Life Sciences 231 (2019) 116422

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Evaluation of wound healing activity of plumbagin in diabetic rats

Yi Shao[a], Minyan Dang[b], Yukiat Lin[b], Feng Xue[c,*]

[a] Department of Dermatology, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, School of Clinical Medicine, Henan University, Zhengzhou, Henan, 450003, China
[b] Innoscience Research Sdn Bhd, Subang Jaya, 47650, Selangor, Malaysia
[c] Department of Plastic & Cosmetic Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021, China

ARTICLE INFO

Keywords:
Plumbagin
Antidiabetic
Anti-inflammatory
Nrf2
Wound healing

ABSTRACT

This study was performed to evaluate the antidiabetic and wound healing activity of plumbagin in diabetic rats by macroscopical, biochemical, histological, immunohistochemical and molecular methods. Percentage of wound closure and contraction was delayed in diabetic rats when compared to non-diabetic group. There was significant reduction in period of epithelialization, collagen and protein content. Serum insulin level was significantly lowered together with increase in glucose level in diabetic rats. Lipid levels were increased significantly with concomitant decrease in HDL level. The mRNA levels of Nrf2, collagen-1, TGF-β and α-SMA were significantly lowered whereas Keap-1 levels were increased in diabetic rats. The level of lipid peroxides was increased while the levels of antioxidants were lowered significantly. ELISA results reveal upregulated levels of inflammatory markers. Western blot result shows upregulated levels of CD68 and CD163 proteins in wound area of diabetic rats. Histopathological observation revealed increased inflammatory cells infiltration in diabetic control. Immunofluorescent staining and immunohistochemical analysis also displayed delayed wound healing in diabetic groups. Diabetic rats treated with 10% and 20% plumbagin showed increased epithelialization, collagen deposition, increased serum insulin level and increased antioxidant status. Lipid peroxides and lipid levels were lowered significantly with increase in HDL level. Inflammatory markers were lowered, and growth factors expressions were increased markedly. Thus, the results of the study indicated that plumbagin administration could improve wound healing activity and could serve as a potent antidiabetic and anti-inflammatory agent.

1. Introduction

Diabetes Mellitus (DM), the most common and prevalent disease which is increasing at an alarming rate worldwide and the complications of diabetes accounts for the disease burden among diabetic patients. The health burden resulting from diabetes is because of the lifestyle changes [1]. Diabetes mellitus is a chronic metabolic disease with unenthusiastic effects on wound healing. Diabetes has the effect of decreasing wound and the levels of hydroxyproline. The troubles encountered through wound healing in diabetic patients include granulation of tissues, decreased cellular infiltration, the amount and formation of collagens and angiogenesis. This leads to a worsen infectious complications. Although, the causes of these problems seen in diabetes cannot be fully understood, hyperglycemia is commonly held responsible for these circumstances. Increased levels of blood glucose have been shown to inhibit cell proliferation and collagen production. Furthermore, conditions that cause a decrease in growth factors and fibroblast proliferation, and apoptosis increase injury in the tissue cells. Infections caused by a decrease in chemotaxis and phagocytosis, have shown to have adverse effects of hyperglycemia on wound healing as well [100].

The chronic wounds such as diabetic foot ulcers, pressure ulcers and venous leg ulcers are a major problem to wound healing specialization and use a great deal of healthcare resources around the worldwide [26]. Opening of the normal skin that might have resulted from physical injuries or by injuries from heat is usually referred to as wounds [2,3]. These wounds are capable of causing disruption to the normal anatomy of skin and its functions. Apart from healing wounds, non-healing wounds have emerged as a major and threatening health care concern worldwide [4–6]. Wounds can be categorized into chronic and non-chronic wounds, among which the non-chronic wounds are closely associated with DM which is a common disease worldwide at present [7–10]. To manage the issues that contribute to late wound healing are vital components of an inclusive approach to wound care and at present

* Corresponding author.
E-mail address: drgzhslw@sina.com (F. Xue).

https://doi.org/10.1016/j.lfs.2019.04.048
Received 24 December 2018; Received in revised form 8 April 2019; Accepted 18 April 2019
Available online 03 May 2019

the principal challenges to the therapy of chronic wounds. For wounds that fail to resolve after 4 weeks of standard care, reassessment of the underlying pathology and consideration of the need for advanced therapeutic agents should be undertaken.

Wound healing or repair is a dynamic and complex process which involves four phases such as hemostasis, inflammation, proliferation and remodeling [11–14]. The multifactorial process of wound healing can be possibly delayed by the presence of infectious microbes [15,16] or by the presence of free radicals [11,16]. Inflammation plays a vital role in the d disturbance of the normal healing pathological cascade. Chronic wound caused by various factors including, pressure, arterial, venous, and diabetic ulcers, can be observed during a juxtaposition of normal healing and the rogue inflammatory response created by the common mechanism surrounded by chronic wounds such as hypoxia, ageing, bacterial colonization and ischaemia-reperfusion injury [102] and increased expression of inflammatory cytokines [17–19], elevated matrix metalloproteases (MMPs) and elastases [20,21] disturbed neovascularization, changes in morphology of collagen, decreased collagen deposition, increased proteolytic activity, decreased production of growth factors, impaired endothelial function and impaired angiogenic responses. Decreased or impaired deposition of collagen, high blood glucose level [22] and increased ROS production is found to be positively correlated with delayed response in wound healing [23,24].

Wound healing represents a challenging societal and medical problem which tends to affect the quality of life in majority of the population [25]. The patients with chronic diabetic wound require hospitalization and the treatment options remains unruly because of the complex association of cellular and molecular peculiarity in those persons. Early diagnosis and treatment among those individuals may reduce the risk of morbidity and mortality [26]. Numerous synthetic forms of drugs are available in the market for the treatment of wound, but they are at expensive cost and it also results in adverse effects. Allergic reactions and resistance to drugs are the major problems that limit the usage [4]. This leads to the development of an alternative and effective treatment approach for treating diabetic wounds. The usage of medicinal plants or its bioactive components which possess numerous pharmacological properties might pave way for developing a new therapeutic strategies for treating diabetic wounds [5,29].

Plumbagin (5-hydroxy-2-methyl-naphthalene-1,4-dione) is one such bioactive phytoconstituent which is present in relatively higher concentration in the roots of *Plumbago zeylanica* [30]. It constitutes about 0.03% of dry weight of the roots [31]. The root portion of the plant contains various bioactive constituents such as coumarin, naphthoquinone, binaphthoquinones and anthroquinones [32]. Plumbagin is a naphthoquinone and it has been shown to possess numerous therapeutic properties which is capable of treating diseases of the liver and spleen, diarrhea, dysentery, leprosy, fevers and various infections [33–35]. It has also been shown to have protective effects such as anticarcinogenic, anti-atherosclerotic, anticoagulant, antimutagenic, hypolipidemic, antibacterial, antifungal, anti-fertility, cardioprotective, antioxidant, antimalarial, antihyperglycemic and anti-inflammatory properties [36–40]. Besides these pharmacological properties, the studies related to diabetes and wound healing property of plumbagin has not been done extensively and the literature evidences is also limited. Hence, with this background, our present study aimed to investigate the protective role of plumbagin on wound healing properties in diabetic rats.

2. Materials and methods

2.1. Chemicals and reagents

Streptozotocin (STZ), Plumbagin from *Plumbago indica* (CAS Number 481-42-5; Fig. 1) were procured from Sigma Aldrich. All other chemicals used were analytical grade.

2.2. Animal procurement and maintenance

Male wistar albino rats of body weight around 200 ± 20 g were procured from Shandong Provincial Hospital Affiliated to Shandong University and used for the study. The rats were maintained in standard conditions in animal house under relative humidity and temperature. Rats were fed with commercial rat feed (pellets) and water ad libitum. Prior 7 days to the start of the experiment, the rats were acclimatized to the animal house environment. All the experimental procedures were approved by the Institutional Animal Ethics Committee and the guidelines are followed strictly throughout the experimental period (ethical committee approval number for animal sanction).

2.3. Diabetes induction

Single intraperitoneal injections of streptozotocin (STZ) and nicotinamide was used to induce experimental diabetes in overnight fasted rats. First, nicotinamide was dissolved in sterile water and injected at a concentration of 110 mg/kg body weight. After 15 min, STZ, dissolved in 0.1 M of cold citrate buffer (pH 4.5), was injected at a concentration of 55 mg/kg body weight. After 72 h, diabetes was confirmed by measuring the tail vein blood glucose using a glucometer (Bayer Contour TS Blood Glucose Monitor). After three days of induction, rats with blood glucose level > 250 mg dL^{-1} were considered as diabetic and used for the in vivo experiments. Plumbagin was dissolved in 0.1 M DMSO and topically applied on rats with excision wounds. The rats were divided into four groups, each group comprising six rats:

2.3.1. Incisional wound model

The rats were anesthetized with 3% isoflurane during surgery. The hairs on the back were shaved using an electric clipper after the rats were unconscious. The dorasal hair of rats was removed and then two 10 mm diameter full thickness wounds were created with sterile biopsy punch. For wound healing, the rats were randomly divided into four groups each contains 6 rats (n = 6):

Group I: Normal control group.
Group II: Diabetes control group.
Group III: Diabetic animals treated with 10% plumbagin applied topically on excision wounds.
Group IV: Diabetic animals treated with 20% plumbagin applied topically on excision wounds.

2.3.2. Collection of blood samples

After 14 days of plumbagin administration, the rats were anesthetized, and the 2 ml of blood samples were collected into ethylene diamine tetra-acetic acid (EDTA) embedded tubes for hematological analysis. Another 5 ml of blood sample was collected and centrifuged and the serum was aspirated carefully for various biochemical assays.

2.3.3. Percentage of wound contraction and period of epithelialization

Wound contraction percentage was calculated as the percentage of wound that has been reduced from the original size of the wound. The wound area was marked on a transparent sheet and the surface area of the wound was measured [41]. During the wound healing process, the epithelialization period was also measured which represents the number of days taken for healing of the wound.

2.4. Estimation of collagen

The granulated tissues were collected on day 8 which is used for estimation of collagen content. The fat layer in the tissue samples was removed using chloroform and methanol mixture (2:1). Then the tissues which are removed from fat were frozen in acetone. The frozen tissues were weighed, hydrolysed and dried. The treated samples were made up to the final volume using double distilled water. From this the

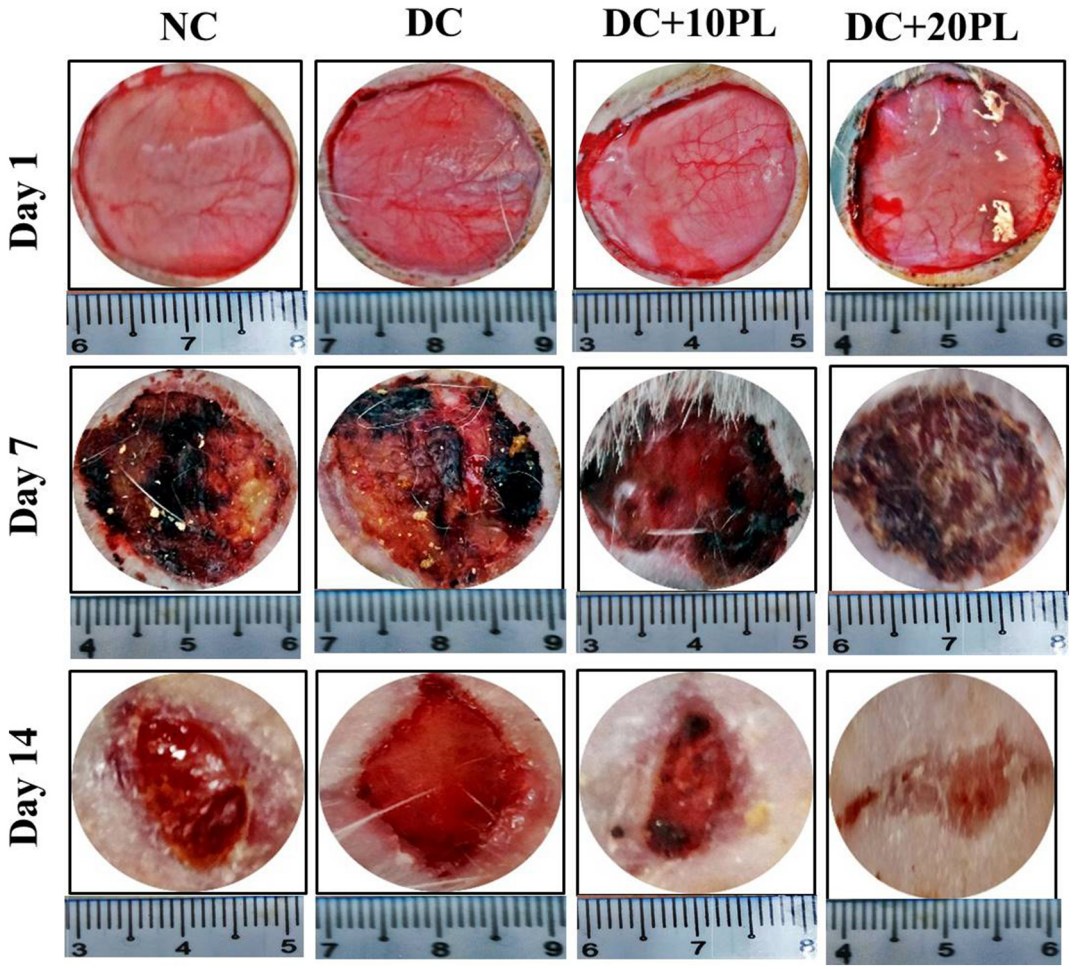

Fig. 1. Effects of plumbagin treatments on wound contraction.
Photographic representation of contraction rate on different days (1, 7, 14) of normal control (NC), diabetic control (DC), 10 and 20% plumbagin (DC + 10PL & DC + 20PL) gel applied diabetic wounds.

aliquot was taken and measured at 557 nm spectrophotometrically to estimate the hydroxyproline content [42]. The assays were performed in triplicates and the collagen content was calculated [43].

