Topical combined *Phyllanthus emblica* Linn. and simvastatin improves wound healing in diabetic mice by enhancing angiogenesis and reducing neutrophil infiltration

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Abstract. The present study aimed to investigate the effects of combined *Phyllanthus emblica* Linn. (PE) and simvastatin (SIM) on diabetic wounds in male BALB/C mice. Bilateral full thickness wound excisions were performed in the control and diabetic groups (45 mg/kg streptozotocin, intraperitoneally injected daily for 5 days). The diabetic mice received daily treatment with four different types of cream: Vehicle [diabetes mellitus (DM) + Vehicle group], 100% PE (DM + PE group), 5% SIM (DM + SIM group) and combined 100% PE + 5% SIM (DM + Combination group) for 4, 7 and 14 days. The tissue malondialdehyde (MDA) and IL-6 protein levels, the number of infiltrated neutrophils, and the percentages of wound closure (%WC), capillary vascularity (%CV) and re-epithelialization (%RE) were subsequently measured. The results indicated that in the DM + Combination group, %CV and %WC were significantly increased when compared with the DM + Vehicle group on days 7 and 14. The tissue MDA content on day 14, and the number of infiltrated neutrophils on days 4 and 7 were significantly reduced in the DM + Combination group compared with those in the DM + Vehicle group. Furthermore, a strong positive correlation was revealed between %CV and %WC in the five groups on day 7 (r=0.736; P=0.0003). These findings indicated that topical application of combined PE and SIM could enhance wound healing by upregulating angiogenesis and reducing neutrophil infiltration in mice with diabetic wounds.

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Introduction

Chronic diabetic wounds are characterized by a prolonged inflammation stage, impaired proliferation stage and long-lasting remodeling stage. During the impaired proliferation stage, VEGF protein expression, angiogenesis and re-epithelialization (RE) are impaired due to prolonged inflammation and hyperglycemia-induced excessive oxidative stress (1). Therefore, the resolution of prolonged inflammation is a key point in the transition to the impaired proliferation stage that may improve wound healing (1-3). Notably, promoting angiogenesis is one of the gold standard treatments for diabetic foot ulcers (1-3). Bitto *et al* (4) demonstrated that topical application of simvastatin (SIM) enhanced wound healing in diabetic mice. Recently, pleiotropic effects of SIM on wound healing have been discovered, in addition to its classic lipid-lowering effect (4-8). The possible pleiotropic effects of SIM on wound healing have been reported to be induced by reducing inflammation, and upregulating VEGF production, angiogenesis and RE (4-8). *Phyllanthus emblica* Linn. (PE) is a medicinal plant of the *Phyllanthus* genus, which is distributed in tropical and subtropical areas, and is widely used in Ayurvedic medicine (9). PE extracts have been shown to exert wound-healing effects *in vitro* and in rat wound models (9-11). Based on the effective chemical constituents in PE used in various models, the wound-healing benefit of PE has been reported to be associated with its antioxidant and anti-inflammatory effects (9-14).

To the best of our knowledge, no research has yet been conducted on the combined treatment of diabetic wounds with topical PE and SIM. Therefore, the present study aimed to determine the effect and the potential associated mechanisms of topical application of PE and SIM in a mouse model of diabetic wounds.

Materials and methods

Animal preparation. Male BALB/C mice (total number of mice, 75; number of mice/group, 5); age, 7-8 weeks; weight, 20-25 g) were purchased from Nomura Siam International

Co. Ltd. The experimental procedures and daily care of the mice were approved by the Animal Care and Use Ethics Committee, Faculty of Medicine, Chulalongkorn University (Bangkok, Thailand; IRB no. 007/2562). The procedures were conducted according to the experimental animal guidelines of The National Research Council of Thailand (15). All mice were housed at 25±3˚C and 55+5% humidity, with *ad libitum* access to standard chow and sterilized water under a 12-h light/dark cycle.

For the induction of diabetes, the mice were intraperitoneally injected with streptozotocin (STZ; MilliporeSigma) in citrate buffer (pH 4.5; MilliporeSigma), at a dose of 45 mg/kg daily for 5 consecutive days (16). The control mice received an equal volume of citrate buffer (pH 4.5) intraperitoneally for 5 consecutive days (16). A total of 2 weeks after the first day of STZ injections, the fasting blood glucose (FBG) level was detected in 1 μl tail-vein blood from each mouse. The diabetic mouse model was established successfully when the FBG level was ≥200 mg/dl (14). The diabetic mice were divided into four subgroups: Mice treated daily with vehicle [diabetes mellitus (DM) + Vehicle (PMA2 cream)], mice treated daily with 100% PE cream (DM + PE), mice treated daily with 5% SIM cream (DM + SIM) and mice treated daily with a combination of 100% PE + 5% SIM cream (DM + Combination). The diabetic mice in each group were further divided into three minor subgroups according to experimental period, as follows: Days 4, 7 and 14. The mice in the early and intermediate groups (day 4 and 7) were used to assess the inflammation and proliferation phases (3). The parameters measured on days 4 and 7 included the number of infiltrated neutrophils, and the tissue levels of IL-6 and VEGF proteins. On day 14, the percentages of wound closure (%WC), capillary vascularity (%CV) and RE (%RE) were measured.

PE extraction. A total of 10.0 kg fresh PE fruits were purchased from Kru-La-Or Farm (Sai Yok District, Kanchanaburi, Thailand). PE fruits were squeezed to obtain 3.4 kg minced pulp. Subsequently, the pulp was extracted with distilled ethanol (1:2, w/v) three times (72 h each time) at room temperature (10). Subsequently, the extracted solutions were collected and pooled together. The pooled alcoholic extraction solution was passed through filter paper (pore size, 11 μm) and dried under vacuum rotary evaporation (Rotavapor R-114; Buchi AG) below 45˚C to yield 219.81 g PE ethanol extract. The dried PE ethanol extract was kept in an airtight and light-protecting container at 4˚C in a refrigerator.

Combination drug preparation. For the combined PE and SIM cream, the exact amount of dried PE extract and SIM powder [lot no. 116M4716 V; MilliporeSigma; >97% purity (HPLC grade)], were weighed according to the calculation of 100% PE cream and 5% SIM (w/v) cream. PMA2 cream (Paragon Aesthetic Co., Ltd.) was used as a base cream [10 μl PMA2 base cream contained 100% (w/v) PE, and 5% (w/v) SIM] (17). The well-mixed combined cream was kept in tightly closed light-proof brown glass bottle at 4˚C.

