CRITICAL MEDICAL AND SURGICAL NURSING JOURNAL
Vol. 14, no. 1, March 2025

Journal Homepage: https://e-journal.unair.ac.id/CMSNJ

Effectiveness of Turmeric Rhizome Extract and Egg Shell Waste Ointment in Healing Diabetic Wounds in Mice With DDY Strain

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ARTICLE HISTORY
Received: July 29, 2024
Accepted: February 27, 2025
Published: February 28, 2025

KEYWORDS
Diabetes mellitus, eggshell, ointment, turmeric extract

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ABSTRACT

Introduction: One of the complications of diabetes is wounds on the feet. The use of curcumin as a natural ingredient can accelerate the healing of diabetic wounds with its anti-inflammatory and antioxidant properties. However, curcumin has low solubility, so it requires a conductor such as hyaluronic acid in eggshells to increase hydration and bioavailability. The aim of this research was to determine the effectiveness of turmeric rhizome and eggshell extract ointment in healing diabetic wounds.

Methods: True experimental research was carried out using a pre-post test control group design. This research was carried out using 36 mice, which were divided equally into 6 control and treatment groups. This study used Kruskal-Wallis analysis to determine differences between groups with a significance level used of $p<0.05$. Macroscopic monitoring by measuring the diameter of the wound and microscopically monitoring the wound using histopathological tests was carried out in this study.

Results: The results of macroscopic and histopathological observations showed that the F3 ointment dose group had the best speed of healing diabetic wounds in mice when compared with the other groups. The statistical results significantly show the effect of administering turmeric rhizome and eggshell extract ointment at a p-value of 0.035.

Conclusion: Using a combination of turmeric rhizome and eggshell ointment at a dose of 9:15 was proven to be effective in helping speed healing of diabetic wounds in DDY strain mice. This study has the potential to be continued in the next stage by comparing different doses of eggshells so as to create a more effective formulation in healing diabetic wounds.

Cite this as: Firmansyah, A., Yashuda, S., Pekerti, H.H.H., Zharifah, J.A.N., Nurhadiyanti, E.A., Fauziningtyas, R. (2025). Effectiveness of Turmeric Rhizome Extract and Egg Shell Waste Ointment in Healing Diabetic Wounds in Mice With DDY Strain. Crit. Méd. Surgical. Nurs. J, 14(1),18-25.

1. INTRODUCTION

Diabetes mellitus is a metabolic disease characterized by hyperglycemia that occurs due to abnormalities in insulin secretion. This condition is associated with long-term damage and functional disorders of various tissues and organs. One of the complications of diabetes is the presence of foot wounds. Statistically, according to the International Diabetes Forum (IDF) report, the number of people with diabetes in Indonesia reached 19.5 million in 2021 (Magliano & Boyko, 2021). Indonesia also ranks fifth among countries with the highest number of diabetics in the world. This is supported by Riskesdas data (2018) which shows the prevalence of diabetic ulcers in Indonesia is around 15%, the amputation rate is 30%, besides that the mortality rate 1 year after amputation is 14.8% (Kementerian Kesehatan Republik Indonesia, 2018).

Currently, diabetic wound management focuses on preventing wounds from drying out and hardening, increasing the rate of epithelialization, accelerating the autolysis debridement process, and can reduce the incidence of infection, as well as being easy to apply (Primadani & Safitri, 2021). Turmeric, which is known as an herbal plant, is known to help wound healing. The curcumin content in turmeric can shorten the inflammatory phase and prevent infection in diabetic wounds. However, curcumin has low water solubility so it requires a carrier that can increase bioavailability such as hyaluronic acid (Castro, 2021). Previous research has been conducted by administering curcumin intragastrically to diabetic rats that were given wounds on their feet, with the results that curcumin can increase proliferation, migration, and angiogenesis in the diabetic rat group, thereby accelerating the healing of diabetic wounds (Cao et al., 2024)

