Digital Image Analysis of Angiogenesis in Diabetic Wounds Treated with *Muntingia calabura* L. Extract Cream

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Abstract
Diabetes mellitus is a chronic metabolic disease that causes delayed wound healing due to persistent hyperglycemia, oxidative stress, prolonged inflammation, and angiogenesis disorders. This condition encourages the development of topical therapy based on natural ingredients combined with digital technology to increase the effectiveness of therapy as well as the objectivity of histopathological evaluation. This study aims to analyze the effect of kersen leaf ethanol extract cream (Muntingia calabura L.) on angiogenesis in wound healing of rats (Rattus norvegicus) in the diabetes mellitus model using a digital image analysis approach. The novelty of this study lies in the integration of kersen leaf ethanol extract cream formulation with digital image-based histopathological analysis to quantitatively, objectively, and reproductively identify and count the number of new blood vessels. The study used a Complete Random Design (RAL) consisting of five treatment groups with three replicates. Skin tissue samples were collected on days 3, 7, and 14, then processed using Hematoxilin-Eosin (HE) staining. The preparations were observed using a digital microscope and analyzed through image processing software to measure the number of new blood vessels as an indicator of angiogenesis. The results showed that the administration of kersen leaf ethanol extract cream significantly increased angiogenesis compared to the control group, especially on days 7 and 14, which was characterized by an increase in the density of new blood vessels and an acceleration of the tissue proliferation phase. The digital analysis approach results in more accurate, consistent measurements, and minimizes the subjectivity of microscopic observations. These findings suggest that kersen leaf ethanol extract cream has the potential to be developed as a natural topical therapy to accelerate the healing of diabetic wounds, while also introducing a digital approach in histopathological evaluation that supports the development of digital pathology in biomedical research.

1. Introduction

Diabetes mellitus (DM) is one of the chronic metabolic diseases characterized by hyperglycemia due to impaired insulin secretion, insulin action, or both.a [1]–[7]. Long-lasting hyperglycemia not only causes disruption of carbohydrate, fat, and protein metabolism, but also triggers various chronic complications, one of which is wound healing disorders [8]–[12]. Wounds in diabetics, especially diabetic ulcers, become a serious health problem because the healing process takes place more slowly than normal individuals [13]–[19]. This condition increases the risk of infection, tissue necrosis, limb amputation, and death, thus having a significant impact on the patient's quality of life and the burden of health services [20]–[23], [18], [24]–[28].

The wound healing process is a complex biological mechanism and involves four continuous phases, namely hemostasis, inflammation, proliferation, and remodeling [29], [30]. In people with diabetes mellitus, each of these phases is disrupted due to persistent hyperglycemia, increased oxidative stress, immune cell dysfunction, and microcirculatory damage. One of the most affected phases is the proliferation phase, which is characterized by reduced granulation tissue formation, low fibroblast activity, and disruption of angiogenesis processes [31]–[33]. In people with diabetes mellitus, each of these phases is disrupted due to persistent hyperglycemia, increased oxidative stress, immune cell dysfunction, and microcirculatory damage. One of the most affected phases is the proliferation phase, which is characterized by reduced granulation tissue formation, low fibroblast activity, and disruption of angiogenesis processes [34], [35].

Angiogenesis is the process of forming new blood vessels from pre-existing blood vessels [36]–[38]. This process has an important role in wound healing because it is responsible for the supply of oxygen, nutrients, and various growth factors necessary for cell proliferation, collagen synthesis, as well as tissue regeneration [39]–[41]. In diabetic wounds, angiogenesis is often inhibited due to decreased expression of various proangiogenic factors, such as vascular endothelial growth factor (VEGF), accompanied by increased oxidative stress and chronic inflammatory responses. As a result, granulation tissue is formed suboptimally and the epithelialization process becomes slower. Therefore, increasing angiogenesis is one of the main targets in the development of diabetic wound healing therapies.

