Research Article

The effect of *temulawak* extract (*Curcuma xanthorrhiza* Roxb.) on decreasing erythema and macrophages of diabetic wounds healing process in a male Wistar rat diabetes mellitus model

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ABSTRACT

Context: A diabetic wound in diabetes patients is involved in the peripheral and autonomic nervous systems. The *temulawak* extract as essential oils and curcumin can improving wound healing process. **Aims:** This research was aimed to observe *temulawak* extract in decreasing erythema level and the macrophages present in the healing of diabetic wounds in a male diabetes mellitus (DM) Wistar rat. **Settings and Design:** This study used true experimental laboratory research design with randomized post-test only control group design method. **Subjects and Methods:** DM was induced in white male Wistar rats using streptozotocin 40 mg/kg BW intraperitoneally then divided into five experimental groups. Three groups were administered by various doses of temulawak extract (15%, 20%, and 25%) and two groups were administered by saline and Vaseline solution as a control comparison. **Statistical Analysis Used:** One way ANOVA test can be performed to determine the influence of *temulawak* extract. Furthermore, the *post hoc* test was performed to determine the difference between the control and treatment groups, while the parametric test of linear regression was performed to know the influence of temulawak extract to the erythema level and the macrophage presences. **Results:** This research obtained that Group 2 (*temulawak* 20%) had the most significant influence of erythema level and macrophage presence. *Temulawak* extract had the influence to decrease erythema level ($P = 0.041$) and macrophage presence ($P = 0.002$). **Conclusions:** *Temulawak* (*Curcuma xanthorrhiza* Roxb.) has potential to decrease the level of erythema and the presence of macrophages during the wound healing process of diabetes in a male Wistar rat DM model.

KEY WORDS: *Curcuma xanthorrhiza* Roxb, Diabetic wounds, Erythema, Macrophage

INTRODUCTION

Diabetes is a chronic disease that requires a strategy and treatment to reduce the risks associated with increased glycemic levels.[1] The World Health Organization (WHO) reported 422 million people or 8.5% of the world's population to be suffering from diabetes. This number had increased from 108 million people or about 4.7% in 1980; the disorder is common in adults. In the last period, the prevalence of diabetes had increased more rapidly in developing countries than in developed countries. The WHO predicts that diabetes will be the seventh leading cause of death in the world by 2030.[2]

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Diabetes is a metabolic disease characterized by hyperglycemia due to the influence of pancreatic organs on the induction of impaired insulin secretion, insulin action, or both. This results in the failure of glucose metabolism. The failure of glucose metabolism causes hyperglycemia and glucose intolerance.[2] This may increase the risk of complications in the microvascular system.[3]

Diabetes mellitus (DM) has a major influence on chronic wound healing. The wound will affect pathological changes that occur in the extremities. Diabetic wounds are the most common complications of diabetes; if wound treatment is not performed correctly, the patients will have a high risk of major complications including infection and amputation.[4]

Wound healing is a process combining vascular and cellular activity and the formation of chemicals

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as mediating substances in the wound area. There are three phases in the wound healing process: Inflammation, proliferation, and maturation.[5] The early phase (inflammatory phase) will affect the wounds, causing erythema, swelling, and an increase in temperature which is often associated with pain. Erythema or redness is a physiological manifestation of the body against the most easily observed lesions in an inflamed area.

Cellular activity in the inflammatory phase is the presence of leukocyte cell migration (especially neutrophils) in the extravascular region. After a few days, neutrophils play a role in wound healing and will be inactivated and then replaced by macrophages.[6] Macrophages have an important role in the wound healing phase, particularly in the inflammatory and proliferative phases. Macrophages secrete a number of biologically active products shortly after activation. Macrophages will induce tissue damage as a defense mechanism.[7]

In the state of DM, chronic wounds will develop due to the slow physiological response and the decreased macrophage function; this will cause a decrease in the production of growth factors and affect angiogenesis response and collagen accumulation so that the inflammatory process will lengthen and cause a disruption of wound closure.[8] The wound healing process is influenced by endogenous and exogenous factors. One exogenous factor is wound treatment using modern medicine or traditional medicine.

Temulawak (*Curcuma xanthorrhiza* Roxb.) is a plant that has spread throughout Indonesia and which has various benefits. Xanthorrhizol in *temulawak* has been reported to exhibit various activities as an antidiabetic substance and there are also anti-inflammatory and antioxidant compounds that can counteract free radicals.[9] Therefore, the inflammatory phase does not extend and the wound healing process improves.