2.5. Total protein estimation

The protein content of skin tissues was determined by the method of Lowry et al. [1,44] The tissue lysate was treated with a mixture of sodium tartrate, copper sulfate and sodium carbonate. The mixture was left to stand for 10 min and then treated with Folin-Ciocalteu reagent that resulted in a bluish colour in 20–30 min. The absorbance was taken at 650 nm using Spectrophotometer.

2.6. Assay of blood glucose and serum insulin

To ensure diabetes induction, venous blood samples were collected in a fasting state from jugular veins of rats and the blood glucose levels were determined using a digital glucometer (Accu-Cheks, Roche Diagnostic, Meylan, France). The fasting blood glucose levels are monitored on the 0th, 3rd, 7th and 14th day of the experimental period thoroughly to ensure maintenance. Serum insulin levels were measured by using enzyme linked immunosorbent assay (ELISA) kit (EZRMI-13K, Linco Research; St. Charles, MO, USA) according to manufacturer's guideline.

2.7. Lipid profile analysis

The serum concentrations of total cholesterol, triglycerides, HDL-cholesterol, and LDL-cholesterol were determined by automatic analyser technique (Beckman Coulter Inc., Ireland).

2.8. Determination of lipid peroxidation

Lipid peroxidation rate in the wound tissue homogenate was quantified by measuring (TBARS) reaction as described previously [45]. The wound skin tissue were homogenized in 50 mmol/L Tris-HCL reagent (pH 7.4) containing 180 mmol/L KCl, 10 mmol/L EDTA and 0.02% butylated hydroxytoluene. TO 0.2 ml of the tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid, 1.5 ml of 0.95% thiobarbituric acid and 0.6 ml of distilled water were added and mixed well. The reaction mixture was placed in a water bath at 95 °C for 1 h. Then cooling on ice, 1.0 ml of distilled water and 5.0 ml of butanol/pyridine mixture (15:1, v/v) were added and vortexed. After centrifugation at 10,000 $\times g$ for 10 min, later absorbance of the resulting lower phase was determined at 532 nm. The lipid peroxidation concentration was calculated by 1,1,3,3-tetraethoxypropane as a standard and presented as nonomole TBARS per mg of protein.

2.8.1. Estimation of antioxidant status

Tissues was homogenized by 0.02 M potassium phosphate buffer pH 7.6 and centrifuged at 6000 rpm. The obtained clear supernatant

sample was used for estimation of SOD activity by according to the method of [2,46]. The activity of catalase was estimated by the method of [3,47]. In brief, this estimation was involves the incubation of a test tube containing 0.5 mL of H_2O_2 and 0.1 mL of tissue homogenate. After incubation in water bath at 37 °C for 60 s, after completed the reaction by adding 0.5 mL of ammonium molybdate solution. A yellow complex of ammonium molybdate and hydrogen peroxide was formed. The absorbance was read at 405 nm using spectrophotometer. The enzyme glutathione peroxidase (GPx) [4,48] briefly, To 0.2 ml tissue homogenate, 0.2 ml of 0.8 mM EDTA, 0.1 ml of 10 mM sodium azide, 0.1 ml of 2.5 mM H2O2, 0.2 ml of 4 mM reduced glutathione, 0.4 ml of phosphate buffer (0.4 M, pH 7.0) were added and incubated at 37 °C for 10 min. The reaction was detained by adding 0.5 ml of 10% TCA and the tubes were centrifuged at 2000 rpm for 20 min. To the supernatant, 3 ml of disodium hydrogen phosphate (0.3 M) and 1.0 ml of 40% 5, 5′-Dithiobis-2-nitrobenzoic acid (DTNB) were added and the colour developed was read immediately at 420 nm in spectrophotometer. Glutathione-S-transferase (GST) [5,49]. In brief, 2 mL of 0.3 M potassium phosphate buffer (pH 6.35), 75 μL of 30 mM CDNB solution, 725 μL of distilled water and 0.1 mL of wound tissue sample were added into a test tube. The test was mixed thoroughly and incubated at 37 °C for 10 min. Following by incubation, the reaction was commencing by addition of 100 μL of 30 mM reduced glutathione solution. The reduced in absorbance was read by spectrophotometrically at 340 nm. Glutathione reductase (GR) [6,50] in brief, briefly, 1 mL of 2.728 Mm GSSG solutions and 40 μL of wound tissue homogenate were incubated in a water bath at 37 °C for 5 min. After, the reaction was started by addition of 200 μL of 1.054 mM NADPH solution. The reduced in absorbance was read at 340 nm by spectrophotometer.

2.8.2. Measurement of the NF-κβ p65 activity

The wound healing skin, NF-κβ DNA binding activity was measured by NF-κβ p65 transcription factor assay ELISA kit (Active Motif North America, Carlsbad, CA). NF-κβ activities in the samples were expressed as activity/OD units.

2.8.3. Measurement of the TNF-α, IL-6, and IL-1β

The wound healing skin was grind into 10% homogenate, and then supernatant collected followed by centrifugation at 4000 ×*g* for 15 min. Then, TNF-α, IL-6, and IL-1β levels were estimated by using ELISA kits according to the manufactures guidelines. The results were expressed as pg/mL.

2.8.4. Real time PCR

Wound tissue was kept in RNALater solution (Ambion, Austin, TX, USA) prior to RNA extraction. Trizol was used to extract total mRNA (Invitrogen, Carlsbad, CA). 1 μg of RNA synthesis into cDNA using reverse transcription reaction by Thermo Scientific kit (Burlington, Canada). The cDNA sample was subjected to PCR analyses using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94,404, USA) then reaction mixture was composed of 10 ng of cDNA solution and 9 μl of qPCR Master Mix (EURx Company, Gdańsk, Poland) along with 20 μl of all primers were used. The obtained qPCR data was analyzed by the DDCT method. The obtained PCR results, with the Ct value (elbow value of a PCR amplification curve) as well as the $2^{-\Delta\Delta CT}$ method applied to calculate the relative expression of the target genes. GAPDH was used as a reference gene. PCR primer sequences used in this study are given in Table 1.

2.8.5. Histopathological analysis

Pieces of tissue samples from the skin were taken from area of healed injuries from each group and placed in 10% buffered formaldehyde solution to perform histopathological studies. The healed samples of skin were cut at a thickness of 5 μm and deparaffinized in xylene, rehydrated in series of alcohol, and stained with hematoxylin and eosin (H & E) and periodic acid-Schiff (PAS). The sections were visualized using a light microscope at a magnification of ×200. Histological sections were then observed and the changes in the tissues were recorded in Table 1.

2.8.6. Immunohistochemistry and immunofluorescence staining

For immunohistochemistry, deparaffinized and rehydrated sections were heated with 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval by using a microwave. After washing the sections were blocked with bovine serum albumin (BSA), and then incubated with primary antibody (COX_2 and iNOS (Abcam, Cambridge, UK, 1:100) overnight at 4 °C. Then the sections were incubated with appropriate biotinylated secondary antibodies. Then the sections were stained with *3′-Diaminobenzidine* (*DAB*) and counterstained with hematoxylin. Finally, the sections were viewed under Olympus phase contrast microscope (Tokyo, Japan).

For immunofluorescence, the sections after deparaffinized, rehydrated and antigen retrieval blocked with 5% serum. The sections were incubated with primary antibody (EGF, VEGF, FGF, MMP-2) at dilution of 1:100 in PBS overnight at 4 °C. After 3 times washing with PBS, the sections were incubated with secondary antibody conjugated with Alexa Fluor® 488 or Alexa Fluor 647 (1:500, Invitrogen, Carlsbad, CA, USA) for 60 min at room temperature. For counter stain to visualize nuclei, the sections were mounted using UltraCruz (Santa Cruz, CA, USA) mounting medium with DAPI. Images were captured with a fluorescence microscope (Leica DM IRB, Germany).

2.8.7. Western blot analysis

About 30 μg of protein were electrophoresed on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel for the analysis of CD68 and CD163. After running gel electrophoresis, the gels were wet-transferred on to nitrocellulose membranes. Membranes were blocked using 5% skim milk powder in TBST for 1 h at room temperature prior to antibody incubation overnight. Dilution of antibodies were for CD68 and for CD163 (Abcam, Cambridge, UK, 1:1000). Blots were visualized by chemiluminescence using a Chemidoc XRS imaging system (Bio-Rad, Milan, Italy). Densitometry was performed using ImageJ. The housekeeping protein β-actin (1:1000) was used as the loading control.

2.9. Statistical analysis

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

3. Results

3.1. Effect of plumbagin in wound contraction rate and percentage of wound closure in control and experimental animals

Fig. 1 shows the rate of contraction of the wounds in different days of the experimental rats. Wound closure rate was calculated as the percentage of wounds that has been reduced when compared with the original wound (Fig. 2A). Diabetic control rats (Group II) have decreased rate of wound closure when compared to normal control animals (Group I). The rate of wound closure was significantly increased in both group III and group IV rats receiving treatment with 10% and 20% plumbagin. Diabetic rats receiving 20% plumbagin have a higher rate of wound closure when compared to 10% plumbagin treated diabetic rats. The percentage of wound closure was significantly increased in all groups. The percentage of wound closure was significantly increased in day 14 when compared to day 7 in both control and experimental animals.

Table 1
Sequences of specific primers used for qPCR.

Gene	NCBI accession number	Sequence (5′–3′)	GC%	Melting temperature (°C) (Tm)	Product size (bp)
Nrf2	NM_031789.2	Forward: CATTTGTAGATGACCATGAGTCGC	45.83	60.03	77
		Reverse: ATCAGGGGTGGTGAAGACTG	55.00	59.01	
Keap-1	NM_057152.2	Forward: CTTCGGGGAGGAGGAGTTCT	60.00	60.32	74
		Reverse: CGTTCAGATCATCGCGGCTG	60.00	61.82	
Col1a1	NM_053304.1	Forward: GTACATCAGCCCAAACCCCA	55.00	59.96	87
		Reverse: TCGCTTCCATACTCGAACTGG	52.38	59.87	
Tgfb1	NM_021578.2	Forward: GACCGCAACAACGCAATCTA	50.00	59.21	87
		Reverse: TGCTTCCCGAATGTCTGACG	55.00	60.39	
α-SMA (Acta2)	NM_031004.2	Forward: TGGAAAAGATCTGGCACCACT	47.62	59.57	76
		Reverse: TCCGTTAGCAAGGTCGGATG	55.00	59.83	
Gapdh	NM_017008.4	Forward: AGTGCCAGCCTCGTCTCATA	55.00	60.68	77
		Reverse: GGTAACCAGGCGTCCGATAC	60.00	60.25	

3.2. Effect of plumbagin in period of epithelization, total collagen and total protein content

The mean period of epithelialization in diabetic control group was significantly ($p < 0.05$) longer when compared with non-diabetic control animals (Fig. 2B). The epithelization period was significantly reduced in animals treated with 10% and 20% plumbagin when compared with diabetic control group (Group II). The diabetic rats treated with 20% plumbagin showed even more decrease in period of epithelization when compared to 10% plumbagin treated rats. The treatment groups (Group III and Group IV) showed significant decrease in period of epithelization when compared with the control group and diabetic control rats.

