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Research Article

The wound healing property of ethanolic extract of *Michelia champaca* flowers in diabetic rats

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ABSTRACT

Background: The plant *Michelia champaca* (MC) is widely used in the treatment of inflammation, constipation, dysmenorrhea, ulcers, wounds, fever, and cough. The aim was to evaluate the wound healing property of ethanolic extract of MC flowers in streptozotocin-induced diabetes in rats.
Methods: Wound healing activity was assessed by incision and excision wound models. Five groups of n=6 rats and n=14 rats were used for incision and excision wound models, respectively. Group I rats, non-diabetic control and Group II rats diabetic control, received 1 ml of 0.5% caboxymethylcellolose, which was used to prepare a suspension of ethanolic extracts of MC. Group III, IV and V rats were given MC extract the suspension of 100 mg/kg, 200 mg/kg and 300 mg/kg respectively. Parameters observed were breaking strength of incision wound and wound contraction, epithelialization, hydroxyproline content of excision wound respectively. Results were analyzed using one-way analysis of variance, followed by Tukey's *post-hoc* test.
Results: Breaking strength, rate of wound contraction and hydroxyproline content were significantly increased, and the period of epithelialization was significantly reduced in Group IV and V rats respectively.
Conclusion: Oral administration of ethanolic extract of MC promotes wound healing in diabetic rats. Hence, further study in humans is suggested.

Keywords: Incision wound, Excision wound, Hydroxyproline and diabetic rats

INTRODUCTION

Wound healing is a complex phenomenon that results in the restoration of anatomic continuity and function. Wound healing involves different phases including inflammation, granulation, fibrogenesis, neo-vascularization, wound contraction and epithelialization.[1]

Effective management of the wound requires understanding of the normal repair process and selection of appropriate intervention to optimize the process of healing. Diabetes, even in its early stages can impair the normal course of wound healing, however the underlying mechanisms of defective wound repair in diabetes are not completely understood, but delayed collagen synthesis and accelerated

degradation of newly synthesized collagen, impaired epithelialization and reduced angiogenesis have been described during proliferative phase of healing process.[2]

The plant *Michelia champaca* (MC) belonging to family Magnoliaceae, is widely used in both Ayurveda and Siddha medicine. Root and bark are used as a purgative and in the treatment of inflammation, constipation, and dysmenorrhea. The stem bark has astringent and febrifuge properties.[3] The flower buds of MC are commonly used by many traditional healers in most of herbal preparations for diabetes.[4] Furthermore, the extracts of flower and flower buds have shown anti-inflammatory, antipyretic, and wound healing properties.[5-7]

A survey of the literature revealed that wound healing activity of MC in diabetics has not been documented. Hence, this study was undertaken to evaluate the wound healing activity of MC in diabetic rats.

METHODS

Animals

Healthy, adult albino rats of Wistar strain of either sex, bred locally in the animal house of Kasturba Medical College, Manipal, weighing between 150 and 200 g were used. They were housed under controlled conditions of temperature (23±2°C), humidity (50±5%) and 10-14 hrs of light and dark cycles.[8] The animals were housed individually in polypropylene cages with sterile paddy husk bedding. Food pellets and water were provided *ad libitum*.

The study was carried out after obtaining clearance from the Institutional Animal Ethics Committee, Manipal (IAEC/KMC/44/2010-2011).

Preparation of ethanolic extract of MC

The flowers of MC were procured from a local florist shop and authenticated by the Professor of botany, Mahatma Gandhi Memorial College, Udupi. A voucher specimen was preserved at the department of pharmacology, Kasturba Medical College, Manipal, India. Flowers were shade dried and were finely powdered. The powder was loaded into soxhlet extractor in batches of 200 g each and was subjected to extraction for 30-40 hrs with 95% ethanol.[9] After extraction, the solvent was distilled off and concentrated on a water bath at a temperature below 50°C to syrup consistency. Then it was dried and stored in a desiccator. The yield was about 10%.

Acute toxicity study

The acute toxicity studies were performed in accordance with the Organization for Economic Co-operation and Development Test Guideline 425 (up-and-down procedure).[10] Adult female rats were used and a limit test was done using a limit dose of 2000 mg/kg. Animals were observed individually, for mortality and general behavior, at least once during the first 30 mins after dosing, periodically during the first 24 hrs (with special attention given during the first 4 hrs), and daily thereafter, for a total of 14 days. No death was observed till the end of the study. The test samples were safe up to the dose of 2000 mg/kg, and, from the results, 400 mg/kg dose was chosen as the maximum dose for further experimentation, along with doses of 100 mg/kg and 200 mg/kg.