The purpose of this research was to determine the effect of *temulawak* extract on the decreasing erythema level and the macrophage presences in the healing process of diabetic wounds of a male Wistar rat DM model.

SUBJECTS AND METHODS

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed (Ethical Clearance No. 249/EC/KEPK-S1-PKM/06/2016).

Samples and Research Design

This study was used 25 Wistar rats (*Rattus norvegicus*) due to they are easily adapted and have biological and behavioral responses that are similar to those in humans. There were divided into five experimental groups. The inclusion criteria were male, aged 2.5–3 months, weighed 180–250 g, in healthy condition, and had no previous treatment.

This study used true experimental laboratory research design with randomized post-test only control group design method. The study consisted of five groups of experimental animals included: Negative DM group rats, positive DM without treatment *temulawak* group rats, and three groups of DM rats were administrated by various *temulawak* extracts. The various *temulawak* extract is group treatment 1 (T1) administered by 15% *temulawak* extract, T2 and T3 were administered by 20% and 25% *temulawak* extract, respectively.

Temulawak Extract

A kilogram of *temulawak* was dried using an oven at 80°C, smoothed to a fine powder, and then soaked for 3–4 days in an Erlenmeyer flask containing 1 L of 96% ethanol (Merck, Germany). The resulting precipitate was separated by filtration using filter paper; the evaporation process was performed using an evaporator flask at 70°C. The ethanol flowed into the holding flask for ±2 h. The results obtained in this process were for a quarter of the total amount of dried *temulawak* and the remaining *temulawak* extract was stored in the freezer.

Temulawak Ointment Production

Basic standard for making ointment is Vaseline type hydrocarbons.[10] The base of this hydrocarbon ointment is known as the basic fatty ointment, like Vaseline (Petrolatum) (Unilever, US).[11] The formulation of the ointment was divided into three concentrations 15% v/b: 0.75 mL of *temulawak* extract + 5 g of Vaseline, 20% v/b: 1 mL of *temulawak* extract + 5 g of Vaseline, and 25% v/b: 1.25 mL of *temulawak* extract + 5 g of Vaseline.

Streptozotocin (STZ) Induction

The rats were adapted for 7 days and seen to remain healthy, after which they were fasted for 12 h and then intraperitoneally injected with a single dose of 40 mg/kg BW STZ (Cayman Chemical, USA). The STZ was dissolved in a 0.1 M citrate buffer (BioWORLD, USA) solvent at a pH of 4.5. After that, the rats were given 5% glucose solution for 24 h to prevent hypoglycemia as this can cause death. Seven days later, the measurements of blood glucose levels of rats were taken from the rat tail vein at the tip to avoid dysfunctional rat tails. Rats with blood glucose levels of 200 mg/dl were considered diabetic.[12]

Diabetic Wounds

The creation of diabetic wounds was performed on rats with fasting blood sugar levels reaching >200 mg/dl (the measurement was taken with a blood

glucometer). Rats were generally anesthetized with 25 mg/kg BW of ketamine hydrochloride (Cayman Chemical, USA) intraperitoneally. Then, a 5 cm×3 cm area of the back fur of the rats was shaved. An area of the shaved portion with a size of 1.5 cm×1.5 cm was marked. After that, disinfection was done using 70% alcohol in the injured area with reference to Grade 1 Wagner with excision of the skin part to the depth of the epidermis until the hypodermis using tweezers.

Diabetic Wounds Treatment

Treatment of wounds was conducted for 14 days with a sterile technique using sterile gauze as a secondary dressing to prevent infection. The negative control treatment (healthy rats) used normal saline and the positive control treatment (diabetic rats) used normal saline and Vaseline. The treatment groups used an ointment containing extract of *temulawak*. Wound treatment was conducted once a day.[13]

Erythema Data Collection

To collect data for erythema variables, researchers observed the results of the study repeatedly. Data collection was conducted once a day on the 14th day at the time of wound treatment at the Pharmacology Laboratory, Faculty of Medicine, Brawijaya University, Malang, Indonesia. Then, the documentation data were collected until the last day of the research.

The assessment of the decrease in erythema was performed by taking the average erythema value around the diabetic wound formed in a quadrilateral shape. The whole process of calculating the average erythema value was performed using Corel Photo-Paint Suite Graphic X6 program (Corel Corporation, Canada).

