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The Effectiveness of *Kokang (Lepisanthes Amoena) Leaves* Extract on Wound Healing in Diabetic Rat

Vera Pravitasari Susila,[1]* IGAA. Praharsini,[2] I Made Muliarta,[1] Agung Wiwiek Indrayani[3]

[1]*Magister Biomedical Sciences. Faculty of Medicine, Udayana University, Denpasar, Bali-Indonesia.*
[2]*Departement of Dermatology and Venereology, Faculty of Medicine, Udayana University, Denpasar, Bali-Indonesia.*
[3]*Departement of Physiology Fisiologi, , Faculty of Medicine, Udayana University, Denpasar, Bali-Indonesia.*
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ABSTRACT

Background: Hyperglycemia in Diabetes Mellitus can lead to chronic wound conditions, which can reduce quality of life, increase morbidity and socio-economic burden for sufferers. Kokang (*Lepisanthes amoena*) leaves contains flavonoids, tannins, phenols, and strong antioxidants, often used by Dayak people for wound treatment. This study aims to scientifically prove the efficacy of 5% Kokang (*Lepisanthes amoena*) leaves ethanol extract gel as a wound healing drug on the skin of diabetic male Wistar rats through measurement of vascularization, fibroblast, epithelialization, and wound diameter.

Materials and methods: This experimental study – post.test only design, used 26 diabetic male Wistar rats through STZ-NA induction, a 0.5 cm punch biopsy wound was performed on the rat's back. Rats were divided into six group: two grups of 5% Kokang (*Lepisanthes amoena*) leaves ethanol extract, for days 4 and 14; 2 groups of Bioplacenton® gel as positive control groups, for days 4 and 14; and 2 groups of Basis gel as negative control groups, for days 4 and 14.

Results: The statistical test results showed that on day 4, the wound diameter of the Kokang-treated group was the smallest compared to control groups ($p<0.05$). On day 14, Kokang-treated group epithelialization was equivalent to Bioplacenton® gel-treated group ($p=0.155$), and the wound diameter of Kokang-treated group was also equivalent to Bioplacenton® gel – treated group ($p=0.204$). This study concluded that the administration of 5% Kokang (*Lepisanthes amoena*) leaves ethanol extract gel was shown to increase epithelialization and accelerate wound closure, but did not increase vascularization and fibroblasts.

Keywords: Kokang, *Lepisanthes amoena*, wound in diabetic rat, epithelialization, wound diameter

1. INTRODUCTION

Wound healing is a dynamic and complex biological process, through phases: hemostasis, inflammation, proliferation, and remodeling.[1] Interruptions in the wound healing phase can make the wound enter a chronic wound condition, such as wounds in the elderly and diabetic condition.[2,3] Chronic wound conditions due to skin aging and diabetic condition should be prevented, avoided, and treated so that it can return to its original state, this is in line with concept of Anti-Aging Medicine.[4] There are various therapeutic efforts to speed up the healing process of chronic wounds in person with diabetes, and it is vital to create novel therapy strategies that are more efficient, less expensive, and safer since they require sophisticated, expensive, and risky treatment.[5]

Topical application of herbal ingredients has been known to provide more optimal results in accelerating wound healing because they contain secondary metabolites which are safe and beneficial for the treatment of wounds in DIABETIC conditions but their use is still limited traditionally.[6–8] Kokang (*Lepisanthes amoena*) leaves is a medicinal plant that has been contain secondary metabolites, such as phenol, flavonoids, tannins, and showing strong antioxidant.[9] This traditional medicinal plant is often used for generations by the Dayak people for skin care, wound healing, boils, acne medicine, body cleanser and as a medicine for diseases associated with diabetic condition.[10–12]

This study aims to scientifically prove the efficacy of Kokang (*Lepisanthes amoena*) leaves in wound healing, especially diabetic wounds. The results of this study are expected to be the basis for further research regarding the development of traditional medicinal plants in Indonesia, as an alternative to wound treatment that is safer, more effective and cheaper than what already exists, so as to reduce the socio-economic burden, and improve the quality of life of patients with diabetes.

