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Research Paper

Spirulina platensis Extract Reduces Serum TNF-α, Neutrophils, and Increases Macrophage Count in Skin Incisional Mice Model

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Abstract
Spirulina sp. is an alga that has antioxidant and anti-inflammatory potential. Phycocyanin contained in Spirulina platensis can reduce tumor necrosis factor-α levels, neutrophils, and M2 macrophages in inflammation. This research aims to investigate the anti-inflammatory activity of S. platensis in a mice wound model. Thirty-two male Wistar rats were incised and divided into 4 groups. The first therapeutic group (X1) received S. platensis extract at a dose of 500 mg/kgBW/day, and the second therapeutic group received S. platensis extract at a dose of 750 mg/kgBW/day. The negative control group (C1) received a saline solution and the positive control group (C2) received diclofenac sodium 20mg/kgBW/day. Serum TNF-α levels examined by the Enzyme-Linked Immunosorbent Assay method. Neutrophils and M2 macrophages were calculated by tissue biopsy and hematoxylin-eosin (HE) staining. Data analysis was performed with ANOVA test and LSD Post-Hoc Test. The average levels of serum TNF-α on the 14-day were 394.50; 180.33; 2,980.33; and 607.42 pg/ml for X1, X2, C1 and C2, respectively. The average neutrophils number on the 14-day were 8.00; 6.83; 14.67; and 11.17 for X1, X2, C1 and C2, respectively. The average macrophage number in the 14-day was 15.50; 19.17; 6.33; and 11.17 for X1, X2, C1 and C2, respectively. We found significant differences between TNF-α levels, the number of neutrophils, and the number of M2 macrophages. The administration of S. platensis extract at a dose of 750 mg/ kgBW/day reduces serum TNF-α levels, neutrophil count, and increases M2 macrophages in skin incisional mice model.

Keywords
Spirulina platensis, anti-inflammatory, TNF-α, neutrophils, macrophages.

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1. INTRODUCTION

Skin is the largest organ of the body that works as a first-line barrier to toxins, trauma, and pathogens. Any breach on the skin surface disrupts the skin's protective barrier function and may result in the formation of scars when the wound heals. There are several steps of wound healing, such as hemostasis, inflammation, proliferation, and maturation.(Rohl et al., 2015; Martin and Nunan, 2015) The inflammatory phase in the wound healing process cleans damaged cells, extracellular matrix and helps to inhibit the growth of pathogenic organisms. This phase lasts about 5-7 days. Excessive inflammation phase can cause chronic inflammation, inhibits re-epithelialization and increases the occurrence of fibrosis.(Rohl et al., 2015).

The inflammatory phase of the wound in the skin denotes the entry of mast cells, monocytes, neutrophils, and T cells that originate from capillaries into the tissues. Neutrophils and macrophages enter the wound area in the initial phase of inflammation at different times. Neutrophils arrive at the wound area approximately in the first 6-12 hours, and they reach a peak one day after injury. (Rohl et al., 2015; Gonzalez et al., 2016). Neutrophils migrate through the process of diapedesis in capillary endothelial cells and activated by proinflammatory cytokines such as interleukin (IL)-1β, Tumor Necrosis Factor (TNF)-α and interferon (IFN-γ) .(Karnen, 2012).

Macrophages enter the wound area in large numbers up to 48 hours after injury and the number is stable on days 4-5; furthermore, they continue to decline until the 14-day. Macrophages will differentiate into various kinds of cells with different functions. At the initial phase of inflammation, macrophages will differentiate into proinflammatory M1 macrophages. M1 macrophages are classified as phagocytosing bacteria, which can be produced TNF-α, IL-6, and IL-23.

M1 Macrophages can be differentiated into M2 macrophages after the phagocytic process of apoptosis in response to the cytokines IL10 and IL4. M2 macrophages possess wound repairing abilities and they release growth factors such as TGF-β, Vascular Endothelial Growth Factor (VEGF), and Platelet-Derived Growth Factor (PDGF)-β which recruit endothelial (pro-angiogenic) cells and fibroblasts, promote myofibroblast differentiation and secrete extracellular matrix components.(Rohl et al., 2015).

Pharmacological therapies are useful to reduce symptoms and to speed up the healing process of the wounds. One of the therapeutic modalities that can be used is Phyto therapeutics (Levine, 2017)., which has various potentials, one of them is phytotherapeutic potential.(BPS, 2014; Nur, 2014; Triyono, 2013). Spirulina sp. is a type of cyanobacteria that grows in water, freshwater, brackish water or saltwater. Growth and nutrient content of Spirulina sp. are influenced by factors such as light intensity, temperature, growth media, pH and salinity. Phycocyanin plays a big role in anti-inflammatory activities.(Nur, 2014; Wu et al., 2016).

