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ORIGINAL ARTICLE

Wound healing activity of topical *Phaleria macrocarpa* extract in type 2 diabetic rats

Evy Sulistyoningrum[1], Rosmelia Rosmelia[1], Ika Fidiangingsih[1], Shindy Galuh Narantika[2], Neysa Nurprahutri Cleopatra,[2] and Fathimah Azzahra[2]

[1]Department of Histology, Faculty of Medicine, Islamic University of Indonesia, Sleman, Yogyakarta, Indonesia
[2]Faculty of Medicine, Islamic University of Indonesia, Sleman, Yogyakarta, Indonesia

evy.sulistyoningrum@uii.ac.id

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ABSTRACT

BACKGROUND
Hyperglycemia interrupts wound healing, causing persistent and non-healing wounds. *Phaleria macrocarpa* extract (PME) has anti-diabetic, anti-inflammatory, antimicrobial, and antioxidant properties. This study aimed to assess *P. macrocarpa* activity on skin wound healing in diabetic rats.

METHODS
An experimental study performed on 25 male Wistar rats. Ointments were prepared by adding vehicle (w/w) to PME at the desired concentration. Diabetes was induced by injecting rats with nicotinamide (NAD) 230 mg/kg and streptozotocin (STZ) 65 mg/kg. After hyperglycemia was confirmed, animals were randomly grouped into: i) normal rats, ii) diabetic rats; iii) diabetic rats + 2.5% ointment; iv) diabetic rats +5% ointment; and v) diabetic rats +10% ointment. Full-thickness skin wounds were induced on the dorsum and treatment was applied daily for 3 and 7 days, respectively. On days 4 and 8, wound closure was measured and animals were sacrificed for tissue samples. Wound healing was evaluated by measuring malondialdehyde (MDA) in tissue homogenates of the dermal wounds and analyzing histological changes by hematoxylin-eosin and Sirius-red staining.

RESULTS
PME 10% ointment improved MDA levels and wound closure of inflammatory and proliferation phases. In inflammatory phase, 5% and 10% ointment reduced inflammation severity compared with diabetic rat group ($p<0.05$). In proliferation phase, PME 10% ointment group had a higher wound histological score (characterized by epidermal regeneration, fibroblast count, granulation tissue, and angiogenesis), and higher collagen bundle density compared with untreated groups ($p<0.05$).

CONCLUSIONS
Topical *P. macrocarpa* improves inflammatory and proliferation phases of excision wound healing in type 2 diabetes.

Keywords: Diabetes mellitus, *Phaleria macrocarpa*, inflammation, proliferation, rats

INTRODUCTION

The International Diabetes Foundation (IDF) roughly calculated that the global prevalence of diabetes mellitus among adult people in 2017 was 451 million and will rise to 693 million by the year 2045.[1] Chronic hyperglycemia in diabetic patients can develop into serious complications such as foot ulcers. Diabetic foot ulcers are a serious condition that needs special awareness since they cause not only substantial clinical problems but also have psychological effects and are an economic burden to health systems globally.[2] Diabetic patients have a 25% higher risk to develop foot ulcers [3] and in 2010-2019, the number of amputations among diabetic patients increased significantly from 5,049 to 7,759 per year.[4] Worldwide prevalence of diabetic foot ulcer was 6.3% in 2016 [5] while the Global Burden of Disease (GBD) study revealed that in the same year about 131 million people worldwide (1.8% of the global population) suffered from complications in the lower extremities related to diabetes.[6]

Phaleria macrocarpa (Scheff.*)* Boerl (*Thymelaeaceae*) grows throughout the year and is widely distributed in Indonesia. The ripe fruit of *P. macrocarpa* has been reported to contain certain active components such as flavonoids, glycosides, and tannins. *P. macrocarpa* extract (PME) has antioxidant and antimicrobial activities.[7] Other studies reported that PME has antihyperglycemic activity and protects the pancreas in diabetic rats,[8] and improves renal complications of diabetes.[9] Another study reported the strong antibacterial activity of ethanolic extract of *P. macrocarpa* against *S. aureus* bacteria in vitro.[10] Also, ointments derived from PME were reported to have good physical stability and high antimicrobial potency against *S. aureus* in vivo, as identified by the disappearance of the suppuration and by subsequent healing of the wound.[11] Regarding wound treatment, simple preparations, such as ointments, are still the gold-standard vehicle for topical drug delivery. The efficacy of topical treatment is firmly connected to cutaneous conditions and the biochemical and physical properties of the active substance. Selecting the most suitable vehicle for topical preparations is essential to skin therapy because it can affect the appearance, performance, physical stability, and patient acceptance of the preparation.[12]

Several studies reported that topical *P. macrocarpa* fruit extract augments wound healing under normal conditions. Water-based gel treatment with *P. macrocarpa* fruit extract increased the superoxide dismutase (SOD) and catalase (CAT) activities in the healing wounds, thereby significantly decreasing malondialdehyde (MDA) level. Topical treatment with *P. macrocarpa* fruit extract showed a significant healing effect on excision wounds on the 15th day of wound healing.[13]

The wound healing process consists of many overlapping phases including hemostasis, inflammation, and proliferation, as well as the remodeling phase. Under normal conditions, these phases can be accomplished in a very short time, thereby restoring the normal function of the skin. Unfortunately, skin wound healing in diabetic patients requires longer treatment compared with nondiabetic patients due to persistent and chronic wounds.[14]

Many factors contribute to this condition such as peripheral neuropathy and ischemia which can be complicated by infections, resulting in chronic wound formation.[15] On the other hand, abnormal peripheral microvasculature, impaired connective tissue regeneration,[16] and oxidative stress caused by free radicals [17,18] also bring major contributions to delayed wound healing mechanisms, particularly in the inflammatory and proliferative phases. Abnormal and delayed inflammatory responses mainly in the form of abnormal neutrophil recruitment in the wound area of acute diabetes were markedly observed, thereby possibly increasing the risk of bacterial infection.[19] Thus, early intervention of diabetic wounds is critical to avoid bacterial infection and ameliorate the healing process in diabetic wounds. Our study aimed to examine the effect of PME ointment on excision skin wounds in the early crucial phases of wound healing, namely the inflammatian and proliferation phases, using rat models of diabetes induced by streptozotocin (STZ) and nicotinamide (NAD).

