The Effects of Javanese turmeric (*Curcuma xanthorriza Roxb*) on fibroblasts, granulation, blood vessel density, and contraction in wound healing of STZ-induced diabetic rats

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Abstract

Diabetic ulcers usually occur in the lower extremities of diabetic patients. One in 20 diabetic patients will develop an ulcer, and 10% of those cases lead to amputation. This study aimed to investigate the effects of Javanese turmeric (*Curcuma xanthorriza* Roxb.) extract on the number of fibroblasts, granulation, blood vessel, and the rate of wound contraction in a Wistar rat model of the diabetic ulcer. This was an experimental study with post-test observations only and randomized control group design. Rats were divided into five groups: (1) negative control (KN); (2) positive control (KP); (3) P1; (4) P2; and (5) P3. Every rat was assessed for fibroblast number, granulation, blood vessel density, and wound contraction percentage. Javanese turmeric extract had a significant effect on histological parameters (fibroblast, blood vessels and tissue granulation) ($p < 0.05$) and wound contraction ($p < 0.05$). The double linear test revealed a significant relationship between fibroblasts, granulation, blood vessel quantity, and wound contraction ($p < 0.05$). Correlation and regression tests showed that Javanese turmeric extract explained 91% of the effects on fibroblasts, blood vessels, and granulation. Treatment with Javanese turmeric extract increased the number of fibroblasts, tissue granulation, blood vessel density and wound contraction in male diabetic Wistar rats.

Key words: Blood vessel; contraction; *Curcuma xanthorriza;* fibroblasts; granulation.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disease caused by the interruption of insulin production or the inability of the pancreas to utilize insulin effectively, leading to hyperglycemia or a high level of glucose in the blood (Hinkle *et al.*, 2018). The global prevalence of DM in the adult population (20-79 years old) almost reached 415 million in 2015 and is predicted to increase to 642 million in 2040. Indonesia is one of 10 countries with the highest DM prevalence, ranked 7th with 10 million patients in 2015. It will be in the 6th

place by 2040 with 16.2 million patients if curative and preventative actions are not implemented (Atlas, 2015).

Diabetic wound is defined by an ulcer or wound in a diabetic patient, usually in the lower extremities (Lazzarini *et al.*, 2018). The symptoms are neuropathy, ischemia, infection, ulceration, and/or fibrous tissue destruction in the lower limbs (Mavrogenis *et al.*, 2018). Diabetic patients commonly undergo wound treatment, infection treatment, amputation, and hospital treatment (Vibha *et al.*, 2018). Levine's model of wound treatment should be implemented to increase the quality of services in hospitals (Laksmi *et al.*, 2020).

DM complications are a severe and complex problem that affects multiple vital organs (Singh *et al.*, 2013; Harding *et al.*, 2019). It can be classified as acute or chronic. Chronic complications are divided into two groups, which are micro and macrovascular complications. Microvascular complications can involve angiopathy, neuropathy, and retinopathy. Macrovascular complications can affect the coronary arteries or involve cerebrovascular problems and peripheral vascular diseases (Huang *et al.*, 2017). Peripheral vascular disease and neuropathy occur prior to the onset of diabetic ulcers. In a study on 20 DM patients with diabetic ulcers, 10% of them had to undergo amputation (Stoekenbroek *et al.*, 2014). Amputation as a result of a diabetic ulcer often happens in the lower extremities (Kristianto *et al.*, 2013).

The normal wound healing process is marked by cellular and tissue responses in four phases: hemostasis, inflammation, proliferation, and maturation or remodeling. DM disrupts these phases. Hyperglycemia causes oxidative stress and reactive oxygen species (ROS) production that exceeds tissue antioxidant capacity. This condition also induces the production of advanced glycation end-products (AGE) that will interact with the AGE receptor (RAGE). This eventually disrupts the wound healing process via nuclear factor-κB (NF-κB) pathway activation and extracellular matrix degradation (Prompers *et al.*, 2008). Induction of diabetic wound models with streptozotocin in experimental animals is recommended by several research results (Luan & Wang, 2020; Yan *et al.*, 2020).