Chicken eggshells are one of the household organic wastes that have potential as a basic material for wound healing dressings. The amount of eggshell waste in 2018 is estimated to reach 180.89 tons, potentially polluting the environment if not utilized (Saputra et al., 2021). Chicken eggshells contain calcium active substances that can help prothrombin form fibrin threads. In addition, eggshells also contain collagen, hyaluronic acid, glucosamine, and chondroitin that support wound healing (Sariyana et al., 2018). Hyaluronic acid increases cell infiltration and helps in the mobilization of important proinflammatory cytokines such as TNF-α and interleukin 8 and plays a role in the process of proliferation to keratinocyte (Amsia, 2021). The hyaluronic acid in chicken eggshells can help increase permeability, potentially improving the bioavailability of topical drugs. Hyaluronic acid can function as an enhancer by hydrating the stratum corneum which will change the arrangement of cells that are tightly packed to be more tenuous. As a result, skin permeability to active ingredients will increase (Suzliana et al., 2020).

Ointment is a semisolid preparation that aims for topical therapy. Ointment has the advantage of being able to maintain skin hydration and increase wound absorption, so that it is in line with therapeutic purposes to prevent wounds from drying out and increasing the effectiveness of therapy. Therefore, we propose to conduct research on the potential utilization of chicken eggshell waste and turmeric rhizome extract in ointment preparations as an alternative to diabetic wound healing. The novelty of the research that we propose is the utilization of eggshell waste and turmeric rhizome extract in ointment formulation as an alternative topical medicine for diabetic wound healing. We use the DDY strain of mice because they have skin anatomy and physiology similar to humans, which can help us achieve maximum results.

2. METHODS

Study Design

This study uses a quantitative method with true experimental approach where we look at the pre-test and post-test with 3 control groups and 3 intervention groups. In this study, researchers treated with the independent variable, the ointment formulation from eggshell waste and turmeric extract.

Population, Samples, and Sampling

The population in this study were mice (Mus Musculus) with sample criteria weighing 20-30 grams, 2-3 months old, male, and DDY strain. The sampling technique used simple random sampling. The number of samples used was 36 mice which were divided into 6 different treatment groups, Healthy Control (moist gauze), Negative Control (moist gauze), Positive Control (calcium alginate), Dose 1 (F1 Dose Ointment), Dose 2 (F2 dose ointment), and Dose 3 (F3 dose ointment). There are 5 groups that inducted to be diabetes in accordance with the objectives of the study, namely positive control group, negative control group, dose 1, dose 2, and dose 3.

Instruments

The research instruments were all the tools and materials of the ointment formulation. The materials used in this research are turmeric rhizomes, 96% ethanol, chicken eggshells, vaselin flavum, paraffin wax, liquid paraffin, adeps lanae, BHT, Propylene glycol, alloxan, ketamine. The tools used include grinder, maserator container, buchner funnel, vacuum pump, rotary evaporator, round bottom flask, 250 mesh, beaker glass, glass funnel, stirring rod, spatel, tray, porcelain cup, stamper, thermometer, waterbath, analytical balance, measuring cup, blades, napkin, ointment pot container, Viscometer, spreadability test kit, pH meter. test tube, capillary tube, dropper pipette, silica gel plate, HPLC with Zorbax eclipse XDB-C18, UV lamp, syringe, and mice animal cage (Hanwar et al., 2020; Ningsih et al., 2018)

Procedure

Determination Test

Turmeric rhizome plants obtained from the Mulyorejo market area were then subjected to Determination Test at the UPT Laboratorium Herbal Materia Medica Batu.

Simplisia Preparation

Turmeric rhizome plants are cleaned by washing from impurities and then carried out wet sorting, kneading, drying, and dry sorting. The weight of dry simplisia was 800 grams.

