Wound Medicine 26 (2019) 100160

Contents lists available at ScienceDirect

Wound Medicine

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

Original research article

Wound healing properties of *Epiphyllum oxypetalum* (DC.) Haw. leaf extract in streptozotocin-induced diabetic mice by topical application

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ARTICLE INFO

Keywords:
Diabetic wound
Epiphyllum oxypetalum
Macrophage
Fibroblast

ABSTRACT

Diabetic foot ulcer is a serious complication of diabetes, resulting from peripheral neuropathy, macrovascular and microvascular disease, impaired angiogenesis and chronic inflammation. *Epiphyllum oxypetalum* leaves have been proven to be efficacious in incision wound healing. This project aimed to evaluate the wound healing properties of topical application of *Epiphyllum oxypetalum* leaves in diabetic mice. Diabetes was induced using streptozotocin. Wound healing activity was assessed by incision model. A total of 40 mice were used in this project, divided into four groups: WE10 and WE20 groups were treated topically with 10% and 20% *Epiphyllum oxypetalum* leaf extract ointment, respectively, while the diabetic control and non-diabetic groups were treated topically with the ointment base. The results showed significant wound healing progress in mice treated with *Epiphyllum oxypetalum* extract ointment, as assessed by wound contraction, number of macrophages and fibroblast count. In conclusion, topical application of 96% ethanol extract of *Epiphyllum oxypetalum* leaves could accelerate the wound healing time in diabetic mice, where the best activity was shown by 20% *Epiphyllum oxypetalum* extract.

1. Introduction

People diagnosed with diabetes have a 25% chance of developing a diabetic foot ulcer (DFU). Patients with DFU are more likely to have early death, myocardial infarction and fatal stroke than those without DFU. This disorder is often associated with diabetic neuropathy, peripheral vascular disease, and irregular cellular and cytokine activity [1–4]. Healthcare costs associated with diabetic ulcers and amputations contribute significantly to the economic problem of diabetic patients [5], and the recurrence rate for foot ulcers is more than 50% after 3 years [6].

Some of the important reasons for impaired diabetic wound healing include abnormal keratinocyte and fibroblast migration, proliferation, differentiation and apoptosis, abnormal macrophage polarisation, decreased vascularisation and prolonged expression of TNF- α [7]. The role of macrophages in wound healing includes phagocytosing debris and producing vascular endothelial growth factor, which controls tissue repair and induces the formation of lymphatic and blood vessels. Interference in the activation of these macrophages and depletion of the inflammatory mediator in diabetic wounds could delay the inflammatory process, which is the main cause of wound healing disorder in diabetic patient [8,9]. Furthermore, the delay in inflammatory response could lead to infection and wound aggravation [10].

Early intervention with DFU would reduce the number of patients progressing into further complications, infection and necrosis, and thus reduce the number of major amputations in diabetic patients [11]. Surgical debridement is one of the standard treatments for handling diabetic wounds, but this therapy requires substantial funds and is also very painful [12]. Therefore, early treatment using natural materials would be beneficial for patients. Several plants have been studied for their activity on the diabetic wound healing process, such as *Syzygium mundagam*, *Blechnum orientale* Linn., *Centella asiatica*, *Lepidium meyenii*, *Carthamus tinctorius* and *Avena sativa* [13–18]. *Epiphyllum oxypetalum* (DC.) Haw., with the common name Night Blooming Cereus or known in Indonesia as Wijaya Kusuma [19], has been used by the citizens of Indonesia to heal general incision wounds. Research has shown that Wijaya Kusuma has antimicrobial potential against *Staphylococcus aureus*, a common bacteria that delays wound healing [20,21]. In addition, Wijaya Kusuma leaves have been proven to be efficacious as an anti-inflammatory and antioxidant [22,23]. Other natural products, such as Radix Astragali and Radix Rehmanniae herbs, have been studied to enhance diabetic wound healing through their anti-inflammatory properties [24]. Thus, Wijaya Kusuma leaves extract could also have potential as a diabetic wound healing drug. No studies have been done regarding this, highlighting the need for this current research. This study aimed to determine Wijaya Kusuma efficacy in the

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https://doi.org/10.1016/j.wndm.2019.100160
Received 8 March 2018; Received in revised form 22 March 2019; Accepted 3 June 2019
Available online 05 June 2019

wound healing process in diabetic mice.

