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BLACK TILAPIA FISH (*Oreochromis niloticus*) SKIN EXTRACT INDUCE WOUND HEALING ON *Rattus norvegicus*

Dermatology

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ABSTRACT

Objective: The objective of this research is to evaluate the effect of black tilapia fish/BTF (*Oreochromis niloticus*) skin extract cream application on wound healing by assessing its effect on wound contraction, epithelial thickness, and collagenization rate. **Method:** T*rue experiment* using 36 *Rattus norvegicus* as research object, wounded with punch biopsy sized 8 mm on right side of the back. Research objects were divided into 6 groups (X0: no treatment; X1: cream base application on wound; X2: application of gentamicin cream; X3: application of BTF extract cream 10%; X4: application of BTF extract cream 20%; X5: application of BTF extract cream 30%. Wound contraction was evaluated on day 3, 6, 9, and 11. Tissue sample was biopsied and examined on day 11 to evaluate epithelial thickness (*HE*) and collagenization (*mallory*). **Result:** Application of BTF skin extract cream showed an effect on wound contraction, epithelial thickness, and collagenization rate of new wound, the higher the concentration of BTF cream given, the better the result was. **Conclusion:** BTF skin extract cream can be a new hope and alternative therapy in wound treatment.

KEYWORDS

Black tilapia fish, Wound healling, Wound contraction, Epithelial thickness, Collagenization

1. INTRODUCTION

Wound healing is often overlooked until a problem occurs, among which is wound area infection.[1,2] This will delay and complicate wound healing process, which will cause an increase in wound incidence and prevalence rate each year. In Indonesia, according to the data collected in 2010 in Kariadi General Hospital-Semarang, there were 23 cases of post-surgical wound infection caused by *Staphylococcus aureus*.[3]

Topical antibiotic like gentamycin is often used in wound management in general community, but careless use of antibiotic will cause resistance and side effects.[4-6]One of the many wound management that can be done to patients is by giving collagen-rich agent. Collagen works by initiating cell differentiation, native collagen, and cellular migration.[7-11]

Animal source contains more collagen compared to plant. Mammals like cow may cause diseases like Bovine Spongiform Encephalophaty, Transmissible Spongiform Disease and bovine foot and mouth disease, thus other collagen-rich source that can replace mammal-source is fish skin.[10] Fish skin contains large amounts of collagen type 1, which play an important role on tissue reepithelization. In addition, fish collagen is more stable in structure than collagen in mammals so it is easy to apply to human.[12]

One type of fish that is rich in collagen is black tilapia (*Oreochromis niloticus*). Black tilapia has more beneficial biological properties such as easy breeding, fast growth, high adaptability and tolerance to various environmental conditions so it is more likely to be developed compared to other types of freshwater fishes.[13-20]Tissue reepithelization by adding collagen-rich agent made from black tilapia fish skin can be an alternative mode of therapy in improving wound healing process in terms Tissue reepithelization by adding collagen-rich agent made from black tilapia fish skin can be an alternative mode of therapy in improving wound healing process. Utilization of fish skin as a source of collagen in dermatology can limit fishery dan protect the environment.[12,15,18]

2. METHODS AND MATERIALS

Research design

This study used a true experimental design using the *Rattus norvegicus* test animal as the object of research. Wound was made on test animals before treatment (day 0) with a punch biopsy technique on the back of *Rattus norvegicus* with diameter of 8 mm, post-test was carried out by measuring wound contractions on day 3, day 6, day 9 and day 11 after treatment. All test animals will be biopsied on the 11th day by punch biopsy with diameter of 8 mm in the newly formed tissue and a histopathological examination is carried out by staining with Hematoxylin Eosin (HE) and Mallory to see the thickness of the epithelialization and collagenization, then assessed using the image program J® 1.52. Ethical approval for this study was obtained from Diponegoro University Ethical Committee (31/EC/H/FK-UNDIP/V/2020).

Research Population and Sample

The inclusion criteria were healthy, male, 3-4 months old *Rattus norvegicus* strain *Sprague dawley* weighed 200-250 grams, given same food and placed in the same place. The exclusion criteria were *Rattus norvegicus* which fell ill during adaptation period. Drop out criteria were rats that fell ill or died during research.

