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Topical Administration of Red Gedi Leaves (*Abelmoschus Manihot L. Medik*) Gel Extract Increases Neovascularization, Number of Fibroblasts and Re-Epithelialization on Wounds in Diabetic Rats

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ABSTRACT

Flavonoids (quercetin), saponins, tannins, alkaloids and phenols (gallic acid) in red gedi leaves (Abelmoschus manihot L. Medik) can accelerate wound healing which is often found in diabetes mellitus. This was an experimental randomized post-test only control group design using 30 male Wistar rats with diabetes mellitus that were divided into 6 groups. On the 4th and 14th days after treatment, skin tissue samples were taken, histology preparations were made using Hematoxilin-Eosin (HE) staining to examine neovascularization, number of fibroblasts and re-epithelialization. Comparative analysis showed that the average neovascularization on the 4th day of the treatment I group was higher than treatment group II and the lowest was in the control group. Meanwhile, on 14th day, treatment group I had the highest neovascularization rate (p<0.001). The average of fibroblasts in treatment group I on the 4th day higher than treatment group II and the lowest in the control group. Meanwhile, on 14th day, treatment group I had the highest fibroblast average (p<0.001). The mean re-epithelialization on 4th day was highest in treatment group I and lowest in the control group. Meanwhile, on the 14th day, treatment group I had the highest re-epithelialization average (p<0.001). In conclusion, administration of red gedi leaf ethanol gel extract with concentrations of 12.5% and 25% increased neovascularization, number of fibroblast cells and re-epithelialization in wound healing of male Wistar rats with diabetes mellitus, with a concentration of 12.5% being better for all parameters.

Keywords: anti-aging; red gedi leaf (Abelmoschus Manihot L. Medik); neovascularization; fibroblast, re-ephithelization

INTRODUCTION

Aging is a natural process that occurs in the human life cycle. In the aging process, the function of various body organs decreases or even stops, which causes various signs and symptoms of the aging process to appear. Aging occurs due to the influence of factors from within the body (internal factors) and from outside (external factors). Internal factors include free radicals, decreased hormones, glycosylation processes, methylation, apoptosis, decreased immune system and genes. Meanwhile, external factors include unhealthy lifestyles, unhealthy diets, wrong habits, environmental pollution, stress and poverty (Pangkahila, 2019).

Diabetes is often considered a biological model of premature aging, because in diabetes a glycosylation process occurs where glucose may combine with dehydrated proteins, then causing disruption of the body's organ systems (Pangkahila, 2019).

Diabetes is a group of metabolic disorders characterized by the presence of hyperglycemia without treatment, the heterogeneous etiopathology of which includes defects in insulin secretion, insulin action or both (WHO, 2020). Long-term complications of diabetes are cardiovascular disease, retinopathy, nephropathy, neuropathy. Neuropathy in the feet, ultimately causes diabetic feet, foot ulcers, and even amputation (WHO, 2016).

Characteristics of aging and diabetes are hyperglycemia, which is caused by insulin resistance or insulinopenia in diabetes, and reduced regeneration capacity of pancreatic β cells in old age. High glucose in in vitro systems directs premature senescence in some cell types, causing accelerated replication leading to late replication exhaustion and oxidative stress.

Accelerated replication promotes the accumulation of oxidized proteins (in human fibroblasts) leading to the formation of AGEs, which directly induce cellular senescence. AGEs trigger NF-κB activation via RAGE to exacerbate wound inflammation, and induce oxidative stress through various pathways. Interestingly, hyperglycemia-induced production of AGEs, contributes to various age-related comorbidities, such as reduced tissue elasticity. Furthermore, AGEs cause extensive cellular damage through protein cross-linking and ROS, and promote the pathobiochemistry of many diabetes complications (Wilkinson and Hardman, 2021).

Wounds in diabetes are caused by uncontrolled blood sugar, infection of the feet followed by high pressure on the nerves of the feet, nerve deformities and the inability to move the feet, allowing an increase in the incidence of severe foot ulcers (Mariadoss *et al*., 2022). Diabetic wounds are chronic wounds, which do not undergo a normal healing process but are trapped in the chronic inflammatory stage because hypoxia occurs which is caused by two factors, limited oxygen supply and high oxygen consumption in the wound. Oxygen supply to the wound is limited due to vascular dysfunction and neuropathy. High oxygen supply by wound cells during inflammation, induces hypoxia in the wound that strengthens the inflammatory response, and prolongs the injury by increasing oxygen radical levels. (Bai *et al*., 2020).

