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Acceleration of wound healing activity with syringic acid in streptozotocin induced diabetic rats

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ABSTRACT

Impaired wound healing is a serious concern of uncontrolled hyperglycemia that can lead to gangrene, and even death. There is an urgent need to look for better alternative therapy because of the undesirable side effects of currently available synthetic drugs in the market. Syringic acid (SA) is a natural phenolic compound abundantly available in edible fruits and plants. In this study, wound healing activities of 2.5% and 5.0% SA were evaluated in type 2 diabetic rats using incisional wound model. SA-treated diabetic wounds showed faster rate of wound closure and epithelization with enhanced contents of hydroxyproline and protein compared to diabetic wounds. SA effectively prevents alterations in blood glucose levels, serum insulin and dyslipidemia in diabetic wound rats. The SA-treated diabetic wounds after 14 days of treatment demonstrated inhibition of pro-inflammatory response (NF-κB p65, TNF-α, IL-1β, IL-8 and IL-2) with improvement in anti-inflammatory response (IL-10), inhibited the elevated oxidative stress and decreased the concentrations of matrix metalloproteinases (MMP-2, −8 and −9) and increased the concentrations of TIMP-1 & TIMP- 2. Furthermore, the diabetic wounds were presented with an increase in expression of CD 31 and 68, growth factors (TGF-β1, collagen-I and α-SMA and VEGF) with significant improvement in collagen deposition, re-epithelialization and complete skin structure as revealed by histological analysis after treatment of diabetic wounds with SA for 14 days. Hence, the results of this study designate that SA significantly improves wound healing in diabetic rats and could be used as a potential therapy for treatment of diabetic wounds.

1. Introduction

Diabetes mellitus is a metabolic disorder that resulted mainly due to a defect in insulin secretion, insulin action or both resulting in hyperglycemia. In recent years, the global occurrence of diabetes has been increasing at a greater pace and patients with diabetes had a higher morbidity and mortality risk compared to normal population. According to 2015 estimates of International Diabetes Federation, it was predicted that there were 415 million people living with diabetes aged between 20 and 79 years and this number can rise to 642 million by 2040 [1]. It was also estimated that the global deaths and health expenditure attributable to diabetes were 5 million and 673 billion US dollars, respectively [1].

The wound healing is intricate and dynamic phenomenon involving communication at biochemical, immunological and physiological levels. The wound healing is an innate process that involves important phases, such as inflammatory response, cellular migration, proliferation, matrix deposition and remodeling of tissue [2]. This process is accompanied by many modulators such as cytokines, growth factors, matrix metalloproteinases (MMPs), cellular receptors and extracellular components [3]. The impaired wound healing is a serious complication that resulted mainly due to a combination of intense inflammation by neutrophil infiltration and impacting growth factor interactions (insulin-like growth factors and vascular endothelial growth factors) leading to poor re-epithelialization or angiogenesis in diabetic patients [4–7].

The impairment in wound healing in diabetic patients has been associated with serious complications such as chronic open wounds, gangrene, amputation or even death [8–9]. Earlier it was reported in diabetic wound model that hyperglycemia induced the formation of free radicals and advanced glycation end products (AGEs), reduced the antioxidant enzyme activities such as SOD, CAT and GPX and ultimately delayed the wound healing process [10,11]. During wound healing process in diabetes, the expression of NF-κB pathway genes were upregulated and triggered the downstream expression of inflammatory cytokines (TNF-α, IL-1β and IL-8) and delayed the diabetic

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wound healing. The histo-morphometric analysis after wounding in diabetic group revealed reepithelization was not complete with less amount of collagen [12]. Although antibiotics could be used as a therapy to treat diabetic wounds, the common serious issues associated with it are the unnecessary side effects and drug resistance. Therefore, alternative therapies are urgently needed to overcome the above-mentioned symptoms and shorten the process of healing diabetic wounds using animal models [13].

Now a days, the usage of phytochemicals gained public and scientific interest due to its natural origin and less side effects. One such naturally occurring phenolic compound is syringic acid (SA), derived from shikimic acid pathway in edible plants and fruits. SA is an abundant phenolic compound present in grapes, red wine, honey, acai palm, dates, olives, pumpkin, spices and in other plants [14]. An experimental study conducted in rabbits showed that the absolute bioavailability of SA in blood samples was found to be 86.27% [15]. SA is known for its wide range of applications in biomedical sector such as anti-oxidant, anti-inflammatory, anti-microbial, anti-cancer, anti-adipogenic and anti-diabetic effects, hepato-protective, neuro-protective, cardio-protective [16–23]. SA provided significant protection to wide range of oxidative stress associated diseases through its potent antioxidant capacity [24]. Several *in-vitro*, *in-vivo*, *in-silico* studies have revealed that SA exhibits anti-inflammatory effects through the regulation of various genes and pathways involved in inflammation [25]. Taking into account all reported pharmacological activities, the current study was undertaken to evaluate SA *in-vivo* wound healing potential using macroscopic, biochemical, molecular and histopathological methods on type 2 diabetic rat type incisional wounds.

2. Results

2.1. Effect of SA on percentage of wound closure and epithelialization period

Fig. 1A depicts the variations in wound closure at first, third, seventh and fourteenth day in control and experimental groups. On the third day of experiment, crust was observed in all the groups. The wound closure was significantly delayed ($p < 0.05$) on 3rd, 7th and 14th days in diabetic wounded rats when compared to non-diabetic wounded rats. While, the % wound closure in diabetic rats was significantly improved on day 3rd, 7th and 14th after 2.5% ($p < 0.05$) and 5.0% ($p < 0.05$) SA applied rats when compared to diabetic rats (Fig. 1B).

Additionally, the duration of epithelialization in diabetic wounded rats was significantly ($p < 0.05$) delayed when compared to normal wounded rats. Whereas treatment of diabetic wounds for 14 days with 2.5% ($p < 0.05$) and 5.0% SA ($p < 0.05$) has significantly improved the epithelialization compared to that of diabetic wounds. The epithelialization was faster in diabetic wounds treated with 5.0% SA (Fig. 1C).

2.2. Effect of SA on blood glucose, lipid profile and serum insulin levels

Blood glucose levels were higher in diabetic wounded rats as evidenced by higher levels of blood glucose levels on days 3, 7 and 14 when compared to normal control wounded rats (Fig. 2A). On the other hand, the treatment of diabetic wounded rats with SA 2.5% and 5.0% showed significant improvement with diminution in the levels of blood glucose on 7th and 14th day when compared to diabetic wounded rats.

Serum levels of TC, TG and LDL were significantly ($p < 0.05$) higher with significant ($p < 0.05$) lower in the levels of HDL in diabetic wound rats as compared to normal control wounded controls. Meanwhile, the treatment of diabetic wounded rats with 2.5% and 5.0% SA caused a significant decrease ($p < 0.05$) in the serum levels of TC, TG and LDL levels with significant improvement ($p < 0.05$) in the HDL levels as compared to the corresponding diabetic wound controls (Fig. 2B).

The serum levels of insulin on the other hand were significantly decreased ($p < 0.05$) in diabetic wounded rats when compared to only wounded control rats (Fig. 2C). Whereas the diabetic wounded rats subjected to 2.5% and 5.0% SA treatment for 14 days exhibited significant increase ($p < 0.05$) in insulin levels compared to diabetic wounded rats.