Extraction with Maceration Method

The method is by adding 4 liters of 96% ethanol to 800 grams of simplisia powder (1:5 ratio). Extraction was done in a maserator container by soaking for 3 days and occasionally stirring. After 3 days, filtering was conducted with a buchner funnel and vacuum pump, followed by evaporating at temperature of 60 °C with a rotary evaporator until a thick extract was obtained.

Phytochemical Test

Flavonoid Test

0.1 gram of ethanol extraction of turmeric rhizome in a test tube added with 0.1 mg of magnesium powder, 0.4 mL of amyl alcohol and 4 mL of ethanol then shaken gently, the presence of flavonoid compounds is indicated by a change in color to red, orange and yellow (Ningsih et al., 2018)

Polyphenol Test

Ethanol extraction of turmeric rhizome of 1 mL, reacted with 0.5 mL of 1% $FeCl_3$ solution in a test tube. Positive results contain polyphenols if a blackish or dark blue color is formed (Hasan et al., 2023)

Alkaloid Test

1 mL ethanol extraction of turmeric rhizome, placed in a test tube, added 0.1 mL HCl 2N then added Dragendorff reagent. Positive contains alkaloid compounds with a reddish brown precipitate (Hasan et al., 2023)

Anthraquinone Test

Extracting 0.3 grams of ethanol extract of turmeric rhizome with 10 mL of distilled water and the filtrate was separated. The filtrate is then extracted again using 3 mL of toluene and added with ammonia. The extract is positive for anthraquinone if it shows a red color change (Ningsih et al., 2018)

Saponin Test

Ethanol extract of turmeric rhizome as much as 0.1 gram is added with 10 mL of distilled water and then shaken vigorously, positive if foam forms as high as ± 1 cm (Ningsih et al., 2018)

Triterpenoid or steroid assay

Weigh as much as 0.1 gram of turmeric rhizome extract then add 3 drops of anhydrous acetate and concentrated H_2SO_4. Positive for steroids if there is a green change, while triterpenoids are positive if there is purple or red (Ningsih et al., 2018)

Curcumin Assay

The curcumin content test on turmeric rhizome extract was carried out qualitatively and quantitatively. Qualitative tests were carried out by KLT method with stationary phase, Silica Gel GF 254 and mobile phase Chloroform: Methanol (9.5:0.5). The presence of curcumin was identified by comparing the Rf between Curcumin Standard and Turmeric Rhizome Ethanol Extract. Meanwhile, quantitative tests were carried out with High Performance Liquid Chromatography (HPLC) instruments because they have high separability, sensitivity, and have good accuracy and precision for curcuminoid compounds (Hanwar et al., 2020)

Preparation of Chicken Egg Shell Powder

Eggshell waste was collected and soaked using hot water, then dried in an oven at 105^{0}C for 30 minutes. After drying, the eggshells were pulverized with a blender until they became a fine powder.

Qualitative Test of Eggshell Content

Qualitative tests of eggshell content were carried out by benedict test and calcium test. Benedict test was conducted to see the hyaluronic acid content of eggshell powder (Novita et al., 2023). Hyaluronic acid is a class of heteropolysaccharides so that it can show

Table 1. Phytochemical Test Results

	Turmeric rhizome extract
Alkaloid	+
Polyphenol	+
Flavonoid	+
Triterpenoid	+
Anthraquinone	+

Table 2. Qualitative Test Results of Chicken Egg Shell

	Hyaluronic acid	Calcium
Chicken eggshell powder	+	+

Table 3. Qualitative Test Results of Chicken Egg Shell

Formulation	F1 (7:15)	F2 (8:15)	F3 (9:15)
Color	dark brown	dark brown	dark brown
Smell	typical turmeric	typical turmeric	typical turmeric
Texture	greasy	greasy	greasy
Ease of application	easy to apply	easy to apply	easy to apply
Ease of purification	easy to wash off	easy to wash off	easy to wash off