The wound healing phase consists of hemostasis, inflammation, proliferation, and remodeling (Raziyeva *et al*., 2021). The proliferation process begins in the lesion microenvironment within the first 48 hours and can progress until the 14th day after the appearance of the lesion. In the proliferation phase, neovascularization (angiogenesis), fibroplasia and reepithelialization occur, and (Gonzalez *et al*., 2016). Neovascularization or the formation of new blood vessels occurs through the processes of vasculogenesis and angiogenesis. Where vasculogenesis is new vascularization without previous blood vessels, while angiogenesis is new vascularization from pre-existing blood vessels. In wound repair, what occurs more often is the angiogenesis process (Moreira and Marques, 2022). Neovascularization is essential for wound healing. It is required for nutrient delivery and maintenance of oxygen homeostasis, to allow cell proliferation and tissue regeneration (Rodrigues *et al*., 2019). Fibroplasia is the proliferation and differentiation of fibroblasts into myofibroblasts, deposition of extracellular matrix, and wound contraction (Dorantes and Ayala, 2019). Re-epithelialization occurs migration, proliferation, and differentiation of keratinocytes, to produce more cells for wound closure, rebuilding damaged basement membranes, and ultimately reconstructing the skin barrier. In this process keratinocytes interact with fibroblasts, endothelial cells, immune cells, growth factors and cytokines (Tan and Dosan, 2019).

Impaired wound healing is caused by hypoxia, nutrition, infection, stress, age, sex hormones, chronic diseases (such as diabetes), drugs, smoking, alcohol, and genetic predisposition (Gushiken *et al*., 2021). Diabetes causes vascular disease through molecular changes, the deposition of glycation products that stiffen blood vessels through increased basement membrane permeability, which disrupts the basic processes of wound healing. Increased vascular resistance with changes in vessel diameter, leads to increased tissue ischemia and microvascular sedimentation at the capillary level. The combined effects of these disorders make diabetes patients fragile, and susceptible to infection, amputation, and delayed wound healing (Stiehl, 2020).

The wound healing process can be helped by both chemical and natural treatments (Canpolat and Başa, 2017). Natural treatment is assisted by using medicines from natural ingredients such as medicinal plants (Lawal *et al*., 2021). The ingredients in medicinal plants have been researched to have significant effects on wound healing, skin tissue regeneration and therapeutic applications (Barku, 2019). The antidiabetic effects of plants are attributed to mixtures of phytochemicals or single components of plant extracts. The phytochemicals responsible for antidiabetic properties are mainly flavonoids, saponins, tannins, alkaloids and phenolic acids (Bahare *et al*., 2019) which are mostly contained in red gedi leaves (Samantha and Almalik, 2013).

Red gedi leaves are known to contain antioxidants and active compounds such as flavonoids (quercetin), tannins, phenols, saponins and alkaloids (Integrated Research Laboratory, Faculty of Mathematics and Natural Sciences, Udayana University, 2023). Compounds in this plant can lower blood glucose, are anti-diabetic, anti-inflammatory, anti-microbial, anti-septic, antioxidant, and play a role in healing burns and ulcers (Selvaraj, Subramanian and Samuel, 2020).

METHODS
This was experimental research with post-test-only control group design. This research has been approved by the ethics commission of Udayana University, Bali, (B/278/UN14.2.9/PT.01.04/2022).

A. Red Gedi Leaves Extract and Base Gel Processing
The 12.5% and 25% red gedi leaves ethanol extract gel formulation is red gedi leaves ethanol extract with a concentration of 12.5% (12.5 grams of red gedi leaves ethanol extract in a total mixture of 100 grams of gel base), and 25% (25 grams of red gedi leaves ethanol extract in a total mixture of 100 grams of gel base). The base gel is a base cream (1% Carbomer 940, 5% Propylene glycol, 0.81% Triethanolamine, 0.8% Methylparaben, 25% Glycerin). Red gedi leaves extract 12.5%, 25% and base gel were made at the Integrated Laboratory Unit, Faculty of Medicine, Udayana University, Bali.

B. Experimental Animals
The experimental animals in this study were healthy male Wistar rats (*Rattus norvegicus*), 2 to 3 months old weighing 180 to 200 grams from the Integrated Laboratory Unit, Faculty of Medicine, Udayana University, Bali.

This study used 30 experimental animals randomly divided into 6 groups: namely 2 control groups (P0) which were given gel base, 2 treatment groups I (P1) which were given 12.5% red gedi leaf extract gel and 2 treatment groups II (P2) which were given 25% red gedi leaf extract gel for 4 and 14 days. The number of neovascularization, fibroblasts and re-epithelialization were observed. Experimental animals were kept in special cages at the Integrated Laboratory Unit, Faculty of Medicine, Udayana University, Bali.

