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The Effectivity of *Muntingia calabura* L. Leaf Extract Cream on Skin Incision Wound Healing Day 7 on White Rat (*Rattus Norvegicus*) with Diabetes Mellitus

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ABSTRACT

Wounds are damage to tissue integrity and function that occurs in the body. High blood glucose levels in diabetes mellitus can inhibit wound healing. This study aimed to determine the effectiveness of kersen leaf extract cream (Muntingia calabura L.) on the healing process of incision wounds on the skin of white rats (Rattus norvegicus) with diabetes mellitus on day 7. The experimental animal used were 12 male white rats weighing 150-200 g, aged 2-3 months old which were induced by streptozotocin 45 mg/kg BW intraperitonially. This research method used a completely randomized design (CRD) consisting of K1 as a negative control given a cream base and distilled water, K2 as a positive control given 0,1% silver sulfadiazine cream and metformin 4.5 mg/kg BW, K3 and K4 given 5% and 15% kersen leaf extract cream and 450 mg/kg BW kersen leaf extract orally. Incision wounds were made in the paravetebral area along 2 cm with a depth of 2 mm. Wound care was performed twice a day for 7 days. ANOVA test result showed that the administration of cream and kersen leaf extract significantly affected the number of fibroblast cells, collagen, and angiogenesis. The results showed that K1 was significantly different (P<0,05) from K2, K3 and K4. K3 and K4 groups had higher average numbers of fibroblast cells, collagen, and angiogenesis. The application of kersen leaf extract cream was able to increase the number of fibroblast cells, collagen, and angiogenesis so that it could accelerate the healing of incision wound on day 7 in white rats with diabetes mellitus

Keywords: *Angiogenesis, kersen leaf extract, collagen, wound, fibroblast cells*

INTRODUCTION

Skin is a vital and essential organ that lies superficially covering and protecting the body (Hasliani, 2019). One of the conditions that can cause skin damage is injury. Wound is a condition of damage to tissue function and integrity (Suriadi, 2007). When injured, the body will naturally perform a wound healing process through continuous cellular and biochemical activities (Purnama et al., 2017). Wound healing consists of the inflammatory stage, proliferation stage, and remodeling or maturation stage. The inflammatory stage begins at the onset of injury until the third day. The proliferation stage occurs on the fourth day characterized by an increase in fibroblast cells and angiogenesis until the seventh day. The maturation stage is the stage of wound healing that takes place over a long period (3-6 months to years) (Theoret, 2017).

The cells that play a role in wound healing are fibroblasts. Fibroblasts are the main collagen-producing cells such as type I collagen which plays a role in forming fibrosis, type III collagen which forms granulation tissue and type VIII collagen which integrates tissue. Fibroblast cells together with endothelial cells also produce vascular endothelial growth factor (VEGF) as the main growth factor in the angiogenesis stage (Masir et al., 2012). In diabetic conditions, oxygen levels in the blood are relatively less than normal, which could inhibit wound healing processes such as angiogenesis (Frisca et al.,

2009). Hyperglycemia decreases the wound healing process by inhibiting the perfusion of oxygen and nutrients through blood vessels, as well as affecting lymphocyte activity by reducing the ability of chemotaxis, diapedesis and phagocytosis which can increase infection (Hess, 2009).

Kersen (Muntingia calabura L.) is known as a plant that absorbs air pollution, has low economic value, grows wild in the tropics and has medicinal benefits (Sadino et al., 2022). The kersen plant is believed to contain phytochemical compounds that are efficacious as antioxidative, antimicrobial, antiseptic, anti-inflammatory, antidiabetic, antitumor, and also anti-gout (Zakaria et al., 2011). The chemical content of kersen leaves are flavonoids, saponins and tannins (Bamasri, 2021).

Based on the content of phytochemical compounds, kersen plants have the potential to accelerate the wound healing process and reduce blood sugar levels (Rahman et al., 2017). Therefore, research on the effectiveness of kersen leaf extract cream (Muntingia calabura L.) on day 7 incision wound healing on the skin of white rats (Rattus norvegicus) with diabetes mellitus is necessary.

