Trop J Nat Prod Res, February 2023; 7(2):2382-2387 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Biological Activity of Catfish (*Clarias gariepinus*) Mucus Gel on the Wistar Rats (*Rattus norvegicus*) Wound Healing Process

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ARTICLE INFO

Article history:
Received 08 January 2023
Revised 19 February 2022
Accepted 21 February 2023
Published online 01 March 2023

ABSTRACT

Clarias gariepinus (North African catfish) is a preferred freshwater fish for consumption in Indonesia. Catfish mucus by-product is considered to have no economic value and is not optimally utilized. Even though, recent studies have shown that the mucus contains various bioactive molecules that support fish immunity. The purpose of this study was to determine the effect of *C. gariepinus* catfish strain *Mutiara* mucus gel on the Wistar rat wound healing process. This study was conducted by formulating catfish mucus into a gel preparation. Mucus gel was evaluated for physicochemical characteristics and organoleptics. Mucus gel was applied in vivo to the incision wound of rats for 14 days and compared with the positive control using povidone iodine and placebo control using gel base. Wound morphology and length were observed every day, whereas wound histology and immunohistochemistry were observed once on the 15th day. The qualitative data was analyzed descriptively. The physicochemical characteristics of the gel revealed that the gel has a low viscosity, an effective adhesion time, and a neutral pH. Based on organoleptic evaluation, it has a moderate texture, a fishy and strong aroma, and a cloudy color, making it less favorable to the panelists. In an in vivo study, catfish mucus gel significantly accelerated wound closure and increased epithelial tissue thickness but had no significant effect on increasing fibroblast and blood vessel numbers, and VEGF-A expression. More in-depth studies of the catfish mucus gel is needed for further development.

Keywords: *Clarias gariepinus* catfish, mucus, gel, wound healing, VEGF-A

Introduction

Clarias gariepinus (North African catfish) is an important fishery commodity in Indonesia. This catfish is widely cultivated almost everywhere in the country.[1] It is a preferred consumption freshwater fish, which has several advantages, including high nutritional content, fast growth, being easy to breed, being easy to adapt to all water conditions, being easy to cultivate, and being easy to market with relatively low capital requirements and therefore having a relatively affordable price.[2] According to Indonesia Central Bureau of Statistics data, national production of *Clarias gariepinus* catfish commodities has increased rapidly, reaching over 1 million tons with a value of more than 17 billion IDR.[3] In 2017, production increased by 131 percent from the previous year.[4]
Clarias gariepinus catfish has a mucus by-product that is considered to have no economic value. Catfish mucus is produced by the skin mucous layer and provides a physical barrier against the dynamic aquatic environment.[5] Mucus plays a role in reducing friction with water, covering wounds, and acting as the body's defense system against aquatic environments that contain various microorganisms. Fish skin is at high risk of infection because it is always exposed directly to microorganisms carried in water.

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Citation: Widiyani T, Iriansyah AH, Listyawati S. Biological Activity of Catfish (*Clarias gariepinus*) Mucus Gel on the Wistar Rats (*Rattus norvegicus*) Wound Healing Process. Trop J Nat Prod Res. 2023; 7(2):2382-2387 http://.www.doi.org/10.26538/tjnpr/v7i2.12

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Compared to land organisms and amphibians, the adaptive defense system of fish is relatively underdeveloped and therefore relies heavily on innate mechanical immunity.[6] Mucus is the first line of defense against water-borne microorganisms.
Several studies have shown that fish mucus inhibits microorganism colonization in specific parts of the body, including the skin, gills, and mucosal tissues. Previous study resulted in *Clarias gariepinus* mucus having antibacterial activity against methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* ATCC 27853.[7] Another study stated that fish mucus contains natural antibodies that fight pathogens and play a role in the immune system. In general, teleost and mammalian mucus consist of mucin.[8] However, there is a difference in the bioactive molecule content of mucus. The composition of fish mucus varies based on species, endogenous factors (sex and development phase), and exogenous factors (temperature, pH, infection, and environmental stress).[9]
The molecule contents of fish mucus include lysozymes, lectins, immunoglobulins, and antimicrobial peptides.[10-12] A number of essential amino acids, such as leucine and lysine, which function in the formation of various new tissues during growth and replace damaged tissue, are also found in the catfish mucus.[13] Leucine is known to be useful in the overhaul and formation of muscle protein, while lysine is known to be needed for the growth and repair of damaged tissue. The biological activity of various fish mucus, such as that of eels, cod, snapper, salmon, and catfish itself, has been studied.[13,14] They indicated that fish mucus has the potential to be developed and applied for health and aquaculture purposes. However, further studies are still needed to characterize the biological activity of *Clarias gariepinus* catfish mucus, especially its function in the wound healing process. There has never been a study of catfish mucus in gel preparations for wound healing. Due to the volatile nature of the gel, topical gel preparations can improve the effectiveness and comfort of their use, including their ability to deliver medicinal ingredients well and dry quickly. Gel preparations also have the advantage of spreading easily on the skin,