2. Materials and methods

2.1. Wijaya Kusuma extraction and ointment preparation

The leaves of Wijaya Kusuma used in this research were obtained from *Balai Penelitian Tanaman Rempah dan Obat* (Balitro) Bogor, Indonesia. Based on the results of determination in the Herbarium Bogoriense Botanical Field Centre Biological Research – LIPI Cibinong, the plant species is *Epiphyllum oxipetalum* (DC) Haw. from the family *Cactaceae.* Three kilograms of fresh Wijaya Kusuma leaves were used in the extraction process. The leaves were dried and mashed into coarse powder form, then extracted by maceration method using 96% ethanol. The extract was then concentrated using a vacuum rotary evaporator at ± 50 °C. The Wijaya Kusuma extract ointment was prepared using sterilised Vaseline flavum base.

2.2. Animals and experimental design

A total of 40 12-week-old male Swiss Webster mice weighing between 25 and 30 g were used in this study. The study protocol was approved by the Research Ethics Commission, Faculty of Medicine, University of Indonesia by applying the principles of replacement, reduction and refinement (the 3Rs) with approval under ethics number 510/UN2.F1/ETIK/2017. The mice were allowed to acclimatise for 7 days prior to the experiment. The mice were kept under standard laboratory conditions (22–25 °C and a 12-h light/dark cycle) and fed with standard pellets diet and water ad libitum. After overnight fasting, all the mice were induced with a single intraperitoneal (i.p.) injection of 60 mg/kg body weight (BW) streptozotocin (STZ) in 0.01 M citrate buffer (pH 4.5), except for the non-diabetic group [15,25].

The animals were divided into four experimental groups with ten animals in each group, consisting of:

WE10: treated topically with 10% Wijaya Kusuma extract ointment
WE20: treated topically with 20% Wijaya Kusuma extract ointment
DC: diabetic control group, treated topically with the ointment base
N-DC: non-diabetic group, treated topically with the ointment base.

2.3. Excisional wound preparation and macroscopic examination

The mice in each group were wounded 5 days after STZ injection. In this research, STZ induction increased the blood glucose concentration in the animals to ≥ 200 mg/dl, which was considered as hyperglycaemic. Briefly, the mice were anesthetised with a single i.p. injection of ketamine (2 mg/25 g of BW). The hair on the back of each mouse was shaved and wiped with 70% ethanol. A full-thickness wound (1 cm diameter) was made on the back of each mouse by excising the underlying panniculus carnosus of the skin [26]. A day after being wounded, the WE10 and WE20 groups were treated topically once daily with Wijaya Kusuma extract ointment, while the diabetic control (DC) and non-diabetic (N-DC) groups were treated topically with the ointment base for 14 days. Skin biopsy specimens were obtained from the animals (2 mice of each group) 3, 7, 10 and 14 days post-wounding. An area that included the scab, the complete epithelial and dermal compartments of the wound margins, the granulation tissue, parts of the adjacent muscle and subcutaneous fat tissue was excised from each individual wound at each time point. Each wound site was digitally photographed every day. The photos were then used to determine the wound area using ImageJ software (open source ImageJ software available at https://imagej.nih.gov/ij/) [27]. Wound area was used to calculate the percentage of wound contraction.

$$\text{Wound Contraction (\%)} = \frac{\textit{Initial wound area} - \textit{wound area day n}}{\textit{initial wound area}} 100\%$$

2.4. Histopathological studies

A specimen sample was isolated from each group of mice on day 3, 7, 10 and 14 days after wounding for histopathological examination. The skin specimens were fixed immediately in 10% (v/v) neutral buffered formalin. Each specimen was embedded in a paraffin block and thin sections (5 μm) were prepared and stained with haematoxylin and eosin for general morphological observations [28].

2.5. Quantification of macrophages and fibroblasts

Histologic observation was done in 10-images view with 400× objective magnification.

2.6. Blood glucose analysis

The blood glucose levels were determined using a glucometer (Easy Touch).

2.7. Statistical analysis

The data were first tested for normality and for variance homogeneity prior to any further statistical analyses. The data were normally distributed and were expressed as the mean ± SEM (standard error of the mean). The differences between the groups were analysed by one-way analysis of variance (one-way ANOVA) (for more than two groups) followed by Tukey's post-test.