MATERIALS AND METHODS

Ethical Approval

The research was conducted after obtaining approval to conduct research given by the Ethics Commission for the Use of Experimental Animals Faculty of Veterinary Medicine, Syiah Kuala Universitywith number 186/KEPH/XI/2022.

Research Procedure

This study was a laboratory analysis study using a one-way randomized complete design (RAL). A total of 12 mice were divided into 4 groups and each group consisted of 3 mice. The extraction process started with washing the kersen leaves, then drying them in the shade. The leaves collected were whole leaves, not brownish, and not too yellowish with various sizes (Syahara and Siregar, 2019). A total of 2 kg of dried kersen leaves were pulverized with a blender to form 600 g of simplicial powder.

Ground kersen leaves were put into a container along with the solvent. The maceration method was carried out using 96% ethanol solvent. The ratio between simplisia powder and solvent was 1:5. A total of 600 g of simplicial powder was put into the macerator along with 3 liters of 96% ethanol until fully submerged and macerated for five days with stirring once per day. The results obtained in the form of macerate will then be concentrated in a vacuum rotary evaporator with a temperature of 50 ℃ until a thick extract was formed. The extract was then processed into cream and oral preparations.

Preparation of kersen leaf extract cream is carried out with two phases, namely the oil phase with the formulation of stearic acid and cetyl alcohol put in a porcelain cup, then propyl paraben was added and dissolved on a waterbath. For the water phase, glycerin, propylenglycol, TEA, and distilled water were put into a beaker glass and then added with methyl paraben. The oil phase was poured into a mortar and stirred until homogeneous. The water phase was added little by little while stirring slowly until a creamy mass was formed. Kersen leaf extract was then added to the cream mass and stirred until homogeneous. Oral preparation of kersen leaf extract was conducted by adding kersen leaf extract into 1% CMC-Na solvent.

The glucose level of the rats was examined 3 times, before streptozotocin induction, 3 days after streptozotocin induction and before sample collection. The glucose level was examined using GlucoDr

procedure, the tip of the rat's tail was stabbed with a GlucoDr lancet to collect the blood, and then the blood was dripped onto a GlucoDr strip. After 11 seconds the blood sugar level in mg/dL was displayed. Rats with blood sugar levels ≥ 250 mg/dl were categorized as hyperglycemia (Purnamasari et al., 2014). Before treatment, rats were adapted for approximately 2 weeks so that rats had the same body weight ranging from 150-200 g. Before injecting streptozotocin, the rats were placed in a cage and first fed for 12 hours (Saputra et al., 2018). Then all rats were induced with streptozotocin intraperitoneally at a dose of 45 mg/kg BW (Islam and Loots, 2009). Streptozotocin injection at a dose of 45 mg/kg had the highest success rate of 100% (Zhang et al., 2008).

Incision wounding was performed after the rats developed diabetes mellitus with blood sugar levels ≥ 250 mg/dl. White rats were anesthetized by injecting ketamine and xylazine with a dose of ketamine 40 mg/kg body weight and xylazine 5 mg/kg body weight intramuscularly in the femoral musculus. After the rats were anesthetized, the dorsal fur of the rats was shaved. Then the rat skin was sterilized using 70% alcohol and 2% iodine tincture. An incision wound was made in the paravertebral region 2 cm long and subcutaneous in depth. After that, treatment was carried out for 7 days according to the treatment group. K1 was given cream base (negative control), K2 was given silver sulfadiazine 0.1% cream and metformin at a dose of 4.5 mg/kg BW (positive control), K3 and K4 were given oral extract of kersen leaves at a dose of 450 mg/kg BW and 5% and 15% kersen leaf extract cream, respectively.