Fig. 2C represents the collagen content of diabetic control animals

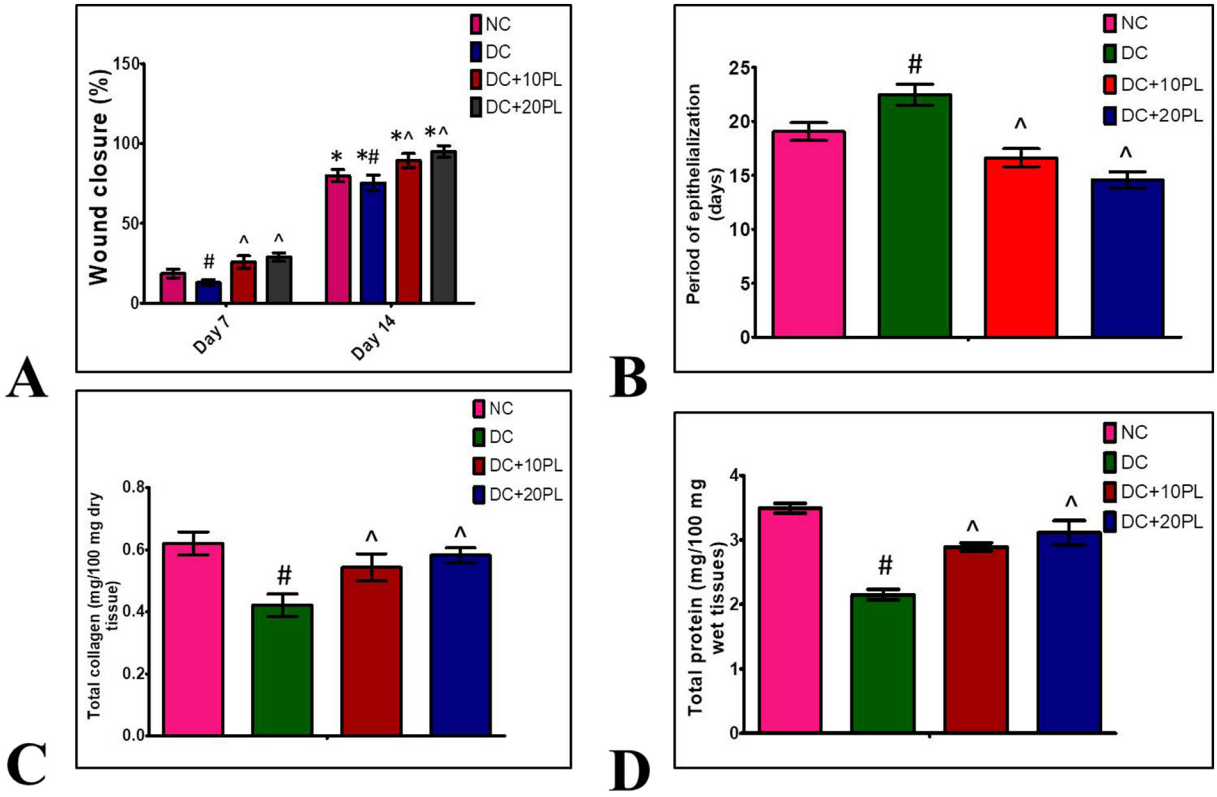

Fig. 2. Effects of plumbagin treatments on (A). Percentage of the wound closure (B) period of epithelization (C) Total collagen (D) Total protein in normal control (NC), diabetic control (DC), 10 and 20% plumbagin (DC + 10PL & DC + 20PL) gel applied diabetic rats. Values (mean ± SEM) were obtained from each group of 6 animals. *$p < 0.05$ compared to the values of day 7. #$p < 0.05$ compared to NC. ^$p < 0.05$ compared to DC.

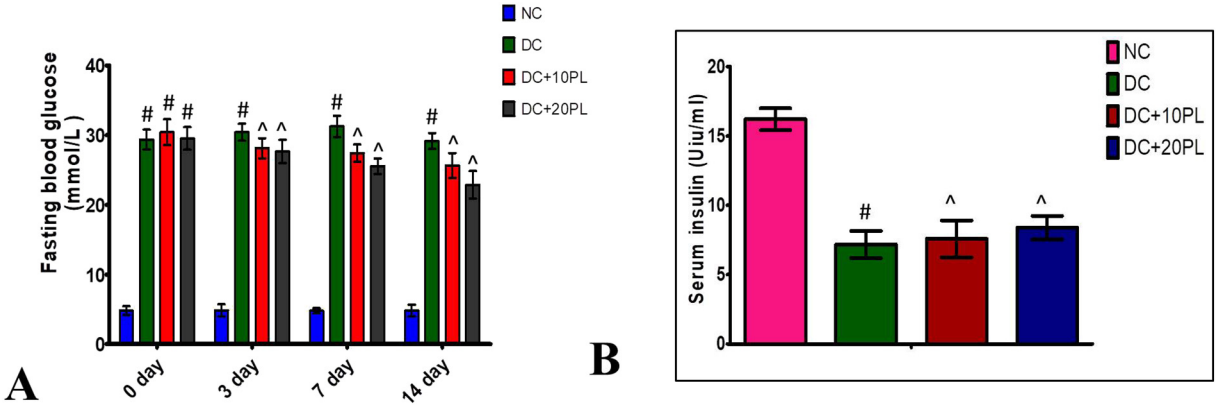

Fig. 3. Effects of plumbagin treatments on (A) fasting blood glucose (3,7,14 days). (B) Insulin levels in normal control (NC), diabetic control (DC), 10 and 20% plumbagin (DC + 10PL & DC + 20PL) gel applied diabetic rats. Values (mean ± SEM) were obtained from each group of 6 animals. $^{\#}p < 0.05$ compared to NC. $\hat{}p < 0.05$ compared to DC.

which was significantly lowered when compared with group I. Diabetic animals treated with plumbagin (10% and 20%) displayed significant ($p < 0.05$) improvement in collagen content when compared to diabetic control rats and the levels were brought back to near normalcy in group IV animals.

Total protein content of control and experimental animals was shown in Fig. 2D. The protein content of the diabetic control animals was significantly decreased when compared with normal control group. The diabetic animals treated with 10% and 20% plumbagin displayed significant improvement in protein content when compared with diabetic control rats. However, treatment with 20% plumbagin showed that the protein content was brought back to near normalcy.

3.3. Effect of plumbagin in fasting blood glucose and serum insulin levels of control and experimental animals

Fasting blood glucose levels of untreated diabetic rats was significantly increased when compared with normal control rats (Fig. 3A). Significant decrease in blood glucose levels were observed in diabetic rats treated with plumbagin (Group III and Group IV). The treatment with plumbagin (10% and 20%) showed significant reduction in blood glucose levels on day 14 in diabetic rats when compared with normal control animals. Serum insulin levels of control and experimental animals were shown in Fig. 3B. The insulin levels in serum of diabetic control rats was significantly decreased when compared with group I animals. Treatment with 10% and 20% plumbagin to diabetic rats showed significant improvement in insulin levels in serum of group III and group IV animals when compared to diabetic control animals (Group II).

3.4. Effect of plumbagin treatment in serum total cholesterol, triglycerides, HDL and LDL

The serum lipid profile (TC, TG, HDL and LDL) of control and experimental animals is represented in Fig. 4. The levels of TC, TG and LDL were significantly elevated, whereas, the HDL levels were significantly lowered in diabetic control rats (Group II) when compared to normal control animals (Group I). Plumbagin treatment (10% and 20%) showed improvement in HDL levels together with significant decrease in serum levels of TC, TG and LDL in group III and group IV animals when compared with diabetic control animals.

3.5. Effect of plumbagin treatment on mRNA expression of Nrf2, Keap 1, collagen-1, TGF-β and α-SMA of control and experimental animals

Fig. 5(A–E) illustrates the effect of plumbagin on mRNA expression of Nrf2, Keap 1, collagen-1, TGF-β and α-SMA. The mRNA expression of Nrf2, collagen-1, TGF-β and α-SMA were significantly decreased when compared to normal control animals. However, the mRNA expression of Keap 1 was found to be significantly increased in group II animals when compared to group I animals. Upon treatment with plumbagin, at 10% and 20%, showed significant improvement in mRNA expression of Nrf2, collagen-1, TGF-β and α-SMA, while, the expression of Keap 1 was found to be significantly lowered in group III and group IV animals when compared to group II animals.

3.6. Effect of plumbagin treatment on lipid peroxidation and antioxidant status of control and experimental animals

Fig. 6A represents the extent of lipid peroxidation and Fig. 6(B–F) represents the antioxidant status of control and experimental animals. The levels of lipid peroxides in diabetic control rats was significantly ($p < 0.05$) increased when compared with normal control animals (Group I). Treatment with plumbagin significantly lowered the levels of lipid peroxides in group III and group IV animals when compared to diabetic control animals. The antioxidant enzymes such as SOD, CAT, GPx, GR and GST were found to be lowered to a significant extent in group II animals when compared to normal control group. The plumbagin treatment improved and brought back the activities of antioxidant enzymes to near normalcy. The 10% and 20% treated diabetic rats showed significant improvement in activity of antioxidants when compared to its untreated counterpart.

3.7. Effect of plumbagin in histopathological examination of control and experimental animals

Fig. 7(A–B) represents the histopathological changes of wound healing rats in experimental groups. The epidermal thickness was lower in diabetic control rats when compared to normal control rats. However, in diabetic rats to which 10% and 20% plumbagin was administered displayed increased thickness of epidermal tissues when compared to diabetic control rats. Moreover, epidermal regeneration, granulation tissue, angiogenesis, proliferation of fibroblast cells and collagen deposit was lower in diabetic control rats when compared to normal control rats. On the other hand, the diabetic rats to which 10% and 20% plumbagin was administered, showed higher epidermal regeneration, granulation tissue, angiogenesis, proliferation of fibroblast cells and collagen deposit when compared to diabetic control rats. In contrast, inflammatory cells infiltration was higher in diabetic control rats when compared to normal control rats. Moreover, diabetic rats to which 10% and 20% plumbagin was administered also showed lower inflammatory cells infiltration when compared to diabetic control rats

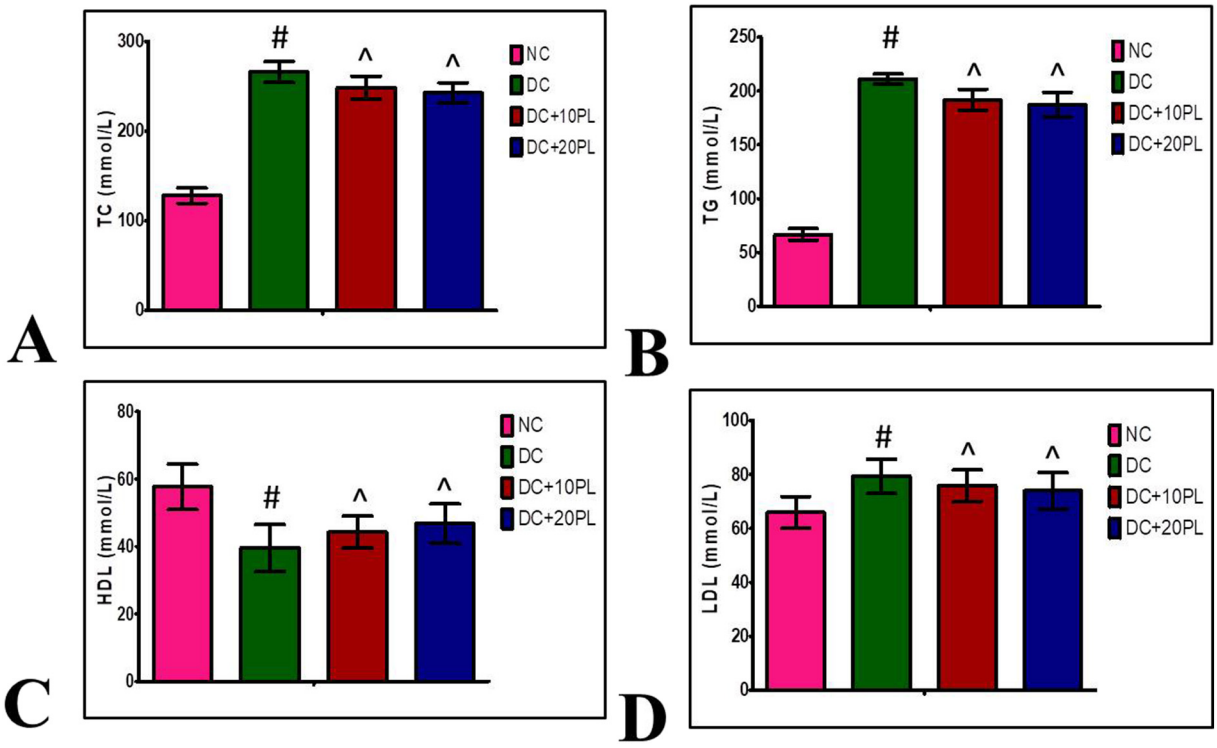

Fig. 4. Effects of plumbagin treatments on serum (A) Total cholesterol (B) Triglycerides (C) High density lipoproteins (D) Low density lipoproteins in normal control (NC), diabetic control (DC), 10 and 20% plumbagin (DC + 10PL & DC + 20PL) gel applied diabetic rats. Values (mean ± SEM) were obtained from each group of 6 animals. $^{\#}p < 0.05$ compared to NC.