3. Results

The wound healing observation results showed that both groups treated with Wijaya Kusuma extract at concentrations of 10% (WE10) and 20% (WE20) presented a rapid decline of wound area, while the diabetic control (DC) group tended to decrease more slowly in spite of the greater wound area of the WE10 and WE20 groups in the early days after the incision (Fig. 1.). Mice treated with WE10 and WE20 showed a significant ($p < 0.05$) wound contraction compared to the DC group starting from day 3 of the wound healing process (Table 1.). Nevertheless, from day 7, only the WE20 group showed activity comparable ($p > 0.05$) to that of the non-diabetic control (N-DC) group (Table 1.), implying the treatment capability in accelerating the wound healing was similar to the normal process, despite the high glucose condition.

Table 2. shows that the groups treated with WE10 and WE20 exhibited more macrophages than DC on day 3. In the DC group, the number of macrophages was lower in the early wound phase (day 3) but increased significantly on day 7. The high number of macrophages on day 7 indicated that the inflammation continues to a chronic condition, which is a common diabetic problem [8]. In the meantime, on day 7 the WE20 group showed a better result with a lower number of macrophages compared to the WE10 group, and comparable to the N-DC group.

Table 3. shows that the peak number of fibroblasts was seen on day

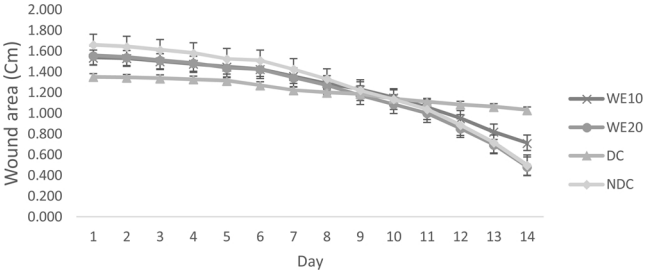

Fig. 1. Wound areas for mice: Wijaya Kusuma extract (WE), diabetic control (DC) and non-diabetic control (N-DC) groups.

Table 1
Wound contraction percentage.

Day	Wound contraction (%)			
	WE10	WE20	DC	N-DC
3	2.22[a]	2.85[a]	1.04[b]	2.98[a]
7	11.56[ab]	13.57[a]	9.57[b]	14.16[a]
10	25.03[ab]	30.16[a]	15.48[b]	31.67[a]
14	53.50[ab]	68.86[a]	23.93[b]	70.05[a]

Wijaya Kusuma Extract (WE), diabetic control (DC) and non-diabetic control (N-DC).
[a] Significantly different from DC at the 0.05 level.
[b] Significantly different from N-DC at the 0.05 level.

Table 2
Total macrophages numbers.

Groups	Day 3		Day 7	
WE10	68.15	± 3.572[a]	67.10	± 2.208[b]
WE20	75.75	± 4.335[a]	57.15	± 3.307[a]
DC	50.40	± 3.408[b]	74.25	± 3.313[b]
N-DC	81.05	± 3.631[a]	54.50	± 2.811[a]

Wijaya Kusuma extract (WE), diabetic control (DC) and non-diabetic control (N-DC).
[a] Significantly different from DC at the 0.05 level.
[b] Significantly different from N-DC at the 0.05 level.

Table 3
Total fibroblast numbers.

Groups	Day 7		Day 10		Day 14	
WE10	31.10	± 7.656[b]	38.80	± 8.256[a]	24.40	± 8.444[a]
WE20	33.05	± 9.506[a]	40.70	± 12.12[a]	23.10	± 10.82[a]
DC	22.55	± 8.690[b]	29.85	± 7.849[b]	32.60	± 7.096[b]
N-DC	35.15	± 8.652[a]	44.25	± 11.76[a]	21.60	± 10.66[a]

Wijaya Kusuma extract (WE), diabetic control (DC) and non-diabetic control (N-DC).
[a] Significantly different from DC at the 0.05 level.
[b] Significantly different from N-DC at the 0.05 level.

10, with the highest number of fibroblasts observed in the N-DC group, followed by the WE20 and WE10 groups. High numbers of fibroblasts were observed in the WE20 and WE10 groups started from day 7, which confirmed the beginning of the proliferation phase, and the fibroblast numbers decreased on day 14 where this phase ended.

Fig. 2A.c shows that were more macrophages on day 7 in the DC group compared to the WE10, WE20 and N-DC groups (Fig. 2A. a, b and d). Fig. 2B shows a high fibroblast density in all groups except for the DC group (Fig. 2.B.c), which is beneficial for the proliferation phase of the wound healing process.