Javanese turmeric (*Curcuma xanthorrhiza Roxb*) contains major metabolites such as curcuminoid, terpenoid, and xanthorrhizol essential oil that affect human organs, such as the gallbladder, liver, pancreas, rheumatism, stomach disease, and skin inflammation (Musfiroh *et al.*, 2013). Xanthorrhizol from *Curcuma xanthorrhiza Roxb* has been reported as having antimicrobrial activity for S.aureus resistance (Kesumayadi *et al.*, 2021). Xanthorrhizol also has the potential to inhibit Matrix Metalloproteinase -1 (MMP-1), in which excessive MMPs induced chronic wounds (Oh *et al.*, 2009; Caley *et al.*, 2015).

Javanese turmeric has the effect of reducing oxidative stress during diabetic wound treatment. Oxidative stress occurs when free radical molecules are produced to a greater extent than the body's ability to neutralize them (Pizzino *et al.*, 2017). The antioxidant activity of Javanese turmeric is supported by its bioactive components, such as tannins, alkaloids, flavonoids, and polyphenols. A preclinical study demonstrated that Javanese turmeric functions as as an antioxidant, hepatoprotective, anti-inflammatory, anti-cancer, anti-diabetic, anti-microbial, anti-hyperlipidemic, anti-choleric, and anti-bacterial agent (Fatmawati, 2008; Rahmat *et al.*, 2021; Rukayadi *et al.*, 2013). Another study revealed that Javanese turmeric contains flavonoids with strong antioxidant properties (Labban, 2014).

The tannins in Javanese turmeric can also influence the wound healing process via multiple cellular mechanisms, such as fibroblast proliferation, angiogenesis, and increased wound contraction (Singh *et al.*, 2013).

This study aimed to investigate the effect of Javanese turmeric extract on the number of fibroblasts, granulation, blood vessel density, and wound contraction rate in a male Wistar rat model of the diabetic ulcer.

2. Material and Methods

2.1 Study Design

This was an experimental study with post-test only observations and randomized control group design. Twenty-five rats were divided into five groups: (1) negative control rats without DM (KN); (2) positive control rats with DM (KP); (3) rats with diabetic wounds treated with 15% Javanese turmeric extract (P1); (4) rats with diabetic wounds treated with 20% Javanese turmeric extract (P2); and (5) rats with diabetic wounds treated with 25% Javanese turmeric extract (P3). Ethical clearance for this study was granted by Ethic Committee for Health Research of the Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia.

2.2 Animal Model

Male Wistar rats (*Rattus norvegicus*) were used in this study. Each experiment used at least five rats, 2.5-3 months of age, weighing around 180-200 g per rat.

2.3 Study Location

This study was conducted at the Pharmacology Laboratory, Medical Faculty, Universitas Brawijaya, Malang, Indonesia. Treatment was conducted for 21 days. Skin histopathology and assessments were performed at the Anatomy and Pathology Laboratory and Micro Anatomy Laboratory, Medical Faculty, Universitas Brawijaya, Malang, Indonesia.

2.4 Javanese Turmeric Extraction

The Javanese turmeric was obtained from Materia Medica, Batu, Malang East Java Indonesia. A kilogram of Javanese turmeric was washed and sliced. It was then dried at 80°C in an incubator then powdered. The powder of Javanese turmeric (100 g) was soaked and shaken in 900 mL 98% ethanol for 24 by maceration method. The supernatant was filtered and evaporated at 70 °C. The extraction obtained about one third and it was stored in dark bottle at 0°C until it dissolved and it could be used for treatment.

2.5 Javanese Turmeric Ointment Production

The standard formulation for the ointment comprised extract paste and Vaseline. Three formulations were prepared in this study: (1) 15% v/b Javanese turmeric: 0.75 mL paste + 5 g Vaseline; (2) 20% v/b Javanese turmeric: 1 mL paste + 5 g Vaseline; and (3) 25% v/b Javanese turmeric: 1.25 mL paste + 5 g Vaseline.

