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Gel of *Monascus purpureus* JmbA rice accelerate wound healing in diabetic rats

Ika Rahmawati SUTEJO [1] *, Desie Dwi WISUDANTI [2], Alfira Khoirun NISA [3], Alya Febriana GOESTIEN [3], Latiefah Noer WIDIASTUTI [3], Novik NURHIDAYAT [4], Sugiyanta SUGIYANTA [1], Ali SANTOSA [3,5]

1 Department of Biochemistry, Faculty of Medicine, Universitas Jember, Jember, Indonesia.
2 Department of Pharmacology, Faculty of Medicine, Universitas Jember, Jember, Indonesia.
3 Faculty of Medicine, Universitas Jember, Jember, Indonesia.
4 Center for Applied Microbiology, National Research and Innovation Agency-BRIN, Bogor, Indonesia.
5 Department of Internal medicine, Soebandi Regional Hospital, Jember, Indonesia.
* Corresponding Author. E-mail: ikarahmawati.fk@unej.ac.id (I.R.S.); Tel. +62-331-337-877.

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ABSTRACT: The most frequent complication of diabetes mellitus (DM) is diabetic ulcer, with a prevalence of 15-20% and an amputation rate of 30%. There is no effective and easy-to-apply treatment for acute-phase open wounds of patients with DM to prevent their development into ulcers. *Monascus purpureus* jmbA Rice (MJR) from Jember is superior to similar products from other regions in Indonesia because it has the highest monacolin content with an antioxidant effect. The study aimed to prove the effectiveness of MJR gel in accelerating the healing of acute wounds of diabetic rats. This true experimental laboratory research was a posttest-only control group design. We used *Rattus norvegicus* Wistar rats, 3-4 months old, weighing 200-300 grams. The 36 rats were divided into four treatment groups. The control group was given a gel of MJR 0%. The positive control group was given the antibiotic and placenta extract gel combination, and treatment groups T1 and T2 were given gel MJR40% and MJR80%. Excision wounds were made as deep as the subcutaneous layer on the back of the hyperglycemic rat. Four rats were terminated from each group on days 4, 11, and 18, and skin tissue was taken to observe the percentage of wound area shrinkage, epithelial thickness, and fibroblast count and analyze the levels of hydroxyproline and MDA. The MJR80% group had the highest percentage of wound area shrinkage, epithelial thickness, and fibroblast count compared to other groups. MDA levels decreased with increasing days in all treatment groups. Hydroxyproline levels increased on days 4 and 11, then decreased on day 18 in all treatment groups. We conclude that the 80% MJR group revealed the best acute diabetic wound healing. It was not statistically different from the combination of antibiotic and placenta extract gel treatment group.

KEYWORDS: Diabetes Mellitus; Fibroblast; Hydroxyproline; MDA, *Monascus purpureus*; Wound Healing

1. INTRODUCTION

Diabetic ulcers represent a significant complication of diabetes mellitus, adversely affecting the wound-healing process. Diabetes interferes with various phases of healing, including hemostasis, inflammation, proliferation, and remodeling, leading to long-term negative impacts on quality of life, morbidity, and mortality [1]. The presence of hyperglycemia in diabetes impairs cellular function, resulting in the production of cytokines, growth factors, and chronic inflammation. Furthermore, individuals with diabetes experience macrovascular and microvascular changes, leading to tissue hypoxia caused by the accumulation of advanced glycosylation end products (AGEs). Elevated AGE levels inhibit angiogenesis, vasculogenesis, and leukocyte counts, hindering wound healing.

Moreover, hyperglycemia exacerbates oxidative stress, further impairing wound healing. Increased production of reactive oxygen species (ROS) occurs through pathways such as polyol, hexosamine, protein kinase C, and advanced glycation end products (AGEs) [2]. Elevated ROS levels disrupt the various stages of wound healing and cause damage to the blood supply, metabolism, and peripheral nerve structures. Additionally, oxidative stress, characterized by an elevation in malondialdehyde (MDA) levels, is observed in this context.

