Wound healing activity of neferine in experimental diabetic rats through the inhibition of inflammatory cytokines and nrf-2 pathway

Juan Li, Haiyan Chou, Lei Li, Hao Li and Zhengjun Cui

Department of Plastic surgery, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, China; Department of Plastic surgery, Henan Provincial People’s Hospital, Zhengzhou, China; Department of Burn surgery, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan Province, China

ABSTRACT

The diabetic foot ulcer (DFU) may be associated with late healing and septic manifestation, subsequently lead to amputation which is an overpriced incident. Neferine is an alkaloid found lotus. Neferine possesses many physiological functions such as anti-inflammatory, antioxidant, antimicrobial activity and anticancer effect. The aim of the present study was to evaluate the effect of topical application based on neferine, in streptozotocin-induced diabetic incision wound models rats. The data demonstrated wound healing activities via macroscopic, biochemical, histological, immuno-histochemical, immunofluorescent and molecular methods. There was significant acceleration in wound closure rate, decrease in the period of re-epithelization, higher amount of collagen and protein content in neferine treated group when compared with diabetic wound control. Histological data evidence collagen formation in skin and marked granulation with more connective tissue markers. The augmentation of serum insulin and HDL was dissimilar with blood glucose reduction and decreased lipid level (TC, TG and LDL). The healing effect was additionally validated by decreased lipid peroxidation and enhanced antioxidants. Concurrently, the mRNA level of Nrf-2, collagen-1, TGF-β and α-SMA were decreased with Kaepp-1 increased significantly. This enhancement was achieved through downregulation of inflammatory mediators such as nuclear factor kappa-light-chain-enhancer of activated B cells, tumour necrosis factor-α, interleukin-1β, interleukin-8, inducible nitric oxide synthase, and cyclooxygenase-2, and upregulation of growth factor such as in groups treated with neferine. The western blot results reveal the macrophage (CD 68 and CD 163) involved in wound healing markedly elevated. Hence, the results indicate that neferine significantly promotes a fast and efficient wound healing in diabetic rats.

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Introduction

Diabetes mellitus (DM) is a metabolic condition clinically expressed by hyperglycaemia. Being the systemic character of diabetes, the incidence of diabetic foot syndrome (DFS) continues to be the major health problem in diabetic patients. DFS or foot ulceration (between the knee and the ankle) is linked to lower-extremity amputation, not only in the final stages of DM yet also in diabetic patients who is newly diagnosed [1]. It has been estimated that 5–24% of diabetic patients with amputation history develop high risk of mortality, with a 5-year survival rate of 40–48% post-amputation. Furthermore, the risk of DFS increases with duration of diabetes and age approaching 50–59%. Long-standing diabetes in patient may lead to chronic complication affecting organs such as heart and kidney, which may profoundly end in severe disability, poor life quality and high financial burden. Regardless of the protocols to standardize care, the physiological impairments caused in a DFS, obfuscate the healing progression [2].

The non-healing phenotype due to systemic effects of DFS is neuropathy, peripheral vascular disease, and impaired immune system components, a circumstance that impedes proper wound healing in diabetes with hyperglycaemic condition. Hyperglycaemia or high blood glucose creates oxidative stress on neurological cells which cause neuropathy. These cellular changes on nerve cells lead to damage to autonomic nerves such as impairment of sensory components (pressure and heat), and sweat gland functions to decrease the capacity to nourish and moisturize the skin, which eventually lead to skin ulcers [3]. Damage to motor neurons cause insufficient blood supply to maintain intact skin. Thus, inadequate perfusion of lower-extremity, causing further ischaemia and abridged healing response due to deranged immunity (reduced leucocytes), and crucially unhealed wound may provide a site vulnerable to chronic microbial ulcers. Considering the negative influence on a patient’s life quality and the accompanying economic load on the healthcare system, quest for therapies to treat the
chronic, non-healing wounds in diabetic patient has been substantial [4].

The long duration of treatment as well as high costs to treat DFS make it imperative to employ effective therapies that prevent wounds from developing and accelerate healing rates once wounds occur. While there has been research that has progressed from laboratory, to clinical trials, and finally to clinical practice, these treatments have failed to be the silver bullet that will heal chronic diabetic wounds. One remedy that has been greatly trusted upon since prehistoric time is medicinal plants. The plant materials medicinal effects are from the amalgamations of secondary metabolites found in the plant and they are alleged to be harmless because they ease adaptation to the biological systems. Many medicinal plants are still widely used in developing countries especially in the form of crude or potions to treat common wound infections, however without any scientific evidence of efficacy. Thus, a greater accomplishment in decreasing the diabetic foot syndrome using medicinal plants yet to be accomplished using a systemic classification diagnosis, and treatment of diabetes.