2.6 DM Induction

The rats were DM-induced with a single dose of 40 mg/kg body weight streptozotocin (STZ) by intraperitoneal injection with 0.1 M citric acid (pH 4.5) as the solvent after a 12-hour fasting period. Research results stated that rats with more than 200 mg/dL blood glucose level after 7 days of STZ injection can be considered diabetic (Arokiyaraj *et al.*, 2011). Seven days after STZ injection, the blood glucose level was measured with a glucometer and every rat with more than 200 mg/dL blood glucose was considered diabetic (Winarsih *et al.*, 2012). After injection, every rat was given 5% glucose solution for 24 hours in order to prevent death by hypoglycemia.

2.7 Diabetic Ulcer Induction

The rats were anesthetized with 25 mg/kg body weight ketamine hydrochloride intra peritonial injection. The ventral hair on a 5 x 3 cm area was shaved and the shaved area was disinfected with 70% alcohol. A 1.5 x 1.5 cm incision wound was introduced in the shaved area (Li *et al.*, 2011).

2.8 Diabetic Ulcer Treatment

Wound treatment progressed for 14 days using sterile technique and a secondary dressing of sterile gauze to prevent infection. Control groups were treated with Vaseline and the treatment groups were treated with Javanese turmeric ointment, based on their respective group. The diabetic ulcer was treated once a day (Lodhi *et al.*, 2013).

2.9 Histopathology Preparation Procedure

The histological staining was based on Lodhi, *et al.* (2013). Briefly, all specimens from skin tissue were fixed in 4% buffer formalin for less than 24 hours, embedded in paraffin, sectioned into 4-6 μm thick slices, and stained with hematoxylin and eosin (Ijaz *et al.*, 2021).

2.10 Fibroblast Enumeration and Assessment of Granulation

Fibroblasts were observed in the skin dermis layer using an Olympus XC10 series light microscope with OlyVIA (Viewer for Imaging Application) software. The observations were made at 400x magnification in every observation field. Five observation fields were taken from each slide and the average was taken. Fibroblasts were counted manually (Melo *et al.*, 2011). Tissue granulation was assessed by measuring the granulated tissue from the wound surface to the lower dermis where fibroblast proliferation stopped and fibrocytes were first observed as purple cells by HE staining. The thickness was measured from the lower edge to the upper edge of the wound with a sequential mark every 2 mm. The values were averaged to determine the mean granulated tissue thickness. HE-stained slides were scanned with OlyVIA software by competent personnel (single-blinded study), then magnified by 40x on the screen, and analyzed using AutoCAD software (Kusuma *et al.*, 2016).

2.10 Blood Vessel Density Measurements

The blood vessel density was measured by the average of the vessel in each specimen. Blood vessels were shown by erythrocyte cells within the vessels (Yuhernita, 2014). The observation was done under Olympus microscope 400 magnification, then scanned using OlyVia software. Every specimen was observed ten times in different areas including in the skin wound and surrounding area (Melo *et al.*, 2011).

2.11 Wound Contraction Measurements

Diabetic ulcer healing was assessed by a physical parameter, namely wound contraction. This parameter was based on the progressive difference in the wound area, excluding not including the day of wound induction, and was measured by the following formula (Bairy *et al.*, 2012).

$$\%\ Contraction = \frac{Initial\ wound - Wound\ at\ n\ day}{Initial\ wound} \times 100\%$$

AutoCAD was used to measure the wound area as it is more precise for obtaining quantitative data (Ashkani-Esfahani *et al.*, 2012; Wibawani *et al.*, 2016) The wound area was measured by clicking the polyline and then making a line corresponding to the wound area. The area, in mm^2, was calculated after right-clicking on the line in the property tab.

