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> An: info@soc-bdr.org
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>
> First Name : Maharani
> Middle Initial : Laillyza
> Last Name : Apriasari
> Title : Dr
> Institution : University of Lambung Mangkurat
> Department : Oral Medicine
>
> Address 1 : Veteran street no. 128B
> Address 2 :
> City : Banjarmasin
> State :
> ZIP/Postal Code : 70239
> Country : Indonesia
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> Date of Birth (MM/DD/YYYY):
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> Phone Number :
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> E-mail : maharaniroxy@gmail.com
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Follow-Up: Online Paper Submission

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On Fri, Jul 8, 2022 at 8:15 AM Rohan JCF Corp <rohan@jcfcorp.com> wrote:

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Manuscript: RDS-2022-7-2 - Anti Inflammatory Effect of Channa micropeltes Extract in Angiogenesis of Diabetes Wound Healing

Authors: Maharani Laillyza Apriasari (Corresponding Author), Dewi Puspitasari (Co-author), Juliyatin Putri Utami (Co-author)
Date submitted: 2022-07-08

Dear Dr. Apriasari
Thank you very much for submitting the above manuscript. Please refer to the manuscript number in all correspondence concerning the manuscript as listed above.

The manuscript will now be forwarded to our Editors and reviewers and we shall inform you as soon as a decision has been made by the editorial board.

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Cc: dewident@ulm.ac.id, juliyatin.utami@ulm.ac.id

Dear Rohan,

Thank you for the information. Yes, i agree to pay it.

Best regards,

Maharani L. Apriasari
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Greetings,

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Review comments:

I went through the manuscript and found it to have a very good concept with basic laboratory experimental research, however it needs to be revised based on the comments and suggestions I put as Review points on the corresponding points in the manuscript.

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1. Introduction

Indonesia ranks sixth among countries with a high prevalence of diabetes mellitus (DM). In an uncontrolled state, DM will result in various oral problems such as xerostomia, candidiasis, stomatitis, gingivitis and periodontitis. The healing of such conditions is often complicated due to hyperglycemia that initiates chronic inflammation [1-3]. Previous study revealed that *Channa micropeltes* ointment at 20% concentration or *Channa striata* ointment at 10% concentration applied topically can accelerate wound healing in a DM rat model. Both species are categorized in the same genus and contain albumin as well as omega-6 that acts as an antioxidant and anti-inflammatory [3,4]. As an antioxidant, *Channa micropeltes* extract elevates superoxide dismutase (SOD) activity and lowers malondialdehid (MDA) level on Day 7 [2]. As an anti-inflammatory, the topical application of *Channa micropeltes* at 20% concentration on the back skin of a diabetic rat model can increase the number of macrophage and lymphocyte cells on Day 8 and gradually reduce it on Day 14 [1].

Macrophage is the key inflammatory process in wound healing. Reduction in macrophage number at the end of inflammatory stage demonstrates tissue recovery by producing growth factors and cytokines and inducing as well as terminating angiogenesis [5,6]. Macrophage is also produced Nuclear Factor kappa B (NF-κB) that regulates the inflammatory response of metabolic disease such as DM. A state of hyperglycemia in DM will increase reactive oxygen species (ROS) and advanced glycation end products (AGEs) that elevate chronic inflammation through the activation of NF-κB . This will change vascular endothelial growth factor (VEGF) expression that will generate the damage to blood vessels in the angiogenesis process [7,8].

The extract of *Channa micropeltes* has shown to promote wound healing on the skin of diabetic rat model by reducing macrophage number at the end of inflammatory stage [3]. There has been no study that explores the effect of *Channa micropeltes* application on NF-κB , VEGF, and neovascular cells that are pivotal components in the DM wound healing process. Based on that, a study to analyze the expression of NF-κB , VEGF and neovascular cells number at the inflammatory stage of the DM wound healing process was warranted.

2. Methods

This study was an experimental laboratory research study incorporating a post-test only control group design. It was approved by Ethical Clearance Committee, Faculty of Dentistry, Universitas

Lambung Mangkurat, Banjarmasin, South Kalimantan, Indonesia, with number 111/KEPKG-FKGULM/EC/III/2020.

2.1 Manufacturing Channa micropeltes and Channa striata extract

Preparing both *Channa micropeltes* and *Channa striata* extract used fresh fish weighing 600-1000 grams. Each extract was later steamed in a pan for 25-35 minutes at a temperature of 60^{o} Celsius. The flesh was enclosed with flannelette and pressed in a hydraulic device. Furthermore, *Channa micropeltes* and *Channa striata* were centrifuged for 15 minutes at a speed of 6000 rpm. Each extract was kept inside a dark glass bottle and then covered with aluminum foil and clean pack.

2.2 Formulation of Channa micropeltes and Channa striata ointment

Adeps lanae (Asian chemicals, Semarang) in a weight of 16.875 grams and vaselin flavum (PT. Brataco, 1295578) in a weight of 23.125 grams were used in the formulation of the *Channa micropeltes* ointment. Meanwhile, a combination of 16.875 grams adeps lanae and 28.125 grams vaselin flavum were combined in the formulation of *Channa striata* ointment. Adeps lanae was initially poured into different tubes for each extract and later added gradually with either *Channa micropeltes* at 20% concentration or *Channa striata* at 10% concentration. After the extract was fully absorbed by adeps lanae, the mixture was then mashed to obtain a homogenous consistency. Subsequently, the composition was further mixed with vaselin flavum and mashed again until homogenous.

2.3 In vivo study

This study included 2-3 months old male Wistar (*Rattus novergicus*) rats (weight, 250-300 grams) obtained from an animal laboratory at the Faculty of Medicine, University of Lambung Mangkurat. The 24 rat specimens were kept in cages, and the temperature and humidity were set within ±25 °C and 60%, respectively. They were fed standard BR-II, and they had access to boiled water *ad libitum*. Rats with hyperglycemia were obtained by injecting streptozotocin (STZ) at 35 mg/kg dosage until the blood glucose level was over 126 mg/dL-1; non-diabetic rats were ones without intervention. All animals were divided into 3 treatment groups consisting of 20% *Channa micropeltes* extract ointment, 10% *Channa striata* extract ointment, and placebo

ointment as control. Each substance was applied topically 3 times daily (every 6-8 hours). An incisional wound was made on the back of the rats with 1 cm length and 1 mm depth using sterile scalpel under inhaled anesthesia of 5 ml diethyl ether.

After the 4th and 8th day of application, rats were euthanized by inhaling a lethal dosage of diethyl ether. The back skin was then biopsied for histopathology examination using hematoxylin eosin (HE) to evaluate macrophages and neovascular cells, and immunohistochemistry (IHC) to evaluate NF-κB and VEGF. The number of macrophages and neovascular cells were calculated in 3 different field locations using a light microscope (Olympus, WA) at 400 magnifications and subsequently calculated for its average. IHC staining was performed using anti-mouse NF-kB monoclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, NF-kB p65 (F-6): sc 8008) and anti-mouse VEGF monoclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, VEGF (C1): sc 7269). Positivity NF-κB expression was defined as only distinct nuclear immunostaining, which is considered as activated NF-κB in the studied field at 100 magnifications.

2.4 Data analysis

The results were analyzed using a 2-way analysis of variance parametric test based on the Shapiro-Wilk normality test and Levene's variance homogeneity test. The results showed normal data distribution and homogenous data variances. Consequently, further analysis by means of a *post hoc* Bonferroni test was conducted with a statistical significance of $p < 0.05$.

3. Results

Table 1 shows the results of NF-κB analysis. The highest expression of NF-κB was observed in the control group (Group III) on Day 4 (15.5 ± 2.38), and the lowest was in the treatment of 20% *Channa micropeltes* extract ointment (Group I) (4.75 ± 0.96) and 10% *Channa striata* extract ointment (Group II) (5.75 ± 1.71) on Day 8. The expression of NF-κB between Group I and 10% *Channa striata* extract ointment (Group II) treatment groups did not show any difference ($p > .05$; Table 1). The statistical significance value between treatment group and day was $p > 0.05$, demonstrating no interaction between treatment groups and days on NF-κB expression.

The highest expression of VEGF was observed in Group I (14.75 ± 0.96) on Day 8, whereas the lowest was in Group III (7.00 ± 1.41) on Day 4. The expression of VEGF showed statistically

significant differences in all groups ($p < 0.05$; Table 1). The significance value between treatment groups and days was $p > 0.05$, demonstrating no interaction between treatment group and day on VEGF expression.

The highest count of neovascular cells was observed in Group I (11.00 ± 2.16) on Day 8, while the lowest was in Group III (5.50 ± 0.58) on Day 4. The count of neovascular cells showed statistically significant differences in all groups ($p < 0.05$; Table 1). The statistical significance value between treatment groups and days was $p > 0.05$, demonstrating no interaction between treatment groups and days on the number of neovascular cells.

4. Discussion

DM is characterized with an increase in blood glucose level that induces glycation reaction. This process will result in amadory production to formulate toxic proteins (AGEs). Interaction between AGEs and a receptor advanced glycation end product (RAGE) will increase the signal for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which produces superoxide anion. This process elevates the production of reactive oxygen species (ROS) which are the key for molecular signaling as well as the development of inflammatory disorders such as DM. Excessive production of ROS will complicate the healing process of wounds in DM [2,9].

Channa micropeltes contains albumin and omega-6 fatty acid. Albumin can decrease ROS by cutting chained oxidative reaction in the ROS formation's process. Albumin can bind metal ions and also catch oxygen that processing hydrogen peroxide into non radical compound. Omega-6 fatty acid, especially arachnodic acid are the keys to anti-inflammatory processes. It plays the role in stimulating macrophages to release growth factors, such as VEGF. Arachidonic acid will be metabolized through an enzymatic mechanism such as the 5-lipoxygenase and cyclo-oxygenase pathways that produce leukotrienes, prostaglandins, and thromboxane A2. These can stimulate cell migration and new local vascularization in the wound healing process of DM [4,10].

In previous studies, *Channa micropeltes* extract ointment at 20% concentration and *Channa striata* extract ointment at 10% concentration were shown to promote the wound healing process in DM. *Channa micropeltes* and *Channa striata* are categorized in the same genus. Both species contain albumin, the secondary antioxidant that can bind metal ion in ROS formation [1,2]. ROS induces an inflammatory response through the activation of Nuclear Factor kappa B (NF-κB).

NF-κB signal is the main key of chronic inflammation in DM [7,8]. This is demonstrated by the study result on Day 4 that reveals the highest expression of NF-κB in control group, while the result for 20% *Channa micropeltes* extract application was comparable to 10% *Channa striata* extract application.

Our study result on Day 8 demonstrates the reduction of NF-κB expression in both *Channa micropeltes* extract ointment at 20% concentration and *Channa striata* extract ointment at 10% concentration when compared to the control. Topical application of *Channa micropeltes* extract at 20% concentration or *Channa striata* extract at 10% concentration may reduce excessive ROS, thereby suppressing NF-κB expression. Both extracts possess potential as natural substances that may inhibit the expression of NF-κB . Previous studies reveal that the impediment of pro-inflammatory NF-κB from therapeutical application of several natural and synthetic ingredients will be a good target to manage vascular complication in DM [7]. A prolonged inflammatory response can be resolved by the inhibition of NF-κB. As an anti-inflammatory substance, *Channa micropeltes* will reduce inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) that suppress the NF-κB gene regulator. This will prevent prolonged inflammation in the wound healing process of DM [11,12].

Excessive activation of NF-κB will cause abnormal DNA transcription which includes various gene expression of vascular complications occurring in VEGF, Platelet Derived Growth Factor (PDGF), Endothelin-1 (ET-1) and Transforming Growth Factor beta (TGF-β) that cause vascular cell damage [7]. VEGF as pro-angiogenic modulators encounter down regulation in DM, disturbing the angiogenesis process [13,14]. Prior studies demonstrate Vascular Endothelial Growth Factor A (VEGF-A) protein and messenger ribonucleic acid (mRNA) level in the wound of a diabetic rat model which shows reduction when compared to the group with a normal wound. DM leads to the decrease of angiogenesis in wound healing, so that it lessens vascular and capillary density [13].

The angiogenic effect is initiated by VEGF-A binding to Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2). Angiogenesis stimulation through PI3K-Akt-eNOS will cause endothelial cell to migrate, proliferate, and differentiate. Molecular signals will be commenced by phosphatidylinositol 3 kinase from serine/threonine kinase Akt/protein kinase B. Akt/PKB through the phosphorylation of endothelial nitric oxide synthesis on Ser 1177, and will stimulate NO production, vasodilatation, and endothelial cell migration [15,16]. This result presents that

Channa micropeltes extract ointment at 20% concentration increases the highest expression of VEGF when compared to *Channa striata* extract ointment at 10% concentration or the control on Day 8. This exhibits the potential of *Channa micropeltes* extract ointment at 20% concentration to promote angiogenesis on the DM wound healing process.

Previous study by Carabelly et al. [1] reveals that the application of *Channa micropeltes* extract ointment at 20% concentration may elevate macrophage number on Day 8 and reduce them on Day 14. Macrophage regulates angiogenesis signals in neovascular along the formation of granulation tissue process [17]. This concept is in accordance with this study as the number of macrophages was observed the highest on Day 8 and followed by the increase of neovascular cell number by the application of *Channa micropeltes* extract ointment at 20% concentration on Day 8. The highest number of neovascular cells also was observed in the application of *Channa micropeltes* extract ointment at 20% concentration when compared to *Channa striata* extract ointment at 10% concentration or the control in Day 8. This study did not continue to further days to limit the parameters.