The Nelumbo nucifera (sacred lotus) is a traditional medicinal plant used throughout India and China primarily as a food and medicine. On the dietary aspect, all parts of the plant are all edible, demonstrating the non-toxic nature of it. Further, they are also medically applicable because they harbour a massive array of bioactive complexes. Flowers, leaves, seeds and fruit of the plant have been utilized traditionally to treat a wide range of conditions, comprising diarrhoea, abnormal bleeding, poor digestion, fever and insomnia. However, there is lack of not scientific research on the use of lotus to treatment of any this condition [5]. Neferine is a dibenzylisoquinoline alkaloid extracted from lotus seed. A previous study exerted significant efficacy antioxidant, anti-depressant, anti-tumour and anti-inflammatory properties [6–8]. Collectively, these pharmacologic properties may provide a foundation to investigate the studies related to diabetic wound healing activity of neferine. Therefore, grounded on the existed evidence and understanding, the present study was subjected to scrutinize the protective role of neferine in wound healing activity in diabetic rat model.

Materials and methods

Chemicals and reagents

Streptozotocin (STZ), Neferine from N. nucifera was purchased from Sigma-Aldrich, USA. All chemicals in the experiment were analytical grade.

Experimental animals

Healthy wistar rats (male) weighing between 200 and 220 g were obtained and kept in standard laboratory conditions in cross-ventilated animal house at relative humidity 44–56%, and light and dark cycle of 12:12 h for acclimatization. The animals were fed with standard diet and water during the experiment. The study protocol was agreed by the Institutional Animal Ethics Committee as per Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines, China.

Induction of diabetes

Diabetes was induced by administration of Streptozotocin (STZ), freshly prepared with citrate buffer (pH 4.00) at the dose of 55 mg/kg b.w. via single intraperitoneal injections to the overnight fasted rats [9,10]. After 72 h, the blood glucose level was estimated using standard glucometer (Bayer Contour TS Blood Glucose Monitor). Animals with blood glucose level above 250 mg dL$^{-1}$ were chosen as wound models.

Experimental design

Grouping of animals was carried out 72 h after Streptozotocin (STZ) administration. The animals were randomly divided into 4 groups ($n = 6$):

- **Group I**: Normal control group
- **Group II**: Diabetes control group
- **Group III**: Diabetic animals treated with 10% neferine on excision wounds
- **Group IV**: Diabetic animals treated with 20% neferine on excision Wounds

Incision wound model

Excision wounds were used to investigate the wound contraction rate and epithelization in both normal and diabetic animals for this study. The dorsal skin of anaesthetised animals (3% isoflurane) was shaved with an electric clipper. Circular pieces (10 mm) of full-thickness skin were cut off from predetermined areas on the back of the rats d with sterile biopsy punch. Animals were inspected for sign of infection, and those with sign of infection were excluded from the experiment and substituted. Wound areas were measured and recorded on day 1, 7 and 14 for all groups on a graph paper.

Preparation of the test compound

The test compound was evaluated for wound healing effect in excision wound model. Neferine was daily formulated freshly in 0.1 M dimethyl sulphoxide (DMSO) and applied topical at the wound region of group 3 and 4 experimental animals.

Blood samples collection

The rats were anaesthetised after 14 days of Neferine administration. Blood samples using anticoagulant, ethylene diamine tetra-acetic acid (EDTA) tube, were collected, for haematological analysis. Additional blood sample (5 ml) was collected to obtain the serum for biochemical assays

Estimation of wound contraction and epithelialization period

The progressive reduction in the wound area is measured each day by mark out the wound area using transparent
Sheet graph paper. Wound contraction was represented as percentage of healing wound area:

\[
\text{Percentage of wound closure (\%)} = \frac{\text{Wound area on day } 0 - \text{Wound area on day 14th}}{\text{Initial area of wound}} \times 100
\]

**Estimation of collagen**

Granulation tissues were obtained on 8th day to assess the collagen content. Concisely, the fat layer was removed from the tissue samples by using mixture of chloroform and methanol (2:1 v/v). Then, the tissues were then frozen using acetone. The frozen tissue samples were weighed, hydrolysed and dried. Distilled water was added to the treated samples to a known volume of double. An aliquot was taken from this mixture, and the absorbance was taken spectrophotometrically at 557 nm to evaluate the hydroxyproline content. The collagen content was calculated:

\[
\text{Concentration of the sample} = \frac{\text{OD of the sample}}{\text{OD of the standard}} \times \text{Concentration of the standard}
\]

**Estimation of total protein**

The content of protein in skin tissues was evaluated according to of Lowry et al. [11]. The homogenate of granulated tissue was treated with sodium tartrate, copper sulphate and sodium carbonate mixture and then with Folin-Ciocalteu reagent for 10 min. A bluish colour was formed in 20–30 min and the absorbance reading was taken using spectrometry readings against the blank with water at 650 nm.

**Determination of biochemical parameters (blood glucose, serum insulin and lipid profile)**

The level of blood glucose and serum insulin was measured to verify diabetes induction. The blood samples were collected via jugular veins from fasting animal. Blood glucose level for all rats was determined was carried out using digital glucometer (AccuCheks, Roche Diagnostic, Meylan, France). The serum insulin level for all the rats who was determined was carried out using enzyme linked immunosorbent assay (ELISA) kit (EZRMI-13K, Linco Research; St. Charles, MO, USA) in accordance with manufacturer instructions. The total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) serum concentrations were measured using automatic analyser (Beckman Coulter Inc., Ireland) technique.