2.12 Data Analysis

Data analysis used SPSS 21 for windows. The statistical tests included the Shapiro-Wilk normality test and Levene's homogeneity test. After that, one-way ANOVA and the Tukey HSD post hoc tests were performed to determine significance compared to other variables. A p-value below 0.05 was considered significant. A double linear regression test was performed to determine the relationship between the dependent and independent variables.

3. Results

3.1 The effect of Javanese turmeric extract on fibroblast number.

Javanese turmeric extract had a significant effect on the number of fibroblast ($P < 0.05$) in the wound tissue of diabetic rats based on the one-way ANOVA (Figure 1 and 2). The fibroblast number was significantly higher in the treatment groups compared to the control groups on day 14 of treatment. Fibroblast in negative control (11.68 ± 2.03); positive control (6.48 ± 1.64); 15% Javanese turmeric treatment (15.12 ± 1.20); 20% Javanese turmeric treatment (22.64 ± 1.51); and 25% Javanese turmeric treatment (23.64 ± 3.36). The dose of Javanese turmeric (25%) has the highest fibroblast.

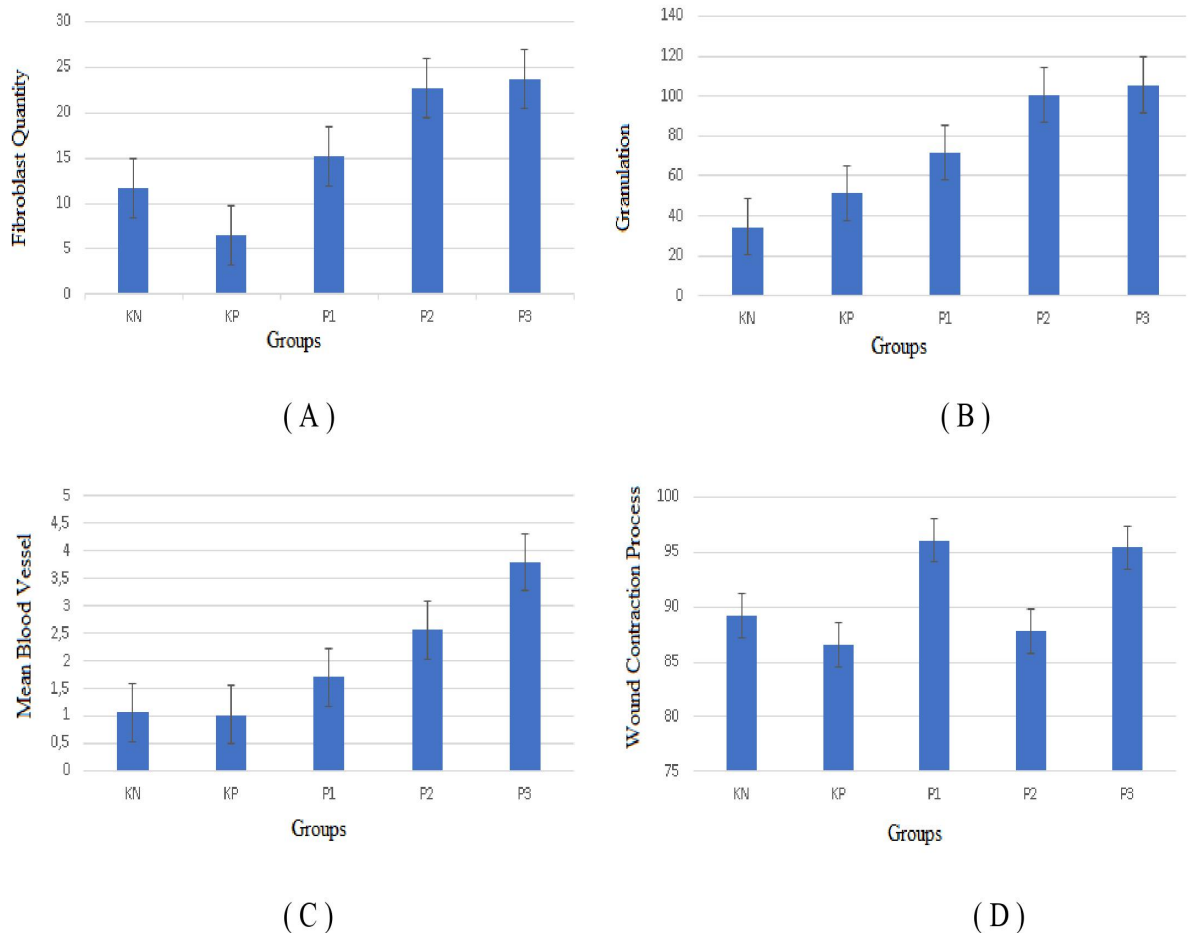

Fig. 1. Mean fibroblast (A); Mean granulation (B); Mean blood vessel (C); Mean wound contraction (D). KN: negative control; KP: positive control; P1: 15% Javanese turmeric treatment; P2: 20% Javanese turmeric treatment; and P3: 25% Javanese turmeric treatment.

3.2 The effect of Javanese turmeric extract on granulation.

One-way ANOVA revealed that *Curcuma xantorrhizza Roxb* treatment had a significant effect on wound tissue granulation compared to untreated wounds ($P < 0.05$). The treated groups had significantly more granulation than the control group (figure. 1 and figure. 2). Granulation thickness in negative control (51.21 ± 1.74); positive control (34.50 ± 3.69); 15% Javanese turmeric treatment (71.93 ± 21.86); 20% Javanese turmeric treatment(100.45± 14.86); and 25% Javanese turmeric treatment (105.607 ± 17.12). The dose of Javanese turmeric (25%) has the highest granulation thickness.