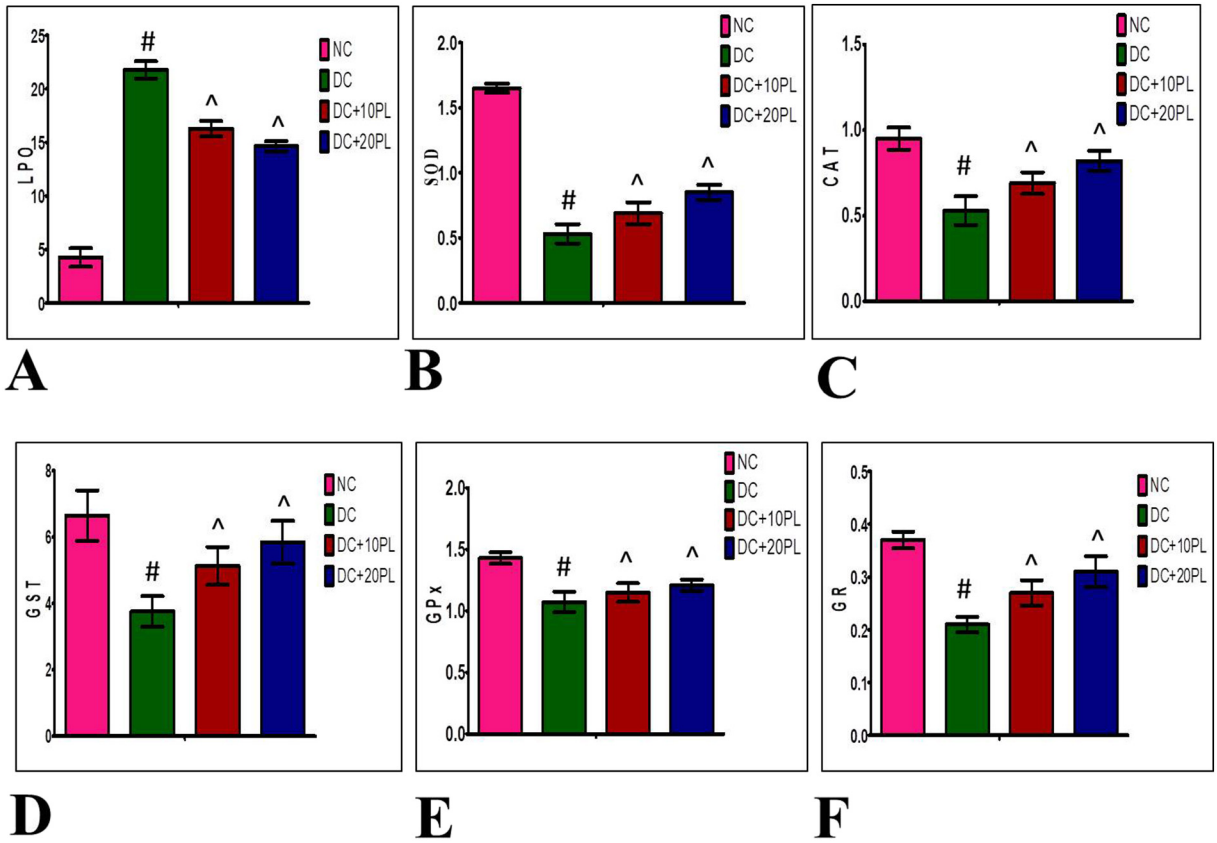

Fig. 5. Effects of plumbagin treatments on (A) LPO (B) SOD (C) CAT (D) GST (E) GPx (F) GR in normal control (NC), diabetic control (DC), 10 and 20% plumbagin (DC + 10PL & DC + 20PL) gel applied diabetic rats. Values (mean ± SEM) were obtained from each group of 6 animals. $^{\#}p < 0.05$ compared to NC. $^{\wedge}p < 0.05$ compared to DC.

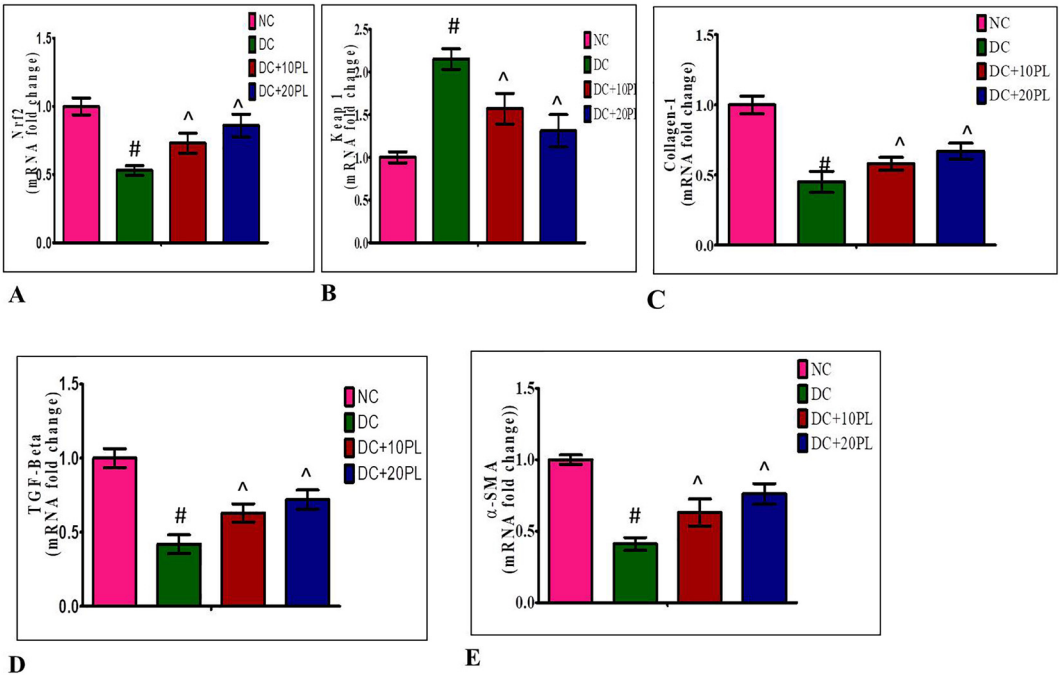

Fig. 6. Effects of plumbagin treatments on (A) Nrf2 mRNA (B) Keap1 (C) Collagen-1 mRNA (D) TGF-β mRNA (E) α-SMA mRNA in normal control (NC), diabetic control (DC), 10 and 20% plumbagin (DC + 10PL & DC + 20PL) gel applied diabetic rats. Values (mean ± SEM) were obtained from each group of 6 animals. $^{\#}p < 0.05$ compared to NC. $\hat{}p < 0.05$ compared to DC.

(Table 2).

3.8. Effect of plumbagin in immunofluorescent staining of EGF, VEGF, FGF and MMP-2

EGF is one of the growth factors involved in wound healing. In this present study, immunofluorescence images show that lower EGF distribution was mainly in the epidermal layer of wound healing diabetic control rats when compared to normal control rats. However, diabetic rats to which 10% and 20% plumbagin administered displayed higher distribution of EGF protein in epidermal layer of the skin when compared to diabetic control rats (Fig. 8A).

VEGF is one of the most prominent angiogenic markers determined by immunofluorescence. The VEGF protein distribution was lower in diabetic control rats as compared to the normal control rats. However, diabetic control rats injected with 10% and 20% plumbagin showed higher VEGF protein distribution when compared to diabetic control rats (Fig. 8B).

One more growth factor, FGF induces the activation and proliferation of fibroblast. Fig. 9A represents immunofluorescence results of FGF

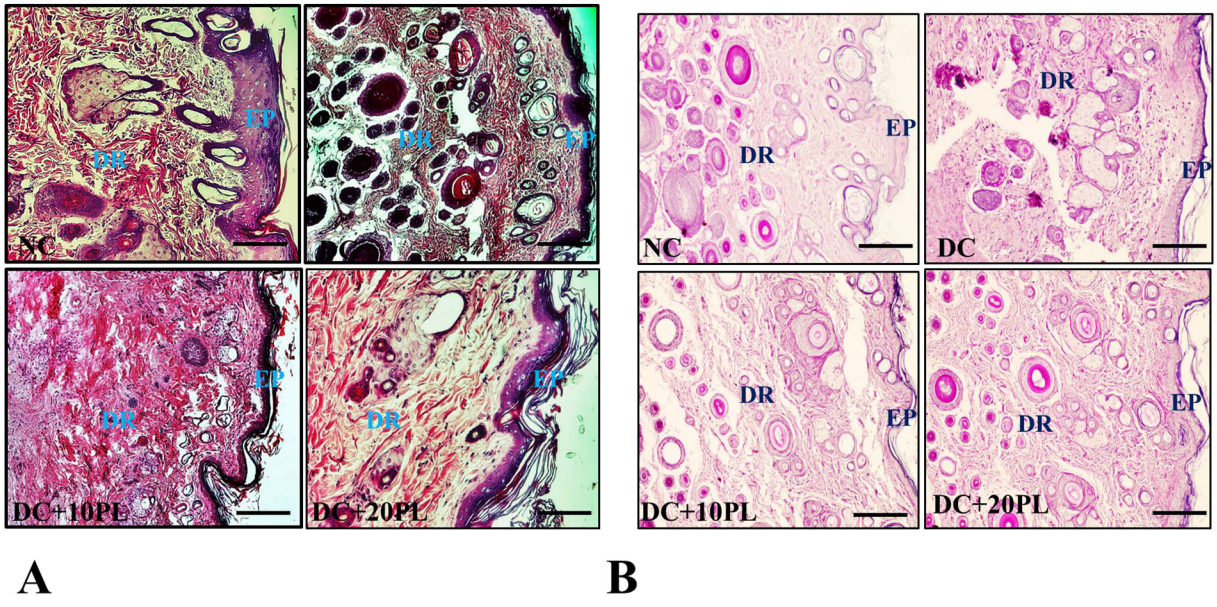

Fig. 7. A–B illustrate the histological changes of wound healing rats in experimental groups. A. shows the microphotographs of normal control (H & E) (B) periodic acid-Schiff (PAS) view of wound-healing in normal control (NC), diabetic control (DC), 10 and 20% plumbagin (DC + 10PL & DC + 20PL) gel applied diabetic rats. Scale bar = 50 μm. Original magnifications at 100×.
EP: epidermis; DR: dermis.

Table 2
Semiquantitative evaluation of histological parameters for the assessment of wound healing.

Histological parameter	NC	DC	DC + 10PL	DC + 20PL
Epidermal regeneration	+ + +	–	+ +	+ + +
Granulation tissue	+ +	–	+ +	+ + +
Inflammatory cells infiltration	–	+ + +	+	–
Angiogenesis	+ +	–	+ +	+ + +
Proliferation of fibroblast cells	+ +	–	+ +	+ +
Collagen deposit	+ +	–	+ +	+ +

Notes: +, slight; + +, moderate; + + +, extensive; –, absence.

in the experimental rats. The FGF protein distribution was lower in diabetic control rats when compared to the normal control rats. Moreover, the experimental rat to which 10% and 20% plumbagin was administered showed higher FGF protein distribution when compared to diabetic control rats.

MMP regulating extracellular matrix degradation and deposition that is essential for wound re-epithelialization. In this studies, immunofluorescence results indicated that MMP-2 protein distribution was higher in diabetic control rats when compared with normal control rats. Moreover, diabetic rats injected with 10% and 20% plumbagin showed lower MMP-2 distribution when compared with diabetic control rats (Fig. 9B).

3.8.1. Effect of plumbagin on inflammatory markers in control and experimental animals

The skin-wound-healing nuclear NF-κβ p65 levels were higher in diabetic control rats when compared to normal control rats. Moreover, diabetic control rats injected with 10% and 20% plumbagin showed lower NF-κβ p65 levels when compared to diabetic control rats (Fig. 10A). On the other hand, ELISA results of skin-wound-healing TNF-α, IL-6, and IL-1β levels were higher in diabetic control rats when compared to normal control rats. Meanwhile, skin-wound healing TNF-α, IL-6, and IL-1β levels were decreased in the diabetic rats injected with 10% and 20% plumbagin when compared to diabetic control rats (Fig. 10B–D).

3.8.2. Effect of plumbagin in immunohistochemical analysis of COX-2 and iNOS in the control and experimental animals

Fig. 11A represents immunohistochemistry results of COX-2 in experimental rats. The COX-2 protein distribution was higher in diabetic control rats when compared to normal control rats. In addition, diabetic rats injected with 10% and 20% plumbagin showed lower COX-2 protein distribution when compared to diabetic control rats. On the other hand, iNOS protein distribution was higher in diabetic control rats compared to normal control rats. Meanwhile, diabetic rats injected with 10% and 20% plumbagin showed lower distribution when compared to diabetic control rats (Fig. 11B).