METHODS

Research design

This was an experimental laboratory study of post-test only control group design. The research was executed at the Integrated Research Laboratory, Universitas Islam Indonesia from February to June 2018.

Study subjects

We used 25 healthy male albino rats (*Rattus norvegicus* strain *Wistar*) aged 8-10 weeks (175 ± 25 g). The animals were obtained from the Integrated Research Laboratory, Universitas Islam Indonesia.

Sample size determination

The number of subjects was calculated according to research equation by Charan and Kantharia.[20] Animals were caged in typical laboratory conditions (12 hours of dark and light cycles, 22 ± 4°C temperature, and 50 ± 5% relative humidity) in the Integrated Research Laboratory, Universitas Islam Indonesia. Animals were given standard feed and tap water during acclimatization and intervention.

Preparation of *P. macrocarpa* extract ointment

Ripe fruits of *P. macrocarpa* (1,000 g) were collected from Merapi Herbal (Yogyakarta, Indonesia) (GPS coordinates -7°.37'41.176"S, 110°.25'30.38"E) and identified by the Laboratory of Plant Systematics, Faculty of Biology, Universitas Gadjah Mada (Register Number 01245/S.Tb/2018). The mesocarp was sliced, dried at 70°C, and then pulverized in a milling machine (Fritsch Universal Cutting Mill-Pulverisette 19, Germany). Ground material (855 g) was macerated with 5 liters of methanol for 24 hours and all filtrates from 3 x 24 h methanol extraction were gathered and then evaporated by rotary machine resulting in raw extract, which was kept in a refrigerator (4ºC) until needed. To obtain PME ointments, the crude extract was mixed with white vaseline and span 80 [sorbitan monooleate] (1:1) to yield PME concentrations of 2.5%, 5%, and 10%, respectively (w/w).

Intervention

To induce diabetic conditions, all animals were fasted for 12 h and then a single dose of nicotinamide (NAD) [Sigma Aldrich; USA] 230 mg/kg was injected intraperitoneally (i.p). After a 15-minute interval, an injection of 65 mg/kg streptozotocin (STZ) (Nacalai Tesque; Japan) was also given i.p. All chemicals were freshly dissolved in citrate buffer at pH 4.5. After three 24-hour intervals, blood glucose (BG) from the tail capillary was measured using a glucose meter (GlucoDr® AGM 2100; South Korea), and hyperglycemia was confirmed if the fasting BG level was above 130 mg/dL.[21] Prior to treatment, the rats were randomly assigned to five groups: (i) normal controls; (ii) diabetic controls; (iii) diabetic rats + 2.5% PME ; (iv) diabetic rats + 5% PME ; and (v) diabetic rats + 10% PME.

All animals were given ketamine 25 mg/kg i.p. (Sandoz; Canada) and shaved at 2 x 2 cm^2 dorsum area. Two full-thickness skin wounds (5 mm in diameter, 1 cm interval) were made on the shaved area by biopsy punch (Acu-Punch; USA) and remaining skin was excised until subcutaneous tissue was exposed. Proper disinfection of the skin area using 1% povidone-iodine and sterilization of the medical instruments were performed before wound excision. Topical treatments were applied once daily with a sterile cotton bud and then each wound was dressed with modern transparent film for 3 days (inflammatory phase). On day 4, the animals were sacrificed to obtain skin tissue. For the proliferation phase, treatments were applied for 7 days, and on day 8, animals were terminated for the same reason. During scheduled treatments, animals were subjected to well-being observations including wound condition, food and water intake, abnormal discharges, and body weight. All skin tissue was isolated for the next process.

Measurement

Wound assessment

Length and width of the wound was measured using graph paper, and total wound contraction was estimated by the formula:

$$(\%\ TWC) = [(A_0 - A_T)/ A_0] \times 100\%$$

Where TWC = total wound contraction, A_0 = wound area on day 0 and A_T = wound area at time T.[22]

Tissue collection

Before termination, animals were injected with pentobarbital sodium 100 mg/kg i.p. (MSD; New Jersey, USA). Sedated animals were then decapitated by guillotine.[23] Two areas of 1x 1 cm^2 wounded skin were dissected, one of which was then washed carefully with cold saline. The skin was then gently blotted between layers of Whartman filter paper and then weighed. The method for determining the MDA level was based on the previous method conducted by Abood et al.[13] and Saleh et al.[24] One piece of skin tissue (1 x 1 cm^2) was weighed, consisting of 48.5 mg tissue. The skin was then homogenized in a cold mixture of PBS and potassium phosphate 50 mM at pH 7.3 to obtain 10% concentration (w/v). The homogenate was then centrifuged at 3,000 rpm at

4°C for 20 minutes to obtain the supernatant, which was subjected to malondialdehyde (MDA) analysis.[25] About 200 µl of trichloroacetic acid (TCA) 20% and 400 µl thiobarbituric acid (TBA) 0.67% were added to 400 µL supernatant, mixed thoroughly, and then warmed up at 95°C for 1 hour and cooled down to room temperature. Sample adsorbance was measured at 532 nm, the result being expressed in nmol/g.