 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

and providing a cooling sensation.[15] Therefore, the purpose of our study was to determine the biological activity of *Clarias gariepinus* catfish mucus gel in the wound healing process.

Materials and Methods

Source of mucus material

The catfish samples belong to *Clarias gariepinus* strain *Mutiara.* They were obtained from the Aquaculture Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Mucus was isolated from 56 catfish ranging in age from 1 to 2.5 months and measuring an average of 20 cm in length. Catfish were placed in dry conditions an hour before mucus isolation. The mucus was scraped off the dorsal catfish body surface using a spatula. Collected mucus was centrifuged for 10 minutes at 3500 rpm to separate the dirt and liquid. The supernatant (pure mucus) was stored in a common refrigerator at 4^0C before further preparation. Mucus collection was carried out on January 2022.

Gel preparation

The formulation of catfish mucus gel consisted of pure mucus, hydroxypropyl methylcellulose (HPMC), glycerin, methyl paraben, citrus oleum, and distilled water. These ingredients were formulated in certain ratios,[14] as follows:

Catfish pure mucus	Active ingredient	15.5%
Hydroxy propyl methylcellulose (HPMC)	Gelling agent	2%
Glycerin	Humectan	10%
Methyl paraben	Preservative	0.2%
Citrus oleum	Fragrance	0.5%
Distilled water	Solvent	Added up to 100%

The HPMC was stirred in hot water until it expanded and became homogeneous. Meanwhile, the glycerin, methylparaben, citrus oleum, and catfish pure mucus were mixed together. Then, this mixture was added to the previously homogenous HPMC. The finished catfish mucus gel was evaluated, which included measurements of pH, adhesion strength, viscosity,[16] and organoleptics.

Organoleptic testing was carried out by the panelists on several sensing aspects. In this study, 20 panelists participated to provide an assessment based on their likes and dislikes on the texture/consistency, aroma, and color aspects of the catfish mucus gel. Panelists were asked to give a score ranging from 1-7 on the aspects being assessed. Score 1 means really dislike while score 7 means really like.[17]

Bioactivity testing of catfish mucus gel on wound healing process

Experimental animals preparation

Eighteen male Wistar rats (*Rattus norvegicus*) weighing 150-200 grams were used. Rats were randomly divided into three groups of six individuals each. The three experimental groups were placebo control, positive control, and catfish mucus gel. They were acclimated for five days and fed and drank ad libitum prior to the experiment. Permission of the Universitas Muhammadiyah Surakarta Ethics Committee has been obtained for the use of experimental animals, as proven by Ethical Clearance Letter No. 4165/A.1/KEPK-FKUMS/III/2022 dated March 25, 2022.

In vivo assessment

After acclimation, the rat's back hair was shaved, and the back skin was cleaned using 70% ethanol. Rats were anesthetized by chloroform inhalation. An incision was made parallel to the os vertebrae, 1 cm long in the subcutis layer, using a scalpel by stretching the skin with the index finger and thumb of the left hand.[18] Exposure was carried out by dripping 3 ml testing material on the 3x3 cm^2 cotton pad and affixing it to the wound surface. Every 24 hours, the gauze was replaced with a new one. This treatment was performed for 14 days. Group I (the placebo control) used a gel base; group II (the positive control) used 10% povidone-iodine gel; and group III used catfish mucus gel.