Monascus purpureus jmbA Rice (MJR) is rice that undergoes fermentation by the mold *Monascus purpureus*, producing red pigments as secondary metabolites. In Indonesia, MJR is commonly used as a

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coloring agent without altering taste, texture, or odor. In the realm of health, MJR has been found to have cholesterol-lowering and blood sugar-reducing properties [3, 4]. Lin's research in 2019 further demonstrated that MJR possesses antioxidant and antibacterial abilities [3, 5]. The increased presence of antioxidants in MJR contributes to antiatherosclerotic and hepatoprotective effects. Additionally, the antibacterial compound Monascidin present in MJR inhibits the growth of specific bacteria on the skin, such as *Staphylococcus epidermidis*, *Staphylococcus aureus,* and *Propionibacterium acnes* [6]. Furthermore, *Monascus purpureus* exhibits potential as an antidiabetic agent, as it has shown the ability to reduce hyperglycemia in rats induced with streptozotocin [3]. In bone healing, MJR demonstrates anabolic effects on bone mass and acts as an HMG-CoA reductase inhibitor, increasing the expression of VEGF. VEGF, in turn, induces angiogenesis and integrates it into the remodeling process [7]. MJR is native to Jember and outperforms 18 isolates from other regions in Indonesia [8]. Moreover, the antioxidant and antibacterial properties of *Monascus purpureus* jmbA potentially expedite the healing of diabetic wounds [9].

Several parameters were measured to evaluate the effectiveness of bioactive compounds with antioxidant properties in accelerating wound healing, including wound area shrinkage, epithelial thickness, fibroblast count, MDA levels, hydroxyproline, and VEGF. Hydroxyproline and fibroblast count indicate skin collagen formation, crucial in wound healing. These variables were monitored serially to observe the progress of wound healing phases over time. The findings of this study underscore the health benefits of MJR, particularly its ability to enhance the healing process of acute diabetic wounds.

2. RESULTS

This research obtained 271.11 grams of MJR extract from 750 grams of simplicial, resulting in a rendement extraction yield of 36.15%. This percentage yield reflects the value of the natural product as an extract, with higher yields indicating a greater extract value. A minimum yield of at least 9.6% is desirable for good natural products [10]. The acute wound in diabetic rats produced immediately after excision is an open wound of grade II, according to Wagner-Meggit, with a size of 2x2 cm². The excised wound looks reddish, with the subcutaneous layer retained. The results of Wagner-Meggit second-degree acute wound excision in diabetic rats can be seen in Figure 1.

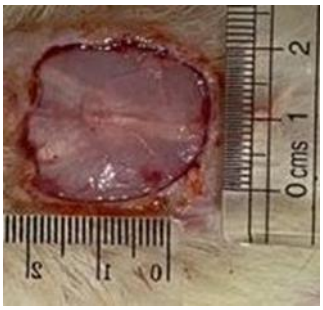

Figure 1. Wagner-Meggit second-degree acute wound excision in diabetic rats

2.1. Wound Area

Table 1 shows the average wound area values on days 4, 11, and 18. The MJR 0% treatment group produced the largest final wound area, while the MJR gel 80% had the smallest final wound area. The percentage of wound area shrinkage was calculated based on the wound area on the final day of the study. The group treated with antibiotics and placenta exhibited a shrinkage of 76.6%, the MJR 0% group showed a shrinkage of 63.42%, the MJR 40% group had a shrinkage of 79.25%, and the MJR 80% group achieved the highest shrinkage at 81.25%. Figure 2 shows wound area shrinkage observed macroscopically on days 4, 11, and 18.

