) high in comparison to the control group of animals. Table 2 shows the wound area and other biochemical observations of all the four group of animals in excision wound model.

Histological studies of the tissue obtained from the Compound 2 treated group showed significant increase in collagen deposition, few macrophages, tissue edema and more fibroblasts. It was more or less equal to the animals treated with sulphathiazole. In the case of Compound 1 treated animal groups, moderate collagen deposition, macrophages, tissue edema and fibroblasts were observed. The histological studies of the granulation tissue of the control group of animal showed more aggregation of macrophages with lesser collagen fiber. The wound healing was more significant in Compound 2 treated group of animals.

Wound healing normally involves an initial inflammatory phase followed by fibroblast proliferation, formation of collagen fibres and shrinking and drying of the scar. These phases are concurrent but independent of each other.

In resutured wounds, wound breaking strength is determined, which indirectly speaks collagenation phase of healing an parameter is commonly used to assess the healing, perhaps because surgeons are specially interested and concerned with the strength of healed incision wounds[16]. The granulation tissue of the wound is primarily composed of fibroblast, collagen, edema and small new blood vessels. The undifferentiated mesenchymal cells of the wound margin modulate themselves into fibroblast, which start migrating into the wound gap along with the fibrin strands. The collagen composed of amino acid (hydroxyproline) is the major component of extra cellular tissue, which gives strength and support. Breakdown of collagen librates free hydroxyproline could be used as an index for collagen turnover. The data depicted in table 2 showed that the hydroxyproline content of the granulation tissue of the animals treated with Compound 2 of *Ipomoea carnea* was significantly increased when compared to the control and the group of animals treated with Fraction1 indicating increased collagen turnover. In addition, increase in dry tissue weight also indicated the presence of higher protein content. Flavonoids are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving vascularity. Hence any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibres, increasing the circulation preventing the cell damage and by promoting the DNA synthesis. Flavonoids[17] to promote the wound healing process mainly due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelialisation. Similar types of wound healing activity were reported on *Vernonia arborea*[18] and *Pentas lanceolata*[19].

CONCLUSION

Compound 1 and Compound 2 compound of *Ipomoea carnea* demonstrates potent wound healing activity comparable to Sulphathiazole and significantly improved than untreated wounds.

TABLE-1
EFFECT OOF DRUG ON THE *IPOMOEA CARNEA* IN INCISION WOUND

Parameter	Control	Standard	Compound 1	Compound 2
Breaking Strength	318.1 ± 3.2	460.1 ± 4.5	390.0 ± 3.4	440.0 ± 4.53
Granulation tissue wet weight(mg)	87.1 ± 5.2	128.2 ± 4.1	107.0 ± 2.8	125.6 ± 4.11
Granulation tissue dry weight(mg)	12.0 ± 2.3	18.0 ± 0.6	15.0 ± 0.4	20.0 ± 2.30
Hydroxyproline (mg g^{-1})	170.0 ± 2.9	220.0 ± 3.30	183.6 ± 3.0	216.1 ± 3.42

Values are expressed as mean ± SE, n=10 animals in each group, P < .001 when compared to control.
Compound 1 = Kaempferol, Compound 2 = Kaempferol 3-O-â-D –glucoside

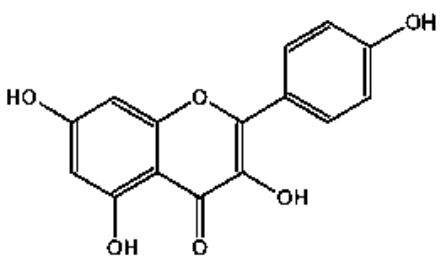
TABLE-2
EFFECT OF DRUG ON THE *IPOMOEA CARNEA* IN EXCISION WOUND

Parameter	Control	Standard	Compound 1	Compound 2
Wound area (mm^2)				
Day 1	190.0 ± 1.83	190.0 ± 1.83	190.0 ± 1.83	190.0 ± 1.83
Day 5	151.3 ± 1.50	122.4 ± 4.14	125.8 ± 5.3	141.6 ± 5.6
Day 15	72.2 ± 1.24	40.5 ± 4.4	43.5 ± 2.1	48.0 ± 4.33
Period of epithelialisation	14.1 ± 0.12	9.8 ± 0.13	10.2 ± 0.13	11.7 ± 0.15
Hydroxyproline (mg g^{-1})	32.0 ± 2.11	57.1 ± 1.73	67.1 ± 7.39	49.5 ± 4.6

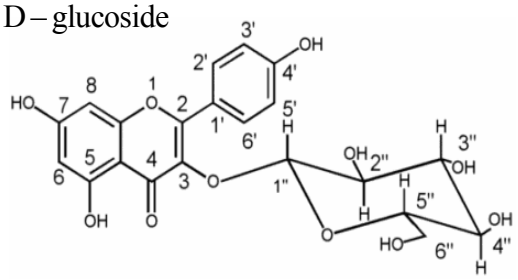
Compound 1- (Kaempferol), Compound 2- (Kaempferol 3-O-â-D - glucoside).
Values are expressed as mean ± SE, n=10 animals in each group, P < .001 when compared to control.

Fig 1: Structure

a. Kaempferol.



D – glucoside



b. Kaempferol 3-O-â-