Necropsy of the rat skin was carried out 1 day after the last treatment, namely on day 8. The euthanasia method chosen was cervical dislocation. The skin was collected by cleaned it from fur, then the skin was cut in the paravertebral area with a thickness of ± 3 mm to subcutaneous with an area of 1-1.5 cm2. Furthermore, skin specimens were cleaned with 0.9% NaCl and fixed with 10% neutral buffer formalin (BNF) solution for 18-24 hours. Then the samples were placed in a tissue basket and labeled. After that, the skin samples were dehydrated using graded alcohol 80%, 90%, 95%, absolute alcohol I and absolute alcohol II for ± 2 hours. Skin specimens were put into xylol solution (I, II and III) for 1 hour each for impregnation, also known as the clearing process (Murti et al., 2018). Then proceed with infiltration in paraffin I, II and III at 60 oC for 1 hour each. The skin sample is then embedded in paraffin and tissue blocking. After blocking, sectioning was performed using a microtome with a thickness of 4-5 microns. The samples were placed on glass objects that have been smeared with adhesive materials such as polysilane. Specimens were incubated in an incubator at 56-58 oC to remove paraffin. Then, the skin specimens were stained with HE stain to observe the wound healing process of skin tissue on the 7th day.

Staining of skin specimens begins with the process of removing paraffin or paraffinization using xylol three times for 2 minutes each. Then rehydration was carried out by introducing water into the tissue using alcohol solution with decreasing concentration (absolute, 95%, 90% and 80%) for 5 minutes each and rinsed with running water for 10 minutes. After that, the tissue was stained with hematoxylin staining for 5 minutes and rinsed with water again for 10 minutes. Furthermore, it was stained with eosin for 2 minutes followed by using graded alcohol solutions, clearing with xylol and ending with closing the slide using cover glass (mounting) using Entellan® adhesive material.

Histopathological observations were carried out on the healing process of incision

wounds on the skin of rats on the 7th day using a light microscope (Olympus CX31) with a magnification of 100 times and 400 times, followed by taking micrograph photos with software toupview program and image J. Histopathological parameters observed were the number of fibroblast cells, collagen and angiogenesis formed on the 7th day incision wound healing in the skin of white rats with diabetes mellitus. Histopathology scoring parameter for collagen density was performed based on the calculation of 5 fields of view, at 400x magnification object. The score for collagen observation was as follows:

0 = No collagen found

+1 = Low collagen density (less than 10% per field of view)

+2 = Medium collagen density (10% - 50% per field of view)

+3 = Dense collagen (50% - 90% per field of view)

+4 = Collagen density is very tight (90% - 100% per field of view) (Rizka et al., 2013).

Data Analysis

Data were analyzed using Analysis of variance (ANOVA) and continued with Duncan's test at 5% significance level.

RESULTS AND DISCUSSION

Fibroblast Cells

The average number of fibroblast cells in all treatment groups of K1 cream base, 0.1% silver sulfadiazine cream K2, 5% kersen leaf extract cream K3, and 15% kersen leaf extract cream K4 respectively are 98.40 ± 18.97a, 140.66 ± 12.07b, 135.53 ± 6.35b, 143.20 ± 3.99b can be seen in table 1.

Table 1. Mean and standard deviation of fibroblast cell counts.

Treatment Groups	Average fibroblast cell count
K1	98.40 ± 18.97^{a}
K2	140.66 ± 12.07^{b}
K3	135.53 ± 6.35^{b}
K4	143.20 ± 3.99^{b}

Notes: Different superscript letters in the same column indicate significant differences (P<0.05); K1: Negative control group: oral administration of cream base and distilled water; K2: Positive control group: administration of silver sulfadiazine 0.1% cream and metformin dose 4.5 mg/kg BW orally; K3: Treatment group I: topical administration of 5% kersen leaf extract cream and oral administration of 2 ml kersen leaf extract at a dose of 450 mg/kg BW; K4: Treatment group II: topical administration of 15% kersen leaf extract cream and oral administration of 2 ml of kersen leaf extract at a dose of 450 mg/kg BW.