3.9. Effect of plumbagin on protein expression of CD68 and CD163 in various experimental groups

Fig. 12 display the impact of plumbagin on protein expression of CD68 and CD163 in wound area of control and experimental animals. Results obtained from the present study revealed that there was a significant ($p < 0.05$) increase in the protein levels of CD68 and CD163 in diabetic control rats when compared to normal control rats. Plumbagin supplementation brought down the levels of CD68 and CD163 to near normal values in group III and group IV rats when compared to their respective untreated counter parts. 20% plumbagin treated diabetic rats showed even lower levels of protein expression than 10% plumbagin treated rats and group IV rats displayed near normalcy values when compared to that of normal control animals.

4. Discussion

This present study was performed to investigate whether plumbagin supplement improved wound healing process in diabetic rats. The results obtained from this study revealed that the wound healing process is hindered in diabetic rats. Interestingly, plumbagin supplement significantly increased the wound healing rate and the time taken for healing of wounds was also reduced. These results reveal that plumbagin supplement accelerated the process of wound healing in diabetic rats.

Wound healing is characterized by three stages which are inflammation, proliferation, and remodeling. The proliferative phase

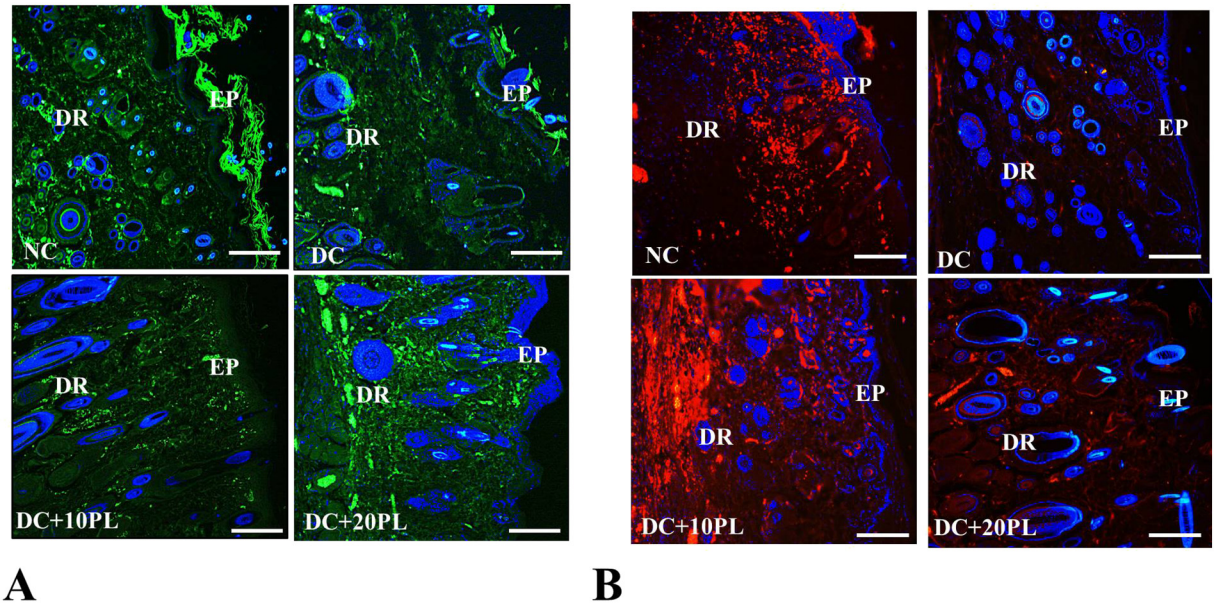

Fig. 8. (A) Representative immunofluorescence staining (green) of epidermal growth factor (EGF) (B) vascular endothelial growth factor expression (VEGF; red colour) in normal control (NC), diabetic control (DC), 10 and 20% plumbagin (DC + 10PL & DC + 20PL) gel applied diabetic rats. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

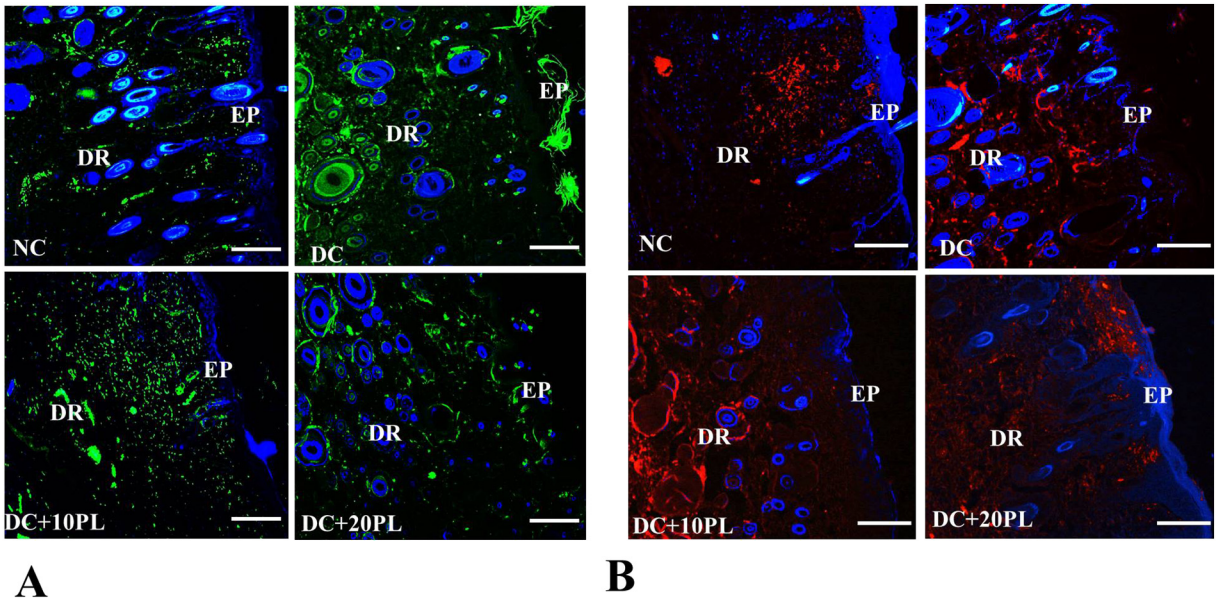

Fig. 9. (A) Representative immunofluorescence staining (green) of fibroblast growth factors (FGF) (B) matrix metalloproteinase-2 (MMP-2) (red) in normal control (NC), diabetic control (DC), 10 and 20% plumbagin (DC + 10PL & DC + 20PL) gel applied diabetic rats. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

typically demonstrates angiogenesis, collagen deposition, granulation tissue formation, epithelialization and wound contraction [103]. Wound contraction is the major evaluating factor in the healing process of large open wounds [51]. In this study, a significant difference was noted in both the healthy and diabetic animals. The wound contraction rate was even faster in diabetic animals which are treated with plumbagin when compared with untreated diabetic rats and normal control animals. In angiogenesis, new blood vessels grow from endothelial cells. In fibroplasia and granulation tissue formation, fibroblasts grow and form a new provisional extracellular matrix by excreting collagen and fibronectin. The increased rate of wound contraction reported in this study might be due to enhanced activity of fibroblasts which is mediated by specialized myofibroblasts located in the granulated tissues [52]. In epithelialization, epithelial cells crawl across the wound bed to cover it. Fibronectin, the major glycoprotein secreted by fibroblasts, has important functions of chemo-attraction for macrophages, fibroblasts and endothelial cells, promoting re-epithelialization and acting as a transduction agent in wound contraction [104].Wound contraction occurs by myofibroblasts, which establish a grip on the wound edges, bringing them in apposition. Thus, the rate at which the contraction of the wound occurs determines the period of epithelialization which facilitates the wound closure [53]. This supports the findings of the current study wherein a correlation is derived at these two junctures. The results reveal that the plumbagin treated diabetic rats have increased rate of wound closure and decreased period of epithelialization, while it was the reverse in the diabetic control rats.

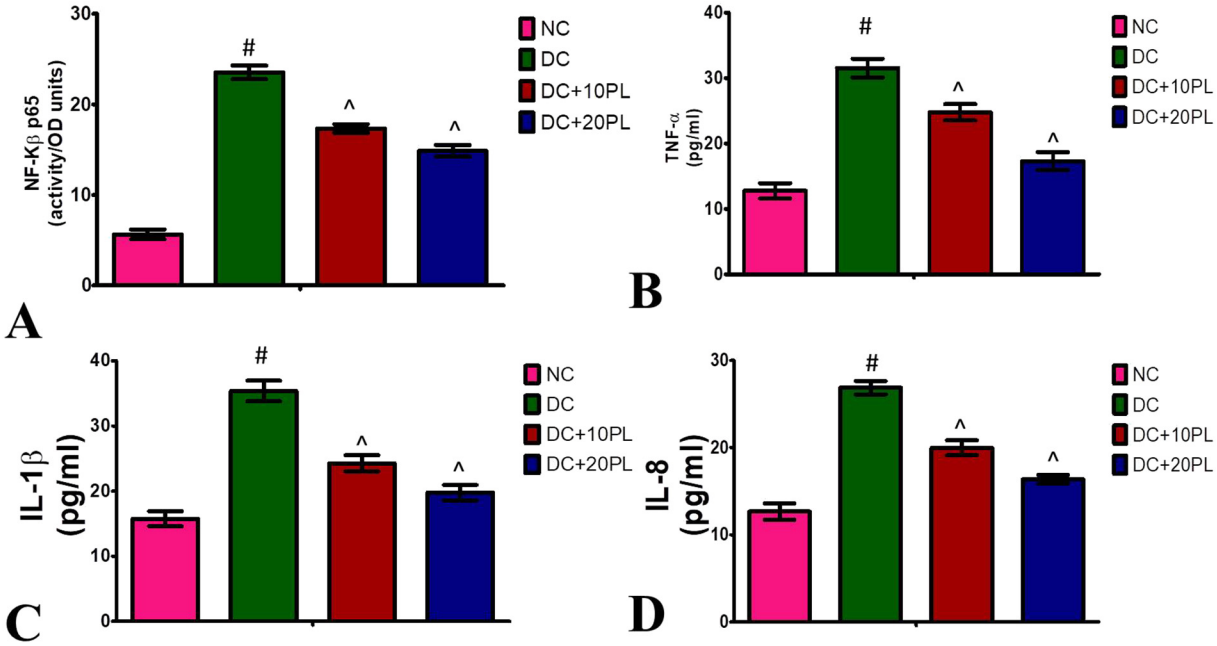

Fig. 10. Effects of plumbagin treatments on inflammatory markers in normal control (NC), diabetic control (DC), 10 and 20% plumbagin (DC + 10PL & DC + 20PL) gel applied diabetic rats. Values (mean ± SEM) were obtained from each group of 6 animals. $^{\#}p < 0.05$ compared to NC. $\hat{}p < 0.05$ compared to DC.

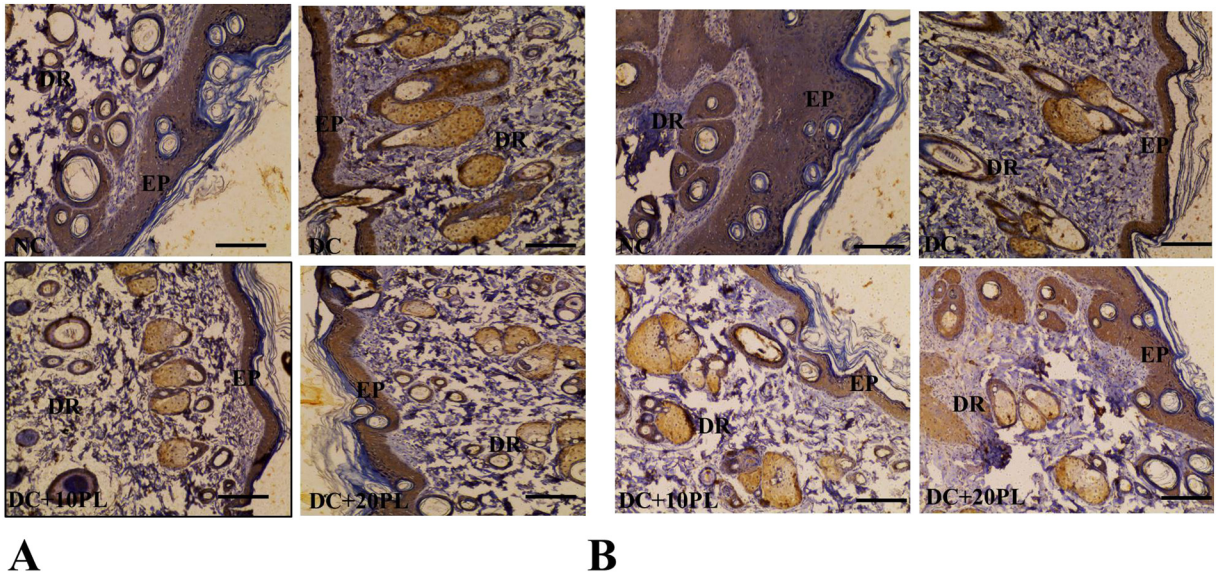

Fig. 11. (A) Representative immunohistochemistry staining of cyclooxygenase (COX)-2 (B) Nitric oxide synthase (iNOS) in normal control (NC), diabetic control (DC), 10 and 20% plumbagin (DC + 10PL & DC + 20PL) gel applied diabetic rats. Scale bar = 50 μm.