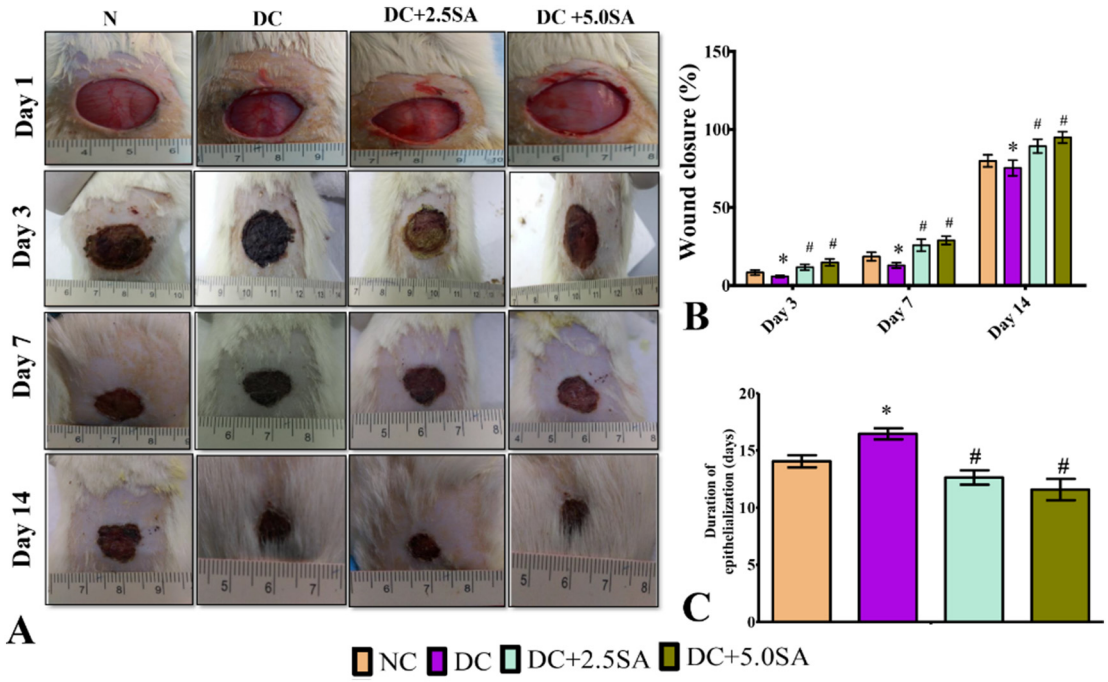

Fig. 1. (A) Effects of syringic acid treatments on wound contraction. Photographic representation of contraction rate on different days of normal control (NC), diabetic control (DC), 25 and 50% syringic acid (DC + 25SA & DC + 50SA) applied diabetic wounds; B) percentage of the wound closure; (C) duration of epithelization (days). Values (mean ± SD) were obtained from each group of six animals. Four animals from each group were used for the evaluation of period of epithelialization. $^{*}p < 0.05$ compared to NC. $^{\#}p < 0.05$ compared to DC.

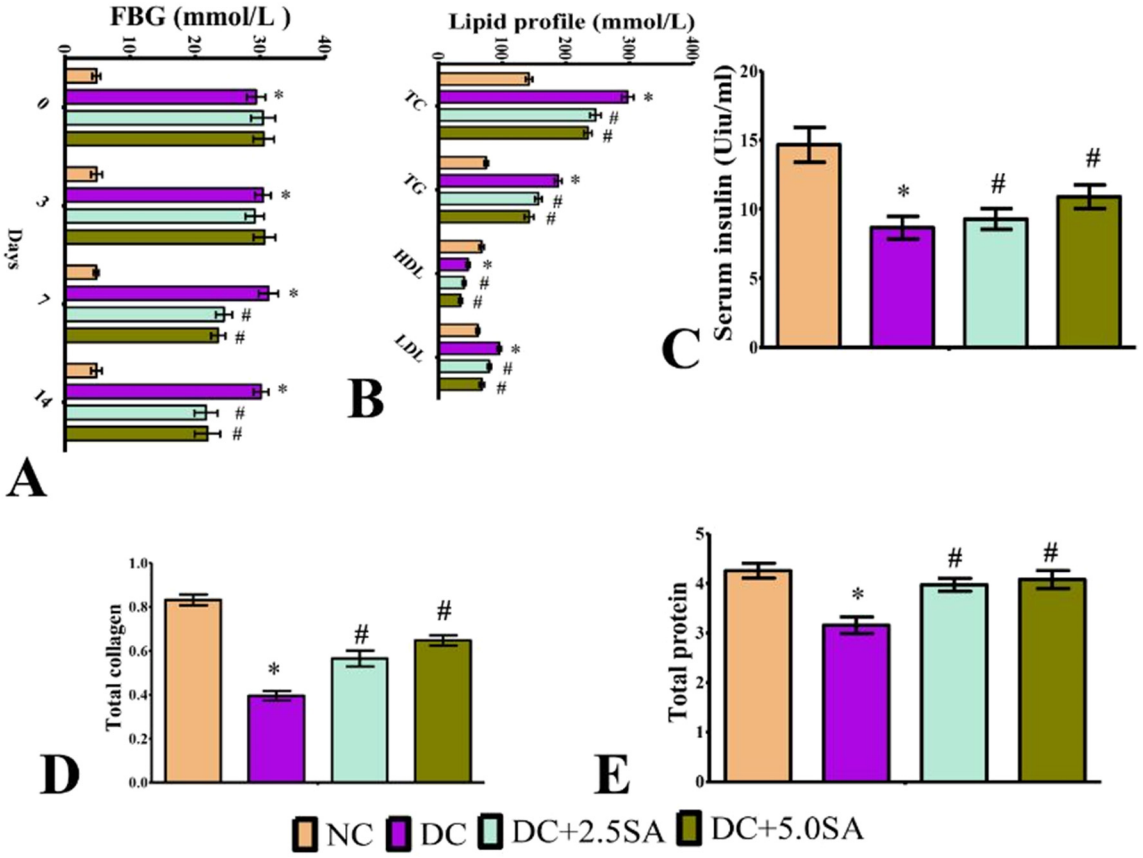

Fig. 2. Effects of syringic acid treatments on (A) fasting blood glucose (FBG) (B) lipid profile such as total cholesterol (TC), (B) Triglycerides (TG), (C) High density lipoproteins (HDL), (D) Low density lipoproteins (LDL) (C) Serum insulin levels (D) Total collagen (wound-healing skin) (E) Total protein (wound-healing skin) in normal control (NC), diabetic control (DC), 25 and 50% syringic acid (DC + 25SA & DC + 50SA) applied diabetic rats. Values (mean ± SD) were obtained from each group of six animals. $^{*}p < 0.05$ compared to NC. $^{\#}p < 0.05$ compared to DC. Total collagen units: mg/100 mg dry tissue; Total protein: mg/100 mg wet tissues.

2.3. Effect of SA on hydroxyproline content and total protein

The concentration of hydroxyproline is a direct measure of collagen content. As shown in Fig. 2D and E, the hydroxyproline content and total protein in wound healing tissue taken from diabetic wounded rats were significantly decreased ($p < 0.05$) compared to that of wounded control rats. While, the hydroxyproline content and total protein were significantly increased ($p < 0.05$) in diabetic wounded rats treated with 2.5% and 5.0% SA 14 days after wounding when compared to diabetic wound rats.

2.4. Effect of SA on antioxidant status

The mRNA expression level of *Nrf2* was significantly downregulated ($p < 0.05$) associated with an upregulation ($p < 0.05$) in the mRNA expression level of *Keap1* in wound healing tissue from wounded diabetic rats when compared to wound control rats. Whereas treatment of wounded diabetic rats with 2.5% and 5.0% SA results in a significant improvement ($p < 0.05$) in the *Nrf2* and *Keap 1* expression when compared to same expression levels in diabetic wound rats (Fig. 3A). Furthermore, the levels of MDA were significantly increased with a significant decrease ($p < 0.05$; Fig. 3B) in the activities of antioxidant such as SOD, CAT, GPx, GST and GR in the wounded tissue from diabetic rats compared to control rats. The SA 2.5% and 5.0% gel application to diabetic wound rats significantly reduced ($p < 0.05$) the MDA levels with significant improvement ($p < 0.05$) in the antioxidant enzyme activities when compared to diabetic wound control rats (Fig. 3C).

2.5. Effect of SA on expression levels of collagen-1, α-SMA and TGF-β

The wound healing tissues taken from the diabetic wound rats have shown significant reduction ($p < 0.05$) in the mRNA expression of *collagen-1*, *α-SMA* and *Tgf-β*, when compared to normal wound rats. On the other hand, the diabetic wounds treated with 2.5% and 5.0% SA has resulted in a significant elevation in the mRNA expression levels of *collagen-1, α-SMA* and *TGF-β* compared to that of diabetic wound controls (Fig. 3A).

2.6. Effect of SA on NF-κβ p65 DNA binding activity and levels of TNF-α, IL-8, and IL-1β

The NF-κB p65 DNA binding activity was significantly increased ($p < 0.05$; Fig. 4A) in association with an increase in the levels of TNF-α, IL-1β and IL-8 in the diabetic wounded skin homogenate compared to same expression levels in wounded control skin of rats (Fig. 4B). However, the application of 2.5% and 5.0% SA gel to diabetic wounded skin of rats for 14 days has resulted in a significant decline ($p < 0.05$) in the NF-κB p65 binding activity and levels of TNF-α, IL-1β and IL-8 compared to that of diabetic wounded skin.