The study was conducted using test animals: 36 male Rattus norvegicus. The research subjects will be grouped randomly into 6 groups with different treatments, namely: group I without treatment, group II cream base, group III gentamicin cream, group IV black tilapia fish skin extract 10% cream, group V black tilapia fish skin extract 20% cream, and group VI black tilapia tilapia fish skin extract 30% cream. The treatments given to each group II, III, IV, V, and VI were carried out routinely every day topically until day 10. On the 11th day the tissue samples were examined microscopically for, epithelial thickness, and collagenization rate.

Cream of Black Tilapia Fish Skin Extract

Preparation of 1x1 cm dry black tilapia tilapia skin. The removal of non-protein content was carried out by immersing the sample in 0.1 M NaOH (sodium hydroxide) solution for 24 hours with skin weight and solution volume ratio 1:20 (w/v). Then, the sample was washed using distilled water until the pH of the washing water was close to 7 (neutral). The next process was hydrolysis using CH3COOH (acetic acid) solution with a concentration of 0.75 M (w/v 1:10) for 2 hours and then neutralized again with distilled water to neutral pH. The

sample was then extracted using distilled water with a ratio of 1: 2 (w/v) for 3 hours at 40°C. The extract was filtered with a dacron cloth in a plastic sieve to separate the residue and extract (supernatant). The extracted liquid collagen is then freeze dried and collagen is obtained in solid form. Afterwards, collagen powder is mixed with cream base with a concentration of 10%, 20%, and 30%. The extraction process stages can be seen in Figure 1.

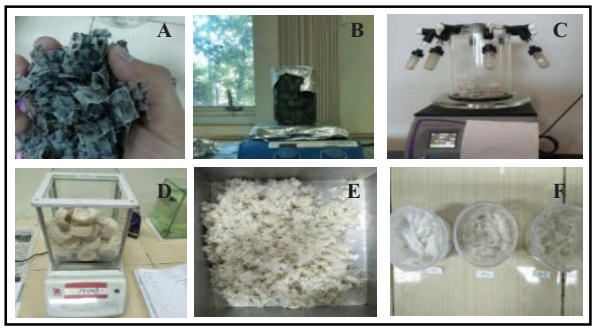

Figure 1. Tilapia fish collagen extraction; A. Dried tilapia fish skin, B. Collagen in liquid form, C. Drying process of liquid collagen with freeze drier, D. Collagen in solid form, E. Collagen in powdered form, F. Collagen extract cream with concentration of 10%, 20%, 30%.

Histopathology

Measurement of wound length and area was carried out on the 11th day, the tissue was then fixed with formalin buffer and made into a paraffin block then stained with Hematoxylin-Eosin (HE) to evaluate the thickness of the epithelium and Mallory's stain to assess tissue collagenization. Epithelial thickness and tissue collagenization were observed using a microscope and compared in all groups and images were taken directly under a microscope.

STATISTIC ANALYSIS

Data analysis was done using the Analysis of Variance (Anova) test to compare treatment and control groups on days 0, 3, 6, 9, 11. To see the significance between groups, the analysis was continued with LSD (Least Significant Difference).

3. RESULTS

Biochemical Composition of Black Tilapia Fish Skin Extract

The composition of the black tilapia fish skin collagen extract in the form of a solution was analyzed for its chemical compound content to determine its safety when used topically, the composition of the content can be seen in table 1 below:

Table 1. Biochemical composition of black tilapia fish skin collagen extract

Composition	Unit	Result
pH	-	5.2
Protein	%	3.40
Ash	%	0.090
Fat	%	3.64
Water	%	95.6
Cadmium (Cd)	mg/kg	ND
Timbal (Pb)	mg/kg	ND
Arsen (As)	mg/kg	ND
Merkuri (Hg)	mg/kg	ND
Escherichia coli	MPN/g	0
Salmonella spp	/25g	Negative

Note
ND : Non Detected

We assumed the protein content in tilapia fish skin extract as collagen because collagen composed the largest percentage of protein found in the fish skin. The total ash obtained was in small amount, this means that the quality of the collagen contained in the extract was good. Heavy metals such as Cadmium, lead, arsenic, and mercury were not found in this extract, *Escherichia coli* and *Salmonella* spp were also not found, this proves that BTF skin collagen extract is safe when applied topically.

The Effect of Tilapia Fish Skin Extract on Wound Contraction

Wound contraction on day 3, 6, 9, 11 in the treatment group showed improvement compared to the positive and negative control groups. The macroscopic wound contractions of each group on day 11 are shown in Figure 2 below.