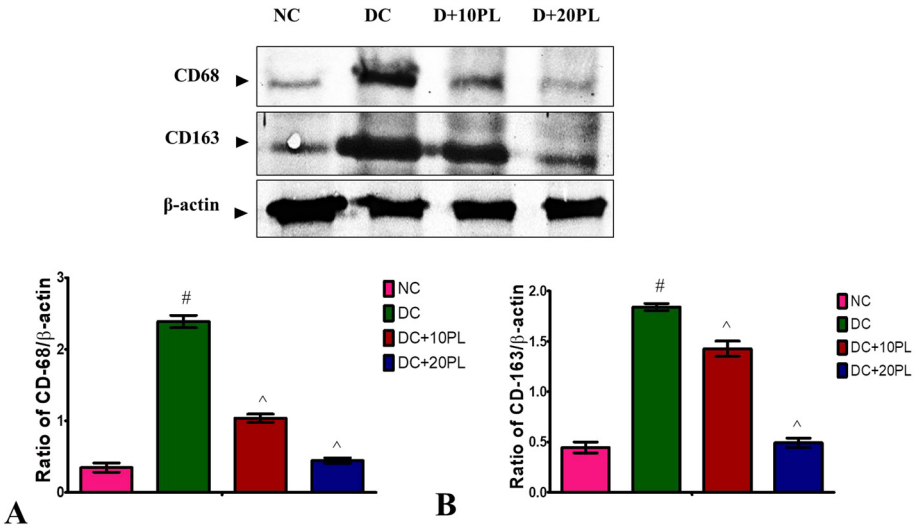

Fig. 12. Representative western blotting for (A) CD68 (B) CD163 in wound area of normal control (NC), diabetic control (DC), 10 and 20% plumbagin (DC + 10PL & DC + 20PL) gel applied diabetic rats. Values (mean ± SEM) were obtained from each group of 6 animals. $^{\#}p < 0.05$ compared to NC. $\hat{}p < 0.05$ compared to DC.

The wound healing activity of plumbagin reported by Devender Rao Kodati et al. [54] might have resulted in the contraction and closure of wounds in diabetic animals.

Collagen is the most abundant protein present in the blood vessels, skin and connective tissues [57]. During normal wound healing process, cross-linking of collagen molecules creates strength and stability [58]. The process of wound healing requires either direct or indirect involvement of collagen. Therefore, patients with diabetes are reported to have defects in tissue repair which might to be due to alterations in collagen metabolism [59]. The total protein content of the diabetic control animals was decreased significantly when compared to normal control animals. The plumbagin treated diabetic rats showed significant improvement in protein content and their values are near normalcy. Increased protein content in the treatment groups indicated increased synthesis and deposition of matrix proteins [60]. Increased protein content also supported increased collagen content in the treatment groups. Antidiabetic, antihyperglycemic [61] and wound healing property of plumbagin [54] might have decreased blood glucose levels and increased wound healing rate in the treatment groups.

Wound healing process is impaired in diabetes due to high levels of glucose in the blood which hinders proliferation of cells and decreases the production of collagen resulting in decline in phagocytosis and chemotaxis. Higher blood glucose levels also result in significant reduction in levels of growth factors and inhibits the proliferation of fibroblast all of which are suggestive contributors responsible for impaired wound healing [55]. Insulin is the regulator of blood glucose levels and it plays a potent beneficial role in wound healing process [56]. The evidences are in correlation with our present findings which also reveals that animals with diabetes have high blood glucose levels, decreased insulin secretion and decreased collagen content which might have attributed to impaired wound healing in that group.

Diabetes is mainly associated with the presence of high levels of lipids in the blood which is due to increased lipid mobilization to adipose tissue and decreased level of lipoprotein lipase in blood. Results from this present study also display a significant increase in the levels of total cholesterol, triglycerides and LDL together with significant decline in HDL level in diabetic control animals. After plumbagin administration, the level of HDL was significantly elevated, while the levels of

cholesterol, triglycerides and LDL was lowered in group III and group IV animals. Our findings are supported by earlier evidences that plumbagin supplement in hyperlipidemic rabbits, significantly reduced the levels of total cholesterol, LDL cholesterol, ratio of cholesterol/phospholipid and decreased HDL cholesterol in serum. Reports also revealed that plumbagin supplement significantly reduced cholesterol and triglycerides accumulation in the aorta and the liver. It was also found to prevent the atheromatous plaques of abdominal and thoracic aorta [62].

Nuclear transcription factor 2 (Nrf2), is a novel target for the action of keratinocyte factor It regulates the expression of genes and the inflammation involved in wound healing [63]. Nrf2 deficient diabetic mouse model show delayed wound closure rates [64]. Hence, Nrf2 activation plays a major role in healing diabetic wounds and improves overall health of the mouse. Recent literature evidences has demonstrated that diabetes associated with high blood glucose levels is closely associated with dysfunction of Keap 1 which in turn prevents Nrf2 localization [65]. Thus, studies have reported that over-expression of Keap1 and suppression of Nrf2 disturbs redox homeostasis and results in impaired wound healing in diabetes [66]. Thus, the results from this present study also reveals the over-expression of Keap 1 mRNA and downregulated Nrf2 mRNA expression which might have resulted in impaired wound healing in diabetic animals, while diabetic animals treated with plumbagin showed protective effect by upregulating mRNA levels of Nrf2 and downregulating mRNA levels of Keap 1, eventually resulting in accelerated wound healing.

Diabetic rats have impaired wound healing which is mainly due to dysfunction of fibroblast and epidermal cells, hypoxia, neovascularization, impaired angiogenesis and augmented levels of metalloproteases [7]. Besides all these factors, there is an increased reactive oxygen species production (ROS), lipid peroxidation (LPO), modulation of fibroblast proliferation and defective antioxidant defensive mechanism which triggers impaired wound healing. Increased ROS accumulation results in excessive oxidation of lipids, proteins and nucleic acids which ultimately results in damage to cellular membranes [68,69]. The findings are in correlation with the findings of this research work which also showed that the diabetic control rats have increased lipid peroxidation rate when compared to control animals. Plumbagin treated diabetic rats also displayed significant reduction in lipid peroxidation rate when compared to diabetic control animals. Imbalance in antioxidant defensive enzymes and increased lipid peroxidation rate resulted in further increase in the production of free radicals [70].

Various studies also reported significant increase in free radical formation and decreased potential of antioxidants in diabetes mellitus which ultimately results in worsened condition of wounds. Increased antioxidant defense was shown to increase accelerated wound healing and decrease the ROS production [69]. The results of the current study also portrayed significant decline in antioxidant enzymes such as SOD, CAT, GPx, GR and GST in diabetic rats, while the plumbagin supplementation improved antioxidant enzymes levels to a significant extent which resulted in an accelerated wound healing. Lower antioxidant levels in diabetic rat results from increased oxidative stress in a glucose enriched environment [71]. High blood glucose levels might have resulted in increased production of free radicals and decreased levels of antioxidants [72,73]. The beneficial effect of plumbagin in diabetic rats is due to its multi-potent property such as free radical scavenger, antioxidant and inhibition of lipid peroxides [74]. The results were further confirmed by histopathological findings. The histopathological study from the diabetic group displayed significant changes such as prominent fibrosis, cellular infiltration and regeneration of epithelium. This prominently showed impaired wound healing in rats induced with diabetes. Plumbagin treatment showed well-organized collagen fibers, reduce cellular infiltration and improve contraction of wounds in the treatment groups. These findings are in correlation with the reports of Katsuhiro et al. (2018) [75] who also revealed similar histological findings in diabetic rats.

Growth factors are proteins which play a major role in healing process by stimulating and activating cell proliferation via activation of various reactions such as myelogenesis, angiogenesis and other gene transcription factors [76,77]. Among various growth factors, the growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF-β) and vascular endothelial growth factor (VEGF) plays a major role in healing process [78,79]. Presence of numerous growth factors and cytokines has been reported at wound site. Alteration of one growth factor expression eventually can affect the expression or production of other cytokines and growth factors. During the early phase of healing process, various proinflammatory cytokines and growth factors are released into the serum which in turn act as stimulator for expression of other growth factors. At the wound site, FGF7 is produced by fibroblasts and VEGF is produced by macrophages and keratinocytes [80]. EGFR signaling results in increased rate of epithelialization of wounds and promotes keratinocyte migration to the wound [81]. FGFs stimulate the proliferation and migration of various cells involved in wound healing. It was also shown to stimulate collagen synthesis, epithelialization and neovascularization [82]. VEGF plays a key role in wound healing by stimulating endothelial cell migration and it also improves angiogenesis by increasing endothelial cell permeability [83,84]. The evidences reported are in unison with the results of this present study. Numerous plants are reported to have wound healing property and therefore, they have gained considerable attention for therapeutic use in accelerating the wound healing process by promoting the release of growth factors [85]. In this present study, plumbagin has also proven its efficacy in promoting the wound healing process. However, the mechanism by which plumbagin has promoted the release of growth factors for promoting the wound healing process remains unknown.

The MMPs are a family of structurally related class of zinc endopeptidases which degrades the components of extracellular matrix (ECM) [86]. The MMP family members have been classified into three types such as gelatinases, stromelysis, collagenases and membrane type MMPs depending on their substrate specificity and structural properties. However, like TGFβ, MMPs influence normal physiological processes such as wound healing, tissue remodeling, angiogenesis and embryonic development, as well as pathological conditions such as rheumatoid arthritis, atherosclerosis and tumor invasion [105,106]. They also play a major role in influencing the activities of growth factors. Under normal conditions, these MMPs can interact with growth factors and results in the maintenance of the balance of ECM [87]. The imbalance of matrix proteins in the ECM results in degradation of the matrix and increases the risk of disease development [88,89]. Interaction results in downregulation of growth factors and increases the levels of MMP-1, MMP-2, MMP-8, MMP-9 and proinflammatory cytokines such as Interleukin-1 (IL-1) and Tumor Necrosis Factor alpha (TNF-α). The impact is higher in chronic wounds than normal wounds [90]. Hyperglycemia results in increased activities of MMP-2, MMP-1 and MMP-9 which stimulates the ECM degradation and creates imbalance in diabetes [91]. The expression of MMPs under normal conditions in skin is low and it is upregulated in inflammatory cells such as macrophages, T cells and eosinophils [92]. The literature correlates with the findings of this present study wherein MMP-2 has played a major role in promoting wound repair.

Inflammation is a normal process in wound healing which results in the release of cytokines such as cyclooxygenase-2 (COX-2). Hyperglycemia caused by diabetes increases oxidative stress and it also results in secretion of COX-2 [92]. Increased COX-2 expression decreases the synthesis of collagen and delay the process of wound healing. Diabetes also results in significant increase in levels of TNF-α, IL-6, iNOS and COX-2 [93]. Transforming Growth Factor-beta (TGF-β), plays a major role in healing process by influencing the inflammatory response, re-epithelialization, extracellular matrix deposition and promotes healing. TGF-β, plays a vital role in wound healing by acting at

the specific site of the injury following tissue damage. In this present study, the mRNA levels of TGF-β decreases in diabetes and the mechanism by which plumbagin enhances TGF-β level in the wounded skin remains unknown and it still needs to be explored. Antioxidant and anti-inflammatory properties of plumbagin [94] might have improved the wound healing process through regulation of blood glucose, preventing inflammatory response, decreasing oxidative stress and reducing the expression levels of iNOS and COX-2. Alpha smooth muscle actin (α-SMA) is a well-known marker and it plays a major role in healing process of wounds [95]. Increased mRNA expression of α-SMA in plumbagin treated groups might have resulted in promoting the change of fibroblast to myofibroblast via upregulated expression of TGF-β. We also found increased expression of COX-2, iNOS and decreased mRNA levels of TGF-β, collagen-1 and α-SMA in diabetes group which is responsible for prolonged inflammatory response and delay in wound healing. Significant decrease in mRNA levels of COX-2, iNOS and increased mRNA levels of TGF-β, collagen and α-SMA were observed in diabetic animals treated with plumbagin which proved its beneficial effect on rate of wound closure.