Table 1. Average wound area

Day	MJR 0% (mm² ± SD)	MJR 40% (mm² ± SD)	MJR 80% (mm² ± SD)	Antibiotic and placenta extract gel (mm² ± SD)
4	388.67 ± 4.50^{b}	345 ± 5.56^{ab}	328.67 ± 8.02^{ab}	361 ± 4.00^{a}
11	216 ± 6.55^{b}	175 ± 10.44^{b}	135.3 ± 12.50^{ab}	190 ± 4.35^{a}
18	146.3 ± 11.67^{b}	83 ± 4.00^{b}	75 ± 4.00^{ab}	93.67 ± 3.51^{a}

Data presented as mean ± SD. significant value at $p < 0.05$. ^{a}significant value at $p<0.05$ compare to MJR 0% on the same day. ^{b}significant value at $p<0.05$ compare to antibiotic and placenta extract on the same day

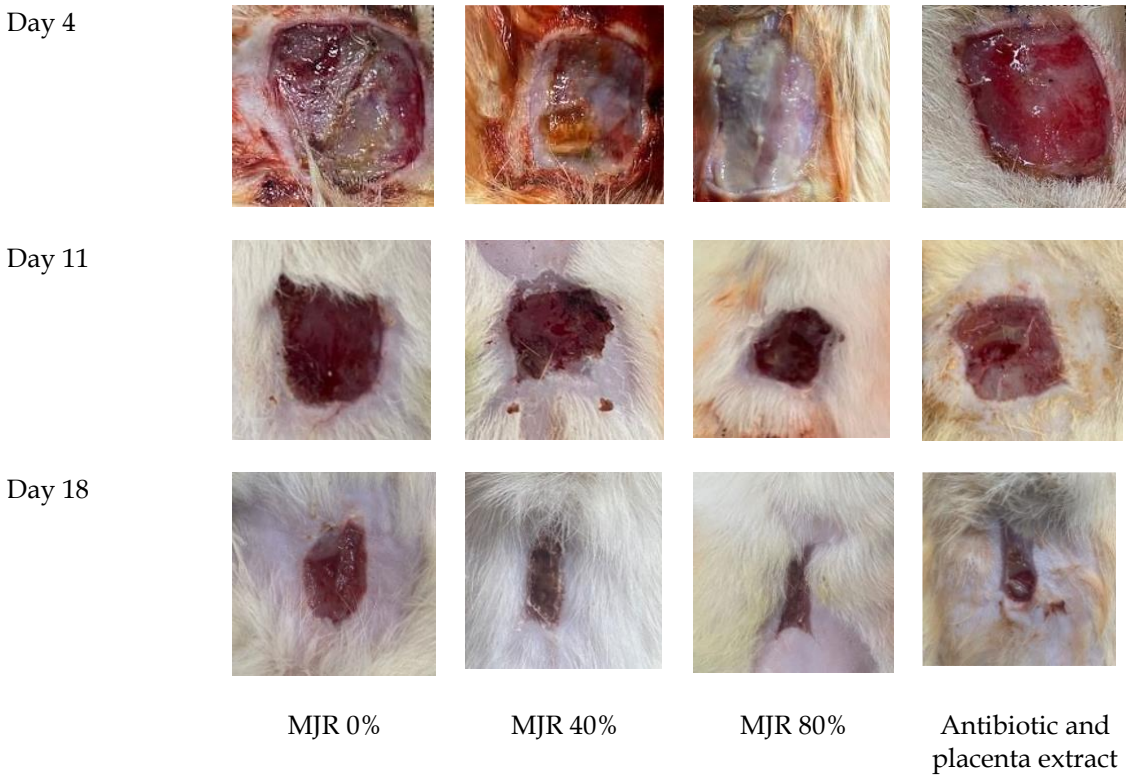

Figure 2. The healing process in the treatment group

From day four, skin healing began around the wound edges, gradually detaching from the skin. By the 18th day, the groups treated with MJR 40%, MJR 80%, and the combination of antibiotics and placenta had fully closed wounds. However, the group treated with MJR 0% gel (K-) still had a wet, reddish wound that had not completely closed. Notably, the MJR 80% group demonstrated the highest percentage of wound shrinkage compared to the other groups.