**Determination of lipid peroxidation**

Wound tissue was collected and tissue homogenates were prepared for the measurement of lipid peroxidation rate by measuring the level of malondialdehyde (MDA) using the TBA reaction. The homogenization mixture, 50 mmol/L Tris-HCl reagent (pH 7.4) were prepared using 10 mmol/L EDTA, 0.02% butylated hydroxytoluene and 180 mmol/L KCl. The tissue homogenate (0.2 ml) was added with 1.5 ml of 20% acetic acid, 0.2 ml of 8.1% sodium 1.5 ml of 0.95% thiobarbituric acid, dodecyl sulphate and 0.6 ml of distilled water and mixed well. The reaction mixture was then kept in a water bath at 95 °C for 1 h. Later, mixture of 1.0 ml of distilled water and 5.0 ml of butanol/pyridine (15:1, v/v) were added and vortexed. The vortexed mixture was centrifuged (10,000 x g) for 10 min, to obtain absorbance reading at 532 nm. The lipid peroxidation concentration was calculated using 1, 1, 3, 3-tetraethoxypropane as a standard and presented as nonomole TBARS per mg of protein.

**Determination of antioxidant status**

Tissue homogenate (10%) of the granulation tissues was prepared in 0.02 M phosphate-buffered saline and was used for the estimation of super oxide dismutase (SOD) and catalase (CAT) and reduced glutathione (GSH), Glutathione reductase (GR) and the enzyme glutathione peroxidase (GPx) [12–15]. The supernatant from the sample was estimated for SOD activity. The sample was mixed in test tube containing tissue homogenate (0.1 ml) with H$_2$O$_2$ (0.5 ml) and incubated in water bath for 60 s at 37 °C to estimate the catalase activity. Then, the reaction was added with 0.5 ml of ammonium molybdate solution to form yellow complex. The absorbance using spectrophotometer was read at 405 nm. For GPx, 0.2 ml tissue homogenate were mixed together with 0.2 ml of 4 mM reduced glutathione, 0.2 ml of 0.8 mM EDTA, 0.1 ml of 2.5 mM H$_2$O$_2$, 0.1 ml of 10 mM sodium azide, 0.4 ml of phosphate buffer (0.4 M, pH 7.0). The mixture was set for incubation for 10 min at 37 °C. The mixture was detained by adding 0.5 ml of 10% TCA followed by centrifugation for 20 min at 2000 rpm. 3 ml of disodium hydrogen phosphate (0.3 M) and 1.0 ml of 40% 5, 5-dithiobis-2-nitrobenzoic acid (DTNB) were added to the supernatant. The reading was immediately taken at 420 nm in spectrophotometer once the colour developed. The activity GST was measured by adding 75 µL of 30 mM CDNB solution, 2 ml of 0.3 M potassium phosphate buffer (pH 6.35), 725 µL of distilled water and 0.1 ml of wound tissue sample into a clean test tube. The mixture was incubated at 37 °C for 10 min. The reaction was then initiated by adding of 100 µL of 30 mM reduced glutathione solution. The absorbance was read by spectrophotometrically at 340 nm. The GR activity in brief, 1 ml of 2.728 Mm GSSG solutions and 40 µL of wound tissue homogenate were incubated at 37 °C in a water bath for 5 min. The reaction was then started by addition of 200 µL of 1.054 mM NADPH solution. The solution was then read spectrophotometrically for absorbance at 340 nm.

**Determination of the NF-κβ p65 activity**

The NF-κβ DNA binding activity was evaluated using NF-κβ p65 transcription factor assay ELISA kit (Active Motif North America, Carlsbad, CA) using the tissue homogenate. NF-κβ activities were articulated as activity/OD units.
**Determination of the of proinflammatory cytokines (TNF-α, IL-6, and IL-1β)**

The supernatant 10% homogenate centrifuged for 15 min at 4000 × g. Pro-inflammatory cytokines (IL-6 and TNF-α) and anti-inflammatory cytokines (IL-10) were measured using ELISA kits according to the manufactures guidelines. The cytokines concentration was expressed in pg/ml.

**Real time PCR**

The expression patterns of Nrf2, Keap 1, collagen-1, TGF-β and α-SMA were investigated using quantitative real-time PCR. The total RNA extraction from the wound tissue sample was carried out using TRIzol (Ambion, Austin, TX, USA). The conversion of RNA synthesis into cDNA was carried out by reverse transcription reaction (Thermo Scientific kit, Burlington, Canada). Then, the PCR analyses of cDNA sample were carried out using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA). The reaction mixture comprise of 9 µL of qPCR Master Mix (EURx Company, Gdansk, Poland), 10ng of cDNA solution and 20 µL of all primers. The qPCR data obtained was scrutinized using the DDCT technique. The PCR results, with the Ct value (elbow value of a PCR amplification curve) and the 2 − ΔΔCT method applied to calculate the relative expression of the target genes. The data were normalized with GAPDH of the same sample against controls.

**Histopathological analysis**

The wound sample was fixed in 10% buffered formalin and paraffin, according to the standard laboratory techniques for histopathological analysis. Next, a serial of 5 µm thick sections was achieved and stained with haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) dye. Slides were examined qualitatively under light microscope (magnification ×200) for changes in the tissues.

