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**THE EFFECT OF TOPICAL TOMAN (*Channa micropeltes*) FISH EXTRACT ON
 THE NUMBER OF NEUTROFIL CELLS IN DIABETES MELITUS WOUND
 HEALING**

(In Vivo Study on Male Wistar (*Rattus novergicus*) Rat's Back)

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ABSTRACT

Backgrounds: Diabetes mellitus forges the wound healing process to last longer. Toman fish which contains albumin and omega-6 fatty acid is proven to enhance wound healing process in this systemic condition. Patients who suffer from diabetes mellitus retain high neutrophil number. Omega-6 fatty acids contained in Toman fish can decrease the number of neutrophil cells. **Objectives:** To prove the effect of Toman fish (*Channa micropeltes*) extract at 20% concentration topically on the number of neutrophil in diabetes mellitus-induced Wistar Rat (*Rattus novergicus*) injuries on day 4, 8 and 14. **Methods:** This research was a true experimental study with posttest-only and control group with complete random design. Thirty six male Wistar rats (*Rattus novergicus*) was divided evenly for three treatments comprised of negative control given BR2 comfeed, positive control treated with Haruan fish (*Channa striata*) extract at 10% concentration topically and treatment group given with Toman fish (*Channa micropeltes*) extract at 20% concentration topically. **Results:** The data was analyzed using One Way ANOVA and was presenting the result of the 4th day ($p=0.000$), 8th day ($p=0.001$) and 14th day ($p=0.000$). Post-hoc Least Significant Difference (LSD) showed that p value was less than 0.05 which means that there was a significance difference in the mean of all treatment groups. **Conclusion:** Toman fish (*Channa micropeltes*) extract possesses the capacity in decreasing the number of neutrophil on 4th, 8th and 14th day in diabetic wound healing of Wistar Rat (*Rattus novergicus*).

Keywords : *Channa micropeltes*, diabetic wound healing, neutrophil cell

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder in the form of carbohydrates, proteins and fats intolerance which is characterized by the presence of chronic hyperglycemia.^{1,2} Indonesia is reported with high diabetes mellitus prevalence and positioned 6th among other populations in the world.³ According to the 2013 Basic Health Research (RISKESDAS) data, there was an increase in the prevalence of diabetes mellitus in South Kalimantan province from 1.0% in 2007 to 1.4% in 2013.⁴ Oral manifestations in the form of periodontitis, gingivitis and xerostomia are reported among patients with this condition.² Consumption of toman fish and haruan fish is believed by the people of South Kalimantan to accelerate wound healing however the availability of haruan fish becomes a problem due to the high demand from the community.⁵ Nevertheless, it can be replaced by utilizing toman fish which are very

abundant in number.^{6,7} The wound healing process of diabetes mellitus has been proven to be accelerated by toman fish extract concentrates 20% topically.⁸

Toman fish extract contains albumin, omega 3 fatty acids and omega 6 fatty acids.⁵ The albumin content in toman fish is proven to be high so that it can be an alternative to Human Serum Albumin (HSA) which has high economic value.⁹ Toman fish also contains omega 6 fatty acids which have derivatives namely arachidonic acid (AA).¹⁰ Categorized in the same *Channidae* family, toman fish and haruan fish are claimed to possess similar active contents.^{11,12} Empirically, public is currently familiar with the supplement products made from raw fish such as haruan fish.⁶ Haruan fish can speed up the healing process of wounds in the feet of diabetic rat.¹³ It is affirmed by Andrie and Sihombing (2017) study which revealed that the healing of wounds in wistar

rats can be accelerated by the aid of 10% topical extract of haruan fish.¹⁴

Hyperglycemia in people with diabetes mellitus makes the healing process last longer.⁹ Albumin and omega 6 fatty acids contained intoman fish can assist the acceleration of wound healing.^{5,12} Albumin plays an important role in the inflammatory phase because it can maintain the osmotic pressure between the fluid inside and outside the cell that occurs in that phase.¹⁴ Omega 6 fatty acids have derivatives, namely AA (arachidonic acid), and metabolize lipoxin.¹⁰ Lipoxin promotes the acute inflammatory phase into chronic inflammation because it shortens the period of neutrophil cell infiltration into the tissue. This is intended so that the acute inflammatory phase is not prolonged.^{8,10,12} Neutrophil cells play a role in phagocytosis to kill pathogens and help eliminating cell debris in phagosomes.^{15,16} Fajriani *et al* (2018) mentioned that a decrease in neutrophil number was presented on day 4 of acute inflammatory phase in diabetic wounds and it is continued to decline until the 8th day of wound healing process. According to previous studies, it has also been shown that neutrophil cells are still found on the 15th day even though the numbers are greatly decreased.⁹

Based on the background above, The number of research which proves the use of topical toman fish extract in 20% concentration in wound healing of patients with diabetes mellitus are still very limited. This study aims to prove the effect of giving Toman fish (*Channa micropeltes*) extract in 20% concentration topically to the number of neutrophil cells in the back wounds of wistar (*Rattus Novergicus*) rat induced by diabetes mellitus on day 4, 8 and 14.