Phycocyanin and β-carotene of Spirulina sp. play an important role in the anti-inflammatory effects.(Wu et al., 2016). The blue pigment phycocyanin in S. platensis works as an anti-inflammatory agent, by acting as a selective inhibitor of the cyclooxygenase-2 (COX-2) enzyme, inhibiting the production of nitric oxide (NO) and prostaglandin E2.(Wollina et al., 2018). Besides, phycocyanin is also able to inhibit the expression of TNF-α, and IL-6.9 β-carotene also has antioxidant and anti-inflammatory effects by inhibiting the production of inducible nitric oxide synthase (iNOS), COX-2, TNF-α, and IL-1β.(Sorg et al., 2017). The previous study had concluded that S. platensis has an anti-inflammatory effect on acute and chronic inflammation.(Quader et al., 2013). Besides that, S. platensis extract significantly influences wound healing through granulation tissue formation and neovascular enhancement in the wound area.

Wound healing, especially chronic wounds, is not easy to heal due to various disturbances and chronic inflammation in the wound bed. Therefore, we aimed to evaluate the potency of S. platensis extract as a novel wound-healing enhancement agent. We hypothesized that S. platensis could reduce inflammation that happened in the wound bed, which can be measured by TNF-α, macrophage count, and neutrophil count as inflammation indicators.

2. EXPERIMENTAL SECTION

2.1 Materials

The extraction of S. platensis requires 95% ethanol. The materials needed for the maintenance of experimental animals and incision procedures are AD-shaped pellet type II, reverse osmosis drinking water, 70% alcohol and 10% ketamine. For the examination of serum TNF-α levels, we used the rat TNF-α ELISA kit (catalog no. E-EL-R0019: Elab Science Biotechnology, Texas, USA). For examination of neutrophil and macrophage counts, we used formalin as a natural buffer, alcohol with multilevel concentration (10%, 70%, 80%, 90%, 95% and 100%), paraffin, xylol, and Mayer's hematoxylin stain.

2.2 Collection and Extraction of S. platensis

The S. platensis used in this study were obtained as S.platensis powder with US FDA registration number of 15594742028 and CERES number of 50OGA1200043 (9241). The powdered S.platensis microalgae were macerated in 95% ethanol solution with 1:10 concentration (one part of S.platensis powder macerated in 10 parts of 95% ethanol solution). The maceration process done for five days in a glass container. The glass container was stirred every day to make sure the uniformity of the maceration process. After five days, the solution was filtered through Whatman and was evaporated using a rotary evaporator machine at the ethanol boiling point temperature until a thick extract was obtained. This extract of S.platensis was used as the material in the subsequent tests, further explained below.

2.3 Experiment subjects

Thirty-two male pure-breed laboratory-grade Wistar rats, 2-3 months obtained from a local laboratory rat breeder with a lineage certificate of with a bodyweight of 100-200 grams, were acclimatized for 7 days. The animal was fed with water and rat pellets ad libitum at the cage. The animals were randomly divided into 4 groups and were incised to make a wound on the skin of their backs. The first therapeutic group (X1) received S. platensis extract at a dose of 500 mg/ kgBW/ day (X1) orally, the second therapeutic group (X2) received S. platensis extract at a dose of 750 mg/ kgBW/ day orally, the negative control group (C1) received saline solution 0.9 % 0.5 mL and the positive control group (C2) received diclofenac sodium 20mg/ kgBW/ day orally using oral dosing (gavage) method. This study has been approved by Health Research Ethics Committee, Faculty of Medicine, Sultan Agung Islamic University with Ethical Exemption certificate number 241/V/2019/Komisi Bioetik.

2.4 Procedure of incision

The mice were anesthetized using an intramuscular injection of 60 mg/kgBW ketamine. The hair of the rats was shaved about 1.5 cm to the right of the centerline on the back of the head and cleaned with 70% alcohol. The skin was incised using a 4 cm long scalpel parallel to the paravertebral lines until reaching the muscle layer. The wound was sutured with a simple interrupted suture technique at 0.5 cm intervals, and the suture was removed on the 7th day.

2.5 Collection of blood and tissue samples

One milliliter of the blood sample was obtained from the retroorbital blood vessels of the experimental rats on 7 and 14-days. We harvested the skin tissue nearest to the wound incision site, put it into a paraffin block, sliced and was processed for further histopathological analysis.