Microscopic observation of epithelial thickness further confirms this finding. On days 4, 11, and 18, the MJR 80% group exhibited the thickest epithelial layer, whereas the MJR 0% group had the thinnest. Remarkably, the epithelial thickness in the MJR 80% group even surpassed that of the positive control group, which received a combination of antibiotics and placenta (Table 2). Greater epithelial thickness indicates better wound healing.

Table 2. Epithelial Thickness

Day	MJR 0% (mm^2 ± SD)	MJR 40% (mm^2 ± SD)	MJR 80% (mm^2 ± SD)	Antibiotic gel and placenta extract (mm^2 ± SD)
4	1.61±0.23[b]	2.29±0.14[a]	3.04±0.11[ab]	2.50±0.17[a]
11	9.69±0.22[b]	12.04±0.33[ab]	13.49±0.44[ab]	10.72±0.15[a]
18	10.19±1.06[b]	14.09±1.01[a]	20.48±2.37[ab]	12.93±0.55[a]

Data presented as mean ± SD. significant value at $p < 0.05$. [a]significant value at $p<0.05$ compare to MJR 0% on the same day. [b]significant value at $p<0.05$ compares to antibiotic and placenta extract on the same day

2.2. Hydroxyproline Levels

Hydroxyproline levels in all groups increase until the 11th day and then decrease on the 18th. Hydroxyproline of MJR 0% (K-) showed the smallest increase at the beginning to the middle of the phase proliferation. It decreased more slowly at the end of the proliferative phase than the other groups. This can be seen from the highest levels of hydroxyproline in the MJR0% group on day 18 compared to other groups. the MJR80% group showed the highest increase in hydroxyproline levels until day 11. Even though it decreased on day 18, the levels were maintained higher than the MJR40% group and the combination of antibiotics and placenta. The average hydroxyproline levels of each group can be seen in Table 3.

Table 3. Average hydroxyproline levels

Day	MJR 0% (µg/100 mg ± SD)	MJR 40% (µg/100 mg ± SD)	MJR 80% (µg/100 mg ± SD)	Antibiotic and placenta extract (µg/100 mg ± SD)
4	933 ± 75.49[b]	1283 ± 86.60[ab]	4203 ± 65.57[ab]	2606 ± 244.40[a]
11	7523 ± 395.85[b]	10026 ± 390.17[a]	10899 ± 235.01[ab]	9863 ± 81.85[a]
18	2516 ± 236.92[b]	1066 ± 189.26[a]	1676 ± 102.14[ab]	1023 ± 228.69[a]

Data presented as mean ± SD. significant value at p<0.05. [a]significant value at p<0.05 compare to MJR 0% on the same day. [b]significant value at p<0.05 compare to antibiotic and placenta extract on the same day

2.3. MDA Levels

MDA is the end product of oxidative stress, with higher MDA levels indicating increased oxidative stress. In all groups, MDA levels decreased as the number of days increased. However, the MJR0% gel treatment group exhibited higher MDA levels than the others. On day 4, the MJR40% group showed the lowest MDA levels, while on days 10 and 18, the MJR80% group exhibited the lowest MDA levels. Detailed data can be found in Table 4.

Table 4. Average MDA Levels

Day	MJR 0% (nmol/mL ± SD)	MJR 40% (nmol/mL ± SD)	MJR 80% (nmol/mL ± SD)	Antibiotic gel and placenta extract (nmol/mL ± SD)
4	6.135 ± 0.614[b]	4.329 ± 0.304[a]	4.865 ± 0.720[a]	4.704 ± 0.277[a]
11	5.976 ± 1.007[b]	3.887 ± 0.816[a]	3.453 ± 1.156[a]	3.994 ± 0.765[a]
18	5.696 ± 1.711[b]	3.437 ± 0.492[a]	3.084 ± 0.738[a]	3.620 ± 0.453[a]

Data presented as mean ± SD. significant value at p<0.05. [a]significant value at p<0.05 compare to MJR0% on the same day. [b]significant value at p<0.05 compare to antibiotic and placenta extract on the same day.