Induction of diabetes

Rats were fasted overnight, and their fasting blood glucose was measured. Single dose of streptozotocin solution (STZ, 30 mg/kg) in sodium citrate buffer, pH 4.5, freshly prepared was injected intraperitoneally, and 10% glucose water was supplied to avoid sudden hypoglycemia post-injection.[11] Blood glucose measurement was performed 7 days after STZ injection.[12] Blood was drawn from the tail vein, and glucose level was determined using a glucometer. Rats with blood glucose levels >250 mg/dL were considered as diabetic.[13] Rats in non-diabetic group were injected with a single dose of saline, intraperitoneally.

Study design

A total of 100 animals were used. They were divided randomly into five groups for each of incision (n=6) excision (n=14) wound models. In each of the model the dose and method of drug administration were as follows;

Group I was non-diabetic control animals, which received 1 ml of 0.5% carboxymethylcellolose (CMC) orally. Group II was diabetic control animals received 1 ml of 0.5% (CMC) orally. Group III, IV and V were diabetic animals, which received MC extract the suspension of 100 mg/kg, 200 mg/kg and 300 mg/kg, respectively. The incision and excision wounds were created on 7th day after induction of diabetes.[13]

Wound models

Incision wound model

The animals were anesthetized by injecting ketamine, 80 mg/kg, intraperitoneally. The back of the rats were shaved. Two 6 cm long paravertebral straight incision were made, 1 cm lateral to the vertebral column on either side through the entire thickness of the skin. Wounds were closed with intermittent sutures, 1 cm apart, with black silk thread and curved needle.[14] Animals were treated once daily with the drugs from day 0 (day of wounding) to day 9, sutures were removed on day 7. On day 10, breaking strength of the wound was measured by applying tearing force in the form of continuous water flow technique of Lee.[15]

Excision wound model

Under ketamine anesthesia, the back of the rats were shaved. A round seal of 2.5 cm diameter was impressed on the dorsal interscapular region, 5 cm away from the ears of the rat. Full thickness skin from the demarcated area was excised to get a wound approximately measuring 500 mm^2.[16] Animals received the drugs from day of wounding to 21st post-operative day. Wound contraction rate was monitored in six animals from each group on every alternate day starting from day 0 by planimetric measurement. The wound tracings were then transferred to a 1 mm^2 graph paper, to determine the wound area. Wound contraction was calculated as a percentage of the original wound size.[17] Epithelialization period was monitored by noting the number of days required for eschar to fall away, leaving no raw wound behind.

On the 10th post-operative day, granulation tissue from the wound area was collected using a punch biopsy needle (5 mm), for histopathological and biochemical analysis.[18] The animals from which punch biopsy was taken were not included for the assessment of wound contraction and period of epithelialization. On the 10th post-operative day, granulation tissue was collected. The granulation tissue was dried in an oven for 24 hrs and the dry weight was noted. Acid hydrolysate of the dry tissue was used for the determination of hydroxyproline content.

Statistical analysis

The results were analyzed for statistical significance using one-way analysis of variance, followed by Tukey's *post-hoc* test, using SPSS computer software, version 15.0 (Statistical Package for the Social Sciences, IBM, NY, USA). $p<0.05$ was considered to be statistically significant.

RESULTS

Incision wound model

In this wound model, the breaking strength of 10 day old incision wound was measured. The mean breaking strength OD wound in non-diabetic control group was 256.56±6.54 g, and diabetic control group it was 232.61±4.62 g, but was not statistically significant. In animals treated with 100 mg/kg and 200 mg/kg doses of MC extract, the mean breaking strength was better than the diabetic control group, 238.17±5.56 g and 247.78±6.97 g, respectively, which are not significantly higher. It was significantly ($p<0.05$) increased to 271.5±4.4 g in diabetic rats, which received 400 mg/kg dose of MC extract when compared with the diabetic control group and the group that received 100 mg/kg dose of MC extract (Table 1).

Table 1: Effect of oral MC flower extract on breaking strength of 10 days old incision wound.