Table 4. Homogeneity test results

	Top	Middle	Bottom
F1 (7:15)	Homogeneous	Homogeneous	Homogeneous
F2 (8:15)	Homogeneous	Homogeneous	Homogeneous
F3 (9:15)	Homogeneous	Homogeneous	Homogeneous

Table 5. Viscosity test results

Replication	F1 (7:15)	F2 (8:15)	F3 (9:15)
1	25.135	23.412	20.156
2	24.892	23.114	20.012
3	25.025	22.830	20.312
Average ± RSD	25.017,33 ± 121,68	23.118,67 ± 291,03	20.160,00 ± 150,04

positive results in the benedict test which is indicated b y a color change from blue to yellow, green, orange, or red.

Calcium was tested by weighing 1 gram of eggshell, then given 10 mL of HNO3 which was heated until white acid formed and a clear filtrate appeared. The filtrate is then filtered with filter paper and given distilled water up to 10 mL. The test was continued by taking 2 mL of filtrate solution which was added with 10 drops of 5% ammonium oxalate solution. The sample is said to be positive for calcium if a white precipitate appears (Raya et al., 2023)

Level Determination

Determination of curcumin content was carried out using the High Pressure Liquid Chromatography (HPLC) method conducted by the Airlangga University Research Service Unit.

Ointment Making

In the first stage, weighing each ingredient then melting the ointment base with a waterbath temperature of ± 65oC. The second stage, mixing turmeric rhizome extract and eggshell powder until homogeneous, then adding antioxidant additives. The last stage is mixing the base in stage I with stage 2 ingredients using a mortar and stamper until homogeneous.

Physical Properties Test of Ointment

Organoleptical Test

The test is carried out by observing the color, odor, and texture of the preparation using the five senses.

Acceptability Test

The acceptability test was carried out by collecting a number of respondents to provide responses to the preparation in the form of, ease of application and ease of washing.

Homogeneity Test

The homogeneity test was carried out by applying the ointment preparation on an object glass and then covering it with another object glass and visually observing whether the preparation was homogeneous.

pH test

The pH test was carried out by weighing 1 gram of ointment preparation and then adding 10 mL of CO2-free water stirred until homogeneous, then checked using a pH meter (Sudjarwo et al., 2023)

Viscosity Test

The viscosity test was conducted using a brookfield viscometer with spindle number 64. The ointment was placed in a beaker glass, tested at a speed of 50 rpm.

Spreadability Test

The ointment spreadability test was carried out by weighing 1 gram of ointment placed in the center of the glass of the spreadability test tool and covered with another glass then given a load and waited for 1 minute. Measurement of the diameter of the spread of the preparation was carried out vertically and horizontally, with the addition of weights every 50 grams to a total weight of 500 grams.