**Western blot analysis**

Protein sample of 30 µg was electrophoresed in 10% SDS-PAGE gel to analyze CD68 and CD163. Then, the gel was blocked on Polyvinylidene fluoride or polyvinylidene difluoride (PVDF) membrane with a blocking solution for 1 h (5% skim milk powder in TBST) at room temperature. Membranes were then probed with primary antibodies (Abcam, Cambridge, UK, 1:1000). The next day, membranes were washed and incubated with secondary antibodies (horseradish peroxidase-conjugated antimouse or antirabbit at 1:2000). Proteins were enhanced with chemiluminensce substrate and scanned using Chemidoc XRS imaging system (Bio-Rad, Milan, Italy). The results were expressed in standard units and intensity of bands analyzed using image J software (Biotech Inc). β-Actin used as housekeeping gene.

**Statistical analysis**

All the results are expressed as the mean ± SEM (n = 6) and were evaluated by Student's t-test. Statistical analyses were achieved using Graph Pad Prism (version 5.0; Graph Pad software Inc. San Diego CA, California, USA). The values of p < 0.05 were deliberated as statistically significant.

**Results**

**Effect of neferine on wound contraction rate and mean epithelialization time of wound closure in experimental rats**

The following results were obtained during study of wound healing in control and diabetic experimental rats (Figure 1). The parameter studied was percentage wound closure and the wound closure rate was valued as the percentage of wounds reduced from the initial wound. It was observed that the rate of wound contraction in diabetic control rats (Group II) has decreased in comparison with the normal control animals (Group I). This effect was obvious from 7th day onward. The wound contracting ability of experimental rats receiving neferine topical administration (Group III and IV) showed significant wound healing from the 7th day onward compared with normal control animals (Group I). The maximum percentages (rate) of wound contraction were observed in 20% neferine treated animals (Group IV) compared to other groups at day 14 (Figure 2A). Hence, the 20% neferine treated animal’s revealed better observable effect.

**Effect of neferine on period of epithelization, total collagen and total protein content in experimental rats**

The results of the wound contraction study indicated that process in the experimental rats proceeded almost identically by showing relatable period of epithelialization (Figure 2B) total collagen (Figure 2C) and total protein content (Figure 2D) on the 14 day of experiment. Throughout the experiment, the time for complete epithelialization was slightly prolonged in diabetic control rats (Group II) equated to normal control animals (Group I). However, animals treated with 10% and 20% neferine (Group III and IV) showed faster rate of epithelialization in comparison to diabetic control group (Group II). Similarly, 10% neferine treated animals showed significant difference of epithelialization period as compared to 20% neferine treated animals. The collagen content of diabetic control animals (Group II) was significantly lower than with normal control animals (Group I). Animals treated with 10% and 20% neferine (Group III and IV) showed significant improvement in collagen content compared to diabetic control rats (Group II). In protein content analysis, the diabetic control animals (Group II), was significantly reduced in comparison to normal control group (Group I). The 10% and 20% neferine treated diabetic animals (Group III and IV) improvised the protein content when compared with diabetic control rats (Group II).
Figure 1. The wound-healing effect of neferine topical administration on excision wound model on different days (1, 7, 14).

Figure 2. The effect of neferine treatments on (A) percentage of the wound closure rate (B) period of epithelization (c) total collagen (d) total protein in normal. Values (mean ± SEM) were obtained from each group of 6 animals. *p < .05 compared to the values of day 7. #p < .05 compared to NC.
Effect of neferine in fasting blood glucose and serum insulin levels in experimental rats

Figure 3 recapitulates the blood glucose and serum insulin level together with body weight changes in normal and experimental rats. The baseline of fasting blood glucose in normal control rats were between 45 and 55 mmol/L. Diabetic control group (Group II) showed significant \( p < 0.05 \) increases in blood glucose levels from day 1 when associated with normal control animals (Group I). The administration of neferine, 10% and 20% treated diabetic animals (Group III and IV) significantly reduced glucose insulin levels as compared with diabetic control rats. There was no significant difference among 10% and 20% neferine treated diabetic animals. After 14 days, the insulin levels in serum of diabetic control group (Group II) were reduced significantly when compared with normal control animals (Group I). Treatment with 10% and 20% neferine to diabetic rats (group III and group IV) showed a significant elevation in insulin levels when compared to diabetic control animals (Group II).

Effect of neferine treatment in serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol in experimental rats

The determined concentrations of TC, TG, HDL and LDL after 14 days are presented in Figure 4. There was a significant decline in serum lipid levels (TC, TG and LDL), whereas HDL level was significantly elevated in diabetic control group (Group II) as compared to normal control animals (Group I). In the group treated with 10% and 20% neferine (Group III and IV), the HDL and LDL were restored to near normal control animals (Group I) together with significant reduction in the levels of TC and TG serum.