Group	Drug with dose	Breaking strength (g) mean±SEM
Non-diabetic control	0.5% CMC - 1 ml	256.56±6.54
Diabetic control	0.5% CMC - 1 ml	232.61±4.62
Test Group I	MC extract suspension 100 mg/kg	238.17±5.56
Test Group II	MC extract suspension 200 mg/kg	247.78±6.79
Test Group III	MC extract suspension 400 mg/kg	271.5±4.4[a,b]

Values are mean±SEM (n=6), [a]$p<0.05$ versus diabetic control, [b]$p<0.05$ versus test Group I, One-way ANOVA followed by Tukey's *post-hoc* test, SEM: Standard error of mean, MC: *Michelia champaca*, CMC: Carboxymethylcellolose

Excision wound model

The percentage of wound contraction measured on day 4, 8, 12 and 16 in non-diabetic control group was 18.62±3.66, 40.53±2.47, 60.88±5.21 and 81.24±4.12, respectively. This was significantly ($p<0.05$) reduced in diabetic control group to 28.26±5.27, 43.92±3.76 and 64.1±4.04 on day 8, 12 and 16, respectively, but there was no significant decrease in the wound contraction on day 4 (Table 2).

In animals that received 100 mg/kg of MC extract it was 17.45±4.23, 31.57±1.45, 49.28±5.46 and 70.97±3.13 on day 4, 8, 12 and 16, respectively. The rate of wound contraction in this group was better than the diabetic control group, but it was not significant. The percentage of wound contraction in animals that received 200 mg/kg of MC extract it was 18.36±4.64, 35.6±3.26, 52.32±4.1 and 73.77±5.26 on day 4, 8, 12 and 16, respectively, no significant change from the diabetic control group was observed. In animals, which were treated with 400 mg/kg dose of MC extract, the percentage of wound contraction was significantly ($p<0.05$) increased to 62.94±3.48 and 82.42±3.58 on day 12 and 16, respectively, when compared with diabetic control group. However, there was no significant change seen on day 4 and 8, post-operatively (Table 2).

The mean period of epithelialization was significantly ($p<0.05$) shorter in non-diabetic control group (18.86±0.82 days) when compared with diabetic control group (22.16±0.45 days) and the group that received 100 mg/kg dose of MC extract it was (21.67±0.22 days). Although the group treated with 200 mg/kg dose of MC extract demonstrated a hastened mean period of epithelialization (20.72±0.34 days), it was not significant. The mean period of epithelialization was significantly ($p<0.05$) shortened in group treated with 400 mg/kg dose of MC extract (19.31±0.37 days) when compared with diabetic control group and animals that received 100 mg of MC extract (Table 2).

The granulation tissue from 10 day old excision wound demonstrated a mean hydroxyproline content

Table 2: Effect of oral MC flower extract on wound contraction rate, hydroxyproline content and period of epithelialization of excision wound in diabetic rats.

Group	Drug with dose	Wound contraction (%) (mean±SEM)				Hydroxyproline	POE
		Day 4	Day 8	Day 12	Day 16		
Non-diabetic control	1 ml of l0.5% CMC	18.62±3.66	40.53±2.47[a]	60.88±5.21[a]	81.24±4.12[a]	145.65±3.36[a,b,c]	18.86±0.82[a,b]
Diabetic control	1 ml of 0.5% CMC	15.62±1.72	28.26±5.27	43.92±3.76	64.1±4.04	111.5±1.79	22.16±0.45
Test Group I	100 mg/kg MC extract	17.45±4.23	31.57±1.45	49.28±5.46	70.97±3.13	116.87±2.56	21.67±0.22
Test Group II	200 mg/kg MC extract	18.36±4.64	35.6±3.26	52.32±4.1	73.77±5.26	118.35±2.75[a]	20.72±0.34
Test Group III	400 mg/kg MC extract	21.86±2.7	37.97±4.87	62.94±3.48[a]	82.42±3.58[a]	132.96±2.36[a,b,c]	19.31±0.37[a,b]

Values are mean±SEM (n=6), Hydroxyyproline content in mg/g dry tissue weight, [a]p<0.05 versus diabetic control, [b]p<0.05 versus test Group I, [c]p<0.05 versus test Group II, One-way ANOVA, followed by Tukey's *post-hoc* test. POE: Period of epithelialization, CMC: Carboxymethylcellolose, MC: *Michelia champaca*, SEM: Standard error of mean

of 145.65±3.36 mg/g dry tissue weight in non-diabetic control group, which was significantly (p<0.05) higher than the mean hydroxyproline content of granulation tissue seen in diabetic control group (111.5±1.79 mg/g) In test Group I it was (116.87±2.56 mg/g) and test Group II it was (118.35±2.75 mg/g). It was significantly (p<0.05) higher in test Group II when compared with diabetic control group. In animals treated with 400 mg/kg dose of MC extract (test Group III) there was a significant (p<0.05) increase in mean hydroxyproline content of granulation tissue (132.96±2.36 mg/g) when compared with diabetic control group and groups treated with 100 mg/kg and 200 mg/kg doses of MC extract (Table 2).