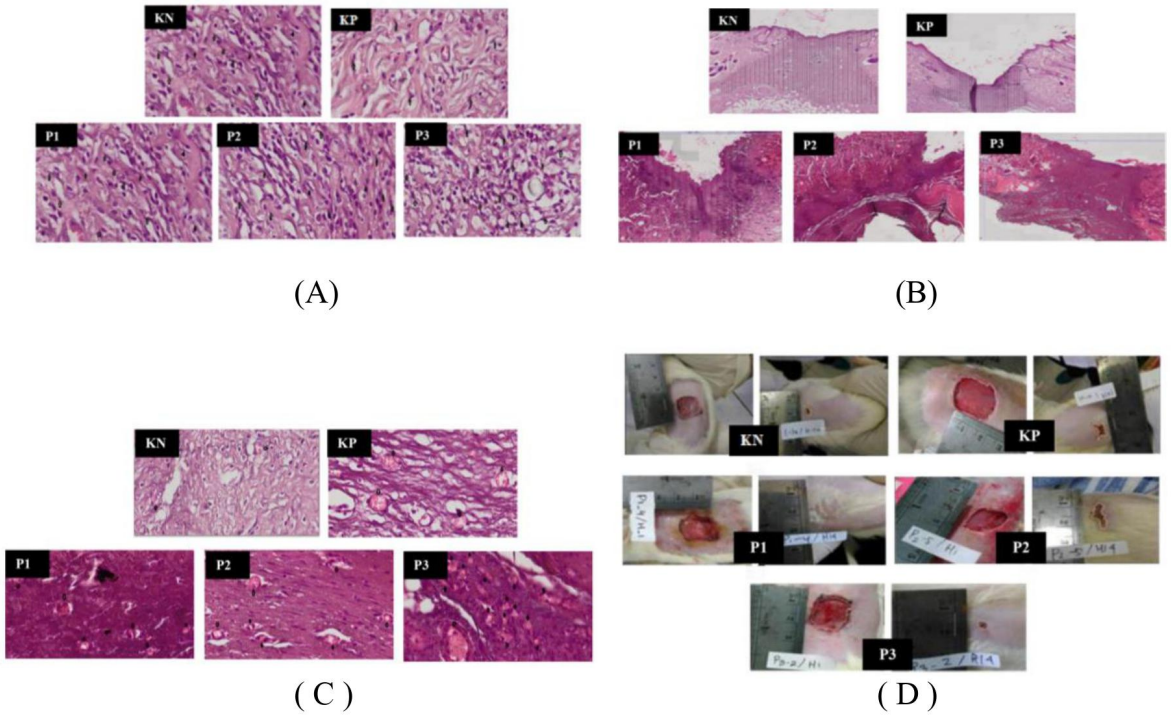

Fig. 2. Scanning results of each observational field in HE staining and 400x magnification. (A) Fibroblast (dark cells) quantity difference of each group; (B) Granulation tissue thickness; (C) Blood vessels are marked by black arrows; (D) AutoCAD 2010 analysis on diabetic ulcer area. KN: negative control; KP: positive control; P1: 15% Javanese turmeric treatment; P2: 20% Javanese turmeric treatment; and P3: 25% Javanese turmeric treatment.

3.3 The effect of Javanese turmeric extract on blood vessel density.

The blood vessel density is determined by vessel quantity. Javanese turmeric extract significantly increased blood vessel formation in the wounded tissue based on the statistical analysis ($P < 0.05$). The control groups had significantly fewer blood vessels compared to the treated groups on day 14 of treatment (Figure 1 and 2). Blood vessels in negative control (1.06 ± 0.54); positive control (1.02 ± 0.31); 15% Javanese turmeric treatment (1.70 ± 0.41); 20% Javanese turmeric treatment (2.56 ± 0.79); and 25% Javanese turmeric treatment (3.80 ± 0.78). The dose of Javanese turmeric (25%) has the highest density of blood vessels.

3.4 The effect of Javanese turmeric extract on wound contraction.

Javanese turmeric extract significantly increased the wound contraction rate compared to the control groups ($P < 0.05$). The wound contraction process was significantly faster in the treated group than that in the control group on day 14 of treatment (Figure 1 and 2). Wound contraction analysis revealed that wound contraction in negative control (89.22 ± 5.92); positive control (86.51 ± 6.09); P1: 15% Javanese turmeric treatment (96.07 ± 2.99), ; P2 20% Javanese turmeric treatment(87.76 ± 3.08); and P3 25% Javanese turmeric treatment (95.42 ± 1.15). The dose of Javanese turmeric (15%) has the highest wound contraction.

3.5 The relationship between fibroblast number, granulation, blood vessel density, and wound contraction during diabetic ulcer healing