Rats were made diabetic with injection of 230 mg/kg body weight of nicotinamide (NA) intraperitoneally, which was done 15 minutes before administering 65 mg/kg body weight of streptozotocin (Furman, 2021), but in this study, the researchers reduced the dose by considering the condition of the rats and the risk of death, so the STZ dose given was 40 mg/kg intraperitoneally (Asali, 2021). Fasting blood glucose levels were examined before and the day after STZ-NA induction (Sayeli and Shenoy, 2021). Examination of fasting blood glucose level was done with a Nesco brand glucotest by taking blood from the tails (Asali, 2021). After 4 days of STZ and NA induction (Sayeli and Shenoy, 2021), rats with fasting blood glucose levels > 150 mg/dl (8.3 mmol/L) were used for research (Furman, 2021).

The rats were anesthetized with 10% ketamine (40-50 mg/kg), xylazine (2.5-8 mg/kg) and acepromazine (0.75-4 mg/kg) intramuscularly before wound incision (Flecknell *et al.*, 2015). The dose of ketamine given in this research was 25-33.5 mg/kg after assessing the rats. The wound was made using a 0.5 cm disposable punch biopsy on the back to obtain a full thickness wound (Brubaker, Carter and Kovacs, 2015).

The control group was given 0.1 g of base gel 3 times a day for 4 days and 14 days for the 4th day and 14th day control group respectively. In treatment group 1, 0.1 g of 12.5% red gedi leaves ethanol extract was given 3 times a day for 4 days and 14 days for the 4th day and 14th day control group respectively. In treatment group 2, 0.1 g of 25% red gedi leaves ethanol extract was given 3 times a day for 4 days and 14 days for the 4th day and 14th day control group respectively. The gel was applied directly to the wound evenly at 09.00, 13.00 and 17.00 WITA.

On 4th and 14th day, the rats were anesthetized to take samples from the wound tissue by making an excisional wound using a scalpel with a diameter of 1.5 cm thickness ± 5 mm. The skin obtained was then fixed with 10% neutral buffer formalin solution with a penetration speed of 1 mm/hour and left at room temperature for ± 24 hours.

An examination was carried out to see neovascularization, number of fibroblasts, and epithelialization histologically, with Hematoxylin-Eosin (HE) staining.

C. Histological Examination
• *Neovascularization Calculation*
Neovascularization was calculated from the number of new blood vessels in injured dermis area, in the form of an endothelium with a very thin intima (Prastica, 2020). The number of new blood vessel was counted, shifted from left to right until 3 fields of view were obtained, using an Olympus CX-21 microscope connected to an Optilab Viewer 2.2, then saved in J-PEG, with magnification 400x, then the number of fibroblasts was counted using Image Raster 3. The calculation results were then added up and divided by 3 to get the average neovascularization per field of view.

• *Fibroblast Count*
The number of fibroblasts was calculated by counting the number of fibroblast cells in injured dermis area (Prastica, 2020), shifted from left to right 3 fields of view were obtained, using an Olympus CX-21 microscope connected to the Optilab Viewer 2.2 tool, then saved in J-PEG, with 400x magnification, then the number of fibroblasts was counted using Image Raster 3. The calculation results were added up and divided by 3 to get the average fibroblast cells per field of view.

• *Re-epithelization Count*
Measurement of epithelial density was done by measuring the width of the epithelium in the wound edge area using the histomorphometric method and in micrometers using microphotographs (Prastica, 2020). Re-epithelialization was counted and the length of both ends of the wound was measured using an Olympus CX-21 microscope connected to an Optilab Viewer 2.2 and stored in J-PEG, with 40x magnification, then re-epithelialization was calculated using Image Raster 3.

D. Data Analysis
Data obtained was statistically analyzed using One Way Anova, post hoc LSD (Least Significant Different), and follow-up tests with T-Paired with SPSS Version 22 for Windows at a confidence level of 95%. A significance value <0.05 stated a significant difference.

RESULTS
A. Neovascularization
The mean neovascularization in the 6 experimental groups with significance analysis using One Way Anova is presented in Table 1.

TABLE 1: Average of Neovascularization.

Variable	Group	n	Average	SD	p
Neovascularization	P0 (4)	5	0.26	0.31	
	P1 (4)	5	2.58	1.45	
	P2 (4)	5	0.94	0.71	0.001
	P0 (14)	5	0.46	0.55	
	P1 (14)	5	3.06	1.85	
	P2 (14)	5	1.94	0.84	

The significance analysis using One-Way ANOVA test showed a p value <0.001, meaning that the mean vascularization in the six groups after being given treatment was significantly different (p<0.05). The results of this comparative analysis show that the mean neovascularization in the 4th day treatment group 1 was 2.58 blood vessels/HPF, higher than the 4th day treatment group 2 and the lowest was the control group.