Histopathological evaluation of the granulation tissue obtained on day 10 from the experimental animals was performed under a light microscope. Fewer macrophages and fibroblasts were observed in diabetic control group when compared with non-diabetic control group. The diabetic control group also showed poorly formed granulation tissue and sparse distribution of collagen fibers. A significant increase in a number of macrophages and fibroblasts was observed in treated animals that were treated with MC extract. The treated animals also showed a denser distribution and better organization of collagen fibers within the granulation tissue. These changes were more prominent in group that was treated with 400 mg/kg MC extract (Figure 1).

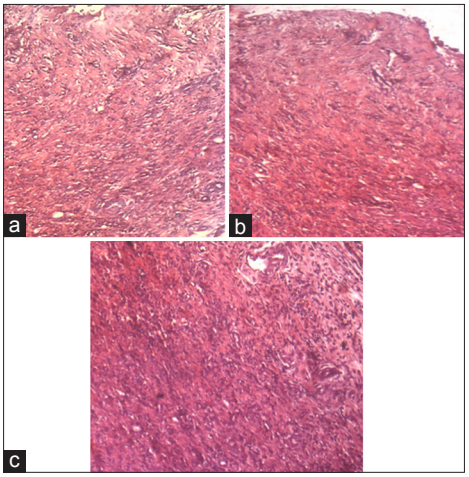

Figure 1: (a) Non-diabetic control group: normal granulation tissue with inflammatory cells and fibroblasts; well laid collagen fibers, (b) diabetic control group: fewer inflammatory cells and fibroblasts; sparse distribution of collagen fibers, (c) test group treated orally with *Michelia champaca* (MC) extract: significant increase in number of inflammatory cells and fibroblasts; denser distribution and better organization of collagen fibers. The changes are more evident in group treated with topical 400 mg/kg MC extract.

DISCUSSION

Cutaneous wound repair *in vivo* is a dynamic and complex process that shepherds the different elements of inflammatory cells, especially macrophages, platelets, coagulation factors, fibroblasts, endothelial cells, and keratinocytes. This is a highly coordinated and regulated process, which normally ends in repair of the dermal defect by collagen deposition and regeneration of new epidermis. Intrinsic factors comprising cell-surface receptors, growth factors such as platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-1), transforming growth factor (TGF-β), and cell adhesion molecules such as integrins play a significant role in the coordination of intricate events during normal wound healing.[19]

Diabetes is one of the factors affecting the normal course of wound healing. Diabetic wound healing is characterized by a delay in cellular infiltration and formation of granulation tissue, decreased wound collagen content, diminished wound tensile strength and prolonged epithelialization time.[20,21]

Experimental diabetes has shown to impair wound healing by decreasing collagen concentration and formation of granulation tissue;[22,23] by increasing activities of protease and collagenase.[24] It has been suggested that alterations in the wound healing process are present even at the onset of diabetes that can be associated with deficiencies in the defense cells involved in normal wound healing;[25] also with a marked decrease in the production of collagen.[26]

In our study, the mean breaking strength of the repaired tissue was decreased in the diabetic control animals. Oral preparations of (MC) enhanced the mean breaking strength of the wound. The collagen molecules synthesized are laid down at the wound site and become cross-linked to form fibers. Wound strength is acquired from both remodeling of collagen, and the formation of stable intra- and intermolecular crosslinks.

Similar observations were made by another study, where, in dexamethasone suppressed wound model, the mean breaking strength of the incision wound was increased with MC (oral, 250 mg/kg), but was not significant.[6]

Full-thickness cutaneous wounds which heal by secondary intention form granulation tissue which serves to fill the dermal defect and provide a structural framework for the deposition of newly synthesized collagen.[19] The full-thickness wound in diabetic rats is clinically relevant and comparable with impaired wound healing in human diabetes mellitus.[27] In this study, the rate of wound contraction was significantly ($p<0.05$) reduced in diabetic controls; MC extract showed better rate of wound contraction at 400 mg/kg oral dose. In a previous study, the results were closely similar to the current study. Oral dosage showed a significant increase in the rate of wound contraction only on 12th day.[7]

In the present study, oral administration of MC accelerated the wound healing in diabetic animals. The rats treated with 400 mg/kg dose required significantly ($p<0.05$) shorter duration for complete epithelialization. These results are in accordance with the other studies, where the period of epithelialization was reduced in orally and topically treated animals, when dexamethasone suppressed excision and burn wound models were used.[6,7]