CD68 is a glycoprotein and a marker which binds to low-density lipoprotein and it is highly expressed on monocytes and macrophages, especially in wounds. The expression of CD68 peaks initially and it is decreased over time. CD163 is the hallmark of wound healing macrophages [96]. In gout model of monosodium urate crystal phagocytosis, CD163 is expressed when monocytes or macrophages switch from proinflammatory to anti-inflammatory cytokines [97]. CD163 has been found to reduce/minimize oxidative injury in atherosclerotic plaques [98]. Thus, CD163 plays a vital role in anti-inflammatory and wound healing macrophages [99]. The western blot results also reveal a higher protein expression of CD68 and CD163 in diabetes induced group and its expression is reduced to a significant extent in diabetic animals treated with plumbagin.

To conclude, plumbagin was found to be beneficial in healing diabetic wounds. The wounds treated with plumbagin showed significant contraction rate when compared with that of untreated wounds. Histopathological findings also revealed faster re-epithelialization, collagen deposition and inflammation is also reduced in plumbagin treated groups. In addition to this, plumbagin minimized the oxidative stress and improved antioxidant status of diabetic animals. To large extent, plumbagin has proved to be a potent anti-lipidemic, anti-inflammatory and anti-diabetic agent. However, further studies are expected to explore the mechanism of the anti-diabetic and wound healing action of plumbagin and the active principle involved which may contribute to the development of effective therapeutic strategies for treatment of diabetic wounds.

Conflict of interest

The authors declare no conflict of interest.

Ethical statement

All the animal procedures were approved by the Institutional Ethical committee and all the experiment performed on mice was done with extra care and concern followed by the National Institutes of Health guide for the care and use of Laboratory animals.

References

[1] M.I. McCarthy, Genomics, type 2 diabetes, and obesity, N. Engl. J. Med. 363 (24) (2010) 2339–2350.

[2] J. Wedler, T. Daubitz, G. Schlotterbeck, V. Butterweck, In vitro anti-inflammatory and wound-healing potential of a Phyllostachys edulis leaf extract-identification of isoorientin as an active compound, Planta Med. 80 (2014) 1678–1684.

[3] L.T. Fox, A. Mazumder, A. Dwivedi, M. Gerber, J. Plessis, J.H. Hamman, In vitro wound healing and cytotoxic activity of the gel and whole-leaf materials from selected Aloe species, J. Ethnopharmacol. 200 (2017) 1–7.

[4] J. Kim, C.M. Lee, Wound healing potential of a polyvinyl alcohol-blended pectin hydrogel containing Hippophaerahmnoides L. extract in a rat model, Int. J. Biol. Macromol. 99 (2017) 586–593.

[5] U. Das, S.S. Behera, K. Pramanik, Ethno-herbal-medico in wound repair: an incisive review, Phytother. Res. 31 (2017) 579–590.

[6] X. Su, X. Liu, S. Wang, B. Li, T. Pan, D. Liu, F. Wang, Y. Diao, K. Li, Wound-healing promoting effect of total tannins from Entadaphaseoloides (L.) Merr. in rats, Burns 43 (2017) 830–838.

[7] S. Lodhi, A.K. Singhai, Wound healing effect of flavonoid rich fraction and luteolin isolated from Martyniaannua Linn. on streptozotocin induced diabetic rats, Asian Pac J Trop Med (2013) 253–259.

[8] L. Yarmolinsky Budovsky, S. Ben-Shabat, Effect of medicinal plants on wound healing, Wound Repair Regen. 23 (2) (2015) 171–183.

[9] S. Babaei, H. Ansarihadipour, M. Nakhaei, M. Darabi, P. Bayat, M. Sakhaei, M. Baazm, A. Mohammadhoseiny, Effect of Omegaven on mast cell concentration in diabetic wound healing, J. Tissue Viability 26 (2017) 125–130.

[10] X.Y. Ji, Y. Chen, G.H. Ye, M.W. Dong, K.Z. Lin, J.G. Han, X.P. Feng, X.B. Li, L.S. Yu, Y.Y. Fan, Detection of RAGE expression and its application to diabetic wound age estimation, Int. J. Legal Med. 131 (2017) 691–698.

[11] Kasuya, Y. Tokura, Attempts to accelerate wound healing, J. Dermatol. Sci. 76 (2014) 169–172.

[12] T.K. Biswas, S. Pandit, S. Chakrabarti, S. Banerjee, N. Poyra, T. Seal, Evaluation of Cynodondactylon for wound healing activity, J. Ethnopharmacol. 197 (2017) 128–137.

[13] G. Gainza Garcia-Orue, F.B. Gutierrez, J.J. Aguirre, C. Evora, J.L. Pedraz, R.M. Hernandez, A. Delgado, M. Igartua, Novel nanofibrous dressings containing rhEGF and Aloe vera for wound healing applications, Int. J. Pharm. 523 (2017) 556–566.

[14] S. Negahdari, H. Galehdari, M. Kesmati, A. Rezaie, G. Shariati, Wound healing activity of extracts and formulations of Aloe vera, Henna, Adiantum capillus-veneris, and Myrrh on mouse dermal fibroblast cells, Int. J. Prev. Med. 8 (2017) 18–27.

[15] Y. Park, Y.K. Lee, D.S. Lee, J.E. Yoo, M.S. Shin, N. Yamabe, S.N. Kim, S. Lee, K.H. Kim, H.J. Lee, S.S. Roh, K.S. Kanga, Abietic acid isolated from pine resin (ResinaPini) enhances angiogenesis in HUVECs and accelerates cutaneous wound healing in mice, J. Ethnopharmacol. 203 (2017) 279–287.

[16] M. Jridi, S. Sellimi, K.B. Lassoued, S. Beltaief, N. Souissi, L. Mora, F. Toldra, A. Elfeki, M. Nasri, R. Nasri, Wound healing activity of cuttlefish gelatin gels and films enriched byhenna (Lawsoniainermis) extract, Colloids Surf. A Physicochem. Eng. Asp. 512 (2017) 71–79.

[17] N.J. Trengove, H. Bielefeldt-Ohmann, M.C. Stacey, Mitogenic activity and cytokine levels in non-healing and healing chronic leg ulcers, Wound Repair Regen. 8 (1) (2000) 13–25.

[18] H.J. Wallace, M.C. Stacey, Levels of tumor necrosis factor-alpha (TNF-alpha) and soluble TNF receptors in chronic venous leg ulcers—correlations to healing status, J. Invest. Dermatol. 110 (3) (1998 Mar) 292–296.

[19] R.W. Tarnuzzer, G.S. Schultz, Biochemical analysis of acute and chronic wound environments, Wound Repair Regen. 4 (3) (1996) 321–325.

[20] G. Lauer, S. Sollberg, M. Cole, I. Flamme, J. Stürzebecher, K. Mann, et al., Expression and proteolysis of vascular endothelial growth factor is increased in chronic wounds, J. Invest. Dermatol. 115 (1) (2000) 12–18 Jul.

[21] D.R. Yager, S.M. Chen, S.I. Ward, O.O. Olutoye, R.F. Diegelmann, C.I. Kelman, Ability of chronic wound fluids to degrade peptide growth factors is associated with increased levels of elastase activity and diminished levels of proteinase inhibitors, Wound Repair Regen. 5 (1) (1997) 23–32.

[22] Y. Ozay, S. Guzel, I.H. Erdogdu, Z. Yildirim, B. Pehlivanoglu, B.A. Turk, S. Darcan, Rec. Nat. Prod. 12 (2018) 4.

[23] H. Brem, M. Tomic-Canic, Cellular and molecular basis of wound healing in diabetes, J. Clin. Invest. 117 (2007) 1219–1222.

[24] F. Sabino, U. Keller, Matrix metalloproteinases in impaired wound healing, J. Metalloproteinases Med. 2 (2015) 1–8.

[25] C.W. Hicks, S. Selvarajah, N. Mathioudakis, B.A. Perler, J.A. Freischlag, J.H. Black 3rd, C.J. Abularrage, Trends and determinants of costs associated with the inpatient care of diabetic foot ulcers, J. Vasc. Surg. 60 (5) (2014) 1247–1254.e2 Nov.

[26] R.G. Frykberg, J. Banks, Challenges in the treatment of chronic wounds, Adv. Wound Care 4 (9) (2015) 560–582.

[29] S.F. Nabavi, N. Braidy, O. Gortzi, E. Sobarzo-Sanchez, M. Daglia, K. Skalicka-Wozniak, S.M. Nabavi, Luteolin as an anti-inflammatory and neuro-protective agent: a brief review, Brain Res. Bull. 119 (2015) 1–11.

[30] K. Navneet, B.B. Mishra, V.K. Tiwari, V. Tripathi, Difuranonaphthoquinones from Plumbago zeylanica roots, Phytochem. Lett. 3 (2010) 62–65.

[31] M. Chauhan, A review on Morphology, Phytochemistry and Pharmacological activities of medicinal herb Plumbago Zeylanica Linn, J. Pharmacogn. Phytother. 3 (2) (2014) 95–118.

[32] A.M. Melo, M.L. Jardim, C.F. Santana, Y. Lacet, J.L. Filho, I. Lima, O.G. Eoncio, First observations on the topical and use of primin, plumbagin and mayteni in patients with skin cancer, Revista do Instituto de Antibioticos 14 (1974) 9–16.

[33] E. Blatter, J.R. Cauis, K.S. Mhaskar, L.M. Basu, K.R. Kirtikar, B.D. Basu (Eds.), Indian Medicinal Plants, vols I & II, 1984 Allahabad, India.

[34] R.S. Thakur, H.S. Puri, A. Husain (Eds.), Major Medicinal Plants of India, Central Institute of Medicinal and Aromatic Plants, Lucknow, India, 1989.

[35] K.D. Arunachalam, P. Velmurugan, R.B. Raja, Anti-inflammatory and cytotoxic effects of extract from plumbago zeylanica, Afr. J. Microbiol. Res. 4 (12) (2010) 1239–1245.

[36] P. Luo, Y.F. Wong, L. Ge, Z.F. Zhang, Y. Liu, L. Liu, H. Zhou, Anti-inflammatory

