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**THE EFFECT OF TOMAN FISH EXTRACT (*Channa Micropeltes*)
 ON NEUTROPHILIN DIABETES MELLITUS WOUND HEALING**

(In Vivo Study in the Back of Male Wistar Mice (*Ratus Novergicus*))

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Abstract

Background: Toman fish contains albumin and unsaturated fatty acids which can be an alternative of diabetes mellitus wounds healing process. People with diabetes mellitus have their neutrophil decreased. Albumin increases the number of neutrophil. Unsaturated fatty acid decreases the number of neutrophil. **Purpose:** To prove the effect of toman fish extract of 16 ml/Kg BW mice orally on the number of neutrophil in day 2, 4, and 8 of male wistar mice with diabetic wound. **Materials and Method:** This research applied pure experimental research method with posttest only control design. This research took 36 wistar mice which were divided into negative control group which is given BR2 feed, treatment group which is given toman fish extract in 16 mL/Kg BW mice and positive control group which is given haruan fish extract in 13.54 mL/Kg BW mice. **The Result:** One-way ANOVA showed that there was significant difference of all groups in day 2 ($p=0.000$), day 4 ($p=0.000$) and day 8 ($p=0.000$). Post-Hoc LSD test for the average of neutrophil number showed that there was significant difference ($p<0.05$) between the negative control group, treatment group and the positive control group. **Conclusion:** Toman fish extract has an effect which increases the number of neutrophil in day 2 and decreases the number of neutrophil in day 4 and 8 on the healing of diabetic wound in mice.

Keywords: diabetes mellitus wound healing, number of neutrophil, toman fish extract.

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INTRODUCTION

Diabetes Mellitus is a metabolic disorder which is characterized by the rising of blood glucose level (hyperglycemia) and the loss of carbohydrate tolerance.^{1,2} The number of Diabetes Mellitus in South Kalimantan province is ranked 13th with the prevalence of 1.4%.³ According to International Diabetes Federation (IDF), the number of patients with Diabetes Mellitus in Indonesia that occurred in the year of 2040 will be 2.125 million people.⁴ Diabetes mellitus patients often suffer from slow wound healing process and depict on the higher cost for their treatment.⁵ South Kalimantan communities from generation to generation have believed that consuming toman fish and haruan fish can help them to accelerate the wound healing process.^{6,7} Toman

fish extract with a dosage of 16mL/Kg BW can accelerate wound healing without systemic disease.⁸

Toman fish contains albumin, zinc, omega-6 fatty acids, and omega-3 fatty acids.^{9,10} Albumin content in toman fish is the highest of albumin level from five other *channidae* family which is 5.35%.⁹ Albumin can also be used as an alternative of *Human Serum Albumin* (HSA) that helps to fulfil the needs of albumin in the wound healing process.⁷ Omega-6 fatty acids content in toman fish have derivative which is called *Arachidonic Acid* (AA) which plays important role for neutrophil in inflammatory phase of wound healing.^{11,12} Winarsihet *al*, 2012 stated that giving natural substances which contain antioxidant to mice with diabetic wound also increased the number of neutrophil in the 2nd day and decreased the neutrophil in the 4th and 7th day. Giving toman fish

extract orally with the dosage of 16mL/Kg BW to mice with diabetes mellitus incised wound could accelerate the wounds closure in the 11th day.^{13,14}

Toman fish, which is in the same genus of haruan fish, also has the same content just like haruan fish. The contents are albumin, fatty acids, and zinc.^{9,15} According to the previous study, albumin content in haruan fish is 4,53% which is on the same level to 13.54 mL/Kg BW.⁹ Empirically, patent drugs have been circulated among society which are made from haruan fish in the form of capsules.¹⁶ Haruan fish is one type of fish that has potential to be used as medicine for wound healing.¹⁷ Haruan fish extract could decrease the number of neutrophil in the 3rd day on normal wound and accelerated wound healing in mice with diabetes mellitus in the 8th day.¹⁸⁻²⁰