We can conclude that the application of *Channa micropeltes* extract ointment at 20% concentration on the wound of a diabetic rat model can reduce the expression of NF-κB. The anti-inflammatory effect of *Channa micropeltes* can elevate the expression of VEGF and the number of neovascular cells in angiogenesis process of diabetic wound healing. Our findings require further research using different parameters, adding time of evaluation, and using larger experimental animals.

Acknowledgements: This study was financed by the Faculty of Dentistry, University of Lambung Mangkurat. The authors express their gratitude.

Conflicts of interest statement: The authors have no conflicts of interest to report.

Ethical approval: Approval for this study was provided by the Ethical Clearance Committee, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, South Kalimantan, Indonesia (#111/KEPKG-FKGULM/EC/III/2020).

Gmail maharani roxy <maharaniroxy@gmail.com>

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ABSTRACT

Background: *Channa micropeltes* extract contains albumin and Omega-6 which possess anti-oxidant and anti-inflammatory agent that can promote macrophages in wound healing process of diabetes mellitus (DM). **Objective**: to analyze Nuclear Factor kappa B (NFkB) and Vascular Endothelial Growth Factor (VEGF) expression as well as neovascular cells in the inflammatory stage of DM wound healing. **Materials and methods**: The twenty-four males Rattus novergicus were divided into three groups that were 20% *Channa micropeltes* ointment (Group I), 10% *Channa striata* extract ointment (Group II), and placebo ointment as control (Group III). Ointments were applied three times daily. **Results**: The highest expression of NFkB was observed in Group III on day 4 (15.50 ± 2.38), while the lowest was in treatment of Group I and Group II on day 8 (4.75±0.96). The highest expression of VEGF was observed in Group I on day 8(14.75±0.96), while the lowest was Group III on day 4 (7.00±1.41). The highest count of neovascular cells was observed in Group I on day 8 (11.00±2.16), while the lowest was in Group III on day 4 (5.50±0.58). **Conclusion**: *Channa micropeltes* have an anti-inflammatory effect by regulating NFkB expression and elevating VEGF expression in angiogenesis process of DM wound healing.

Keywords: *Channa micropeltes, nfkb, vegf, neovascular, wound healing, diabetes mellitus*

Introduction

Indonesia is ranked sixth among the countries with high prevalence of Diabetes mellitus in the world. In uncontrolled state, Diabetes mellitus will result in the variance of oral problems such as xerostomia, candidiasis, stomatitis, gingivitis and periodontitis. The healing of such conditions is often complicated due to hyperglycemia that initiates chronic inflammation.[1,2,3] Previous study revealed that *Channa micropeltes* ointment at 20% concentration or *Channa striata* ointment at 10% concentration that was applied topically can accelerate wound healing in diabetes mellitus rat model. Both species are categorized in the same genus and contain albumin as well as omega 6 that act as anti-oxidant and anti-inflammation.[3,4] As antioxidant, *Channa micropeltes* extract elevate Superoxide dismutase (SOD) activity and lower Malondialdehid (MDA) level on day 7.[2] As anti-inflammation, the topical application of *Channa micropeltes* at

20% concentration on the back skin of diabetic rat model could increase the number of macrophage and lymphocyte cells on day 8 and gradually reduce it on day 14.[1]

Macrophage is the key of inflammatory process in wound healing. Reduction in macrophage number at the end of inflammatory stage demonstrates tissue recovery by producing growth factors, cytokines, and induce as well as terminate angiogenesis.[5,6] Macrophage is also produced Nuclear Factor kappa B (NFkB) that regulates inflammatory response of metabolic disease such as diabetes mellitus. Hyperglycemia state in diabetes mellitus will increase Reactive Oxygen Species (ROS) and Advanced Glycation End Products (AGEs) that elevate chronic inflammation through the activation of NFkB. This will change Vascular Endothelial Growth Factor (VEGF) expression that will generate the damage of blood vessel cell in angiogenesis process.[7,8]

The extract of *Channa micropeltes* has been proven to promote wound healing on the skin of diabetic rat model by reducing macrophage number at the end of inflammatory stage.[3] There has been no study that explore the effect of *Channa micropeltes* application on NFkB, VEGF, and neovascular cells that are pivotal components in diabetes mellitus wound healing process. Based on that, a study to analyze the expression of NFkB, VEGF and neovascular cells number at the inflammatory stage of diabetes mellitus wound healing process should be conducted.

Materials and Methods

This study was an experimental laboratory research incorporating post-test only control group design. It was approved by Ethical Clearence Commitee, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, South Kalimantan, Indonesia, with number 111/KEPKG-FKGULM/EC/III/2020.

Manufacturing *Channa micropeltes* and *Channa striata* extract

Preparing both *Channa micropeltes* and *Channa striata* extract used fresh fish weighed 600-1000 g. Each of it was later steamed in a pan for 25-35 minutes under 60^{o} temperature. The flesh were enclosed with flannelette and pressed in hydraulic device. Further, *Channa micropeltes* and *Channa striata* were centrifuged for 15 minutes within 6000 rpm speed. Each extract was kept inside dark glass bottle and then covered with aluminum foil and clean pack.

Formulation of *Channa micropeltes* and *Channa striata* ointment

Adeps lanae (Asian chemicals, Semarang) in a weight of 16.875 g and vaselin flavum (PT. Brataco, 1295578) in a weight of 23.125 g were used in the formulation of *Channa micropeltes* ointment. Meanwhile, a combination of 16.875 g adeps lanae and 28.125 g vaselin flavum were utilized in the formulation of *Channa striata* ointment. Adeps lanae was initially poured into different tubes for each extract and later added gradually with either *Channa micropeltes* at 20% concentration or *Channa striata* at 10% concentration. After the extract was fully absorbed by adeps lanae, the mixture was then mashed to obtain homogenous consistency. Subsequently, the composition was further mixed with vaselin flavum and mashed again until homogenous.

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In Vivo Study

This study included 2-3 months-old male Wistar (*Rattus novergicus*) rats (weight, 250–300 g) obtained from animal laboratory, Faculty of Medicine, University of Lambung Mangkurat. The total of 24 rats were kept in cages, and the temperature and humidity were set within ±25 °C and 60%, respectively. They were fed standard BR-II, and they had access to boil water ad libitum. Rats with hyperglycemia was obtained by injecting Streptozotosin (STZ) at 35 mg/kg dosage until the blood glucose level was over 126 mg/dL-1, while non Diabetic Rats is rats without intervention. All animals were divided into 3 treatment groups consisting of 20% *Channa micropeltes* extract ointment, 10% *Channa striata* extract ointment, and placebo ointment as control. Each substance was applied topically three times daily (every 6-8 hours). Incisional wound was made on the back of the rats with 1 cm length and 1 mm depth using sterile scalpel under inhaled anesthesia of 5 ml diethyl ether.

After the 4th and 8th day of application, rats were euthanized by inhaling lethal dosage of diethyl ether. The back skin was then biopsied for histopathology examination using Haematoxyllin Eosin (HE) to evaluate macrophages and neovascular cells, and Immunohistochemistry (IHC) to evaluate NF-κB and VEGF. The number of macrophages and neovascular cells were calculated in three different field locations using a light microscope that were counted using light microscope (Olympus, United States) at 400 magnifications and subsequently calculated for its average. Immunohistochemistry (IHC) staining was performed using anti-mouse NF-kB monoclonal antibody (Santa Cruz

Biotechnology Inc. NF-kB p65 (F-6) : sc 8008) and anti-mouse VEGF monoclonal antibody (Santa Cruz Biotechnology Inc. VEGF (C1) : sc 7269). Positivity NF-κB expression was defined as only distinct nuclear immunostaining, which is considered as activated NF-κB in the studied field at100 magnification.

Statistical Analysis

The results were analyzed using two-way analysis of variance parametric test based on Shapiro Wilk normality test and Levene's variance homogeneity test. The results showed normal data distribution and homogenous data variances. Consequently, further analysis by means of a Post Hoc Bonferroni test was conducted with significance $p < 0.05$.

Results

The results of NFkB analysis were shown in table 1. The highest expression of NFkB was observed in the control group (Group III) on day 4 (15.5±2.38), while the lowest was in the treatment of 20% *Channa micropeltes* extract ointment (Group I) (4.75±0.96) and 10% *Channa striata* extract ointment (Group II) (5.75±1.71) on day 8. The expression of NFkB between Group I and 10% *Channa striata* extract ointment (Group II) treatment groups did not show any difference ($p > 0.05$; Table 1). The significance value between treatment group and day were $p>0.05$ that represents no interaction between treatment groups and days on NFkB expression.

The highest expression of VEGF was observed in Group I (14.75±0.96) on day 8, where as the lowest was in Group III (7.00±1.41) on day 4. The expression of VEGF showed significant differences in all groups ($p < 0.05$; Table 1). The significance value between treatment groups and days were $p>0.05$ that represents no interaction between treatment group and day on VEGF expression.

The highest count of neovascular cells was observed in Group I (11.00±2.16) on day 8, while the lowest was in Group III (5.50±0.58) on day 4. The count of neovascular cells showed significant differences in all groups ($p < 0.05$; Table 1). The significance value between treatment groups and days were $p>0.05$ that represents no interaction between treatment groups and days on the number of neovascular cells.

Discussion

Diabetes mellitus is characterized with an increase in blood glucose level that induces glication reaction. This process will result in amadory production to formulate toxic protein called Advanced Glication End Products (AGEs). Interaction between AGE and Receptor Advanced Glication End Product (RAGE) will increase the signal for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which produces superoxide anion. This process elevates the production of Reactive Oxygen Species (ROS) which are the key for molecular signaling as well as the development of inflammatory disorders such as diabetes mellitus. Excessive production of ROS will complicate the healing process of wound in diabetes mellitus.[2,9]

Channa micropeltes contains albumin and omega-6 fatty acid. Albumin can decrease ROS by cutting chained oxidative reaction in the ROS formation's process. Albumin can bind metal ions and also catch oxygen that processing hydrogen peroxide into non radical compound. Omega-6 fatty acid, especially arachnodic acid are the key of anti inflammatory. It plays the role in stimulating machropages to release growth factors, such as VEGF. Arachinodic acid will be metabolized through an enzymatic mechanism such as the 5-lipoxygenase and cyclo-oxygenase pathways that produce leukotrienes, prostaglandins, and thromboxane A2. These can stimulates the cell migration and new local vascularization in wound healing process of diabetes mellitus.[4,10]

In previous studies, *Channa micropeltes* extract oinment at 20% concentration and *Channa striata* extract ointment at 10% concentration were proven to promote wound healing process in diabetes mellitus. *Channa micropeltes* and *Channa striata* are categorized in same genus. Both species contain albumin, the secondary antioxidant that can bind metal ion in ROS formation.[1,2] ROS induce inflammatory response through the activation of Nuclear Factor kappa B (NFkB). NFkB signal is the main key of chronic inflammation in diabetes mellitus.[7,8] This is proven by this study result on Day 4 that reveals the highest expression of NFkB in control group, while the result for 20% *Channa micropeltes* extract application was comparable to 10% *Channa striata* extract application.

Study result on day 8 demonstrates the reduction of NFkB expression in both *Channa micropeltes* extract ointment at 20% concentration and *Channa striata* extract ointment at 10% concentration when compared to control. Topical application of *Channa micropeltes* extract at 20% concentration or *Channa striata* extract at 10% concentration may reduce excessive ROS,

thus suppressing NFkB expression. Both extract possess potential as natural substance that may inhibit the expression of NFkB. Previous study reveals that the impediment of proinflammatory NFkB from therapeutical application of several natural and synthetic ingredients will be a good target to manage vascular complication in diabetes mellitus.[7] Prolonged inflammatory response can be resolved by the inhibition of NFkB. As anti-inflammatory substance, *Channa micropeltes* will reduce inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase 2 (COX2) that suppress NFkB gene regulator. This will prevent prolonged inflammation in wound healing process of diabetes mellitus.[11,12]

Excessive activation of NFkB will cause abnormal DNA transcription which include various gene expression of vascular complication occurring in VEGF, Platelet Derived Growth Factor (PDGF), Endothelin-1 (ET-1) and Transforming Growth Factor beta (TGF-b) that cause vascular cell damage.[7] VEGF as pro-angiogenic modulators encounter down regulation in diabetes mellitus, thus disturbing the angiogenesis process.[13,14] Prior study demonstrates Vascular Endothelial Growth Factor A (VEGF-A) protein and messenger Ribonucleic Acid (mRNA) level in the wound of diabetic rat model which shows reduction when compared to the group with normal wound. Diabetes mellitus leads to the decrease of angiogenesis in wound healing, so that it lessens vascular and capillary density.[13]

The angiogenic effect is initiated by VEGF-A binding to Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2). Angiogenesis stimulation through PI3K-Akt-eNOS will cause endothelial cell to migrate, proliferate and differentiate. Molecular signal will be commenced by Phosphatidylinositol 3 kinase from serine/threonine kinase Akt/protein kinase B. Akt/PKB through the phosphorylation of endothelial Nitric Oxide synthesis on Ser 1177 will stimulate NO production, vasodilatation, and endothelial cell migration.[15,16] This result presents that *Channa micropeltes* extract ointment at 20% concentration increase the highest expression of VEGF when compared to *Channa striata* extract ointment at 10% concentration or control on day 8. This exhibits the potential of *Channa micropeltes* extract ointment at 20% concentration to promote angiogenesis on diabetes mellitus wound healing process.