In Vivo Effectiveness Test

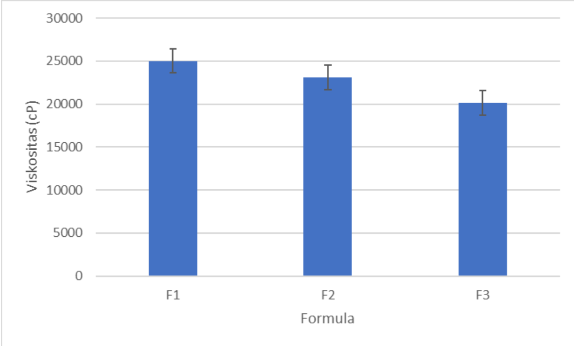

Figure 1. Histogram of viscosity test results

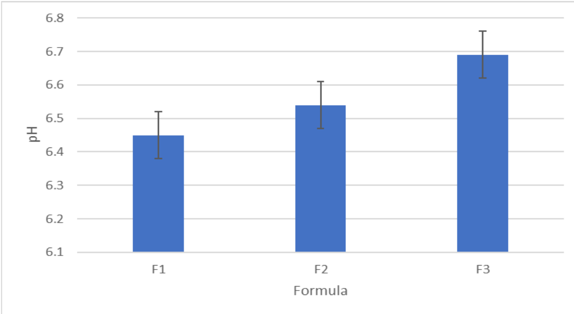

Figure 2. Histogram of acidity level test results

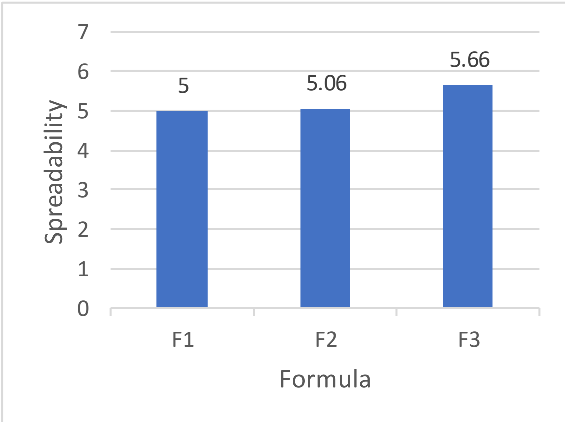

Figure 3. Histogram of spreadability test results

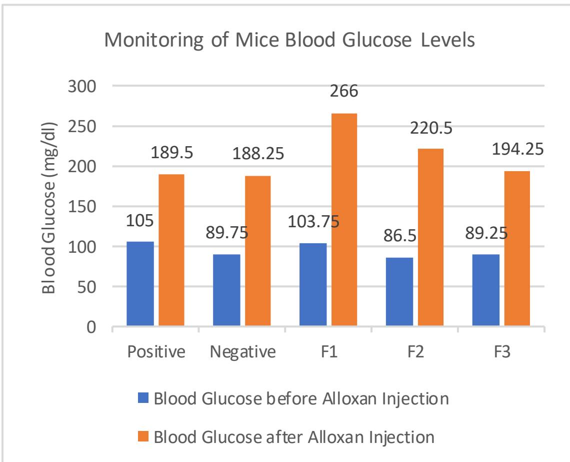

Figure 4. Blood glucose monitoring of mice

In this study, 36 male mice of DDY strain with the age of 2-3 months and body weight between 20-30 grams were divided into 6 groups. The groups consisted of healthy group (moist gauze), negative control group (moist gauze), positive control group (calcium alginate dressing), and three treatment groups with turmeric rhizome and eggshell extract ointment at several concentrations (F1 = 7%; F2 = 8%; F3 = 9%). The research was conducted on mice that had been induced by Alloxan as much as 3 mg/20 grams of BW with the Multiple Low Dose method. If the mice have blood sugar levels > 175 mg/dl, then the mice are already diabetic (Pertiwi et al., 2020). After the mice are confirmed to be diabetic, then the mice are injected with ketamine and a diabetic wound model is made. Makingdiabetic wounds is done by excision with a length of 1 cm x 1 cm. Ointment was applied every 2 days for 8 days. Observation of wound healing effectiveness was done macroscopically and histopathologically. Macroscopic observations were made by measuring the wound diameter on days 2, 4, 6, 8 and compared with the control group. After that, mice were euthanized and tissue preparations were made, microscopic wound healing observations were made by observing indicators of fibroblast cell formation, re-epithelialization, and angiogenesis. All research methods used are in accordance with the 5 freedoms of animal welfare. The experiment was approved by the animal ethics commission of the Faculty of Veterinary Medicine, Universitas Airlangga with letter number 2.KEH.076.05.2024.

Data Analysis

The data obtained through testing were processed and analyzed using SPSS 25 testing. Normality test was conducted with Shapiro Wilk and continued with homogeneity test using Levene Test. Data analysis continued using One Way ANOVA if proven normally distributed and Kruskal Walls if not normally distributed. Further analysis was carried out with the Post Hoc test.