2.7. Effect of SA on MMP-2, MMP-9, TNF-α, IL-10, TIMP-1 and TIMP-2 gene expression

The quantitative real-time PCR analysis has revealed that compared to the wounded control group of rats, the *MMP-2*, *MMP-9, TNF-α, TIMP-1* and *TIMP-2* mRNA expression levels were upregulated ($p < 0.05$) with downregulation in the mRNA expression level of IL-10 in diabetic

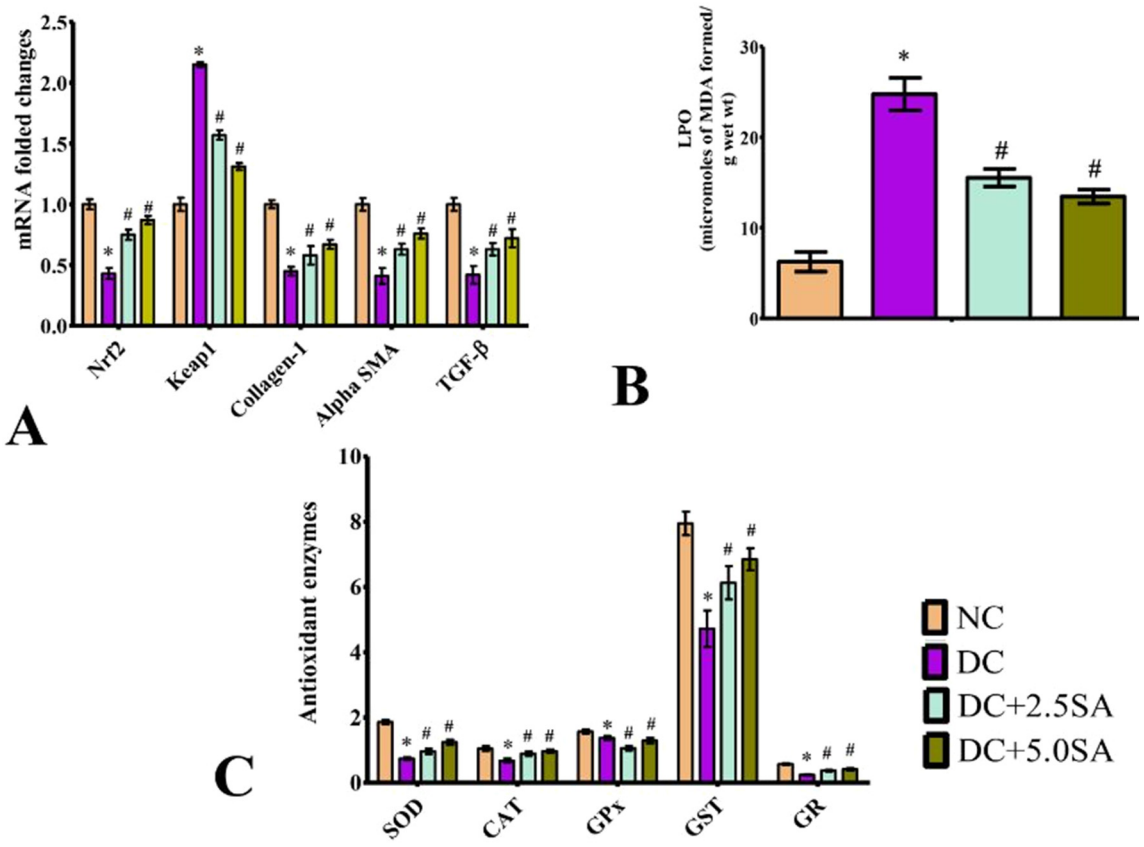

Fig. 3. Effects of syringic acid treatments on wound-healing skin of (A) *Nrf2, Keap1, collagen-1, α-SMA, TGF-β* mRNA levels (B) LPO product malondialdehyde (MDA) levels, (C) SOD, CAT, GPx, GST and GR in normal control (NC), diabetic control (DC), 25 and 50% syringic acid (DC + 25SA & DC + 50SA) applied diabetic rats. Values (mean ± SD) were obtained from each group of 6 animals. $^{*}p < 0.05$ compared to NC. $^{\#}p < 0.05$ compared to DC.

wound group of rats. Moreover, application of 2.5% and 5.0% SA gel to diabetic wounded tissue caused downregulation ($p < 0.05$) in the mRNA expression levels of MMP-2, MMP-9, TNF-α, TIMP-1 and TIMP-2 with an upregulation mRNA expression level of IL-10 when compared to that of diabetic wound controls (Fig. 4C).

2.8. Effect of SA on protein expression of TNF-α, IL-2, MMP-2, MMP-8, TGF-β, VEGF, TIMP-1 and TIMP-2

The immunofluorescence analysis of TNF-α (Fig. 5A) and immunohistochemistry analysis of IL-2 (Fig. 5B) showed an increased protein distribution of both TNF-α and IL-2 in diabetic wounded rats compared with the normal wound rats. However, SA 2.5% and 5.0% treatments for 14 days to diabetic wounds resulted in a downregulated distribution of TNF-α and IL-2 compared to that of only diabetic wounds.

The immunofluorescence results of MMP-2 (Fig. 6A) and immunohistochemistry results of MMP-8 (Fig. 6B) revealed an upregulation in the protein distribution of MMP-2 and MMP-8 with a downregulation in the protein distribution of TGF-β (Fig. 7A) and VEGF (Fig. 7B) in diabetic wounded rats. While the treatment of diabetic wound rats with SA 2.5% and 5.0% for 14 days has resulted in a downregulated distribution of MMP-2 and MMP-8 with an upregulated distribution of TGF-β and VEGF compared to that of only diabetic wounded controls.

The immunofluorescence results of TIMP-1 (Fig. 8A) and immunohistochemistry results of TIMP-2 (Fig. 8B) revealed an upregulation in the protein distribution of TIMP-1 and TIMP-2, while treatment of diabetic wounds with SA 2.5% and 5.0% for 14 days has resulted in a downregulation of TIMP-1 and TIMP-2 protein distribution when compared with diabetic wounded controls.

2.9. Effect of SA on protein expression of CD 31 and CD68

The western blotting results have revealed a decreased protein expression of CD 31 and CD 68 in diabetic wounds compared to that of normal wounds. Whereas treatment of diabetic wounds with SA 2.5% and 5.0% caused an increased protein expression of CD 31 and CD 68 in diabetic wound rats when compared to diabetic wound rats (Fig. 9).

2.10. Effect of SA on histopathology

Microscopic analyses helped us to determine wound healing events in the wound healing tissues collected on day 15 and represented in Fig. 10. Photomicrographs of skin sections from control group showed well stratified and thick epithelium, complete remodeling of dermis and well developed skin appendages (hair follicles and sebaceous gland). Diabetic wound group presented with complete epidermis shedding with infiltrated inflammatory cells in dermis and tissue area loss as shown by empty spaces. Rat skin section photomicrographs from diabetic wound with application of 2.5% SA gel showed regenerating epithelial layer, thick collagen bundles with mixed arrangement pattern, formation of blood vessels and mild spaces. While the application of 5.0% SA to diabetic wounds has resulted in a complete regeneration as evidenced by a prominent reepithelization and well organized collagen bundles with horizontal pattern of arrangement, abundant blood vessels and complete dermal reconstruction.

3. Discussion

The global prevalence of diabetes has been increasing drastically and attracting the attention of both scientific and public community. It has substantial effect on patient's health, expenditure and quality of life.