Wound model. After 4 weeks of successful diabetic mouse model establishment, the mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (55 mg/kg). Subsequently, the fur on the dorsal area of the mice was sheared using electric clippers. To minimize the number of sacrificed mice, two 6x6 mm^2 full-thickness wounds were made on the dorsal skin of both sides of the vertebral column along the spine, 30 mm from the middle of the ears and 15 mm from the spine (16,17). The wound skin collected from the left side of mice was used for the measurement of MDA content, IL-6 and VEGF protein levels, whereas the wound skin collected from the right side of the mice was used for measurement of re-epithelialization and neutrophil infiltration. The number of animals in each group was 4-5, whereas the number of wounds in each group was 3-5; the number of wounds in each group was sometimes <5 if some of the wounds were scratched and could therefore not be used to assess wound area and other parameters. A Tegaderm™ frame was sutured to each wound to minimize the confounding factor of mouse skin contraction (16,17). Subsequently, 10 μl PMA2 base cream, 100% PE, 5% SIM (w/v) or a combination of PE and SIM was gently applied to the wound area once a day every day until the end date of the experiment. At the end of the experiment, the mice euthanized by intraperitoneal injection of an overdose of sodium pentobarbital (100 mg/kg). After euthanasia was confirmed by the absence of cardiovascular function and no vital signs (IRB no. 007/2562), the wound tissues were collected for histological analysis and ELISA.

Determination of %WC. On days 7 and 14 post-wounding, after the mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (55 mg/kg), images of each wound area were captured using a stereoscopic zoom microscope (Nikon SMZ800; Nikon Corporation). The unhealed wound area was determined from each microscopic image using digital imaging software (Image-Pro II 6.1 software; Media Cybernetics, Inc.). %WC was calculated using the following equation: %WC=[(area of the original wound-area of actual wound)/area of the original wound] x100 (16,17).

Determination of %CV. On days 7 and 14 post-wounding, after the mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (55 mg/kg), the jugular vein was cannulated for injection of 0.1 ml 5% FITC-labeled dextran (molecular weight, 250,000; MilliporeSigma). By using confocal fluorescence microscopy at x100 magnification (Nikon Eclipse E800; Nikon Corporation), the surrounding capillary vasculature in the wound area was recorded. To avoid tissue damage from fluorescent light, each image was captured in a few seconds. From the fluorescent images of the capillaries (diameter <15 μm), %CV was calculated using Image-Pro II 6.1 software (Media Cybernetics, Inc.) (16,17). %CV located in each area of interest (40x40 pixels) was determined using the following equation: %CV=(number of pixels within capillaries/total number of pixels in the entire frame) x100 (16,17).

Determination of neutrophil infiltration. Wound tissues were collected from each mouse and fixed in 10% formaldehyde at room temperature (25±2˚C) for 24 h. The centers of the wound specimens were cut and embedded in paraffin. Sections (5 μm) were then stained with hematoxylin [Modified Hematoxylin Solution (progressive stain); C.V. Laboratories Co., Ltd.] for 5 min and eosin [Eosin Solution (Working Solution); C.V.

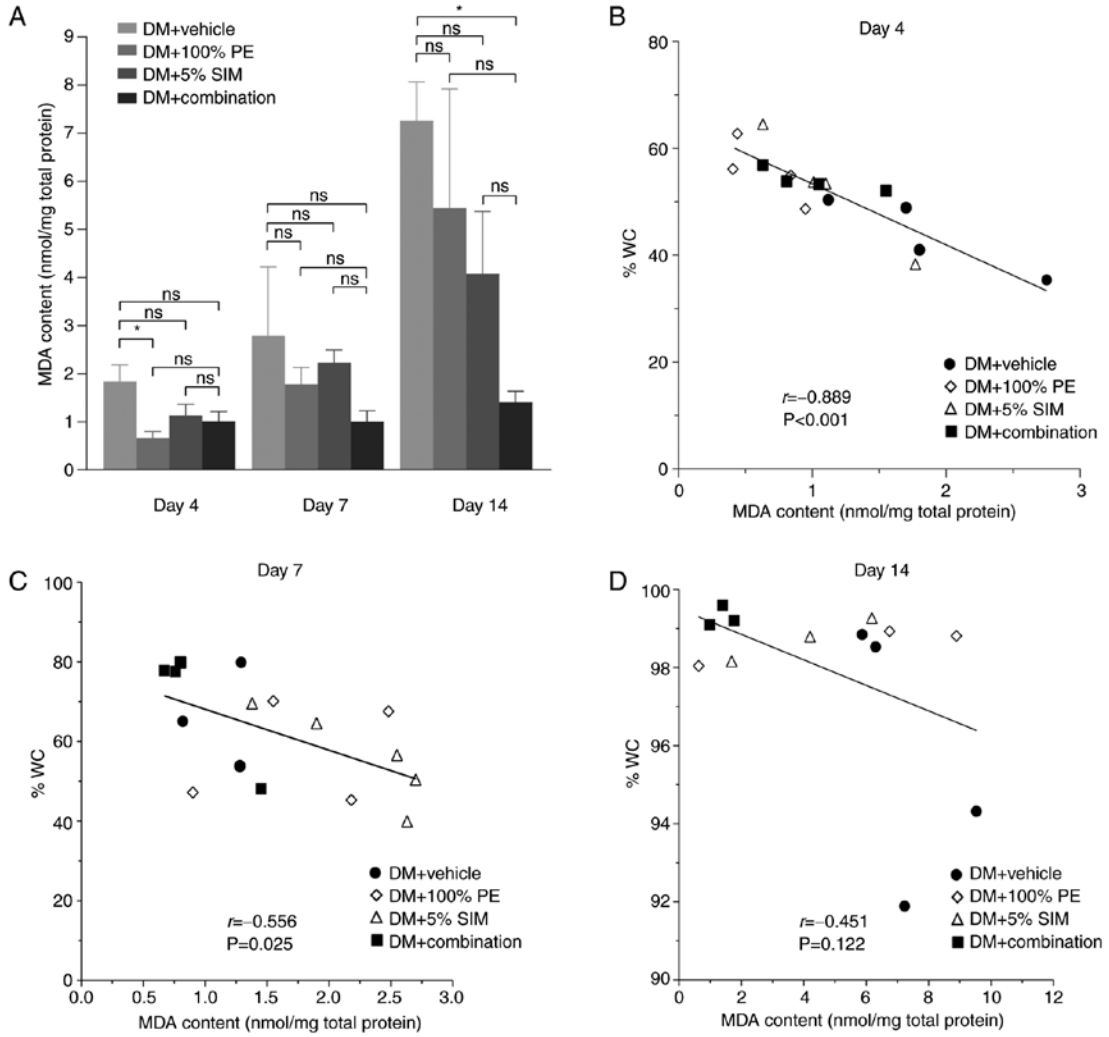

Figure 1. MDA content and correlation analysis between MDA and %WC. (A) Tissue MDA levels in the various groups at day 4, 7 and 14. Data are presented as the mean ± SEM. Pearson's correlation analyses between MDA contents (nmol/mg total protein) and %WC on day (B) 4, (C) 7 and (D) 14. Number of animals in each group, 4-5; number of wounds in each group, 3-5. *P<0.05. %WC, percentage wound closure; DM, diabetes mellitus; MD, malondialdehyde; ns, not significant; PE, *Phyllanthus emblica* Linn.; SIM, simvastatin.