Macrophage Data Collection

Data collection for macrophage variables was conducted by observing and measuring the macrophage presences after treatment on the 14th day in each treatment or animal group. Data collection for macrophage variables was conducted by counting the macrophage presences using the Olympus XC10 series microscope (Olympus Corporation, US) (M=×400) equipped with digital cameras and documented by taking 5 fields of view and analyzed using OlyVia software (Olympus Corporation, US), where each preparation was examined in 5 viewing areas and then averaged. The scan results of the preparations using a microscope were calculated manually using Corel Photo-Paint 12 (Corel Corporation, Canada) in each field of view that was being averaged.

Data Analysis

This research used parametric test data analysis. After calculating the average erythema value in the diabetic wound and the macrophage presence cells in each experimental animal, statistical tests were performed using SPSS 16.0 (IBM Corporation, US), statistics for windows with a normality test and homogeneity test. The normality test data were assessed using the Shapiro–Wilk test with $\alpha = 0.05$ because the number of samples was <50; if $P > 0.05$, then the data are normally distributed. The homogeneity test was performed using the Levene statistic test with $\alpha = 0.05$; if the test table of homogeneity of variance shows $P > 0.05$, then the data are homogeneous and have the same variant so that the next test (one way ANOVA) can be performed to determine the influence of *temulawak* extract. Furthermore, the *post hoc* test was performed to determine the difference between the control and treatment groups, while the parametric test of linear regression was performed to know the influence of *temulawak* extract to the erythema level and the macrophage presences.[14]

RESULTS

Effect of *Temulawak* Extract to Erythema Level

The one way ANOVA test result of the erythema level given with *temulawak* extract data showed a significance value ($P = 0.000$). The result of the Tukey *post hoc* test showed that the group with the most significant difference was the T2 administered with 20% *temulawak* extract. The average erythema level is presented in Table 1.

The result of erythema average calculation showed that there was a difference between treatment group and control group. The positive control group had average macrophages higher than T1 and T2. T3 had higher average erythema than the positive control group. Based on Table 1, there is also a difference in average value between treatment group (T1, T2, and T3) with the lowest average result in treatment Group 2 with 20% *temulawak* extract administered topically.

Effect of *Temulawak* Extract to the Presences of Macrophage

One way ANOVA test results obtained a significance value of $P = 0.000$. The Tukey *post hoc* test showed that the group with the most significant difference was the group treated with 20% *temulawak* extract. This indicates that giving topical extracts of ginger may affect the macrophage presences in diabetic wounds. The average macrophages are presented in Table 2.

Based on the calculation in Table 2, there is a difference between the treatment group and the control group, where the positive control group has average macrophages that are higher than T1, T2, and T3. There is also a difference in average value between treatment group (T1, T2, and T3); it was obtained the lowest average result in treatment 2 with 20% *temulawak* extract topically.

Table 1: Average value of erythema (average±SD)

Group	Average of erythema (average±SD)
Negative (KN)	97.40±8.79
Positive (KP)	101.40±5.41
Treatment 1 (P1)	87.66±7.00
Treatment 2 (P2)	81.87±8.01
Treatment 3 (P3)	102.34±5.51

Table 2: Average macrophage presence in each sample (average±SD)

Group	Average macrophage presenceb (average±SD)
Negative (KN)	57.40±13.27
Positive (KP)	64.40±9.76
Treatment 1 (T1)	44.40±9.01
Treatment 2 (T2)	39.40±7.43
Treatment 3 (T3)	64.20±9.14

Observations of the macroscopic diabetic wound were conducted on the 15th day or after the rats were executed. Observations were made after taking photos (taken on the 14th day) using a 14-megapixel resolution digital camera. The macroscopic pictures of diabetic wounds (erythema) on the 14th day are presented in the following figures [Figure 1].

The result showed that there were differences of macrophages number between negative control group (KN), positive control (KP), treatment with concentration 15% (T1), concentration 20% (T2), and concentration 25% (T3), KP> T3> KN> T1> T2 [Figure 2].

Erythema Level and Macrophage Presences in the Healing of DM Wounds

Correlation and regression analysis of erythema level with concentration of temulawak extract in the proliferation phase

Data measurement results will be analyzed using the correlation analysis to determine the relationship of *temulawak* extract to decrease the erythema level. Based on the results of statistical analysis using simple linear regression tests, there was a significant correlation with $P = 0.041$. The results of correlation analysis between *temulawak* extract and erythema level are presented in Table 3.

Correlation analysis between regression of macrophages number with concentration of temulawak extract on proliferation phase

Data measurement results were analyzed using the correlation analysis to determine the relationship of *temulawak* extract to decrease the macrophage presences. Based on the results of statistical analysis using a simple linear regression test, there was a significant relationship with $P = 0.002$. The results of the correlation analysis between *temulawak* extract and macrophages are presented in Table 4.