Diabetic wound increases the formation of free radicals / Reactive Oxygen Species (ROS).²¹ The increased number of ROS inside the body can damage cells and cause the wounds to become chronic.²²⁻²⁴ Toman fish extract contains albumin and omega-6 fatty acids. Albumin functions as an antioxidant that can reduce and destroy the formation of ROS.^{9,25,26} Omega-6 fatty acids has derivative which is called Arachidonic Acid (AA). Arachidonic Acid (AA) can be converted to be lipoxin in inflammatory phase. Lipoxin has role to stop constant inflammation in neutrophil.^{1,12} Neutrophil can produce ROS in normal amount which can kill and digest bacteria during the phagocytosis.^{24,27}

The result of the study that supports the use of toman fish extract with dosage of 16 mL/Kg BW in wound healing to diabetes mellitus patients are still very limited. This study is aimed to prove the effect of toman fish extract (*channamicropeltes*) of 16mL/kg body weight orally on the number of neutrophil on the wound of wistar mice (*Rattus Novergicus*) which was induced to diabetes mellitus in the 2nd, 4th, and 8th day.

MATERIAL AND METHODS

The researcher began the research by asking the ethical clearance and permission to conduct the research from The Ethic Committee of Faculty of Dentistry, Lambung Mangkurat University No. 022/KEPKG-FKGULM/EC/VIII/2017. This study applied the true experimental design with posttest-only control group design. The population of this study was wistar mice.

The sample inclusion criteria of this study was male wistar mice with the weight of 250-300-gram, age of 2-3 months and in healthy condition. The sample exclusion criteria of this study found that there were more than 10% weight loss of mice after the adaptation in laboratory, the condition of unhealthy mice, abnormal mice, and dead mice. The

researcher used animal testing (in vivo testing) which was divided into nine groups; three groups of negative control which were given BR2 feed, three treatment groups which were given BR2 feed plus toman fish extract with the dosage of 16mL/Kg BW and three groups of positive control which were given BR2 feed plus haruan fish extract with the dosage of 13.54mL/Kg BW.

Treatment for each mice was given orally by using feeding tube. It was given twice a day for eight days. Each group consisted of four mice and were sacrificed in 2nd, 4th, and 8th day.

The Process of Extracting Toman Fish and Haruan Fish

This study began by taking sample of toman fish and haruan fish. Toman fish or haruan fish which were used in this study were 11 kg in total. The part used in this study was toman flesh or haruan flesh. First of all, the fishes' scales were cleaned and eviscerated. Then, the scale of the flesh was weighed for 9.84kg. The flesh was put inside a container and steamed approximately 30 minutes. The pale-yellow liquid which was emerged from the flesh as much 750 ml was taken and set aside. Toman flesh or haruan flesh was wrapped with flannel and was put into hydraulic press tool. Toman fish extract or haruan's was put into test tube for 7.5 ml and centrifuged for 15 minutes in 6000 rpm speed. The result of centrifugation was obtained 700 ml of fluid and 50 ml of precipitants were separated. The extract of either toman fish or haruan fish was kept in a dark glass bottle and sealed with aluminum foil and clean pack.

Procedures Induction of Diabetes Mellitus in Mice (Induction of *Streptozotocin* (STZ))

The mice model of diabetes was obtained by injecting STZ at a dose of 35 mg/kg BW. The mice were given feed and then examined after seven days. The glucose level on the mice were measured using glucometers before and after given STZ. The mice were diagnosed with diabetes when the blood glucose level were ≥ 126 mg/dL.^{1,28} The physical condition of mice with diabetes were appeared to be lethargic, underweight, and inactive.

Making Incision Wound in Wistar Mice

The treatment began with adapting the mice for one week in the laboratory, then divided into nine treatment groups using thirty-six mice. The mice were taken and given sedative substance using diethyl ether. Incised wound was made 1 cm long with 2 mm depth on the back of the mice using sterile scalpel, then blood was cleaned with aquadest. Wound on the back of the mice was bandaged.

Sacrificing Mice Using *Diethyl Ether*

On the 2nd, 4th, and 8th day, all mice in every group were sacrificed to see if there was the process of healing with 5 ml of diethyl ether inhalational anesthetic. The inhalational process began by putting a white mice inside a beaker then covered so that the ethyl did not evaporate. Then, waited for a while until the white micewas dead.