2. MATERIAL AND METHODS

2.1 Method Research

This experimental study with a post-test only control group design, was carried out for 8 weeks from December 2022 to January 2023 and was approved by the Animal Ethics Committee - Faculty of Veterinary Medicine, Udayana University (No.B/232/UN14.2.9/PT.01.04/2022). The research was conducted at the Integrated Biomedical Laboratory Unit, Division of Drug and Experimental Animal Development, Faculty of Medicine, Udayana University, Denpasar - Indonesia.

2.2 Plant Collection and Extraction

Kokang (*Lepisanthes amoena*) leaves were taken from Pampang Cultural Village, North Samarinda District, Indonesia. The authentication of Kokang leaves was from Ecology and Conservation laboratory of Tropical Forest, Mulawarman University, Indonesia. The Kokang (*Lepisanthes amoena*) leaves then processed into 5% ethanol extract gel.

2.3 Phytochemical Analysis

Phytochemical screening of Kokang Leaves was performed using standard procedure in Integrated Biomedical Laboratory, Udayana University, Indonesia. Examination of the phytochemical content of Kokang (*Lepisanthes amoena*) leaves found: phenol (928.34 mg GEA/100g), flavonoids (226.28 mg QE/100g), tannins (717.22 mg TAE/100g), and IC50 test was 76.2 ppm showing strong antioxidants.

2.4 Animal Study

Thirty (30) diabetic male Wistar rats through Streptozotocin-nicotinamide-induction, with the inclusion criteria of glucose levels above 150 mg/dl, 2-3 months old, and 180-200 gr body weight, were selected and randomized into six groups. [13,14] Excisional Wound was created using a 0.5 cm punch biopsy in the area of the rat's back.[15] Group P1 and P4 served as the negative control and received base gel. Group P2 and P5 as the positive control, received Bioplacenton® gel. Group P3 and P6 as Kokang-treated group (5% Kokang (*Lepisanthes amoena*) leaves ethanol extract gel). The material according to the group was applied topically 3 times a day, at a dose of 0.1 mg/cm2. P1, P2, and P3 received for four day, and P4, P5, and P6 received for fourteen day.

2.5 Histopathology

In end of the study, all rats in each group were euthanized. The diameter of the wound was measuring and a histology slide of the wound tissue were made using Hematoxylin-eosin stained. The slides were examined under microscope, then the number of vascularization, fibroblast cells, and epithelialization was calculated.

2.6 Statistical Analysis

Data were expressed as the Mean ±Standard Error of the Mean (SEM). Data were analyzed statistically using one-way Analysis of Variance (ANOVA) followed by Least Significant Different (LSD) or Dunnett's post hoc test for multiple comparisons between the control groups and treated groups. Values of $P \leq 0.05$ were considered significant.

3. RESULT

3.1 Gross observation of wound appearance

Wound appearance and closure characteristic were observations in all groups on day 4 and day14 after wounding. The groups were: negative control group on day 4 (gel base, P1), positive control group on day 4 (Bioplacenton® gel, P2), Kokang-treated group on day 4 (*Lepisanthes amoena* 5%, P3), negative control group on day 14 (gel base, P4), positive control group on day 14 (Bioplacenton® gel, P5), and Kokang-treated group on day 14 (*Lepisanthes amoena* 5%, P6). Gross evaluation of wound revealed that on day 4, showed that the condition of the wound on P1 was bad, the scab was harder than on P2 and P3, and on palpation there was a swelling that felt dense and slightly hard in the area around the wound in group P1. Groups P2 and P3 got clean wounds and no swelling was found, but in P2 there was a slight redness in the area around the wound. Observations on day 14, the wounds in all study groups on day 14 had begun to close properly. The macroscopic picture of the wound condition of the study group is shown in Figure 1.

3.2 H&E staining

The images of H&E-stained wound slide are presented in Figure 1 for vascularization, Figure 2 for fibroblasts, and epithelialization in Figure 3. There was no increase of vascularization in Kokang-treated group on day 4 or day 14 compared to negative control group and positive control group. Whereas in the fibroblast, the average number of fibroblast cells in P3 appeared low, but the average number of fibroblast cells appeared to increase in P6 compared to P4 and P5. Examination of the epithelialization was carried out by measuring the thickness of the epithelium, analysis of the epithelialization was only carried out in P4, P5 and P6, this was because epithelialization had not yet been seen in all day 4 study groups (P1, P2, and P3). The average epithelialization in P6 appeared to be more thickness than P4 and P5.