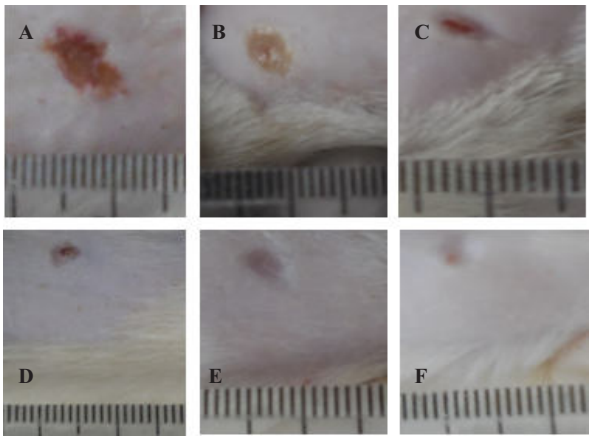

Figure 2. Wound contraction in each group on day-11; A. No treatment; B. Cream base; C. Gentamycin cream; D. BTF skin extract 10%; E. BTF skin extract 20%; F. BTF skin extract 30%.

The wound contraction data on day-11 showed the mean wound contraction is directly proportional to the type of treatment and is dose-dependent, in which wound contraction in positive control and treatment is better than without treatment and the higher the treatment concentration, the better wound contractions were, as we can see in the table 2 below

Table 2. Wound contraction on day-11

Group	N	Min (%)	Max (%)	Mean ± SD (%)	Median (%)
X0	6	0,88	5,81	2,38 ± 1,791	1,83
X1	6	1,32	2,94	1,89 ± 0,591	1,66
X2	6	0,54	1,47	0,99 ± 0,303	0,99
X3	6	0,37	1,81	0,89 ± 0,587	0,70
X4	6	0,00	1,03	0,29 ± 0,383	0,22
X5	6	0,00	0,22	0,10 ± 0,887	0,13

From the data above, the comparison value of p was obtained, where each p value is X0 and X3, p = 0.004; X0 and X4, p = 0.000; X0 and X5, p = 0.000; X1 and X3, p = 0.047; X1 and X4, p = 0.002; X1 and X5, p = 0.001; X2 and X3, p = 0.834; X2 and X4, p = 0.153; X2 and X5, p = 0.073. As we can see in the box plot image in Figure 3 below.

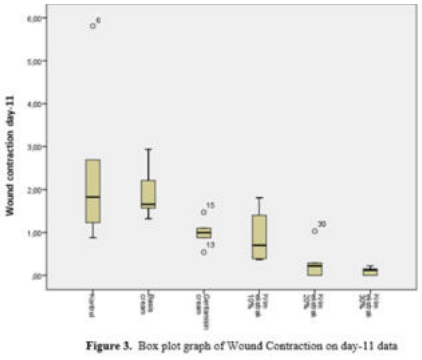

Figure 3. Box plot graph of Wound Contraction on day-11 data

Figure 3. Box plot graph of Wound Contraction on day-11 data

The result of the ANOVA test showed differences between groups where the value of p = 0.000, so it can be concluded that there was a significant difference in wound contraction between the six groups on the 11th day as shown in Table 3 below.

Table 3. Wound contraction improvement on day-11 analysis

Group	Mean ± SD	P	Information
X0	2,38 ± 1,791		
X1	1,89 ± 0,591		
X2	0,99 ± 0,303	0,000	Significant
X3	0,89 ± 0,587		
X4	0,29 ± 0,383		
X5	0,10 ± 0,887		

* Tested using *ANOVA* (significant $p < 0,05$)

Wound contraction (on day 11) analysis between group was then tested with LSD, as shown in table 4 below.

Table 4. Wound contraction (on day 11) analysis between group

Group	Comparison between treatment group	P	Information
	X1	0,321	
	X2	0,007	
X0	X3	0,004	Significant
	X4	0,000	
	X5	0,000	
	X0	0,321	
	X2	0,072	
X1	X3	0,047	Significant
	X4	0,002	
	X5	0,001	
	X0	0,007	
	X1	0,072	
X2	X3	0,834	Significant
	X4	0,153	
	X5	0,073	
	X0	0,004	
	X1	0,047	
X3	X2	0,834	Significant
	X4	0,220	
	X5	0,110	
	X0	0,000	
	X1	0,002	
X4	X2	0,153	Significant
	X3	0,220	
	X5	0,695	
	X0	0,000	
	X1	0,001	
X5	X2	0,073	Significant
	X3	0,110	
	X4	0,695	

* Tested with LSD (significant p < 0,05)

The LSD test results showed significant differences between groups X0, X1, X2 with X3, X4, and X5. Differences were also obtained in accordance to dose response between X3, X4, and X5. No significant differences were found between groups X0, X1, and X2.