4. Discussion

The diabetic wound could not progress through the normal healing process, which leads to a prolonged inflammation phase and infection [29]. In order to prevent delayed wound healing in diabetics, early treatment should be applied, including the use of plant extracts. This work showed that topical application of both 10% and 20% Wijaya Kusuma extract ointment could decrease wound healing time in the diabetic condition. The healing activity of Wijaya Kusuma was seen to start from day 3 onwards, which was shown by the higher wound contraction, incomparable to the DC group (Table 1). This finding showed that Wijaya Kusuma leaf extract could heal diabetic wounds faster than *Lepidium meyenii* (black maca) root extract ointment, which showed a significant wound contraction starting on day 10 [15]. In addition, wound contraction rate with Wijaya Kusuma showed dose-dependent activity.

In the wound healing process, the first to third days are critical for the early inflammatory stage, in which macrophages play an important role in preventing infection in the wound area [30]. Depletion of macrophages during this phase results in a significant delay to wound repair, which is common in diabetics [7]. In addition, macrophages can generate growth factors that are useful for starting the proliferative phase [31]. On day 3, in the diabetic state, the number of macrophages is less than that in normal wounds, which interferes with the early inflammatory process. This was confirmed by the DC group, where the macrophage number was significantly lower than that of the N-DC group by 37.82%. On the other hand, mice treated with WE10 and WE20 exhibited a normal inflammatory phase, which was confirmed by a similar number of macrophages with N-DC. The macrophage numbers of the WE10 and WE20 groups on day 3 were higher than those in the DC group by 26.04 and 33.47%, respectively. Dysfunctional macrophages have been proven to increase apoptotic cell burden at the wound site, thus prolonging inflammation and obscuring wound healing [9].

The DC group showed less macrophages in the early wound phase but this increased significantly later on day 7. Consequently, the longer continuous inflammation time caused chronic inflammation of the

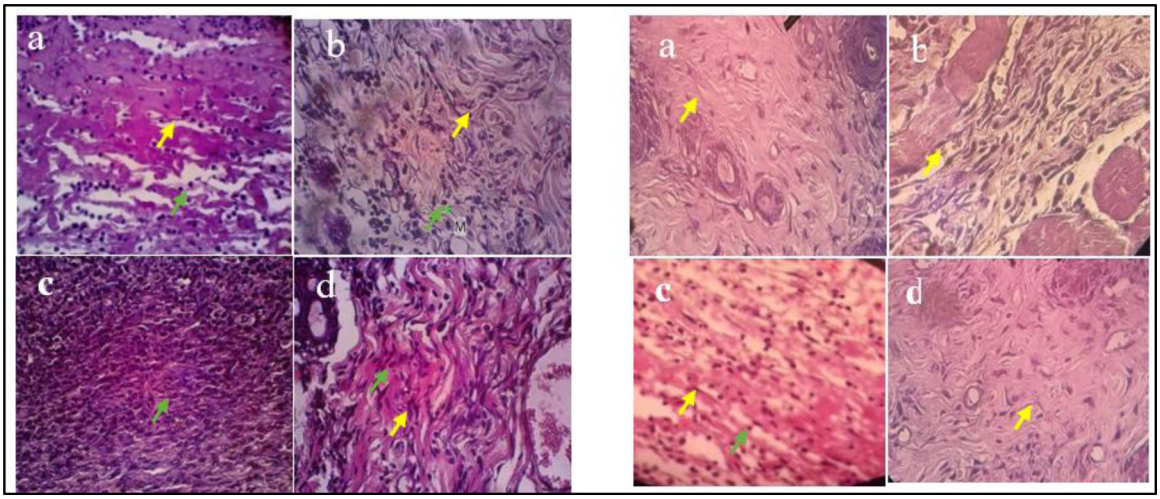

Fig. 2. Histological changes in the wounds on day 7 (A) and day 10 (B) (haematoxylin and eosin staining; 400× magnification). a) Diabetic group treated with 10% WE (WE10); b) diabetic group treated with 20% WE (WE20); c) Diabetic Control (DC); and d) Non-Diabetic control (N-DC). Macrophage (↑), Fibroblast (↑).