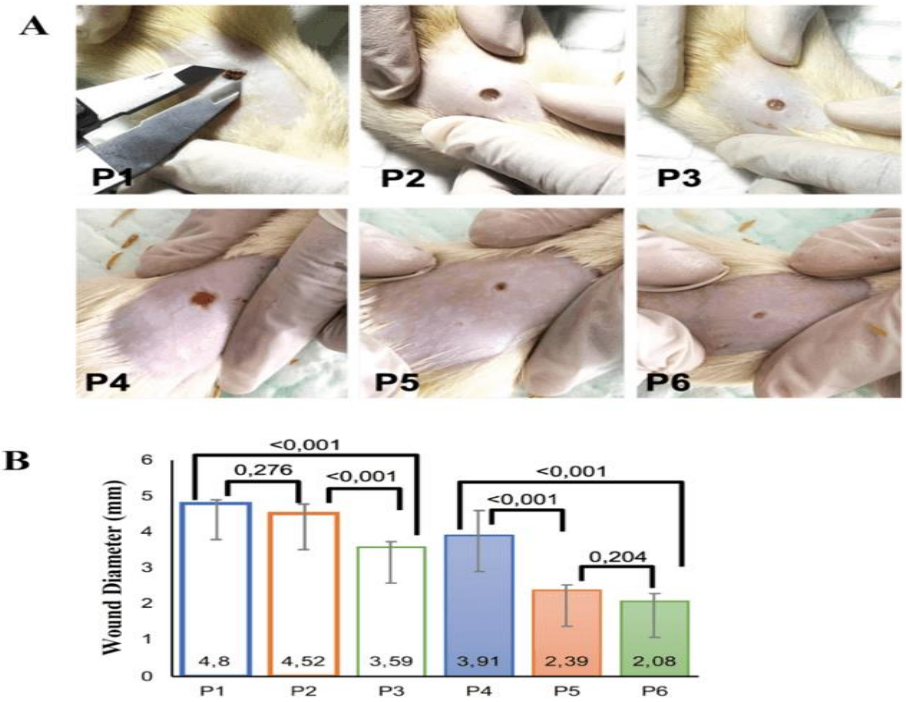

Figure 1. (A) Photographs of wound appearance. Clean wound conditions without scab were seen in P2 and P3, while in P1 it was seen a thick, hard scab. In groups P5 and P6, wound closure was almost complete, seen from the small diameter of the wound, whereas in P4 the wound diameter is still wide and redness. (B) Wound diameter (mm) in all groups after wounding. Data are expressed as means ± standard error of the mean. The results of the Statistical analysis showed that there was a significant difference in wound diameter between the study groups on day 4, the average wound diameter in P3 (3.59±0.15 mm) was the smallest compared to P1 (4.8±0.1 mm) and P2 (4.52±0.26 mm). On day 14, the average wound diameter in P6 (2.08±0.22 mm) was smaller than the average wound diameter in P4 (3.91±0.69 mm), but not significantly different with P5 (2.39±0.15 mm).