Previous study by Carabelly (2019) reveals that the application of *Channa micropeltes* extract ointment at 20% concentration may elevate macrophage number on Day 8 and reduce them on Day 14.[1] Macrophage regulate angiogenesis signal in neovascular along the formation of granulation tissue process.[17] This concept is in accordance with this study as the number of

macrophage was observed the highest on Day 8 and followed by the increase of neovascular cell number by the application of *Channa micropeltes* extract ointment at 20% concentration on Day 8. The highest number of neovascular cell was also observed in the application of *Channa micropeltes* extract ointment at 20% concentration when compared to *Channa striata* extract ointment at 10% concentration or control in Day 8. This study did not continue to further day to limit the parameters.

It can be concluded that the application of *Channa micropeltes* extract ointment at 20% concentration on the wound of diabetic rat model can reduce the expression of NFkB. Anti-inflammatory effect of *Channa micropeltes* can elevate the expression of VEGF and the number of neovascular cells in angiogenesis process of diabetic wound healing. This study requires further research using different parameters, adding time of evaluation, and using larger experimental animals.

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Acknowledgements

The authors thank to Faculty of Dentistry, University of Lambung Mangkurat which have supported this work.

Funding Information

This study was financed by Faculty of Dentistry, University of Lambung Mangkurat which have supported this work.

Conflicts of Interest statement

The authors of the work have no conflict of interest.

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Dear Dr. Apriasari

We are pleased to inform you that your manuscript Anti Inflammatory Effect of Channa micropeltes Extract in Angiogenesis of Diabetes Wound Healing has been accepted for publication and passed on to our Production Editing Department. You will hear from a production editor in due course.

We hope you will continue to submit work from your group to the Review of Diabetic Studies in the future and thank you for your valuable contribution .

Sincerely,

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Anti Inflammatory Effect of *Channa micropeltes* Extract in Angiogenesis of Diabetes Mellitus Wound Healing

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Abstract~~**BSTRACT**~~

OBJECTIVE~~**Background**~~: *Channa micropeltes* extract contains albumin and o~~O~~mega-6 which possess anti-oxidant and anti-inflammatory agents that can promote macrophages in the wound healing process associated with~~of~~ diabetes mellitus (DM). In this study, we ~~**Objective: to**~~ analyzed Nuclear Factor kappa B (NFkB) and Vascular Endothelial Growth Factor (VEGF) expression as well as neovascular cells in the inflammatory stage of DM wound healing.

~~-~~**METHODS**~~**aterials and methods**~~: The 24~~twenty four~~ male~~s~~ *Rattus novergicus* were divided into 3~~three~~ groups that were 20% *Channa micropeltes* ointment (Group I), 10% *Channa striata* extract ointment (Group II), and placebo ointment as a control (Group III). Ointments were applied 3~~three~~ times daily.

~~-~~**RESULTS**~~**esults**~~: The highest expression of NFkB was observed in Group III on D~~d~~ay 4 (15.50 ± 2.38), and~~while~~ the lowest was in treatment of Group I and Group II on ~~day~~ Day 8 (4.75 ± 0.96). The highest expression of VEGF was observed in Group I on ~~day~~ Day 8 (14.75 ± 0.96),

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andwhile the lowest was Group III on day Day 4 (7.00 ± 1.41). The highest count of neovascular cells was observed in Group I on day Day 8 (11.00 ± 2.16), andwhile the lowest was in Group III on day Day 4 (5.50 ± 0.58).
-CONCLUSIONSonclusion: *Channa micropeltes* hasve an anti-inflammatory effect by regulating NFkB expression and elevating VEGF expression in the angiogenesis process of DM wound healing.

Keywords: Channa micropeltes ; NFnfkBb ; VEGFvegf ; neovascular ; wound healing ; diabetes mellitus

1. INTRODUCTIONntroduction

Indonesia is ranksed sixth among the countries with a high prevalence of dDiabetes mellitus (DM) in the world. In an uncontrolled state, DMiabetes mellitus will result in variousthe variance of oral problems such as xerostomia, candidiasis, stomatitis, gingivitis and periodontitis. The healing of such conditions is often complicated due to hyperglycemia that initiates chronic inflammation [1-3].[1,2,3] P Previous study revealedsed that *Channa micropeltes* ointment at 20% concentration or *Channa striata* ointment at 10% concentration that was applied topically can accelerate wound healing in a DMdiabetes mellitus rat model. Both species are categorized in the same genus and contain albumin as well as omega-6 that acts as an anti-oxidant and anti-inflammatory [3,4]ion.[3,4] As an antioxidant, *Channa micropeltes* extract elevates sSuperoxide dismutase (SOD) activity and lowers mMalondialdehid (MDA) level on day Day 7 [2].[2] A As an anti-inflammatoryion, the topical application of *Channa micropeltes* at 20% concentration on the back skin of a diabetic rat model canould increase the number of macrophage and lymphocyte cells on day Day 8 and gradually reduce it on day Day 14 [1].[1]

Macrophage is the key of inflammatory process in wound healing. Reduction in macrophage number at the end of inflammatory stage demonstrates tissue recovery by producing

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growth factors and, cytokines, and inducingce as well as terminatinge angiogenesis [5,6].[5,6]. Macrophage is also produced Nuclear Factor kappa B (NFkB) that regulates the inflammatory response of metabolic disease such as DMdiabetes mellitus. A state of hHyperglycemia state in DMdiabetes mellitus will increase rReactive oOxygen sSpecies (ROS) and aAdvanced gGlycation eEnd pProducts (AGEs) that elevate chronic inflammation through the activation of NFkB. This will change vVascular eEndothelial gGrowth fFactor (VEGF) expression that will generate the damage toof blood vessels cell in the angiogenesis process [7,8].[7,8]

The extract of *Channa micropeltes* has shownbeen proven to promote wound healing on the skin of diabetic rat model by reducing macrophage number at the end of inflammatory stage [3].[3] There has been no study that explores the effect of *Channa micropeltes* application on NFkB, VEGF, and neovascular cells that are pivotal components in the DMdiabetes mellitus wound healing process. Based on that, a study to analyze the expression of NFkB, VEGF and neovascular cells number at the inflammatory stage of the DMdiabetes mellitus wound healing process was warrantedshould be conducted.

2. METHODSaterials and Methods

This study was an experimental laboratory research study incorporating a post-test only control group design. It was approved by Ethical Clearaence Committee, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, South Kalimantan, Indonesia, with number 111/KEPKG-FKGULM/EC/III/2020.

2.1 Manufacturing Channa micropeltes and Channa striata extract

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Preparing both *Channa micropeltes* and *Channa striata* extract used fresh fish weighinged 600-1000 grams. Each extractof it was later steamed in a pan for 25-35 minutes at a temperature of under 60^{o} Celsiustemperature. The flesh wasere enclosed with flannelette and pressed in a hydraulic device. Furthermore, *Channa micropeltes* and *Channa striata* were centrifuged for 15 minutes at a speed ofwithin 6000 rpm speed. Each extract was kept inside a dark glass bottle and then covered with aluminum foil and clean pack.

2.2 Formulation of Channa micropeltes and Channa striata ointment

Adeps lanae (Asian chemicals, Semarang) in a weight of 16.875 grams and vaselin flavum (PT. Brataco, 1295578) in a weight of 23.125 grams were used in the formulation of the *Channa micropeltes* ointment. Meanwhile, a combination of 16.875 grams adeps lanae and 28.125 grams vaselin flavum were combinedutilized in the formulation of *Channa striata* ointment. Adeps lanae was initially poured into different tubes for each extract and later added gradually with either *Channa micropeltes* at 20% concentration or *Channa striata* at 10% concentration. After the extract was fully absorbed by adeps lanae, the mixture was then mashed to obtain a homogenous consistency. Subsequently, the composition was further mixed with vaselin flavum and mashed again until homogenous.

2.3 In vVivo sStudy

This study included 2-3 months- old male Wistar (*Rattus novergicus*) rats (weight, 250—300 grams) obtained from an animal laboratory at the, Faculty of Medicine, University of Lambung Mangkurat. The total of 24 rat specimens were kept in cages, and the temperature and humidity were set within ±25 °C and 60%, respectively. They were fed standard BR-II, and they

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had access to boiled water *ad libitum*. Rats with hyperglycemia wereas obtained by injecting sStreptozotocsin (STZ) at 35 mg/kg dosage until the blood glucose level was over 126 mg/dL-1;, while non-d-Diabetic rRats were ones is rats without intervention. All animals were divided into 3 treatment groups consisting of 20% *Channa micropeltes* extract ointment, 10% *Channa striata* extract ointment, and placebo ointment as control. Each substance was applied topically 3three times daily (every 6-8 hours). An iIncisional wound was made on the back of the rats with 1 cm length and 1 mm depth using sterile scalpel under inhaled anesthesia of 5 ml diethyl ether.

After the 4th and 8th day day of application, rats were euthanized by inhaling a lethal dosage of diethyl ether. The back skin was then biopsied for histopathology examination using Haematoxyllinhematoxylin eEosin (HE) to evaluate macrophages and neovascular cells, and iImmunohistochemistry (IHC) to evaluate NF-κB and VEGF. The number of macrophages and neovascular cells were calculated in 3three different field locations using a light microscope that were counted using light microscope (Olympus, WA United States) at 400 magnifications and subsequently calculated for its average. Immunohistochemistry (IHC) staining was performed using anti-mouse NF-kB monoclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, -NF-kB p65 (F-6) -: sc 8008) and anti-mouse VEGF monoclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, VEGF (C1) : sc 7269). Positivity NF-κB expression was defined as only distinct nuclear immunostaining, which is considered as activated NF-κB in the studied field at 100 magnification.

2.4 DataStatistical aAnalysis

The results were analyzed using a 2two-way analysis of variance parametric test based on the Shapiro--Wilk normality test and Levene's variance homogeneity test. The results showed

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normal data distribution and homogenous data variances. Consequently, further analysis by means of a *post hoc*~~*Post Hoc*~~ Bonferroni test was conducted with a statistical significance of $p < 0.05$.

3. RESULTS~~esults~~

Table 1 shows the~~he~~ results of NFkB analysis~~ were shown in table 1~~. The highest expression of NFkB was observed in the control group (Group III) on ~~day~~ Day 4 (15.5 ± 2.38), and~~while~~ the lowest was in the treatment of 20% *Channa micropeltes* extract ointment (Group I) (4.75 ± 0.96) and 10% *Channa striata* extract ointment (Group II) (5.75 ± 1.71) on ~~day~~ Day 8. The expression of NFkB between Group I and- 10% *Channa striata* extract ointment (Group II) treatment groups did not show any difference ($p > 0.05$; Table 1). The statistical significance value between treatment group and day was~~ere~~ $p > 0.05$~~, that~~ demonstra~~represent~~ings~~ no interaction between treatment groups and days on NFkB expression.

The highest expression of VEGF was observed in Group I (14.75 ± 0.96) on ~~day~~ Day 8, where as the lowest was in Group III (7.00 ± 1.41) on ~~day~~ Day 4. The expression of VEGF showed statistically significant differences in all groups ($p < 0.05$; Table 1). The significance value between treatment groups and days was~~ere~~ $p > 0.05$~~, that~~ demonstrating~~represents~~ no interaction between treatment group and day on VEGF expression.

The highest count of neovascular cells was observed in Group I (11.00 ± 2.16) on ~~day~~ Day 8, while the lowest was in Group III (5.50 ± 0.58) on ~~day~~ Day 4. The count of neovascular cells showed statistically significant differences in all groups ($p < 0.05$; Table 1). The statistical significance value between treatment groups and days was~~ere~~ $p > 0.05$, demonstrating~~ that~~ ~~represents~~ no interaction between treatment groups and days on the number of neovascular cells.

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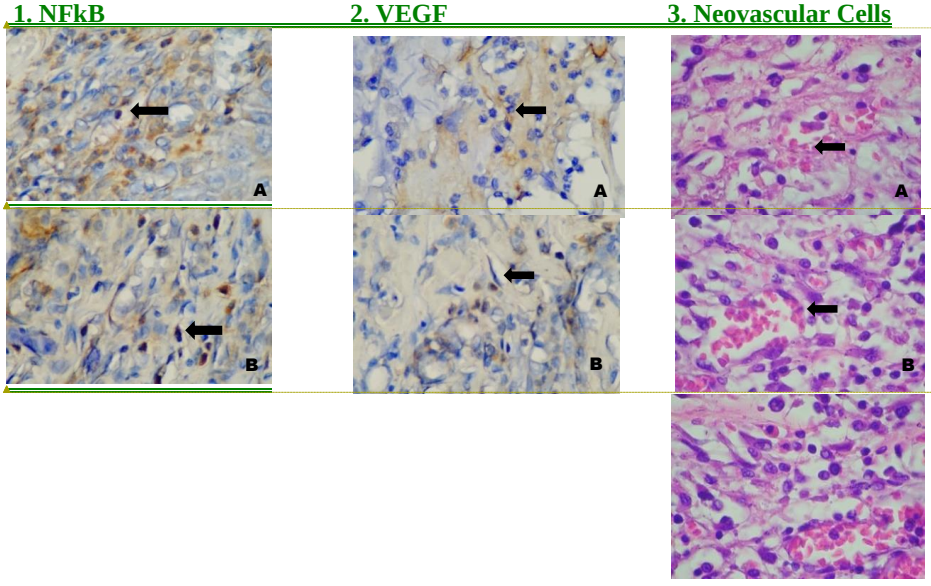
~~Table 1 about here~~**Table 1.** The expression of NFkB, expression of VEGF and count of neovascular cells