2.4. Number of Fibroblasts

The number of fibroblasts in all groups increased with increasing days. However. the MJR 80% gel treatment group had a higher amount of fibroblast than the other groups on observation day 18 can be seen in Table 5. Active myofibroblasts or myofibroblasts have an apparent nucleus and extensive cytoplasmic projections. Inactive fibroblasts have a dense. oval-shaped nucleus and slender cytoplasm. Myofibroblasts have a function to contract in the area around the wound so that a large number of myofibroblasts will increase wound shrinkage. Pictures of fibroblasts from the results of this study can be seen in Figure 3.

Table 5. The average number of fibroblasts

Day	MJR 0% (cell/field of view ± SD)	MJR 40% (cell/field of view ± SD)	MJR 80% (cell/field of view ± SD)	Antibiotic gel and placenta extract (cell/field of view ± SD)
4	33.43±1.80[b]	52.1±4.55[ab]	72.5±2.45[ab]	40.3±1.60[a]
11	96.8±1.96[b]	125.5±1.90[ab]	145.47±1.89[ab]	117.7±1.01[a]
18	131.56±2.31[b]	164.23±8.59[ab]	202.93±5.15[ab]	144.53±2.27[a]

Data presented as mean ± SD. significant value at p <0.05. [a]significant value at p<0.05 compare to MJR0% on the same day. [b]significant value at p<0.05 compare to antibiotic and placenta extract on the same day.

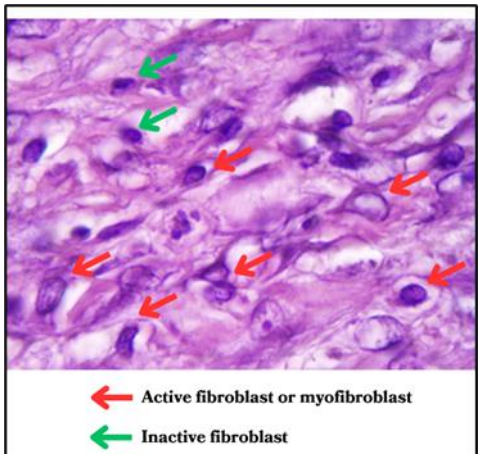

Figure 3. Histology observation of fibroblast cells

3. DISCUSSION

The results of wound area measurements on the 4th, 11th, and 18th day of observation revealed that the group treated with 80% MJR gel exhibited the smallest wound area and the highest percentage of wound shrinkage compared to the other treatment groups on day 18. These results indicate that the MJR gel accelerates the healing of diabetic acute wounds. Microscopic observation of epithelial thickness further confirms this finding. Re-epithelialization serves as the foundation of the wound-healing process. Epithelial regeneration commenced on the 3rd day following the injury and exhibited consistent growth until the 15th day. The efficacy of this process can be determined by assessing the thickness of the epithelial layer. This effect can be attributed to the multiple properties of MJR gel, including its antioxidant, antibacterial, and angiogenesis-inducing activities. Specifically, the antibacterial component in MJR, Monascidin, effectively inhibits the growth of certain skin bacteria such as *Staphylococcus epidermidis, Staphylococcus aureus*, and *Propionibacterium acnes* [6]. Moreover, MJR acts as an HMG-CoA reductase inhibitor, which enhances the expression of VEGF (vascular endothelial growth factor). VEGF plays a vital role in blood vessel development, and the endothelium facilitates its production. This molecule induces permeability and vascular leakage and initiates differentiation, proliferation, extracellular matrix synthesis, migration, and blood vessel formation, thereby accelerating wound healing [11, 7].