- Observation of the wound morphology was carried out every day for 14 days, including measuring the wound length.[19] The length was measured using a Vernier caliper with an accuracy of 0.01 cm. On the 15^{th} day, rats were euthanized. The wound skin tissue was sectioned using the microtechnical paraffin method and stained in two different ways. Mayer's Hematoxyllin-Eosin staining was used to examine the general structure of wound tissue and to count the number of epithelial tissue layers, blood vessels, and fibroblasts. In contrast, a specific immunohistochemical staining with monoclonal antibody VEGF-A (ABclonal Technology®) was used to examine the distribution of vascular endothelial growth factor A (VEGF-A). When there is a brown color in the cell's cytoplasm, this indicates a positive reaction.[18,20] Microscopic data collection was carried out by observing six different fields of view under a light microscope with a magnification of 400×.

ImageJ software was used to compare color intensity to determine VEGF-A expression. The higher the percentage of VEGF-A expression, the darker the brown color. The levels of VEGF-A expression were classified as follows: 0% unexpressed, 0%-33.33% weak positive expression, 33.36%-66.67% moderate positive expression, and 66.67%-100% strong positive expression.[20,21]

Data analysis

Quantitative data included incisional wound length, epithelial thickness, number of fibroblasts, number of blood vessels, and VEGF-A expression percentage. The first three variables were analyzed statistically, using one-way ANOVA and Duncan's multiple range test for further analysis, while the expression of VEGF-A was analyzed using chi-square. Qualitative data on wound skin tissue morphology and histology, were analyzed descriptively. Catfish gel mucus pH, adhesiveness, viscosity, and organoleptic characteristics were also analyzed descriptively and compared to the gel standard.

Result and Discussion

Physicochemical parameters

Pure catfish mucus samples were made into a gel preparation to facilitate its application to wound tissue. The gel preparation should meet the standards of several physicochemical parameters, including pH, adhesiveness (gel adhesion strength), and viscosity (gel consistency). Data on the physicochemical characteristics of the catfish mucus gel are shown in Table 1.

The soft gel consistency makes it easy for the gel to spread evenly and cover the wound's skin tissue surface. A factor that affects the gel's consistency is the viscosity factor. Its value is inversely proportional to the gel's spread ability. The lower the gel viscosity, the higher the gel spreadability. The catfish mucus gel viscosity was 15033.82 ± 95.46 cps. Compared to the gel standard, our gel has low viscosity; therefore, the gel's consistency is too aqueous. A good gel preparation viscosity is between 2000-4000 cps.[22] This might be caused by the low concentration of HPMC, which acts as a gelling agent. HPMC forms a gel base by absorbing solvents, thereby increasing the resistance of the liquid and producing a compact liquid mass. For further research, it is necessary to review the gel formulation in order to obtain the appropriate gel viscosity.

The adhesiveness of a gel preparation is related to the length of contact time between the gel and the skin surface. A good gel preparation is able to provide an effective contact time with the skin, but it is not too sticky, which is related to user comfort. The adhesiveness of a gel preparation can also have an impact on the efficacy of its active substance contents. The longer the adhesion strength, the more effective the active substance at the exposure site. The adhesion of the catfish mucus gel was 350.50 ± 30.52 seconds, meeting the gel standard requirements.

Table 1: Physicochemical parameters of *Clarias gariepinus* catfish mucus gel

No	Parameters	Mean ± standard deviation
1	Viscosity 9cps)	15033.82 ± 95.46
2	Adhersiveness	350.50 ± 30.52
3	pH	6.83 ± 0.04

An effective gel adhesion time on the skin is more than 1 second, so that the active substance content can work effectively at the administration site.[23]

Gel pH measurements were carried out to determine the safety level during the gel's use. To avoid skin irritation, the pH of gel preparations should match the skin pH, which is between 5-7.[13] The pH of the catfish mucus gel preparation was 6.83±0.04 and classified as neutral. Based on morphological observation, after conducting an *in vivo* experiment, the rat skin tissue did not experience irritation.

Table 2 summarizes the organoleptic evaluation of *Clarias gariepinus* catfish mucus gel. The gel texture aspect got the highest average score (4.10 ± 0.99 compared to other aspects), with a neutral interpretation. It means being in the middle position between being liked and disliked. According to the panelists, the texture of the catfish mucus gel is similar to the texture of gel products in general, which is neither too watery nor too dense. This moderate texture makes it easy to apply the gel to the skin's tissue surface.

