The Immune Responses of DDY Mice infected S. aureus After Treatment of M. citrifolia Fruit Crude Extract

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ABSTRACT

Infection disease that caused by bacteria is one of the illness in several developing countries including in Indonesia, with high mortality rate. One of the plants that can be used as medical herbal is Morinda citrifolia. The aim of this study is known the immune response of mice with S. aureus infection by change to quantitatives’ lymphocytes and neutrophils after administration of M. citrifolia fruit crude extract. Mice were divided into two groups there are Non-Infection and infection. Non Infection is without S. aureus and than infection has S. aureus. The each groups are including control, dose 1 (25 mg/kg BW), dose 2 (100 mg/kg BW), and dose 3 (300 mg/kg BW). Oral treatment carried out for 20 days and injection of S. aureus at day 21 after with a concentration is 10⁶ cell/ml. Relative number of neutrophil cell (Gr-1) and T cell subsets was measured using the BD FACSCalibur™ Flowcytometer. Data were analyzed using two way ANOVA (p<0.05). The result showed that administration of M. citrifolia crude extract was significantly changes in the relative amounts Gr-1⁺, CD4⁺, and CD8⁺ T cells.

Keywords: immunomodulators, Morinda citrifolia, Staphylococcus aureus

Introduction

Infection disease that caused by bacteria is one of the illness in several developing countries including in Indonesia, with high mortality rate. This includes the incidence of infectious diseases caused by normal flora in humans such as Staphylococcus aureus. S. aureus is also able to form small-colony variants (SCVs), which may contribute to persistent and recurrent infection. In vitro, SCVs are able to hide in host cells without causing significant host-cell damage and are relatively protected from antibiotics and host defenses. They can later revert to the more virulent wild type phenotype, possibly resulting in recurrent infection [¹].

Immune response that occurs as a result of invasion of bacteria S. aureus when it enters the body will be eliminated by neutrophils and macrophages as its role in the innate immune system. Besides, macrophages may also act as antigen presenting cells (APC). In the macrophage, the bacteria will phagocytes later recognize by MHC II, and will be presented in the form of antigen peptides. Furthermore, MHC II binds to T helper lymphocytes (CD4) in the T Cell Receptor (TCR) section [²]. CD4⁺ T cells are activated will be lose CD62L and express a variety of surface molecules such as CD25, CD44, CD69 as a purpose to regulate the activity of effector cells are activated as a result of exposure bacterial antigens [³]. Effector T cells CD4⁺ will secrete IFN-γ⁺, which serves as the activation of macrophages, phagocytes, and bacterial killing. CD4⁺ T cells will produce cytokines IL-2 were result in activation of cytotoxic T cells (CD8⁺) and regulator T cells (CD4⁺CD25⁺) [²].

Treatment of infection caused by S. aureus has become more problematic since the development of antimicrobial resistant S. aureus [⁴]. The prevalence of bacterial resistance to existing drugs was encouraging the importance of searching a source of antibacterial from natural materials. Indonesian society has used various types of natural materials as a traditional medicine. Medicinal plants known can potentially be further developed for prevention or treatment of infectious diseases. Based on the above, the aims of this study are determine the immunomodulator activity of the fruit extract of M. citrifolia in mice infected with S. aureus. Immunomodulator activity of aqueous extract of fruits M. citrifolia can be seen by observing the relative amounts of neutrophil cell (Gr-1⁺) and lymphocyte T cell subsets (CD4⁺, CD8⁺).
and CD8^+) in spleen organs of mice. The results of this research can be used as an alternative material for immunomodulator in addressing a variety of diseases associated with the infection of microbial pathogens such as S. aureus.

Methods

This study used female murine pathogen free strain Deutschland Denken Yonken (DDY), age 6 weeks with an average weight 25 grams and obtained from LPPT Gajah Mada University. Using of experimental animals has received ethical clearance no.89, from the Ethics Committee of Brawijaya University. Herbs were tested is the fruit of M. citrifolia obtained from Joyotambaksari Malang, and S. aureus obtained from Laboratory of Medical Microbiology Brawijaya University. Experimental design used in this study was completely randomized design factorial with two factors, namely the group of mice not infected with S. aureus (non-infectious), and groups of mice were infected S. aureus (infection).