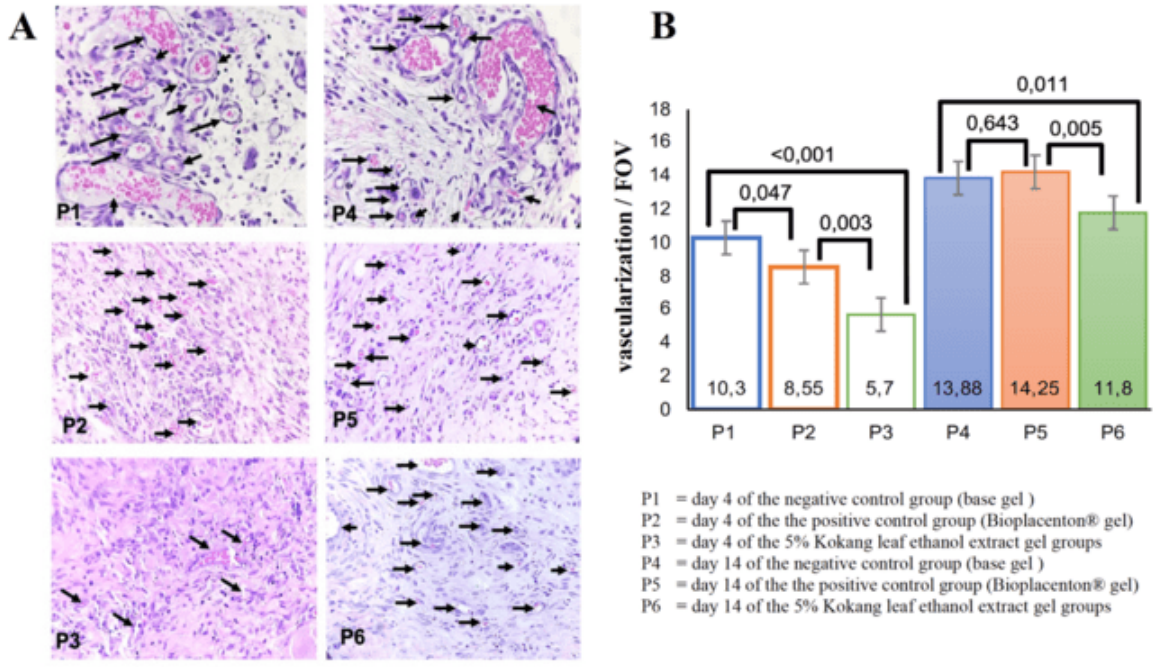

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Figure 2. (A) Photomicrographs of vascularization histological study of all groups on day 4 and day 14 post-wounding (stained with H&E; magnification 400x), Black arrows indicate blood vessels. The smallest amount of blood vessels was seen in the P3, whereas in the other groups many blood vessels were seen evenly distributed throughout the field of view. This indicated an increase in the formation of new vascularization in the wound tissue of diabetic rats on day 4 and day 14. (B) The results of the Statistical analysis showed that the vascularization amount in P3 was 5.71.25/FOV, the lowest amount compared to P1 (10.3±0.96/FOV), and P2 (8.55±0.89/FOV). On day 14, the average vascularity P6 (11.8±1.14/LP), significantly lower than P4 (13.99±1.1/FOV) and P5 (14.25±1.59/FOV).

