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Ointment Formulation from Collagen Extract of Tilapia Fish Skin (*Oreochromis niloticus*) for Healing Burns in *Mus musculus*

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ABSTRACT

Introduction: Burns are injuries that caused by contact with a heat source. The natural alternative burn treatment can use hydrolyzed collagen derived from aquatic ecosystems, one of them is fish.
Objectives: The aim of this research was to find the optimum formulation of tilapia fish collagen extract ointment.
Methods: Fish skin that has been separated was prepared using the solution of NaOH 0.1 N and Butyl Alcohol 10%.Then the fish skin was extracted using a 0.5 M acetic acid solution and precipitated with a 0.9 M NaCl solution. Then, dialysis was performed using a plastic membrane (14 KDa) in a 0.1 M acetic acid solution and distilled water to obtain a wet collagen extract which was then in the FreezeDryer to reduce the water content.
Results: The collagen extract obtained was used for ointment formulations with different concentrations of collagen extract that is 5%, 10%, and 15%. The results of the formulation were tested on burns with a diameter of 0.715 cm in mice. Collagen has a better wound closure diameter than the negative control containing only base.
Conclusion: From the results of the effectiveness test, the formulation with 15% collagen extract showed effective results with wound closure diameter on the last day of 0.11±0.02 cm, significantly different from the negative control of 0.22±0.01 cm. This is indicated by the rate of wound healing in mice observations. Furthermore the ointment has met the standards of homogeneity, spreadability, and pH.

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1. INTRODUCTION

Burns are injuries due to contact with heat sources such as fire, hot water, electric currents, chemicals, and radiation that hit the skin, mucosa, or deeper tissues. Burns can stimulate trauma and are the third leading cause of accidental death in all age groups in the world (1). Burns are injuries with the highest morbidity and degree of disability in hospitals which are still the main of death caused (2). The World Health Organization (WHO) was reported that burns belong to earnest public health problems worldwide. In one country there are 70% of burns occur in households, 25% in industry, and about 5% due to traffic accidents. In Asia, there around 195,000 people died from burns (3). An alternative treatment for natural burns is using hydrolyzed collagen derived from aquatic ecosystems (marine collagen). One of which comes from fish (4). In a previous study, it was known that hydrolyzed collagen was found in snakehead fish (5). In addition, it was also found on the skin of tilapia (6). Collagen is part of the protein fibrin (fiber-shaped), which plays a role in the formation of the largest cell structure in the extracellular matrix that maintains tissue shape. Collagen is a protein constituent found in the skin, tendons, bones (cartilage and hard bone), and other tissues (7). Hydrolyzed collagen is a protein that can close wounds because it is the result of the denaturation of collagen sourced from bone, skin, and tissue in fish where fish collagen has good biocompatibility and low antigenic properties (8).

Generally, collagen comes from the bones and skin of mammals, such as cows and pigs. However, there are serious problems regarding the raw materials from pigs, which are a critical point for Muslims and raw materials from cows for Hindus, as well as concerns about disease issues. Nowadays, the researchers also conducted a study on the discovery of collagen sources, both synthetic and natural, to find out new sources of collagen whose production can be accepted by the wider community. There are study succeeded in obtaining collagen hydrolyzate from salmon fish which has antioxidant and antifreeze activity (9). Moreover, another study showed that tilapia skin collagen hydrolyzate had the lowest antioxidant IC_{50} value after hydrolysis of 93.32 g/mL (0.093 mg/mL) with the addition of 8.000 U of enzymes while before hydrolysis the value was 836.2 g/mL. The antioxidant activity value belongs in the category of strong antioxidant (<100 ppm). The lowest of the IC50 value shows the stronger the antioxidant activity of a compound. Therefore, it is concluded that fish waste can be used as an alternative raw material for making collagen which can increase the use-value of fishing industry waste and reduce the negative impact of environmental pollution (10).

In this study, Tilapia skin collagen was used in ointment preparations with various collagen concentrations, 5%, 10%, and 15%. Moreover, the positive control in the form of a commercial ointment, namely bioplacenton, and negative control in the form of ointment formulation without collagen which serve as a comparison. Various formulations hypothesize that formulations with 15% collagen have effective burn healing properties. Utilization of tilapia skin to be used as collagen extract as a basic ingredient for making burn ointment can increase the effectiveness of tilapia, and can assist the government in raising and developing local tilapia livestock commodities, especially in Jambi Province. This is due to the maximum utilization of local commodities that can provide benefits to the community at large and become new potential findings in increasing fish livestock production, especially tilapia. This finding also has the potential to improve the Indonesian economy with the production of ointments and collagen extracts from tilapia skin which are relatively cheaper.