Effect of neferine treatment on mRNA expression of Nrf2, keap 1, collagen-1, TGF-\( \beta \) and \( \alpha \)-SMA in experimental rats

As shown in Figure 5, the mRNA expression of Nrf2, Keap 1, collagen-1, TGF-\( \beta \) and \( \alpha \)-SMA was illustrated. In diabetic control group (Group II), the mRNA expression of Nrf2, Keap 1, collagen-1, TGF-\( \beta \) and \( \alpha \)-SMA decreased whereas the mRNA expression of Keap 1 was increased markedly respectively when compared to normal control animals (Group I). The 10% and 20% neferine treatment (Group III and IV) restored the mRNA levels of Nrf2, collagen-1, TGF-\( \beta \) and \( \alpha \)-SMA, whereas the levels of Keap 1 was found to be significantly lowered when compared to group II animals.
Effect of neferine treatment on lipid peroxidation and antioxidant enzymes in experimental rats

The activities of lipid peroxidation (TBARS) concentrations in the serum of all animal groups were illustrated in Figure 6A. A significant increase of lipid peroxides was observed in diabetic control group (Group II) when related to the normal control animals (Group I). Administration of neferine to diabetic rats (group III and group IV) significantly reduced the levels of lipid peroxides which are similar to that of diabetic control group (Group II). The activities of enzymic antioxidants SOD, CAT, GPx, GR and GST in the serum of all animal groups were measured and depicted in Figure 6B. As compared to normal group, diabetic control group (Group II) caused significant decrease for all the antioxidant enzymes. However, administration of neferine to diabetic rats (group III and group IV) significantly reduced the levels of lipid peroxides which are similar to that of diabetic control group (Group II). The activities of enzymic antioxidants SOD, CAT, GPx, GR and GST in the serum of all animal groups were measured and depicted in Figure 6B. As compared to normal group, diabetic control group (Group II) caused significant decrease for all the antioxidant enzymes. However, administration of neferine to diabetic rats (group III and group IV) exhibited antioxidant effect by restoring the antioxidants enzymes, close to normalcy. Both 10% and 20% treated diabetic rats (group III and group IV) displayed significant improvement in activity of antioxidants when compared to diabetic control group (Group II).

Effect of neferine on inflammatory markers in experimental rats

Results presented in the Figure 8 indicated that the inflammatory markers activity (NF-κB, TNF-α, IL-6 and IL-1β) was markedly upregulated in diabetic control rats (Group II) compared to the levels in normal control rats (Group I). Though, the treated diabetic rats with 10% and 20% neferine (group III and group IV) significantly decreased the induction of inflammatory markers activity (NF-κB, TNF-α, IL-6 and IL-1β).

Effect of neferine on protein expression of CD68 and CD163 in experimental rats

Western blotting revealed the protein expression of CD68 and CD163 in wound skin area of all experimental groups (Figure 9). An up-regulation of CD68 and CD163 levels was observed in diabetic control rats (Group II) was observed when compared to normal control rats (Group I). Conversely, in the treated diabetic rats with 10% and 20% neferine (group III and group IV), a significant downregulation of
CD68 and CD163 protein expression was noticed in respect-
ive to diabetic control rats (Group II).

**Discussion**

Diabetes and wound healing have an intricate relationship, particularly because numerous symptoms impact the body’s ability to recover at an average pace. When injured, a wound is formed on the normal epithelial layer, known as skin. Skin, the largest organ of the human body, is made of various layers of the ectodermal tissues shielding the muscles, ligaments, bones, and internal organs. It is a protective wall to harmful agents outside including microorganisms, light, heat and injury. The wound healing process is strongly controlled by three overlapping phases; inflammatory reaction, proliferation, and remodelling. During the inflammatory phase, the wound site is characterized by exudation and blood coagulation. The blood vessels release varieties of immune cells to the wound and pro-inflammatory cytokines. Substantial amounts of reactive oxygen species (ROS) is released to protect body against developing infection. Within few days, a reduction of immune cells and inflammatory cytokines occur followed by migration of fibroblasts, endothelial cells and keratinocytes to secrete several growth factors. The proliferative phase begins with the establishment of the epithelium on the wound, superficially with granulation tissue to fill the gap of wound with fibroblasts, deposition of collagens, extracellular matrices (ECM) and new blood vessels (angiogenesis). The process aid in reduction of the wound size and gradually converted into a mature scar. The remodelling process will re-establish tissue’s structural integrity and functional competence [16,17].

Acute wound healing occurs in orderly overlapping processes. However, once this development is disturbed, a non-healing chronic wound will advance. Therefore, a chronic wound healing is disruption in the pathway of normal healing, triggered by the underlying disease such as diabetes. Diabetic patients are often associated with impaired healing and are resistant to existing wound management that eventually lurks limb viability [18]. Managing chronic wound encompasses the usage of anti-inflammatory agents and antibiotics clinically but nearly all drugs require months to years for full-thickness chronic wound healing completion, often expensive and exhibit major adverse effects. This offers a foremost burden for the patient’s health and substantial financial weight on the health care systems. In need of other alternatives, scientists are trying to develop newer drugs for wound care based on natural sources because of their lesser side effects. Plants are rich in various bioactive agents, such as flavonoids, alkaloids, steroids and tannins. These bioactive compounds usually can affect one or more phases of wound healing. Assessment of various plant products conferring to their traditional value and medicinal efficacy leads to the discovery of new and cost-effective drugs for treating various disorders [19]. This forms the base for selection of neferine as a potential wound healing agent. Therefore, the present...
study evaluated the *in-vivo* efficacy of neferine in promoting wound healing in diabetic animal.