Tissue Retrieval

The research retrieved tissues which would be observed with excisional biopsy technique. The dermis of incised wound, which was 1 cm long and 2 mm in-depth, on the back of wistar micewas the area in which the biopsy would be done. The tissue which had been observed with biopsy was fixed in 10% of Buffer Neutral Formalin (BNF), made for histopathologic preparations and colored with HE dye. Mice which tissues had been taken were buried.

Animal Handling and Care After Tissue Retrieval

Unutilized animal testing organs were buried. The burial of animal testing organs was done by cleaning them, then wrapped with fabric and buried under ± 25 – 50 cm depth.

Calculating the Number of Neutrophil

The neutrophil number of the wound on the back of wistar mice were calculated with light microscope using the Haematoxyllin Eosin (HE) dye on the 2nd, 4th, and 8th day. The neutrophil calculation were undertaken at 400x magnification with each treatment group result was input to a table and data processing was processed using SPSS.

RESULT

The test result on one-way Anova shows that there is significant difference (p<0,05) where p=0.000 in each of the treatment group in 2nd, 4th, and 8th day. Post-Hoc LSD for the average number of neutrophil in 2nd, 4th, and 8th day shows that there is significant difference (p<0,05) in each of the treatment group.

The result of the average number of neutrophil in diabetes mellitus wound healing can be seen in table 1. The graphic of average neutrophil amount on the back injuries of wistar mice for eight days in each group can be seen in Figure 1.

Table1. Average (Mean±SD) of Neutophils Counts On Diabetes Wound Healing on Back of Wistar Mice.

Group	Mean ± SD The Number of Cell		
	Day-2	Day-4	Day-8
Toman Fish Extract	22,7 ± 0,9 (22,0 – 24,0 cells)	7,7 ± 0,9 (7,0 – 9,0 cells)	4,0 ± 0,8 (3,0 – 5,0 cells)
Haruan Fish Extract	20,2 ± 1,2 (19,0 – 22,0 cells)	10,0 ± 0,8 (9,0 – 11,0 cells)	8,0 ± 0,8 (7,0 – 9,0 cells)

Feed BR 2	19,8 ± 2,8 (15,0 – 18,0 cells)	14,7 ± 0,9 (14,0 – 16,0 cells)	10,5 ± 1,2 (3,0 – 12,0 cells)
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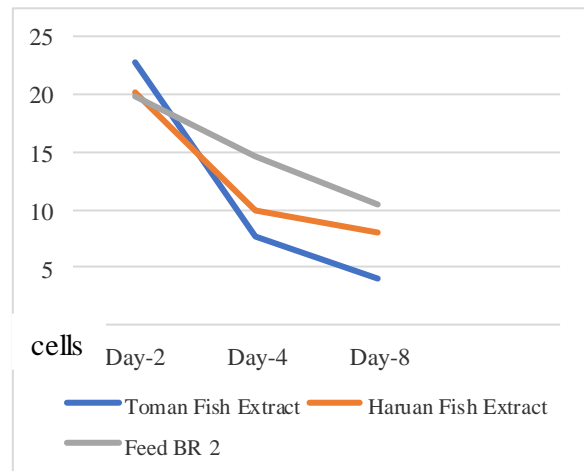


Figure 1. Graphic of The Average of Neutrophil Counts on Wistar Mice Back Injuries during eight days for each grup.

According to Figure 1, it can be concluded that the lower the average number of neutrophil, the better wound healing process is. The number of neutrophil in the 2nd day was increased, while in the 4th and 8th day was decreased. The highest average number of neutrophil was found in the 2nd day between three groups in sequence which weretoman fish extract, haruan fish extract and feed only.The lowest number of neutrophil was found in the 4th and 8th day between three groups in sequence which were toman fish extract, haruan fish extract and BR2 feed.