Group	Mean ± SD (cells)					
	Expression of NFkB		Expression of VEGF		Count of Neovascular	
	Day 4	Day 8	Day 4	Day 8	Day 4	Day 8
Channa micropeltes (Group 1)	7.00 ± 1.41^{A}	4.75 ± 0.96^{A}	11.75 ± 1.71^{C}	14.75 ± 0.96^{C}	7.75 ± 0.96^{C}	11.00 ± 2.16^{C}
Channa striata (Group II)	7.7 5± 1.26^{A}	5.75 ± 1.71^{A}	10.50 ± 1.29^{B}	12.50 ± 1.29^{B}	6.25 ± 0.96^{B}	9.00 ± 0.816^{B}
Control (Group III)	15.50 ± 2.38^{B}	10.00 ± 2.58^{B}	7.00 ± 1.41^{A}	9.75 ± 0.96^{A}	5.50 ± 0.58^{A}	6.25 ± 0.96^{A}

Note.
Abbreviations: The different superscript character in each variable shows the differences for each group ($p < .05$).
A in Expression of NFkB has value $p = .044$
B in Expression of NFkB has value $p = .000$
A, B and C in Expression of VEGF has value $p = .000$
A, B and C in Count of Neovascular has value $p = .000$

~~Figure 1 about here~~

Figure 1. Macrophage expression of NFkB on the control group, Neovascular's expression of VEGF on the control group, and Neovascular cell's count on the control group



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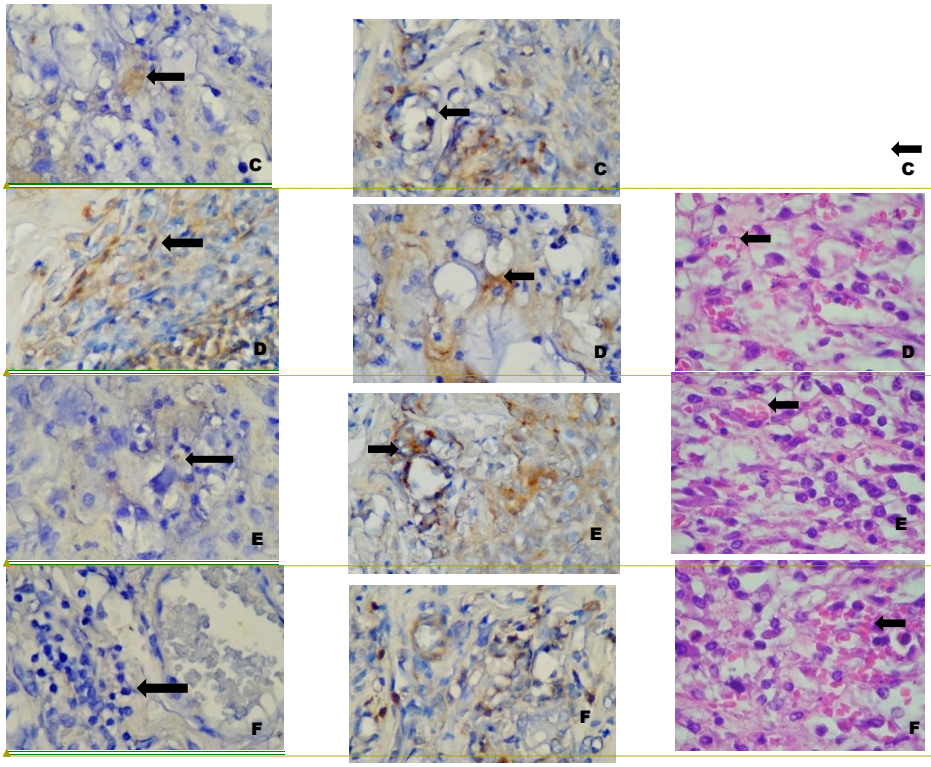

Note.
Macrophage's expression of NFkB on control group (A), *Channa micropeltes* extract concentrations of 20% (B), *Channa striata* extract concentrations of 10% (C) on Day 4. Macrophage's expression of NFkB on control group (D), *Channa micropeltes* extract concentrations of 20% (E), *Channa striata* extract concentrations of 10% (F) on Day 8. **2.** Neovascular's expression of VEGF on control group (A), *Channa micropeltes* extract concentrations of 20% (B), *Channa striata* extract concentrations of 10% (C) on Day 4. Neovascular's expression of VEGF on control group (D), *Channa micropeltes* extract concentrations of 20% (E), *Channa striata* extract concentrations of 10% (F) on Day 8. **3.** Neovascular cell's count on control group (A), *Channa micropeltes* extract concentrations of 20% (B), *Channa striata* extract concentrations of 10% (C) on Day 4. Neovascular cell's count on control group (D), *Channa micropeltes* extract concentrations of 20% (E), *Channa striata* extract concentrations of 10% (F) on Day 8.

4. DISCUSSION~~iscussion~~

DM~~iabetes mellitus~~ is characterized with an increase in blood glucose level that induces gly~~i~~cation reaction. This process will result in amadory production to formulate toxic proteins ~~called Advanced Glication End Products~~ (AGEs). Interaction between AGEs and a r~~R~~eceptor a~~A~~dvanced g~~G~~ly~~i~~cation e~~E~~nd p~~P~~roduct (RAGE) will increase the signal for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which produces superoxide anion. This process

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elevates the production of rReactive oOxygen sSpecies (ROS) which are the key for molecular signaling as well as the development of inflammatory disorders such as DMdiabetes mellitus. Excessive production of ROS will complicate the healing process of wounds in DM [2,9]diabetes mellitus.[2,9]

Channa micropeltes contains albumin and omega-6 fatty acid. Albumin can decrease ROS by cutting chained oxidative reaction in the ROS formation's process. Albumin can bind metal ions and also catch oxygen that processing hydrogen peroxide into non radical compound. Omega-6 fatty acid, especially arachnodic acid are the keys toof anti–inflammatory processes. It plays the role in stimulating machrophages to release growth factors, such as VEGF. Arachidnondic acid will be metabolized through an enzymatic mechanism such as the 5-lipoxygenase and cyclo-oxygenase pathways that produce leukotrienes, prostaglandins, and thromboxane A2. These can stimulates the cell migration and new local vascularization in the wound healing process of DM [4,10]diabetes mellitus.[4,10]

.

In previous studies, *Channa micropeltes* extract ointment at 20% concentration and *Channa striata* extract ointment at 10% concentration were shownproven to promote the wound healing process in DMdiabetes mellitus. *Channa micropeltes* and *Channa striata* are categorized in the same genus. Both species contain albumin, the secondary antioxidant that can bind metal ion in ROS formation [1,2].[1,2] ROS induces an inflammatory response through the activation of Nuclear Factor kappa B (NFkB). NFkB signal is the main key of chronic inflammation in DM [7,8]diabetes mellitus.[7,8] This is demonstratedproven by theis study result on DayDay 4 that

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reveals the highest expression of NFkB in control group, while the result for 20% *Channa micropeltes* extract application was comparable to 10% *Channa striata* extract application.

Our sStudy result on ~~day~~ Day 8 demonstrates the reduction of NFkB expression in both *Channa micropeltes* extract ointment at 20% concentration and *Channa striata* extract ointment at 10% concentration when compared to the control. Topical application of *Channa micropeltes* extract at 20% concentration or *Channa striata* extract at -10% concentration may reduce excessive ROS, thereby~~us~~ suppressing NFkB expression. Both extracts possess potential as natural substances that may inhibit the expression of NFkB. Previous studies~~y~~ reveal~~s~~ that the impediment of pro-inflammatory NFkB from therapeutical application of several natural and synthetic ingredients will be a good target to manage vascular complication in DM [7]~~diabetes mellitus~~.[7] A p~~P~~rolonged inflammatory response can be resolved by the inhibition of NFkB. As an anti-inflammatory substance, *Channa micropeltes* will reduce inducible n~~N~~itric o~~O~~xide s~~S~~ynthase (iNOS) and c~~C~~yclooxygenase 2 (COX2) that suppress the NFkB gene regulator. This will prevent prolonged inflammation in the wound healing process of DM [11,12]~~diabetes mellitus~~.[11,12]

Excessive activation of NFkB will cause abnormal DNA transcription which includes various gene expression of vascular complications occurring in VEGF, Platelet Derived Growth Factor (PDGF), Endothelin-1 (ET-1) and Transforming Growth Factor beta (TGF-b) that cause vascular cell damage [7]. V[7] ~~V~~EGF as pro-angiogenic modulators encounter down regulation in DM~~diabetes mellitus~~, ~~thus~~ disturbing the angiogenesis process [13,14].[13,14] ~~P~~ Prior studies~~y~~ demonstrate~~s~~ Vascular Endothelial Growth Factor A (VEGF-A) protein and messenger r~~R~~ibonucleic a~~A~~cid (mRNA) level in the wound of a diabetic rat model which shows reduction

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when compared to the group with a normal wound. DMiabetes mellitus leads to the decrease of angiogenesis in wound healing, so that it lessens vascular and capillary density [13].[13]

The angiogenic effect is initiated by VEGF-A binding to Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2). Angiogenesis stimulation through PI3K-Akt-eNOS will cause endothelial cell to migrate, proliferate, and differentiate. Molecular signals will be commenced by pPhosphatidylinositol 3 kinase from serine/threonine kinase Akt/protein kinase B. Akt/PKB through the phosphorylation of endothelial nNitric oOxide synthesis on Ser 1177, and will stimulate NO production, vasodilatation, and endothelial cell migration [15,16].[15,16] T. This result presents that *Channa micropeltes* extract ointment at 20% concentration increases the highest expression of VEGF when compared to *Channa striata* extract ointment at 10% concentration or the control on day Day 8. This exhibits the potential of *Channa micropeltes* extract ointment at 20% concentration to promote angiogenesis on the DMdiabetes mellitus wound healing process.

Previous study by Carabelly, et al. [1] (2019) reveals that the application of *Channa micropeltes* extract ointment at 20% concentration may elevate macrophage number on Day 8 and reduce them on Day 14.[1] Macrophage regulates angiogenesis signals in neovascular along the formation of granulation tissue process [17].[17] This concept is in accordance with this study as the number of macrophage was observed the highest on Day 8 and followed by the increase of neovascular cell number by the application of *Channa micropeltes* extract ointment at 20% concentration on Day 8. The highest number of neovascular cells was also was observed in the application of *Channa micropeltes* extract ointment at 20% concentration when compared to *Channa striata* extract ointment at 10% concentration or the control in Day 8. This study did not continue to further days to limit the parameters.

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We can~~It can be~~ conclude~~d~~ that the application of *Channa micropeltes* extract ointment at 20% concentration on the wound of a diabetic rat model can reduce the expression of NFkB. The a~~A~~nti-inflammatory effect of *Channa micropeltes* can elevate the expression of VEGF and the number of neovascular cells in angiogenesis process of diabetic wound healing. Our findings require~~s~~ ~~This study requi res~~ further research using different parameters, adding time of evaluation, and

using larger experimental animals.

Acknowledgements:

~~The authors thank to Faculty of Dentistry, University of Lambung Mangkurat which have supported this work.~~

~~Funding Information~~

This study was financed by the Faculty of Dentistry, University of Lambung Mangkurat. ~~which have supported this work.~~ The authors express their gratitude.

Conflict~~s~~ of i~~I~~nterest statement:

The authors ~~of the work~~ have no conflict of interest to report.

Ethical approval: Approval for this study was provided by the Ethical Clearance Committee, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, South Kalimantan, Indonesia (#111/KEPKG-FKGULM/EC/III/2020).~~.~~

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Table 1. The expression of NFkB, expression of VEGF and count of neovascular cells

Group	Mean ± SD (cells)					
	Expression of NFkB		Expression of VEGF		Count of Neovascular	
	Day 4	Day 8	Day 4	Day 8	Day 4	Day 8
Channa micropeltes (Group I)	7.00 ± 1.41[A]	4.75 ± 0.96[A]	11.75 ± 1.71[C]	14.75 ± 0.96[C]	7.75 ± 0.96[C]	11.00 ± 2.16[C]
Channa striata (Group II)	7.75± 1.26[A]	5.75 ± 1.71[A]	10.50 ± 1.29[B]	12.50 ± 1.29[B]	6.25 ± 0.96[B]	9.00 ± 0.816[B]
Control (Group III)	15.50 ± 2.38[B]	10.00 ± 2.58[B]	7.00 ± 1.41[A]	9.75 ± 0.96[A]	5.50 ± 0.58[A]	6.25 ± 0.96[A]

Note.
Abbreviations: The different superscript character in each variable shows the differences for each group ($p < .05$).
A in Expression of NFkB has value p = .044
B in Expression of NFkB has value p = .000
A, B and C in Expression of VEGF has value p = .000
A, B and C in Count of Neovascular has value p = .000