Based on ANOVA test of Completely Randomized Design (CRD) unidirectional pattern showed that the treatment of cream base, silver sulfadiazine cream 0.1%, kersen leaf extract cream 5%, and kersen leaf extract cream 15% significantly affected (P <0.05) the number of fibroblast cells formation during treatment. Duncan test results showed a significant difference (P< 0.05). K1 group was significantly different from K2, K3 and K4 groups, but between groups K2, K3 and K4 were not

significantly different (P>0.05). Histopathological picture of fibroblast cells healing incision wounds on day 7 of post-treatment diabetes mellitus white rat skin can be seen in Figure 1.

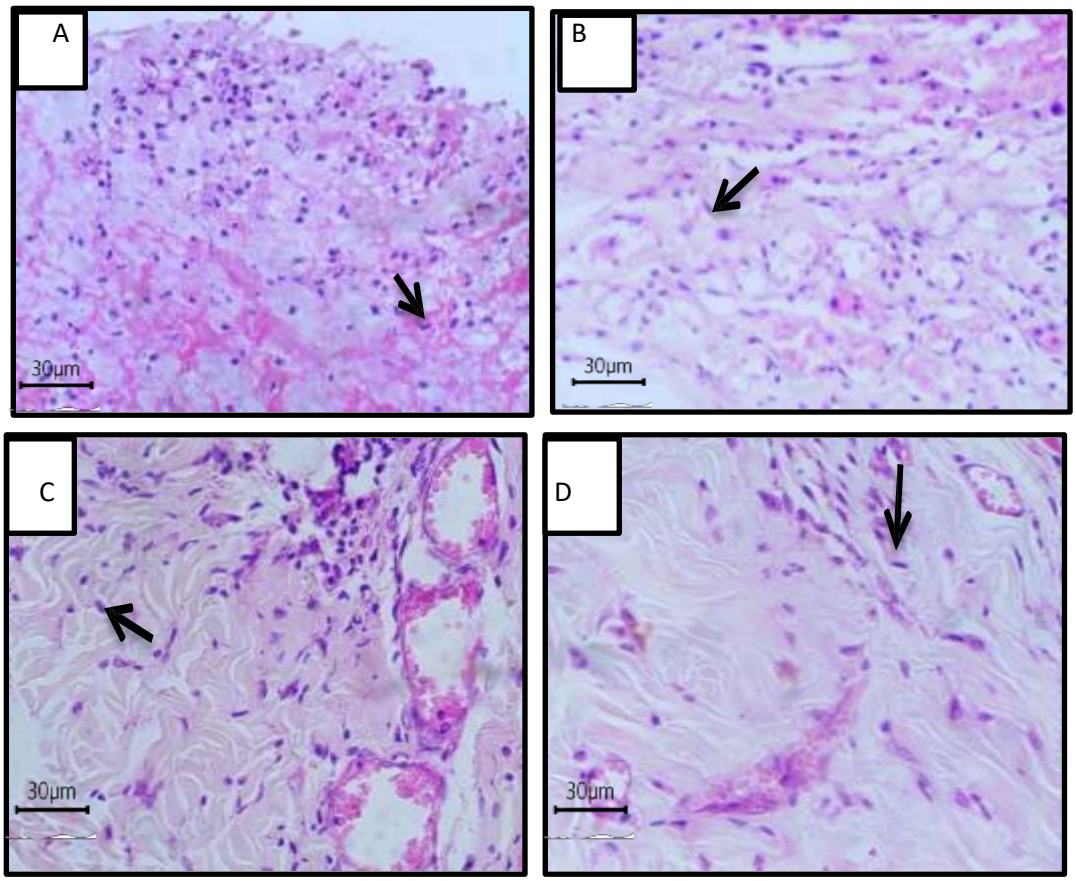

Figure 1. Microscopic image of fibroblast cells in the skin of white rats with diabetes mellitus day 7 with HE staining. A). K1, topical administration of cream base, few fibroblast cells (negative control);. B) K2, topical administration of sulfadiazine ointment 0.1%, fibroblast cells were many (positive control);. C) K3, topical administration of 5% kersen leaf extract cream, many fibroblast cells. D) K4, 15% kersen leaf extract cream, numerous amounts of fibroblast cells (400x).