Laboratories Co., Ltd.] (H&E) for 3 min at room temperature (25±2˚C) at the Department of Pathology, Faculty of Medicine, Chulalongkorn University. The H&E-stained slides were used to measure neutrophil infiltration at x400 magnification under a stereoscopic zoom microscope (Nikon SMZ800). The x400 magnification wound images were captured using a digital camera (Nikon DS-L2; Nikon Corporation). The number of neutrophils per 1,000 cells counted from the wound edge was recorded. Digital imaging software (Image-Pro II 6.1 software) was used to count the numbers of infiltrated neutrophils. The results were confirmed by blinded assessment (15,17).

Determination of %RE. The H&E-stained slides were also used for the measurement of RE. The wound edge and RE tip judgment were conducted in images at x400 magnification, which were captured using a digital camera (Nikon DS-L2). The lengths of the curved lines from both wound edges to the RE tips and the wound gap between the RE tips were also measured. The %RE of the wound was calculated using the following equation: %RE=distance covered by epithelium/distance between wound edges) x100 (5,16).

Determination of VEGF and IL-6 protein levels. Wound tissue was collected from each mouse and kept at -80˚C until processing. The wound samples were weighed and homogenized in RIPA buffer (Cell Signaling Technology, Inc.) and phosphatase inhibitor cocktails (MilliporeSigma). The samples were sonicated (20 kHz; Model VCX750; Sonics & Materials Inc., USA) three times (each time, 15 sec; interval time, of 10 sec), and centrifuged (Sorvall™ Legend™ X1R; Thermo Fisher Scientific, Inc.) at 13,416 x g for 10 min at 4˚C. The supernatants of each sample were used to determine the levels of total protein using the BCA protein assay kit (Thermo Fisher Scientific, Inc.), VEGF using the VEGF ELISA kit (mouse VEGF quantikine ELISA kit; cat. no. MMV00; R&D Systems, Inc.), and IL-6 using the IL-6 ELISA kit (mouse IL-6 quantikine ELISA kit; cat. no. M6000B; R&D Systems, Inc). Subsequently, the OD value of the solution in each well was measured using a colorimetric microplate reader (Model 860; Bio-Rad Laboratories, Inc.), setting the measured wavelength at 450 nm and the reference wavelength at 570 nm. The VEGF and IL-6 protein levels were expressed in units of pg/mg total protein. All standard solutions and samples were duplicated in a second plate (5,16).

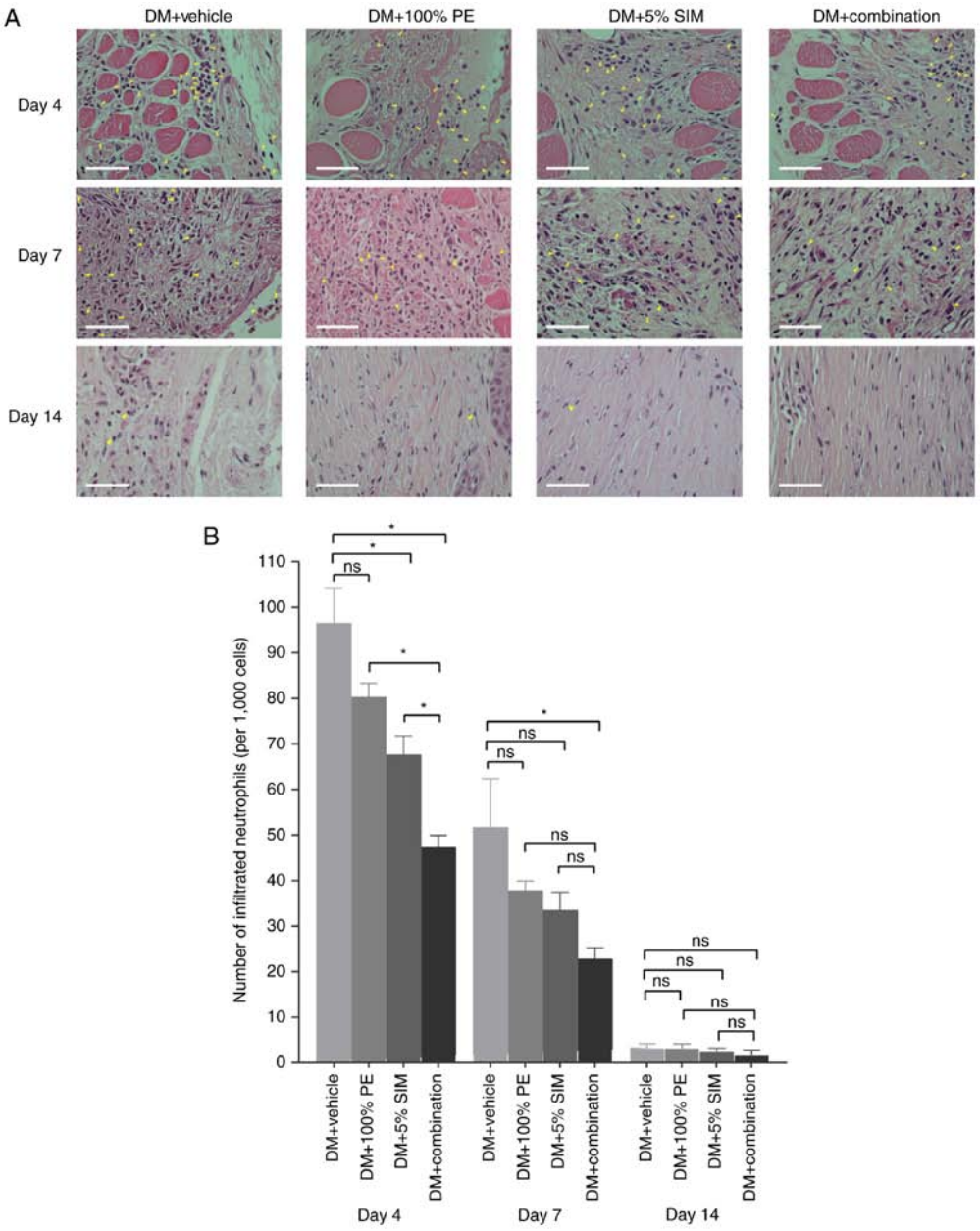

Figure 2. Effects of topical application of combined PE and SIM cream on the number of infiltrated neutrophils in diabetic wounds. (A) Positive infiltrated neutrophils were indicated by yellow arrows (hematoxylin and eosin staining; x400 magnification; scale bar, 100 μm). (B) Number of infiltrated neutrophils (per 1,000 cells) in each group on days 4, 7 and 14. Data are presented as the mean ± SEM. Number of animals in each group, 4-5; number of wounds in each group, 3-5. *P<0.05. DM, diabetes mellitus; ns, not significant; PE, *Phyllanthus emblica* Linn.; SIM, simvastatin.

