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Research Article

Evaluation of Myricetin loaded Nanoemulsion Formulation for the Management of Diabetic Wound Healing in Experimental Animals

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ABSTRACT

Myricetin is considered a flavonols under the category of flavonoids. According to recent research, myricetin has different curative effects on diabetes, cancer, and the heart. It has been suggested that myricetin is a more potent antioxidant than quercetin. The present study aimed to investigate the wound healing effect of myricetin loaded nanoemulsion (MYCT-NE) gel formulation in diabetic animals. Myricetin-loaded nanoemulsion was converted in nanoemulsion gel using carbopol 934 and evaluated for diabetic wound healing effect against wound contraction measurement, hydroxyproline estimation, protein estimation, antioxidant assay and histopathological study. On day 18th of treatment, the wound contraction of Faster wound healing was observed in the MYCT-NE gel treated groups compared to the control group, as indicated by a shorter epithelialization duration. Increased collagen turnover was shown by increased hydroxyproline levels in MYCT-NE gel-treated tissue, which sped up the healing of treated wounds. MYCT-NE gel possesses effective antioxidant activity by restoration of the superoxide dismutase SOD, GSH and catalase level in the wound tissues after treatment and healing. The observations showed that the original tissue regeneration was found efficiently in the wound treated with MYCT-NE gel and reference group without edema and congestion. The results obtained in the present study were indicated that MYCT-NE gel accelerates cutaneous diabetic wound healing through reducing oxidative status in experimental animals.

Introduction

A chronic wound often results in tissue damage that is accompanied by inflammation, oxidative stress caused by the production of free radicals, lipid peroxidation, and the inactivation of enzymes. Several causes, including diabetes, infection, or metabolic abnormalities, might cause a wound to fail to heal.[1] Different therapeutic modalities have been researched in both clinical and experimental settings to speed up wound healing.[2] Numerous variables that lead to thickening of the basement membrane of the capillaries and arterioles hinder wound healing in diabetics. It frequently happens in people with diabetes, impairing wound healing and causing forceful ulcer development.[3] The creation of advanced glycation end products, which trigger the release of inflammatory molecules (TNF, IL-1), and interfere with collagen synthesis, have been found to have a detrimental influence on wound healing.[4] High glucose levels also affect cellular shape, granulation tissue's lack of collagen, keratinocytes' aberrant differentiation and decreased proliferation.[3] However, the risk of major side effects or the disadvantage of the drug's early inactivation might accompany administering medications for treating wounds via oral and parenteral routes.[5]

Clear, thermodynamically stable, isotropic mixtures of oil, water, and a surfactant/cosurfactant combination are referred to as NEs.[6] After topical administration, lipophilic medicines often concentrate in the uppermost layers of the skin. According to recent studies, lipophilic medicines included into NEs effectively enter the skin. NEs can increase the local or systemic distribution of a

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medicine through a variety of ways when used as topical vehicles.[7] First, compared to other traditional topical formulations like ointments, creams, gels, and lotions, their composition and structure allow them to contain more medicine. The finely dispersed oil droplet phase of NEs can improve the solubility of non-water soluble medications. Second, depending on the NE's composition, the diffusional barrier of the skin may change.[8] Third, a drug's higher thermodynamic activity might favor its skin partitioning.[9] As a result, the drug's formulation will allow it to penetrate the stratum germinativum and dermis, two areas of the wound's underlying skin layers where wound healing and epithelialization occur.