Histological preparation and examination

Skin tissue (1 x 1 cm^2) was fixed in buffered formalin 10% for 24 h and then dehydrated in serial alcohol solutions. The tissue was then embedded in a hot paraffin block, sectioned at 5-mm thickness, and stained with hematoxylin-eosin (HE) to evaluate morphological changes in the inflammatory and proliferation phases. Regarding the inflammatory phase, the severity of inflammation and inflammatory infiltrates was scored as follows: 0 (no observed inflammatory reaction), 1 (giant cells, lymphocytes, and plasma cells present), 2 (neutrophils and presence of cells in score 1), and 3 (formation of micro-abscess).[14] Representative photomicrographs were made with a CX22 microscope (Olympus, Japan) provided with Optilab camera (Miconos, Indonesia). Regarding wound score in proliferation phase, wound healing was scored into three or four scales according to Altavilla histologic score of wounds of 3 components: epidermal and dermal regeneration (scored 1-3; score 1 = minimal organization of epidermis and dermis, score 2= moderate organization of epidermis and dermis, score 3 = complete remodeling of epidermis and dermis), granulation tissue thickness (scored 1-4; 1 = thin granular layer, 2 = moderate granulation layer, 3 = thick granulation layer, and 4 = very thick granulation layer); and angiogenesis (scored 1-4; score 1 = disturbed angiogenesis (1-2 vessels/site) delineated by severe edema and hemorrhage, and infrequent congestion and thrombosis; score 2 = a small number of newly-formed capillaries (3-6/site), moderate edema and hemorrhage, occasional congestion and intervascular deposition of fibrin, absence of thrombosis, score 3 = moderate number of capillaries (7-10/site), moderate perivascular and interstitial edema and congestion, absence of thrombosis and hemorrhage; score 4 = numerous newly formed and well-developed capillaries (>10/site) which are vertically disposed toward the epithelium and at the wound margins, slight perivascular edema.[26, 27]

The proliferation phase tissue sections were also stained with Sirius Red to quantify collagen bundle density. The dermal papilla area in a circle of 3 mm radius of the wound occupied by collagen bundles, was compared with the total area and multiplied by 100%. The morphometric analysis was performed in 3 fields of view at 400× magnification using Image J. The examinations were performed by two observers.

Statistical analysis

Statistical analyses were executed with IBM SPSS Statistics version 26.0 (SPSS Inc.; USA). Normality of data distribution was determined by the Shapiro-Wilk test. One-way ANOVA and Tukey High Significant Difference (HSD) posthoc test were used for multiple comparison using p-value <0.05.

Ethical clearance

Ethical approval for our study was received from the Health Research Ethics Committee, Faculty of Medicine, Universitas Islam Indonesia (Approval letter number 24/Ka.Kom.Et/70/KE/VIII/2017).

RESULTS

Induction of type 2 DM by intraperitoneal injection of 65 mg/kg STZ and 230 mg/kg NAD in groups II-V significantly elevated fasting glucose level as compared with group I. The baseline glucose level between diabetic rat groups (II-V) were not significantly different ($p>0.05$, ANOVA test, data not shown). The tissue MDA levels were measured on the 4^{th} and 8^{th} days. Untreated diabetic rats had the highest MDA level, which was significantly lower in all three PME-treated groups ($p<0.05$, Figure 1). Interestingly, the MDA level of rats treated with PME 10% did not differ from that of the healthy controls ($p> 0.05$), while the other concentrations of PME exhibited higher MDA levels if compared with the healthy controls on both 4^{th} and 8^{th} days.

Wound closure assessment

During inflammatory (4^{th} day) and proliferation (8^{th} day) phases, the wound areas were measured and wound contraction was calculated (Figure 2). Wound closure with PME 10% on day 4 was better than in the diabetic rat group, while with the other PME doses, wound closure was not significantly different. On day 8, the PME 5% and 10% treated groups had

significantly higher wound contraction compared with the untreated diabetic rat group ($p<0.05$). On day 8, the PME 10 % treated group showed almost complete recovery of the skin, and the wound contraction did not differ from that of the normal healthy rat group ($p>0.05$).

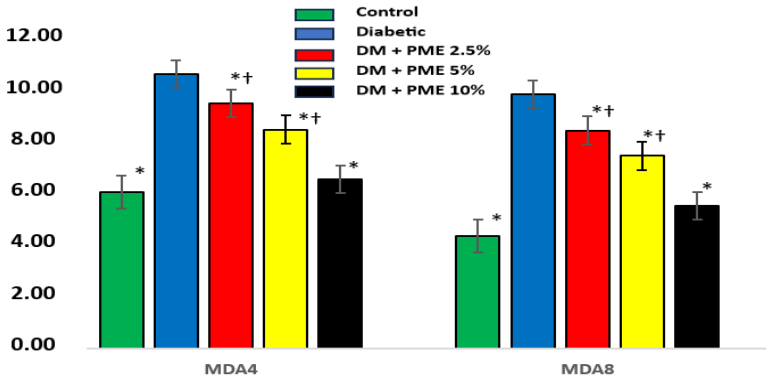

Figure 1. MDA level (nmol/g) on days 4 and 8.
Control: healthy rats, DM: diabetic rat group, DM + PME 2.5%: diabetic rats + PME ointment 2.5%, DM + PME 5%: diabetic rats + PME ointment 5%, DM + PME 10%: diabetic rats + PME ointment 10%, n = 5, values are mean ± standard deviation, *p < 0.05 compared with diabetic rat group, †p < 0.05 compared with healthy control group, from ANOVA and posthoc Tukey HSD analyses. X axis represents group comparison on representative variables, while Y axis represents the level of tissue MDA.