The aroma aspect has the lowest average score compared to the other aspects. The score aspect was 2.75 ± 0.76 with a quite disliked interpretation. This shows that the aroma of catfish mucus gel is not liked by the panelists. Panelists considered that the smell of catfish mucus gel was too fishy and strong. Even though the catfish mucus gel formulation has been enhanced with citrus oleum as a fragrance, it cannot reduce the unpleasant odor. The color aspect was also interpreted negatively by the panelists with an average score of 3.52 ± 0.82. This shows that visually, the catfish mucus gel is less attractive in color because it looks cloudy (Figure 1). Therefore, it is necessary to add coloring or clearing agents so that the gel is more attractive for future application.

Bioactivity Catfish Mucus Gel on Wound Tissue

Wound Morphology

The reduction of wound length is a characteristic of wound healing activity. Figure 2 shows that the wound length of the rats treated with the catfish mucus gel for 14 days decreases faster. With ANOVA, a significant difference ($p<0.05$) in the average wound length was observed in the rats treated with the catfish mucus gel compared with those who received the placebo control treatments. Nevertheless, the catfish mucus gel group was not significantly different from the positive control group ($p>0.05$). Based on these results, it is known that the ability of catfish mucus gel to promote wound healing is equivalent to using standard drugs (povidone iodine).

The wound healing process was also explained descriptively. On the first day, the wound was still wet and filled with fresh blood (Figure 3). In this phase, the wound experiences a homeostatic mechanism due to trauma to the skin tissue. The bleeding stopped after a few minutes. On the second and third days, the blood looked thickened and dry, but the wound was still open. On the fourth day, the blood remnants hardened and began to peel off. By the fifth day, the wound had closed, and the dead tissue as well as blood clots had detached from the skin.

Starting on the sixth day, the remaining wound was a closed hollow, but in the placebo control group, dried red blood was still visible (Figure 4). The proliferation process occurs in wound tissue, which is characterized by the regeneration of new epithelial and connective tissue. Thus, not only the wound length but also the wound depth decreased. On the 11th day, the wound is flat against the surrounding tissue, but the hairs are still absent. On the 14th day, the wound is considered to have healed because the incision has completely closed. In healed wound, either new tissue covered the wound or there was no erythema and swelling. The process of tissue formation has started, but it's still marked by the absence of hair on the new tissue. This is in accordance to the study of Song et al., that the wound healing process is divided into 4 phases; homeostasis which occurs within seconds to minutes, inflammation for 3-5 days, proliferation for 4-14 days and remodeling phase which occurs for 8 days to 1 year.[24]

Wound Histology

After morphological observations, on the 15th day, the rats were dissected. The incision wound tissue was observed microscopically through histological preparations to observe the wound healing process at the cellular level. The parameters taken into account in this observation included the epithelial tissue thickness, the number of fibroblasts and blood vessels, and the distribution of vascular endothelial growth factor A (VEGF-A). Epithelial tissue is the outermost layer of the skin that is in direct contact with the external environment and acts as a physical barrier. In the incisional wound, the epithelial tissue is exposed, causing the underlying tissue to be directly connected to the external environment. The process of regenerating new epithelial cells is very important in the process of wound closure. In addition to observing the epithelial tissue, fibroblasts and blood vessel were also evaluated. Fibroblasts, the most common cells found in connective tissue, play a role in the proliferative phase, providing protein structures that are used for tissue reconstruction. Extracellular matrix is synthesized by fibroblasts and can stimulate cell attachment to substrates.[25] The presence of blood vessels plays a role in the distribution of nutrients so that homeostatic conditions in wound tissue are maintained.