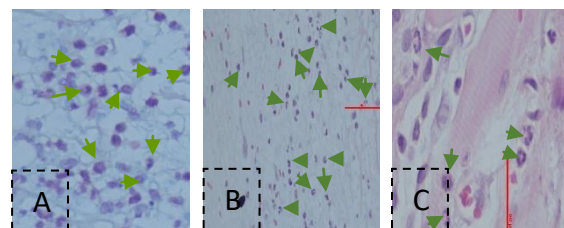


Figure2. Histopathology of Neutrophil Counts on Wistar Mice Back Wound Healing in Feed Only Group (A), Toman Fish Extract (B), and Haruan Fish Extract (C) in the 2nd day with 400x magnification using Light Microscope.

Based on Figure 2, it can be seen the depictionof neutrophil with purplecell coreand U-Shaped nuclei. Picture 2 shows the highest number of neutrophil was found in the 2nd day. The number of neutrophil in the group given toman fish extract

was higher than the group given haruan fish extract and feed only.

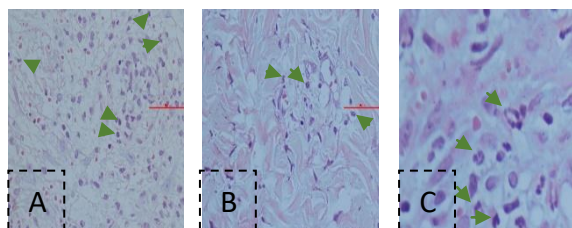
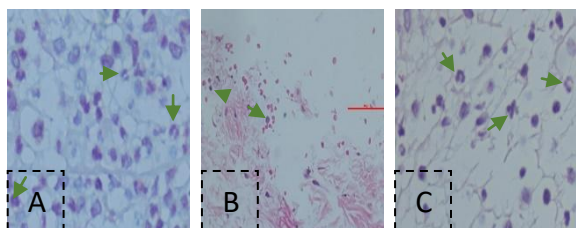


Figure 3. Histopathology of Neutrophil Counts on Wistar Mice Back Wound Healing in Feed Only Group (A), Toman Fish Extract (B), and Haruan Fish Extract (C) in the 4th day with 400x magnification using Light Microscope.

Picture 3 shows the histopathology description with the same observation as Picture 2, but in Picture 3 we can see that the number of neutrophil was decreased in the 4th day. The number of neutrophil with toman fish extract (B) was lower than haruan fish extract (C) and feed (A).



Gambar 4. Histopathology of Neutrophil Counts on Wistar Mice Back Wound Healing in Feed Only Group (A), Toman Fish Extract (B), and Haruan Fish Extract (C) in the 8th day with 400x magnification using Light Microscope.

Picture 4 shows histopathology depiction with the same observation with Picture 2 and Picture 3, but in Picture 4 the lowest decreasing number of neutrophil which found in the 8th day. The number of neutrophil in the group given toman fish extract was lower than the group given haruan fish extract and feed only.

DISCUSSION

This research proves that there was an increase in the number of neutrophil on the 2nd day and a decrease on its number on the 4th day and 8th day. It showed that toman fish extract groups was the most influential group compared with haruan fish extract group and feed only group. It was because of the difference between albumin content found in toman fish and haruan fish.²⁹ According to Firliyanti (2016), toman fish has the highest albumin level among five *channidae* family because albumin in toman fish has the most complete amino acids formation which is the composer of albumin.³⁰

According to Firliyanti et al (2013), albumin level of toman fish was the highest level among the five *channidae* family which is 5,35%, while haruan fish has only 4,53% of albumin level.⁹ Albumin in toman fish extract and haruan fish extract is an antioxidant which is potential in DM wound healing process.^{9,14,25,29}

The highest increasing number of neutrophil was found on the 2nd day in the group which was given toman fish extract. Neutrophil were the first defense cell which its number increased when wound was occurred.^{27,31,32} In DM wound, *Reactive Oxygen Species* (ROS) is increased which can inhibit the DM wound healing process^{21,33,34,35}. Albumin in toman fish has a role as animal antioxidant^{9,18} Antioxidant can inhibit the increase of ROS by bonding the heavy metal ion which is involved in ROS formation through Cu²⁺ bond, vanadium ion, cobalt and nickel with high affinity. According to Perdana *et al* research (2015), Vip Albumin® use could increase the number of neutrophil in diabetes patient. It was happened because the decreasing number of ROS which could trigger granulocytes development, especially neutrophil³⁸⁻⁴⁰. Neutrophil could utilize the neutralized ROS to assist in killing pathogens in phagosome through phagocytic process.^{24,27,41}