Hydroxyproline levels increased on days 4 and 11 and then decreased on day 18 in all groups. The treatment group and the combination of antibiotics and placenta extract showed higher hydroxyproline levels, indicating optimization of the wound healing process. This finding is supported by the data on wound area reduction, which was greater in the treated groups than in the non-treated group. The underlying mechanism is that MJR enhances the expression of VEGF, increasing the production of hydroxyproline by fibroblasts [4]. High levels of hydroxyproline in the tissue indicate increased collagen content, which is crucial for wound healing. Fibroblasts play a key role in collagen synthesis during granulation tissue formation, leading to elevated hydroxyproline levels. However, once the wound healing progresses and granulation tissue is formed, hydroxyproline levels start to decrease [13]. In the maturation phase, type III collagen is degraded and replaced by type I collagen. The recovery of skin strength begins during this phase, as the structure of type I collagen undergoes modifications, resulting in larger and thicker fiber sizes than type III collagen.

On the 18th day, the 80% MJR gel treatment group exhibited the highest hydroxyproline level compared to the 40% MJR gel treatment group and the combination of antibiotic and placenta extract gel treatment group. This finding presents two possibilities. First, high hydroxyproline levels are beneficial as they accelerate the wound-healing process. Second, excessively high hydroxyproline levels can lead to keloid scar formation. However, in this study, the hydroxyproline content in the 80% MJR gel treatment group (1676 ± 102.14 µg/100 mg) was much lower than the levels associated with hypertrophic scarring (6575 ± 1461.19 µg/100 mg), as mentioned in Evani's research (2020). Other studies suggest that hydroxyproline levels in hypertrophic scars can be up to twice as high as those in normal scars [15]. Therefore, based on this study, it can be predicted that treatment with an 80% MJR gel will not cause hypertrophic scars.

Administration of MJR effectively accelerated the wound healing process, as evidenced by the smaller wound area and decreased levels of MDA in the wound tissue during treatment. This is because MJR has an antioxidant effect that reduces tissue MDA levels. This finding aligns with the research conducted by Kasim

et al. (2012), which indicates that *Monascus purpureus* JmbA flour containing lovastatin, specifically monacolin K, possesses antioxidant activity. Monacolin K exhibits pleiotropic effects as an antioxidant, similar to other statins. It activates the antioxidant system, including GPx, which reduces the concentration of H2O2 [9]. GPx is vital in neutralizing H2O2 and lipid hydroperoxides by utilizing reduced glutathione (GSH), thus preventing oxidative stress and promoting wound healing [16]. Furthermore, another source of antioxidants present in MJR is the secondary metabolite called dimerumic acid. Dimerumic acid effectively scavenges radical compounds and inhibits the activity of metal ions that contribute to lipid peroxidation [17]. Additionally, dimerumic acid inhibits NADPH, which is involved in the production of reactive oxygen species (ROS) by the enzyme NADPH oxidase (NOXs), converting NADPH to NADP [18]. The interesting finding of this study was that on day 4 or the proliferation phase, low doses of MJR were more effective in reducing MDA than higher doses. The reasons why this happens need to be investigated further.

Fibroblasts consistently increase during the proliferative phase on days 4, 11, and 18 [19]. Fibroblasts produce collagen from the first day of the wound and reach its peak in the first to third week. Upon completion of the inflammation phase, fibroblasts transition into an active state known as myofibroblasts, initiating collagen synthesis and wound contraction [20]. The antioxidant component of MJR plays a modulatory role during the inflammatory phase of wound healing, regulating excessive cell activity and facilitating smooth transitions between inflammation, proliferation, and remodeling phases [21]. Compared to the control group, the MJR40% and 80% groups demonstrated increased fibroblasts. In contrast, the control group exhibited a lower number of fibroblasts.

Fibroblasts play a crucial role during the proliferation phase by responding to growth factors and producing matrix metalloproteinases (MMPs). These MMPs break down the fibrin clot and extracellular matrix components like type I collagen, facilitating re-epithelization. The stimulation of fibroblasts is regulated by growth factors, particularly TGF-β [22], which influences fibroblast differentiation and re-epithelialization. Disruptions in TGF-β signaling mechanisms often contribute to chronic wound conditions [23]. During the proliferation phase, the presence of flavonoids acts as antioxidants, helping to reduce free radicals [24]. Excessive production of free radicals can interfere with TGF-β activity during this phase [23].