Likewise, the highest mean neovascularization was in the 14th day treatment group I (3.06 blood vessels/HPF) followed by treatment group 2 and control group. The t-paired test was carried out to compare the average neovascularization between the 4th and 14th day groups, and the results showed no significant differences were found between the three groups (p>0.05). The result is presented in Table 2.

TABLE 2: Average of Neovascularization in 4th And 14th Day Groups.

Variable	Group	Mean ± SD		p
		4th day	14th day	
Vascularization	P0	0.26±0.31	0.46±0.55	0.553
	P1	2.58±1.45	3.06±1.85	0.373
	P2	0.94±0.71	1.94±0.84	0.197

B. Fibroblast Count

The average number of fibroblasts in the 6 groups were examined using One-Way Anova and is presented in Table 3.

TABLE 3: Average of Number of Fibroblasts.

Variable	Group	n	Average	SD	p
Fibroblast	P0 (4)	5	4.88	6.05	
	P1 (4)	5	24.44	3.02	
	P2 (4)	5	19.76	5.05	<0.001
	P0 (14)	5	19.40	7.19	
	P1 (14)	5	43.78	7.30	
	P2 (14)	5	31.20	3.83	

The significance analysis using One-Way ANOVA test showed a p value <0.001, meaning that the mean fibroblasts in the six groups after being given treatment were significantly different (p<0.05). The results of the comparative analysis showed that the average number of fibroblasts in the 4th day treatment group 1 was 24.44 cells/LBP, higher than the 4th day treatment group 2, and the lowest being the control group.

Likewise, the average number of fibroblasts in the 14th day treatment group 1 was the highest with 43.78 cells/LBP, followed by the treatment group 2 and control group. T-paired test was carried out after to compare the average number of fibroblasts between 4th and 14th day groups and the result showed a p value of <0.05 (significantly different) and is presented in Table 4.

TABLE 4: Average of Number of Fibroblasts in 4th and 14th Day Groups.

Variable	Group	Mean ± SD		p
		4th day	14th day	
Fibroblast	P0	4.88±6.05	19.40±7.19	0.006
	P1	24.44±3.02	43.78±7.30	0.011
	P2	19.76±5.05	31.20±3.83	0.015

C. Re-epithelialization

The significance analysis of the mean re-epithelialization in 6 groups were sone using One-Way Anova and is presented in Table 5.

TABLE 5: Average of Re-Epithelialization.

Variable	Group	n	Average	SD	p
Re-epithelialization	P0 (4)	5	13.52	13.83	
	P1 (4)	5	52.69	32.24	
	P2 (4)	5	32.28	21.68	<0.001
	P0 (14)	5	56.73	35.15	
	P1 (14)	5	99.60	25.14	
	P2 (14)	5	61.95	17.72	

The significance analysis using One-Way ANOVA test shows a p value <0.001, meaning the mean re-epithelialization in the six groups after treatment was significantly different (p<0.05). The results of the comparative analysis showed that the mean re-epithelialization in 4th day treatment group 1 was 52.69 μm, longer than 4th day treatment group 2 and control group. Likewise, on the 14th day, the 14th day treatment group 1 was the longest with 99.60 μm, followed by the 14th day treatment group 2 and the control group.

T-paired test was carried out next to compare the average re-epithelialization between 4th and 14th day. The result showed that in control group and treatment group 2, the p value was> 0.05, while in the treatment group 1 the p value was <0.05, meaning that the mean epithelialization between 4th and 14th day in treatment group 1 after being given treatment was significantly different (p<0.05), whereas in the control group and treatment group 2 the difference was not significant (p>0, 05). The result is presented in Table 6.

TABLE 6: Average of Re-Epithelialization in 4th And 14th Day Groups.

Variable	Group	Mean ± SD		p
		4th day	14th day	
Epithelialization	P0	13.52±13.83	56.73±35.15	0.100
	P1	52.69±32.24	99.60±25.14	0.032
	P2	32.28±21.68	61.95±17.72	0.107