Determination of tissue MDA. After the wound tissue was sonicated (20 kHz; Model VCX750) three times (each time, 15 sec; interval time, 10 sec) and centrifuged (Sorvall Legend X1R) at 13,416 x g for 10 min at 4˚C, the MDA content was determined using the TBARS assay kit (cat. no. 10009055, Cayman Chemical Company). The OD value was measured using a colorimetric microplate reader (Model 860), setting the wavelength at 540 nm. The MDA content was expressed in nmol/mg total protein. All standard solutions and samples were run in duplicate (16).

Statistical analysis. The results are presented as the mean ± standard error of the mean. The significance of differences between groups was determined by one-way analysis of variance followed by the Tukey's post hoc test. Correlation analysis was performed using the Pearson's correlation coefficient. Statistical analyses were conducted using SPSS (version 22; IBM, Corp.) and GraphPad Prism 6 software (GraphPad Software; Dotmatics). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of topical application of combined PE and SIM on wound oxidative stress. The MDA content (nmol/mg total protein) of the wound site on days 4, 7 and 14 is shown in Fig. 1A. The results indicated that the tissue MDA content seemed to increase during the progression of diabetes.

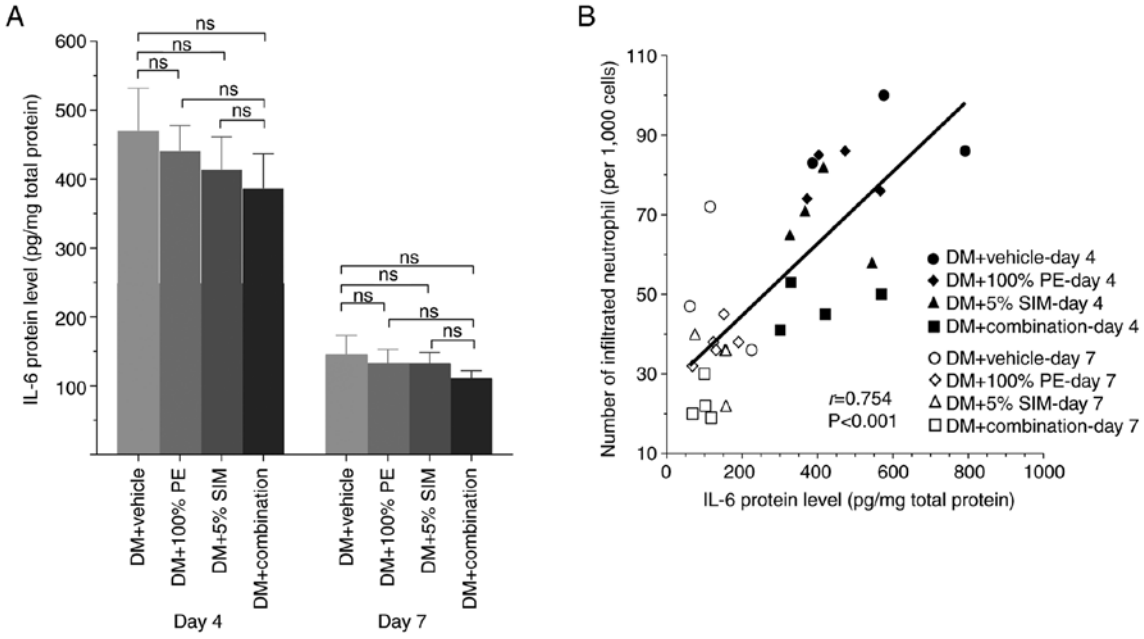

Figure 3. Effects of topical application of combined PE and SIM cream on IL-6 protein levels (pg/mg total protein) in diabetic wounds. (A) IL-6 protein levels in diabetic groups on days 4 and 7. Data are presented as the mean ± SEM. Number of animals in each group, 4-5; number of wounds in each group, 3-5. (B) Pearson's correlation analysis between number of infiltrated neutrophils (per 1,000 cells) and IL-6 protein levels on days 4 and 7. DM, diabetes mellitus; ns, not significant; PE, *Phyllanthus emblica* Linn.; SIM, simvastatin.

However, in the DM + Combination group, the tissue MDA content was demonstrated to be significantly decreased on day 14 compared with that in the DM + Vehicle group (P<0.05). A negative correlation was identified between MDA level and %WC on day 4 (r=-0.889; P=0.001; Fig. 1B) and day 7 (r=-0.556; P=0.025; Fig. 1C), but not on day 14 (r=-0.451; P=0.122; Fig. 1D).

Effects of topical application of combined PE and SIM on wound neutrophil infiltration. Images of neutrophil infiltration at the wound site (H&E staining; x400 magnification) on days 4, 7 and 14 post-wounding are shown in Fig. 2A. The number of infiltrated neutrophils detected during the inflammation and proliferation phases of the wound (days 4 and 7) were significantly downregulated in the DM + Combination group compared with those in the DM + Vehicle group (P<0.05; Fig. 2B).

Effects of topical application of combined PE and SIM on wound IL-6 protein levels. The levels of IL-6 (pg/mg total protein) in the wound tissue on days 4 and 7 post-wounding are shown in Fig. 3A. The results showed that the IL-6 protein levels in all groups seemed to increase during the wound inflammation phase on day 4 and appeared to be downregulate on day 7, the proliferation phase of wound healing. However, there was no statistically significant difference among all groups in each phase (P<0.05). Notably, a strong positive correlation was detected between IL-6 protein levels and neutrophil infiltration (r=0.754; P=0.001; Fig. 3B).