Preparation of M. citrifolia fruit crude extract

M. citrifolia fruit used is not too old and not too young, whitish green and still a little hard. Solvent extraction is done using distilled water. 5 grams of M. citrifolia fruit dissolved in 150 ml of water in a glass beaker. Then heated using a water bath until the temperature obtained extract in the glass beaker at 80^o C, and then maintained for 15 min. Furthermore, the filtering performed using filter cloth to obtain the crude extract as a stock solution. Giving crude extracts of M. citrifolia fruit into the body of mice based on body weight of mice were weighed every day orally, in accordance with the prescribed dose once for 20 days with a volume of 200 µl of extract.

Infections of S. aureus

S. aureus in medium Nutrient agar (NA) which has been confirmed previously cultured in medium Nutrient broth (NB) liquid and incubated for 1 x 24 hours. Furthermore, as many as 1 ml was taken and added to 9 ml fresh NB medium. Then do the process of counting bacteria by using haemacytometer every 1 hour, until a cell concentration of 10^9 bacterial cells/ml. After getting bacterial cell concentration 10^8 cells/ml, then performed centrifuge with speed 10.000 rpm for 10 min at 25^oC. The next pellet obtained was suspended with 1 ml of PBS. The suspension then injected in animals intraperitoneally with a volume of 100 µl. Injection performed on day 21 after treatment of extract of fruit M. citrifolia.

Isolation of lymphocytes from spleen

Isolation cells performed on day 25, which is 4 days after the treatment of infections S. aureus. Spleen taken from mice had been dissected and cleaned with phosphate-buffered saline (PBS). Isolation of lymphocytes from the spleen is done by crush the spleen and resuspended with 6 ml PBS. Furthermore, the cells obtained are filtered using wire. Then the results obtained centrifuged at 2500 rpm at 4°C for 5 min. Supernatant was discarded and the pellet obtained resuspended with 1 ml PBS. Further homogeneous to obtain homogenates. 200 µl homogenate was transferred to a new tube and giving 500 µl PBS. Then do the centrifuge at 2500 rpm, 4°C for 5 min. Supernatant was discarded and the pellet further incubated with antibodies for the subsequent analysis.

Flowcytometry analysis

Flowcytometry analysis performed to detect populations of cells that express Gr-1^+, CD4^+ and CD8^+. In this study, cells were isolated from spleen were incubated with antibodies for 15 minutes in the ice box. Antibodies used were rat anti-mouse anti-Gr-1 FITC conjugated, rat anti-mouse anti-CD4 FITC conjugated, rat anti-mouse anti-CD8 PE conjugated. The samples were incubated with antibody plus 300 µl PBS and placed in the cuvette flowcytometer. Furthermore chosen acquire and flowcytometer will calculate the total number of cells and the number of cells detected by antibody labeling. The results obtained further processed with BD cellquest ProTM.
Data analysis

The result using CellQuest further tabulated, and statistically tested using two way Analysis of Variance (ANOVA) with SPSS 16.0. If obtained significant result (p<0.05), the test was done using Tukey (HSD) analysis.

Result and Discussion

Analysis of Relative Number of Gr-1\(^{+}\) Cells in the Bone marrow using Flowcytometry

Neutrophils are cells that play an important role in the innate immune system. Mature neutrophils are distinguished by the characteristic nuclear morphology segments and composed of organelles that are responsible for phagocytosis, bacterial clearance and inflammatory response. Differentiated neutrophils in the bone marrow and can be classified into subsets based on the characteristics of mature morphology (promyleocytes, myelocytes, metamyelocytes, and band-segmented neutrophils) \(^{[5]}\). This figure show that the relative number of neutrophil (Gr-1\(^{+}\)) in bone marrow mice after treatment of *M. citrifolia* fruit crude extract with a few doses based on flowcytometry analysis. This result show that the percentage of the relative number of neutrophil (Gr-1\(^{+}\)) in bone marrow using flowcytometry show changes the relative amount of Gr-1\(^{+}\) on all doses when compared with the control normal (K-) and it is significant (p<0.05). Treatment of *M. citrifolia* fruit crude extract and then infection with *S. aureus* can changes the relative amount of Gr-1\(^{+}\) when compared with positive control (K+) although it is not significant (p>0.05).