2. METHODS

Chemicals and Equipments.

The main material used in this research is *Oreochromis niloticus,* which is purchase from Fish Farmer Jambi City, the part of the fish used for this research is the skin. The chemicals used were 0.1 N NaOH solution, 0.5 M

CH_3COOH solution, 0.9 M NaCl (Merck), distilled water, 10% butyl alcohol, lanolin, methylparaben, yellow vaseline, 10% ether for anesthesia, PEG, and 70% alcohol (Sigma-Aldrich). In this study also used 30 test animals, *Mus musculus*, with bodyweight, 20-30g, 2-3 months, and healthy. The experimental animals were adapted to the environment for ± one week for in vivo experiment. Some of the instruments used are glassware (Pyrex), Freeze Dryer (Sigma-Aldrich), pH meter, Centrifuge, and Plastic membrane (Repligen's SpectraPor®)

Collagen Extraction Preparation

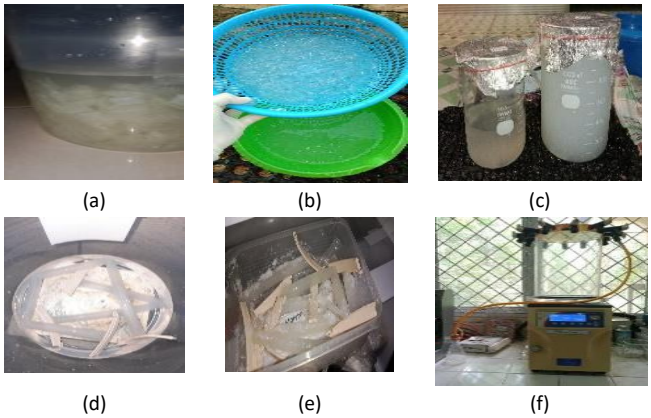
The fish skin samples were first washed using cold water (± 5°C), then dried by aerating (RT) for two days. After two days, the dried sample was weighed as much as 100 g and cut into small pieces (3-5 cm). The sample was then soaked for 12 hr using 0.1 N NaOH solution with a sample and solvent ratio of 1:10 (w/v) with the solution changing every 2 hours. After that, the sample was dried in RT, for 2 hr. Next, the sample was rinsed with distilled water until the pH of the rinse water was close to neutral, and dried again for 2 hours to reduce the water content. Then the sample was immersed in a 10% 1:10 (w/v) butyl alcohol solution for 24 hours by changing the solution every 12 hr (11).

(a) (b) (c) (d)

Figure 1. Sample preparation (a) Immersion in 0.1 N NaOH; (b) Washing with distilled water; (c) pH test of the bath; (d) Immersion in 10% Butanol.

Collagen Extraction

Crude collagen fibers from the pre-treatment phase were dried (RT) for 2 hr. Then the samples were extracted in 0.5 M 1:30 (w/v) acetic acid solution for 48 hr (RT) with stirring and separating the fish skin samples so that they were not mixed with 0.5 M acetic acid solution every 4 hours. The extraction results were then centrifuged at 4700 rpm for 40 min at 4°C. The supernatant was separated and stored at 4°C and then precipitated using NaCl with a concentration of 0.9 M for 12 hr. The sample was then centrifuged again at 4700 rpm for 20 minutes. The precipitate was collected and dissolved in 0.5 M (1:1) acetic acid solution, then dialyzed using a dialysis membrane (14 KDa) against 0.1 M acetic acid for 24 hr, followed by dialysis with distilled water for 24 hr by replacing the acetic acid solution. and distilled water every 4 hours. The resulting wet collagen was then dried using PEG 4000 for 30 hr and drying using a Freeze Dryer for ±3 hr (11). Collagen extraction steps are revealed in Figure 2.