Effect of topical application of combined PE and SIM on wound VEGF protein levels and angiogenesis. The VEGF protein levels (pg/mg total protein) in all groups on days 7 and 14 post-wounding are shown in Fig. 4A. The results showed that VEGF protein levels were significantly upregulated in the DM + Combination group compared with those in the DM + Vehicle group on days 7; however, there was no significant difference among groups on day 14 post-wounding. Microscopic images of CV near the wound area are shown in Fig. 4B on days 7 and 14 post-wounding. Notably, %CV was significantly increased in the DM + Combination group compared with those in the DM + Vehicle group on days 7 and 14 post-wounding (P<0.05; Fig. 4C); however, there was no significant difference in the DM + Combination group when compared with the CON + Vehicle group. These results indicated that the combination cream could enhance wound healing in diabetic mice as good as the spontaneous wound healing observed in non-diabetic control mice. As shown in Fig. 4D, on day 7 post-wounding, there was a moderate positive correlation (r=0.635; P=0.003) between VEGF protein levels and %CV in the mouse wounds.

Effect of topical application of combined PE and SIM on wound RE. Images of RE at the wound site (H&E staining; x400 magnification) on days 4, 7 and 14 post-wounding are shown in Fig. 5A. The results showed that %RE at the wound site in the DM + 5% SIM and DM + Combination groups were slightly upregulated compared with that in the DM + Vehicle group on day 4; however, by day 14 post-wounding, all groups had completed 100% RE except for the DM + Vehicle group (Fig. 5B).

Effect of topical application of combined PE and SIM on WC. Images of WC in all groups on days 7 and 14 are shown in Fig. 6A. Regarding %WC, none of the treatment groups had significant differences when compared with the

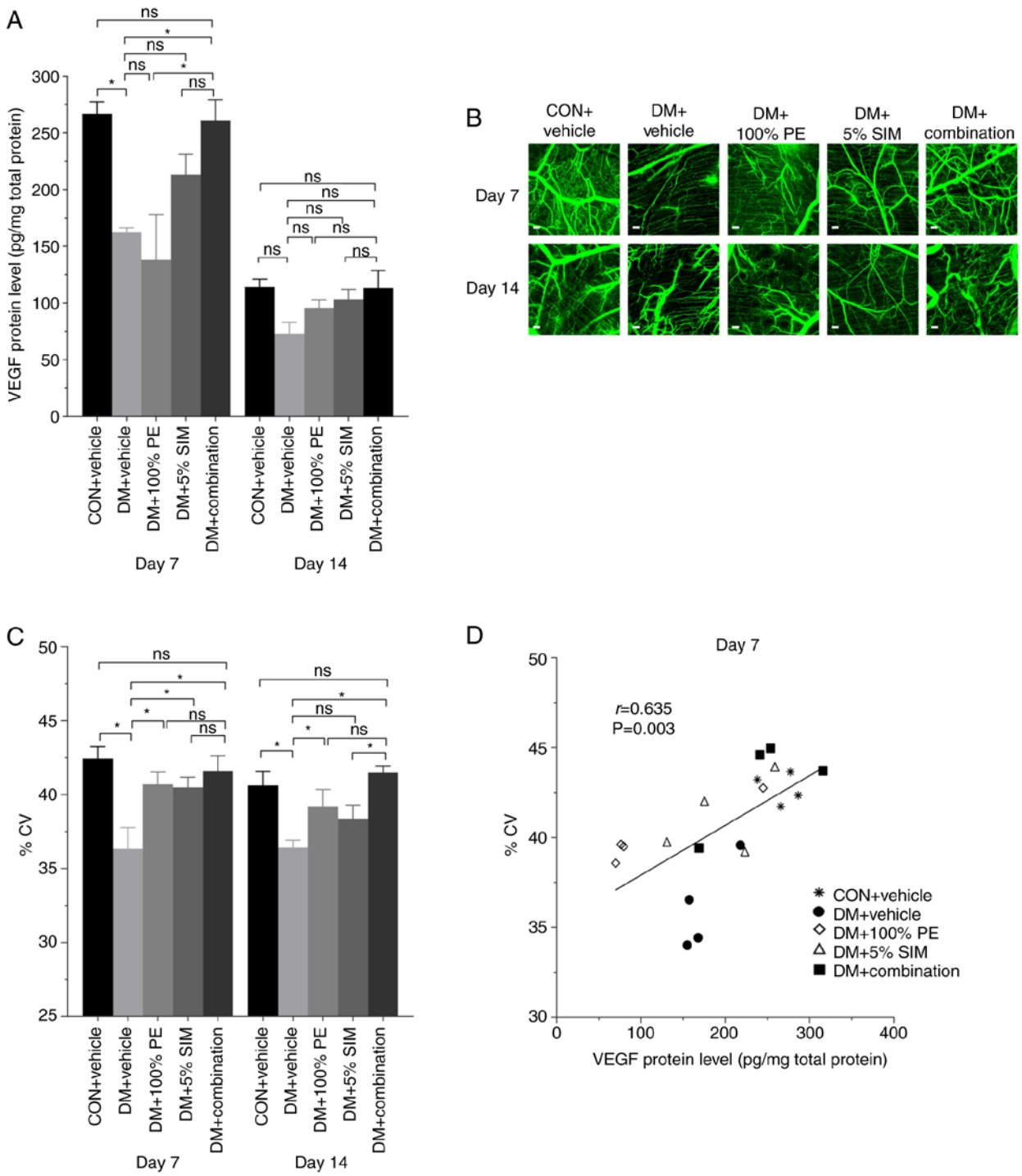

Figure 4. Effects of topical application of combined PE and SIM cream on tissue VEGF protein levels (pg/mg total protein). (A) VEGF protein levels in the groups on day 7 and 14 (n=5). Data are presented as the mean ± SEM. (B) Confocal image of capillary microvasculature. Scale bar, 100 μm. (C) %CV on days 7 and 14. Number of animals in each group, 4-5; number of wounds in each group, 4. Data are presented as the mean ± SEM. *P<0.05. (D) Pearson's correlation analysis between VEGF levels and %CV on day 7. %CV, percentage of capillary vascularity; CON, control; DM, diabetes mellitus; ns, not significant; PE, *Phyllanthus emblica* Linn.; SIM, simvastatin.

DM + Vehicle group on day 7, except the DM + Combination group. However, on day 14 all of the treatment groups exhibited increased %WC compared with the DM + Vehicle group (Fig. 6B). Furthermore, %WC in the DM + Combination group was not significantly different from that in the CON + Vehicle group. As shown in Fig. 6C, there were positive correlations between %CV and %WC on days 7 (r=0.736; P=0.0003) and 14 (r=0.457; P=0.006).