Wound healing is a reaction to the injured tissue that results in the re-establishment of tissue's integrity. Thus, wound contraction and closure are important markers in the healing process in open wounds. The form of damage plays an important factor in wound healing. Thus, healing phases of wound models such as excision, incision, dead-space and burn are influenced differently during the process. Based on the length of the experimental period (14 days), excision wound models were selected due to the wider morphological variations happening during the process of wound healing. When compared to normal control rats (Group I) and diabetic control rats (Group II), the 10% and 20% neferine treated diabetic rats (group III and group IV) showed significant wound closure ratios in incision wound models on day 14. Diabetic rodent models exhibited impaired wound repair, with decreased tensile strength with poor collagen deposition. Numerous mechanisms have might be suggested for neferine's wound healing effects which contain guarding the wound from moist, increasing migration of epithelial cell, more rapid maturation of collagen, and reduction in inflammation. Its valuable impact on the healing of wounds in both normal and diabetic rats was also perceived by Ahmad et al. [20].

It is testified that an excision skin wound is cured by the growth of granulation tissue and re-epithelialization. Collagen is the major extracellular protein in the granulation tissue of a wound site and there is a prompt upsurge in the synthesis collagen in the wound site shortly after an injury. Besides providing strength and support to the tissue matrix, collagen also involves in haemostasis process. Subsequent epithelialization also requires collagen. Results obtained suggested that neferine treatment in diabetic wound rats may be probably beneficial for the control of wound healing because it improves the maximum levels of collagen in the granulation tissues [21]. The events of collagen synthesis and intensification of contraction process must have been stimulated by the topical administration of neferine in 10% and 20% neferine treated diabetic rats (group III and group IV). Increased collagen content also supported increased protein in the treated groups. It has been proposed that application of wound dressings based on natural compound mimic the initial cellular environment and extracellular matrix (ECM). It has been reported that collagen improved the efficacy of collagen application can decrease infection by bacteria [22]. Since collagen is the most abundant protein of ECM, collagen-based dressings can considered as ideal biomaterial for wound dressing applications to promote faster wound healing.

Glucose homeostasis in blood circulation is regulated by the balance between the rate of incoming glucose and outgoing glucose. Under normal condition, insulin suppresses enhances glucose removal in the peripheral tissues; but, in a diabetic condition, glucose production is raised, thus glucose appearance surpasses glucose disappearance in the circulation, causing hyper-glycaemia. Similarly in our study, normal control rats (Group I) have basal levels of blood glucose and insulin secretion [23]. Opposing, diabetic rats (group II, III and group IV) have high blood glucose levels and decreased insulin secretion. However, upon administration of 10% and 20% neferine in diabetic rats (group III and group IV), significantly improved the blood glucose and insulin secretion. The beneficial effects of the neferine could be attributed to improved insulin sensitivity which stimulates glucose metabolism and contributing to storage of glucose as glycogen in adipocytes. The improvement is consistent with reduced levels of TC, TG and LDL in 10% and 20% neferine treated diabetic rats (group III and group IV). Fatty acids (FAs) play a critical role in the development insulin resistance, a major risk factor for diabetes. The improvement of insulin sensitivity by neferine may lead to the reduction of lipids, indicating that neferine has a good effect in lowering blood lipids. This observation is in consonance with the findings that most hypoglycaemic plants have potentials of ameliorating diabetic lipid metabolism anomalies [24].

Under physiological condition, reactive oxygen species (ROS) are beneficial to battle the invasion of microbes and also to control few intracellular signalling pathways. However, in diabetic patients, high blood glucose induces oxidative stress to subsequently halt the process of wound healing in an unregulated phase of inflammation [25]. Nuclear related factor 2 (Nrf2) is a part of cellular defence mechanism, in retort to severe oxidative stress. Upon oxidative insult, Nrf2 is dissociated from its cytoplasmic defence repressor Keap1, where it binds to cyto-protective genes encoding antioxidant enzymes (SOD, CAT, GR, and GST) in the nucleus to stimulate the transcription of its ECM-related target genes, including collagen-1, TGF-β and α-SMA [26]. In our study, the rate of wound healing in diabetic control rats (group II) was slower because deprivation of Nrf2 leads to inadequacy in antioxidant defence response, raising the lipid peroxidation level. However in 10% and 20% neferine in diabetic rats (group III and group IV), the nuclear Nrf2 was activated, followed by augmentation of antioxidant enzymes (SOD, CAT, GPx, GR and GST) which further resulted in faster reduction of wound via the ECM related genes (collagen-1, TGF-β) and α-SMA. Chartoumpekis et al. [27] confirmed crucial positive effect of Nrf2 in diabetic wound healing.

Wound healing activity has been attributed to increased collagen deposition and angiogenesis which provides nutrients and promotes tissue granulation. Defective angiogenesis and collagen metabolism in diabetes is thought to be a factor in delayed wound healing [28]. The histopathological study revealed well-organized collagen fibres, increase in fibroblast cells, and new blood vessel formation after the wound treated with 10% and 20% neferine in diabetic rats (group III and group IV) compared to diabetic control rats (group II). The topical application of neferine resulted in significant increase in wound contraction by enhanced epithelialization. It has been reported that topically administered drugs are known to be effective in faster wound contraction, wound closure, and overall healing due to the desired local effect directly at the wound site [29]. Similarly, the possible mechanism of improved healing by neferine could be due to the ability of neferine to sustain the concentration at the site of injury with limited potential for systemic absorption and toxicity. This histopathological observation also provided
additional evidence for the experimental wound-healing studies based on the contraction value of wound areas and period of re-epithelization. Comparable findings were reported where topical application of Moringa oleifera aqueous extract enhanced the wounds healing in diabetic rats through enhancements of wound contraction [30].