Based on histopathological observations on day 7, the administration of only base cream (K1) on the wound area showed fewer fibroblast cells than the administration of 0.1% silver sulfadiazine cream (K2). K2 had more fibroblast cell compared to K3, but less fibroblast cells compated to K4. Between treatment groups, K4 had the highest fibroblast cells than K3 (Figure 1).

Kersen leaves have several pharmacological activities including antioxidant, antidiabetic, antibacterial, antihelmintic, anti-inflammatory, and antihyperlipidemia. These pharmacological activities are influenced by several secondary metabolites of kersen leaves in the form of flavonoids, alkaloids, tannins, saponins and terpenoids, where flavonoids are the main content (Sadino et al., 2022). In wound healing process, flavonoids can accelerate the migration and proliferation of fibroblast cells to the wound area and increase collagen synthesis for the wound epithelialization process (Ricardo et al., 2015). Flavonoids also play role in

increasing the number of fibroblast cells because they contain anthocyanins. Anthocyanins are flavonoid compounds found in the form of glycosides that have the effect of increasing collagen synthesis by fibroblast cells (Kunnaryo and Wikandari, 2021).

Saponins will activate the function of Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF), Transforming Growth Factor β (TGF- β) and Epidermal Growth Factor (EGF) in order to stimulate fibroblast cell proliferation and migration (Kimura, et al., 2006). Tannin also functions to induce (TGF-β) for fibroblast cell proliferation and increase macrophage migration through lymphokine induction (Fakim,

2008).

The fibroblast cells that are formed will move towards the opening and synthesize the basic substance of collagen in large compositions to fill the wound area and accelerate wound closure (Irwandi et al., 2022). Fibroblast cells are the main unit in the proliferation phase that appears on day 3 and reaches a peak on day 7 so that the number of fibroblast cells becomes a parameter in wound healing. Fibroblast cells then synthesize collagen which plays a role in holding the wound together, assisting the re-epithelialization stage, proliferating and migrating to form new connective tissue, and producing collagen which affects the strength of the wound healing site and tensile strength. Increased macrophage cells in the wound area affect fibroblast cell proliferation, connective tissue and epithelialization because macrophage cells produce growth factors such as EGF, FGF, PDGF and TGF-β which play a role in the proliferation of fibroblast cells and blood vessels (Murti et al., 2018).

Based on the results, the distribution of fibroblast cells was lower in the negative control compared to the positive control and treatment because the cream base only contains no bioactive compounds that act as anti-inflammatory component resulting in the inflammatory phase becoming longer so that the proliferation phase also lasts long due to the inhibition of fibroblast cell formation (Salim et al., 2019). Positive control (K2) and treatment groups (K3 and K4) statistically showed no difference because silver sulfadiazine 0.1% is a gold standard topical drug of the sulfonamide group to prevent bacterial folic acid synthesis because it has antibacterial properties. Silver sulfadiazine 0.1% can also induce macrophage cells to release growth factors and cytokines in accelerating the wound healing process (Esfahani et al., 2012). Silver sulfadiazine has a positive effect on fibroblast cell proliferation as a producer of collagen and fibronectin (Fuadi et al., 2015). In K3 and K4 treatment groups, there was an increase in the number of fibroblast cells due to secondary metabolites contained in kersen leaf extract which have an influence on the wound healing process. The number of fibroblast cells is higher than the control, especially in the 5% and 15% concentrations, indicating the density of collagen and fibroblast cells equivalent to standard drugs.

Collagen

The average number of collagen scores in all treatment groups of cream base (K1), silver sulfadiazine 0.1% cream (K2), kersen leaf extract cream 5% (K3), and kersen leaf extract cream 15% (K4) were 36.08 ± 3.09a, 50.51 ± 6.44b, 55.67 ± 7.78b, 57.73 ± 3.09b, respectively (Table 2).