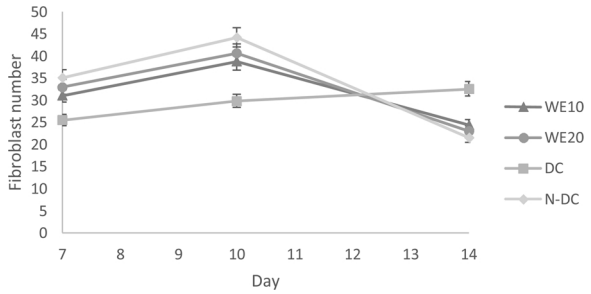

Fig. 3. Fibroblast numbers during the wound healing process for Wijaya Kusuma extract (WE), diabetic control (DC) and non-diabetic control (N-DC) groups.

wound. In contrast, the WE20 group showed a significantly lower number of macrophages compared to the DC group on day 7. This confirmed the activity of WE20 in accelerating the healing of diabetic wounds in the inflammatory phase.

Another important parameter of wound healing progression is fibroblasts. This component of skin tissue is the primary source of extracellular matrix proteins such as collagen and fibronectin. In a diabetic wound, there is a decrease in migration and proliferation of fibroblasts [7]. An increase in the local inflammatory response is attributed to the lack of growth factor production, which is important for fibroblast activation [32]. In the normal wound healing process, the proliferation phase would start between days 5 and 7, which is indicated by the increase in the number of fibroblasts [30].

Fig. 3. illustrates the changes in the fibroblast numbers in the tested groups. Wounds treated with WE10 and WE20 showed a similar trend to the N-DC group, where the fibroblast number increased from day 7 to reach a peak on day 10. The higher the fibroblast number at the peak, the better the healing process occurs in the proliferation phase [32]. The highest peak is seen in the N-DC group, followed by WE20 and WE10, sequentially. The number of fibroblasts for these groups then fall rapidly on day 14, showing that the proliferation phase was almost completed, and continued to the remodelling phase, the final phase of the wound healing process. Meanwhile, the DC group showed late activation of fibroblasts, despite the steady incline. It is possible that diabetic wound treatment with WE might increase the activity of inflammatory cells, leading to the proper inflammation response and accelerating the proliferation phase in wound healing process. This acquaintance possibly underlies the role of this plant in elevating the fibroblast numbers at the wound site.

Phytochemicals examination of WE showed that the extract contains alkaloid, flavonoid, tannin, saponin and steroid. Several studies have shown flavonoid mechanism in wound healing by modulating the expression of cytokines and nitric oxide in the Inflammation phase [33]. Recruitment of macrophages by *Epiphyllum oxypetalum* was suspected due to these mechanisms. The similar mechanisms could also be expected to occur in wounds in humans [34].

Lower macrophage density was obviously seen in the WE10 and WE20 groups on day 7 compared to that in the DC group (Fig. 2A). The histopathological results in Fig. 2B. b demonstrated better regeneration in the skin wound for the WE20 group. The presence of numerous fibroblasts in the tissue in the WE groups, particularly at 20% concentration, indicates that the wound healing process reached the proliferation phase on day 7 and continued to day 10. A similar observation was made in an analogue wound healing study using other plants, such as *Sida cordifolia* Linn [35]. However, the methanolic extract of *Sida cordifolia* in hydrogel form exhibited 100% wound contraction at day 14, while in this research Wijaya Kusuma showed only 70% wound contraction. Wound healing in rats mostly occurs through wound contractions. This mechanism also plays a major role in acute and chronic wounds in humans. Failure on rapid contraction appears to be a significant problem in diabetic ulcers [36]. However, infection, the location of the ulcer, and foot deformities which are important factors that influence the healing process in diabetic patients are not applied in this diabetes wound model. Thus, in future research investigations could be attempted with other types of preparations to improve the effectiveness of Wijaya Kusuma extract in diabetic wounds.

5. Conclusion

The groups treated with 10% and 20% WE showed significant wound healing compared to the DC group. The best activity was shown by the WE20 group, which was comparable to that of the N-DC group. From the results above, it can be concluded that topical application of 96% ethanol extract of Wijaya Kusuma (*Epiphyllum oxypetalum (DC.) Haw.*) leaves could accelerate the wound healing process in diabetics.

Conflict of interest

None declared.

Acknowledgements

The authors thank Mr. Dan Mugisidi for the valuable comments on the article and also thank Unit Pembina dan Pengembang Publikasi Ilmiah (UPPI) UHAMKA for facilitating proofreading for this article.

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