Table 1. Serum TNF-α levels of experimental groups

Post-injury day	Groups	Mean ± SD (pg/mL)	Min-Max (pg/mL)	p Values
7th Day	X1	377.83 ± 145.98	109.50-522.00	$p < 0.001$
	X2	232.42 ± 40.54	164.50-277.00	
	C1	4634.08 ± 1956.02	2094.50-6707.00	
	C2	658.67 ± 35.38	612.00-719.50	
14th day	X1	394.50 ± 24.70	364.50-429.50	$p < 0.001$
	X2	180.33 ± 39.10	134.50-229.50	
	C1	2980.33 ± 892.50	1707.00-4207.00	
	C2	607.42 ± 80.89	502.00-744.50	

2.6 Measurement of serum TNF-α levels

Measurement of serum TNF-α levels from blood samples was done using the Elabscience Rat TNF-α ELISA kit, (Catalog number E-EL-R0019, Elabscience Biotechnology, Houston, TX, USA). Blood was left to clot for 2 hours at room temperature and centrifugated for 15 minutes. The supernatant was collected and was stored in a clean container. One hundred microliters of sample was put into the wells and was incubated for 90 minutes at 37 C. One hundred microliters of Biotinylated detection antibody were added to the solution, and the plate was aspirated and washed three times. Afterward, 100 μL of HRP conjugate working solution and 90 μL of substrate reagent was added and incubated according to the factory provided procedure. Finally, 50 μL of stop solution was added and the plate was read using microplate reader at 450nm.

2.7 Histopathology

Tissue samples were obtained through tissue biopsy on the 14th day. Subsequently, the histological preparations and hematoxylin-eosin (H&E) staining were made. Histological slides were analyzed by a pathologist by using a 40x magnification binocular light microscope (Olympus CX23, Shinjuku, Japan).

2.8 Statistical analysis

The statistical analysis was done using SPSS for Windows version 21. We used One-Way ANOVA and Post Hoc LSD Test for further analysis. The statistical significance used in this study was set at $p < 0.05$.

3. RESULTS AND DISCUSSION

All of the mice used in this research were kept alive until the end of the measurement period. No mice were dropped out of the study. All of the mice were terminated after samples were taken following ethical termination rules.

The mean baseline value of serum TNF-α levels on the pre-wounded mice was 34.89 ± 22.47 pg/mL. One-way ANOVA analysis showed significant differences ($p < 0{,}001$) in serum TNF-α levels between study groups on both 7 and 14-days. On the 7th day, LSD post hoc tests showed significant differences were found between the X1 group and C1 group and between X2 group and the C1 and C2 groups. There were also significant differences between C1 and C2 groups. We also found significant differences on the 14th day between X1 group and X2, C1, and C2 group, between X2 group and both control groups, and between C1 and C2. (Table 1)

Table 2. The changes of the number of neutrophils per field of view in experimental groups

Groups	Mean ± SD	Min-Max	p Values
X1	8.00 ± 2.366	4-11	$p < 0.001$
X2	6.83 ± 1.722	4-9	
C1	14.67 ± 1.862	12-17	
C2	11.17 ± 2.483	8-15	

The results from Table 2 showed that there was a significant difference between the negative control group and the first therapeutic group, which received S. platensis extract group with a dose of 500 mg/kg of body weight/day. Moreover, significant differences were also found between the positive control group and both of the therapeutic groups. Table 3 also shown the results of the changes in the number of macrophages in experimental groups.

Table 3. The changes in the number of macrophages in experimental groups

Groups	Mean ± SD	Min-Max	p Values
X1	15.50 ± 2.429	12-19	$p < 0.001$
X2	19.17 ± 2.317	16-22	
C1	6.33 ± 1.862	4-9	
C2	11.17 ± 1.329	9-13	

Analysis test showed that there was a significant increase in the number of M2 macrophages in the both of the therapeutic groups which received S. platensis extract compared to the negative and positive control groups. A significant difference was also found between the two ther-

apeutic groups, where the second therapeutic group had a significantly higher M2 macrophage compared to the first therapeutic groups.

Analysis test showed that there was a significant increase in the number of M2 macrophages in the both of the therapeutic groups which received S. platensis extract compared to the negative and positive control groups. A significant difference was also found between the two therapeutic groups, where the second therapeutic group had a significantly higher M2 macrophage compared to the first therapeutic groups.

In this study, we found a significant difference in TNF-α levels between the experimental groups. Normal levels of TNF-alpha in non-wounded rats were around 32 pg/mL, and on wounded rats was around 124.6 pg/mL.(Rohl et al., 2015; Martin and Nunan, 2015) These results are in accordance with the previous study found that the administration of S. platensis extract orally at a dose of 500mg/ kgBW/ day was able to suppress acute inflammation induced by carrageenan and the administration duration of 7 days has the potential to inhibit chronic inflammation which was induced by granuloma. Another study also shows that the administration of S. platensis could reduce TNF-α levels under the inflammatory process condition (Wu et al., 2016). Another study shows that the administration S. platensis extract at a dose of 20 mg/kg of body weight and 30 mg/kg of body weight in diabetic-induced mice decreases serum TNF-α levels (Liu et al., 2016).