MATERIALS AND METHODS

This study used true experimental with a posttest-only control group and complete randomized design. This research began with processing research permits and obtaining ethical feasibility statement N0.107/KEPKG-FKGULM/EC/1/2019 from Health Ethics Committees of Dental Faculty, Lambung Mangkurat University. The population in this study was Wistar rats. The inclusion criteria in this study were healthy male rats aged 2-3 month with 250-300 grams in weight. The exclusion criteria in this study were dead or abnormal rats (presented with wounds or incomplete organs), unhealthy rats (inactive, no appetite and weakness) and rat with weight loss more than 10% after a period of adaptation in the laboratory. Thirty six male Wistar rats were divided into 9 groups, namely 3 negative control groups given BR2 comfeed, 3 positive control groups given 10% of haruan fish extract and 3 treatment control groups given topical application of toman fish extract. The administration of toman fish extract and herbal extract using a cotton bud was done twice a day for 14 days.

Each group comprised of 4 male wistar rats to be sacrificed and the number of neutrophil was observed and quantified on day 4, 8 and 14.

The Making Process of Toman Fish and Haruan Fish Extract

Six hundred to a thousand grams of toman fish and haruan fish used in this study were taken from Martapura Traditional Market, South Kalimantan. This study only used toman fish and haruan fish that had been washed cleanly. Toman fish and haruan fish were steamed at 60° C for 25-35 minutes which were then wrapped in flannel cloth and pressed with a hydraulic press to obtain extracts from the two fish. The extract was obtained and put into a test tube and centrifuged at 6000 rpm for 15 minutes. This was done so that toman fish extract and haruan fish extract can be separated from the contaminant. Toman fish extract and haruan fish extract were then stored in dark glass bottles and covered with clean pack and aluminum foil.

The Making Process and Storing of Toman Fish and Haruan Fish Extract Ointment

Ointment bases, adeps lanæ and vaselin flavum, were weighed using analytical scale to obtain 16.875 grams and 23.125 grams base respectively for toman fish extract ointment while 16.875 grams and 28.125 grams for haruan fish extract ointment. Toman fish extract in 20% concentration topically and haruan fish extract in 10% concentration topically was poured into each mortar filled with adeps lanæ which has been weighed and scoured homogenously. After that, vaselin flavum was added to each mortar and was grinded again until mixed properly. Toman fish extract ointment and haruan fish extract ointment were inserted into each ointment pot and stored into the refrigerator.

Induction of Diabetes Mellitus in Wistar Rat (The administration of Streptozotocin (STZ))

Glucose levels of wistar rats were previously examined before STZ induction using a glucometer. The wistar mouse model was injected with STZ with the composition of 35 mg/kg. After induction, the rats were examined for glucose every day for 3 days. Rat suffer from diabetes mellitus if the blood sugar level is 6126 mg/dL with the presentation of weak body condition, less activity and no appetite.

Rat Back Injury Process

Before processing the injury, rats were adapted in a laboratory for 1 week. The rats were taken and the wound was designed by measuring the length of injury on the back of rats. Hand-washing procedure was performed and sterile gloves were applied prior to the incision. In order for rats to fall asleep, a sedative procedure was conducted using 5 ml diethyl ether exerting inhalation technique. The fur on the back was

shaved with 3 cm diameter and then was cleaned with 70% ethanol. The wound was made for about 1 cm in length and 1 cm in-depth to the dermis using sterile scalpel and disposable blade no. 11. Overflowing blood was cleaned using distilled water. Application of toman fish extract ointment and herbal extract ointment on injured area was done using a cotton bud twice a day for 14 days. After that, the wound was wrapped with a sterile gauze that had been given NaCl and layered with a dry sterile gauze.