3. RESULTS

Determination Test Results

Determination test on plant samples obtained the results of turmeric plant determination which has the species name Curcuma longa (L.).

Turmeric Rhizome Extraction Result

The turmeric rhizome symplisia obtained was 800 grams obtained from drying and pulverizing 2.5 kg of turmeric rhizomes. Turmeric powder was extracted with 96% ethanol and produced 79 grams of thick extract with a yield of 9.88%.

Phytochemical Test Results

Table 1 showed that turmeric rhizome extract contained alkaloids, polyphenols, flavonoids, triterpenoids, and anthraquinones.

Curcumin Assay Results

Based on the results of qualitative tests on turmeric rhizome extract, it was found that the extract was positive for curcumin while

Table 6. Ointment pH test results

Replication	F1 (7:15)	F2 (8:15)	F3 (9:15)
1	6,49	6,65	6,70
2	6,46	6,43	6,73
3	6,41	6,55	6,65
Average ± RSD	6,45 ± 0,04	6,54 ± 0,11	6,69 ± 0,33

Table 7. Ointment spreadability test results

Replication	F1 (7:15)	F2 (8:15)	F3 (9:15)
1	5,10	5,15	5,60
2	5,00	5,10	5,65
3	4,90	4,95	5,66
Average ± RSD	5,00 ± 0,10	5,06 ± 0,10	5,64 ± 0,03

Table 8. Wound Closure Results after Treatment

Group	Diameter of Wound (cm)		
	H4	H6	H8
Positive Control	0,77 ± 0,15	0,77 ± 0,15	0,77 ± 0,15
Negative Control	0,60 ± 0,07	0,60 ± 0,07	0,60 ± 0,07
F1 (7:15)	0,48 ± 0,09	0,48 ± 0,09	0,48 ± 0,09
F2 (8:15)	0,64 ± 0,05	0,64 ± 0,05	0,64 ± 0,05
F3 (9:15)	0,64 ± 0,14	0,64 ± 0,14	0,64 ± 0,14

Table 9. Assessment results of histopathology test parameters

Groups	Indicators		
	Fibroblast	Angiogenesis	Squamous Epithelium
Healthy	16,3	1,3	++
Positive Control	24,3	3,6	++
Negative Control	14,3	0,67	+
F1 Dose	17,3	1,6	++
F2 Dose	16,3	1,3	++
F3 Dose	24,3	4	+++

Description :
+ : low amount of ephitelization
++ : standard amount of ephitelization
+++ : high amount of epithelization

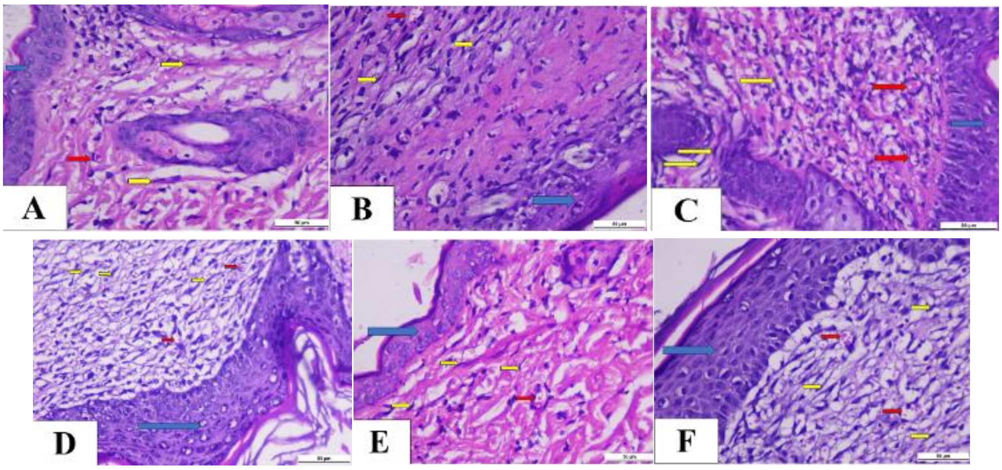

Figure 5. Histology of mice skin
Description: A) Healthy, B) Positive Control, C) Negative Control, D) F1, E) F2, F) F3
fibroblast cell formation (→), angiogenesis (→), and reepitalization (→)

quantitatively the curcumin content in the extract showed an average RPD of 27.0 ± 0.03%.