Cytokines play an important role in the inflammatory process through the production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, which initiates the immune system in the inflammatory process.(Liu et al., 2016). TNF-α is mainly produced by macrophages, endothelial cells, and mast cells. TNF-α increases adhesion molecules in inflammatory cells (neutrophils, monocytes) and endothelial cells.(Owen et al., 2013; Abbas et al., 2016). Tumor Necrosis Factor-α is needed in the wound healing, especially in the inflammatory phase, because the inflammatory process is a physiological process in wound healing. However, if the inflammation in the wound healing process occurs too long, the wound may become a chronic wound. On the 7th day, TNF-α was still needed in the inflammatory phase of the wound healing process. However, the increase of TNF-α levels which continued for a prolonged period than normal indicated an extension of the inflammatory phase.

Anti-inflammatory agents produced by S. platensis are phycocyanin and β-carotene.(Wu et al., 2016) As an anti-inflammatory agent, phycocyanin contained in S. platensis works a selective inhibitor of the cyclooxygenase-2 (COX-2) enzyme which is regulated during the inflammatory process and has the ability to induce apoptosis in macrophages. Other studies also concluded that phycocyanin was able to inhibit the expression of iNOS, COX-2, TNF-α, and IL-6 while β-carotene was able to inhibit the production of iNOS, COX-2, TNF-α, and IL-1β. (Wu et al., 2016; Sorg et al., 2017).

In this study, we found that the mean TNF-α level of the control group was negative on the 7th day compared to the treatment group, which was given S. platensis extract. This result shows that there was a possibility that the administration of S. platensis extract may disrupt the wound healing process. This might happen because the average TNF-α levels in the treatment group were much lower than the negative control group. Therefore, the timing of S. platensis administration is important to prevent the disruption of the wound healing process.

The measurement of the number of neutrophils in wound tissue was done to analyze how the development of the inflammatory phase in the wound healing process. The presence of neutrophils at the wound inflammation area usually peaks within one day after the initiation of tissue injury, because the neutrophils are needed in the wound healing process to phagocytose bacteria and to secrete proteinase which works as an antimicrobial agent.(Rohl et al., 2015; Gonzalez et al., 2016) In acute wounds, the inflammatory phase lasts for less than 7 days. If the number of neutrophils continues to increase or persist until the 14th day, the inflammatory phase might be prolonged and the wound might become chronic wounds.(Rohl et al., 2015).

The results of this study indicate that the administration of S. platensis extract has the potential to reduce the number of neutrophil inflammatory cells compared to the control group. These results are consistent with a previous study that found that the phycocyanin content possessed by S. platensis has an anti-inflammatory effect. One of the anti-inflammatory effects possessed by S. platensis extract is the inhibition of neutrophil migration to the area of inflammation. (Liu et al., 2016).

Macrophages are involved in the inflammatory and proliferation phase of the wound-healing phase. Macrophages entered the area of injury in large numbers up to 48 hours after injury and the number was stable on days 4-5. Furthermore, they continued to decline until the 14th day. Macrophage assessment in this study was conducted on the 14th day of wound healing, which was purposely done to let the macrophages differentiate into M2-type anti-inflammatory macrophages. The therapeutic groups which received S. platensis extract show a significant effect on increasing M2 macrophages compared to the control group. Therefore, the administration of S. platensis extract helped the wound healing process by increasing the amount of M2 macrophages.

The results of this study differ from a previous study, which might be attributed to the difference in data collection time. Their previous research showed that the number of macrophages in the group which received S. platensis extract increased significantly on the 3rd day. However, it started to decrease in the 7, 12 and 13-days. Therefore, an immunohistochemical examination should be carried out to ascertain the type of macrophage. Besides, It is necessary to examine proinflammatory, other anti-inflammatory cytokines, and

the growth factors to see the effectiveness of S. platensis in the healing of proliferative and remodeling phases. There are several limitations in this study that can be studied in the future, such as the measurement of wound healing markers, preferably should have been done together with the microscopic (histopathological) analysis to accurately measure the wound healing process and its correlation with the wound healing markers.

4. CONCLUSIONS

S. platensis extract was found to have the capability to reduce serum TNF-α levels, reduce the number of neutrophils, and significantly increase the amount of anti-inflammatory M2 macrophages compared with the control groups. The administration of S. platensis extracts at a dose of 750 mg/kg body weight/day on the second experimental group has better potential to reduce serum TNF-α levels and increases the number of M2 macrophages when compared to the first experimental group which was given with S. platensis extract at a dose of 500 mg/kg body weight/ day.

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