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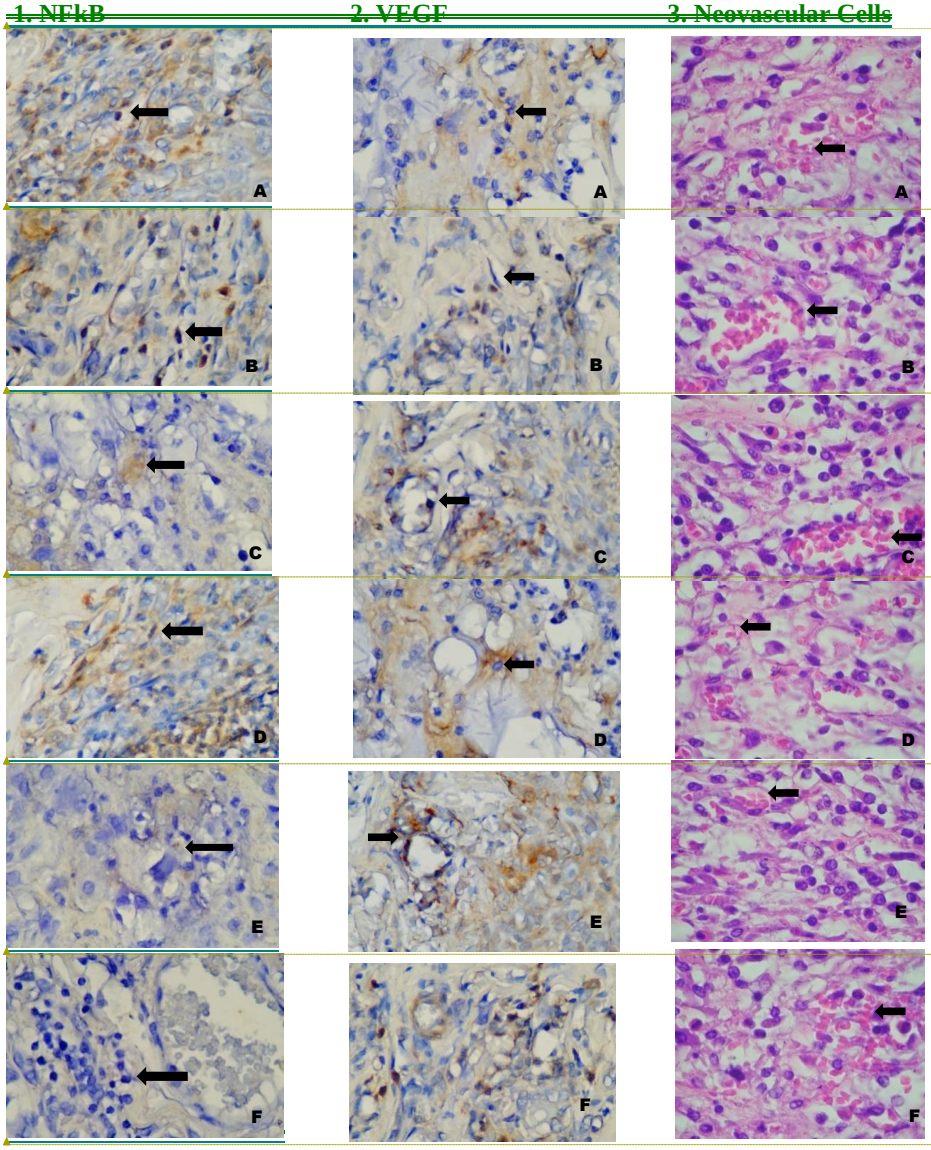
Figure 1. Macrophage expression of NFkB on the control group, Neovascular's expression of VEGF on the control group, and Neovascular cell's count on the control group

1. NFkB **2. VEGF** **3. Neovascular Cells**



Note.

Macrophage's expression of NFkB on control group (A), *Channa micropeltes* extract concentrations of 20% (B), *Channa striata* extract concentrations of 10% (C) on Day 4. Macrophage's expression of NFkB on control group (D), *Channa micropeltes* extract concentrations of 20% (E), *Channa striata* extract concentrations of 10% (F) on Day 8. 2. Neovascular's expression of VEGF on control group (A), *Channa micropeltes* extract concentrations of 20% (B), *Channa striata* extract concentrations of 10% (C) on Day 4. Neovascular's expression of VEGF

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~~on control group (D), *Channa micropeltes* extract concentrations of 20% (E), *Channa striata* extract concentrations of 10% (F) on Day 8.~~ ~~**3.**~~ ~~Neovascular cell's count on control group (A), *Channa micropeltes* extract concentrations of 20% (B), *Channa striata* extract concentrations of 10% (C) on Day 4. Neovascular cell's count on control group (D), *Channa micropeltes* extract concentrations of 20% (E), *Channa striata* extract concentrations of 10% (F) on Day 8.~~

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Gmail maharani roxy <maharaniroxy@gmail.com>

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maharani roxy <maharaniroxy@gmail.com> 21 Juli 2022 19.09
Kepada: Rohan JCF Corp <rohan@jcfcorp.com>

Dear Rohan,

Thank you for the response. Here i send my manuscript that have been revised.

Regards,

Dr. Maharani

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Rohan JCF Corp <rohan@jcfcorp.com> 22 Juli 2022 10.47
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Dear Dr. Maharani,

Thank you for sending us the updated file.

Within 10 business days, you will receive the copyediting of your article.

Regards,
Rohan Reddy
RDS
[Kutipan teks disembunyikan]

maharani roxy <maharaniroxy@gmail.com> 29 Juli 2022 10.48
Kepada: Rohan JCF Corp <rohan@jcfcorp.com>

Dear Rohan,

Thanks for the response. I am waiting it.

Regards,

Maharani L.A

[Kutipan teks disembunyikan]

Rohan JCF Corp <rohan@jcfcorp.com> 30 Juli 2022 10.50
Kepada: maharani roxy <maharaniroxy@gmail.com>

Dear Dr. Maharani,

Greetings.

We are lacking two submissions for the upcoming issue. We would be grateful if you could submit your other research article (if any) by August 12 2022, or we request you to kindly suggest our Journal to your friends/colleagues to submit their article .

Awaiting your reply.

Regards,
Rohan Reddy
RDS
[Kutipan teks disembunyikan]

Article Title

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Abstract~~**BSTRACT**~~
OBJECTIVE~~**Background**~~: *Channa micropeltes* extract contains albumin and o~~O~~mega-6 which possess anti-oxidant and anti-inflammatory agents that can promote macrophages in the wound healing process associated with~~of~~ diabetes mellitus (DM). In this study, we ~~**Objective: to**~~ analyze~~d~~ Nuclear Factor kappa B (NFkB) and Vascular Endothelial Growth Factor (VEGF) expression as well as neovascular cells in the inflammatory stage of DM wound healing.
~~**M**~~**METHODS**~~**aterials and methods**~~: The 24~~twenty four~~ male~~s~~ *Rattus novergicus* were divided into 3~~three~~ groups that were 20% *Channa micropeltes* ointment (Group I), 10% *Channa striata* extract ointment (Group II), and placebo ointment as a control (Group III). Ointments were applied 3~~three~~ times daily.
~~**R**~~**RESULTS**~~**esults**~~: The highest expression of NFkB was observed in Group III on D~~d~~ay 4 (15.50 ± 2.38), and~~while~~ the lowest was in treatment of Group I and Group II on ~~day~~ Day 8 (4.75 ± 0.96). The highest expression of VEGF was observed in Group I on ~~day~~ Day 8 (14.75 ± 0.96), and~~while~~ the lowest was Group III on ~~day~~ Day 4 (7.00 ± 1.41). The highest count of neovascular cells was observed in Group I on ~~day~~ Day 8 (11.00 ± 2.16), and~~while~~ the lowest was in Group III on ~~day~~ Day 4 (5.50 ± 0.58).
~~**C**~~**CONCLUSIONS**~~**onclusion**~~: *Channa micropeltes* has~~ve~~ an anti-inflammatory effect by regulating NFkB expression and elevating VEGF expression in the angiogenesis process of DM wound healing.

Keywords: Channa micropeltes ~~,~~ NF~~nf~~kB~~b~~ ~~,~~ VEGF~~vegf~~ ~~,~~ neovascular ~~,~~ wound healing ~~,~~ diabetes mellitus

1. INTRODUCTION~~ntroduction~~

Indonesia ~~is~~ rank~~sed~~ sixth among ~~the~~ countries with a high prevalence of d~~D~~iabetes mellitus (DM) ~~in the world~~. In an uncontrolled state, DM~~iabetes mellitus~~ will result in various~~the variance of~~ oral problems such as xerostomia, candidiasis, stomatitis, gingivitis and periodontitis. The healing of such conditions is often complicated due to hyperglycemia that initiates chronic

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inflammation [1-3]. [1,2,3] P Previous study revealsed that *Channa micropeltes* ointment at 20% concentration or *Channa striata* ointment at 10% concentration that was applied topically can accelerate wound healing in a DMdiabetes mellitus rat model. Both species are categorized in the same genus and contain albumin as well as omega--6 that acts as an anti-oxidant and anti-inflammatory [3,4]ion. [3,4] As an antioxidant, *Channa micropeltes* extract elevates sSuperoxide dismutase (SOD) activity and lowers mMalondialdehid (MDA) level on day Day 7 [2]. [2] A As an anti-inflammatoryion, the topical application of *Channa micropeltes* at 20% concentration on the back skin of a diabetic rat model canould increase the number of macrophage and lymphocyte cells on day Day 8 and gradually reduce it on day Day 14 [1]. [1]

Macrophage is the key of inflammatory process in wound healing. Reduction in macrophage number at the end of inflammatory stage demonstrates tissue recovery by producing growth factors and, cytokines, and inducingce as well as terminatinge angiogenesis [5,6]. [5,6] Macrophage is also produced Nuclear Factor kappa B (NFkB) that regulates the inflammatory response of metabolic disease such as DMdiabetes mellitus. A state of hHyperglycemia state in DMdiabetes mellitus will increase rReactive oOxygen sSpecies (ROS) and aAdvanced gGlycation eEnd pProducts (AGEs) that elevate chronic inflammation through the activation of NFkB. This will change vVascular eEndothelial gGrowth fFactor (VEGF) expression that will generate the damage toof blood vessels cell in the angiogenesis process [7,8]. [7,8]

The extract of *Channa micropeltes* has shownbeen proven to promote wound healing on the skin of diabetic rat model by reducing macrophage number at the end of inflammatory stage [3]. [3] There has been no study that explores the effect of *Channa micropeltes* application on

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NFkB, VEGF, and neovascular cells that are pivotal components in the DM~~diabetes mellitus~~ wound healing process. Based on that, a study to analyze the expression of NFkB, VEGF and neovascular cells number at the inflammatory stage of the DM~~diabetes mellitus~~ wound healing process was warranted~~should be conducted~~.

2. METHODS~~aterials and Methods~~

This study was an experimental laboratory research study incorporating a post-test only control group design. It was approved by Ethical Clearaence Committee, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, South Kalimantan, Indonesia, with number 111/KEPKG-FKGULM/EC/III/2020.

2.1 Manufacturing Channa micropeltes and Channa striata extract

Preparing both *Channa micropeltes* and *Channa striata* extract used fresh fish weighinged 600-1000 grams. Each extract~~of it~~ was later steamed in a pan for 25-35 minutes at a temperature of ~~under~~ 60^{o} Celsius~~temperature~~. The flesh wasere enclosed with flannelette and pressed in a hydraulic device. Furthermore, *Channa micropeltes* and *Channa striata* were centrifuged for 15 minutes at a speed of~~within~~ 6000 rpm ~~speed~~. Each extract was kept inside a dark glass bottle and then covered with aluminum foil and clean pack.

2.2 Formulation of Channa micropeltes and Channa striata ointment

Adeps lanae (Asian chemicals, Semarang) in a weight of 16.875 grams and vaselin flavum (PT. Brataco, 1295578) in a weight of 23.125 grams were used in the formulation of the *Channa micropeltes* ointment. Meanwhile, a combination of 16.875 grams adeps lanae and

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28.125 grams vaselin flavum were combined~~utilized~~ in the formulation of *Channa striata* ointment. Adeps lanae was initially poured into different tubes for each extract and later added gradually with either *Channa micropeltes* at 20% concentration or *Channa striata* at 10% concentration. After the extract was fully absorbed by adeps lanae, the mixture was then mashed to obtain a homogenous consistency. Subsequently, the composition was further mixed with vaselin flavum and mashed again until homogenous.

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2.3 In v~~V~~ivo s~~S~~tudy

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This study included 2-3 months- old male Wistar (*Rattus novergicus*) rats (weight, 250~~–~~-300 grams) obtained from an animal laboratory at the~~,~~ Faculty of Medicine, University of Lambung Mangkurat. The ~~total of~~ 24 rat specimens were kept in cages, and the temperature and humidity were set within ±25 °C and 60%, respectively. They were fed standard BR-II, and they had access to boiled water *ad libitum*. Rats with hyperglycemia were~~as~~ obtained by injecting sStreptozotocsin (STZ) at 35 mg/kg dosage until the blood glucose level was over 126 mg/dL-1;~~,~~ ~~while~~ non-d-Diabetic rRats were ones ~~is rats~~ without intervention. All animals were divided into 3 treatment groups consisting of 20% *Channa micropeltes* extract ointment, 10% *Channa striata* extract ointment, and placebo ointment as control. Each substance was applied topically 3~~three~~ times daily (every 6-8 hours). An i~~I~~ncisional wound was made on the back of the rats with 1 cm length and 1 mm depth using sterile scalpel under inhaled anesthesia of 5 ml diethyl ether.

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After the 4th and 8th ~~day~~ day of application, rats were euthanized by inhaling a lethal dosage of diethyl ether. The back skin was then biopsied for histopathology examination using ~~Haematoxyllin~~hematoxylin e~~E~~osin (HE) to evaluate macrophages and neovascular cells, and i~~I~~mmunohistochemistry (IHC) to evaluate NF-κB and VEGF. The number of macrophages and

neovascular cells were calculated in 3~~three~~ different field locations using a light microscope ~~that were counted using light microscope~~ (Olympus, WA ~~United States~~) at 400 magnifications and subsequently calculated for its average. ~~Immunohistochemistry (~~IHC~~)~~ staining was performed using anti-mouse NF-kB monoclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, ~~-~~NF-kB p65 (F-6) ~~-~~: sc 8008) and anti-mouse VEGF monoclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, ~~-~~ VEGF (C1) : sc 7269). Positivity NF-κB expression was defined as only distinct nuclear immunostaining, which is considered as activated NF-κB in the studied field at 100 magnification.