Based on the result of flowcytometry analysis followed by ANOVA test (Figure 1.) is known that *M. citrifolia* fruit extract can increase the relative number of GR-1\(^{+}\) in bone marrow mice. In the non-infection group (F1), the data show that in control group (P0F1) has a number of relatively GR-1\(^{+}\) as much as 31.0% and when treated with the fruit extract *M. citrifolia* orally increased the relative amount of GR-1\(^{+}\) 1.2% but not significantly different (p> 0.05) to 32.2% at the treatment dose of 25 mg/kg BW (P1F1). Giving fruit extracts of *M. citrifolia* dose II and III can increase the relative number of GR-1\(^{+}\) and significantly different from the negative control (P0F1). In the infection group (F2), the treatment group were given fruit extracts *M. citrifolia* dose of 25 mg/kg BW (P1F2), 100 mg/kg BW(P2F2) and 300 mg/kg BW(P3F2) for 20 days and then infected with the *S. aureus* on day 21 had an increase in the relative number of GR-1\(^{+}\) but not significantly different when compared to the positive control treatment (the treatment group were infected with *S. aureus* without fruit extract of *M. citrifolia* (P0F2)). The increase of relative number GR-1\(^{+}\) on the infection groups (F2) after treatment of *M. citrifolia* fruit extract and infected with *S. aureus* in this study was thought to be due to some of the active compounds in fruit *M. citrifolia* that can stimulate the formation of neutrophils (GR-1\(^{+}\)) in the bone marrow.

![Figure 1](image)

Figure 1. The relative number of neutrophil cells (GR-1\(^{+}\)) and HSD test results on any treatment results analysis using flowcytometry in bone marrow (F1 = non infections factor, F2 = infection factor, P0 = dose 0 (- and + control), P1 = dose of 25 mg/kg BW, P2 = Dose 100 mg/kg BW, P3 =dose 300 mg/kg BW).
Neutrophils will respond to bacteria that enter the body. In response to microbial pathogen infection, neutrophils in the bone marrow will be removed and the peripheral control of invading pathogens through phagocytosis, oxidative agents, enzymatic digestion, and the formation of extracellular traps. Neutrophils will die in the process of killing bacteria and then granulocyte colony stimulating factor (G-CSF) to be up-regulated to induce granulopoiesis. Thus, the increase in GR-1+ population in the bone marrow as a result of the suspected active compounds in *M. citrifolia* fruit extracts affecting activity increased cytokine granulocyte colony stimulating factor (G-CSF) so as to increase the population of the relative number of neutrophils (GR-1+) [6]. The active compounds from the fruit *M. citrifolia* were including polysaccharides, alkaloids and anthraquinone can increase the phagocytic activity of neutrophils in vitro. When an infection, neutrophils will issue chemoattractants as cathepsins and Defensins that stimulate T cell accumulation at the point of inflammation. Neutrophils are also going to trigger the activation of T cells to release cytokines [7,8].