D. Histologic Preparation

The histological picture showed differences in neovascularization and the number of fibroblasts in the groups (Figure 1).
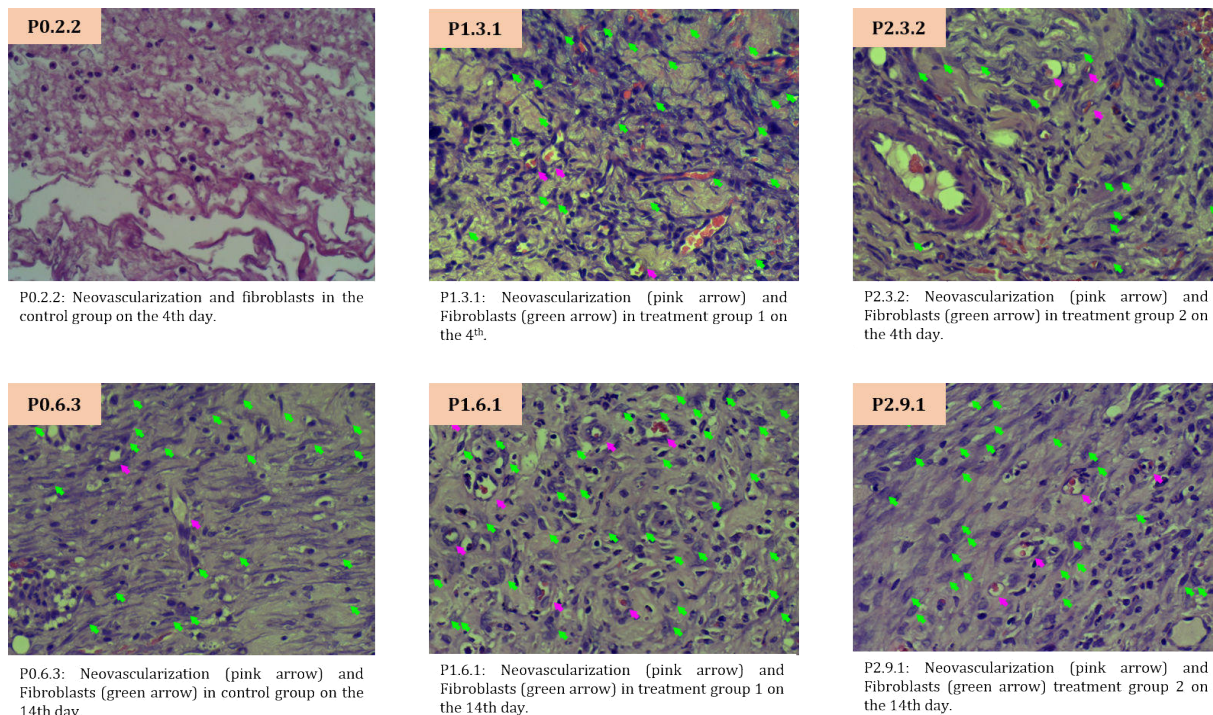

P0.2.2: Neovascularization and fibroblasts in the control group on the 4th day.

P1.3.1: Neovascularization (pink arrow) and Fibroblasts (green arrow) in treatment group 1 on the 4th.

P2.3.2: Neovascularization (pink arrow) and Fibroblasts (green arrow) in treatment group 2 on the 4th day.

P0.6.3: Neovascularization (pink arrow) and Fibroblasts (green arrow) in control group on the 14th day.

P1.6.1: Neovascularization (pink arrow) and Fibroblasts (green arrow) in treatment group 1 on the 14th day.

P2.9.1: Neovascularization (pink arrow) and Fibroblasts (green arrow) treatment group 2 on the 14th day.

FIGURE 1: Histological Features of Mean Neovascularization and Number of Fibroblasts in Control Group (P0), Treatment Group 1 (P1) and Treatment Group 2 (P2), both in 4th and 14th day each group (H&E 400X).

Pink arrows indicate neovascularization. There was no neovascularization in the control group on day 4 (P0.2.2), in contrast to the treatment group 1 on day 4 (P1.3.1) which had more neovascularization than the treatment group 2 on day 4 (P2.3.2). There was an increase in neovascularization in all groups on day 14. The control group on day 14 (P1.6.3) had less neovascularization than the treatment group 2 days 14 (P2.9.1) and the most neovascularization in the treatment group 1 day 14 (P1.6.1).