Rat Sacrifice and Tissue Retrieval

Each group was sacrificed to see the number of neutrophil cells on day 4, 8 and 14. The rats that would be sacrificed was put into a bottle that had been filled with cotton given 5 mL of diethyl ether. The beaker bottle was closed so that ethyl did not evaporate and it was anticipated until the the dead of the rat. Furthermore, biopsy was taken for tissue extraction using an exclusive biopsy technique. Wistar rats which have been sacrificed were then buried.

Managing Trial Animals After Tissue Retrieval

Unused organs of experimental animals were buried in the depth of $\pm 25 - 50$ cm after previously cleaned and wrapped with cloth.

Preparation of Making Process and Stanning

The incision tissue was inserted into a 10% Neutral Formalin (BNF) solution to be fixed for ± 24 hours. Fixation tissue was cut ± 1 cm and was loaded in embedding cassettes. After that, the tissue inside embedding cassettes would be screened for ± 18 hours using tissue processing equipment. The tissue was put into a mold or a base mold filled with melted paraffin and placed back into embedding cassette until it is cool and stored them into refrigerant. The block was cut into 5 microns thickness using microtome. The best cuttings were selected and placed on a waterbath with a temperature of $37-47^\circ$, the specimens were kept until no wrinkles observed and then taken using a glass object to be dried. Staining of preparations was done using the Hematoxylin Eosin (HE) method.

Observation and Calculation of Neutrophil Cells in Histopathological Preparations

A light microscope was used to observe and count the number of neutrophil cells with 40×10 magnification in 5 observing fields. Observation and calculation of neutrophil cell were carried out on day 4, 8 and 14 which then compared among all treatment groups. The data obtained was processed using SPSS.

RESULTS

The mean value of neutrophil cell number obtained from all groups on day 4, 8, and 14 were tabulated and analyzed using Saphiro-Wilk normality test and Levene's homogeneity of variance test. The result obtained $p>0.05$ which means that the data was normally and homogenously distributed. One way

ANOVA test was subsequently conducted and was resulted in $p<0.05$. This result depicted a significant difference between treatment groups so that the LSD Post-hoc analysis was carried out. The average results of neutrophil cells in wound healing of diabetes mellitus-induced wistar rat on day 4, 8 and 14 can be seen in table 1. The mean value of neutrophil cell number in wound healing of diabetic wistar rat on day 4,8 and 14 is illustrated in Figure 1.

Table 1. Mean \pm SD value of Neutrophil Cell Number on the Back Wound Healing of Diabetes Melitus-induced Wistar Rats

Group	Mean \pm SD Number of Cell		
	Day - 4	Day - 8	Day - 14
Feed	13,5 \pm 1,3 (12,0 - 15,0 cells)	11,2 \pm 1,2 (10,0 - 13,0 cells)	8,7 \pm 0,9 (8,0 - 10,0 cells)
Topical haruan fish extract ointment	11,0 \pm 0,8 (10,0 - 12,0 cells)	8,7 \pm 0,9 (8,0 - 10,0 cells)	5,0 \pm 0,8 (4,0 - 6,0 cells)
Topical toman fish extract ointment	9,0 \pm 0,8 (8,0 - 10,0 cells)	6,2 \pm 1,2 (5,0 - 8,0 cells)	3,0 \pm 0,8 (2,0 - 4,0 cells)

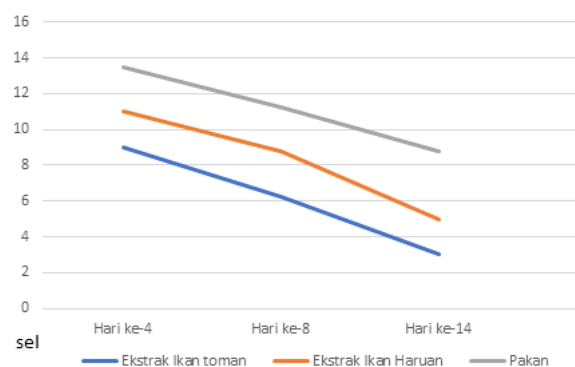


Figure 1 A Diagram depicting the Average Number of Neutrophil Cells On The Back of Diabetic Wistar Rats on Day 4,8 And 14 In Feed-only, Haruan Fish Extract, And Toman Fish Extract Group.

Based on Figure 1, it can be interpreted that the lower the average number of neutrophil cells, the better the wound healing process may occur. Figure 1 shows a diagram which reveals the average number of neutrophil cells on the back wounds of wistar rat induced with diabetes mellitus in each group on day 4, 8 and 14. Group of wistar rats which were given feed-only had the highest average number of neutrophils compared to haruan fish extract at 10% concentration topically and toman fish extract at 20% concentration topically on day 4, 8 and 14.