2.4 Data~~Statistical~~ a~~A~~nalysis

The results were analyzed using a 2~~two~~-way analysis of variance parametric test based on the Shapiro~~-~~-Wilk normality test and Levene's variance homogeneity test. The results showed normal data distribution and homogenous data variances. Consequently, further analysis by means of a *post hoc~~Post Hoc~~* Bonferroni test was conducted with a statistical significance of $p < 0.05$.

3. RESULTS~~esults~~

Table 1 shows the~~he~~ results of NFkB analysis ~~were shown in table 1~~. The highest expression of NFkB was observed in the control group (Group III) on ~~day~~ Day 4 (15.5 ± 2.38), and~~while~~ the lowest was in the treatment of 20% *Channa micropeltes* extract ointment (Group I) (4.75 ± 0.96) and 10% *Channa striata* extract ointment (Group II) (5.75 ± 1.71) on ~~day~~ Day 8. The expression of NFkB between Group I and- 10% *Channa striata* extract ointment (Group II) treatment groups did not show any difference ($p > 0.05$; Table 1). The statistical significance value between

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treatment group and day wasere p > 0.05, that demonstrarepresentings no interaction between treatment groups and days on NFkB expression.

The highest expression of VEGF was observed in Group I (14.75 ± 0.96) on day Day 8, where as the lowest was in Group III (7.00 ± 1.41) on day Day 4. The expression of VEGF showed statistically significant differences in all groups ($p < 0.05$; Table 1). The significance value between treatment groups and days wasere $p > 0.05$, that demonstratingrepresents no interaction between treatment group and day on VEGF expression.

The highest count of neovascular cells was observed in Group I (11.00 ± 2.16) on day Day 8, while the lowest was in Group III (5.50 ± 0.58) on day Day 4. The count of neovascular cells showed statistically significant differences in all groups ($p < 0.05$; Table 1). The statistical significance value between treatment groups and days wasere $p > 0.05$, demonstrating that represents no interaction between treatment groups and days on the number of neovascular cells.

Table 1 about here

Figure 1 about here

4. DISCUSSIONiscussion

DMiabetes mellitus is characterized with an increase in blood glucose level that induces glyication reaction. This process will result in amadory production to formulate toxic proteins called Advanced Glication End Products (AGEs). Interaction between AGEs and a rReceptor aAdvanced gGlyication eEnd pProduct (RAGE) will increase the signal for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which produces superoxide anion. This process

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elevates the production of rReactive oOxygen sSpecies (ROS) which are the key for molecular signaling as well as the development of inflammatory disorders such as DMdiabetes mellitus. Excessive production of ROS will complicate the healing process of wounds in DM [2,9]diabetes mellitus.[2,9]

Channa micropeltes contains albumin and omega-6 fatty acid. Albumin can decrease ROS by cutting chained oxidative reaction in the ROS formation's process. Albumin can bind metal ions and also catch oxygen that processing hydrogen peroxide into non radical compound. Omega-6 fatty acid, especially arachnodic acid are the keys toof anti–inflammatory processes. It plays the role in stimulating machrophages to release growth factors, such as VEGF. Arachidnondic acid will be metabolized through an enzymatic mechanism such as the 5-lipoxygenase and cyclo-oxygenase pathways that produce leukotrienes, prostaglandins, and thromboxane A2. These can stimulates the cell migration and new local vascularization in the wound healing process of DM [4,10]diabetes mellitus.[4,10]

.

In previous studies, *Channa micropeltes* extract ointment at 20% concentration and *Channa striata* extract ointment at 10% concentration were shownproven to promote the wound healing process in DMdiabetes mellitus. *Channa micropeltes* and *Channa striata* are categorized in the same genus. Both species contain albumin, the secondary antioxidant that can bind metal ion in ROS formation [1,2].[1,2] ROS induces an inflammatory response through the activation of Nuclear Factor kappa B (NFkB). NFkB signal is the main key of chronic inflammation in DM [7,8]diabetes mellitus.[7,8] This is demonstratedproven by theis study result on DayDay 4 that

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reveals the highest expression of NFkB in control group, while the result for 20% *Channa micropeltes* extract application was comparable to 10% *Channa striata* extract application.

Our sStudy result on ~~day~~ Day 8 demonstrates the reduction of NFkB expression in both *Channa micropeltes* extract ointment at 20% concentration and *Channa striata* extract ointment at 10% concentration when compared to the control. Topical application of *Channa micropeltes* extract at 20% concentration or *Channa striata* extract at -10% concentration may reduce excessive ROS, thereby~~us~~ suppressing NFkB expression. Both extracts possess potential as natural substances that may inhibit the expression of NFkB. Previous studies~~y~~ reveal~~s~~ that the impediment of pro-inflammatory NFkB from therapeutical application of several natural and synthetic ingredients will be a good target to manage vascular complication in DM [7]~~diabetes mellitus~~.[7] A p~~P~~rolonged inflammatory response can be resolved by the inhibition of NFkB. As an anti-inflammatory substance, *Channa micropeltes* will reduce inducible n~~N~~itric o~~O~~xide s~~S~~ynthase (iNOS) and c~~C~~yclooxygenase 2 (COX2) that suppress the NFkB gene regulator. This will prevent prolonged inflammation in the wound healing process of DM [11,12]~~diabetes mellitus~~.[11,12]

Excessive activation of NFkB will cause abnormal DNA transcription which includes various gene expression of vascular complications occurring in VEGF, Platelet Derived Growth Factor (PDGF), Endothelin-1 (ET-1) and Transforming Growth Factor beta (TGF-b) that cause vascular cell damage [7]. V[7] ~~V~~EGF as pro-angiogenic modulators encounter down regulation in DM~~diabetes mellitus~~, ~~thus~~ disturbing the angiogenesis process [13,14].[13,14] ~~P~~ Prior studies~~y~~ demonstrate~~s~~ Vascular Endothelial Growth Factor A (VEGF-A) protein and messenger r~~R~~ibonucleic a~~A~~cid (mRNA) level in the wound of a diabetic rat model which shows reduction

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when compared to the group with a normal wound. DMiabetes mellitus leads to the decrease of angiogenesis in wound healing, so that it lessens vascular and capillary density [13].[13]

The angiogenic effect is initiated by VEGF-A binding to Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2). Angiogenesis stimulation through PI3K-Akt-eNOS will cause endothelial cell to migrate, proliferate, and differentiate. Molecular signals will be commenced by pPhosphatidylinositol 3 kinase from serine/threonine kinase Akt/protein kinase B. Akt/PKB through the phosphorylation of endothelial nNitric oOxide synthesis on Ser 1177, and will stimulate NO production, vasodilatation, and endothelial cell migration [15,16].[15,16] T. This result presents that *Channa micropeltes* extract ointment at 20% concentration increases the highest expression of VEGF when compared to *Channa striata* extract ointment at 10% concentration or the control on day Day 8. This exhibits the potential of *Channa micropeltes* extract ointment at 20% concentration to promote angiogenesis on the DMdiabetes mellitus wound healing process.

Previous study by Carabelly, et al. [1] (2019) reveals that the application of *Channa micropeltes* extract ointment at 20% concentration may elevate macrophage number on Day 8 and reduce them on Day 14.[1] Macrophage regulates angiogenesis signals in neovascular along the formation of granulation tissue process [17].[17] This concept is in accordance with this study as the number of macrophage was observed the highest on Day 8 and followed by the increase of neovascular cell number by the application of *Channa micropeltes* extract ointment at 20% concentration on Day 8. The highest number of neovascular cells was also was observed in the application of *Channa micropeltes* extract ointment at 20% concentration when compared to *Channa striata* extract ointment at 10% concentration or the control in Day 8. This study did not continue to further days to limit the parameters.

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We can~~It can be~~ conclude~~d~~ that the application of *Channa micropeltes* extract ointment at 20% concentration on the wound of a diabetic rat model can reduce the expression of NFkB. The a~~A~~nti-inflammatory effect of *Channa micropeltes* can elevate the expression of VEGF and the number of neovascular cells in angiogenesis process of diabetic wound healing. Our findings requires~~This study requires~~ further research using different parameters, adding time of evaluation, and

using larger experimental animals.

Acknowledgements:

~~The authors thank to Faculty of Dentistry, University of Lambung Mangkurat which have supported this work.~~

~~Funding Information~~

This study was financed by the Faculty of Dentistry, University of Lambung Mangkurat.~~ which have supported this work.~~ The authors express their gratitude.

Conflict~~s~~ of i~~I~~nterest statement:

The authors ~~of the work~~ have no conflict of interest to report.

Ethical approval: Approval for this study was provided by the Ethical Clearance Committee, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, South Kalimantan, Indonesia (#111/KEPKG-FKGULM/EC/III/2020).~~.~~

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Table 1. The expression of NFkB, expression of VEGF and count of neovascular cells

Group	Mean ± SD (cells)					
	Expression of NFkB		Expression of VEGF		Count of Neovascular	
	Day 4	Day 8	Day 4	Day 8	Day 4	Day 8
Channa micropeltes (Group 1)	7.00 ± 1.41^{A}	4.75 ± 0.96^{A}	11.75 ± 1.71^{C}	14.75 ± 0.96^{C}	7.75 ± 0.96^{C}	11.00 ± 2.16^{C}
Channa striata (Group II)	7.7 5± 1.26^{A}	5.75 ± 1.71^{A}	10.50 ± 1.29^{B}	12.50 ± 1.29^{B}	6.25 ± 0.96^{B}	9.00 ± 0.816^{B}
Control (Group III)	15.50 ± 2.38^{B}	10.00 ± 2.58^{B}	7.00 ± 1.41^{A}	9.75 ± 0.96^{A}	5.50 ± 0.58^{A}	6.25 ± 0.96^{A}

Note.
Abbreviations: The different superscript character in each variable shows the differences for each group ($p < .05$).
A in Expression of NFkB has value $p = .044$
B in Expression of NFkB has value $p = .000$
A, B and C in Expression of VEGF has value $p = .000$
A, B and C in Count of Neovascular has value $p = .000$

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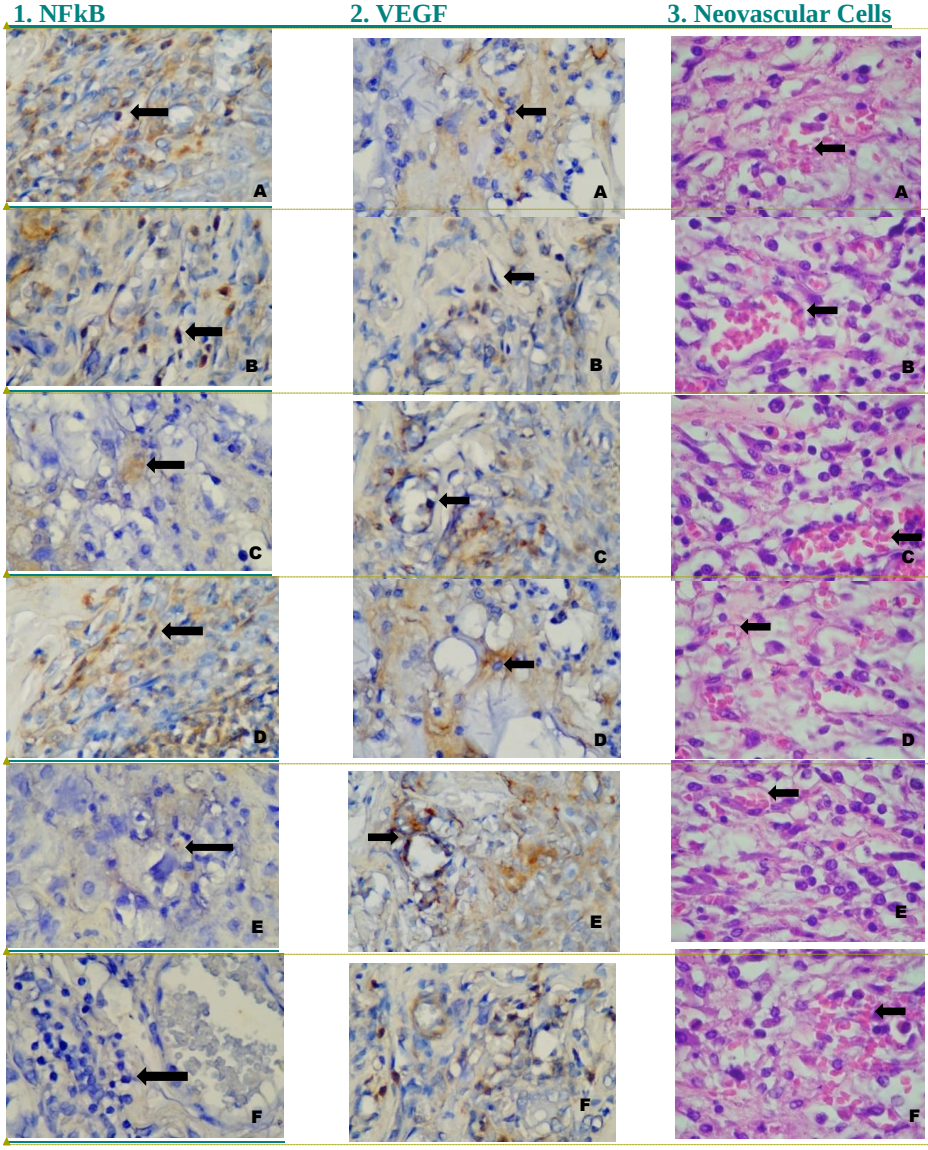
Figure 1. Macrophage expression of NFkB on the control group, Neovascular's expression of VEGF on the control group, and Neovascular cell's count on the control group



Note.