The use of natural ingredients as an alternative to wound healing therapy is growing because it is considered to have good effectiveness with relatively low side effects. One of the plants that has the potential to be developed is the kersen leaf (Muntingia calabura L.), which has long been traditionally used as a medicinal plant in various tropical countries. Various studies report that kersen leaves contain bioactive compounds such as flavonoids, phenolics, tannins, saponins, triterpenoids, and tocopherols that have antioxidant, anti-inflammatory, antidiabetic, and antimicrobial activities. These biological activities have the potential to accelerate the wound healing process through reducing oxidative stress, inhibiting excessive inflammation, as well as stimulating cell proliferation and the formation of new blood vessels.

The use of kersen leaf ethanol extract in the form of cream is one of the promising approaches because it is able to provide direct contact between the active ingredient and the wound surface, thereby increasing local bioavailability. The cream formulation also provides optimal moisture to the wound area, maintains an environment that supports tissue regeneration, and makes application easier than oral forms. Although various studies have reported the antidiabetic and antioxidant activity of kersen leaves, studies on the effect of kersen leaf ethanol extract cream on the angiogenesis process in the healing of diabetic wounds based on histopathological evaluation are still relatively limited.

Based on histopathological data obtained in this study, angiogenesis was observed through the number of new blood vessels in the wound tissue at several healing time intervals, so that it can provide an overview of the effectiveness of kersen leaf ethanol extract cream in accelerating the tissue proliferation phase. The histopathological approach provides more objective morphological evidence of tissue changes than just macroscopic observations of wound closure.

Based on this description, this study aims to analyze the effect of kersen leaf ethanol extract cream (Muntingia calabura L.) on angiogenesis in the wound healing process of rats (Rattus norvegicus) in the diabetes mellitus model through histopathological evaluation. The results of this study are expected to provide scientific evidence regarding the potential of kersen leaf ethanol extract cream as a candidate for topical therapy based on natural ingredients to accelerate the healing of diabetic wounds, as well as enrich the development of phytopharmaceuticals in the field of chronic wound healing.

2. Research Methods

Research Design

This study is a laboratory experimental study using a Complete Random Design (RAL) with five treatment groups and three replications. The study aims to evaluate the effect of giving kersen leaf ethanol extract cream (Muntingia calabura L.) on angiogenesis in the wound healing process of mice in the diabetes mellitus model through histopathological examination.

Research Time and Place

The research was carried out in the Pharmacology Laboratory and the Histopathology Laboratory. All stages of research include diabetes induction, wound making, treatment, skin tissue extraction, histopathological preparations, and microscopic analysis carried out according to laboratory operational procedures.

Experimental Animals

The test animals used were male white rats (Rattus norvegicus) aged 8–12 weeks with a body weight of 180–250 g. The mice were kept under standard laboratory conditions with a temperature of 22–25°C, a relative humidity of 50–60%, a light–dark cycle of 12 hours, and were given standard feed and drinking water ad libitum. Before treatment, all animals were acclimatized for seven days.

Induction of Diabetes Mellitus

The diabetes mellitus model was created through intraperitoneal administration of streptozotocin (STZ) according to the study dose. Blood glucose levels were measured after induction, and rats with fasting blood glucose levels of ≥200 mg/dL were declared successful in developing diabetes mellitus and used as experimental animals.

Wound Making

After the diabetic model was formed, the mice were anesthetized using ketamine and xylazine as per the laboratory anesthesia dose. The back is shaved and sterilized using 70% alcohol. A round-shaped excision wound is made in the dorsal region using a sterile biopsy punch with a diameter of ±1 cm until it reaches the dermis layer.

Treatment

Experimental animals are divided into five treatment groups, namely:

1. K1 : Negative control (cream base)
2. K2 : Positive control (standard wound healing medication)
3. K3 : Low concentration kersen leaf ethanol extract cream (15%)
4. K4 : Medium concentration kersen leaf ethanol extract cream (10%)
5. K5 : High concentration kersen leaf ethanol extract cream (5%)

The cream is applied topically to the wound surface once a day until the end of the observation period.