All data from the three variables were analyzed regarding their relationships with each other. Double linear regression to all variables revealed a significant relationship between fibroblast number, granulation, blood vessel density, and wound contraction rate ($P < 0.05$). Correlation and regression analyses for fibroblast number, granulation, blood vessel density, and wound contraction rate in relation to the dose of Javanese turmeric extract were also done (Table 1).

Table 1. Correlation and Regression analysis of all variables to Javanese turmeric doses

Variable	r	R^2	P *value*
Fibroblast Blood vessel Granulation	0.959	0.919	0.000

Correlation and regression analysis on fibroblast number, blood vessel density, and granulation revealed that Javanese turmeric ointment explained 91.9% of the changes in fibroblasts, blood vessel density, and tissue granulation, while the other 8.1% was explained by other unobserved variables. In other words, all of the assessed variables could explain 91.9% of the dose variable. A requirement for double linear regression is significant. So, the regression model was considered significant for predicting the dose variable. The wound contraction variable had a p-value of 0.67 in the coefficient table. So, it was not used for double linear regression. The analysis resulted in the following equation:

$$\text{Dose} = 11.93 + 0.710\ (\text{fibroblast}) + 2.149\ (\text{blood vessel}) + 0.108\ (\text{granulation})$$

The equation explains that every additional fibroblast will increase the required dosage by 0.710 after controlling the blood vessel density and granulation variables. Every increase in blood vessel density will increase the required dosage by 2.149 after controlling fibroblast number and granulation. Every increase in tissue granulation will increase the required dosage by 0.108 after controlling blood vessel density and fibroblast number. Fibroblasts had the greatest influence on the required dosage, based on the value of 0.473 in the beta column of the coefficient.