The wound contraction data above produces a graph depicting the comparison of wound contractions between groups where there are significant differences between the control group and the treatment group, as shown in Figure 4 below.

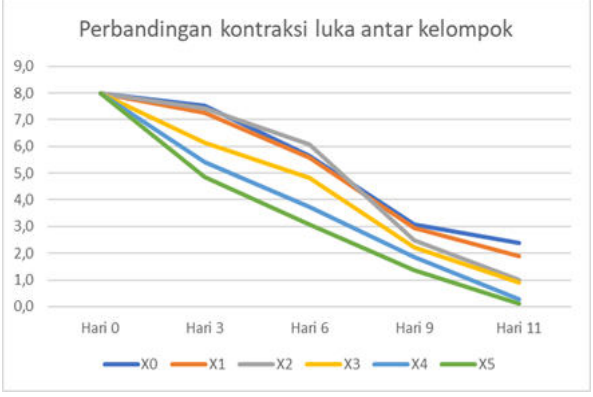

Figure 4. Comparison graph of wound contraction between groups

Figure 4 showed that all groups the improvement in wound contractions from day 0 to day 11 in all groups, but there was a variation of wound contractions where the control group showed an average of 1-2 mm, whereas in the treatment group wound contractions were better with average wound area 1 mm. An even better result was shown in the 20% and 30% extract cream group where most of the samples showed wounds that had completely closed and the numbers of such samples were found more in the 30% extract cream treatment group than in the 20% extract cream treatment group. The wound repair in the treatment group was directly proportional to the dose response: the greater the concentration of black tilapia skin extract cream, the better the wound repair result was, in which the wound constricted more, the epithelium became thicker, and the amount of collagen also increased.

The Effect of Tilapia Fish Skin Collagen Extract on Epithelial Thickness

Epithelial thickness data showed that the mean of epithelial thickness was directly proportional to the treatment and dose given, in which epithelial thickness in treatment group was better compared to control group, as shown in table 5 below.

Table 5. Epithelial Thickness Data Characteristics

Group	N	Min (%)	Max (%)	Mean ± SD (%)	Median (%)
X0	6	16,82	56,67	36,89 ± 12,651	36,87
X1	6	17,31	57,39	37,36 ± 12,728	37,34
X2	6	17,57	57,83	37,70 ± 12,779	37,45
X3	6	18,02	58,36	38,71 ± 12,806	38,57
X4	6	42,36	72,99	52,07 ± 11,460	49,56
X5	6	46,65	88,89	65,48 ± 18,305	58,52

The data above were obtained from microscopic examination with HE staining and then converted into millimeters. The epithelial thickness improvement of each group is shown in A - F is drawn black

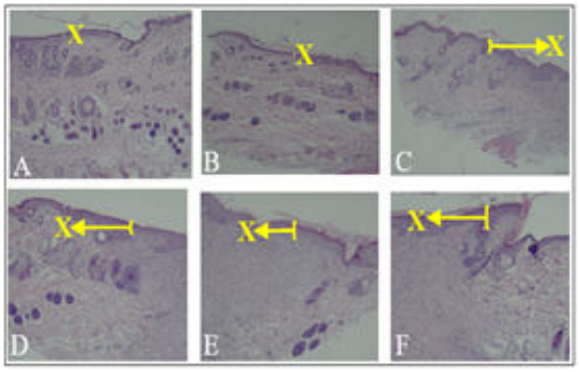

Figure 5. Depiction of epithelial thickness from histologic preparations stained with HE; A. No treatment; B. Cream base; C. Gentamycin cream; D. BTF skin extract 10%; E. BTF skin extract 20%; F. BTF skin extract 30%, X. Epithel thickness

The picture above shows that the preparations of the 10%, 20%, and 30% tilapia fish skin extract cream group have thicker epithelium compared to the no treatment, cream base, and gentamicin cream group. Epithelial thickness is measured perpendicularly from the corneum layer to the basal layer

The comparison value of p was obtained, where each p value is X0 and X3, p = 0.819; X0 and X4, p = 0.063; X0 and X5, p = 0.001; X1 and X3, p = 0.865; X1 and X4, p = 0.072; X1 and X5, p = 0.001; X2 and X3, p = 0.899; X2 and X4, p = 0.078; X2 and X5, p = 0.001, as shown in the box plot graph in Figure 6 below.