Qualitative Test Result of Chicken Egg Shell

The test results (table 2.) show that chicken eggshell powder contains hyaluronic acid and calcium.

Ointment Physical Properties Test Results

Organoleptical and Acceptability

The organoleptical test results of the three ointments met the desired specifications, namely having a dark brown color, smelling typical of turmeric with a greasy texture, easy to apply and easy to wash off.

Homogeneity

Good ointment homogeneity includes the absence of lumps when applying, flat structure, and has a uniform color from the beginning to the end of the application (Lasut et al., 2019). The homogeneity test showed that the ointment was homogeneous.

Viscosity

The viscosity test is carried out to see the level of viscosity of the ointment preparation, the lower the viscosity level, the faster the release of the drug from its base (Sawiji & Sukamdiani, 2021). Based on the test results, the viscosity of the ointment preparation meets the requirements in accordance with the quality requirements for the viscosity of a good ointment preparation, which ranges from 2000-50,000 cP (Putri et al., 2020)

The results of One Way Anova statistical analysis, the viscosity value in each formula has a significant difference (P> 0.05). This shows that different concentrations of turmeric rhizome extract have an effect on the viscosity of the combined ointment preparation of eggshell and turmeric rhizome extract.

pH

pH testing is carried out to assess the safety and comfort of the preparation when used topically. If the preparation has a pH that is too acidic it will cause irritation, while if it is too alkaline it will cause dry scaly skin (Suzliana et al., 2020). Based on the results of pH testing, the ointment preparation meets the requirements in accordance with the pH balance.

The results of the pH test analysis using the One Way Anova statistical method showed a significant difference (p> 0.05), which means that different concentrations of turmeric rhizome extract have an effect on the pH of the ointment preparation of a combination of eggshell and turmeric rhizome extract.

Spreadibility

The spreadability test is intended to see the quality of the ointment preparation through the ease of spreading when applied on the surface of the skin. Based on the results of the spreadability test, the ointment preparation meets the requirements for a good spreadability diameter in ointments, which is 5-7 cm (Lasut et al., 2019).

From the results of One Way Anova statistical analysis, it was found that there were significant differences (p> 0.05) indicating that different concentrations of turmeric rhizome extract combined with eggshell had an effect on the spreading capacity of the ointment preparation.

In Vivo Effectiveness Test Results

The results of monitoring the average blood glucose levels of all treatment groups of mice showed a significant value of p>0.05, which means that the data was normally distributed and homogeneous.

After the mice were confirmed to be diabetic, ketamine was injected and a diabetic wound model of 1 cm x 1 cm was made. Mice were then treated with healthy control, positive control, negative control, and three treatment groups with different doses of ointment. Administration of ointment preparations was carried out every 2 days for 8 days.

Observations of wound healing effectiveness were made macroscopically and histopathologically. Macroscopic observations were made by measuring the wound diameter on days 2,4,6,8 and compared with the control group. As a result, the F3 treatment group had the best wound healing speed compared to the other treatment groups.

Histopathological testing was performed on skin organs to determine the wound healing effect based on indicators of fibroblast cell formation (➔), angiogenesis (➔), and reepitalization (epithelial formation around the wound) (➔) as shown in Figure 5. Results of the assessment of histopathological test parameters after observations made in three fields of view with 400x magnification presented in Table 8.