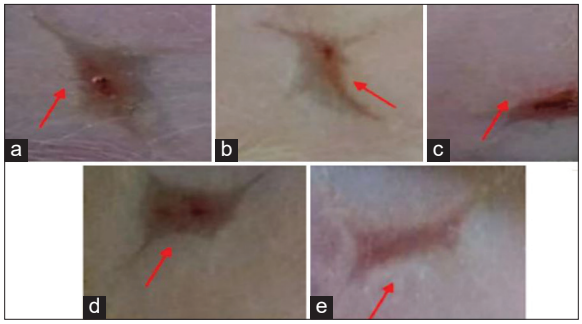

Figure 1: Observation of macroscopic diabetic wounds on the 14th day. There are five groups (a) Normal wound treated with normal saline and Vaseline, (b) diabetic wound treated with normal saline and Vaseline, (c) diabetic wound treated with *temulawak* 15%, (d) diabetic wound treated with *temulawak* 20%, (e) diabetic wound treated with *temulawak* 25%

DISCUSSION

Based on the measurements, the group of DM rats treated with normal saline and Vaseline was significantly different from the treatment group of *temulawak* extract 15% and 20%. The average value of erythema in the 15% treatment group was 87.66 ± 7.00 and in the 20% treatment group was 81.87 ± 8.01, while in the group of DM rats with normal saline and Vaseline, it was 101.40 ± 5.41. This was because the *temulawak* extract contains anti-inflammatory activity that can help the wound healing process.[9] The results were in line with the previous research on the activity of *temulawak* as an anti-inflammatory ingredient by direct inhibition of the enzyme activity of cyclooxygenase-2 (COX-2) so that the COX-1 enzyme is not inhibited and still produces prostacyclin which serves as a gastric protector. COX-2 is a type of inflammatory mediator that can activate nociceptors which causes the appearance of pain. This study found that wound treatment using 25% *temulawak* extract was significantly different from the 15% to 20% treatment groups. Experimental animals treated with 25% *temulawak* extract had a higher average erythema value than the treatment groups using 15% and 20% *temulawak* extract. This was consistent with the theory stating that certain doses given in the treatment will cause a response in the body, depending on the dose given (the dose-dependent response).[15] Therefore, the treatment using 20% *temulawak* extract can decrease the intensity of erythema effectively on the area around the wound.

Groups of DM rats treated with normal saline and Vaseline were significantly different from the treatment group of 15% and 20% *temulawak* extract. The macrophage presences in the group given normal saline