Myricetin is chemically 3,5,7-trihydroxy-2-(3, 4, 5-trihydroxy phenyl)-4-chromenoneunder the category of flavonoids called flavonols. It is widely distributed in fruit, berries, red wine, vegetables and tea. The pyrogallol B-ring, which distinguishes myricetin from other flavonols, is recognized to be the cause of its more hydroxylated structure and its increased biological characteristics.[10, 11] Myricetin is good antioxidant agent and have ability as stronger antioxidant than quercetin.[12,13] Myricetin has been shown to have an efficient radical scavenging action in a variety of radical producing conditions. The superoxide anion produced by phenazine methosulfate-NADH is one of these systems.[14]; hydroxyl radical (OH) is generated either by hydrogen peroxide or tert-butyl hydroperoixde,[15] and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical.[16] Myricetin had an inverse relationship with the risk of type II diabetes among different flavonoids, indicating that it may have potential anti-diabetic activity.[17] Additionally a study found that myricetin reduced plasma glucose levels in rats with diabetes brought on by streptozotocin[18] and in insulin resistance.[19] A study reported that 0.12% myricetin supplementation effectively reduced hypertriglyceridemia and hypercholesterolemia in animal fed a high-fat and high-sucrose diet. This suggests that that myricetin may have an anti-obesity and anti-insulin resistance impact.[20] Based on these reported facts, present work was aimed for detail investigation for wound healing effect of myricetin loaded nanoemulsion gel formulation in diabetic animals.

Materials and Methods

Materials

Myricetin was purchased from Yucca Enterprises, Mumbai. Peanut oil, arachis oil, castor oil, olive oil, carbopol 934, Tween20 (Polyoxyethylene (20) sorbitan monolaurate), Tween 40 (Polyoxyethylene sorbitan monopalmitate), Tween 60 (Polyoxyethylene sorbitan monostearate), Tween 80 (Polyoxyethylene sorbitan monooleate), Span 20 (Sorbitan laurate) (Sorbitan monododecanoate), Span 80 (Sorbitan monooleate) (Sorbitan (2)- mono- a-octadecanoate), Isopropyl alcohol, Polyethylene glycol 400, propylene glycol were procured from Loba Chemie Pvt. Ltd., Mumbai, India. The orthophosphoric acid (HPLC grade—88%), acetonitrile (HPLC grade, 99.9%) and methanol (HPLC grade, 99.9%) were obtained from Merck Specialties Pvt Ltd., Mumbai, India. Furthermore, all the other chemicals, reagents, solvents used in the present study was of analytical grade. Water used in whole experimental work was deionized water purchase from Millipore Corporation, Bedford, MA. Animal diet i.e., pellet diet was purchased from Hindustan Lever Pvt, Bangalore, India. Betadin obtained from Win-Mdicare Pvt. Ltd. New Delhi, India.

Preparation of Nanoemulsion Nanoemulsion Gel

Surfactant and co-surfactant were chosen for nanoemulsion preparation of myricetin based on solubility assessment. An accurately weighed amount of myricetin (30 mg) was incorporated through a spontaneous emulsification method with slight modification.[21] The organic phase consists of myricetin dispersed in peanut oil (0.5 mL), whereas aqueous phase contains a mixture of tween 20 as surfactant and polyethylene glycol 400 as co-surfactant with best ratio from the ternary phase diagram. Organic phase was poured into the aqueous phase dropwise, followed by continuous stirring using a magnetic stirrer (IKA India Private Limited, Bengaluru, India) at 5000 rpm for 5 minutes to obtained primary emulsion. Further primary emulsions were reduced into nanoemulsion by high presser homogenization (T-25 digital ULTRA-TURRAX®, IKA India Private Limited, Bengaluru, India) at 11000 rpm for 20 minute. The resulting transparent, easily flowable nanoemulsion was allowed to stand for 2 hours for equilibration before being characterized. All detail procedure of ternary phase diagram and characterization of nanoemulsion formulations has been mentioned in previous work already submitted for publication.

Optimized nanoemulsion formulation (NEF) was converted into nanoemulsion gel (NE gel) using carbopol 934 polymer. Prepared myricetin loaded nanoemulsion (MYCT-NE) gel formulations were evaluated for spreadability, pH, viscosity and drug release study. Spreadability is another important gel parameter that can affect topical formulation's therapeutic efficacy.