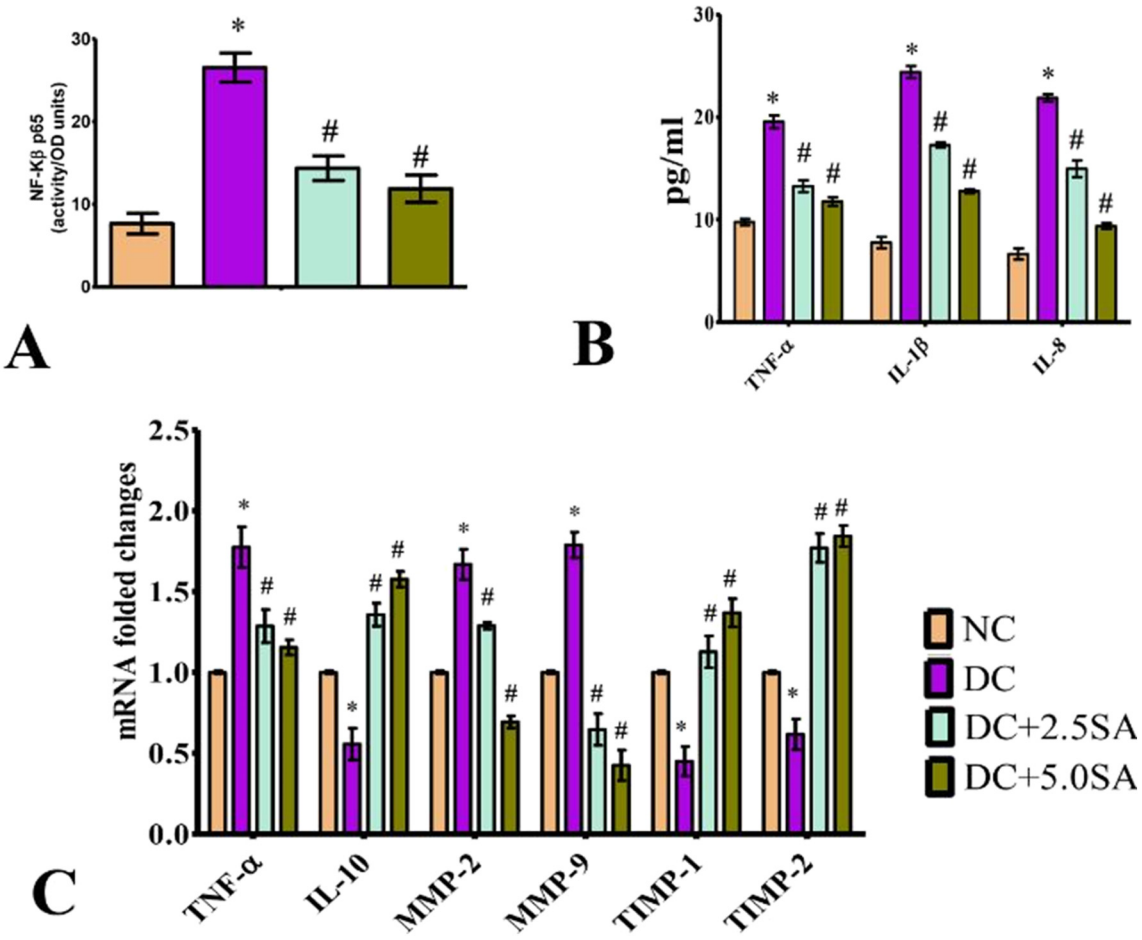

Fig. 4. Representative ELISA results of (A) nuclear NF-κB p65 (B) TNF-α, IL-1β, IL-8; representative mRNA results of (C) TNF-α, IL-10, MMP-2, MMP-9, TIMP-1, TIMP-2 in wound healing skin of normal control (NC), diabetic control (DC), 25 and 50% syringic acid (DC + 25SA & DC + 50SA) applied diabetic rats. Values (mean ± SD) were obtained from each group of 6 animals. $^{*}p < 0.05$ compared to NC. $^{\#}p < 0.05$ compared to DC.

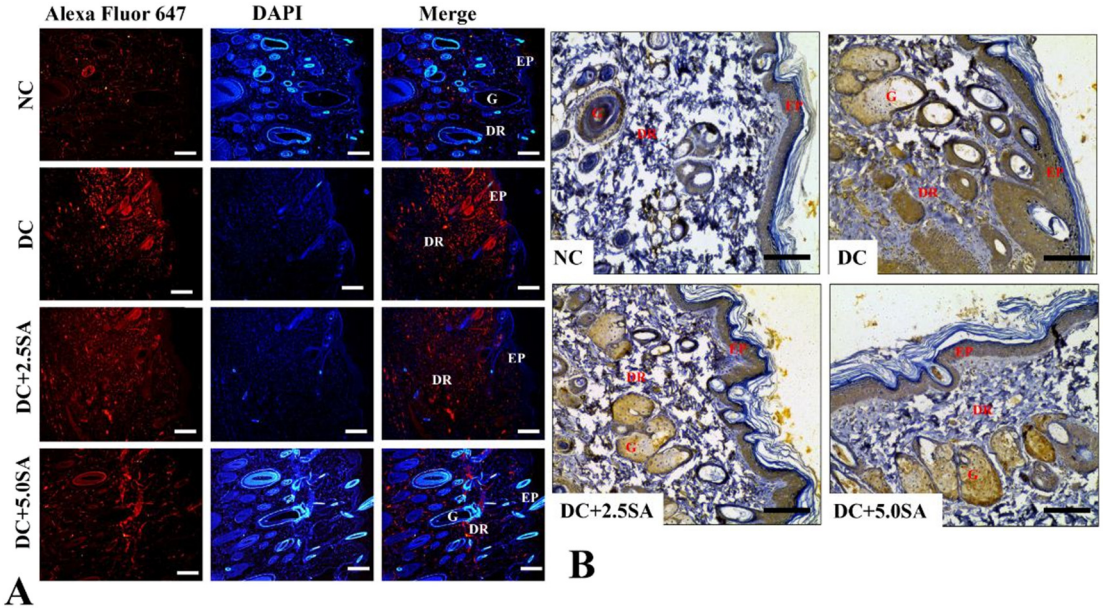

Fig. 5. Representative immunofluorescence staining (red color) of (A) TNF-α and immunohistochemistry staining (brown color) of (B) IL-2 in normal control (NC), diabetic control (DC), 25 and 50% syringic acid (DC + 25SA & DC + 50SA) applied diabetic rats. Scale bar = 50 μm. EP: epidermis; Dr.: dermis; G: gland. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

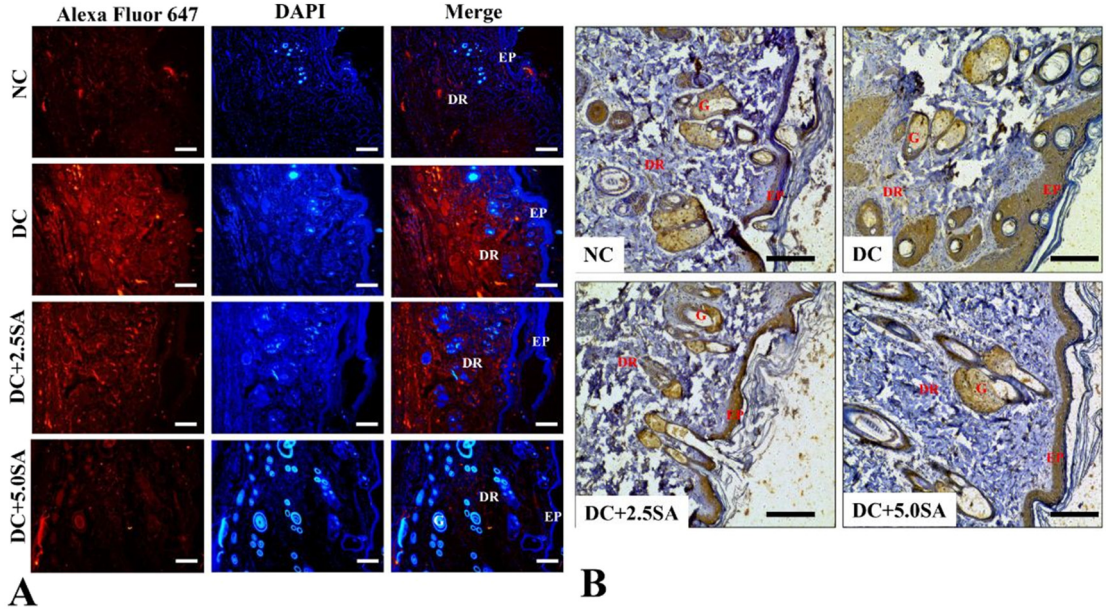

Fig. 6. Representative immunofluorescence staining (red color) of (A) matrix metalloproteinase-2 (MMP-2) and immunohistochemistry staining of (B) matrix metalloproteinase-2 (MMP-8) (brown color) in wound-healing skin of normal control (NC), diabetic control (DC), 25 and 50% syringic acid (DC + 25SA & DC + 50SA) applied diabetic rats. Scale bar = 50 μm. EP: epidermis; Dr.: dermis; G: gland. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The impaired wound healing is a serious concern of uncontrolled hyperglycemia that can lead to gangrene and amputation if left untreated [26]. On an estimate worldwide out of the one million people who undergo leg amputation annually, 75% out of them are having type 2 diabetes. There are various synthetic drugs available in the market for wound management, but due to the undesirable side effects there is an urgent need for the detection of an alternate remedy with less side effects and more effectiveness. Now days, the usage of bioactive compounds has got the attention for the effective management of wound healing during hyperglycemia. One such compound is Syringic acid (SA) which is well known for its various pharmacological effects, so in this study we attempted to investigate the possible protective role of SA in wound healing in type 2 diabetes using rat as an experimental model. This is the first report to show that SA accelerates the process of wound healing in type 2 diabetic rats through its anti-diabetic, anti-lipidemic, anti-inflammatory and anti-oxidative effects.