Macrophage's expression of NFkB on control group (A), *Channa micropeltes* extract concentrations of 20% (B), *Channa striata* extract concentrations of 10% (C) on Day 4. Macrophage's expression of NFkB on control group (D), *Channa micropeltes* extract concentrations of 20% (E), *Channa striata* extract concentrations of 10% (F) on Day 8. **2.** Neovascular's expression of VEGF on control group (A), *Channa micropeltes* extract concentrations of 20% (B), *Channa striata* extract concentrations of 10% (C) on Day 4. Neovascular's expression of VEGF

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on control group (D), *Channa micropeltes* extract concentrations of 20% (E), *Channa striata* extract concentrations of 10% (F) on Day 8. **3.** Neovascular cell's count on control group (A), *Channa micropeltes* extract concentrations of 20% (B), *Channa striata* extract concentrations of 10% (C) on Day 4. Neovascular cell's count on control group (D), *Channa micropeltes* extract concentrations of 20% (E), *Channa striata* extract concentrations of 10% (F) on Day 8.

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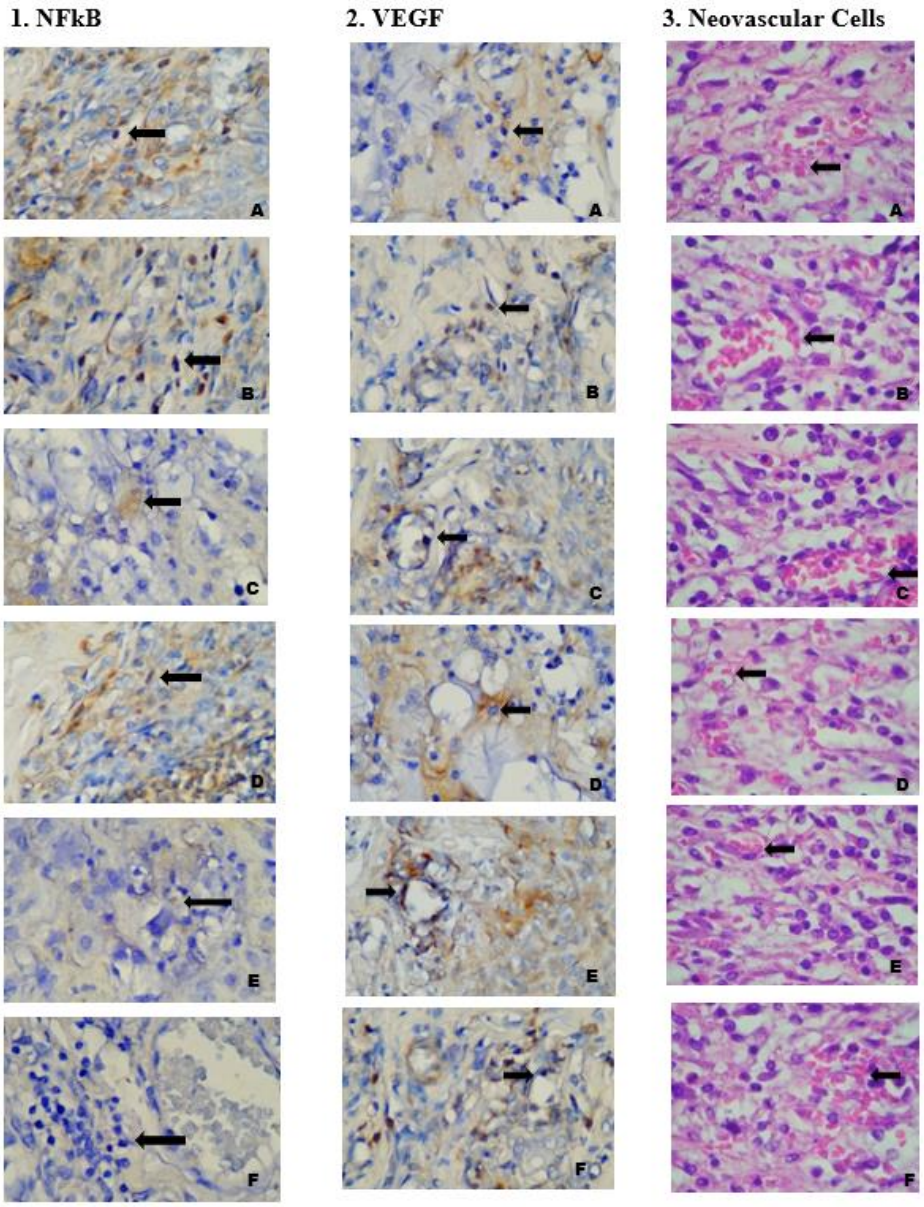
Figure 1. Macrophage expression of NF-κB on the control group, Neovascular's expression of VEGF on the control group, and Neovascular cell's count on the control group.



Note:
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on control group (D), *Channa micropeltes* extract concentrations of 20% (E), *Channa striata* extract concentrations of 10% (F) on Day 8.

Anti-Inflammatory Effect of *Channa micropeltes* Extract in Angiogenesis of Diabetes Mellitus Wound Healing

Maharani Laillyza Apriasari[1], Dewi Puspitasari[2], Juliyatin Putri Utami[3]

[1]Department of Oral Medicine, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, Kalimantan Selatan, Indonesia, [2]Department of Dental Material, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, Kalimantan Selatan, Indonesia, [3]Department of Biomedicine, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, Kalimantan Selatan, Indonesia. Address correspondence to: Maharani Laillyza Apriasari. email: maharaniroxy@gmail.com

Abstract:

OBJECTIVE: *Channa micropeltes* extract contains albumin and omega-6 which possess antioxidant and anti-inflammatory agents that can promote macrophages in the wound healing process associated with diabetes mellitus (DM). In this study, we analyzed Nuclear Factor kappa B (NF-κB) and Vascular Endothelial Growth Factor (VEGF) expression as well as neovascular cells in the inflammatory stage of DM wound healing. **METHODS**: The 24 males *Rattus novergicus* were divided into 3 groups that were 20% *Channa micropeltes* ointment (Group I), 10% *Channa striata* extract ointment (Group II), and placebo ointment as a control (Group III). Ointments were applied 3 times daily. **RESULTS**: The highest expression of NF-κB was observed in Group III on Day 4 (15.50 ± 2.38), and the lowest was in treatment of Group I and Group II on Day 8 (4.75 ± 0.96). The highest expression of VEGF was observed in Group I on Day 8 (14.75 ± 0.96), and the lowest was Group III on Day 4 (7.00 ± 1.41). The highest count of neovascular cells was observed in Group I on Day 8 (11.00 ± 2.16), and the lowest was in Group III on Day 4 (5.50 ± 0.58). **CONCLUSIONS**: *Channa micropeltes* has an anti-inflammatory effect by regulating NF-κB expression and elevating VEGF expression in the angiogenesis process of DM wound healing.

Keywords: *channa micropeltes*, NF-κB, VEGF, neovascular, wound healing, diabetes mellitus

List of abbreviations:

AGEs Advanced Glycation End products

COX2 cyclooxygenase 2

DM Diabetes mellitus

ET-1 Endothelin-1

HE Hematoxylin eosin

IHC immunohistochemistry

iNOS nitric oxide synthase

MDA malondialdehid

mRNA messenger ribonucleic acid

NADPH nicotinamide adenine dinucleotide phosphate

NF-κB Nuclear Factor Kappa Beta

PDGF Platelet Derived Growth Factor

RAGE receptor advanced glycation end product

ROS reactive oxygen species

SOD superoxide dismutase

STZ streptozotocin

VEGF Vascular Endothelial Growth Factor

Manuscript submitted: July 09, 2022

Resubmitted: August 01, 2022

Accepted: August 06, 2022

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The Review of DIABETIC STUDIES

RESEARCH

Anti-Inflammatory Effect of *Channa micropeltes* Extract in Angiogenesis of Diabetes Mellitus Wound Healing

Maharani Laillyza Apriasari[1], Dewi Puspitasari[2], Juliyatin Putri Utami[3]

[1]*Department of Oral Medicine, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, Kalimantan Selatan, Indonesia,*
[2]*Department of Dental Material, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, Kalimantan Selatan, Indonesia,*
[3]*Department of Biomedicine, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, Kalimantan Selatan, Indonesia.*
Address correspondence to: Maharani Laillyza Apriasari, e-mail: maharaniroxy@gmail.com

Manuscript submitted July 09, 2022; resubmitted August 01, 2022; accepted September 06, 2022

■ Abstract

OBJECTIVE: *Channa micropeltes* extract contains albumin and omega-6 which possess antioxidant and anti-inflammatory agents that can promote macrophages in the wound healing process associated with diabetes mellitus (DM). In this study, we analyzed Nuclear Factor kappa B (NF-κB) and Vascular Endothelial Growth Factor (VEGF) expression as well as neovascular cells in the inflammatory stage of DM wound healing. **METHODS:** The 24 males *Rattus novergicus* were divided into 3 groups that were 20% *Channa micropeltes* ointment (Group I), 10% *Channa striata* extract ointment (Group II), and placebo ointment as a control (Group III). Ointments were applied 3 times daily. **RESULTS:** The highest expression of NF-κB was observed in Group III on Day 4 (15.50 ± 2.38), and the lowest was in treatment of Group I and Group II on Day 8 (4.75 ± 0.96). The highest expression of VEGF was observed in Group I on Day 8 (14.75 ± 0.96), and the lowest was Group III on Day 4 (7.00 ± 1.41). The highest count of neovascular cells was observed in Group I on Day 8 (11.00 ± 2.16), and the lowest was in Group III on Day 4 (5.50 ± 0.58). **CONCLUSIONS:** *Channa micropeltes* has an anti-inflammatory effect by regulating NF-κB expression and elevating VEGF expression in the angiogenesis process of DM wound healing.

Keywords: *channa micropeltes* · NF-κB · VEGF · neovascular · wound healing · diabetes mellitus · retinopathy

1. Introduction

Indonesia ranks sixth among countries with a high prevalence of diabetes mellitus (DM). In an uncontrolled state, DM will result in various oral problems such as xerostomia, candidiasis, stomatitis, gingivitis and periodontitis. The healing of such conditions is often complicated due to hyperglycemia that initiates chronic inflammation [1-3]. Previous study revealed that *Channa micropeltes* ointment at 20% concentration or *Channa striata* ointment at 10% concentration applied topically can accelerate wound healing in a DM rat model. Both species are categorized in the same genus and contain albumin as well as omega-6 that acts as an antioxidant and anti-inflammatory [3,4]. As an antioxidant, *Channa micropeltes* extract elevates superoxide dismutase (SOD) activity and lowers malondialdehid (MDA) level on Day 7 [2]. As an anti-inflammatory, the topical application of *Channa micropeltes* at 20% concentration on the back skin of a diabetic rat model can increase the number of macrophage and lymphocyte cells on Day 8 and gradually reduce it on Day 14 [1].

Macrophage is the key inflammatory process in wound healing. Reduction in macrophage number at the end of inflammatory stage demonstrates tissue recovery by producing growth factors and cytokines and inducing as well as terminating angiogenesis [5,6]. Macrophage is also produced Nuclear Factor kappa B (NF-κB) that regulates the inflammatory response of metabolic disease such as DM. A state of hyperglycemia in DM will increase reactive oxygen species (ROS) and advanced glycation end products (AGEs) that elevate chronic inflammation through the activation of NF-κB. This will change vascular endothelial growth factor (VEGF) expression that will generate the damage to blood vessels in the angiogenesis process [7,8].

The extract of *Channa micropeltes* has shown to promote wound healing on the skin of diabetic rat model by reducing macrophage number at the end of inflammatory stage [3]. There has been no study that explores the effect of *Channa micropeltes* application on NF-κB, VEGF, and neovascular cells that are pivotal components in the DM wound healing process. Based on that, a study to analyze the expression of NF-κB, VEGF and neovascular cells number at the inflammatory stage of the DM wound healing process was warranted.

2. Methods

This study was an experimental laboratory research study incorporating a post-test only control group design. It was approved by Ethical Clearance Committee, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, South Kalimantan, Indonesia, with number 111/KEPKG-FKGULM/EC/III/2020.

2.1 Manufacturing Channa micropeltes and Channa striata extract

Preparing both *Channa micropeltes* and *Channa striata* extract used fresh fish weighing 600-1000 grams. Each extract was later steamed in a pan for 25-35 minutes at a temperature of 60° Celsius. The flesh was enclosed with flannelette and pressed in a hydraulic device. Furthermore, *Channa micropeltes* and *Channa striata* were centrifuged for 15 minutes at a speed of 6000 rpm. Each extract was kept inside a dark glass bottle and then covered with aluminum foil and clean pack.

2.2 Formulation of Channa micropeltes and Channa striata ointment

Adeps lanae (Asian chemicals, Semarang) in a weight of 16.875 grams and vaselin flavum (PT. Brataco, 1295578) in a weight of 23.125 grams were used in the formulation of the *Channa micropeltes* ointment. Meanwhile, a combination of 16.875 grams adeps lanae and 28.125 grams vaselin flavum were combined in the formulation of *Channa striata* ointment. Adeps lanae was initially poured into different tubes for each extract and later added gradually with either *Channa micropeltes* at 20% concentration or *Channa striata* at 10% concentration. After the extract was fully absorbed by adeps lanae, the mixture was then mashed to obtain a homogenous consistency. Subsequently, the composition was further mixed with vaselin flavum and mashed again until homogenous.