Many types of pro-inflammatory cytokines and growth factors are responsible during the wound healing process. The growth factors such as (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF-β) and vascular endothelial growth factor (VEGF) are responsible in all phases of wound healing. As for pro-inflammatory cytokines (NF-κβ, IL-1β, IL-8 and TNF-α), only necessary in the first response of skin wound healing (inflammatory phase) Adequate expression of pro-inflammatory cytokines (NF-κβ, IL-1β, IL-8 and TNF-α) is important to recruit neutrophils and removing bacteria, other contaminations from the wound site and also potent inducers of Metalloproteinase (MMP) synthesis in inflammatory and fibroblasts cells. In wound healing, MMP degrade and remove damaged Extracellular Matrixes (ECM) to supports wound restoration [31]. But, a long duration of inflammatory phase causes a delay in healing process. These cytokines and proteinase destroy the tissue lead to the development of chronic wounds. Thus, all the growth factors are suppressed during chronic wound. Previous studies have reported, impaired wound healing diabetic rats was associated with the significant upregulation of pro-inflammatory cytokines (NF-κβ, IL-1β, IL-8 and TNF-α) [32]. Upregulation of growth factors and downregulation of cytokines were also expressed in the skin wound lysate. Our results are similar to Abu Bakar et al. [33], who successfully promoted diabetic excisional wound healing by suppression of pro-inflammatory cytokines.

Macrophages play a pivotal role in the regulation of wound healing and tissue regeneration. These cells are producing pro-inflammatory cytokines to eliminate pathogens and foreign material. Also, these macrophages secrete various growth factors involved in the regulation of inflammation, wound tissue repair and healing. Macrophages have been operationally defined into M1 and M2 macrophages [34,35]. CD68+ (M1) and CD163+ (M2) are a glycoprotein and a marker of wound healing macrophages. In our study, we observed the protein expression of CD68 and CD163 in diabetic control rats (Group II). The expression os contractedly is reduced to a significant measure in 10% and 20% neferine in diabetic rats (group III and group IV).

Conclusion

We have shown that the neferine has demonstrated a promising wound healing activity in a diabetic animal model through improved wound contraction, epithelialization, and modulation of inflammatory mediators. There is a need for further studies in order to isolate the active ingredients in the plant that are responsible for its biological activities and to elucidate the mechanisms of actions of these active ingredients. Hence, there is a need for further studies into the stability of the compound to ensure an efficacious formulation of products for wound healing.

Disclosure statement

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References

[1] Alexiadou K, Doupijs J. Management of diabetic foot ulcers. Diabetes Ther. 2012;3(1):4.
[2] Tuttolomondo A, Maida C, Pinto A. Diabetic foot syndrome: immune-inflammatory features as possible cardiovascular markers in diabetes. WJO. 2015;6(1):62–76.
[3] Okonkwo UA, DiPietro LA. Diabetes and wound angiogenesis. JMS. 2017;18(7):1419.
[4] Demidova-Rice TN, Durham JT, Herman IM. Wound healing angiogenesis: innovations and challenges in acute and chronic wound healing. Adv Wound Care. 2012;1(1):17–22.
[5] Wan Mohd Azizi WS, Sunzida NK, Azad AK. The screening of local herbs in treating non healing wounds and diabetic foot ulcers complications using nih 3T3 mouse fibroblast and RAW 264.7 mouse macrophage cells. Pharmacol Online. 2016;14:139–145.
[6] Kadioglu O, Law BYK, Mok SWF, et al. Mode of action analyses of neferine, a bisbenzylisoquinoline alkaloid of lotus (Nelumbo nucifera) against multidrug-resistant tumor cells. Front Pharmacol. 2017;8:238.
[7] Tang XQ, Co JG. Review on the pharmacological research of neferine. Chinese Pharmacol Bull. 2004;20(1):8–10.
[8] Jung HA, Jin SE, Choi RJ, et al. Anti-amnesic activity of neferine with antioxidant and anti-inflammatory capacities, as well as inhibition of ChEs and BACE1. Life Sci. 2010;87(13-14):420–430.
[9] Tan WS, Arulselvan P, Ng SF, et al. Improvement of diabetic wound healing by topical application of Vinicenin-2 hydrocolloid film on Sprague Dawley rats. BMC Complement Altern Med. 2019;19(1):20.
[10] Phatak RS, Khanwellkar CC, Matule SM, et al. Antihyperglycemic activity of Murraya koenigii Leaves extract on blood sugar level in streptozotocin-nicotinamide induced diabetes in rats. Biomed Pharmacol J. 2019;12(2):597–602.
[11] Lowry OH, Rosebrough NJ, Farr AL, et al. The colorimetric determination of phosphorus. J Biol Chem. 1951;193:265.
[12] Fox LT, Mazumder A, Dwivedi AM, et al. In vitro wound healing and cytotoxic activity of the gel and whole-leaf materials from Aloe species. J Ethnopharmac. 2017;200:1–7.
[13] Kim J, Lee CM. Wound healing potential of a polyvinyl alcohol-blended pectin hydrogel containing Hippophae rhamnoides L. extract in a rat model. Int J Biol Macromol. 2017;99:586–593.
[14] Das U, Behera SS, Pramanick PK. Ethno-herbal-medico in wound repair: an incisive review. Phytother Res. 2017;31(4):579–590.
[15] Su X, Liu X, Wang S, et al. Wound-healing promoting effect of total tannins from Entada phaseoloides (L.) Merr. in rats. Burns. 2017;43(4):830–838.
[16] Chokpaimam J, Chusri S, Amnuaiikt T, et al. Potential wound healing activity of Quercus infectoria formulation in diabetic rats. Peegl. 2017;5:e3608.
[17] Muhammad AA, Arulselvan P, Cheah PS, Abas F, et al. Evaluation of wound healing properties of bioactive aqueous fraction from Moringa oleifera Lam on experimentally induced diabetic animal model. Drug Design Ther. 2016;10:1715–1730.
Anurag S, Pramod KS, Rakesh KS. Antidiabetic and wound healing activity of Catharanthus roseus L. in streptozotocin induced diabetic mice. Am J Phytomed Clin Ther. 2014;2(6):686–692.