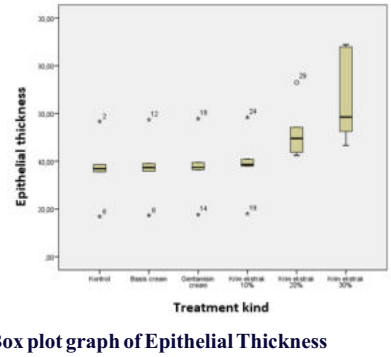

Figure 6. Box plot graph of Epithelial Thickness

The ANOVA test results showed differences in data between groups where the p value = 0.004, which can be concluded that there is a significant difference in epithelial thickness between the six groups as shown in Table 6 below.

Table 6. Epithelial Thickness Improvement Analysis

Group	Mean ± SD	P	Information
X0	36,89 ± 12,651		
X1	37,36 ± 12,728		
X2	37,70 ± 12,779	0,004	Significant
X3	38,71 ± 12,806		
X4	52,07 ± 11,460		
X5	65,48 ± 18,305		

* Tested with *ANOVA* (significant $p < 0,05$)

Significant data differences were shown between each after the ANOVA test was carried out, then LSD test was used to determine the differences between two groups. Epithelial thickness analysis between groups by LSD test is shown in Table 7 below.

Table 7. Analysis of Epithelial Thickness Between Groups

Group	Comparison between treatment group	P	Information
	X1	0,953	
	X2	0,918	
X0	X3	0,819	Significant
	X4	0,063	
	X5	0,001	
	X0	0,953	
	X2	0,966	
X1	X3	0,865	Significant
	X4	0,072	
	X5	0,001	
	X0	0,918	
	X1	0,966	
X2	X3	0,899	Significant
	X4	0,078	
	X5	0,001	
	X0	0,819	
	X1	0,865	
X3	X2	0,899	Significant
	X4	0,100	
	X5	0,002	
	X0	0,063	
	X1	0,072	
X4	X2	0,078	Significant
	X3	0,100	
	X5	0,099	
	X0	0,001	
	X1	0,001	
X5	X2	0,001	Signifikan
	X3	0,002	
	X4	0,099	

* Tested with *LSD* (significant $p < 0,05$)

The Effect of Tilapia Fish Skin Collagen Extract on Collagenization Rate

Histological depiction of collagen density of each group (stained with Mallory staining) can be seen in Figure 7 below.

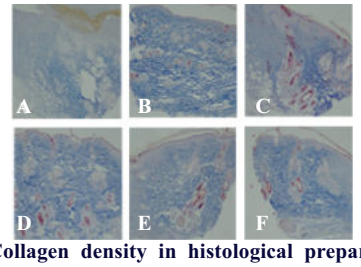

Figure 7. Collagen density in histological preparation using Mallory staining; A. No treatment; B. Cream base; C. Gentamycin cream; D. BTF skin extract 10%; E. BTF skin extract 20%; F. BTF skin extract 30%. Note: Colagen intensity: Blue appearance

The histological picture clearly shows that all preparations in the treatment group showed denser collagen density compared to the control group, both the positive control group and the negative control group. The increase in collagen intensity is shown by blue colouration that spreads in the dermis. The histological picture in the treatment group shows greater collagen density compared to the control group.

Data on the collagenization rate showed that the mean collagenization rate was directly proportional to the type of treatment and dose response. The higher the concentration of BTF skin extract cream, the greater the amount of collagen was. Data characteristic on collagenization rate can be seen in table 8 below.