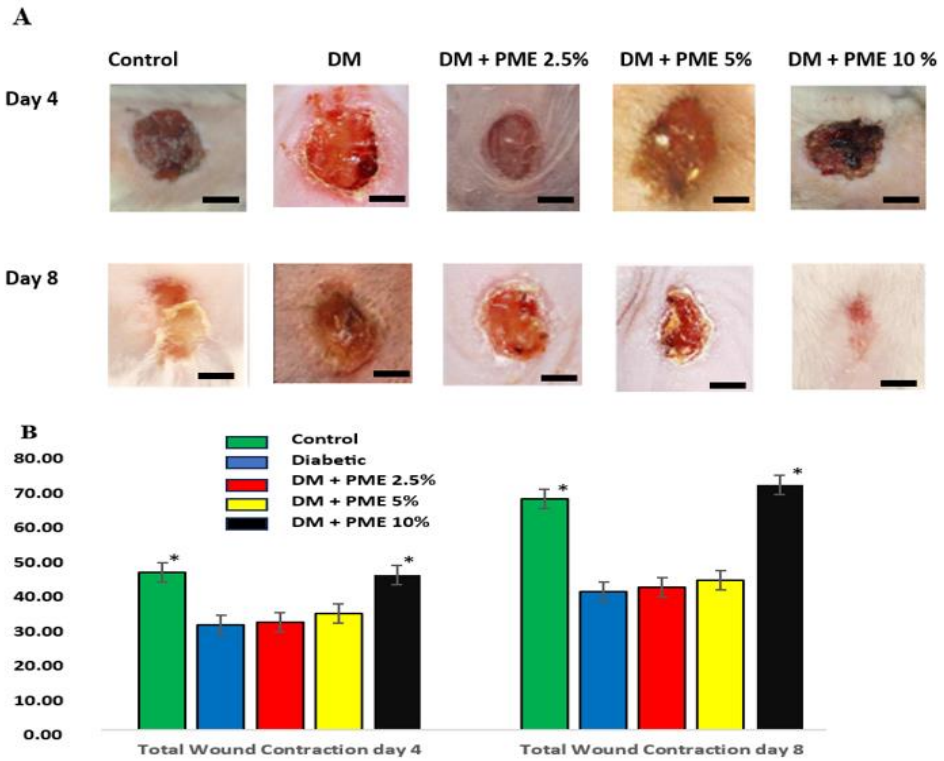

Figure 2 A. Representative picture of wound closure on days 4 and 8, B. Total wound contraction (%) on days 4 and 8
Control: healthy rats, DM: diabetic rat group, DM + PME 2.5%: diabetic rats + PME ointment 2.5%, DM + PME 5%: diabetic rats + PME ointment 5%, DM + PME 10%: diabetic rats + PME ointment 10%, n = 5, values are mean ± standard deviation, *p<0.05 compared with diabetic rat group from ANOVA and posthoc Tukey HSD analyses, bar indicated 2 mm. X axis represents group comparison on representative variables, while Y axis represents the percentage of wound closure

Histological examination

Photomicrographs of the slides stained with HE are shown in Figure 3. On day 4, histological analysis of the wound tissues showed that the untreated diabetic rat group had the highest inflammation score (Figure 4), characterized by blood vessel congestion and inflammatory cell infiltrates (shown in Figure 3F) in the perivascular or interstitial areas. Wound healing parameters of the proliferation phase were scored according to the modified Altavilla score consisting of 3 parameters: re-epithelization, thickness of granulation tissue, and angiogenesis.

The normal healthy rat group had the highest wound healing score, and the most recognizable characteristics were the re-epithelization and the granulation tissue (Figure 3A). The healthy control group showed a thick epidermis with visible epidermal layers and a deep dermal-epidermal junction. The dermal compartment was also occupied by connective tissue, with thick bundles of fibers and newly-formed capillaries. The untreated diabetic rat group had the lowest score, with almost no epidermal layer and minimal granulation tissue. The diabetic rat groups receiving PME 2.5% and 5% had varying degrees of epithelization, fibroblast cell counts, and some granulation tissue thickness. Only the PME 10% group showed improvement in wound healing similar to the normal rat control group, and the score was higher than in the untreated diabetic rat group ($p<0.05$, Figure 4). The dermal compartment of the wounds treated with PME 10% also showed dermal regeneration of connective tissue components including connective tissue fibers and blood vessels.

Collagen bundle density assessment

The normal rat control group was comprised of high-density collagen bundles, that appeared as red, thick, wavy fibers with Sirius Red staining, while the untreated diabetic rat group had the lowest collagen density. The diabetic rats group receiving PME showed enhancement in collagen bundle density compared with the untreated diabetic group ($p<0.05$) but among the PME-treated groups, the group receiving 10% PME had the highest collagen density similar to the controls (Figure 5). In the photomicrographs taken at higher magnification, we can also find hair follicles in the PME-treated group, representing dermal remodeling.

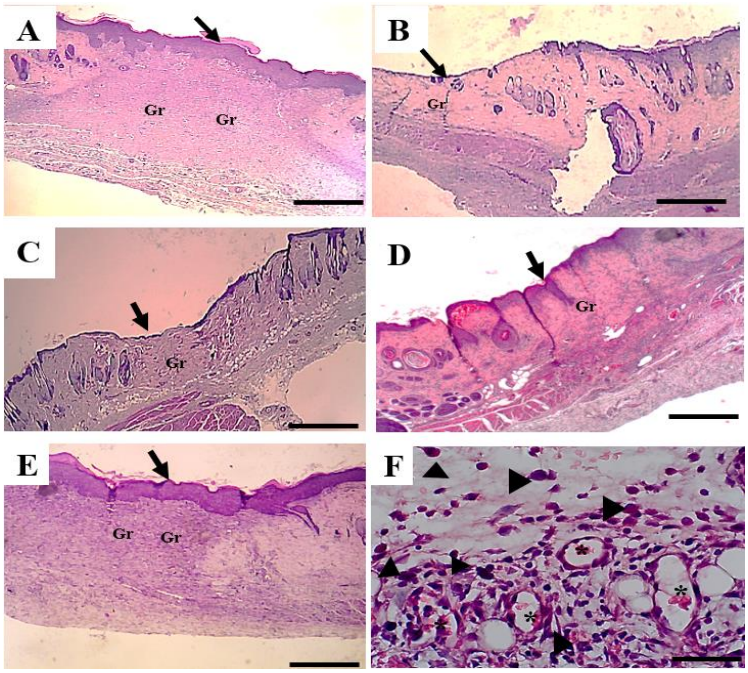

Figure 3. Photomicrographs of the wound healing process
(A). shows healthy controls exhibiting epithelization up to the corneal layer (arrow), thick granulation tissue (Gr), Diabetic rat group showing no epithelization, minimal granulation tissue (B), DM + PME 2.5% and 5% showing various levels of epithelization and moderate granulation tissue (C and D), while the 10 % PME (E) shows epithelization and granulation tissue similar to healthy controls. (F): higher magnification showing dilated blood vessels (asterisks) and inflammatory cell infiltrates (arrowheads). Hematoxylin eosin staining, A-E 100x magnification, F: 400x magnification