On the 18th day of observation, the number of fibroblasts reached its highest level. Fibroblasts continue proliferating until complete wound closure, eventually transitioning to the remodeling phase, where the wound cavity is filled with a collagen matrix. During the remodeling phase, fibroblast proliferation decreases due to apoptotic cell death. A previous study reported a decline in fibroblast numbers on the 21st day of the remodeling phase [25].

The wound healing process in individuals with diabetes mellitus (DM) follows the same phases as general wounds, namely the hemostasis phase, the inflammatory phase, the proliferative phase, and the remodeling phase. However, wounds in DM patients exhibit specific characteristics, including sustained inflammation, elevated levels of reactive oxygen species (ROS), and persistent bacterial colonization. Consequently, the wound-healing process takes longer in these individuals. Hyperglycemia in DM patients contributes to impaired wound closure by affecting the circulation of nutrients reaching the wound. Additionally, hyperglycemia hinders wound healing due to increased oxidative stress resulting from reduced activity of the antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase [2]. To address these challenges, applying MJR membrane, which possesses antioxidant, anti-inflammatory, and antibacterial properties, can expedite wound healing in individuals with DM.

4. CONCLUSION

MJR exhibits antioxidant activity, which contributes to the acceleration of wound healing in diabetic patients through various mechanisms such as modifying hydroxyproline levels, increasing the number of fibroblasts, and enhancing epithelial thickness. Statistical tests indicate no significant differences in several parameters when comparing the effects of MJR40% gel to the control group treated with a combination of antibiotics and placenta extract. On the other hand, MJR80% gel demonstrates superior wound healing effects compared to the control group, as supported by statistical tests showing significant improvements in several parameters.

5. MATERIALS AND METHODS

5.1. Chemicals and Reagents

Ethanol 70%, HPMC, methyl paraben, propyl paraben, aquades, DMSO5%, and propylene glycol were purchased from Merck [Indonesia]. Other chemicals such as ketamine, xylazine, pentobarbital, hydrochloric

acid (HCl), Phosphate Buffer Saline (PBS), TCA 20%, TBA 1% were purchased from Guardian Pharmatama [Indonesia], and the General Hydroxyproline Assay Kit® [imported from, China]. Hematoxylin-eosin (HE) stain, citrate buffer solution, and Streptozotocin (STZ) were purchased from Sigma-Aldrich [Indonesia]. All other chemicals and reagents used for the analysis were analytical grade.

5.2. Extract Collection and Preparation

Extract of *Monascus purpureus* jmbA Rice (MJR) was collected from the Center for Applied Microbiology, National Research and Innovation Agency-BRIN [Indonesia]. Dimethyl sulfoxide (DMSO) 5 mL was dissolved in 100 mL of distilled water, then HPMC was dispersed in 50 mL of distilled water containing DMSO. After the gelling agent expands, it lasts 24 hours at room temperature. After 24 hours, a mixture of methyl and propyl paraben in propylene glycol 15 mL was added to the HPMC. The mixture was stirred until homogeneous, and each *Monascus purpureus* jmbA Rice (MJR) extract was added, then the remaining distilled water containing DMSO up to 100 g was added. The gel was poured onto a glass plate with a sterile gauze of 3x3 cm and a thickness of ±1 mm and stored in a refrigerator at 4 °C [26].

5.3. Animal and Experimental Control

The population used is Rattus norvegicus, Wistar type, 3-4 months old, weighing between 200-300 grams, that are healthy and have normal skin. The 36 rats were divided into four treatment groups. The negative control group was given a gel without MJR extract, the positive control group was given a combination of antibiotic and placenta extract, and the P1 and P2 treatment groups were given a gel with 40% and 80% concentrations of MJR extract. Excision wounds were made as deep as the subcutaneous layer on the back of rats, covering an area of 2x2 cm^2. On days 4, 11, and 18, the rats were terminated, and the skin tissue was taken to analyze wound area shrinkage, epithelial thickness, hydroxyproline levels, MDA levels, and fibroblast numbers.