Table 2. Mean and standard deviation of collagen fibre density score

Treatment Groups	Average number of collagen scores
K1	36.08 ± 3.09^{a}
K2	50.51 ± 6.44^{b}
K3	55.67 ± 7.78^{b}
K4	57.73 ± 3.09^{b}

Notes: Different superscript letters in the same column indicate significant differences (P<0.05); K1: Negative control group: topical administration of cream base and distilled water orally; K2: Positive control group: topical administration of 0.1% silver sulfadiazine cream and metformin dose of 4.5 mg/kg BW orally; K3: Treatment group I: topical administration of 5% kersen leaf extract cream and oral administration of 2 ml kersen leaf extract at a dose of 450 mg/kg BW; K4: Treatment group II: topical administration of 15% kersen leaf extract cream and oral administration of 2 ml kersen leaf extract at a dose of 450 mg/kg BW.

Based on the analysis of variance (ANOVA) test, the results showed that the all groups had significant effects (P<0.05) on the number of collagen scores. Duncan test results showed a significant difference (P <0.05) of K1 and other groups. However, among groups K2, K3 and K4, there were not significantly different (P>0.05). The histopathological image of collagen density of incision wound healing on the 7th day can be seen in Figure 2.

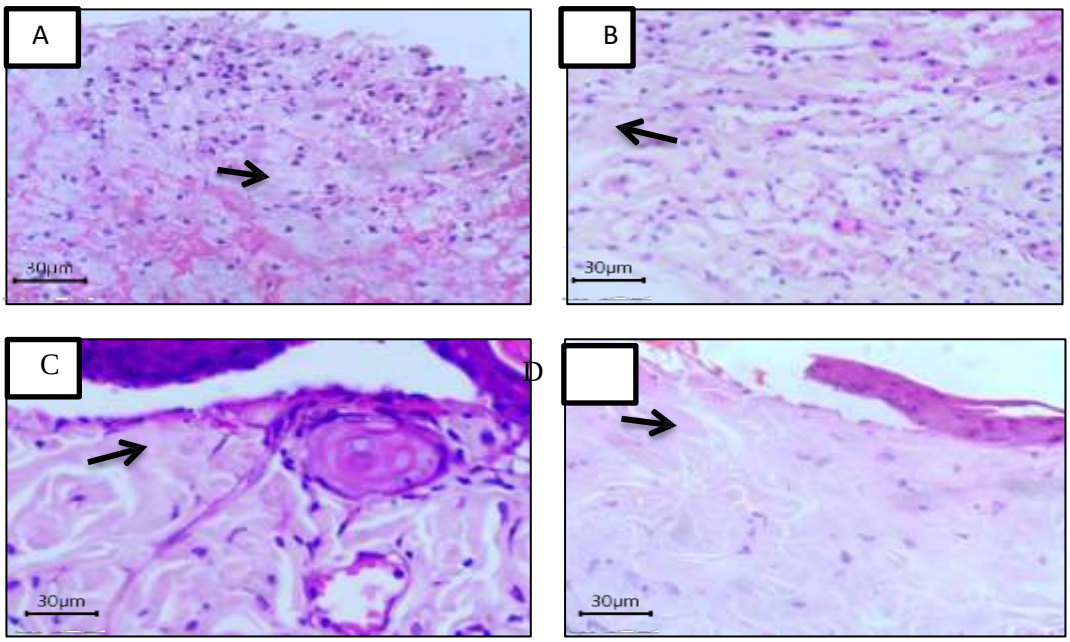

Figure 2. Microscopic image of collagen on the skin of white rats with diabetes mellitus day 7 with HE staining. A). K1 had low collagen production, B) K2 had moderate collagen production, C) K3 had dense collagen production, D) K4 had highly dense collagen production (400x).
K1: cream base
K2: sulfadiazine ointment 0.1%
K3: extract kersen cream 5%
K4: extract kersen cream 15%

Based on histopathological observations on day 7, the administration of base cream on the wound area showed the lowest number of collagen scores compared to other group
(Figure 2). Collagen is the main protein that makes up the extracellular matrix of the skin and is widely distributed in the body. Collagen plays a very important role in every wound healing. Collagen will be formed on the 3rd day after the wound, will be clearly visible on the 7th day, will be stable and regular on the 14th day (Sumbayak, 2015). Normal scarring will result from the balance between collagen synthesis and degradation. From the collagen score based on the results of the study (Table 2), it can be seen that K4 had the highest score compared to K1, K2, and K3. Collagen comes from fibroblast cells, therefore the acceleration of collagen growth is caused by the growth of relatively more fibroblast cells in K4. The growth of fibroblast cells is influenced by the content of secondary metabolites, namely flavonoids, saponins and tannins contained in kersen leaf extract.