and analgesic effect of plumbagin through inhibition of nuclear factor-κB activation, J. Pharmacol. Exp. Ther. 335 (2010) 735–742.
[37] B.R. Subramaniya, G. Srinivasan, S.S. Sadullah, N. Davis, L.B. Subhadara, D. Halagowder, N.D. Sivasitambaram, Apoptosis inducing effect of plumbagin on colonic cancer cells depends on expression of COX-2, PLoS One 6 (2011) e18695.
[38] H. Yen-ju, L. Lei-Chwen, T. Tung-Hu, Measurement and pharmacokinetic study of plumbagin in a conscious freely moving rat using liquid chromatography/tandem mass spectrometry, J. Chromatogr. 844 (2006) 1–5.
[39] W. Yuan-chuen, H. Tung-liang, High-performance liquid chromatography for quantification of plumbagin, an anti-Helicobecter pylori compound of plumbago zeylanica L. J. Chromatogr. 1094 (2005) 99–104.
[40] M. Vanisree, T. Hsin-Sheng, Plant cell culture- an alternative and efficient source for theproduction of biologically important secondary metabolites, Int. J. Appl. Sci Eng. 2 (2004) 29–48.
[41] T. Ponrasu, S. Jamuna, A. Mathew, K.N. Madhukumar, M. Ganeshkumar, K. Iyappan, L. Suguna, Efficacy of L-proline administration on the early responses during cutaneous wound healing in rats, Amino Acids 45 (1) (2013) 179–189 Jul.
[42] P. Banerjee, L. Suguna, C. Shanthi, Wound healing activity of a collagen-derived cryptic peptide, Amino Acids 47 (2) (2015) 317–328 Feb.
[43] J.F.Jr. Woessner, The determination of hydroxy proline in tissue and protein samples containing small proportions of this imino acid, Arch. Biochem. Biophys. 93 (1961).
[44] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, G. Grynkiewicz, M. Poenie, R. Tsien, J. Folch, M. Lees, G.S.J.J.b.C. Stanley, 26. The Colorimetric Determination of Phosphorus, vol. 193, (1951), p. 265.
[45] H. Ohkawa, N. Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95 (2) (1979) 351–358.
[46] H.P. Misra, I. Fridovich, The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase, J. Biol. Chem. 247 (10) (1972) 3170–3175.
[47] A.C. Maehly, B. Chance, The assay of catalases and peroxidases, Methods Biochem. Anal. 1 (1954) 357–424.
[48] J.T. Rotruck, A.L. Pope, H.E. Ganther, A.B. Swanson, D.G. Hafeman, W.G. Hoekstra, Selenium: biochemical role as a component of glutathione peroxidase, Science 179 (4073) (1973) 588–590.
[49] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione S-transferases. The first enzymatic step in mercapturic acid formation, J. Biol. Chem. 249 (22) (1974) 7130–7139.
[50] L. Flohe, W.A. Gunzler, Assays of glutathione peroxidase, Methods Enzymol. 105 (1984) 114–121.
[51] M.A. Latif, M.Z.M. Zaki, T.M. Leng, et al., Alocasia denudata Engler treatment enhance open wound healing activities in Wistar rat's skin, J. Ethnopharmacol. 176 (6) (2015) 258–267.
[52] V. Moulin, F.A. Auger, D. Garel, L. Germain, Role of wound healing myofibroblasts on re-epithelization of human skin, Burns 26 (2000) 3–12.
[53] L. Suguna, S. Singh, P. Sivakumar, P. Sampath, G. Chandrakasan, Influence of Terminalia chebula on dermal wound healing in rats, Phytother. Res. 16 (2002) 223–227.
[54] Devender Rao Kodati, Shashidher Burra, P. Kumar Goud, Evaluation of wound healing activity of methanolic root extract of Plumbago zeylanica L. in wistar albino rats, Asian J. Plant Sci. Res. 1 (2) (2011) 26–34.
[55] R.V. Gonçalves, R.D. Novaes, M.M. Sarandy, et al., 5α-Dihydrotestosterone enhances wound healing in diabetic rats [published online ahead of print March 20, 2016], Life Sci. 152 (2016) 67–75.
[56] M. Hrynyk, R.J. Neufeld, Insulin and wound healing, Burns 40 (8) (2014) 1433–1446.
[57] K.S. Silvipriya, K.K. Kumar, A.R. Bhat, B.D. Kumar, A. John, P. Lakshmanan, Collagen: animal sources and biomedical application, J. Appl. Pharm. Sci. 5 (3) (2015 Mar) 123–127.
[58] S. Chattopadhyay, R.T. Raines, Review collagen-based biomaterials for wound healing, Biopolymers 101 (8) (2014) 821–833. Aug.
[59] R.G. Spanheimer, G.E. Umpierrez, V. Stumpf, Decreased collagen production in diabetic rats, Diabetes 37 (4) (1988) 371–376. Apr.
[60] K.G. Nevin, T. Rajamohan, Effect of topical application of virgin coconut oil on skin components and antioxidant status during dermal wound healing in young rats, Skin Pharmacol. Physiol. 23 (2010) 290–297.
[61] C. Sunil, V. Duraipandiyan, P. Agastian, S. Ignacimuthu, Antidiabetic effect of plumbagin isolated from Plumbago zeylanica L. root and its effect on GLUT4 translocation in streptozotocin-induced diabetic rats, Food Chem. Toxicol. 50 (12) (2012) 4356–4363 Dec.
[62] I. Sharma, D. Gusain, V.P. Dixit, Hypolipidaemic and antiatherosclerotic effects of plumbagin in rabbits, Indian J. Physiol. Pharmacol. 35 (1) (1991) 10–14 Jan.
[63] S. Braun, C. Hanselmann, M.G. Gassmann, et al., Nrf2 transcription factor, a novel target of keratinocyte growth factor action which regulates gene expression and inflammation in the healing skin wound, Mol. Cell. Biol. 22 (15) (2002) 5492–5505.
[64] M. Long, M. Rojo de la Vega, Q. Wen, et al., An essential role of NRF2 in diabetic wound healing, Diabetes 65 (3) (2016) 780–793.
[65] M.A. Soares, O.D. Cohen, Y.C. Low, et al., Restoration of Nrf2 signaling normalizes the regenerative niche, Diabetes 65 (2016) 633–646.
[66] Joshua A. David, William J. Rifkin, Piul S. Rabbani, Daniel J. Ceradini, The Nrf2/Keap1/ARE pathway and oxidative stress as a therapeutic target in type II diabetes mellitus, J. Diabetes Res. 2017 (2017) 482672415 pages.
[68] P.G. Winyars, D.R. Blake, C.H. Evans, Free Radicals and Inflammation, Birkhauser, Switzerland, 2009, pp. 195–203.
[69] Pulak Majumder, M. Paridhavi, A novel PolyHerbal formulation hastens diabetic wound healing with potent antioxidant potential: a comprehensive pharmacological investigation, Nat. Prod. Chem. Res. 06 (2018), https://doi.org/10.4172/2329-6836.
[70] D.K.U. Auf, A. Kumin, S. Braun, S. Werner, Reactive oxygen species and their detoxification in healing skin wounds, J. Investig. Dermatol. Symp. Proc. 11 (2006) 106–111.
[71] R. Rahimi, S. Nikfar, B. Larijani, M. Abdollahi, A review on the role of antioxidants in the management of diabetes and its complications, Biomed. Pharmacother. 59 (2005) 365–373.
[72] A.M. Rasik, A. Shukla, Antioxidant status in delayed healing type of wounds, Int. J. Exp. Pathol. 81 (2000) 257–263.
[73] G.S. Dave, K. Kalia, Hyperglycemia induced oxidative stress in type-1 and type-2 diabetic patients with and without nephropathy, Cell. Mol. Biol. 53 (2007) 68–78.
[74] S.H. Nile, C.N. Khobragade, Antioxidant activity and flavonoid derivatives of Plumbago zeylanica, J. Nat. Prod. 3 (2010) 130–133.
[75] M. Katsuhiro, S. Hui Teoh, H. Yamashiro, M. Shinohara, F. Fatchiyah, T. Ohta, T. Yamada, Effects on glycemic control in impaired wound healing in Spontaneously Diabetic Torii (SDT) fatty rats, Med. Arch. 72 (1) (2018) 4–8 Feb.
[76] M. Sengupta, P. Banerjee, S. Paul, J. Sengupta, M. Ghosh, Healing effect of phenytoin on excisional wound in experimental albino rats, Muller J. Med. Sci. Res. 6 (2015) 27–30.
[77] D.B. Hom, B.M. Linzie, T.C. Huang, The healing effects of autologous platelet gel on acute human skin wounds, Arch. Facial Plast. Surg. 9 (2007) 174–183.
[78] Z. Xie, C.B. Paras, H. Weng, P. Punnakitikashem, L.C. Su, K. Vu, et al., Dual growth factor releasing multi-functional nanofibers for wound healing, Acta Biomater. 9 (2013) 9351–9359.
[79] A.K. Kutlu, D. C¸ec¸en, S.G. Gürgen, O. Sayin, F. C¸etin, A comparison study of growth factor expression following treatment with transcutaneous electrical nerve stimulation, saline solution, povidone-iodine, and lavender oil in wound healing, Evid. Based Complement. Alternat. Med. 2013 (2013) 1–9.
[80] S. Werner, R. Grose, Regulation of wound healing by growth factors and cytokines, Physiol. Rev. 83 (2003) 835–870.
[81] J.P. Pienimaki, K. Rilla, C. Fulop, et al., Epidermal growth factor activates hyaluronan synthase 2 in epidermal keratinocytes and increases pericellular and intracellular hyaluronan, J. Biol. Chem. 276 (2001) 20428–20435.
[82] W.T. Lawrence, R.F. Diegelman, Growth factors in wound healing, Clin. Dermatol. 12 (1994) 157–169.
[83] N. Ferrara, Role of vascular endothelial growth factor in regulation of physiological angiogenesis, Am. J. Physiol. Cell Physiol. 280 (2001) C1358–C1366.
[84] P.J. Taub, L. Silver, H. Weinberg, Plastic surgical perspectives on vascular endothelial growth factor as gene therapy for angiogenesis, Plast. Reconstr. Surg. 105 (2000) 1034–1042.
[85] A.A. Hemmati, M. Foroozan, G. Houshmand, et al., The topical effect of grape seed extract 2% cream on surgery wound healing, Global J. Health Sci. 7 (2015) 52–58.
[86] K. Vitlianova, J. Georgieva, M. Milanova, S. Tzonev, Blood pressure control predicts plasma matrix metalloproteinase-9 in diabetes mellitus type II, Arch. Med. Sci. 11 (1) (2015) 85–91 Mar 16.
[87] M. Muller, C. Trocme, B. Lardy, F. Morel, S. Halimi, P.Y. Benhamou, Matrix metalloproteinases and diabetic foot ulcers: the ratio of MMP-1 to TIMP-1 is a predictor of wound healing, Diabet. Med. 25 (4) (2008) 419–426. Apr.
[88] C.M. Overall, C. López-Otín, Strategies for MMP inhibition in cancer: innovations for the post-trial era, Nat. Rev. Cancer 2 (9) (2002) 657–672 Sep.
[89] O. Lenz, S.J. Elliot, W.G. Stetler-Stevenson, Matrix metalloproteinases in renal development and disease, J. Am. Soc. Nephrol. 11 (3) (2000) 574–581 Mar.
[90] A.K. Death, E.J. Fisher, K.C. McGrath, D.K. Yue, High glucose alters matrix metalloproteinase expression in two key vascular cells: potential impact on atherosclerosis in diabetes, Atherosclerosis 168 (2) (2003) 263–269 Jun.
[91] S. Loffek, O. Schilling, C.W. Franzke, Series "matrix metalloproteinases in lung health and disease": biological role of matrix metalloproteinases: a critical balance, Eur. Respir. J. 38 (1) (2011) 191–208.
[92] R. Geesala, N.R. Dhoke, A. Das, Cox-2 inhibition potentiates mouse bone marrow stem cell engraftment and differentiation-mediated wound repair, Cytotherapy 19 (6) (2017) 756–770 Jun.
[93] S. Guo, L.A. Dipietro, Factors affecting wound healing, J. Dent. Res. 89 (3) (2010) 219–229 Mar.
[94] A.M. Zaki, D.M. El-Tanbouly, R.M. Abdelsalam, H.F. Zaki, Plumbagin ameliorates hepatic ischemia-reperfusion injury in rats: role of high mobility group box 1 in inflammation, oxidative stress and apoptosis, Biomed. Pharmacother. 106 (2018) 785–793 Oct.
[95] L. Wang, W. Qin, Y. Zhou, B. Chen, X. Zhao, H. Zhao, E. Mi, E. Mi, Q. Wang, J. Ning, Transforming growth factor β plays an important role in enhancing wound healing by topical application of Povidone-iodine, Sci. Rep. 7 (1) (2017) 991. Apr 20.
[96] J.J. Boyle, H.A. Harrington, E. Piper, et al., Coronary intraplaque hemorrhage evokes a novel atheroprotective macrophage phenotype, Am. J. Pathol. 174 (3) (2009) 1097–1108.
[97] D.R. Yagnik, B.J. Evans, O. Florey, J.C. Mason, R.C. Landis, D.O. Haskard, Macrophage release of transforming growth factor β1 during resolution of monosodium urate monohydrate crystal-induced inflammation, Arthritis Rheum. 50 (7) (2004) 2273–2280.
[98] A.V. Finn, M. Nakano, R. Polavarapu, et al., Hemoglobin directs macrophage differentiation and prevents foam cell formation in human atherosclerotic plaques, J. Am. Coll. Cardiol. 59 (2) (2012) 166–177.
[99] D.J. Schaer, C.A. Schaer, P.W. Buehler, R.A. Boykins, G. Schoedon, A.I. Alayash, A. Schaffner, CD163 is the macrophage scavenger receptor for native and chemically modified hemoglobins in the absence of haptoglobin, Blood 107 (1) (2006)

373–380 Jan 1.
[100] R. Blakytny, E. Jude, The molecular biology of chronic wounds and delayed healing in diabetes, Diabet. Med. 23 (2006) 594–608.
[102] R. Zhao, H. Liang, E. Clarke, C. Jackson, M. Xue, Inflammation in chronic wounds, Int. J. Mol. Sci. 17 (12) (2016) 2085 Dec.
[103] A. Singhal, H. Gupta, V. Bhati, Wound healing activity of Argyreia nervosa leaves extract, Int. J. Appl. Basic Med. Res. 1 (1) (2011) 36–39 Jan.
[104] P.K. Agarwal, A. Singh, K. Gaurav, S. Goel, H.D. Khanna, R.K. Goel, Evaluation of wound healing activity of extracts of plantain banana (Musa sapientum var. paradisiaca) in rats, Indian J. Exp. Biol. 47 (1) (2009) 32–40 Jan.
[105] S. Chakraborti, M. Mandal, S. Das, A. Mandal, T. Chakraborti, Regulation of matrix metalloproteinases: an overview, Mol. Cell. Biochem. 253 (1–2) (2003) 269–285 Nov.
[106] M.D. Sternlicht, Z. Werb, How matrix metalloproteinases regulate cell behavior, Annu. Rev. Cell Dev. Biol. 17 (2001) 463–516.