Network Sampling

Wound skin tissue samples were taken on day 3, day 7, and day 14 after treatment. The tissue was fixed using a 10% Neutral Buffered Formalin (NBF) solution for 24–48 hours before being processed into a histological preparation.

Histopathological Preparation

The fixated tissue was processed through a staged dehydration stage using ethanol, clearing using xylol, paraffin infiltration, embedding, tissue cutting with a microtom 4–5 μm thick, then stained using the Hematoxilin-Eosin (HE) method. The preparations were observed using a light microscope with a magnification of 400×.

Angiogenesis Observations

Angiogenesis is observed based on the number of new blood vessels in granulated tissue in multiple fields of microscope view. The assessment was carried out using the following semi-quantitative scoring system:

Shoes	**Criteria**
0	No new blood vessels found
1	Angiogenesis ringan
2	Angiogenesis sedang
3	Angiogenesis tinggi

Observations were made from three points of view for each preparation, and, then the average score of angiogenesis score was calculated. Histopathological assessment is carried out independently by observers who do not know the treatment group (blind assessment). This scoring system corresponds to the histopathological data used in the study.

Data Analysis

The angiogenesis score data was first tested for normality using the Shapiro–Wilk test and the homogeneity of variance using the Levene's Test. If the data is normally distributed and homogeneous, the analysis is continued using One-Way Analysis of Variance (ANOVA) followed by a follow-up test of Tukey HSD to determine the differences between treatment groups.

If the data does not meet the parametric assumptions, the analysis is carried out using the Kruskal–Wallis test, then followed by the Mann–Whitney test using the Bonferroni correction as a follow-up test. All analyses were carried out using statistical software with a significance level of $p < 0.05$.

Research Ethics

All procedures for the use of experimental animals are carried out in accordance with the 3R (Replacement, Reduction, and Refinement) principles and follow the ethical guidelines for laboratory animal research. This research has been approved by the Animal Research Ethics Commission (include the ethics approval number if available).

3. Result and Discussion

Effect of Kersen Leaf Ethanol Extract Cream on Angiogenesis in Wound Healing of Diabetes Mellitus Model Rats

Histopathological observations were performed to evaluate angiogenesis during the wound healing process in diabetic mellitus model mice treated with kersen leaf ethanol extract cream (Muntingia calabura L.). Angiogenesis was observed based on the number of new blood vessels in the granulated tissue using preparations stained with Hematoxilin-Eosin (HE). Observations were carried out on day 3, day 7, and day 14 after treatment with a semi-quantitative scoring system.

In general, all groups experienced increased angiogenesis as the observation time increased. However, the group that received the kersen leaf ethanol extract cream showed better formation of new blood vessels than the control group. The difference began to be seen on day 7 and became more pronounced on day 14, when most of the treatment group showed higher angiogenesis scores.

On day 3, angiogenesis is still in its early stages so the number of new blood vessels is relatively small in the entire group. Most preparations show low to moderate angiogenesis scores that describe the onset of the tissue proliferation phase. The treatment group had shown a better tendency to form new blood vessels than the control group, although the difference was not yet clear.

On the 7th day, there is a fairly noticeable increase in angiogenesis. New capillary formation was seen more in the group given kersen leaf ethanol extract cream than in the control group. Granulated tissue looks more developed with better vascularization, suggesting that the administration of the cream is able to accelerate the tissue proliferation phase. This increase in the formation of new blood vessels indicates that the bioactive compounds in kersen leaf extract play a role in supporting the tissue regeneration process through increased oxygen and nutrient supply in the wound area.

On the 14th day, angiogenesis reaches a more mature condition. The treatment group showed new blood vessels that were better arranged than the control group. In this phase, the remodeling process begins to take place so that the vascularization formed is able to support the formation of more perfect connective tissue. The treatment group with a higher concentration of the cream showed a tendency to have the highest angiogenesis score compared to the other groups.

Overall, the results showed that the administration of kersen leaf ethanol extract cream was able to increase angiogenesis during the wound healing process in mice of the diabetes mellitus model. An increase in the formation of new blood vessels is seen gradually from day 3 to day 14, with the most noticeable development in the proliferation phase and the beginning of tissue remodeling.