(a) (b) (c) (d) (e) (f)

Figure 2. Collagen extraction (a) Extraction with acetic acid 0.5 M; (b) Screening; (c) Precipitation with 0.9 M NaCl; (d) Dialysis; (e) Drying with PEG 4000; (f) Drying with Freeze Dryer

Collagen Extract Ointment Formulation

The ointment was made into three formulations with varying doses of tilapia skin collagen extract with concentrations of 5%, 10%, and 15%. Positive control (C+) was in the form of a commercial ointment, bioplacenton, and negative control (C-) was an ointment formulation without collagen. The preparation of the ointment begins with the addition of lanolin into the mortar and extract of tilapia skin collagen with three variations of concentration. Vaseline and methylparaben were added and crushed until homogeneous. The ointment preparation is put into the ointment pot (Figure 3) (12).

Table 1. Tilapia Fish Skin Collagen Extract Ointment Formulation

No.	Materials (gr)	C (+)	C (-)	F1	F2	F3
1.	Collagen	-	-	5	10.00	15.00
2.	Lanolin	-	22.50	22.50	22.50	22.50
3.	Methyl Paraben	-	6.00	6.00	6.00	6.00
4.	*Vaseline*	-	21.50	19.00	16.50	14.00
5.	Bioplacenton	15.00	-	-	-	-

C+ C- F1 F2 F3

Figure 3. Ointmen formulation

Ointment Characterization

1. Ointment Physics Parameters:

- The organoleptic test: Homogeneity test: apply 0.1 gram of ointment on the surface of the object glass.

- The spreadability: 0.5 g of ointment was placed in the middle of a round glass scale, allowed to stand for 1 minute, and the diameter of the spread was recorded.
- The adhesion: 0.5 g of the ointment was placed on an object glass and another slide was placed on top of the ointment and then pressed with a weight of 1 kg for 5 minutes. An object glass was attached to the test instrument, a load of 80 g was removed and the time was recorded until the two slides were released. pH test: carried out using a pH meter dipped directly into the ointment (13).

2. Invivo studies the effectiveness of the ointment: Thirty male mice were divided into five groups (three mice for each group with duplo repetition) (Certificate number: B/668/UN21.8/PT.01.04/2021). The mice were first anesthetized using 10% ether. The hair around the back is shaved and cleaned with 70% alcohol. Furthermore, burns were made on the back of the mice by placing a metal chip that had been burned on fire for five seconds. Burns in mice were then rinsed using running water for one minute, then burn treatment was given according to the treatment group by applying 0.5 g of ointment to each mouse given once a day every and after that, the wound was bandaged with sterile gauze. When the 3rd day the gauze is opened and left open until the 14th day by giving ointment regularly**.** Wound diameter was measured using a caliper which was carried out once in two days with four repetitions for each mouse measured from the edge of the farthest edge of the wound area. Burns have been declared healed if the burned area is close to zero (14). Moreover to assess burns, classification is done by scoring: 0= normal; 1= white-red; 2= low-intensity redness, 3= moderate-intensity redness, 4= high-intensity redness. (15).

3. Data Analysis*:* One-way ANOVA method was used to determine the value of the ointment evaluation results and burn wound healing results, while the Independent samples t-test method was used to compare the significance value of the effectiveness of the ointment for each treatment (16,17)

3. RESULTS AND DISCUSSION

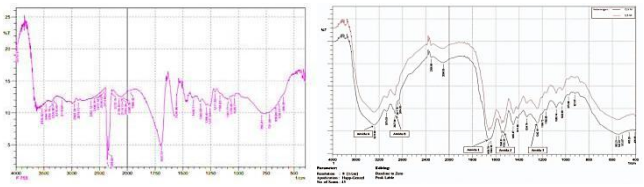
Collagen Extraction Preparation

The process of immersing Tilapia skin samples using 0.1 N NaOH serves to remove minerals and non-collagenous proteins contained in the sample so that the collagen that will be produced meets the standards and is easy to apply. For neutralization of samples from NaOH by rinsing the sample using 5 liters of distilled water, the pH of the rinse water is 7 which means the sample is free from NaOH content. For immersion using butyl alcohol aims to free the sample from fat so as not to affect the extraction process (11).