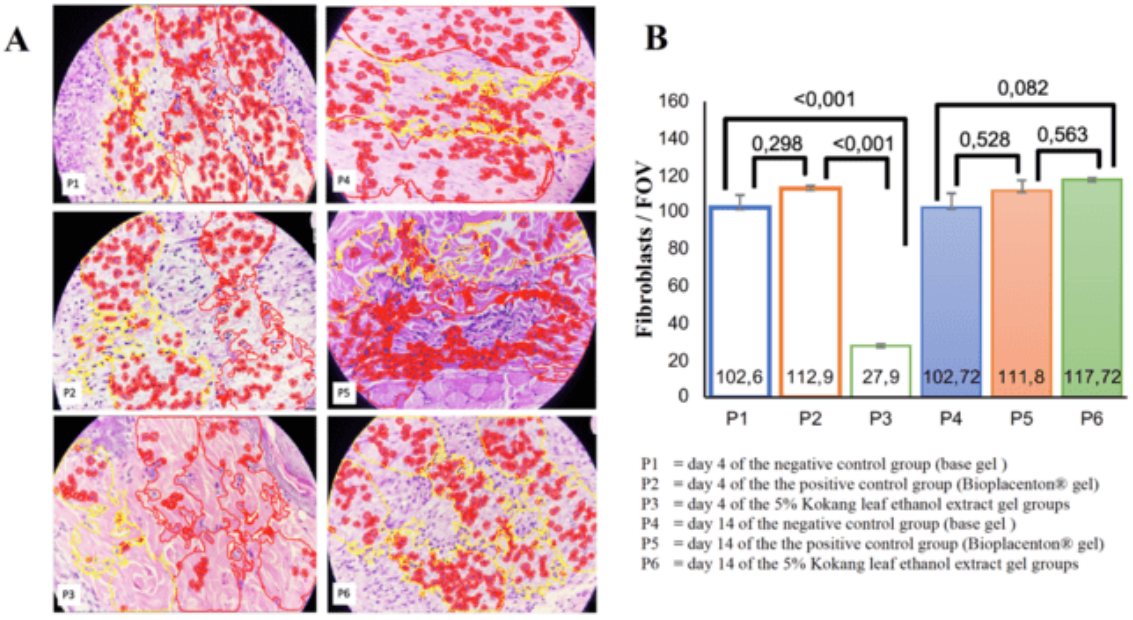

Figure 3. (A) *QuPath* software was used to analyze and enumerate fibroblast cells on histology slides. cells circled in red indicate fibroblast cells. There was a large number of fibroblast cells scattered in the field of view of P1, P2, P4, P5, and P6, but a small number appeared in group P3. (B) The statistical analysis of fibroblasts showed P3 (27.9±0.91 cells/FOV), was the lowest compared to P1 (102.6±6.81 cells/FOV), and P2 (112.9±1.54 cells/FOV). On day 14 showed no significant difference between P4 (102.72±7.57 cells/FOV), P5 (111.8±5.59 cells/FOV), and P6 (117.72±0.86 cells/FOV).

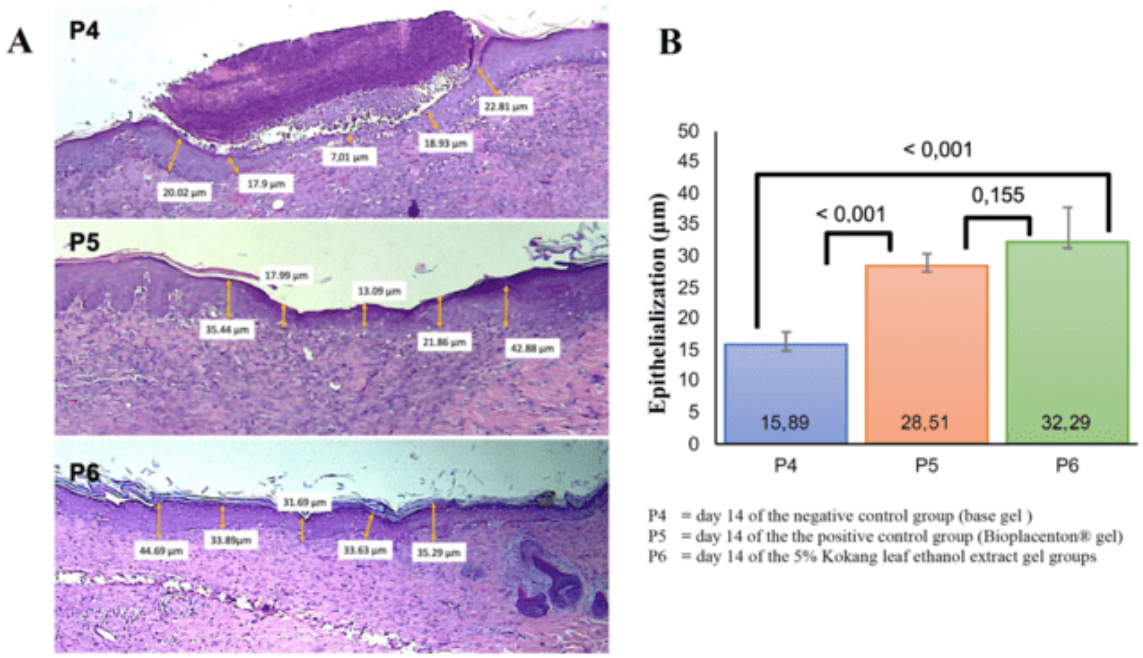

Figure 4. (A) Photomicrographs of epithelialization histological study of control group and Kokang-treated group on day 4 and day 14 post-wounding (stained with hematoxylin–eosin; magnification 100x), P4, P5, and P6 showed good epithelialization, so that the thickness of the epithelium could be measured. At P4, it was seen that the wound had not closed properly, because there was still a gap in the middle of the wound. (B) The results of the Statistical analysis showed that epithelialization thickness (µm) on day 14, was a significant difference in the average epithelialization between P6 (32.29±5.55 µm) compared to P4 (15.89±2.01 µm), but not significantly different compared to P4 (28.51±1.89 µm).