Table 1. Average Angiogenesis Score in Wound Tissue of Rats (Rattus norvegicus) Diabetes Mellitus Model Fed Kersen Leaf Ethanol Extract Cream (Muntingia calabura L.)

Groups	Day 3	Day 7	Day 14
K1	1,56 ± 0,19	0,78 ± 0,38	1,56 ± 0,38
K2	1,44 ± 0,19	1,33 ± 0,58	0,56 ± 0,96
K3	0,89 ± 0,19	1,67 ± 0,88	0,78 ± 0,51
K4	1,11 ± 1,35	2,11 ± 1,02	0,89 ± 0,19
K5	0,78 ± 0,77	1,33 ± 0,33	0,89 ± 0,19

Remarks: The data are presented as an average ± standard deviation (mean ± SD), calculated from three repetitions in each group.

The data is presented as an average ± standard deviation. Statistical analysis was performed using the Shapiro–Wilk normality test and the Levene homogeneity test. Differences between groups were analyzed using One-Way ANOVA if the data were normally distributed and homogeneous, or the Kruskal–Wallis test if the parametric assumptions were not met. Further tests were carried out using Tukey HSD or Dunn to determine the difference between groups at the significance level of $p < 0.05$.

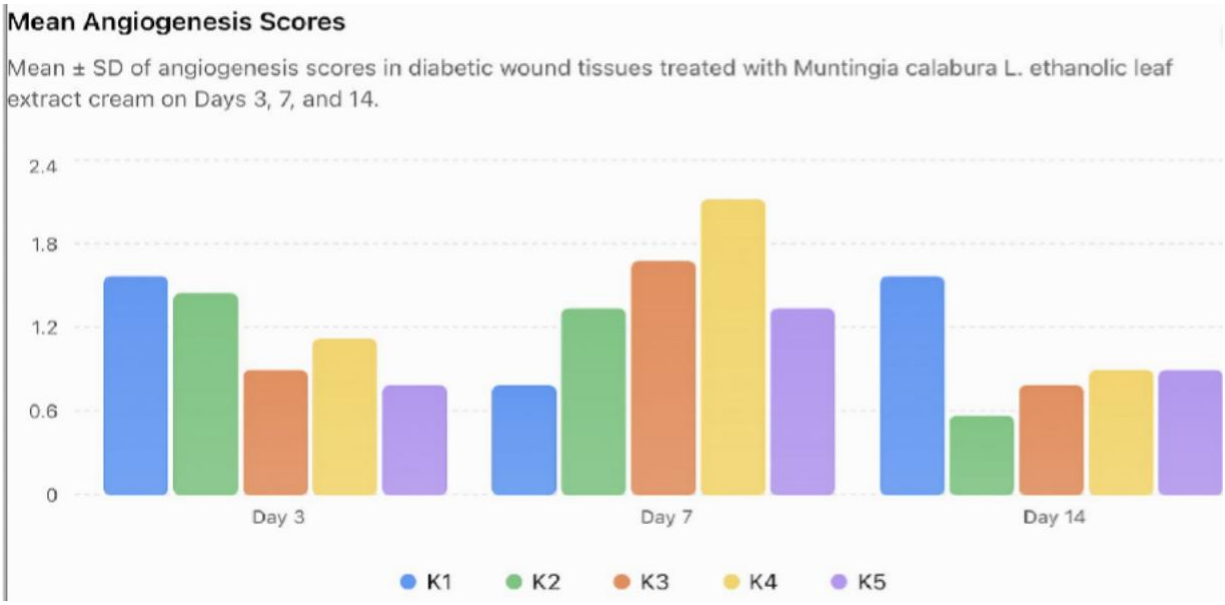

Figure 1. Average Angiogenesis Score Graph

Changes in the mean angiogenesis score in the treatment group during observations on day 3, day 7, and day 14. There was a tendency to increase angiogenesis in the proliferation phase (day 7), especially in the K4 group, then decrease in the remodeling phase (day 14).