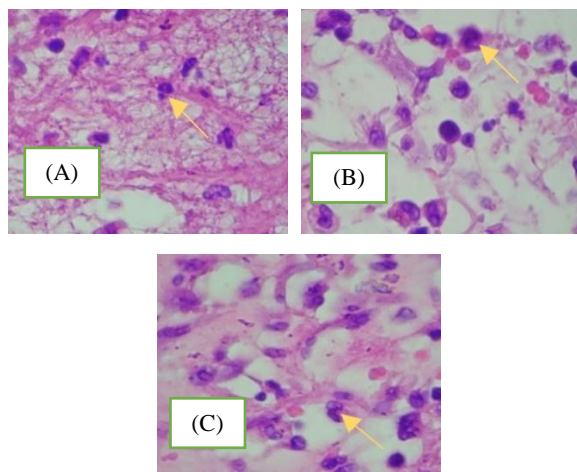


Figure 2. Histopathology of Neutrophil Cells on Back Wound Healing of Diabetic Wistar Rats Using Light Microscope with 40x10 Magnification in Feed Group (A), Haruan Fish Extract in 10% Concentration Topically (B), Toman Fish Extract in 20% Concentration Topical (C) at 4th Day.

Figure 2 shows the histopathology of neutrophil cells number in diabetes mellitus wounds of wistar rats given feed-only with a total of 12.0 - 15.0 cells, those given 10% concentrate of haruan fish extract topically with a total of 10.0 - 12.0 cells and groups of wistar rats given toman fish extract at 20% concentration topically with a total of 8.0 - 10.0 cells.

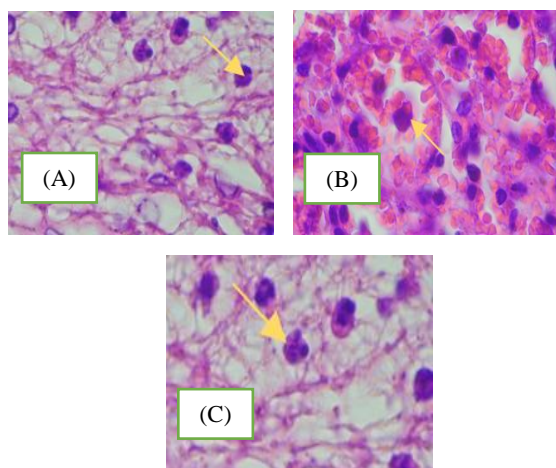


Figure 3. Histopathology of Neutrophil Cells on Back Wound Healing of Diabetic Wistar Rats Using a Light Microscope with 40x10 Magnification in Feed Group (A), Haruan fish extract 10% concentrates topically. (B), Toman fish extract 20% concentrates topically on Day 8.

Figure 3 shows the histopathology of neutrophil cell in diabetic wistar rat wound healing given feed-only with a total of 10.0 - 13.0 cells, those given 10% concentrate of haruan fish extract topically with a total of 8.0 - 10.0 cells and groups of wistar

rats which were given toman fish extract in 20% concentration topically with a total of 5.0 - 8.0 cells.

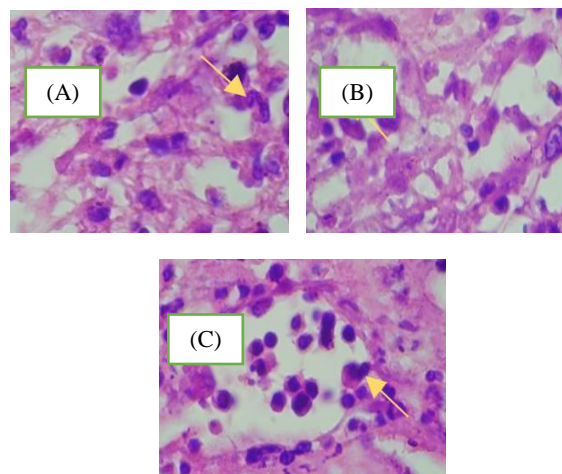


Figure 4. Histopathology of Neutrophil Cells on Back Wound Healing of Diabetic Wistar Rats Using a Light Microscope with 40x10 Magnification in the Feed Group (A), Haruan Fish Extract at 10% Concentration Topically (B), Toman Fish Extract at 20% Concentration Topically (C) at 14th Day.