Flavonoids in kersen leaf extract will activate macrophages to increase the secretion of TGF-β (Transforming Growth Factor-β) to trigger the proliferation of fibroblast cells that will produce large amounts of collagen and stimulate endothelial cells for the formation of new blood
vessels (Giri et al., 2021). Flavonoids are able to accelerate wound healing by increasing the rate of wound contraction, accelerating the epithelialization period, increasing collagen production, and granulation tissue formation (Muralidhar et al., 2013). In addition, saponins also play a role in increasing monocyte proliferation which causes an increase in macrophages to secrete growth factors in the formation of fibroblast cells so that the formation of new fibroblast cells stimulated by saponins also increases the amount of collagen synthesized by fibroblast cells (Sucita et al., 2019). Tannin compounds contained in kersen leaf extract function to increase collagen, epithelium, and angiogenesis for wound closure so that tannins play a role in accelerating the wound healing process (Palumpun et al., 2017).

Angiogenesis

The average number of angiogenesis scores for K1, K2, K3, and K4 were 4.20 ± 0.87a, 7.60 ± 2.40b, 6.70 ± 0.90b, 8.60 ± 0.60b respectively as shown in table 3.

Table 3. Mean and standard deviation of angiogenesis counts

Treatment Groups	Average collagen scores
K1	4.20 ± 0.87^{a}
K2	7.60 ± 2.40^{b}
K3	6.70 ± 0.90^{b}
K4	8.60 ± 0.60^{b}

Different superscript letters in the same column indicate significant differences ($P < 0.05$); K1: Negative control group: topical administration of cream base and distilled water orally; K2: Positive control group: topical administration of 0.1% silver sulfadiazine cream and metformin dose of 4.5 mg/kg BW orally; K3: Treatment group I: topical administration of 5% kersen leaf extract cream and oral administration of 2 ml kersen leaf extract at a dose of 450 mg/kg BW; K4: Treatment group II: topical administration of 15% kersen leaf extract cream and oral administration of 2 ml kersen leaf extract at a dose of 450 mg/kg BW.

Based on analysis of variance (ANOVA) showed that the treatment of cream base, silver sulfadiazine cream 0.1%, kersen leaf extract cream 5%, and kersen leaf extract cream 15% had a significant effect (P <0.05) on the amount of angiogenesis for the four treatment groups. Duncan test results showed a significant difference (P < 0.05). Group K1 was significantly different from groups K2, K3, and K4, but among groups K2, K3 and K4 were not significantly different (P>0.05). The histopathological image of angiogenesis of incision wound healing on the 7th day of post- treatment diabetes mellitus white rat skin can be seen in Figure 3.