Figure 1: *Clarias gariepinus* catfish mucus gel

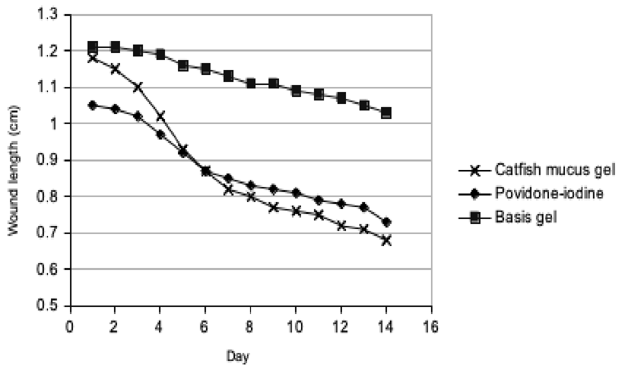

Figure 2: Wound length of catfish mucus gel-treated Wistar strain rats (*Rattus norvegicus*) day 1st to 14th

Table 2: Organoleptic assessment of *Clarias gariepinus* catfish mucus gel

Organoleptic Aspect	Result	Score ± standard deviation	Interpretation
Texture	Moderate (not too watery nor too dense)	4.10 ± 0.99	Neutral
Aroma	Fishy, strong smell	2.75 ± 0.76	Quite dislike
Color	Cloudy	3.52 ± 0.82	Quite dislike

 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Figure 3: Wound morphology of catfish mucus gel (CMG)-treated Wistar strain rats (*Rattus norvegicus*) on days 1, 3, and 5 compared to the positive control (POS) and placebo control (PLA

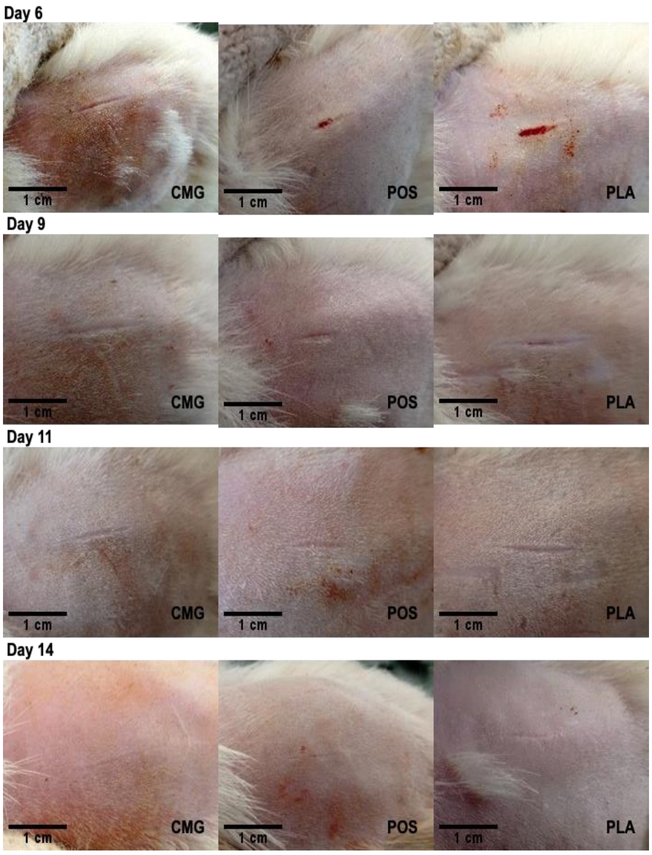

Figure 4: Wound morphology of catfish mucus gel (CMG)-treated Wistar strain rats (*Rattus norvegicus*) on days 6, 9, 11, and 14 compared to the positive control (POS) and placebo control (PLA)

Figure 5 shows that the epithelial tissue in the catfish mucus gel-treated rats and the positive control rats tended to be thicker than that in the placebo control rats. Fibroblasts from both groups, on the other hand, have a more consistent appearance. The appearance of active fibroblasts is more consistent than that of inactive ones because the cell shape is clearly visible due to high internal activity. This indicates that the catfish mucus gel was equivalent in activity to the positive control's. Several other parameters were also observed and analyzed quantitatively, as shown in Table 3.

Based on the histological parameters, the wound tissue of rats treated with catfish mucus gel showed an increase in the epithelial tissue layer thickness, fibroblast numbers, and blood vessel numbers, compared to the rats treated with gel base (placebo control group). However, after statistical analysis using ANOVA, only the epithelial tissue layer thickness was significantly different ($p<0.05$) to the placebo control group. Furthermore, when compared to the positive control group, all parameters of the group treated with catfish mucus gel were significantly different. Therefore, *Clarias gariepinus* catfish mucus gel has little effect on the wound healing process.