Diabetic Wound Healing Activity

Animal and treatment protocol

Albino wistar rats, weighing between 200 and 250 g, were acclimated in a controlled environment with 12-hour light and dark cycles at 23°C for 15 days. They were free access to commercial pellet diet (Hindustan Lever Pvt, Bangalore, India) and water. All animal studies were performed at RKDF University, Bhopal (MP) with prior approval of the Institutional Animal Ethical Committee (Reg. No. 1546/PO/E/S/11/CPCSEA). In order to conduct the experiment, the animals were split into three groups containing six

each group. The control group is vehicle given only base (without myricetin loaded nanoemulsion gel), test group received MYCT-NE) gel. The reference group received a marketed formulation from betadin (Win-Mdicare Pvt. Ltd. New Delhi, India). All groups were treated by topically twice in a day.

Diabetic wound model

After an overnight fast, solution of streptozotocin with a cold citrate buffer (0.1 M, pH 4.5) was prepared for intraperitoneal delivery. Three days before the experiment, a single dosage of streptozotocin (60 mg/kg) was given into test animals to induce diabetes. Three days following the injection, blood sample was taken from the tail vein and calculated using a glucometer (CONTEC BC 300 Auto analyser). Rats' dorsal portions were excised in a circular pattern to reveal increased blood sugar levels (more than 135 mg/dL).[22] Blood glucose levels were evaluated both before and after treatment of the wounds. To assess the effectiveness of healing in diabetic animal, wound contraction, antioxidant levels, and histology studies were carried out.

Wound contraction measurement

During the healing process, wound contraction is the rate at which the unhealed region shrinks. Therefore, a faster rate of wound closure indicates a higher level of therapeutic effectiveness. After creating the wound, translucent paper was used to trace the excision wound margin. During healing process, the %wound contraction was measured every two days and represented as a percentage of the healed area. From the initial day of wound formation, the epithelialization time was calculated.[23] The wound contraction in the percentage, was calculated with the help of following formula:

Percent wound contraction = (measured healed area/ total wound area) x100

Hydroxyproline estimation

Total hydroxyproline content in the wound tissue was determined on 18th day of healing. Tissue samples were collected by following standard procedure and completely dried at 60–70°C for 12–18 hours. Sample of tissue hydrolysate was diluted and filtered with water to 10 mL after chilling. To prepare sample for the colorimetric, 1-mL portions of diluted hydrolysate was further mixed with 5.0 mL assay buffer, and 2.5 mL of chloramine T reagent was added. This mixture of reagents was stood for 20 minutes at 25^{o}C, room temperature. In 2.5 mL of freshly prepared dimethylamino-benzaldehyde reagent solution was added to the previous mixture and mixed thoroughly at 60^{o}C for 15 minutes. This whole content was cool in tap water for 1 to 2 minutes.[24] This whole content was used to take absorbance at 557 nm immediately using UV visible spectrophotometer (Shimadzu) and content estimated with the help of plotting calibration curve of hydroxyproline.

Protein estimation

Protein content in the wound tissue was determined on the post wounding days 18th, by the method of Lowry *et al.* (1951).[25] At the initial step, a sample of tissue lysate was mixed with sodium tartrate, sodium carbonate solution and copper sulfate with manual mixing to determine the amount of tissue protein. Folin-Ciocalteau reagent was applied after the mixture had stood for 10 minutes, and the mixture turned bluish in 20 to 30 minutes. The absorbance was taken at 650 nm using a spectrophotometer. The calculation was done from a standard curve of protein.

Antioxidant assay

Wound tissues were collected from all of the treated animals and the antioxidant assay was performed on them. The tissues were centrifuged in a refrigerated environment after being homogenized in phosphate buffer (pH 7.0). To measure the content of antioxidants, the clear supernatant was collected. Superoxide dismutase (SOD) was measured[26] based on the inhibition of epinephrine autoxidation by the enzyme. Catalase (CAT) was estimated following the breakdown of hydrogen peroxide.[27] Reduced glutathione (GSH) level was determined with the help of standard method of Moron *et al.* (1979).[28] After immediately precipitating the homogenates combination with 25% TCA (0.1 mL), the precipitate was removed by centrifugation. Free-SH groups were assayed by the addition of 0.6 mM DTNB (2 mL) and 0.9 mL of 0.2 mM sodium phosphate buffer (pH 8.0) to the supernatant (0.1 mL). This sample mixture was used to take the absorbance at 412 nm at UV spectrophotometer.