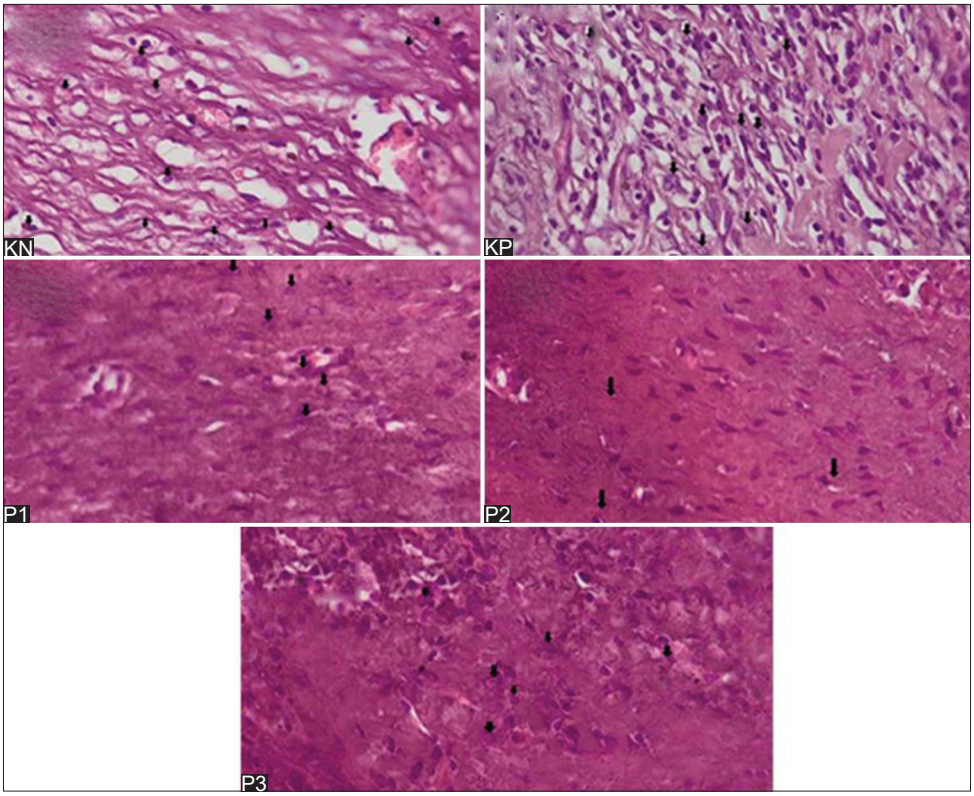

Figure 2: Differences in the macrophage presences (black arrows) with ×400 magnification with HE staining. KN: Normal wound treated with normal saline and Vaseline; KP: Diabetic wound treated with normal saline and Vaseline; P1: Diabetic wound treated with *temulawak* 15%; P2: Diabetic wound treated with *temulawak* 20%; P3: Diabetic wound treated with *temulawak* 25%

Table 3: Correlation analysis between *temulawak* extract and erythema level

Variable	R	R^2	Line equation	*P* value
Erythema	0.501	0.251	81250–1750 (erythema)	0.041

Table 4: The correlation analysis between *temulawak* extract and macrophage presences

Variable	R	R^2	Line equation	*P* value
Macrophage	0.528	0.279	35150–2470 (macrophage)	0.002

and Vaseline were higher than in the treatment groups. This was because normal saline is a physiological fluid that has no antibacterial effect; hence, there is still a high macrophage presence in diabetic wounds, indicating susceptibility to infection.[16] The risk of infection is one of the nursing problems that cannot be resolved optimally if diabetic wound treatment only uses normal saline.[17] Vaseline is a semisolid hydrocarbon known as a fatty ointment base that is difficult to wash, remains unchanged for long periods of time, spreads easily on the skin, can prolong drug contact with the skin, acts as an emollient, and prevents normal moisture evaporation from the skin.[11] The macrophage presences in the 15% treatment group were 44.40 ± 9.01 and in the 20% treatment group was 39.40 ± 7.43, while a group of DM rats with the normal treatment of saline and Vaseline was 64.40 ± 9.76. This was because the *temulawak* extract contains antimicrobial activity and antioxidant activity that can help with the wound healing process.[9,18] Curcumin contained in the rhizome of *temulawak* that contains potent antioxidant compounds.[19] Antioxidant and polyphenol compounds can decrease oxidative stress and decrease TNF-α expression to reduce diabetic complications.[17] Diabetes will also cause an increase in the hormone cortisol, which interferes with the wound healing process.[20]

Based on the results of regression correlation analysis on the erythema level, given that *temulawak* extract had an influence of 25.1%, this means that 74.9% was influenced by other factors not examined in this study, while the effect of *temulawak* extract on the macrophage presences was 27.9%, indicating that 72.1% was influenced by factors not examined in this study. In accordance with the terms of simple linear regression, the results of one way ANOVA analysis of erythema level could be influenced by *temulawak* extract with $P = 0.041$, while the effect of *temulawak* extract on macrophages had $P = 0.002$; this indicates that the regression model had a significant value. The formula of simple regression correlation test

results can be interpreted to show that any decrease of erythema level by 1 unit decreased the concentration by 1750 after being treated with *temulawak* extract. The formula of simple regression correlation test results can be interpreted to show that any decrease of 1 macrophage cell will decrease the concentration by 2470 after treatment with *temulawak* extract.

Inflammation is a normal response of the body at the time of injury or infection; however, if inflammation becomes more widespread and prolonged, it can slow the healing process or cause injury to be difficult to recover from. Therefore, wounds in patients with diabetes will become gangrenous and may lead to amputation. If the inflammatory phase becomes shorter, the wound healing time also shortens.[9] The group treated with *temulawak* extract has a lower average value of erythema and macrophages on the 14th day. This was because there are two main components (curcumin and essential oil) in the *temulawak* extract. The active substance functions as an anti-inflammatory, antimicrobial, and antioxidant compound that can help with the wound healing process.[21] Curcuminoid in *temulawak* contains phenolic compounds, namely curcumin, which causes strong antioxidant activity in biological systems.[9] The essential oil component contains xanthorrhizol, which is a component of essential oils in *temulawak* extract that cannot be found in other *Curcuma* species.[22]

CONCLUSIONS

From this research, it can be concluded that Temulawak (*C. xanthorrhiza* Roxb.) has potential to decrease the level of erythema and the presence of macrophages during the wound healing process of diabetes in a male Wistar rat DM model.

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