The result showed that the lowest count of neutrophil depletion was found on the 4th day in the group which was given toman fish extract. It was because of the difference of the AA contents which know higher in toman fish than haruan fish.⁴² Toman fish contains another unsaturated fatty acid which was omega 6 fatty acids.⁸⁻¹¹ Haruan fish extract also has unsaturated fatty acids contents, too.⁹ Unsaturated fatty acids contents in toman fish (7,2 mg) was higher than haruan fish (3,7 mg). It made the average number of neutrophil in toman fish treatment group was lower than haruan fish.⁴² Omega-6 unsaturated fatty acids in toman fish has AA as its derivative. Arachidonic Acids (AA) has chemical mediators in the form of leukotriene and prostaglandin which have important role in inflammatory phase.^{12,43,44}

In inflammatory phase, neutrophil released chemical mediator to recruit more neutrophil, destroyed and also digested unidentified compounds. This process is called phagocytosis. Cell phagocytosis must be prevented in certain phase because it can damage cell and tissue around the cells.^{12,45,46} This prevention is done by AA by changing leukotriene (pro-inflammation) into lipoxin (anti-inflammation) by regulating 15-LO enzyme (15-Lipoxygenase) which are found in neutrophil.^{12,27,43,44} Lipoxins function is to block neutrophil infiltration to become chronic inflammation, so inflammation can be prevented in time and macrophages can continue the activity of

neutrophil.^{12,43,46} DM condition of mice caused overlong inflammatory phase.⁴⁷ The result of the research is in line with Winarsih *et al* research which stated that there was the decreasing average number of neutrophil in mice wound with DM in the 4th day.¹³

The result shows that, on 8th day there was also the lowest decrease average number of neutrophil in group which was given toman fish extract. It was caused by the large number of albumin and unsaturated fatty acid content in toman fish.^{30,42} The decreasing of the average number of neutrophil which occurred in chronic inflammatory phase was marked by the appearance of macrophages which moved in large scale to the wound zone to take over the neutrophil work.^{31,48,49,50,51} The appearance of macrophages and neutrophil apoptosis indicated the process of tissue proliferation was occurred.^{31,52,53} Agustin *et al* research (2016), stated the depletion in the average number of neutrophil occurred on the 3rd day in mice with normal wound (Agustin *et al*, 2016).¹⁸ In normal wound, proliferation process was started on the 4th day until the 21st day after the wound occurrence.⁵⁴

DM wound is identical with the interference of wound healing in the inflammatory phase and overlong proliferation.^{47,55} This research result is in line with Winarsih *et al*, (2012) research which stated that in the 7th day, the average number of neutrophil was still found, but the number was fewer than DM mice wound in the 4th day.¹³ Perdana *et al*, (2015) research also stated that the use of VipAlbumin® to DM patient toward the average number of neutrophil still could be found in the 15th day.³⁸ In DM patients, frequently, the phagocytosis dysfunction of neutrophil occurred.⁵⁶⁻⁵⁹ Albumin can be used as an alternative *Human Serum Albumin* (HSA) so it can increase neutrophil *azurophilic* degranulation.^{7,60} The research of Mikhalchika *et al* (2013) stated that use of HSA could elevated degranulation of *azurophilic* granule of neutrophil which function in microbial phagocytosis process.⁶⁰⁻⁶² It can be concluded that toman fish extract with the dosage of 16mL/KgBW can increase the number of neutrophil in the 2nd day and decrease the number of neutrophil in the 4th and 8th day in wistar mice (*Rattus norvegicus*) wound which was induced with diabetes mellitus compared with haruan fish extract with the dosage of 13,54mL/Kg body weight and BR2 feed.

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