Day 3 (H3)
On day 3, angiogenesis is still in the early phase of wound healing. Almost all of the groups showed angiogenesis scores of 0–2, which indicates that the formation of new blood vessels is still limited. This condition corresponds to the inflammatory phase, when the migration of inflammatory cells is still dominant and the formation of new capillaries has not taken place optimally. The treatment group did not show any noticeable differences compared to the control group because the process of tissue proliferation was only beginning.

Biologically, the formation of new blood vessels in this phase is influenced by the release of various growth factors such as VEGF (Vascular Endothelial Growth Factor), FGF (Fibroblast Growth Factor), and PDGF (Platelet-Derived Growth Factor). In diabetic mice, increased oxidative stress and hyperglycemia are known to inhibit the activity of these factors so that angiogenesis in the early phase takes place more slowly.

Day 7 (H7)
Observations on day 7 showed an improvement in angiogenesis scores in most treatment groups. Some preparations even get a score of 3, which indicates the formation of more than ten new blood vessels in the field of observation. This condition indicates that the lesion has entered a proliferation phase characterized by the formation of more active granulation and neovascular tissue.

The K4 group showed the highest tendency to angiogenesis scores compared to other groups. This shows that the treatment given is able to accelerate the formation of new blood vessels. Increased vascularity plays an important role in providing the oxygen, nutrients, and growth factors needed to support fibroblast proliferation, collagen synthesis, and epithelialization.
Histology, in this phase it will usually appear:

1. increase in the number of new capillaries,
2. the lumen of the blood vessels that begin to form,
3. proliferation sel endotel,
4. denser granulation tissue,
5. reduced infiltration of inflammatory cells.

Day 14 (H14)
On day 14, the angiogenesis score in most groups decreased compared to day 7. The decline is a physiological phenomenon that shows that the wound healing process has entered the remodeling phase. In this phase, the formation of new blood vessels begins to decrease as the tissue has obtained sufficient blood supply and the tissue regeneration process progresses towards maturation.

Although the number of new blood vessels is decreasing, the tissues that form have generally become more stable with increased collagen deposition as well as more regular reorganization of collagen fibers. Decreased angiogenesis in the remodeling phase indicates that the healing process takes place normally and is not accompanied by excessive vascularization formation.

Biological analysis
The pattern of angiogenesis changes obtained in this study follows the physiological stages of wound healing.

1. Day 3 → the inflammatory phase → low angiogenesis.
2. Day 7 → the proliferation phase → angiogenesis reaches its peak.
3. Day 14 → the remodeling phase → angiogenesis begins to decline.

This pattern shows that kersen leaf ethanol extract cream has the potential to accelerate the transition from the inflammatory phase to the proliferation phase so that the formation of new blood vessels takes place more optimally.

Discussion

Angiogenesis is one of the main processes in the proliferation phase of wound healing that functions to form new blood vessels to provide oxygen, nutrients, and various growth factors needed during tissue regeneration. In patients with diabetes mellitus, the angiogenesis process is often disrupted due to chronic hyperglycemia which increases the formation of reactive oxygen species (ROS), decreases vascular endothelial growth factor (VEGF) expression, and causes endothelial cell dysfunction. This condition results in a delay in the formation of granulated tissue and prolongs the wound healing process [42].

The results showed that the administration of kersen leaf ethanol extract cream (Muntingia calabura L.) gave a tendency to increase the angiogenesis score compared to the control group, especially on day 7 as the proliferation phase. In this phase, the formation of new blood vessels takes place more actively so that the wound tissue obtains sufficient oxygen and nutrient supply to support fibroblast proliferation, extracellular matrix synthesis, and collagen formation. On the other hand, on day 3 angiogenesis is still relatively low because the healing process is still in the initial inflammatory phase. Meanwhile, on the 14th day the angiogenesis score began to decline as part of the remodeling phase, when the blood vessels that had formed matured and some regressed according to the needs of the tissue. The pattern of change is in accordance with the physiological stages of wound healing which consists of the inflammatory, proliferation, and remodeling phases [40].