Wound healing is a complex, dynamic and an orderly process that involves active communication between cell-cell and cell-matrix with various phases such as inflammation, cellular proliferation and tissue remodeling [27,28]. It has been reported that hyperglycemia is the main culprit responsible for the delayed wound healing process in diabetic patients [29]. In the present study, the induction of diabetes

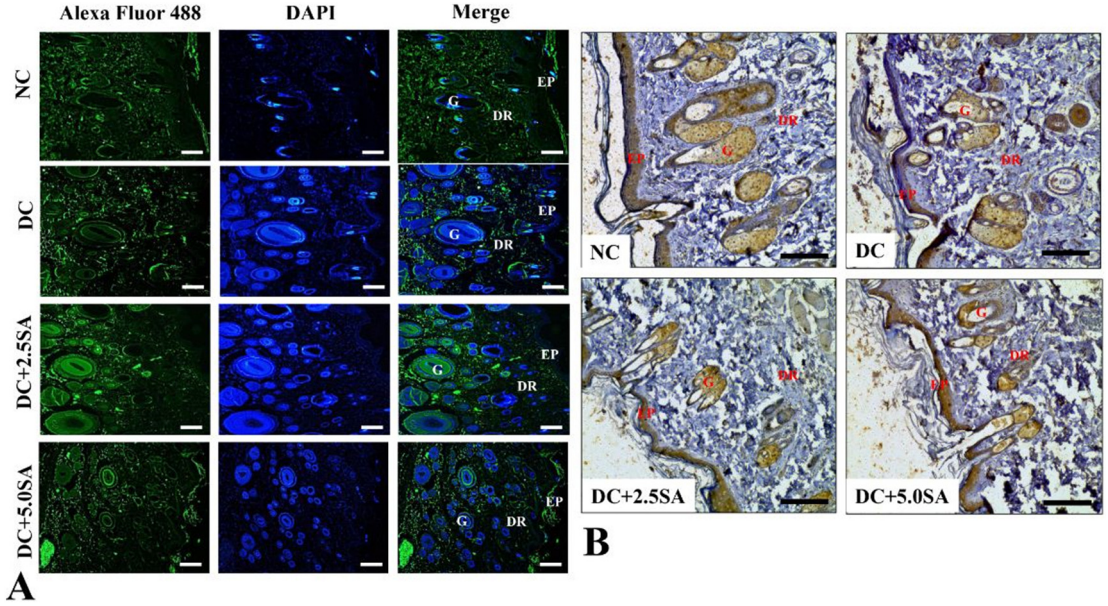

Fig. 7. Represenative immunofluorescence staining (green color) of (**A**) Transforming growth factor beta (TGF-β) and immunohistochemistry staining of (**B**) Vascular endothelial growth factor (VEGF) (brown color) in wound-healing skin of normal control (NC), diabetic control (DC), 25 and 50% syringic acid (DC + 25SA & DC + 50SA) applied diabetic rats. Scale bar = 50 μm. EP: epidermis; Dr.: dermis; G: gland. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

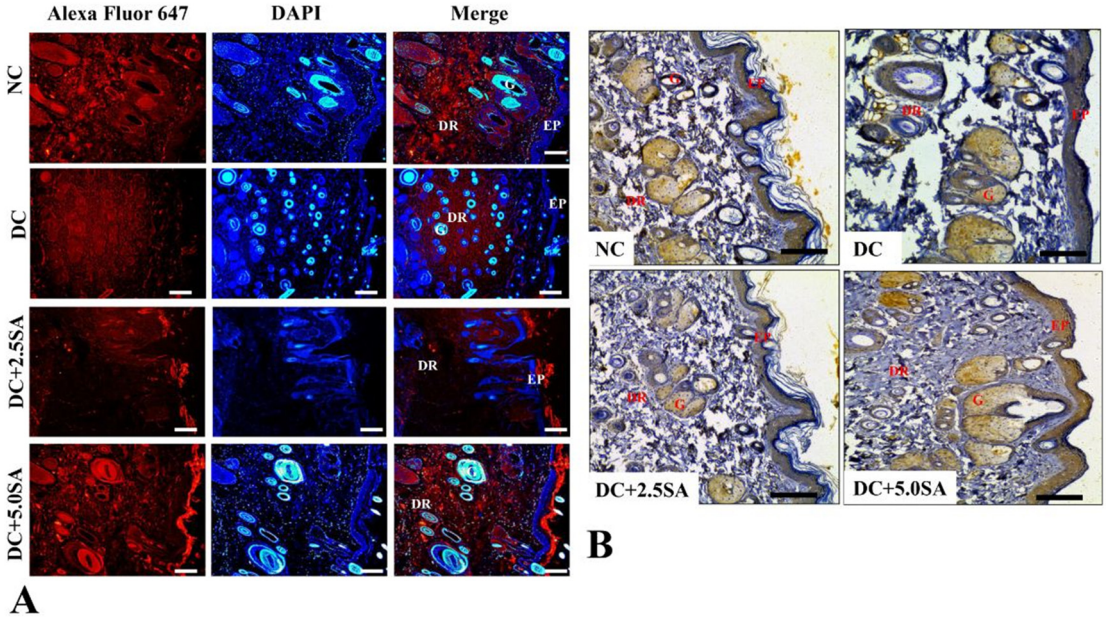

Fig. 8. Represenative immunofluorescence staining (green color) of (**A**) Tissue inhibitors of metalloproteinases-1 (TIMP-1) and immunohistochemistry staining of (**B**) Tissue inhibitors of metalloproteinases-2 (TIMP-2) (brown color) in wound-healing skin of normal control (NC), diabetic control (DC), 25 and 50% syringic acid (DC + 25SA & DC + 50SA) applied diabetic rats. Scale bar = 50 μm. EP: epidermis; Dr.: dermis; G: gland. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

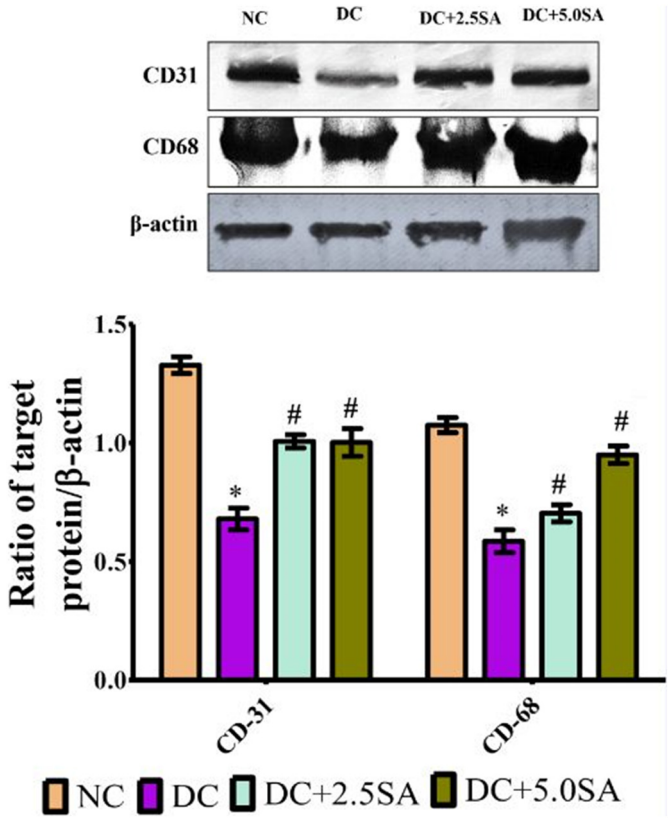

Fig. 9. Representative western blotting for (A) CD31 (B) CD68 in wound area of normal control (NC), diabetic control (DC), 25 and 50% syringic acid (DC + 25SA & DC + 50SA) applied diabetic rats. Values (mean ± SEM) were obtained from each group of 6 animals. $^{*}p < 0.05$ compared to NC. $^{\#}p < 0.05$ compared to DC.

with nicotinamide and streptozotocin, this mimics type 2 diabetes conditions in humans, caused a significant rise in blood glucose levels after 72 h in all the rats and persisted during the entire 14 days experimental period. Further the serum levels of insulin were significantly decreased after 14 days of wounding in diabetic rats compared to same levels in wound control rats. These results are in accordance with earlier reports [30,31]. While treatment of diabetic wounds in rats with either 25 or 50% SA has resulted in significant decrease in blood glucose levels with significant increase in serum insulin levels compared to diabetic wound rats indicating the anti-diabetic effect of SA. The results were well

ted by Srinivasan et al. (2014b) [32], who demonstrated anti-hyperglycemic effect of SA in management of diabetes.

The dyslipidemia and altered metabolism of triglyceride-rich lipoproteins are frequently observed in patients with type 2 diabetes [33]. In the present study, a significant increase in levels of total cholesterol, triglycerides and LDL cholesterol was observed along with a significant decline in the HDL cholesterol in diabetic wound rats. However, treatment of diabetic wounds with 2.5 and 5.0% SA for 14 days has shown a significant reduction in total cholesterol, triglyceride, and LDL cholesterol levels along with a significant increase in the HDL cholesterol level. The results are in accordance with Ramachandran and Raja (2010) [34], who found that treatment with SA shows anti-lipidemic activity by lowering levels of TGL, TG and LDL levels and increasing HDL in acetaminophen-induced hepatic damage in albino rats.