4. DISCUSSION

Based on Table 8, it can be seen that the negative and healthy control groups have few fibroblasts so that the epidermal layer looks still tenuous, group F3 has a very large number of fibroblasts, and positive control groups, F1 and F2 have more fibroblasts. The second histopathology result observed the formation of new blood vessels (angiogenesis). The negative control group had the least amount of angiogenesis and the F3 group had the most new blood vessels in the field of view observation (Pertiwi et al., 2020). Finally, it can be seen that the negative control group has not yet epithelialized and the wound gap is still visible, while F1 has epithelialized but the epidermal tissue has not been fully invaded by granulation tissue. Then in group F2 it has epithelialized but not perfectly and the dermis tissue has been fully invaded by granulation tissue. Meanwhile, in group F3, the epithelium has epithelialized in the epidermal tissue and has invaded granulation in the dermis tissue. Thus, the F3 dose group has the best healing effect compared to the other dose groups.

High glucose levels in diabetic conditions can increase the expression of miRNAs that cause inflammation and can encourage the process of apoptosis in fibroblasts. Curcumin can help suppress miRNA that causes inflammation so that it can increase fibrilin 1 (FBN-1) levels. FBN-1 has an important role in facilitating proliferation, fibroblast migration, and preventing fibroblast apoptosis (Cao et al., 2024). In anti-inflammatory activity, curcumin modulates various signaling pathways such as inhibiting NF-(k)B activation and inhibiting angiotensin II-induced inflammation. In addition, antioxidant activity is caused by the ease of curcumin in transferring its H atoms so that it can prevent oxidative stress due to prolonged exposure to Reactive Oxygen Species (ROS) (Kumari et al., 2022).

The nature of curcumin which has low solubility and permeability correlates with its low bioavailability, while the bioavailability of topical preparations can be seen from the concentration of active substances in the stratum corneum layer (Sudjarwo et al., 2023). The concentration in the stratum corneum can be used as a surrogate of the drug concentration in the viable epidermis (Pellanda et al., 2006). The strong hydration properties of hyaluronic acid can help increase transdermal permeability in the stratum corneum layer (Garg et al., 2015). Therefore, hyaluronic acid may assist curcumin in increasing bioavailability through a hydration mechanism in the epidermal layer. In addition, the ointment base may also affect bioavailability due to its occlusive properties against the stratum corneum. This can increase the flux of drug across the skin and affect the partitioning of drug from ointment to skin (Garg et al., 2015). Curcuwon ointment contains curcumin compounds and hyaluronic acid components from eggshells that can work synergistically in reducing inflammation and increasing proliferation while maintaining skin hydration and maintaining ointment bioavailability. Further research on the combination of turmeric with eggshells is needed to determine the effective dose of eggshells so that they can be used as an alternative treatment in treating diabetic wounds.

However, the limitations of the research are the lack of quantitative testing of hyaluronic acid in eggshells and the same dose of eggshells in all ointments. The quantitative content of hyaluronic acid greatly influences the wound healing process. This factor needs to be considered to produce good outcomes from this research.

5. CONCLUSION

Based on the results of the research and analysis that has been carried out, F3 with turmeric rhizome extract ointment formulation with a concentration of 9% provides the most optimal formula based on the characterization results. Ointment from a combination of eggshell and turmeric rhizome extract is effective as an alternative complementary medicine for diabetic wound healing based on in vivo test results. Based on the findings, it is suggested to conduct further studies exploring different concentrations of eggshell powder combined with turmeric extract to identify the most effective formulation for diabetic wound healing. Additionally, investigating the long-term effects of the combined ointment on diabetic wounds could provide insights into its sustained healing properties. Comparative effectiveness research should also be performed to evaluate the ointment against conventional treatments. Implementing clinical trials with diabetic patients will help validate the ointment's effectiveness in real-world settings. Lastly, exploring the potential benefits of this combination with other therapeutic agents may further enhance healing outcomes.

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