Nagori BP, Solanki R. Role of medicinal plants in wound healing. Res J Med Plant. 2011;5(4):392–405.

Ahmad TB, Liu L, Kotiw M, et al. Review of anti-inflammatory, immune-modulatory and wound healing properties of molluscs. J Ethnopharmacol. 2018;210:156–178.

Daburkar M, Lohar V, Rathore AS, et al. An in vivo and in vitro investigation of the effect of Aloe vera gel ethanolic extract using animal model with diabetic foot ulcer. J Pharm Bioall Sci. 2014;6(3):205–212.

Santosh RK, Rajkiran K, Sunil KP. Evaluation of incisional diabetic wound healing activity of ethanolic leave extract of Mimosa pudica L. in rats. Phyto. 2017;9(8):1143–1147.

Okoduwa S, Umar IA, James DB, et al. Anti-diabetic potential of Ocimum gratissimum leaf fractions in fortified diet-fed streptozotocin treated rat model of type-2 diabetes. Medicines. 2017;4(4):73.

Tang D, Liu L, Ajakber D, et al. Anti-diabetic effect of Punica granatum flower polyphenols extract in type 2 diabetic rats: activation of Akt/GSK-3β and inhibition of IRE1α-XBP1 pathways. Front Endocrinol (Lausanne). 2018;9:586.

Jindam A, Yerra VG, Kumar A. Nrf2: a promising trove for diabetic wound healing. Ann Transl Med. 2017;5(23):469.

Chang AS, Hathaway CK, Smithies O, et al. Transforming growth factor-β1 and diabetic nephropathy. Am J Physiol Renal Physiol. 2016;310(8):689–696.

Chartoumpekis DV, Palliyaguru DL, Wakabayashi N, et al. Nrf2 deletion from adipocytes, but not hepatocytes, potentiates systemic metabolic dysfunction after long-term high-fat diet-induced obesity in mice. Am J Physiol Endocrinol Metab. 2018;315(2):E180–E195.

Ahmad M, Sultana M, Raina R, et al. Hypoglycemic, hypolipidemic, and wound healing potential of quercetin in streptozotocin-induced diabetic rats. Phcog Mag. 2017;13:633–639.

Lipsky BA, Hoey C. Topical antimicrobial therapy for treating chronic wounds. Clin Infect Dis. 2009;49(10):1541–1549.

Tang Y, Choi EJ, Han WC, et al. Moringa oleifera from Cambodia ameliorates oxidative stress, hyperglycemia, and kidney dysfunction in type 2 diabetic mice. J Med Food. 2017;20(5):502–510.

Fard M, Arulselvan P, Karthivashan G, et al. Bioactive extract from Moringa oleifera inhibits the pro-inflammatory mediators in lipopolysaccharide stimulated macrophages. Phcog Mag. 2015;11(44):556.

Ponrasu T, Suguna L. Efficacy of Annona squamosa on wound healing in streptozotocin-induced diabetic rats. Int Wound J. 2012;9(6):613–623.

Abubakar AM, Arulselvan P, Cheah PS, Abas F, et al. Evaluation of wound healing properties of bioactive aqueous fraction from Moringa oleifera Lam on experimentally induced diabetic animal model. Drug Des Devel Ther. 2015;10:1715–1730.

Brochhausen C, Schmitt VH, Mamilos A, et al. Expression of CD68 positive macrophages in the use of different barrier materials to prevent peritoneal adhesions-an animal study. J Mater Sci Mater Med. 2017;28(1):15.

Zuo Q, Wang SC, Yu XK, et al. Response of macrophages in rat skeletal muscle after eccentric exercise. Chin J Traumatol. 2018;21(2):88–95.