Wound contraction is a progressive mechanism to reduce the healing time and to produce adequate granulation tissue to repair damaged tissue [35]. *Re*-epithelialization is one of the crucial stages of wound repairing process which allows keratinocytes to move across the wound bed for the restoration of epidermal layers. The results of the present study revealed a decrease in the rate of wound closure with an increase in the epithelialization period in diabetic wound rats compared to wound control rats. The results were supported by Lan et al. (2008) [36], who reported that hyperglycemia can lead to impaired migration and proliferation of keratinocytes leading to insufficient re-epithelialization. However, treatment of diabetic wounds with 2.5 and 5.0 SA for a period of 14 days has resulted in enhanced rate of wound closure with a decrease in epithelialization period. So it is reasonable to suspect that

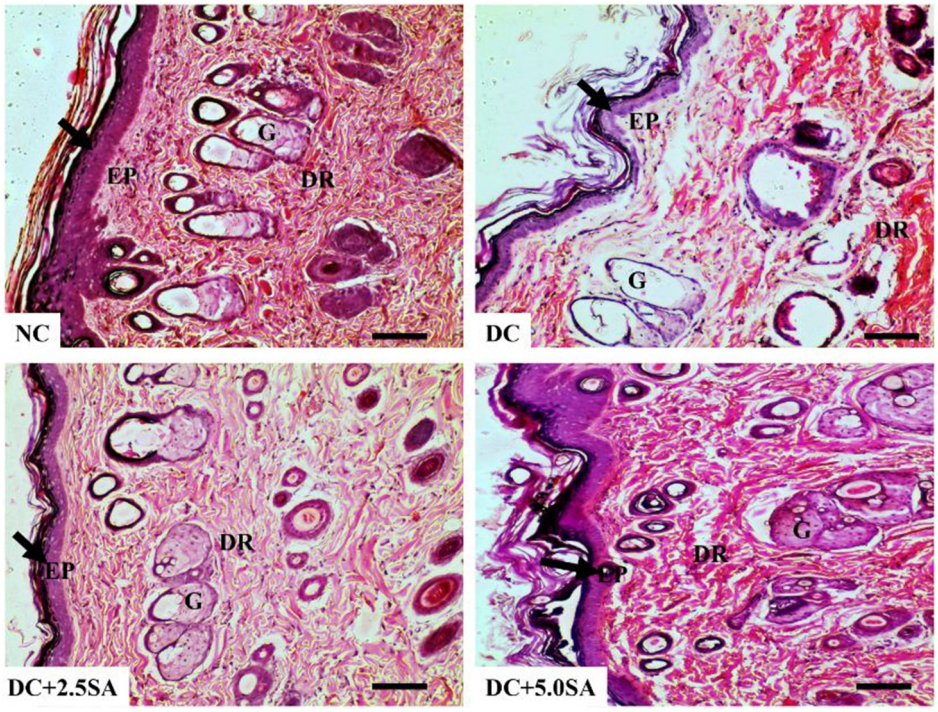

Fig. 10. Effects of syringic acid treatments on (A) hematoxylin and eosin (H & E) pictures of wound-healing skin in normal control (NC), diabetic control (DC), 25 and 50% syringic acid (DC + 25SA & DC + 50SA) applied diabetic rats. Scale bar = 50 μm. EP: epidermis; Dr.: dermis; G: gland.

SA treatment promotes enhanced migration, proliferation of epithelial cells, functioning of myofibroblasts and involved in improvement of wound healing.

Collagen is the main component of the extracellular matrix and its synthesis, deposition, remodeling and maturation are critical steps during tissue repair and regeneration [37]. In this study, hydroxyproline content as a measure of collagen was significantly increased in diabetic wounds tissue after the application of 2.5 and 5.0 SA indicating collagen synthesis and maturation in healing the wound. Further, histopathological studies also confirmed the effectiveness of SA by means of matured collagen fibers in the wound tissue after 14 days of SA treatment to diabetic wounds. These results were further supported by significant increase in total protein content in SA treated diabetic wound group of rats when compared to only diabetic wound group of rats.

Several studies have pointed out a plausible relationship between the elevated levels of oxidative stress and impaired wound healing in diabetic condition [38]. It has also been claimed that an increased free radical generation and lack of antioxidant defenses interferes with fibroblast proliferation and has damaging effects on proteins, lipids and DNA that leads to delaying of healing process [39]. The Nrf2 and Keap1 are key players in antioxidant defense mechanism and are activated by oxidants and electrophiles [40]. During normal biological conditions Nrf2 binds to Keap1 in cytosol, while at induced conditions, dissociation of Nrf2 from Keap1 and translocation into nucleus occurs to bind promoter region of ARE. Nrf2-ARE binding regulates the expression of various antioxidant genes that are involved in antioxidant defense mechanism. In the current study, hyperglycemic wound induces oxidative stress as evidenced by downregulation in the mRNA expression levels of Nrf2 with an upregulation in the expression level of Keap1 mRNA when compared to normal wounds. The results are in consonance with earlier reports of decreased Nrf2 and increased keap1 in diabetic wound healing condition [41]. The changes were effectively reversed in diabetic wound rats by treating SA. The results of this study have been supported by a recent study in which SA therapy reduces ischemia injury by activating Nrf2 [42]. Moreover, hyperglycemia in diabetic wound rats promotes the generation of ROS that leads to delay in the wound healing process. The increased ROS generation leads to increased production of mitochondrial superoxide and auto-oxidation of glucose, resulting in increased lipid peroxidation. The elevated levels of lipid peroxidation are attributed to the enhancement of ROS [43]. The results of the present study showed an increase in lipid peroxidation (MDA) levels with a decrease in antioxidant enzyme activities such as SOD, CAT, GPx, GST and GR in diabetic wounds compared to normal wounds. The results are in par with the earlier reports [44]. While treatment of diabetic wound rats for 14 days with 25 and 50 SA showed significantly decreased levels of MDA in association with decreased activities of antioxidant defenses compared to diabetic wound controls. The SOD plays a vital role in the reduction of oxidative stress in tissue matrix as evidenced by an increased activity of SOD which presumably dismutates the highly toxic superoxide radicals into less toxic hydrogen peroxide and dioxygen free radicals. By increasing CAT and GPx enzyme activities, the resulting hydrogen peroxides were enzymatically neutralized into oxygen and water. In addition, the increased enzyme activities of GR and GST observed in the present study provide reduced glutathione to neutralize toxic electrophiles [45]. The results clearly suggest that SA showed a synergic effect as an effective antioxidant activity by reducing the oxidative stress in wound area, which is a promising effect in wound repairing process.

The sustained oxidative stress can activate various transcription factors including NF-κB and lead to chronic inflammation. Normally in the cytoplasm, NF-κB exists in an inactive form and upon stimulation it translocate into the nucleus to stimulate the transcription of battery of genes involved in inflammation. Previously it has been reported that the stimulation of NF-κB transcription factor leads to the expression of inflammatory cytokines, chemokines and anti-inflammatory molecules [45] and it is reasonable to postulate that NF-κB is involved in wound healing process. In the current study, the binding activity of NF-κB p65, levels of TNF-α, IL-1β and IL-8 mRNA expression levels of TNF-α and protein expression of TNF-α and IL-2 were significantly increased on the day 14 after wounding in diabetic wound rats when compared to diabetic wound control rats. Earlier it was demonstrated that tissue

destruction results in infiltration of neutrophils, monocytes, and mast cells to the injury site and produces cytokines thereby acute inflammatory phase ensues [46]. Previously, Eming et al. (2017) [47] reported that cytokines (TNF-α) and interleukins (IL-1β) trigger the inflammatory response by activating more neutrophils and macrophages, which although are essential for normal repair process, can cause deleterious effects when intense release occurs. The data of the current study also showed that application of 2.5 and 5.0% SA to diabetic wounds for 14 days results in reduction of DNA binding activity of NF-κB, levels of TNF-α, IL-1β and IL-8, mRNA expression levels of TNF-α, IL-10 and protein expression of TNF-α and IL-2 compared to diabetic wounds. Our results supported the assumption of SA involvement in the suppression of NF-κB activation and inflammatory response to improve wound healing process. Earlier it was postulated that suppression of activated NF-kB pathway and downstream inflammatory genes expression is involved in improvement of wound healing process [48].