Discussion

In diabetes, chronic or poor control of hyperglycemia can result in the production of reactive oxygen species (ROS) via both enzymatic and nonenzymatic pathways (18). NADPH oxidase, mitochondrial electron transport chain pathways and other sources of ROS, such as advanced glycation end products and uncoupled nitric oxide synthase (NOS), are the prominent

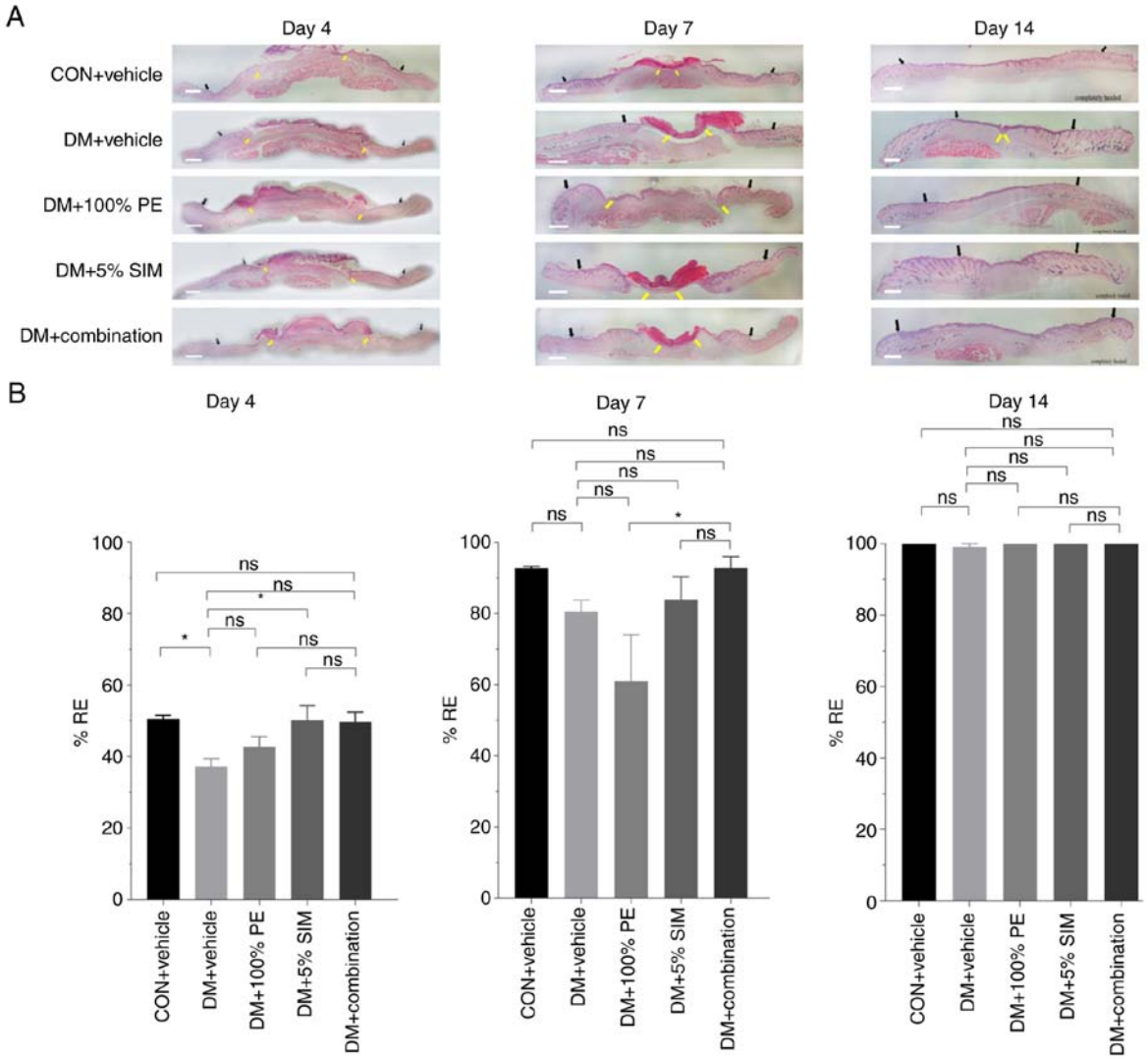

Figure 5. Effects of topical application of combined PE and SIM cream on %RE. (A) RE on days 4, 7 and 14. Black arrows indicate the wound edge with features of increased wound thickness and proliferated epidermis; yellow arrows indicate the tip of the endothelial cell in the wound area (hematoxylin and eosin staining; x400 magnification; scale bar, 1 mm). (B) %RE in diabetic groups on days 4, 7 and 14. Data are presented as the mean ± SEM. Number of animals in each group, 4-5; number of wounds in each group, 4-5. *P<0.05. %RE, percentage of re-epithelialization; CON, control; DM, diabetes mellitus; ns, not significant; PE, *Phyllanthus emblica* Linn.; SIM, simvastatin.

sources of ROS generation in diabetic wounds (18,19). Under this circumstance, excessive ROS serve a critical role in promoting pathophysiological events, prolonging inflammation and preventing diabetic wound healing (19,20). In the present study, the results of a Pearson's correlation analysis between MDA levels and %WC on days 4 and 7 indicated a reduction in oxidative stress at the wound site may markedly increase wound closure. In the DM + Combination group, a significant downregulation in MDA content was observed when compared with DM + Vehicle on day 14, thus confirming the antioxidant effect of combined PE and SIM treatment. Previous studies on animals and patients with diabetes have confirmed the effects of SIM and PE on decreasing MDA levels. Both SIM and PE have been reported for their antioxidant properties, including the restoration of endogenous antioxidant enzymes and scavenging free radicals (13,17,21-25). Therefore, combined PE and SIM cream could improve the healing process in chronic diabetic wounds via its beneficial effects on antioxidant-targeting pathways.

The present study also detected a reduction in the number of infiltrated neutrophils in the DM + Combination group. This may contribute to a reduction in oxidative stress since neutrophil infiltration is recognized as a rich source of ROS during the wound inflammatory phase (18).

Both PE and SIM have been reported to exert anti-inflammatory effects. Nain *et al* (25) proposed that the active components, fisetin and gallic acid, in PE extract may be responsible for reducing IL-6 protein levels in a mouse macrophage model. Furthermore, SIM-induced downregulation of IL-6 protein expression has been observed in both animal models and clinical patients (26,27). In the present study, combined treatment with PE and SIM exhibited a better effect on downregulating IL-6 protein levels in wound tissue compared with single treatment with PE or SIM on days 4 and 7; however, this was not significant. Moreover, in the present study, on days 4 and 7 post-wounding, there was a strong positive correlation (r=0.754; P=0.001) between the IL-6 protein levels and the number of infiltrating neutrophils.