Green arrows indicate fibroblast cells. The number of fibroblasts in the control group on day 4 (P0.2.2) was absent, in contrast to the treatment group 1 on day 4 (P1.3.1) which had more fibroblast cells, compared to the treatment group 2 on day 4 (P2. 3.2). There was an increase in the number of fibroblast cells in all groups on the 14th day. The control group on day 14 (P0.6.3) had fewer fibroblast cells than the treatment group 2 on day 14 (P2.9.1) and the highest number of fibroblast cells in the treatment group 1 on day 14 (P1.6.1).
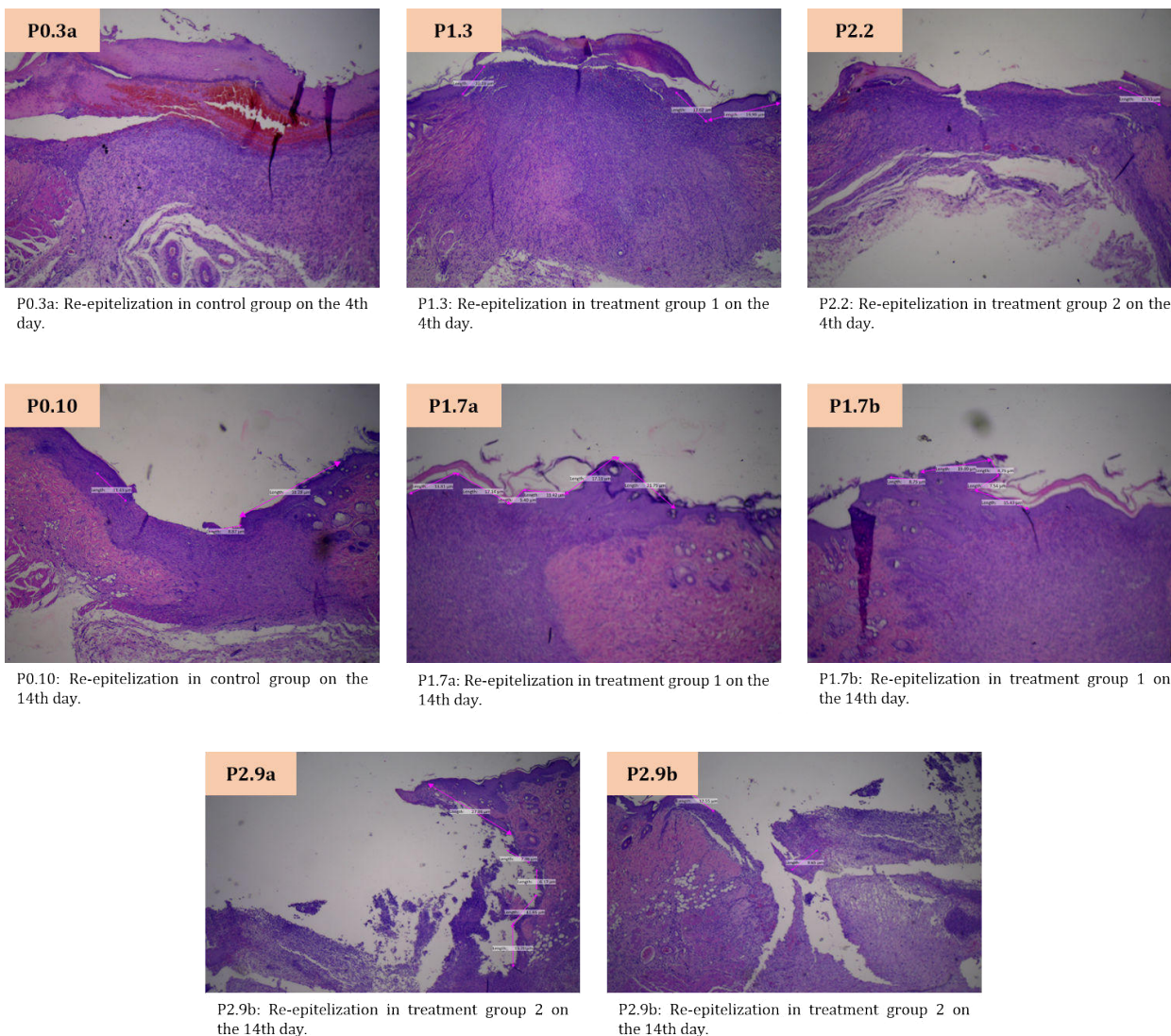

P0.3a: Re-epitelization in control group on the 4th day.

P1.3: Re-epitelization in treatment group 1 on the 4th day.

P2.2: Re-epitelization in treatment group 2 on the 4th day.

P0.10: Re-epitelization in control group on the 14th day.

P1.7a: Re-epitelization in treatment group 1 on the 14th day.

P1.7b: Re-epitelization in treatment group 1 on the 14th day.

P2.9b: Re-epitelization in treatment group 2 on the 14th day.

P2.9b: Re-epitelization in treatment group 2 on the 14th day.

FIGURE 2: Histological Features of Re-epithelialization in Control Group (P0), Treatment Group 1 (P1) and Treatment Group 2 (P2), both in 4th and 14th day each group (H&E 40X).

The pink line indicates re-epithelialization. The mean re-epithelialization of treatment group 1 (P1.3) was 42.19 µm, longer than the control group (P0.3a) 0 µm and treatment group 2 (P2.2) 12.33 µm. The mean re-epithelialization increased in all groups on day 14, with the mean re-epithelialization of the treatment group 1 on day 14 (P17a,b) was 142.48 µm, longer than the control group on day 14 (P0.10) 51.58 µm and treatment group 2 on day 14 (P2.9a,b) 89.04 µm, with 40x H&E staining.