Macrophages displays diverse functional phenotypes depending upon the tissue condition. Under normal wound healing conditions, recruited macrophages demonstrates pro-inflammatory phenotype by recruiting additional inflammatory cells [49]. As wound healing progresses this pro-inflammatory phenotype switches to anti-inflammatory to promote reduction in inflammation, secretion of growth factors and tissue repair [50]. Barros et al. (2013) [51] recognized that the expression of CD 31 and 68 are considered as suitable tools for the detection of macrophages in the tissue sections. In the present study, diabetic wound condition in rats caused a diminution in the mRNA expression of ant-inflammatory cytokine IL-10 and protein expression levels of CD 31 and 68 compared to that of only wound rats. The results were also supported by a study of Werner and Grose (2003) [52], who proposed that ablation of macrophage-produced cytokines leads to decrease in fibroblast proliferation and extracellular matrix (ECM) deposition and thereby impairs healing process and hampers functional recovery. The impairment in the switch from pro-inflammatory to anti-inflammatory has contributed to prolonged inflammation with delay in wound healing process [53,54]. While the treatment of diabetic wound rats with 2.5 and 5.0% SA resulted in a significant upregulation in the protein expression level of CD 31 and 68 indicating the role of SA in modulating macrophages for the improvement of healing process in diabetic wounds.

MMPs are endopeptidases and their primary function is to remodel tissue through selective processes of proteolytic degradation [55]. Under normal conditions, MMPs are expressed in basal level whereas during wound healing process wide range of cytokines and growth factors transcriptionally activate MMPs through different pathways [56]. Uncontrolled MMP activity leads to tissue damage and functional alterations. MMPs are endogenously inhibited by tissue inhibitors of metalloproteinases (TIMPs), which are involved in proteolytic and all other activities of MMPs and thereby regulates ECM remodeling and re-epithelialization [57]. These findings suggest that physiological equilibrium of MMP/TIMP is to be maintained for the normal wound healing process to occur. The results of this study showed significant upregulation in the metalloproteinases mRNA (MMP-2 and MMP-9) and protein (MMP-2 and MMP-8) expression with downregulation in the mRNA and protein expression levels of TIMP-1 and TIMP-2 in diabetic wounds when compared to normal wounds. Earlier Yang et al. (2017) [58] reported compromised wound healing features due to the excessive production of MMPs. The results of the present study are also supported by a study of Lobmann et al. (2002) [56] in which increased concentrations of matrix metalloproteinases (MMP-1, -2, -8 and -9) and decreased concentrations of its inhibitors (TIMP-2) are observed in wounds of diabetic patients compared to non-diabetic patients. Whereas treatment of diabetic wounds with 2.5 and 5.0% SA resulted in downregulated mRNA and protein expression of those MMPs with upregulated mRNA expression of TIMPs, which were involved in ECM remodeling and re-epithelialization.

During wound healing process, TGF-β is secreted and is involved in multiple functions, such as inflammation, fibroblast proliferation, angiogenesis and collagen synthesis, deposition and remodeling of extracellular matrix [60]. In this study, we examined mRNA expression levels of TGF-β1 as well as the collagen-I and α-SMA expression levels of mRNA, which are stimulated by TGF-β1 and are involved in maintaining the cutaneous structure and integrity. The current results showed significant decrease in the expression of TGF-β, collagen 1 and αSMA in diabetic wound rats compared to normal wound rats. While the treatment of diabetic wounds for 14 days with 2.5 and 5.0% SA resulted in significant enhancement in TGFβ, collagen 1 and αSMA expression levels leading to re-epithelialization and wound closure at a faster rate when compared to diabetic wound rats.

The Vascular endothelial growth factor (VEGF), the proangiogenic mediator stimulates endothelial cell functions necessary for the formation of new blood vessels. The VEGF has a role in tissue proliferation, migration, differentiation and survival, which contributes angiogenesis and influences wound repair, closure and granular tissue formation [61]. In the present study, immunofluorescence studies revealed that the protein expression of VEGF was downregulated in diabetic wound rats when compared to normal wound rats. Earlier it was reported in diabetic patients that abnormally low levels of VEGF are associated with insufficient vascularization along with wound closure difficulties, reduction in granular tissue reepithelialization and formation [62,63]. On the other hand topical application of 2.5 and 5.0% SA showed an upregulated expression of VEGF in diabetic wound rats indicating the angiogenic potential of SA.

The results of biochemical and molecular analyses were well supported by histopathological studies. The histopathological studies well supported the results of biochemical and molecular analyses, where diabetic wound group presented with complete epidermis shedding with infiltrated inflammatory cells in dermis and tissue area loss as evidenced by empty spaces. While treatment of diabetic wound with SA gel showed improvement in the wound healing as evidenced by a prominent reepithelization and well organized collagen bundles with horizontal pattern of arrangement, abundant blood vessels and complete dermal reconstruction.

In conclusion, the results of this study indicate that topical application of SA effectively enhanced the wound healing process in diabetic rats through increase in collagen content and aiding in faster wound closure and re-epithelialization. The improvement of wound healing process in hyperglycemic rats may be related to its anti-oxidant (upregulation of Nrf2 mRNA, downregulation of Keap1 mRNA, decrease in MDA levels with increase in SOD, CAT, GPx, GST and GR activities), anti-inflammatory (reduction of NF-κB p65 activity, TNF-α, IL-1β, IL-8 levels, TNF-α mRNA levels and TNF-α,IL-2, CD 31 and 68 protein expression with an increase in IL-10 mRNA levels) properties and assistance of growth factors (increased expression of TGF-β, collagen-1, α-SMA mRNA expression and TGF-β and VEGF protein expression) and inhibition of matrix metalloproteinases (downregulation in MMP-2 and MMP-9 mRNA and MMP-2 and MMP-8 protein expression) with improvement in inhibitors of metalloproteinases (upregulation in TIMP-1 and TIMP-2 mRNA and protein expression). The macroscopic, biochemical and molecular results were well supported by microscopic analysis in the form of improvement in re-epithelialization, collagen deposition, inflammation, neovascularization and complete skin structure. Consequently, the results showed that SA significantly improves wound healing in diabetic rats and this could be a potential therapy to treat diabetic wounds. Although the present research model mimics many features of diabetic wounds, genetically modified db/db mice will be a suitable model to ideally mimic human type 2 diabetes.

4. Materials and methods

4.1. Chemicals and reagents

Streptozotocin (STZ), Syringic acid ($C_9H_{10}O_5$) were procured from

Sigma Aldrich (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade and procured from local commercial sources.

4.2. Animal procurement and maintenance

For this study, male Albino Wistar strain rats of 200 ± 20 g body weight were used. The rats were kept under relative humidity in the animal house (50 ± 5%) and controlled room temperature (23 ± 2 °C) at 12 h light: 12 h dark cycle. Rats were allowed to fed with *ad libitum* commercial rat feed (Research Diets, Inc., New Brunswick, NJ, USA) and water. All the experimental procedures were carried out in accordance with the guidelines of the First Affiliated Hospital of Zhengzhou University. The Institutional Animal Ethics Committee approved the protocols and animal usage. Animals were caged individually to prevent fighting and biting with other animals.

4.3. Induction of diabetes mellitus

Experimental type 2 diabetes was induced in male Wistar rats with the help of a single intraperitoneal injection of nicotinamide (NA; 110 mg/kg body weight) dissolved in sterile water and then after 15 min, animals were administered intraperitoneally with 55 mg/kg body weight STZ dissolved in freshly prepared 0.1 M of cold citrate buffer (pH 4.5). After 72 h of administration, rat's tail vein blood was collected to measure the glucose [64]. The animals showing > 250 mg dL^{-1} fasting blood glucose level were considered as diabetic and used further for the experimentation.

4.4. Incisional wound model

During surgery, the rats were anesthetized with 3% isoflurane. The dorsal region of the animals was clipped off using an electric clipper. One full-thickness round wounds were created on dorsal midline with a 21 mm sterilized Chinese coin. The day on which wound was created was considered as day 0. For wound healing, the rats were randomly divided into four groups of eight in each:

Group I: Normal control group, in which rats were allowed to heal on its own without treatment.

Group II: Diabetes control group, in which rats induced with type 2 diabetes and allowed to heal naturally without treatment.

Group III: Type 2 diabetic animals treated with topical application of 2.5% Syringic acid (SA) on wounds for 14 consecutive days.

Group IV: Diabetic animals treated with topical application of 5.0% Syringic acid (SA) on wounds for 14 consecutive days.

4.5. Determination of the optimal concentration of SA

Several concentrations of SA were dissolved in methanol (0, 312, 625, 1250, 2500, 5000 and 10,000 μg/L). Eighteen normal control rats were used to find the optimum concentration for wound healing. Wound healing rates suggested that optimum healing effects were recognized at concentrations 2500, 5000 μg/L (Supporting File 1). The application of SA was done slowly from outside to inside through circling the wounds and make sure all the wound areas were properly treated.