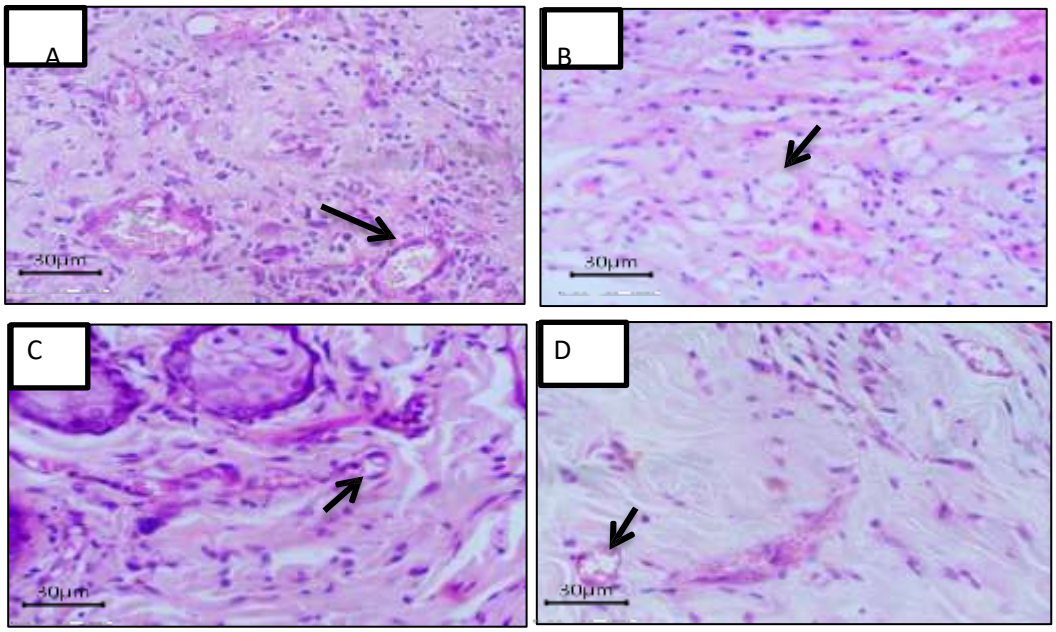

Figure 3. Microscopic image of angiogenesis in the skin of white rats with diabetes mellitus day 7 group A) Negative control; administration of cream base (K1) moderate angiogenesis. B) Positive control; administration of sulfadiazine ointment 0.1% (K2) angiogenesis very much. C) K3, administration of 5% kersen leaf extract cream, high numbers of angiogenesis D) K4, 15% of kersen extract cream, angiogenesis was higher . HE staining (400x).

Based on Figure 3, K1 had the lowest angiogenesis, and K4 had the highest amount of angiogenesis compared to other groups.

Angiogenesis is a physiological process in which new blood vessels will form from existing blood vessels and aims to meet the metabolic needs of cells, namely oxygen and nutrients in injured tissues. The process of angiogenesis is stimulated by several growth factors including VEGF, PDGF, FGF, and TGF-β (Stella and Wahyuningsih, 2021). Angiogenesis is formed through the degradation of old or damaged blood vessels by activating the production of vascular progenitor cells that will migrate and stimulate angiogenesis. Vascular endothelial growth factor (VEGF) produced by macrophages affects the migration of angiogenesis progenitor cells. Vascular endothelial growth factor (VEGF) plays a role in angiogenesis. In the early phase of hypoxic wounds angiogenesis will increase and decrease after neovascularization is complete and perfusion in the wound area is normal (Nofikasari et al., 2016).

The low angiogenesis rate in the negative control (K1) is due to the provision of a cream base which does not contain bioactive compounds that act as anti-inflammatory so that the inflammatory

phase lasts long, this results in low angiogenesis formation in the wound (Salim et al., 2020). The rate of angiogenesis in the positive control (K2) is given sulfadiazine which contains silver and sulfadiazine compounds so that it can stimulate macrophages in producing growth factors for wound recovery so as to stimulate the formation of angiogenesis in the wound recovery process. Silver sulfadiazine has a positive influence on angiogenesis proliferation (Han et al., 2007). The rate of angiogenesis in K3 and K4 treatments has the highest average because the kersen plant contains secondary metabolite compounds in the form of flavonoids and saponins that will increase the activation of macrophages to trigger growth factors. Flavonoids have a vasodilator effect that can facilitate blood flow. Saponins and tannins each have hemostasis and vasoconstrictive effects on capillary blood vessels (Salim et al., 2020).

CONCLUSION

Based on the results of the study, it can be concluded that the administration of kersen leaf extract cream is effective on incision wound healing on day 7 on the skin of white rats with diabetes mellitus indicated by the presence of high fibroblast cells, collagen and angiogenesis counts which will accelerate the wound healing process.

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