Histopathological study

One part of wound tissue collected from each group of animal was taken on 18th day of treatment to perform the histopathological analysis. Samples were fixed using formalin (10.0%), dehydrated alcohol with paraffin blocks. Sections were then cut into 6 µm thickness, stained with Hematoxylin Eosin (HE), and examined under a light microscope.[29]

Statistical Analysis

All data was analyzed statistically using mean values and ANOVA as well as by the multiple comparisons test (Tukey's). GraphPad Instat Software executed a statistical analysis of the results. Presented data were considered statistically significant, if $p < 0.05$. Data were represented as mean ± SD for all statistical analyses.

Results and Discussion

Formulation Preparation of Nanoemulsion Gel

The peanut oil was selected on the basis of solubility with drug as oil phase for the formulation of nanoemulsion on the basis of solubility. The optimized MYCT-NE was converted into the gel formulations using 2.5% of carbopol

Table 1: Effect of myricetin loaded nanoemulsion gel on percent wound contraction area in diabetic wound model

Animal groups	Observations on post wounding days (% wound contraction)										Epithelialization period
	2	4	6	8	10	12	14	16	18	20	
Vehicle control	12.64 ± 0.52	27.45 ± 0.42	35.22 ± 0.65	40.25 ± 0.42	47.23 ± 0.84	54.28 ± 1.75	60.75 ± 1.56	69.51 ± 2.08	78.24 ± 2.42	84.33 ± 2.41	24
Myricetin loaded nano Emulsion gel	13.74 ± 0.75	25.66 ± 0.87	40.98 ± 0.74	48.22 ± 0.42	59.78 ± 1.26	69.37 ± 1.25*	84.29 ± 2.13*	94.21 ± 2.71*	100.20 ± 2.77*	-	18
Betadine cream (Reference)	14.85 ± 0.18	31.29 ± 0.47	46.95 ± 0.76	50.99 ± 1.12	65.39 ± 1.75	70.56 ± 1.41*	86.28 ± 2.08*	95.62 ± 2.14*	100.21 ± 2.06*	-	18

n= 6; Tabular value represents as Mean ± S.D. *$p < 0.05$ when compared each treated group with control group.

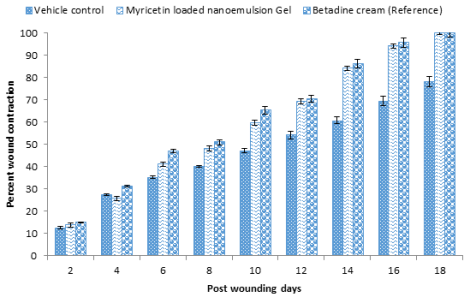

Fig. 1: Effect of myricetin loaded nanoemulsion gel on wound contraction area (in percentage) in diabetic wound animals

934. Preapred nanoemulsion gel showed appropriate spreadability comparable to the market formulation's spreadability. The appropriate spreadability of any topical formulation is support to the easy application on the skin. All characterization parameters were found appropriate and good stability up to three months. The results of these parameters were already communicated for publication in another journal.

In-vivo Diabetic Wound Healing Activity

By drawing the wound area onto translucent paper, the wound area was measured every two days for up to 20 days. In order to compute the healed area, the original wound area was subtracted. On day 6, it was discovered that myricetin-loaded nanoemulsion gel-treated groups had significantly higher wound contraction ($p < 0.05$), which sped up wound healing as seen by a shorter time for epithelialization compared to the control group. On day 18, the animal group treated with myricetin-loaded nanoemulsion gel were in the last stages of healing, but on day 20, the control group had healed to an average of 84.33%. Observation confirmed that the epithelialization period of the myricetin-loaded nanoemulsion gel treated group and standard groups was 18 days which was similar (Table 1 and Fig. 1).