4. DISCUSSION

4.1 Challenges of Wound Healing in Diabetic Conditions

To restore tissue integrity, the physiological process of wound healing involves a complex biochemical process. Hemostasis, inflammation, proliferation, and tissue maturation or remodeling are the four basic stages of this process. This process may be impeded by a variety of factors, leading to higher disease incidence and mortality as well as poor healing. Chronic wounds are those whose healing time is longer than 12 weeks, frequently caused by persistent pathological inflammation.[2,3]

In patients with diabetes, Extrinsic and intrinsic factors in wound-area as well as systemic disturbances, leading chronic wound conditions that are difficult to heal or delayed wound healing, and disrupt wound healing. hyperglycemia can impair wound healing due to alterations in cellular morphology, reduced keratinocyte proliferation, and aberrant keratinocyte differentiation.[2,3] Effects of hyperglycemia through the formation of AGEs are by induced production of inflammatory molecules (TNF-α, IL-1 and IL-6), inhibiting collagen synthesis, inducing apoptosis, causing exaggerated immune responses, and negatively affecting cell physiology.[16] Oxygen levels and tissue nutrient supply may be affected by chronic microvascular damage caused by diabetic disease. Discontinuation of the immune system, due to dysregulation of inflammatory cells, makes patients with diabetes particularly vulnerable to infection and make wounds difficult to heal.[2,17]

4.2 Effectiveness of 5% Kokang (Lepisanthes Amoena) Leaves Ethanol Extract Gel on Wound Healing Process

Angiogenesis is defined as the development of new blood vessels and the remodeling through migration and proliferation of endothelial cells to restore functioning and structure in existing blood vessels.[2] The normal vascularization process does not occur during diabetic condition.[18] It is widely accepted that a strong and dynamic angiogenic response is required to heal wounds, but many of the capillaries that form in the initial wound are immature and highly permeable, and the majority of newly formed blood vessels are perfused ineffectively. The excessive angiogenesis in wounds may not be necessary because excessive angiogenic response may lead to scar tissue development, which restriction of function and causes pain, so excessive angiogenesis that occurs in wounds may not be necessary.[19–21] There was no difference in vascularity between the Kokang-treated group and the control group in this study. According to accepted theory, angiogenesis is an inflammatory reaction by pro-angiogenic factors to the need for nutrition and oxygen in the wound area and also controls infection. However, if the nutrients and oxygen were satisfied and the wound was infection-free, then there was no need for an excessive inflammatory response, so angiogenesis would form adequately.

Due to the stimulating of growth factors, fibroblasts can multiply and move into the wound.3 The fibroblast-produced collagen is essential for giving tissues strength, but too much collagen production can result in hypertrophic scars.[20,22,23] In this study the QuPath software was used to determine the number of fibroblast cells, and it has been found that on days 14, the amount of fibroblast cell increased in the Kokang-treated group equivalent to those given standard medicines (Bioplacenton® gel).

Re-epithelialization is a process of epithelial renewal following injury, including proliferation and migration of epithelial cells into the wounds center as well as wound contraction. The cytokines and growth factors are released during this process.[22,24–26] Due to accumulation of AGEs, which makes diabetic skin more vulnerable to infection, damage and reduces the capacity of epidermal keratinocytes to migrated. The more AGEs are concentrated, the fewer keratinocytes will be activated, so that interventions against AGEs can significantly promote the epithelialization process.[27] Wound healing in the skin is characterised by wound contraction, which occurs at the same time as the epithelialization process.[6,28] Disturbances in this healing phase can lead to deformities and the formation of scars.[3] In the macroscopic assessment of wound healing, visible appearance and diameter measurements are reliable parameters. In this study, Kokang-treated group had thicker epithelialization and smaller wound diameters than the control group. Can be concluded that the ethanol extract gel of Kokang (*Lepisanthes amoena*) leaves at a concentration of 5% has strong wound contraction ability and that the impact of wound closure.

4.3 Kokang (Lepisanthes amoena) Leaves Contains Active Compounds as Wound Healing Medicine

Natural medicinal products derived from a variety of plant species are commonly used to treat wounds. A compound such as flavonoids, phenols, tannins, triterpenoids, oleanolic acid, steroids, alkaloids, saponins and glycosides is commonly contained in plants may have potential therapeutic effects on wound healing. These active substances contribute to the mechanisms of hemostasis, collagenization, enhancement of tensile strength, and epithelialization.[5,29] Increased collagen density, collagenase activity, procollagen production, MMP-1, migration and proliferation of human skin fibroblasts, and/or decreased wound edge distance, wound width, and granulation tissue are all functions of this active substance.[30]