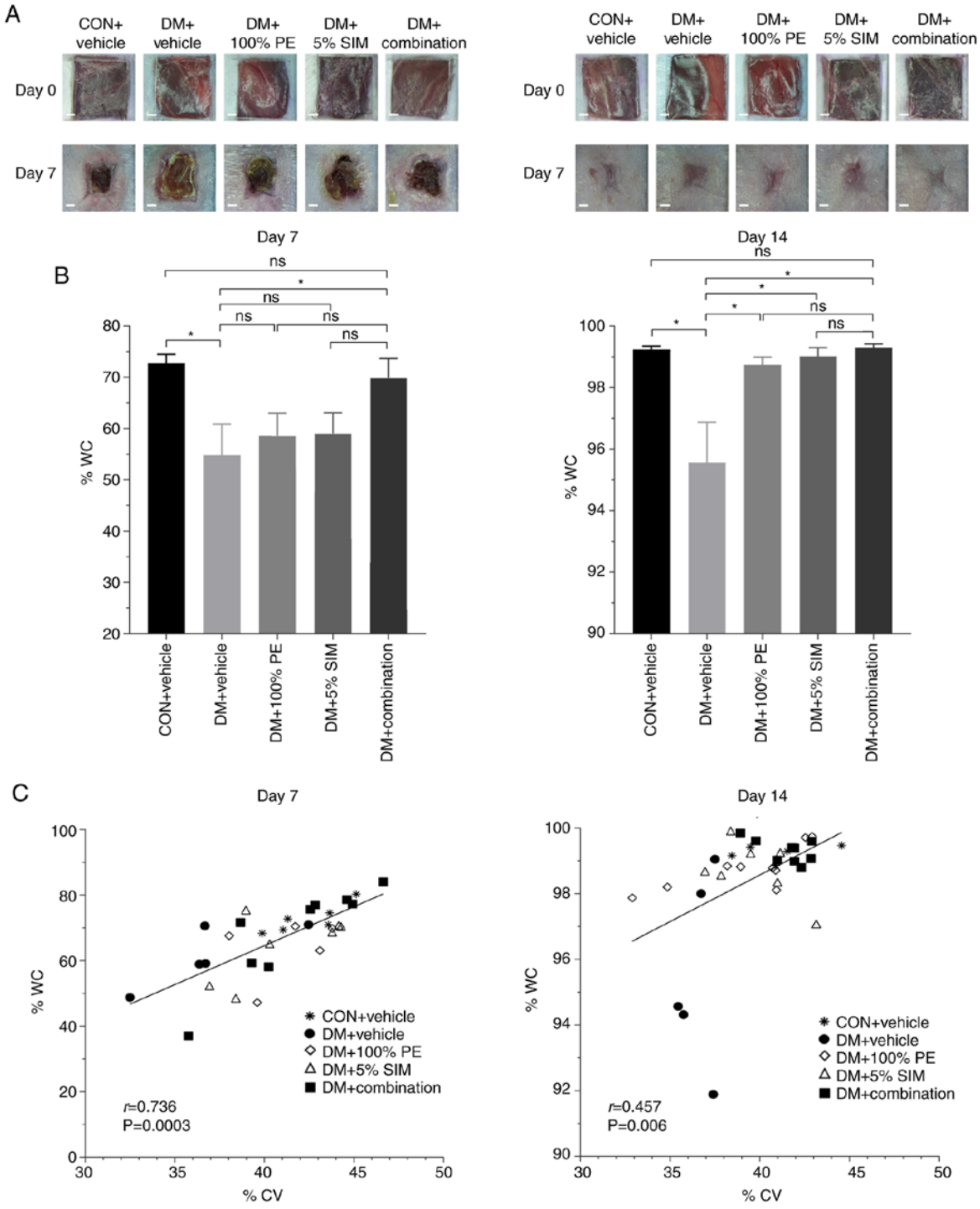

Figure 6. Effects of topical application of combined PE and SIM cream on %WC. (A) Images of wound area (scale bar, 1 mm). (B) %WC in the groups on days 7 and 14 post-wounding. Data are presented as the mean ± SEM. *P<0.05. (C) Pearson's correlation analysis between %CV and %WC in the five groups on days 7 and 14. Number of animals in each group, 4-5; number of wounds in each group, 4-9. %CV, percentage of capillary vascularity; %WC, percentage of wound closure; CON, control; DM, diabetes mellitus; ns, not significant; PE, *Phyllanthus emblica* Linn.; SIM, simvastatin.

In the present study, the results showed that topical application of combined PE and SIM significantly enhanced VEGF protein levels on day 7. In the DM + Combination group, %CV on days 7 and 14 were significantly improved compared with those in the DM + Vehicle group. In addition, there was a positive correlation (r=0.635; P=0.003) detected between VEGF levels and %CV on day 7. In the wound-healing process, PE has been reported to increase wound healing by increasing angiogenesis and VEGF protein levels in an *in vitro* model and in mouse gastric ulcers (12,28). Furthermore, the gallic acid-enriched fraction of PE has been shown to improve indomethacin-induced gastric ulcer healing via an arginine catabolism-related endothelial NOS (eNOS)-dependent pathway while promoting VEGF expression (28). SIM can also enhance wound healing through angiogenesis and VEGF mRNA levels in rats and diabetic mice (5-6,29). In addition, SIM has been reported to enhance eNOS mRNA expression and protein levels in cultured endothelial cells preconditioned