The catfish mucus gel was able to accelerate the wound healing process in Wistar rats probably due to the presence of active compounds in catfish mucus, i.e.: lectins, antimicrobial peptides, and glycoproteins.[24-26] Antimicrobial peptides in catfish mucus will prevent bacterial infection in the wound tissue.[27] Lectins play a role in agglutinating lymphocytes and macrophages, which play an important role in the regulation of tissue repair.[26] Both of them will release growth factors, one of the growth factor is VEGF, which will recruit fibroblasts, keratinocytes, and endothelial cells to repair tissue. Glycoprotein in catfish mucus acts as a building block in the process of repairing new tissue.[28]

Furthermore, immunohistochemical observations were carried out to determine the expression of VEGF-A in the wound tissue. VEGF-A plays a role in inducing endothelial cell movement, endothelial cell proliferation, vasodilation, endothelial cell migration, and the formation of blood vessels (angiogenesis).[27] A positive reaction is marked by a brown color in the cell cytoplasm[18,20] as shown in the Figure 6. In the wound tissue of the group treated with povidone iodine, the expression of VEGF-A is higher than in the other groups. The percentage of VEGF-A expression is summarized in Table 4. It is shown that the VEGF-A expression percentage of the group exposed to catfish mucus gel was significantly lower ($p<0.05$) than that exposed to povidone iodine (the positive control) as well as those exposed to base gel (the placebo control). Their expressions are classified as moderate. This demonstrates that treatment with catfish mucus gel had no effect on increasing VEGF-A expression during the wound healing process. The blood vessel numbers and VEGF-A expression in the positive control group were higher than the other groups. The phospholipid molecule content of povidone iodine is thought to be able to coordinate the process of migrating endothelial cells and smooth muscle, which triggers angiogenesis.[20,30]

Except for epithelial cell thickness, the results revealed no significant differences in any cellular processes during wound closure. This could be due to the wound healing process being complete. The histology samples were collected on the 15th day, when the wound was completely closed. Even after the wound has closed, the healing process continues. The remodeling phase can last from 8 days to a year.[24] This stage is distinguished by the reorganization of collagen structure and tissue. The density of cells and capillaries decreases during this phase.

Conclusion

The study concluded that the administration of *Clarias garipienus* catfish mucus gel was able to accelerate the wound healing in vivo in Wistar rats (*Rattus norvegicus*), which can be observed through the morphological wound closure process. From the results of microscopy preparations, administration of *Clarias garipienus* catfish mucus gel had a significant effect on increasing the epithelial tissue thickness, but did not have a significant effect on increasing fibroblast and blood vessel numbers, and the VEGF-A expressions.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 3: Histological parameters of the wound healing process on Wistar strain rat (*Rattus norvegicus*) treated with *Clarias gariepinus* catfish mucus gel

No	Treatment Group	Histological parameters		
		Epithelial tissue thickness (µm)	Fibroblast number	Blood vessel number
1	Catfish mucus gel	44.16 ± 3.48 b	304 ± 6.46 a	7 ± 0.6 a
2	Positive control	62.51 ± 5.68 c	594 ± 10.16 b	31 ± 2.7 b
3	Placebo control	34.18 ± 6.95 a	277 ± 6.15 a	2 ± 0.32 a

Values followed by different letters are significantly different ($p < 0.05$)

Table 4: VEGF-A expression on the wound tissue of Wistar strain rat (*Rattus norvegicus*) treated with *Clarias gariepinus* catfish mucus gel

No	Treatment Group	Percentage of VEGF-A expression (%)	Interpretation
1	Catfish mucus gel	60.00 ± 10.44 a	Positive, moderate
2	Positive control	73.33 ± 14.97 b	Positive, strong
3	Placebo control	49.16 ± 18.80 a	Positive, moderate