DISCUSSION
Through phytochemical analysis carried out at the Integrated Research Laboratory of the Faculty of Mathematics and Natural Sciences, Udayana University,

results have been found that red gedi leaves contain flavonoids of 763.55 mg QE/100 mL, tannins of 3132.27 mg TAE/100 mL, phenols of 4025.31 mg GAE/100 mL, antioxidant capacity of 1605.09 mgGAEAC/L, IC50 of 311.39 ppm, saponins and alkaloids.

A. Increased Neovascularization with Topical Administration of 12.5 % and 25% Red Gedi Leaves Extract
From the results on histological observations, it was found that the 12.5% concentration of red gedi leaf ethanol extract had the highest mean number of neovascularizations, compared to a concentration of 25% and placebo on the 14th day.

This is because red gedi leaves contain antioxidants which have a role as wound healing agents, such as flavonoids (quercetin), tannins and alkaloids.

Quercetin can inhibit inflammation by modulating macrophage polarization from M1 to M2 phenotype and stimulates angiogenesis in diabetic mouse wounds by increasing CD31 and vascular endothelial growth factor−α (VEGF−α) (Fu *et al*., 2020). The mechanism of the Wnt/β catenin pathway is very important to maintain vascular homeostasis, Wnt3a activates the endogenous Wnt pathway, then there is an interaction between transforming growth factor-beta (TGF-β) and Wnt/β-catenin signaling, specifically the Wnt pathway is involved in modulating factor activity growth. Known as a Wnt target, VEGF has a β-catenin response element in its promoter. It has been found that the T-cell factor-4 (TCF-4) binding region in the VEGF promoter, located upstream of the transcription start site, is a key mediator. This growth factor specifically targets vascular endothelial cells, inducing proliferation and migration and even promoting angiogenesis and increasing vascular permeability (Interdonato *et al*., 2023). The mechanism of the NF-κB pathway is by upregulating the inflammatory response by promoting the expression of various pro-inflammatory cytokines (TNF-α, IL-β) and chemokines, it promotes fibroblast migration and the development and formation of new blood vessels, which is very important for tissue regeneration (Panthi *et al*., 2023).

Tannins have antioxidant, anti-inflammatory, anti-bacterial and diabetic wound healing effects (Lai *et al*., 2016). Tannin can induce the expression of VEGF which is a key factor in angiogenesis which can function as a very specific mitogen to promote endothelial cell proliferation and induce angiogenesis (Lai *et al*., 2016; Qianqian *et al*., 2021).

Alkaloids can inhibit the expression of NF-κB, TNF-α and IL-6 by activating Sirt1, but increase the expression of VEGF, CD-31 and SMA. VEGF and CD-31 play a role in endothelial cell proliferation which ultimately increases angiogenesis (Zhang *et al*., 2020).

B. Increasing the Number of Fibroblasts with Topical Administration of 12.5% and 25% Red Gedi Leaves Extract

From the results on histological observations, it was found that the concentration of 12.5% ethanol extract of red gedi leaves had the highest mean number of fibroblasts, compared to a concentration of 25%, and placebo on day 14. Because red gedi leaves contain flavonoids (quercetin), alkaloids and phenol (gallic acid).

Quercetin can reduce the number of inflammatory cells, increase angiogenesis and collagen distribution and also increase fibroblast production (Fu *et al*., 2020). Through the Wnt/β-catenin pathway mechanism. Epithelial cells are influenced by the interaction between transforming growth factor-beta (TGF-β) and Wnt/β-catenin signaling.

Overexpression of Wnt genes in the epidermis causes the growth of dermal fibroblasts. The Wnt pathway is involved in modulating the activity of growth factors involved in the wound healing process, namely TGF-β. When granulation tissue forms, TGF-β substantially promotes the migration of fibroblasts and endothelial cells as well as fibroblast deposition of the extracellular matrix (Interdonato *et al*., 2023).

Alkaloids can increase collagen deposition and increase fibroblast migration in the wound microenvironment, through increasing SMA which plays a role in the proliferation and migration of fibroblast cells (Azzazy *et al*., 2021; Zhang *et al*., 2020). Phenol (gallic acid) accelerates healing of diabetic wounds by accelerating the migration of keratinocytes and fibroblasts through protecting skin cells against oxidative stress by activating factors that play a relevant role in wound healing, such as focal adhesion kinase (FAK), kinase C-Jun N-terminal (JNK) and extracellular signal-regulated kinase (Erk), which underlie GA's beneficial intervention in diabetic wound repair (Xu *et al*., 2021 ; Melguizo-rodríguez *et al*., 2021 ; Yang *et al.* , 2016). In keratinocytes and Mouse Embryonic Fibroblast (MEF) cells, GA significantly stimulates wound closure in Human Fibroblast (HF21) cells by increasing cell migration. Furthermore, activation of FAK and JNK is also involved in wound healing in HF21 cells. GA was shown to promote wound healing under hyperglycemic conditions in human fibroblasts (Yang *et al*., 2016).