The increase in angiogenesis in the treatment group is thought to be related to the content of secondary metabolites in kersen leaves. Kersen leaves are known to contain flavonoids, phenolic compounds, tannins, saponins, triterpenoids, and tocopherols that have antioxidant and anti-inflammatory activities. Antioxidant activity plays a role in reducing oxidative stress due to hyperglycemia thereby protecting endothelial cells from oxidative damage. Reducing oxidative stress will increase the bioavailability of nitric oxide (NO) and improve endothelial function, so that the process of forming new blood vessels can take place more optimally.

In addition to being antioxidants, flavonoids are also known to increase VEGF expression and transforming growth factor-beta (TGF-β), two growth factors that play an important role in angiogenesis and the formation of granulated tissues. VEGF stimulates the proliferation, migration, and differentiation of endothelial cells to form new capillaries, while TGF-β plays a role in the formation of extracellular matrices as well as coordinating fibroblast activity during wound healing. Therefore, the increase in angiogenesis in the group given kersen leaf ethanol extract cream is likely the result of the synergy of various bioactive compounds acting on several molecular pathways simultaneously.

The triterpenoid compounds contained in kersen leaves are also thought to contribute to increased angiogenesis. Triterpenoids are able to inhibit the formation of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), so that the inflammatory phase does not last too long. Better control of inflammation allows the transition to the proliferation phase to take place more quickly, so that the formation of new blood vessels can occur earlier. Thus, the microenvironment in the wound tissue becomes more conducive to tissue regeneration.

The results of this study are in line with various previous studies that reported that flavonoid-rich plant extracts were able to increase angiogenesis in diabetic wounds through increased VEGF expression and decreased oxidative stress. Increased angiogenesis was then followed by increased fibroblast proliferation, collagen deposition, and accelerated epithelialization, so that the wound healing process took place faster than in the control group. This shows that angiogenesis is one of the important indicators in the success of wound healing therapy based on natural ingredients [43], [42].

However, this study has some limitations. The assessment of angiogenesis was carried out using a semi-quantitative histopathological scoring system so that it still depends on observer interpretation. In addition, this study has not evaluated the expression of molecular biomarkers such as VEGF, CD31, or basic fibroblast growth factor (bFGF) through immunohistochemical techniques or molecular analysis. The use of these biomarkers in future studies will provide stronger evidence regarding the mechanism of action of kersen leaf ethanol extract cream in increasing angiogenesis in the healing of diabetic wounds [15].

Overall, the results of this study show that kersen leaf ethanol extract cream (Muntingia calabura L.) has the potential to increase angiogenesis during the wound healing process in mice model diabetes mellitus. The increased formation of new blood vessels during the proliferation phase indicates that kersen leaf extract is able to improve the microenvironment of wound tissue and support tissue regeneration more effectively. These findings reinforce the potential of kersen leaves as a source of phytopharmaceuticals that can be further developed as topical therapies to accelerate the healing of chronic wounds in people with diabetes.

4. Conclusions

Kersen leaf ethanol extract cream (Muntingia calabura L.) exerts a positive influence on the angiogenesis process in the wound healing of rats (Rattus norvegicus) in the diabetes mellitus model. The increase in angiogenesis observed during the proliferation phase shows that kersen leaf extract is able to support the formation of new blood vessels that play a role in increasing the supply of oxygen and nutrients to the wound tissue, thereby supporting the tissue regeneration process. These findings suggest that kersen leaf ethanol extract cream has potential as a topical phytopharmaceutical candidate in diabetic wound healing therapy.

Further research needs to be carried out with molecular biomarker analysis and clinical trials to confirm the effectiveness and safety of the use of kersen leaf ethanol extract cream in the healing of chronic wounds.

Provide a clear scientific justification for the work and, where relevant, discuss its practical implications, potential applications, and contribution to knowledge. The conclusion may offer recommendations for future research by suggesting directions for further investigation or identifying areas that remain unexplored.

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