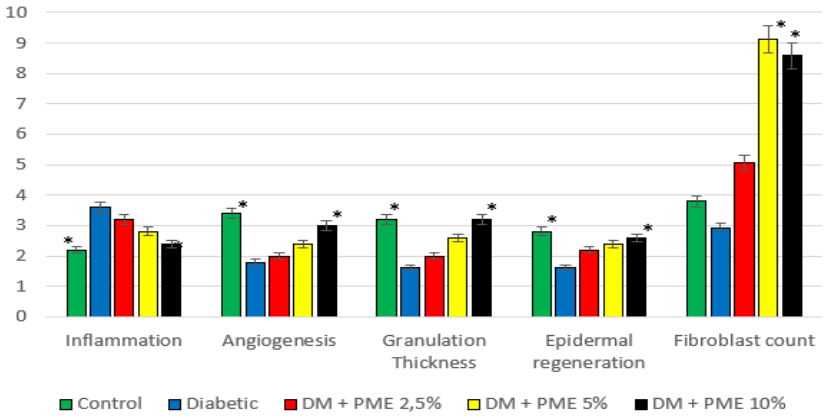

Figure 4. Histological parameters of wound healing on days 4 and 8
The parameters of inflammation severity, angiogenesis, granulation, and dermal-epidermal organization were scored, values are presented as the mean ± standard deviation. The inflammation score was performed on day 4 sections only (inflammatory phase), while the angiogenesis, granulation thickness, epidermal regeneration and fibroblast count were performed on the day 8 section (proliferation phase), *p<0.05 compared with diabetic rat group from ANOVA and posthoc Tukey HSD analyses. Control: healthy rats, DM: diabetic rat group, DM + PME 2.5%: diabetic rats + PME ointment 2.5%, DM + PME 5%: diabetic rats + PME ointment 5%, DM + PME 10%: diabetic rats + PME ointment 10%. X axis represents group comparison on representative variables, while Y axis represents the score of histological parameters.

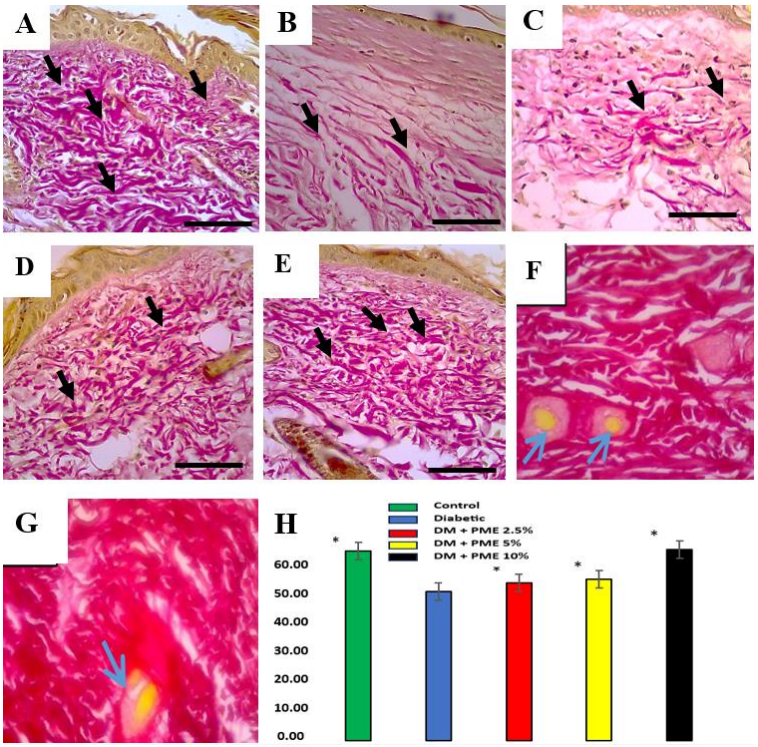

Figure 5. Photomicrographs of dermal collagen and comparative collagen density (%)
Various densities of collagen bundles appear as red-colored thick bundles (black arrows) in (A) healthy controls, with many bundles, high density, (B) diabetic rat group showing scanty collagen bundles, variably density of collagen bundles in DM + PME 2.5% and DM + PME 5% (C and D), while DM + PME 10% (E) shows dense collagen bundle similar to the healthy group. F and G: hair follicles (blue arrows), H: comparative values in % (presented as mean ± standard deviation), magnification 100X (A-E), 400X (F-G), Sirius Red staining, *p<0.05 compared with diabetic rat group, ANOVA and posthoc Tukey HSD analyses. X axis represents group comparison on representative variables, while Y axis represents the percentage of collagen bundle density

DISCUSSION

We have established the role of topical application of PME in rats with STZ-NAD-induced type 2 diabetes. The experiment demonstrated reduced levels of MDA oxidative markers in both inflammatory and proliferation phases. Further, we have examined the histologic sections to observe the detailed morphological changes of both inflammatory and proliferation phases and reported that the PME-treated diabetic rat groups (especially for PME 10%) showed reduced inflammation severity and increased histologic score of wound healing of epidermal organization, angiogenesis, and granulation tissue and also increased collagen bundle density, indicating a potential wound healing effect of PME. It is well-known that diabetes significantly impairs wound healing through several mechanisms, such as abnormalities in extracellular matrix (ECM) differentiation and defective angiogenesis. The wound healing process aims to restore the structural and functional integrity of the tissues as identitfied by the accomplishment of consecutive phases in a specific time frame, and in normal conditions wound healing is highly effective and results in successful wound closure in time.[28] Consistent with our present study, several studies have reported the wound healing activities of various plants containing the same constituents of *P. macrocarpa*. [24,29,30] A study by de Moura et al. [29]reported that phenolic compounds and tannins contained in 4% *Maytenus ilicifolia* extract were effective in promoting wound closure and had anti-inflammatory effects. This conclusion was drawn from the elevated collagen levels in the wounds and matrix metalloproteinase-9 (MMP9) activities. Similar to this result, both in vitro and in vivo studies reported that polyphenols from various plant species intensify the wound healing process via anti-inflammatory and antioxidant activities, and also promote the establishment of granulation tissue and re-epithelization.[30] Saleh et al. [24] also reported that natural substances in topically applied indigo augment wound healing via reduction of dermal inflammation and increased antioxidant enzymes.