The extraction using acetic acid (0.5 M) will trigger the substitution of negative ions in the salt with positive ions and could break the protein structure (18). The decrease of protein content at high acid concentrations because acetic acid will hydrolyze stronger peptide bonds so that protein loss will occur (19). After filtering, the filtrate was obtained as much as ±2.3 liters, then centrifuged to obtain the supernatant. 1850 mL of supernatant was earned, then precipitated using NaCl in the salting-out process to precipitate protein. 700 ml of collagen precipitate was acquired, which was re-centrifuged to earn the high concentrate of supernatant. The supernatant was earned 650 ml. Over and above that, the dialysis process was adhered out to remove impurities, then the concentration of acetic acid used to dissolve the supernatant is higher than that used for dialysis, the impurities agent can draw out of a high concentration to a lower concentration. Moreover, the dialysis was performed using distilled water to neutralize the acetic acid content in the sample. In the final process, the collagen was dried and the results of dry collagen were 69.35 grams (11). The yield value of dry collagen from tilapia skin then found that, yield $= \frac{69.35}{100} x\, 100\% = 69.35\%$.

The pH value of collagen earned around 6.50-7.17. According to the Indonesian National Standard, the standard pH of collagen ranges from 6.5-8 so that the obtained collagen pH is appropriate (20). The results of the collagen extract obtained were then characterized by their functional groups using FTIR.

From the FTIR spectrum (Figure 5), it is known that the type of collagen earned belongs to Type-I-collagen which is indicated by the presence of a triple-helical structure at the absorption of 1,387 (21). The amide region A indicates the presence of an NH group and indicates the presence of hydrogen bonds. The amide B region designated the presence of a CH group. The amide I region designated the presence of a C=O group which is a secondary structure of the protein (21–23). The amide region II shows the presence of NH bonds, and the amide region III shows the presence of N-H bonds which shows the presence of a helical structure (22,23). Collagen FTIR spectrum peaks are listed in Table 2.



(a) (b)

Figure 4. FTIR spectrum of collagen (a) Tilapia skin, and (b) Collagen

Table 2. Collagen FTIR spectrum peak position

Range	Wavenumber (cm^{-1})	Functional Group
Amida A	3336	-NH stretching
Amida B	2949	-CH2- asymmetric stretching
Amida I	1691	-C=O- stretching
Amida II	1558	-NH Bond
Amida III	1257	NH Bond

Ointment Characterization

1. Test the Physical Parameters of the Ointment

The tests carried out were organoleptic tests, homogenity tests, dispersibility, adhesion, and pH tests as shown in Table 3. The requirement for good dispersion of Ointment preparation is 5-7 cm (24). Meantime, for the first week, the ointment made did not meet the requirements yet due to the ointment preparation being slightly more than the proper dosage. That it becomes denser and difficult to spread. As for the adhesion test, it is not less than 4 seconds. An ointment is said to be good if it has great adhesion to the treated site (25). Moreover, the pH value of a good ointment is 4.5-6.5 according to the pH of human skin (26), and the ointment that has been made has met the requirements, namely, the pH obtained is 4-5. The effectiveness of the ointment was tested on male mice with an average weight of 20 grams and obtained second-degree burns which were indicated by red sores, blisters, and slight swelling. After being treated for burns by administering ointment regularly every day for seven days, he has not shown any signs of allergy or infection. Hair growth began to occur in some mice from K-, F1, F2, and F3 treatments on the 5th day of burn treatment.

Table 3. Ointment Physical Parameter Test Results

Formulation	Organoleptic	Homogenity	Spreadability (cm) ± SD	Adhesion (s) ± SD	pH
C+	Consistency : Gel Color: Transparer Smell: Perfume	Y	4.65 ± 0.02	0.85 ± 0.02	5
C-	Consistency : Soli Color: light yellov Smell: Carbo scent	Y	3.60 ± 0.02	2.75 ± 0.03	4
F1	Consistency : Soli Color: light yellov Smell: Carbo scent	Y	3.60 ± 0.01	1.11 ± 0.02	4
F2	Consistency : Soli Color: light yellov Smell: Carbo scent	Y	3.60 ± 0.01	1.25 ± 0.01	4
F3	Consistency : Soli Color: Light Yellov Smell: Carbo scent	Y	3.55 ± 0.01	0.79 ± 0.02	4

2. Wound Closure Wound diameter measurement was carried out to see the wound closure process in mice from the first day to the 13th day (Table 4). It was found that giving the ointment to mice for 13 days, the burn wound that closed the fastest was the F3 ointment formulation with a collagen extract content of 15% and the results were closest to 0, which is calculate by the diameter of burn closure. The wound closure data (Fig 5 and Table 4) shows that the diameter of healing is increasing until the thirteenth day. It shown that the wound is getting smaller with the increase in the percentage of wound healing every day. Descriptive observation shows that the positive control has a diameter of healing 0.15±0.01 cm, while F3 with a dose of 15.00%, 0.11±0.02 cm. Meanwhile, concentrations of 5% and 10% had wound healing diameters of 0.20±0.01 cm and 0.19±0.02 cm were also better than the negative control of 0.22±0.01 cm. statistical tests showed that the difference in the diameter of the control wound closure and the formulation results were not significantly different. The results of the normality test showed that the data were normally distributed ($p>0.05$) and homogeneous ($p>0.05$). Data analysis was continued with the parametric test, namely One Way ANOVA because it has met the requirements of the normal distributed test and homogeneity The results of the parametric test show that the data has a significant difference between groups ($p>0.05$) in control + and F3.