Hydroxyproline Content and Protein Level Measurement

The hydroxyproline level of the animal group treated with myricetin-loaded nanoemulsion gel (58.44 ± 1.31) was

Table 2: Effect of myricetin loaded nanoemulsion gel on biochemical parameters (hydroxyproline and protein content) of wound tissue in diabetic wound model

Animal groups	*Level of hydroxyproline (mg/g tissue)*	*Level of protein content (mg/g tissue)*
Vehicle control	23.86 ± 0.67	29.30 ± 0.84
Myricetin loaded nanoemulsion gel	58.44 ± 1.31*	68.17 ± 1.62*
Betadine cream (Reference)	60.24 ± 1.07	72.20 ± 1.54

n= 6; Tabular value represents as Mean ± S.D. *$p<0.05$ when compared each treated group with control group.

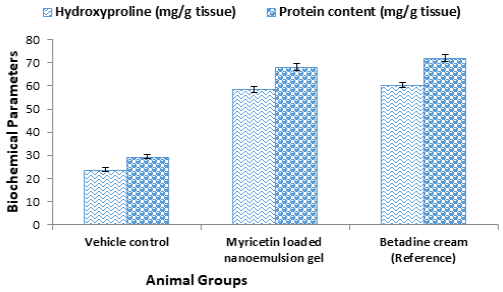

Fig. 2: Effect of myricetin loaded nanoemulsion gel biochemical parameters of wound tissue in diabetic wound model

significantly higher than the control group of animals. Collagen is the major component of extracellular matrix that contributed to wound strength. Collagen is broken down, resulting in the production of hydroxyproline and associated peptides.[30] This hydroxyproline's measurement serves as an indicator of collagen turnover. The newly created collagen molecules are placed at the site of the wound and undergo cross-linking to produce dense fibers. Collagen remodeling and the creation of intra- and intermolecular cross-linking provides good strength to the wounds. Increase hydroxyproline level of myricetin-loaded nanoemulsion treated tissue resulting in increased collagen turnover was seen in the tissue, and this accelerated the healing of treated lesions.

The protein content of myricetin loaded nanoemulsion gel was 68.17 ± 1.62 and reference group was 72.20 ± 1.54 found significantly greater than control group (29.30 ± 0.84) of

Table 3: Effect of myricetin loaded nanoemulsion gel on antioxidant parameters of wound tissue in diabetic wound model

Animal groups	*Level of SOD (µgm/50mg tissue)*	*Level of CAT (µmol/50mg tissue)*	*Level of GSH (µmol/50mg tissue)*
Vehicle control	23.55 ± 0.68	31.62 ± 0.85	41.74 ± 0.88
Myricetin loaded nanoemulsion gel	51.34 ± 0.72*	72.33 ± 1.42*	84.20 ± 1.63*
Betadine cream (Reference)	50.25 ± 0.66*	75.11 ± 1.27*	80.29 ± 1.46*

n= 6; Tabular data represented as Mean ± S.D. *$p < 0.05$ when compared each treated group with control group

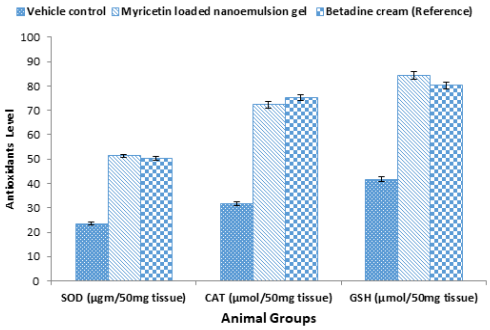

Fig. 3: Effect of myricetin loaded nanoemulsion gel on antioxidant parameters of wound tissue in diabetic wound model

animals (Table 2 and Fig. 2). The protein content of wound tissue indicates cellular proliferation and tissue formation. The fact that the treated wounds had more protein than the untreated ones suggests that myricetin increases cellular proliferation through an unidentified mechanism. For the creation of new granuloma tissue, protein synthesis is necessary. The basic triggers for granuloma development during the inflammation phase include neutrophil and fibroblast infiltration of macrophage proliferation.[31-33]