2.3 In vivo study

This study included 2-3 months old male Wistar (*Rattus novergicus*) rats (weight, 250-300 grams) obtained from an animal laboratory at the Faculty of Medicine, University of Lambung Mangkurat. The 24 rat specimens were kept in cages, and the temperature and humidity were set within ±25 °C and 60%, respectively. They were fed standard BR-II, and they had access to boiled water *ad libitum*. Rats with hyperglycemia were obtained by injecting streptozotocin (STZ) at 35 mg/kg dosage until the blood glucose level was over 126 mg/dL-1; non-diabetic rats were ones without intervention. All animals were divided into 3 treatment groups consisting of 20% *Channa micropeltes* extract ointment, 10% *Channa striata* extract ointment, and placebo ointment as control. Each substance was applied topically 3 times daily (every 6-8 hours). An incisional wound was made on the back of the rats with 1 cm length and 1 mm depth using sterile scalpel under inhaled anesthesia of 5 ml diethyl ether.

Abbreviations:

AGEs	Advanced Glycation End products
COX2	Cyclooxygenase 2
DM	Diabetes mellitus
ET-1	Endothelin-1
HE	Hematoxylin eosin
IHC	Immunohistochemistry
iNOS	Nitric oxide synthase
MDA	Malondialdehid
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear Factor Kappa Beta
PDGF	Platelet Derived Growth Factor
RAGE	Receptor advanced glycation end product
ROS	Reactive oxygen species
SOD	Superoxide dismutase
STZ	Streptozotocin
VEGF	Vascular Endothelial Growth Factor

After the 4th and 8th day of application, rats were euthanized by inhaling a lethal dosage of diethyl ether. The back skin was then biopsied for histopathology examination using hematoxylin eosin (HE) to evaluate macrophages and neovascular cells, and immunohistochemistry (IHC) to evaluate NF-κB and VEGF. The number of macrophages and neovascular cells were calculated in 3 different field locations using a light microscope (Olympus, WA) at 400 magnifications and subsequently calculated for its average. IHC staining was performed using anti-mouse NF-kB monoclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, NF-kB p65 (F-6): sc 8008) and anti-mouse VEGF monoclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, VEGF (C1): sc 7269). Positivity NF-κB expression was defined as only distinct nuclear immunostaining, which is considered as activated NF-κB in the studied field at 100 magnifications.

2.4 Data analysis

The results were analyzed using a 2-way analysis of variance parametric test based on the Shapiro-Wilk normality test and Levene's variance homogeneity test. The results showed normal data distribution and homogenous data variances. Consequently, further analysis by means of a *post hoc* Bonferroni test was conducted with a statistical significance of $p < 0.05$.

3. Results

Table 1 shows the results of NF-κB analysis. The highest expression of NF-κB was observed in the control group (Group III) on Day 4 (15.5 ± 2.38), and the lowest was in the treatment of 20% *Channa micropeltes* extract ointment (Group I) (4.75 ± 0.96) and

10% *Channa striata* extract ointment (Group II) (5.75 ± 1.71) on Day 8. The expression of NF-κB between Group I and 10% *Channa striata* extract ointment (Group II) treatment groups did not show any difference ($p > .05$; Table 1). The statistical significance value between treatment group and day was $p > 0.05$, demonstrating no interaction between treatment groups and days on NF-κB expression.

The highest expression of VEGF was observed in Group I (14.75 ± 0.96) on Day 8, whereas the lowest was in Group III (7.00 ± 1.41) on Day 4. The expression of VEGF showed statistically significant differences in all groups ($p < 0.05$; Table 1). The significance value between treatment groups and days was $p > 0.05$, demonstrating no interaction between treatment group and day on VEGF expression.

The highest count of neovascular cells was observed in Group I (11.00 ± 2.16) on Day 8, while the lowest was in Group III (5.50 ± 0.58) on Day 4. The count of neovascular cells showed statistically significant differences in all groups ($p < 0.05$; Table 1). The statistical significance value between treatment groups and days was $p > 0.05$, demonstrating no interaction between treatment groups and days on the number of neovascular cells.

4. Discussion

DM is characterized with an increase in blood glucose level that induces glycation reaction. This process will result in amadory production to formulate toxic proteins (AGEs). Interaction between AGEs and a receptor advanced glycation end product (RAGE) will increase the signal for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which produces superoxide anion. This process elevates the production of reactive oxygen species (ROS) which are the key for molecular signaling as well as the development of inflammatory disorders such as DM. Excessive production of ROS will complicate the healing process of wounds in DM [2,9].

Channa micropeltes contains albumin and omega-6 fatty acid. Albumin can decrease ROS by cutting chained oxidative reaction in the ROS formation's process. Albumin can bind metal ions and also catch oxygen that processing hydrogen peroxide into non radical compound. Omega-6 fatty acid, especially arachnodic acid are the keys to anti-inflammatory processes. It plays the role in stimulating macrophages to release growth factors, such as VEGF. Arachidonic acid will be metabolized through an enzymatic mechanism such as the 5-lipoxygenase and cyclo-oxygenase pathways that produce leukotrienes, prostaglandins, and thromboxane A2. These can stimulate cell migration and new local vascularization in the wound healing process of DM [4,10].

In previous studies, *Channa micropeltes* extract ointment at 20% concentration and *Channa striata* extract ointment at 10% concentration were shown to promote the wound healing process in DM. *Channa micropeltes* and *Channa striata* are categorized in the same genus. Both species contain albumin, the secondary antioxidant that can bind metal ion in ROS formation [1,2]. ROS induces an inflammatory response through the activation of Nuclear Factor kappa B (NF-κB). NF-κB signal is the main key of chronic inflammation in DM [7,8]. This is demonstrated by the study result on Day 4 that reveals the highest expression of NF-κB in control group, while the result for 20% *Channa micropeltes* extract application was comparable to 10% *Channa striata* extract application (Figure 1).

Our study result on Day 8 demonstrates the reduction of NF-κB expression in both *Channa micropeltes* extract ointment at 20% concentration and *Channa striata* extract ointment at 10% concentration when compared to the control. Topical application of *Channa micropeltes* extract at 20% concentration or *Channa striata* extract at 10% concentration may reduce excessive ROS, thereby suppressing NF-κB expression. Both extracts possess potential as natural substances that may inhibit the expression of NF-κB. Previous studies reveal that the impediment of pro-inflammatory NF-κB from therapeutical application of several natural and synthetic ingredients will be a good target to manage vascular complication in DM [7]. A prolonged inflammatory response can be resolved

Table 1. The expression of NFkB, expression of VEGF and count of neovascular cells

Group	Mean ± SD (cells)					
	Expression of NFkB		Expression of VEGF		Count of Neovascular	
	Day 4	Day 8	Day 4	Day 8	Day 4	Day 8
Channa micropeltes (Group 1)	7.00 ± 1.41^{A}	4.75 ± 0.96^{A}	11.75 ± 1.71^{C}	14.75 ± 0.96^{C}	7.75 ± 0.96^{C}	11.00 ± 2.16^{C}
Channa striata (Group II)	$7.7\ 5\pm 1.26^{A}$	5.75 ± 1.71^{A}	10.50 ± 1.29^{B}	12.50 ± 1.29^{B}	6.25 ± 0.96^{B}	9.00 ± 0.816^{B}
Control (Group III)	15.50 ± 2.38^{B}	10.00 ± 2.58^{B}	7.00 ± 1.41^{A}	9.75 ± 0.96^{A}	5.50 ± 0.58^{A}	6.25 ± 0.96^{A}

Note.
Abbreviations: The different superscript character in each variable shows the differences for each group ($p < 0.05$).
A in Expression of NFkB has value p = 0.044
B in Expression of NFkB has value p = 0.000
A, B and C in Expression of VEGF has value p = 0.000
A, B and C in Count of Neovascular has value p = 0.000

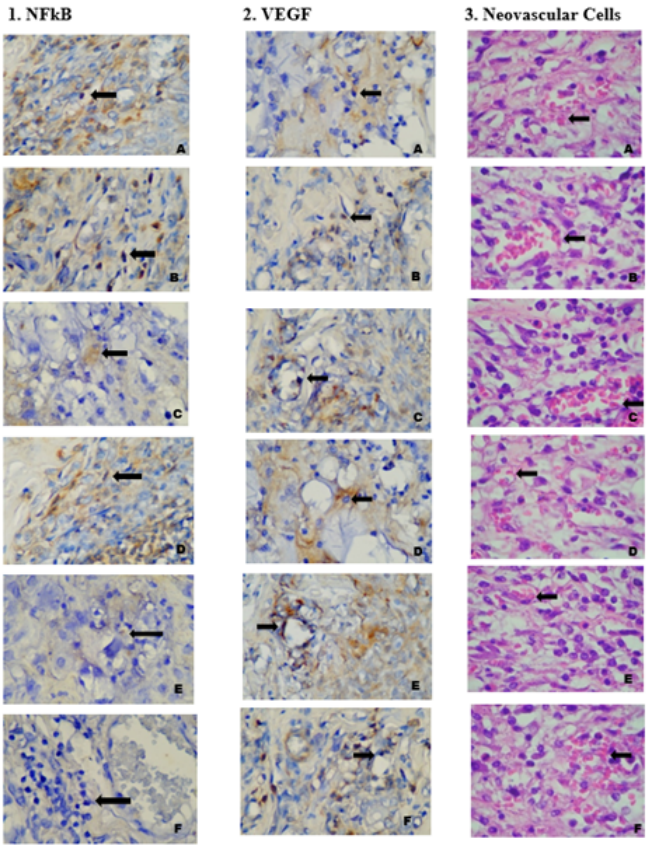

Figure 1. Macrophage expression of NF-κB on the control group, Neovascular's expression of VEGF on the control group, and Neovascular cell's count on the control group.

Note: Macrophage's expression of NF-κB on control group (A), Channa micropeltes extract concentrations of 20% (B), Channa striata extract concentrations of 10% (C) on Day 4. Macrophage's expression of NF-κB on control group (D), Channa micropeltes extract concentrations of 20% (E), Channa striata extract concentrations of 10% (F) on Day 8. 2. Neovascular's expression of VEGF on control group (A), Channa micropeltes extract concentrations of 20% (B), Channa striata extract concentrations of 10% (C) on Day 4. Neovascular's expression of VEGF on control group (D), Channa micropeltes extract concentrations of 20% (E), Channa striata extract concentrations of 10% (F) on Day 8. 3. Neovascular cell's count on control group (A), Channa micropeltes extract concentrations of 20% (B), Channa striata extract concentrations of 10% (C) on Day 4. Neovascular cell's count on control group (D), Channa micropeltes extract concentrations of 20% (E), Channa striata extract concentrations of 10% (F) on Day 8.

by the inhibition of NF-κB. As an anti-inflammatory substance, *Channa micropeltes* will reduce inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) that suppress the NF-κB gene regulator. This will prevent prolonged inflammation in the wound healing process of DM [11,12].

Excessive activation of NF-κB will cause abnormal DNA transcription which includes various gene expression of vascular complications occurring in VEGF, Platelet Derived Growth Factor (PDGF), Endothelin-1 (ET-1) and Transforming Growth Factor beta (TGF-β) that cause vascular cell damage [7]. VEGF as pro-angiogenic modulators encounter down regulation in DM, disturbing the angiogenesis process [13,14]. Prior studies demonstrate Vascular Endothelial Growth Factor A (VEGF-A) protein and messenger ribonucleic acid (mRNA) level in the wound of a diabetic rat model which shows reduction when compared to the group with a normal wound. DM leads to the decrease of angiogenesis in wound healing, so that it lessens vascular and capillary density [13].

The angiogenic effect is initiated by VEGF-A binding to Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2). Angiogenesis stimulation through PI3K-Akt-eNOS will cause endothelial cell to migrate, proliferate, and differentiate. Molecular signals will be commenced by phosphatidylinositol 3 kinase from serine/threonine kinase Akt/protein kinase B. Akt/PKB through the phosphorylation of endothelial nitric oxide synthesis on Ser 1177, and will stimulate NO production, vasodilatation, and endothelial cell migration [15,16]. This result presents that *Channa micropeltes* extract ointment at 20% concentration increases the highest expression of VEGF when compared to *Channa striata* extract ointment at 10% concentration or the control on Day 8. This exhibits the potential of *Channa micropeltes* extract ointment at 20% concentration to promote angiogenesis on the DM wound healing process.

Previous study by Carabelly et al. [1] reveals that the application of *Channa micropeltes* extract ointment at 20% concentration may elevate macrophage number on Day 8 and reduce them on Day 14. Macrophage regulates angiogenesis signals in neovascular along the formation of granulation tissue process [17]. This concept is in accordance with this study as the number of macrophages was observed the highest on Day 8 and followed by the increase of neovascular cell number by the application of *Channa micropeltes* extract ointment at 20% concentration on Day 8. The highest number of neovascular cells also was observed in the application of *Channa micropeltes* extract ointment at 20% concentration when compared to *Channa striata* extract ointment at 10% concentration or the control in Day 8. This study did not continue to further days to limit the parameters.

We can conclude that the application of *Channa micropeltes* extract ointment at 20% concentration on the wound of a diabetic rat model can reduce the expression of NF-κB. The anti-inflammatory effect of *Channa micropeltes* can elevate the expression of VEGF and the number of neovascular cells in angiogenesis process of diabetic wound healing. Our findings require further research using different parameters, adding time of evaluation, and using larger experimental animals.

Acknowledgements: This study was financed by the Faculty of Dentistry, University of Lambung Mangkurat. The authors express their gratitude.

Conflicts of interest statement: The authors have no conflicts of interest to report.

Ethical approval: Approval for this study was provided by the Ethical Clearance Committee, Faculty of Dentistry, Universitas Lambung Mangkurat, Banjarmasin, South Kalimantan, Indonesia (#111/KEPKG-FKGULM/EC/III/2020).

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