Oxidative stress plays a fundamental role in the disturbed wound healing mechanism of diabetes. The redox imbalance because of excessive generation of free radicals and reduction of the antioxidant system, is detrimental to the wound healing process.[31] Semiqualitative analyses of PME showed several bioactive compounds that act as antioxidants such as flavonoids, alkaloids, and phenols. A previous study reported that flavonoids and phenols behave as metal ion chelators and reduce reactive oxygen species (ROS) formation via the stabilization of hydrogen atoms from the hydroxyl group.[32] Flavonoid compounds in *P. macrocarpa* serve as antioxidants that may counteract diabetes-related free radicals, including advanced glycation end products (AGEs). Flavonoids also can act as ROS scavengers via suppression of mitogen-activated protein kinases (MAPK) signaling pathways, comprising extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase, and further will trigger the nuclear-factor kappa-B (NF-κB) pathway, leading to activation of transforming growth factor-β (TGF-β). The latter molecule is known to act as an important regulator of collagen stability via stimulation of procollagens I and III and down-regulation of MMP-1 transcription.[33] Our research also highlighted the antioxidant activities of topical PME in both inflammatory and proliferation phases, as was confirmed by decreased lipid peroxidation, indicated by a low skin tissue MDA level. Our result is in accordance with other studies reporting the antioxidant potency of PME in both *in vitro* [7,34,35] and in various *in vivo* conditions, including wounds.[13]

Topical administration of PME reduces inflammation severity on day 4, establishing the anti-inflammatory effect of PME. Diabetic conditions, mainly uncontrolled hyperglycemia, results in cellular deterioration that interferes with the wound healing process in all phases. Diabetic patients have impaired leucocyte functions, such as reduced activities of neutrophil chemotactic, phagocytic, as well as microbicidal capacities. In addition, fibroblasts in diabetic patients also have a decreased response to the growth factors released during wound healing processes, which increases the risk for delayed wound healing. Peripheral neuropathy in diabetic patients also reduces the function of neuropeptides that are elaborated in wound healing mostly in the inflammatory phase, such as chemoattractant activity, vascular permeability, leucocyte adhesion, cytokine expression, and endothelial cell proliferation.[36]

The prolonged inflammatory phase has been associated with increased inflammatory cell infiltration and increased protease activity, which has damaging effects. As previously stated, in

diabetes, the expression of growth factors accountable for tissue regeneration such as platelet-derived growth factor (PDGF) and TGF-β is reduced. It is also reported that in diabetes, the MMPs, i.e. major enzymes responsible for remodeling and degrading of extracellular matrix (ECM) to form mature tissue, are activated and the excessive effect of these MMPs results in impairment of dermal fibroblastic and keratinocytic functions.[36]

While the untreated diabetic rat group showed severe inflammation, the diabetic rat group receiving PME treatment showed reduced severity of inflammation. This result agrees with previous reports that oral treatment with PME reduces serum tumor necrosis factor-α (TNF-α) levels in a rat model.[13] Tumor necrosis factor-α is a common pro-inflammatory cytokine responsible for the inflammatory response and leucocyte recruitment.[28] On the contrary, TGF-β levels were increased in wounded rats treated with oral *P. macrocarpa*,[13] which emphasizes the inflammatory activity of *P. macrocarpa*.

Phytochemical screenings of methanolic *P. macrocarpa* extracts recognized flavonoids, saponins, tannins, and phenolics in the contents. Preceding studies also highlighted the anti-inflammatory properties of PME in various animal models,[37] including wounds [36] and inflammatory bowel disease.[38] Other studies also reported the anti-inflammatory activity of predimenol, an active substance derived from *P. macrocarpa* which acts via inhibition of the production of nitric oxide (NO), TNF-α, interleukin-1β (IL-1β), IL-2, and IL-6.[39] Results of wound model treatments disclose that saponins have a dominant role in wound healing, by supplementing cellular mediators elaborated in wound healing and generating a microenvironment that encourages tissue repair and accomodates tissue remodeling, which in turn increases wound healing.[40]

Our result reported that the untreated diabetic rat group had the lowest wound healing score and collagen density in the proliferation phase. Diabetes also affects the proliferative phase via impairment of fibroblast signaling which can be followed by disturbed formation of granulation tissue and fibrotic ECM. These conditions will further lead to impeding keratinocyte migration and retarding re-epithelialization. Meanwhile, elevated MMPs and ROS in diabetes will also lead to decreased angiogenesis and poor vascularization due to ECM alternation and reduced sensitivity to vascular endothelial growth factor (VEGF).[36] The diabetic rat groups receiving PME showed varied epithelization and granulation tissue thickness, and the optimum improvement was achieved at 10% PME concentration. Re-epithelization requires the role of keratinocytes and fibroblasts. The latter will proliferate and produce ECM, which bridges the wound margins, whereupon the keratinocytes will coordinate with other integrin molecules and cytoskeletal components to clear a migration path along the interface between the fibrin clot and the underlying dermis. Following the event, keratinocytes will proliferate at the wound margin and thus re-establishing the layered epidermis.[41] The re-epithelization process also involves various cytokines and growth factors, including epidermal growth factor (EGF) and TGF-β.[42] The further remodeling of the dermal compartment resumes after the epithelial barrier is established.[43] In our research, remodeling of dermal components and hair follicle formation has been also established in PME-treated groups. Diabetic conditions can lead to cellular debilitation, that occurs not only in the inflammatory phase but also expands to re-epithelialization and dermal remodeling. Keratinocytes at the non-healing diabetic wound edge show delayed migration and prevent healing in mice. Epidermis at the edge of the diabetic wound also shows the abnormality of several cell cycles, diminished growth factor receptor signaling, and absence of hair follicles.[45]

The limitation of our study is the small number of biochemical and histologic parameters and the fact that all biochemical and histopathological analyses were performed only for the early phases of wound healing. However, this research showed the possibility of using *P.macrocarpa* in the treatment of the diabetic wound. Future research involving more comprehensive parameters is required to furnish real and accurate assertions regarding the impact of PME on the wound healing mechanism.

CONCLUSION

This study demonstrated that topical application of 10% *P. macrocarpa* extract can promote wound healing in rats with STZ and NAD-induced diabetes via reduction of tissue oxidative stress level and improvement of histologic scores in inflammatory and proliferation phases characterized by re-

epithelization, abundant fibroblasts, granulation tissue thickness, angiogenesis, and also dermal collagen formation.