Table 8. Characteristic of Collagenization Data

Group	N	Min (%)	Max (%)	Mean ± SD (%)	Median (%)
X0	6	228,48	229,88	229,37 ± 0,504	229,46
X1	6	230,12	231,99	231,22 ± 0,723	231,23
X2	6	232,14	248,48	238,77 ± 5,789	237,21
X3	6	248,72	263,67	264,25 ± 5,600	259,29
X4	6	256,74	268,39	265,35 ± 4,334	266,57
X5	6	268,02	270,58	269,42 ± 0,964	269,48

Data above showed that there is a significant difference between each group, with a value of p = 0.000. The dose response given is directly proportional to collagenization, so the best collagenization is in the X5 group, as shown in the box plot in Figure 8 below.

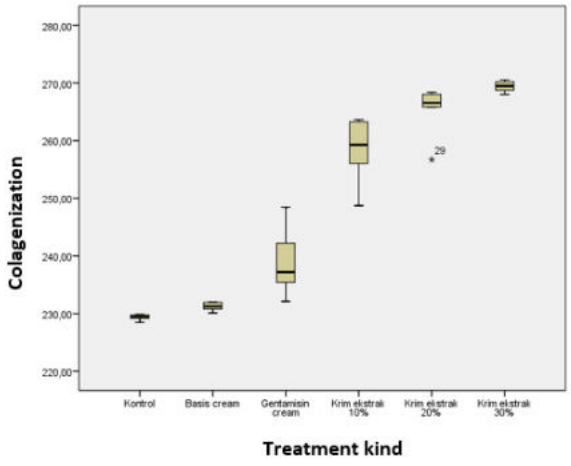

Figure 8. Box plot graph of Collagenization Rate

The results of the ANOVA test showed differences between groups where the p value = 0.000, so it can be concluded that there is a significant difference between the six groups as shown in table 9 below.

Table 9. Analysis of Collagenization Rate

Group	Mean ± SD	P	Information
X0	229,37 ± 0,504		
X1	231,22 ± 0,723		
X2	238,77 ± 5,789	0,000	Significant
X3	264,25 ± 5,600		
X4	265,35 ± 4,334		
X5	269,42 ± 0,964		
X0	229,37 ± 0,504		

* Tested with *ANOVA* (significant $p < 0,05$)

Analysis of collagenization rate between groups using LSD test is shown in table 10 below.

Table 10. Collagenization Analysis between groups

Group	Comparison between treatment group	P	Information
	X1	0,403	
	X2	0,000	
X0	X3	0,000	Significant
	X4	0,000	
	X5	0,000	
	X0	0,403	
	X2	0,002	

X1	X3	0,000	Significant
	X4	0,000	
	X5	0,000	
	X0	0,000	
	X1	0,002	
X2	X3	0,000	Significant
	X4	0,000	
	X5	0,000	
	X0	0,000	
	X1	0,000	
X3	X2	0,000	Significant
	X4	0,003	
	X5	0,000	
	X0	0,000	
	X1	0,000	
X4	X2	0,000	Significant
	X3	0,003	
	X5	0,072	
	X0	0,000	
	X1	0,000	
X5	X2	0,000	Significant
	X3	0,000	
	X4	0,072	

* Tested with *LSD* (significant $p < 0,05$)

4. DISCUSSION

The Effect of Tilapia Fish Skin Extract on Wound Contraction

The main components in the wound healing process are connective tissue or collagen, blood vessels and epithelium. There are three phases in the wound healing process: (1) coagulation and inflammation, (2) the proliferation phase and (3) the remodeling phase.[21] The inflammatory phase starts from the occurrence of the wound until the fifth day. The broken blood vessels constrict and retract, accompanied by hemostatic reactions due to platelet aggregation and formation of a fibrin mesh for blood clots. Inflammation process can be observed in this phase. Besides that, vasodilation and accumulation of PMN leukocytes also occur. Platelet aggregates will release cytokines and inflammatory growth factor mediator TGF-β1 which are also released by macrophages. Macrophages activate MMP in the wound healing process.[15-16,18] BTF skin extract plays a role by binding to the matrix metalloproteinase then stimulating macrophage chemoctasis resulting in a natural antibacterial effect that can prevent infection so that the wound healing process runs faster than the estimated time to heal. The proliferative phase begins at the end of the inflammatory phase (around day 5) and lasts for about 3 weeks. This phase is characterized by proliferation involving matrix production, angiogenesis and epithelialization. This phase is also called fibroplasia because at this time fibroblasts play a very prominent role. Fibroblasts undergo ploriferation and synthesize collagen. The collagen fibers formed will provide strength to connect the wound edges. The fibrin matrix will then be slowly replaced by granulation tissue. Granulation tissue consists of 3 types of cells: fibroblasts, macrophages, and endothelial cells. Fibroblasts produce an extracellular matrix which fills the wound for keratinocytes movement. The movement of keratinocytes can increase wound contraction, so that the area of the wound becomes narrower.[14-15,18-22] This is evident from the mean assessed on observations days-3, 6, 9, and 11 where in the treatment group the wound area narrowed significantly when being compared with the positive control group and the negative control group.