Examination of the phytochemical content of Kokang (*Lepisanthes amoena*) leaves in this study found: phenol 928.34 mg GEA/100g, flavonoids 226.28 mg QE/100g, tannins 717.22 mg TAE/100g, but the negative results for saponins. The IC50 quantitative test value was 76.2 ppm (a strong antioxidant). The content of phenol, flavonoid, and tannin secondary metabolites, as well as the IC50 value in Kokang (*Lepisanthes amoena*) leaves became the basis for research to prove the effectiveness of 5% Kokang (*Lepisanthes amoena*) leaves ethanol extract gel on wound healing in diabetic rats.

Through its active compound content, the ethanol extract of Kokang (*Lepisanthes amoena*) leaves is believed to have antimicrobial and anti-inflammatory abilities that can help the wound healing process. This is also supported by previous studies that *Propionibacterium acnes* and *Staphylococcus epidermidis* bacteria can be inhibited.[31,32] As anti-inflammatories, flavonoids can accelerate the end of the inflammatory phase.[33,34] Tannins as antibacterials can damage bacterial cell membranes as a result of which bacterial growth is inhibited and then dies.[35] A sterile wound environment can support the process of hemostasis so that the inflammatory phase can be controlled.

It has been demonstrated that flavonoids, tannins, and polyphenols boost fibroblast proliferation to create extracellular matrix while avoiding the development of pathological fibrosis.[36–38] Flavonoids can speed up the rate of fibroblast proliferation throughout the healing process by promoting the production of growth factors that promote keratinocyte migration.[39] Flavonoid compounds also exhibit wound healing activity through accelerated wound contraction, shortened epithelialization time, and enhanced collagen deposition.[28] Phenolic compounds and antioxidants can also promote wound healing by accelerating the epithelialization process.[29] Plants with high tannin compounds can be obtained to speed up wound closure by increasing wound contraction. Tannins that are astringent may help improve skin healing, thereby accelerating wound closure by increasing wounds contraction to speed up epithelialization and generate more collagen while promoting fibroblast proliferation.[40] Tannins also can stop light bleeding [30]

Flavonoids and tannins contained in the 5% Kokang (*Lepisanthes amoena*) leaves ethanol extract gel, based on literature and research results, are thought to have a role in fibroblast proliferation and accelerate the process of epithelialization and wound closure, overall help the wound healing process.

4.4 The Benefits of Kokang (Lepisanthes amoena) Leaves in Anti-aging

Chronic wounds healing in diabetic represent a significant health problem that continually increases patient morbidity healthcare costs, and big impacts on quality of life. To achieve physiological wound healing, it is important to manage wound healing in patients with diabetes by preventing chronic wound conditions, reducing tissue damage and ensuring adequate oxygen and nutrient availability for wound healing, including infection control. Relieving pressure, improving mental health, and returning a patient's quality of life to a normal life are consistent with the concept of anti-aging.

The results of this study showed that topical application of 5% Kokang (*Lepisanthes amoena*) leaves ethanol extract gel to the wounds of diabetic rats showed wound healing, with a marked reduction in wound diameter and a clear wound appearance as visible characterized when inspected. Microscopically, we found increased numbers of fibroblasts cells and epithelialization. This is undeniably evidence that Kokang (*Lepisanthes amoena*) leaves, which contain phenolic compounds, flavonoids, tannins, and strong antioxidant might be used as an alternative treatment for wound management in diabetic conditions. However, more research is still required before Kokang (*Lepisanthes amoena*) can actually be used as a treatment in wound management, especially with diabetic conditions. Kokang (*Lepisanthes amoena*) therefore contributes to anti-aging.

5. CONCLUSION

Our data showed that Kokang-treated groups was faster in reduced the wound size and thicker epithelialization, although no significant increase in vascularization and fibroblasts cells. As a main outcome, the current study showed that the topical administration of 5% Kokang (*Lepisanthes amoena*) leaves ethanol extract gel was effective to the wound healing process in diabetic male Wistar rats, and this could be envisioned as a new alternative treatment for accelerating diabetic wound healing. However, there is still a need for further research in order to make sure that Kokang (*Lepisanthes amoena*) can indeed be used as treatment for wound healing in humans, especially with diabetic conditions.

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