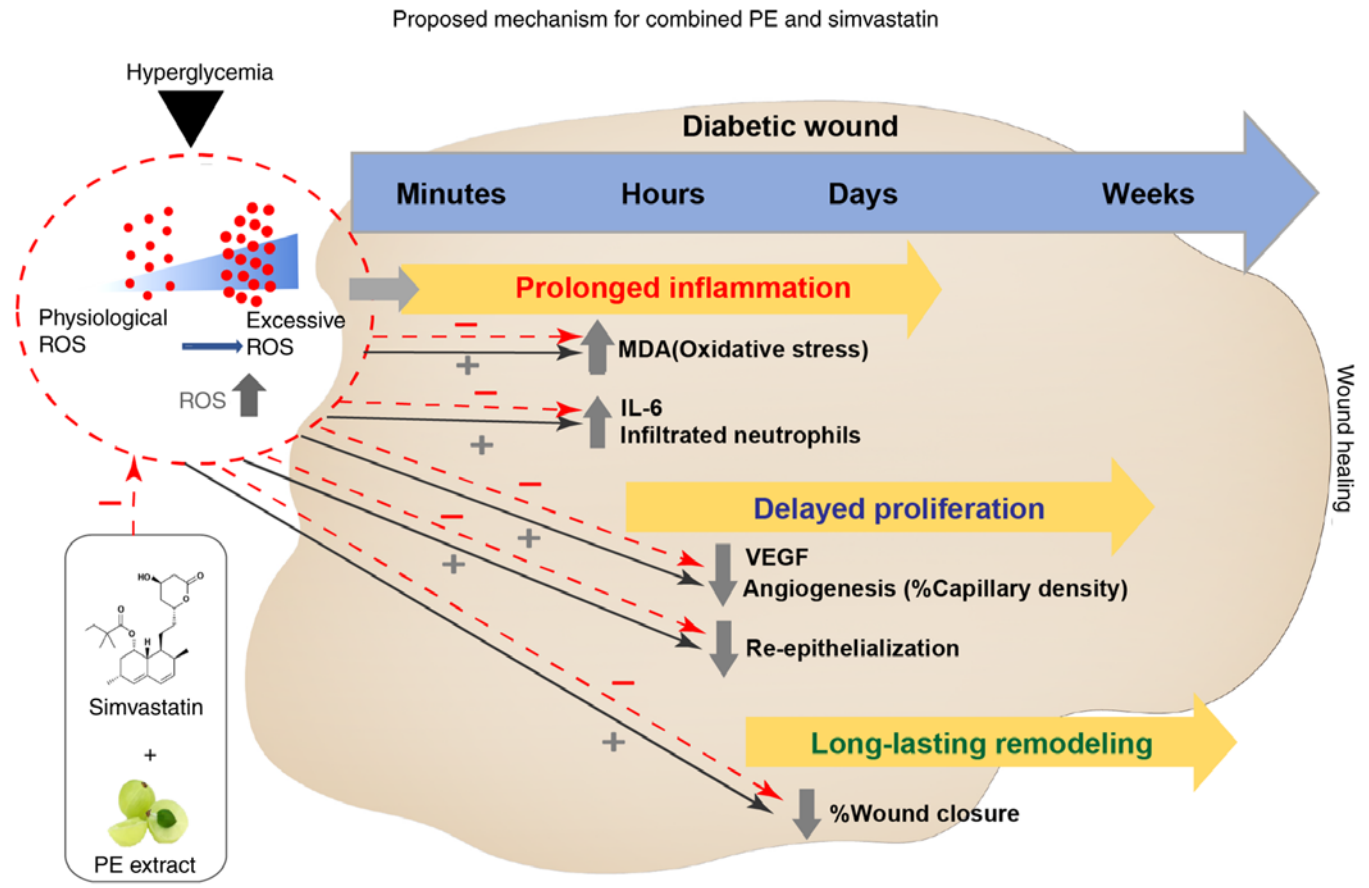

Figure 7. Proposed mechanism of the effect of combined PE and simvastatin on promoting diabetic wound healing. MDA, malondialdehyde; PE, *Phyllanthus emblica* Linn; ROS, reactive oxygen species.

with a steady laminar flow (30). The upregulation of VEGF protein levels may occur through the eNOS/NO pathway; therefore, modulating eNOS activity may enhance angiogenesis in diabetic wound healing.

In the present study, the DM + 5% SIM and DM + Combination groups exhibited a slight promoting effect on RE on day 4; however, there was no significant difference in %RE when compared with the DM + Vehicle group on days 7 and 14 post-wounding. The possible pleiotropic effects of SIM on wound healing may explain the increased RE (5-8). RE is one of the major features that indicates wound healing is successful and the migration of keratinocytes at the wound edge across the wound gap starts the RE process (31). In the present study, %RE was used to determine the degree of new epithelial tissue growing from the edge of the wound.

The present study revealed positive correlations between %CV and %WC on days 7 and 14. These findings indicated that improving angiogenesis may be beneficial to diabetic wound healing. The present findings demonstrated that the combination cream successfully improved diabetic wound healing associated with increased angiogenesis. Moreover, the results of %CV also confirm that the combination cream could enhance wound angiogenesis in diabetic mice comparatively to the spontaneous wound healing process in the control non-diabetic mice.

Notably, SIM has been reported to exert lipid-lowering-independent effects and a pleiotropic effect on wound healing. The pleiotropic effects of SIM are explained well through effective reduction of inflammation, and upregulation of VEGF production, angiogenesis and RE in wound healing (5-8).

Based on the present findings, both PE and SIM had wound healing promoting effects, possibly mediated through their antioxidant and anti-inflammatory properties. PE extract contains polyphenolic compounds, such as gallic acid and ellagic acid, which have been reported to exert antioxidant, anti-inflammatory and wound-healing effects (17,32-35). In previous studies, the wound-healing property of PE extract was revealed to be most likely due to the free radical-scavenging ability of its polyphenols (32-35). Ascorbic acid, emblicanin A, emblicanin B and low molecular weight tannins from PE have been shown to exhibit antioxidant activity at the wound site in dermal wound healing by restoring tissue ascorbic acid, α-tocopherol, SOD, CAT and GPx activities (13,14). The antioxidant and anti-inflammatory properties of statins, which are independent of their lipid-lowering effects, could be partly explained by modulation of the Nrf2/HO-1 pathway. Nrf2 and other proteins involved in the Nrf2/HO-1 signaling pathway have a crucial role in cellular responses to oxidative stress (23). To the best of our knowledge, there is no research addressing the direct effect of PE on the ERK1/2 pathway in wound inflammation. However, the Nrf2 pathway-activating mechanisms of PE have been reported to be associated with the roles of ERK and p38MAPK in directly phosphorylating Nrf2 and promoting Nrf2 nuclear import to increase its nuclear accumulation (24).

The promising effects of the combination of the multi-chemical-containing plant PE and the single structure chemical SIM on diabetic wound healing may offer targets for compounds that effect amelioration of oxidative stress, prolonged inflammation and other impaired healing processes.

Notably, wound healing is a complex process that includes three overlapping phases, as shown in Fig. 7 however, there are a number of transcription factors and signalling pathways associated with cutaneous wound healing (24,27-31). A limitation of the present study is the lack of investigation of all signaling parameters involved in the potential mechanisms; these require further study and verification in the future.

Based on the present findings, the proposed mechanism of topical application of PE and SIM is shown in Fig. 7. The combined effect of PE and SIM on wound healing in diabetic mice may be associated with a reduction in oxidative stress and inflammation, thus leading to improved angiogenesis and wound closure.

In conclusion, to the best of our knowledge, the present study is the first *in vivo* evidence showing that topical application of combined PE and SIM may have beneficial effects on diabetic wound healing through a reduction in excessive oxidative stress and inflammation, and increased angiogenesis. The present study revealed the highly promising topical use of combined PE and SIM for the future treatment of diabetic wound healing.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CC and SP contributed to the conception and design of the study. TTL helped with the acquisition, analysis and interpretation of the data. SS helped in the data analysis and checking the statistical analysis. TTL performed the drafting and writing of the manuscript. TTL and SP confirm the authenticity of all the raw data. CC and SP gave final approval of the article. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental procedures and daily care were approved by the Animal Care and Use Ethics Committee, Faculty of Medicine, Chulalongkorn University, Thailand (IRB no. 007/2562).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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