5.4. Diabetic Induction and Wound Excision

Rats were fed for 4 hours to empty their stomach before induction. Intraperitoneal injection of streptozotocin at 50 mg/kgBB dissolved in citrate buffer (pH 4.5). Induction was done only once. Mice are given 10% sucrose or 10% dextrose solution on the first day after induction to avoid sudden hypoglycemic post-injection [27]. Three days after induction. researchers measured blood glucose levels by pricking the rat's tail using a needle. The blood that came out was checked with an Auto-check glucometer. Rats that have glucose levels ≥200 mg/dL can be excised.

Wound excision was made by Morton's modified method. A wound excision was performed the day after the diabetic condition was confirmed. Rats were given a combination of ketamine HCl at 80 mg/kg BW and xylazine at 10 mg/kg BW intramuscularly. Combining ketamine and xylazine is used as an anesthetic to reduce pain during wound excision. After anesthesia, rat fur is shaved and marked with an area of 2x2 cm^2, then excision is carried out until subcutaneous [28].

5.5. Wound Area and Closure Assessment

This research measured and analyzed the shrinkage of the wound area using the ImageJ application. Each rat wound area was photographed with a ruler on each edge of the wound. The photos obtained were entered into the ImageJ application for area measurement by marking the edges of the rat wound. The wound area was calibrated with a 1 cm ruler on each photo [29].

5.6. Hydroxyproline Analysis

The supernatant obtained from skin tissue was evaporated at 60-80 ˚C for 30-45 minutes. Subsequently, 500 µL of the evaporated solution was combined with 30 µL of Chloramine T and 470 µL of citrate buffer pH 6, and the mixture was thoroughly mixed until homogeneous. The resulting solution was then incubated at room temperature for 20 minutes. To stop the reaction, 250 µL of 0.4 M $HClO_4$ solution and 250 µL of Ehrlich solution were added to the mixture and shaken until homogeneous. The mixture was incubated for 90 minutes at 60 ˚C, forming a black precipitate. Centrifugation was performed at 3000-4000 rpm for 5 minutes to separate the precipitate and the supernatant was carefully transferred to a cuvette. Hydroxyproline content was measured at a wavelength of 557 nm using a spectrophotometer. The hydroxyproline concentration in the samples was then determined by comparing it against the standard curve of 1-hydroxyproline [30].

5.7. Malondialdehyde Analysis

One hundred mg of skin tissue was crushed with a mortar in Phosphate Buffer Saline (PBS) solution. The resulting mixture was centrifuged, and the supernatant was collected. To 1 mL of the skin tissue supernatant, 1 mL of 20% TCA and 1 mL of 1% TBA in 50% glacial acetic acid were added. The mixture was then incubated in a water bath at 95 ˚C for 45 minutes and cooled. After centrifugation at 1000 rpm for 15 minutes, 1 mL of the supernatant was transferred into a cuvette, and the absorbance was read at λ 532 nm using a spectrophotometer. MDA levels were determined by comparing the sample absorbance data with the tetramethoxypropan (TMP) standard curve [31].

5.8. Histopathologic Evaluation for Fibroblast

Microscopic observations showed the number of active fibroblasts characterized by large cytoplasm, fine chromatin, ovoid nucleus, and visible. Fibroblast cell counting was seen with a 400x magnification binocular microscope. Calculations were made in 5 fields of view, and the final results were taken from the average number of fibroblast cells. Cell counting was done using the ImageJ application by marking the presence of active fibroblasts [32].

5.9. Statistic Analysis

Statistical analysis in this study used Shapiro-Wilk for the normality test, and Levene for the homogeneity test, and continued with One Way ANOVA with a 95% confidence level followed by LSD post hoc test.

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