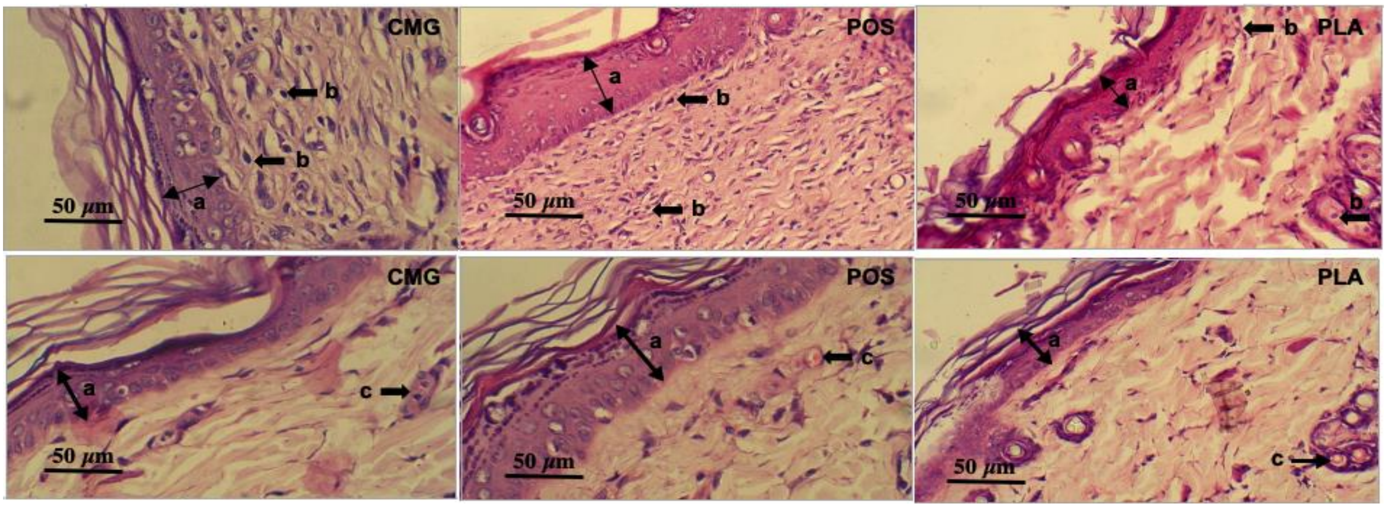

Figure 5: The wound histology of Wistar rats treated with catfish mucus gel (CMG) on the 15th day compared to the positive control (POS) and the placebo control (PLA) using H&E staining, magnification ×400, a = epithelial tissue; b = fibroblast; and c = blood vessel.

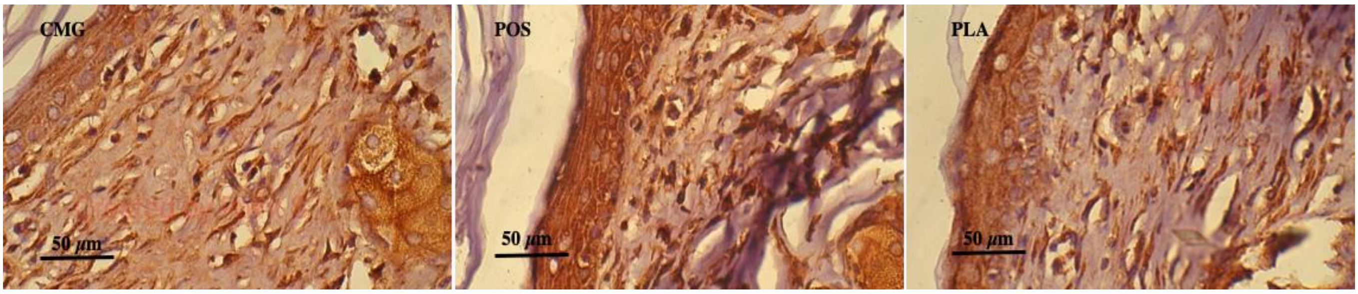

Figure 6: Wound immunohistochemistry of Wistar rats treated with catfish mucus gel (CMG) on the 15th day compared to the positive control (POS) and placebo control (PLA), magnification ×400.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

This publication was supported by the Institute of Research and Community Service Universitas Sebelas Maret Indonesia under Fundamental Grant No. 260/UN27.22/HK.07.00/2021 and No. 254/UN27.22/PT.01.03/2022.

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