Conflict of Interest

None to declare.

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Author Contributions

ES: conceived and designed the experiments; ES, FA, and NNC conducted the experiments and analyzed the experimental data; ES, FA, NNC, RR, and IF contributed to the manuscript draft. All authors contributed to revisions and approved the final version.

Data Availability Statement

Requests for the original data presented in this study can be directed to the corresponding author.

Declaration of Use of AI in Scientific Writing

During the preparation of this work, the authors used Grammarly to improve and correct any typos or missing spelling. After using this tool/service, the authors reviewed and edited the content as needed, for the publication of which they take full responsibility.

REFERENCES

1. Cho HN, Shaw JE, Karuranga S, et al. IDF diabetes atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. Diabetes Res Clin Pract 2018;138:271–81. DOI: 10.1016/j.diabres.2018.02.023.
2. Lo ZJ, Surendra NK, Saxena A, Car J. Clinical and economic burden of diabetic foot ulcers: a 5-year longitudinal multi-ethnic cohort study from the tropics. Int Wound J 2021;18:375–86. DOI: 10.1111/iwj.13540.
3. Brownrigg JRW, Griffin M, Hughes CO, et al. Influence of foot ulceration on cause-specific mortality in patients with diabetes mellitus. J Vasc Surg 2014;60:982-6.e3. doi: 10.1016/j.jvs.2014.04.052.
4. Walicka M, Raczyńska M, Marcinkowska K, et al. Amputations of lower limb in subjects with diabetes mellitus: reasons and 30-day mortality. J Diabetes Res 2021;2021:1–8. DOI: 10.1155/2021/8866126.
5. Zhang P, Lu J, Jing Y, Tang S, Zhu D, Bi Y. Global epidemiology of diabetic foot ulceration: a systematic review and meta-analysis. Ann Med 2017;49:106–16. DOI: 10.1080/07853890.2016.1231932.
6. Zhang Y, Lazzarini PA, McPhail SM, van Netten JJ, Armstrong DG, Pacella RE. Global disability burdens of diabetes-related lower-extremity complications in 1990 and 2016. Diabetes Care 2020;43:964–74. DOI: 10.2337/dc19-1614.
7. Andrean D, Prasetyo S, Kristijarti A, Hudaya T. The extraction and activity test of bioactive compounds in *Phaleria macrocarpa* as antioxidants. Procedia Chem 2014;9:94–101. https://doi.org/10.1016/j.proche.2014.05.012.
8. Ahmad R, Khairul Nizam Mazlan M, Firdaus Abdul Aziz A, Mohd Gazzali A, Amir Rawa MS, Wahab HA. *Phaleria macrocarpa* (Scheff.) Boerl.: an updated review of pharmacological effects, toxicity studies, and separation techniques. Saudi Pharm J 2023;31:874–88. https://doi.org/10.1016/j.jsps.2023.04.006.
9. Sulistyoningrum E, Setiawati S. *Phaleria macrocarpa* reduces glomerular growth factor expression in alloxan-induced diabetic rats. Univ Med 2013;32:71-9. DOI: https://doi.org/10.18051/UnivMed.2013.v32.71-79.
10. Rizki M, Harahap U, Sitorus P. Phytochemical screening of *Phaleria Macrocarpa* (Scheff.) Boerl.) and antibacterial activity test of ethanol extract against *Staphylococcus Aureus* bacteria. Int J Sci 2023;4:422-7. DOI: https://doi.org/10.46729/ijstm.v4i2.781.
11. Widyapranata R. Ointment base for ethanolic seed extract of mahkota dewa (*Phaleria macrocarpa*) as an anti-bacterial against *Staphylococcus aureus* ATCC 25923. J Farmasi Indonesia 2011;8:7–10. DOI: https://doi.org/10.31001/jfi.v8i2.42.
12. Choy YB, Prausnitz MR. The rule of five for non-oral routes of drug delivery: ophthalmic, inhalation and transdermal. Pharm Res 2011; 943–8. DOI: 10.1007/s11095-010-0292-6.
13. Abood WN, Al-Henhena NA, Abood AN, et al. Wound-healing potential of the fruit extract of *Phaleria macrocarpa.* Bosn J Basic Med Sci 2015;15:25–30. https://doi.org/10.17305%2Fbjbms.2015.39.
14. Özay Y, Güzel S, Yumrutaş Ö, et al. Wound healing effect of kaempferol in diabetic and nondiabetic rats. J Surg Res 2019;233:284–96. DOI: 10.1016/j.jss.2018.08.009.