Antioxidant Status in Wound Tissue

Myricetin-loaded nanoemulsion gel possesses potent antioxidant effect through improvement in the SOD to 51.34 ± 0.72 µgm/50 mg tissue, GSH, 84.20 ± 1.63 µmol/50 mg tissue and catalase level to 72.33 ± 1.42 µmol/50 mg tissue in the wound tissues on 18th day of healing process. The significant improvement in antioxidants level (SOD, GSH and CAT) were observed after treatment with myricetin-loaded nanoemulsion gel and marketed formulation on 18th day of the healing process (Table 3 and Fig. 3).

Free radicals are effectively scavenged by reduced GSH which is depleted as a result of increased lipid peroxidation. This may result in increased GSH use, which is linked to a rise in the concentration of oxidized glutathione.[34] Myricetin-loaded nanoemulsion gel therapy causes an increase in GSH levels, which shield cell membranes from oxidative damage by preserving the membrane's redox status. Antioxidant enzymes such as SOD and CAT are essential for an organism's antioxidant defenses, which help to eliminate peroxides. All of these enzymes have overlapping roles. Reduced enzyme activity causes a buildup of lipid peroxides and increased oxidative stress at the site of injury. Treatment with myricetin-loaded nanoemulsion gel increased the activity of these enzymes and thus may help overcome free radicals production during chronic wounds.

Histopathological Observation

Different healing states of the injured tissues were visible through histopathological evaluation of stained sections from different treatment groups. The outcomes show that the nanoemulsion gel formulation is effective at improving wound healing in diabetics. The results revealed that there was effective new tissue regeneration in the wounds treated with myricetin-loaded nanoemulsion gel and the reference group, with no edema or congestion. Epithelial tissue proliferated in the wound region in both groups. The shorter epithelialization time demonstrated the slower dermal modeling process in the vehicle control group (Fig. 4A). Tissue sample from control group exhibited decreased epithelialization, fibrosis, and macrophage aggregation, as well as fewer collagen fibers, indicating inadequate wound healing. In histopathological view, group treated with myricetin loaded nanoemulsion gel shown dense collagen fibers and fibroblast cells (Figs. 4B and C).

Results of present study were confirmed that myricetin loaded nanoemulsion gel found effective for wound healing effect. The results showed that animals treated with myricetin loaded nanoemulsion gel had a faster rate of wound contraction and quicker healing times.

The wound was treated topically with prepared nanoemulsion gel up to 18th days from initial. The reduction in swelling and redness suggests that the developed nanoemulsion gel has a tissue-debriding action at the wound site. The present study showed that the total protein content of the nanoemulsion gel treatment group was increased, indicating that gel was able to stimulate cell proliferation at the wound site. Increases in the SOD, CAT, and GSH level were observed in wound tissues after the 18th day of treatment suggested that healing may occur

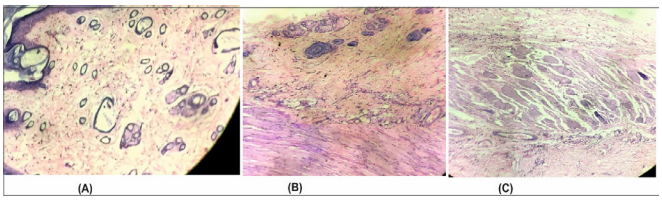

Fig. 4: Photomicrograph of histological observation of wound tissues in diabetic wound model: (A) Vehicle control; (B) Myricetin loaded nanoemulsion gel; (C) Betadine cream (Reference)

through free radical scavenging effect of myricetin. Natural antioxidant molecules can neutralize the superoxide radical, preventing free radicals from damaging cells. Thus, it can be concluded that myricetin-loaded nanoemulsion gel could be used as potential wound-healing formulation, especially in the management of chronic wounds.

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