Figure 4 shows the histopathology of neutrophil in diabetic wistar rat wound healing given feed-only with a total of 8.0 - 10.0 cells, those given 10% concentrate of haruan fish extract topically with a total of 4.0 - 6.0 cells and groups of wistar rats given toman fish extract at 20% concentration topically with a total of 2.0 - 4.0 cells.

DISCUSSION

The application of toman fish extract at 20% concentration reduced the number of neutrophil cells on day 4 and 8 with gradual decline until the 14th day. In this study, it was proven that the topical administration of toman fish extract with a concentration of 20% showed a significant difference compared to feed-only group and haruan fish extract at 10% concentration group. This is due to the different amount of fatty acids and albumin in toman fish extract and haruan fish extract. Toman fish has the highest albumin from *Channidae* family.¹⁷ Albumin is a major protein that plays an important role in maintaining plasma osmotic pressure between the fluid inside and outside the cell during the inflammatory phase.¹⁴ In order to prevent the worsening of edema in inflammatory phase, albumin in toman fish and haruan fish play a role as a regulator of osmotic pressure in the blood so that it can accelerate wound healing.^{8,18}

On the 4th day, Wistar rats which were given toman fish extract at 20% concentration topically had the lowest number of neutrophil cells compared to other groups. The amount of albumin and unsaturated fatty acids (PUFAs) compound including omega-6 fatty acids contained in toman fish contribute to this

result as they are higher than those obtained from haruan fish.^{10,11,19} The content of omega-6 fatty acids in toman fish is 7.2 mg while in haruan fish is only 3.7 mg.¹⁵ Neutrophil cells in the acute inflammatory phase play a role in phagocytosis to kill pathogens and help eliminate cell debris in the fagosome yet more damage and delayed wound healing process will be resulted if the work of neutrophil cells persist.^{15,16} In order to prevent the setback in wound healing process, omega 6 fatty acids in toman fish have a derivative namely arachidonic acid (AA), comprising of leukotrienes and prostaglandins, which has an important role in the inflammatory phase.^{8,9} Lipoxin may shorten the period of neutrophil infiltration so that acute inflammatory phase can be replaced by macrophage to chronic inflammatory phase and can be preceded to the next phase of wound healing process.^{8,10}

There was a decrease in the average number of neutrophil cells on the 8th day in the group of toman fish extract at 20% concentration topically compared to the group of haruan fish extract at 10% concentration topically and feed only. Neutrophil cells release immunoregulation cytokines such as Interferon-gamma (IFN- γ) which aims to recruit macrophages.²⁰ The decrease in the average number of neutrophil cells is characterized by the presence of macrophage cells in the wound. The macrophages will release growth factors that will activate fibroblasts and migrate into the wound to stimulate re-epithelialization of the wound.^{16,21,22} Macrophage cells will produce other growth factors such as the Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor-2 (FGF-2) which will initiate a new blood vessel formation process (angiogenesis). This shows that there has been a proliferation phase in the healing process.^{16,23}

The number of neutrophil cells on the 14th day in this study was still obtained even though the numbers continued to decline in the group wistar rats which were given Toman fish extract at 20% concentration topically. This is because neutrophil cells have a shorter life span than other cells such as macrophage cells.²⁴ Neutrophil cells undergo apoptosis by the induction of macrophage cells. The work of neutrophil cells to do phagocytosis has also been replaced by macrophage cells. This is done so that the healing process is not hampered.^{15,16} In addition, Irwanda *et al*, 2015 has proved that albumin may prevent the hamper of wound healing process by promoting its acceleration.¹⁸ The albumin content in toman fish extract proved to be higher than haruan fish extract. The albumin content in toman fish extract was 5.35% while the albumin content in haruan fish extract was only 4.53%.¹¹ Albumin plays a role in the proliferation and maturation phase as nutrients and oxygen transporter required by the body and as a basic ingredient for new tissue formation in the healing process.^{18,25} It can be concluded that toman fish

extract (*Channa micropeltes*) at 20% concentration can reduce the number of neutrophil higher than haruan fish (*Channa striata*) extract at 10% concentration and feed-only group on day 4, 8 and 14. This shows that the topical use of toman fish extract (*Channa micropeltes*) at 20% concentration is proven as the most effective treatment in enhancing the acceleration of back wound healing process in wistar (*Rattus novergicus*) rat induced with diabetes mellitus compared to haruan fish extract (*Channa striata*) at 10% concentration and feed-only.

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