Control + used bioplacenton, containing 10% placenta extract which plays a role in accelerating cell regeneration and wound healing. While 0.5% neomycin sulfate acts as a bacteriocide (27). The use of Bioplacenton causes the wound to become too dry, resulting in the formation of dry, black, and thick necrotic (dead tissue) scar tissue. This necrotic tissue suppresses the supply of blood and nutrients and slows epithelial migration resulting in a slow healing process. The negative control group which only contained an ointment base had a lower wound healing power value than the formula. The

ointment base acts as an occlusive cover for the skin so that it can hydrate the skin. The hydrating effect of the ointment base increases the absorption of the drug and keeps the wound moist. Moist wound conditions facilitate granulation growth and epithelialization. Wound care in a moist environment is beneficial in preventing tissue dehydration, maintaining optimal temperature, accelerating the breakdown of necrotic tissue, the inflammatory phase, wound contraction and re-epithelialization, accelerating angiogenesis, reducing scar tissue formation, and reducing the risk of infection (28).

Collagen fibers contain amino acids that influence fibroblasts to synthesize collagen, thereby accelerating the process of forming new tissue on proliferation and maturation. In addition, as a carrier of nutrients and oxygen that the body needs in the formation of new tissues (29).

Table 4. The Diameter of Burn Closure

Treatment	Observation of wound diameter on day (cm) ± SD						
	1	3	5	7	9	11	13
C+	0.72±0.01	0.66±0.01	0.53±0.02	0.53±0.02	0.40±0.03	0.28±0.02	0.15±0.01^{A}
C-	0.72±0.02	0.66±0.03	0.62±0.02	0.48±0.03	0.39±0.02	0.30±0.01	0.22±0.01^{B}
F1	0.72±0.01	0.54±0.01	0.52±0.03	0.45±0.03	0.38±0.03	0.34±0.01	0.20±0.01^{AB}
F2	0.72±0.01	0.67±0.02	0.63±0.03	0.48±0.01	0.40±0.03	0.30±0.01	0.19±0.02^{AB}
F3	0.72±0.01	0.69±0.02	0.53±0.02	0.42±0.01	0.35±0.02	0.28±0.03	0.11±0.02^{A}

Superscripts with different lowercase letters on the same line showed a significant difference ($p<0.05$)

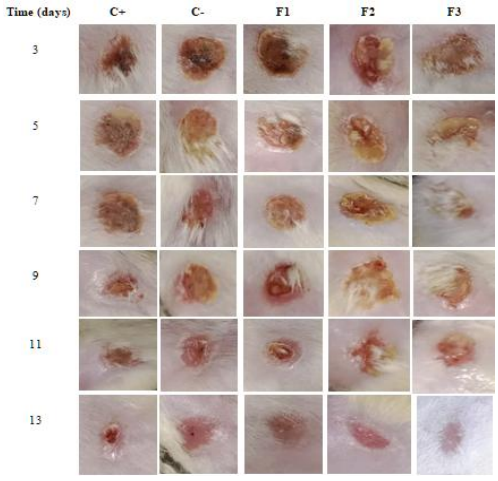


Figure 5. Burn wound closure process on day 1st -13th

4. CONCLUSIONS

Our finding in this research is that we were able to extract collagen from the skin of Tilapia which types I collagen which is indicated by the presence of a triple-helical structure 3. The ointment collagen has a better wound closure diameter than the negative control containing only based. The formulation with 15% collagen extract showed effective results with wound closure diameter on the last day of 0.11±0.02 cm, significantly different from the negative control of 0.22±0.01 cm. This is indicated by the rate of wound healing in mice observations. Furthermore the ointment has met the standards of homogeneity, spreadability, and pH.

5. ACKNOWLEDGEMENTS

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