The major final product of granulation tissue is collagen, of which, hydroxyproline is a main component. It provides strength and support and acts as an indicator of the amount of collagen in a tissue sample. The measurement of hydroxyproline is an index for collagen turnover.[28] In the current study, samples were taken for hydroxyproline only from the center of the wound granulation tissue, and this measure represents predominantly, if not exclusively, newly synthesized collagen. There was a significant ($p<0.05$) reduction in hydroxyproline content of granulation tissue in untreated diabetic rats. Oral administration of MC enhanced the hydroxyproline content, which was significant ($p<0.05$) at 200 mg/kg and 400 mg/kg doses. This is consistent with the study that used dexamethasone suppressed wound model, where animals treated orally with MC showed greater hydroxyproline content when compared with dexamethasone control group, although not significant.[6]

In the present study, the histopathological examination of granulation tissue obtained from MC treated rats have shown an increase in a number of macrophages and fibroblasts. The treated animals also showed a denser distribution and better organization of collagen fibers within the granulation tissue.

The exact mechanism through which MC enhances wound healing not yet known. Although the earlier studies have showed the presence of several phytochemicals in the flower extracts,[29,30] it is not yet clear, which constituents of the extract of MC are responsible for its beneficial effect in diabetic wounds.

Possibly, the constituents such as alkaloids, triterpenoids and tannins of MC may play a major role in the process of wound healing in diabetic rats. Tannins[31] and triterpenoids[32] are known to promote the wound healing process mainly due to their astringent and antimicrobial properties, which seems to be responsible for wound contraction and increased rate of epithelialization.

In this study, it was observed that defective wound repair in diabetic rats is associated with reduced cellular infiltration, formation of granulation tissue and collagen synthesis. The loss of collagen observed in diabetes may be due to decreased synthesis or enhanced catabolism of newly synthesized collagen, or both.[33] Elevated levels of hydroxyproline in the regenerated tissue suggest enhanced collagen synthesis. Collagen not only confers strength and integrity to the tissue matrix but also plays an important role in hemostasis and epithelialization at a later phase of wound healing.[34] Hence, enhanced collagen synthesis by MC in diabetic rats may contribute significantly to healing and also provide the necessary strength to the repaired tissue. Since incisional wounds treated with MC showed greater breaking strength, it may be speculated that it not only increases collagen synthesis per cell, but also aids in cross-linking of the protein, as indicated in the other study.[6] Inhibition of collagenases can also increase the rate of wound filling by granulation tissue and the amount of collagen.[19]

MC may stimulate cellular proliferation and migration through an as yet unknown mechanism. This was evident in the histological studies. The treated group of diabetic rats showed an increase in macrophages and fibroblasts on day 10 after wound creation, which indicates that MC may stimulate macrophage infiltration in the wound environment, collagen synthesis, and re-epithelialization. The macrophages play an important role in activation and recruitment of other cells like fibroblasts via mediators such as TGF-β, VEGF, PDGF, IGF-1, epidermal growth factor and lactate. These mediators regulate cell proliferation, matrix synthesis, and angiogenesis.[35]

Diabetes mellitus involves oxidative stress, which results in the production of free radicals that in turn cause tissue damage and delay wound healing. Many plant extracts and herbs, which have antioxidant effect have proved to be beneficial in treating complications resulting from diabetes.[36] There have been several studies that have reported antioxidant properties of MC,[30] which suggest the beneficial effect of this plant in wound healing in diabetics.

Since control of blood glucose levels have been shown to improve wound healing in diabetics,[37] it is quite possible that the enhanced healing of wounds in diabetic rats by oral administration of MC flower extract is the result of its hypoglycemic activity that has been demonstrated in a previous study.[38] Furthermore, wounds in diabetics have a higher propensity to become infected, which may impede the progress or completion of healing.[39] There have been several studies which have demonstrated the antimicrobial property of flower extracts of MC,[30,40] which may play a role in enhanced wound healing in the treated animals.

MC may, thus achieve the following effects to improve tissue healing: Greater migration of inflammatory cells and fibroblasts; stimulation of collagen synthesis by fibroblasts; extensive reorientation of collagen fibers caused by a stronger cross-linking; hypoglycemic, antioxidant and anti-infective effects.

Since the oral administration of ethanolic extract of MC flowers significantly enhance the wound healing process, it could be made use of clinically, as a supportive therapy to treat wounds in diabetic patients.

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