The Effect of Tilapia Fish Skin Extract on Epithelial Thickness

Epithelial thickness examination was performed using the HE staining method on the tissue taken on day 11 after application of cream for 10 days. The effect of tilapia fish skin extract cream on epithelial thickness, namely BTF (*Oreochromis niloticus*) skin is from its high collagen content. Collagen can stimulate the viability, migration, and differentiation of Human Keratinocytes (HaCaTs) by binding to MMP-9 so that it can increase native collagen production. In addition, collagen also regulates the expression of type I collagen in Human Dermal Fibroblasts (HDFs) either directly or through TGFβ1 secreted by HaCaTs. The formed fibroblasts will stimulate growth factors such as TGF-β, FGF, PDGF, and KGF for the formation of the extracellular matrix. Keratinocyte growth factor will increase the differentiation of epithelial cells resulting in reepithelialization of wounds and wound healing process.[16,21-25] This is evident from the mean that is assessed on the examination of epithelial thickness with HE staining method which is measured perpendicularly from the corneum layer to the basal layer with a micrometer unit where the treatment group obtained a significantly thicker epithelial thickness when compared to the positive control group and the negative control group. The comparison obtained with post hoc statistics with the LSD method between the treatment group and the control group also showed significant differences

The Effect of Tilapia Fish Skin Extract on Collagenization

Collagen intensity examination was carried out by using the Mallory staining method on the tissue taken on the 11th day after 10 days of cream application. Collagenization is the process of forming collagen. The high density of collagen in the proliferation phase is a sign that the wound healing process occurs faster and reduces the potential for bad scar formation. [1,7,9,23-24] The black tilapia fish skin collagen induced HDM to synthesize native collagen. Native collagen which was supposed to bond to MMP in wound physiology, will increase in amount because MMP will be bonded to BTF skin collagen.[16,18-19,25] This is proven as the density of collagen is denser in the treatment group compared to the control group, both the positive control group and the negative control group. The increase in collagen intensity was marked by the blue colouration in the dermis which can be seen in each group.

Dose-dependent Response on Wound Healing

Tilapia fish skin extract cream used in this study was made into three different concentrations, 10%, 20%, and 30%. The higher the concentration given, the results obtained were as follows: wound contraction with a narrower wound area, thicker epithelial thickness, and denser collagen, thus the tilapia fish skin extract cream with a concentration of 30% showed better results than 10% and 20%. Same can be said with tilapia fish skin extract with a concentration of 20%, which showed better results than 10%. In vivo application of tilapia fish skin extract with a concentration of 30% showed no side effects on topical application and wound healing was better than the treatment group with a concentration of 10%, 20%, gentamicin cream, cream base, or without treatment. BTF skin collagen can be an alternative therapy in the wound healing process.

5. CONCLUSION

a. Application of black tilapia fish (Oreochromis niloticus) skin extract cream showed good result in wound healing.
b. Black tilapia fish (Oreochromis niloticus) skin extract cream concentration is related to dose responses, the higher the concentration of the black tilapia fish skin extract cream, the better the result shown in wound healing, in which the wound constricted more, the epithelium got thicker, and the collagenization rate increased. The cream with 30% concentration of black tilapia fish (Oreochromis niloticus) skin extract showed best improvement in wound healing in this research.
c. Black tilapia fish (Oreochromis niloticus) skin extract cream can be an alternative and a new hope for wound healing.
d. Black tilapia fish (Oreochromis niloticus) skin extract cream can be an alternative and a new hope for wound healing.

6. Suggestion

a. Research for human should be developed.
b. Histological examination for epithelial thickness and collagenization was performed in day 2, 5, and 9 post skin punch biopsy in order to monitor wound healing stages.
c. Tilapia fish skin extract contains collagen that has several roles in dermatology, so further research is needed about its' effects in skin aging.

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9. Conflict of Interest

There are no conflicts of interest.

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