4. Discussion

The number of fibroblasts was significantly different in each group. The positive control group had the fewest fibroblasts. This may be the effect of a high glucose content in the blood. The positive control group and P1 did not show any significant differences, possibly because a low dose of Javanese turmeric (15%) had a small effect on normal wound healing process.

During the normal wound healing process, homeostasis occurred. The blood vessel density in the positive and negative control groups was relatively similar. It was slightly lower in the positive control group due to the high blood glucose content, indicating a diabetic condition (Abdennabi *et al.*, 2016). The diabetic condition induced proinflammatory agent release and led to oxidative stress with the production of ROS and TNF-α (Luc *et al.*, 2019). Oxidative stress decreases vascular endothelial growth factor (VEGF) production and inhibits angiogenesis (Tahergorabi *et al.*, 2012). The highest dose of Javanese turmeric (25%) was associated with the highest density of blood vessels. Unfortunately, this condition can lead to excessive tissue granulation (hypergranulation) and eventually produce scars in the wounded area due to continuous collagen synthesis and catabolism. Wound contraction was significantly faster in P1 than in P2. Although the P1 wound closure percentage was higher than that of P3, the mean was not significantly different. 15% Javanese turmeric extract gave a better effect than the higher doses (20% and 25%), supported by the macroscopic observation that the wound was starting to close on day 12.

The inflammatory phase of wound healing was identified from tissue granulation from day 1 until day 10 (Gantwerker *et al.*, 2012). The proliferation phase was indicated by fibroblast proliferation and new granulation tissue characterized by a newly-formed extracellular matrix (collagen) and angiogenesis. An adequate level of oxygen and nutrition is paramount for the formation of healthy granulation tissue and preventing bleeding in the wound area. After that, the epithelialization process continued from day 4 to day 24 (Abdennabi *et al.*, 2016).

Angiogenesis was initiated on day 3 after wound induction and reached a peak on days 3 to 7 (Ram *et al.*, 2016). Angiogenesis occurred on days 3 to 7 after wound induction and ceased by day 13. The peak of angiogenesis was at day 5 (Prasetyo *et al.*, 2010). In the same pattern, angiogenesis declined at day 15 (Ferdinandez *et al.*, 2013). Other studies have shown that angiogenesis starts on day 1 after wound induction, peaks at day 5, and declines from day 7 to day 14 (Setiawan *et al.*, 2015). At the end of the proliferation period, the maturation period began, marked by contraction at the edge of the wound, a decline in vascularity, and an increase in tensile strength (Gantwerker *et al.*, 2012).

Javanese turmeric ointment was observed to have a positive effect on wound contraction in diabetic ulcers. Xanthorrizol, curcumin, flavonoids, and tannins in Javanese turmeric may play a role in this process, as these compounds are known to possess antimicrobial (Ray *et al.*, 2021), anti-inflammatory and antioxidant properties (Kuntorini *et al.*, 2018). Antioxidant activity and wound healing were indicated by increased collagen formation. Collagen is the pivotal extracellular matrix in the proliferative phase of wound healing. The more collagen formed in the wounded tissue, the faster it pulls the fibroblasts to the edge of the wound. Fibroblasts will differentiate into myofibroblasts, which are responsible for the wound contraction process (Kusuma *et al.*, 2016; Vermolen & Van Rijn, 2012). Based on the data of this study, 15% Javanese turmeric extract treatment had positive effects in the homeostasis phase. Higher doses of Javanese turmeric extract (20% and 25%) showed significant differences compared to the control groups. Further research can test pure contents having the most significant effect on wound healing in patients with diabetes mellitus.

5. Conclusion

Javanese turmeric extract has positive effects on the wound healing process of diabetic ulcers. The effects include a higher number of fibroblasts, increased wound tissue granulation, higher blood vessel density, and faster wound contraction in treated diabetic rats.

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