4.6. Collection of blood samples

The rats were given mild anesthesia after 14 days of SA treatment and blood samples were collected into EDTA tubes for hematological analysis. Another set of blood samples without anticoagulant were collected at 3000 *g* centrifuged for 15 min, and serum collected was used for various biochemical assays.

4.7. Percentage of wound contraction and period of epithelialization

The percentage of wound contraction was calculated on the basis of the percentage of wound reduced from the original wound size. The wound area was marked on a transparent tracing sheet and the wound surface was measured [62].

The percentage of wound closure was measured on days 3, 7 and 14.

$$\text{Percentage wound closure} = \frac{\text{Wound area on day 0} - \text{wound area on day (n)}}{\text{Wound area on day 0}} \times 100$$

where n = number of days (3, 7 and 14).

The epithelialization period (number of days required to drop the dead tissue without any sign of raw wound) was also measured during the wound healing process [63].

4.8. Estimation of total protein and collagen content

Animals were sacrificed on day 15 and wound tissues were collected for estimation of total protein and hydroxyproline content which were considered as a measure of collagen content. For the determination of protein from the wound tissues, it was extracted with 5% trichloroacetic acid as described by Porat et al. (1980) [65] and estimation was done based on the method described by Lowry et al. (1951) [66]. The collagen content was determined based on the method of Woessner Jr. and biophysics (1961) [67]. Total collagen units were expressed as mg/100 mg dry tissue.

4.9. Estimation of blood glucose

The levels of fasting blood glucose (FBG) were monitored on the experimental 0th, 3rd, 7th and 14th day on tail vein blood collection to ensure diabetes maintenance by using digital glucometer (Accu-Cheks, Roche Diagnostic, Meylan, France).

4.10. Serum insulin and lipid profile analysis

The level of serum insulin was measured by using ELISA kit (Millipore, USA) according to manufacturer's protocol. The levels of serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-cholesterol were determined by automatic analyser (Beckman Coulter Inc., Ireland).

4.11. Determination of lipid peroxidation

The wounded skin was homogenized (10%w/v), centrifuged at 4000 *g* for 15 min and then collected supernatant was used to estimate lipid peroxidation using thiobarbituric acid (TBA) reaction as described [68]. This reaction measures the level of lipid peroxidation product *i.e.*, malondialdehyde (MDA). The result is expressed as mmol of MDA formed/g wet weight of tissue.

4.12. Estimation of antioxidant status

The enzyme activities of superoxide dismutase (SOD) [69], catalase (CAT) [70], glutathione peroxidase (GPx) [71], glutathione-S-transferase (GST) [72] and glutathione reductase (GR) [73] were estimated using standard protocols. The activities of SOD, CAT, GPx, GST and GR were expressed as units per mg protein per min, μmoles of H_2O_2 metabolized/mg protein/min, μmoles of GSH consumed/mg protein/min, μmoles of CDNB conjugation formed/min/mg protein and μmoles of NADPH oxidized/mg protein/min, respectively.

4.13. Measurement of the NF-κB p65 activity in wound healing skin

The NF-κB DNA binding activity was measured in nuclear fraction by using ELISA kit (Active Motif North America, Carlsbad, CA) following manufactures' protocol. The activities in the samples were represented as activity/OD units.

4.14. Measurement of the TNF-α, IL-8, and IL-1β

TNF-α (EK0526; Biocompare, San Francisco CA, USA), IL-8 (Cloud-Clone Corp; Wuhan, China), and IL-1β (EK0393; Biocompare, San Francisco CA, USA) levels by ELISA based kits (Biocompare, San Francisco CA, USA). The assay was carried out as per manufacturer guidelines and the results were expressed in pg/mL.

4.15. Real time PCR

Total mRNA was extracted from wounded tissue by using Trizol reagent (Invitrogen, Carlsbad, CA) and 1 μg of RNA was synthesized into cDNA by reverse transcription reaction (Thermo Scientific kit, Burlington, Canada). The cDNA samples were subjected to PCR analyses using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Applied Biosystems, Foster City, California 94404, USA). The relative expression of target genes was quantified with the help of Ct values and $2^{-\Delta\Delta CT}$ method. GAPDH was used as a house keeping gene. The PCR primer sequences used were as follows.

Nrf2- F:5′AGCACATCCAGACAGACACCA3′,
R:5′TATCCAGGGCAAGCGACTC3′;
Keap1-F:5′AGCAGGCTTTTGGCATCAT3′,
R:5′CCGTGTAGGCGAACTCAATTAG3′;
Tnf-α- F:5′ GCTCCCTCTCATCAGTTCCA3′,
R:5′ GCTTGGTGGTTTGCTACGAC3′;
Il-10- F:5′ CAGACCCACATGCTCCGAGA3′,
R:5′ CAAGGCTTGGCAACCCAAGTA3′;
Mmp2- F:5′ CCCGTTATGAGACCCTGAGC3′,
R:5′ AGACCAATCGTGCCTCCATC3′;
Mmp9- F:5′ AGGACGGTCGGTATTGGAAG3′,
R:5′ GTACACCCACATTTTGCGCC3′;
Tmp-1- F:5′ TCCTGGTTCCCTGGCATA3′,
R:5′ ATCGCTCTGGTAGCCCTTCT3′;
Tmp-2- F:5′ GCATCACCCAGAAGAAGAGC3′,
R:5′ TGACCCAGTCCATCCAGAG3′;
Tgf-β- F:5′ GCATCACCCAGAAGAAGAGC3′,
R:5′ TGACCCAGTCCATCCAGAG3′;
Collagen-1- F:5′ CAGCCGCTTCACCTACAGC3′,
R:5′ TTTTGTATTCAATCACTGTCTTGCC3′;
α-SMA- F:5′ CCGACCGAATGCAGAAG GA3′,
R:5′ ACAGAGTATTTGCGCTCCGAA3′;
Gapdh- F: 5′AGTGCCAGCCTCGTCTCATA3′,
R:5′ GGTAACCAGGCGTCCGATAC3′;

4.16. Histopathological analysis

The wounded tissues were fixed in 10% neutral buffered formalin solution, embedded in paraffin to perform histopathological studies. The sections were cut at 5 μm thick and stained with hematoxylin and eosin (H&E). Using a light microscope, the sections were visualized at a magnification of ×200.

4.17. Immunohistochemistry and immunofluorescence staining

For immunohistochemistry, deparaffinized and rehydrated sections were antigen retrieved with 10 mM sodium citrate buffer (pH 6.0). The sections were blocked with bovine serum albumin (BSA) and allowed for incubation with primary antibody [IL-2, MMP-8, VEGF and TIMP-2] (Abcam, Cambridge, UK, 1:100) overnight at 4 °C. After, three times washing with phosphate buffer saline (PBS), the sections were incubated with appropriate biotinylated secondary antibodies. The sections were stained with *3′-Diaminobenzidine (DAB)* and counterstained with hematoxylin to view under Olympus phase contrast microscope (Tokyo, Japan).

For immunofluorescence, the sections were incubated overnight at 4 °C with primary antibody (TNF-α, MMP-2, TGF-β and TIMP-1; Abcam, Cambridge, UK) at a dilution of 1:100 in PBS. After three times PBS washing, the sections were incubated for 60 min at room temperature with secondary antibody conjugated with Alexa Fluor® 488 or Alexa Fluor 647 (1:500, Invitrogen, Carlsbad, CA, USA). The sections were mounted using UltraCruz (Santa Cruz, CA, USA) mounting medium with DAPI for visualize nuclei. Images were taken with the help of a fluorescence microscope (Leica DM IRB, Germany).

4.18. Western blot analysis

About 30 microgram of proteins were taken and electrophoresed on a 10% sodium dodecyl sulphate (SDS) polyacrylamide gel. After, the gels were wet-transferred on to PVDF membranes and block with BSA. Then, the membranes were incubated with anti-CD31 (abcam) and anti-CD68 (abcam) primary antibodies overnight at 4 °C in PBST. The membranes were washed 3 times with PBST and incubated with secondary antibodies at room temperature for 1 h. The visualization was done by chemiluminescence kit (Bio-Rad, Milan, Italy). Densitometry was performed using Image J software. The housekeeping protein β-actin was used as a loading control.

4.19. Statistical analysis

All the values were expressed as the mean ± standard deviation and the results obtained were analyzed using Student's *t*-test with raw data. One-way ANOVA was used when multiple comparisons were performed. The values of $p < 0.05$, were considered as statistically significant.

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Declaration of competing interest

None.

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