**Analysis of Relative Number of CD4+ and CD8+ Cells in the Spleen using Flow cytometry**

Lymphocytes were leukocytes that were usually classes from being in the blood [9]. The primary function of lymphocytes is to recognize foreign pathogens that could indicate the presence of viruses, bacteria, parasites or tumor cells [9,10]. Lymphocytes can be grouped into different classes based on its function, Class B-lymphocytes, T-lymphocytes and Natural Killer cells (NK cells). The relative proportions of T cells and B cells in peripheral blood were about 75% and 10%, while the remaining 15% was NK cells [11]. The lymphocytes T and B have their place in different differentiation. T lymphocytes in the thymus differentiate while the B lymphocytes differentiate in the bone marrow. T cells become a central component in a normal immune system. T cells that escaped the next selection differentiate into two sub-species of the CD4 T cells and CD8 T cells. CD4 T cells are known as helper T cells, whereas CD8 T cells are known as killer T cells (cytotoxic) [9]. This figure showed that the relative number of CD4 and CD8 cells in spleen after treatment *M. citrifolia* fruit extract in non-infection and infection groups (Figure 2).

The result show that in the non-infection group (F1), the treatment of the fruit extract *M. citrifolia* at dose 25 mg/kg BW (P1F1) and 100 mg/kg BW (P2F1) is known increase the relative number of CD4+ T cells but not significantly different from the negative control treatment the treated distilled water (P0F1). While the treatment of fruit extracts *M. citrifolia* dose of 300 mg/kg BW (P3F1) lowers the relative number of CD4+ T cells and not significantly different from the negative control treatment group (P0F1). In the treatment of infection group (F2), the data show that treatment *M. citrifolia* fruit extract affect the relative number of CD4+ T cells in mice infected with *S. aureus*. From the test results further using Tukey known that the relative number of CD4+ T cells was reduced in mice infected with *S. aureus* after administration of fruit extracts *M. citrifolia* dose of 25 mg/kg BW (P1F2) 100 mg/kg BW (P2F2) and 300 mg/kg BW (P3F2) and significantly different from the positive control group (P0F2).

In the non-infection group (F1) treatment of *M. citrifolia* fruit extract at dose 25 mg/kg (P1F1) BW can reduce the relative number of CD8+ T cells in the spleen but not significantly different from the positive control (P0F2). However, the *M. citrifolia* fruit extract dose of 100 mg/kg BW (P2F1) and 300 mg/kg BW (P3F1) can increase the relative number of CD8+ T cells in the spleens of mice but the increase was not significantly different from the negative control group (P0F1) except at the dose of 100 mg/kg (P2F2) were known significantly different from the negative control (P0F1). In the infection group (F2), the data show that treatment of *M. citrifolia* fruit extract can reduce the relative number of CD8+ T cells after the spleen of mice infected with *S. aureus* (Figure 2.)

*S. aureus* is known to have the form of superantigen toxins that can increase the production of cytokines massively in response to inflammation and can eventually lead to toxic shock. As with other pathogens that were able to survive in the host. Bacteria *S. aureus* is known to develop different strategies to interfere with host adaptive immune system response. Some research indicates that the bacterium *S. aureus* has TSST (toxic shock syndrome toxins) that can alter the function of T cells through targeting T cell receptor activation pathway (TCR) [12].
Noni fruit extract (M. citrifolia) can inhibit the activation of NFκβ that responsible for the inflammatory process \(^{[13]}\). Transcription factor NF-κβ is a key regulator of inflammatory and acts downstream of the many cell surface receptors, including MHC class II molecules and TLR (toll-like receptors) \(^{[14]}\). Active NF-κβ can induce the formation of proinflammatory cytokines in the immune system such as cytokines TNF-α, IFN-γ, adhesion molecule (VCAM-1, ICAM-1) \(^{[15]}\). This situation can be dangerous if the cytokines produced in excess. Thus, in this study can be presumed that the fruit extract M. citrifolia can decrease the expression of CD4\(^+\) T cells as a result of the active compound from the fruit of M. citrifolia that can inhibit the expression of NFκβ so that proinflammatory cytokines that are not formed, and superantigen activity can be inhibited, so that the activation of T cells also decreased.

**CONCLUSION**

Treatment of Noni (M. citrifolia) fruit crude extract can influence relative amount of neutrophil (GR\(-1^+\)), T cell subset (CD4\(^+\), CD8\(^+\)) on mice that infection and non infection with S. aureus. Treatment of noni fruit extract can increase immune response on mice that infection with S. aureus.

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