15. Edmonds M, Manu C, Vas P. The current burden of diabetic foot disease. J Clin Orthop Trauma 2021;17:88–93. DOI: 10.1016/j.jcot.2021.01.017.
16. Frykberg RG, Banks J. Challenges in the treatment of chronic wounds. Adv Wound Care (New Rochelle) 2015;4:560–82. DOI: 10.1089/wound.2015.0635.
17. Ullah A, Khan A, Khan I. Diabetes mellitus and oxidative stress—a concise review. Saudi Pharm J 2016;24:547–53. DOI: 10.1016/j.jsps.2015.03.013
18. Deng L, Du C, Song P, et al. The role of oxidative stress and antioxidants in diabetic wound healing. Oxid Med Cell Longev 2021;2021:8852759. https://doi.org/10.1155/2021/8852759.
19. Komesu MC, Tanga MB, Buttros KR, Nakao C. Effects of acute diabetes on rat cutaneous wound healing. Pathophysiology 2011;11:63–7. https://doi.org/10.1016/j.pathophys.2004.02.002.
20. Charan J, Kantharia N. How to calculate sample size in animal studies? J Pharmacol Pharmacother 2013;4:303–6. DOI: 10.4103/0976-500X.119726.
21. Ghasemi A, Jeddi S. Streptozotocin as a tool for induction of rat models of diabetes: a practical guide. EXCLI J 2023;22:274–94. DOI: 10.17179/excli2022-5720.
22. Chen L, Mirza R, Kwon Y, DiPietro LA, Koh TJ. The murine excisional wound model: Contraction revisited. Wound Repair Regen 2015;23:874–7. https://doi.org/10.1111/wrr.12338.
23. Hidayat R, Wulandari P. Euthanasia procedure of animal model in biomedical research. J Biomed Translational Res 2021;5:540–4. https://doi.org/10.32539/bsm.v5i6.310.
24. Saleh MA, Shabaan AA, May M, Ali YM. Topical application of indigo-plant leaves extract enhances healing of skin lesion in an excision wound model in rats. J Appl Biomed 2022;20:124-9. DOI: 10.32725/jab.2022.014.
25. Alamoudi AA, Alharbi AS, Abdel-Naim AB, et al. Novel nanoconjugate of apamin and ceftriaxone for management of diabetic wounds. Life 2022;12:1096–120. DOI: 10.3390/life12071096.
26. Tsai HC, Chang GRL, Fan HC, et al. A mini-pig model for evaluating the efficacy of autologous platelet patches on induced acute full thickness wound healing. BMC Vet Res 2019;15:1–13. DOI: 10.1186/s12917-019-1932-7.
27. Karabulut S, Özyaman S, Altintaş A, Keskin I. Histological effect of traditional rose ointment application in the excisional wound model. Acta Pharmac Sci 2022;60:39–47. DOI: 10.23893/1307-2080.APS.6003.
28. Yang F, Bai X, Dai X, Li Y.. The biological processes during wound healing. Regen Med 2021;16:373–90. DOI: 10.2217/rme-2020-0066.
29. de Moura F, Ferreira B, Deconte S, et al. Wound healing activity of the hydroethanolic extract of the leaves of *Maytenus ilicifolia* Mart. Ex Reis. J Tradit Complement Med 2021;11:446–56. DOI: 10.1016/j.jtcme.2021.03.003
30. Melguizo-Rodríguez L, de Luna-Bertos E, Ramos-Torrecillas J, Illescas-Montesa R, Costela-Ruiz VJ, García-Martínez O. Potential effects of phenolic compounds that can be found in olive oil on wound healing. Foods 2021; 10:1642. DOI: 10.3390/foods10071642.
31. Dunnill C, Patton T, Brennan J, et al. Reactive oxygen species (ROS) and wound healing: the functional role of ROS and emerging ROS-modulating technologies for augmentation of the healing process. Int Wound J 2017;14:89-96. DOI: 10.1111/iwj.12557.
32. Petruk G, Del Giudice R, Rigano MM, Monti DM. Antioxidants from plants protect against skin photoaging. Oxid Med Cell Longev 2018;2018 1454936. doi: 10.1155/2018/1454936.
33. Kwon KR, Alam MB, Park JH, Kim TH, Lee SH. Attenuation of UVB-induced photo-aging by polyphenolic-rich Spatholobus suberectus stem extract via modulation of MAPK/AP-1/MMPs signaling in human keratinocytes. Nutrients 2019;11:1341–55. DOI: 10.3390/nu11061341.
34. Hendra R, Ahmad S, Oskoueian E, Sukari A, Shukor MY. Antioxidant, anti-inflammatory and cytotoxicity of *Phaleria macrocarpa* (Boerl.) Scheff fruit. BMC Complement Altern Med 2011;11:110. DOI: 10.1186/1472-6882-11-110
35. Lay MM, Karsani SA, Banisalam B, Mohajer S, Abd Malek SN. Antioxidants, phytochemicals, and cytotoxicity studies on *Phaleria macrocarpa* (Scheff.) Boerl seeds. Biomed Res Int 2014;2014:1–13. DOI: 10.1155/2014/410184.
36. Avishai E, Yeghiazaryan K, Golubnitshaja O. Impaired wound healing: facts and hypotheses for multi-professional considerations in predictive, preventive and personalised medicine. EPMA J 2017;8:23–33. DOI 10.1007/s13167-017-0081-y.
37. Tandrasasmita OM, Sutanto AM, Arifin PF, Tjandrawinata RR. Anti-inflammatory, antiangiogenic, and apoptosis-inducing activity of DLBS1442, a bioactive fraction of *Phaleria macrocarpa*, in a RL95-2 cell line as a molecular model of endometriosis. Int J Womens Health 2015;7:161-9. doi: 10.2147/IJWH.S74552.
38. Kusmardi K, Situmorang NY, Zuraidah E, Estuningtyas A, Tedjo A. The effect of mahkota dewa (*Phaleria macrocarpa*) leaf extract on the mucin 1 expression in mice colonic epithelial cells induced by dextran sodium sulfate (DSS). Pharmacogn J 2021;13:1509–15. DOI:10.5530/pj.2021.13.192.
39. Yuliana Y, Tandrasasmita OM, Tjandrawinata RR. Anti-inflammatory effect of predimenol, a bioactive extract from *Phaleria macrocarpa*,

through the suppression of NF-κB and COX-2. Recent Adv Inflamm Allergy Drug Discov 2021; 15:99–107.
DOI: 10.2174/2772270816666220119122259.

40. Mahmood A, Tiwari AK, Şahın K, Ömer K, Shakir A. Triterpenoid saponin-rich fraction of *Centella asiatica* decreases IL-1β and NF-κB, and augments tissue regeneration and excision wound repair. Turk J Biol 2016;40:399–409. DOI: 10.3906/biy-1507-63.
41. Lindley L, Stojadinovic O, Pastar I, Tomic-Canic M. Biology and biomarkers for wound healing. Plast Reconstr Surg 2017;138:18S-28S. DOI: 10.1097/PRS.0000000000002682.
42. Cialdai F, Risaliti C, Monici M. Role of fibroblasts in wound healing and tissue remodeling on Earth and in space. Front Bioeng Biotechnol 2022;10:958381. DOI:10.3389/fbioe. 2022.958381.
43. Eming SA, Martin P. Tomic-Canic M. Wound repair and regeneration: Mechanisms, signaling, and translation. Sci Transl Med 2014;6:265sr6. DOI: 10.1126/scitranslmed.3009337.
44. Wilkinson HN, Hardman MJ. Wound healing: cellular mechanisms and pathological outcomes: cellular mechanisms of wound repair. Open Biol 2020;10:200223. DOI: 10.1098/rsob.200223