C. Increased Re-epithelialization by Giving Topical Extract of 12.5% and 25% Red Gedi Leaves Extract

From the results on histological observations, it was found that the concentration of 12.5% red gedi leaf ethanol extract had the highest mean re-epithelialization, compared to a concentration of 25%, and placebo on day 14, caused by the content of flavonoids (quercetin), tannins, saponins, alkaloids and phenols (gallic acid) in red gedi leaves.

Flavonoids can increase the migration and proliferation of epithelial cells and increase the synthesis, migration and activity of myofibroblasts (Asali, 2021). Flavonoids (quercetin) increase the expression of α-SMA (a marker of myofibroblasts), the phenotypic transformation of fibroblasts into myofibroblasts upon wound contraction and this conversion is triggered by TGF-β1, the increase in TGF-β1 by quercetin contributes to a greater number of fibroblasts switching to myofibroblasts (Kant *et al*., 2021).

Tannin enhances re-epithelialization and growth of hair follicles in mouse models by increasing the expression of growth factors including basic fibroblast growth factor (bFGF), transforming growth factor-beta (TGF-β), and vascular endothelial growth factor (VEGF), decreasing inflammatory cytokines such as IL-1 and IL-6 and activates the ERK ½ pathway (Jing *et al*., 2022). Saponin reduces the expression of interleukin 1 beta (IL-1β) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$\kappa\beta$),

reduces the expression of matrix metalloproteinase, increases epithelialization and increases collagen synthesis (Chingwaru *et al.*, 2019). Alkaloids can reduce wound epithelial gaps (Azzazy *et al.*, 2021). Through cAMP signaling which regulates keratinocyte proliferation by modulating mitogen-activated protein kinase (MAPK) activity. DBcAMP has been shown to promote the production of transforming growth factor-β by keratinocytes and fibroblasts, as well as keratinocyte proliferation and migration (SlothWeidner and SlothWeidner, 2015).

Synergistically, phenol (gallic acid) increases the rate of cell migration, accelerates wound contraction and shortens the epithelialization phase (Melguizo-rodríguez *et al.*, 2021). The role of GA in skin homeostasis and identified that GA accelerates wound healing by protecting skin cells from oxidative stress and by activating FAK, JNK, and Erk in human keratinocytes. More importantly, GA also significantly improved wound healing under hyperglycemia conditions by promoting cell migration. The increase in FAK activity and wound closure effect in the presence of mitomycin-C suggests that the beneficial effects of GA on wound healing may be mediated by increasing cell migration in epidermal keratinocytes and fibroblasts. Overexpression of FAK can increase cell migration in hyperglycemic conditions. In addition to increasing FAK levels, GA also activates JNK and Erk, which are involved in the wound healing process. Phosphorylation of Erk leads to activation of transcription factors including Elk-1, which is involved in the regulation of matrix metalloprotease–2 (MMP–2) and MMP–9 and induces cell migration (Yang *et al.*, 2016).

D. Antioxidant and Prooxidant Effects of Topical Extract of 12.5% and 25% Red Gedi Leaves Extract

The results of this study show the suitability of the antioxidant effect for wound healing compared to placebo. Where inappropriate antioxidant intake can cause an increase in "antioxidative stress". Antioxidants can neutralize ROS and reduce oxidative stress. However, this is not always beneficial in relation to the development of a disease and/or to delay aging, because antioxidants cannot differentiate between radicals with a beneficial physiological role and radicals that cause oxidative damage to biomolecules. Individuals who overdose on antioxidant supplements may enter an "antioxidative stress" state. If antioxidant supplementation lowers free radical levels, this can interfere with the immune system's ability to fight bacteria and important defense mechanisms for eliminating damaged cells. When antioxidant nutrients are consumed in large amounts, they can also act as prooxidants by increasing oxidative stress. The pro and antioxidant effects of antioxidants are dose dependent, and thus, more is not necessarily better. High levels of antioxidant supplements can disrupt the important physiological balance between ROS formation and neutralization (Poljsak, Šuput and Milisav, 2013).

CONCLUSION

The results of this study show the effectiveness of red gedi leaf ethanol extract gel with concentrations of 12.5% and 25% in increasing neovascularization, number of fibroblast cells and re-epithelialization in wound healing of male Wistar rats with diabetes mellitus, with a concentration of 12.5% being better for all parameters. Meanwhile the control group showed the lowest improvement in neovascularization, number of fibroblasts and re-epithelialization.

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