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Okra Polysaccharides Improves Spleen Weight and B-Lymphocytes Proliferation in Mice Infected by *Staphylococcus aureus*

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B-lymphocytes proliferation; Immunomodulatory activity; Okra polysaccharides; Spleen weight

Abstract

Okra (*Abelmoschus esculentus*) is reported to have various biological functions such as antioxidant, anticancer and anti-inflammation. However, only few studies have been reported immunomodulatory activities of okra to prevent disease caused by bacteria infection. In this study, the immunomodulatory activities of polysaccharides from okra pods were investigated further through the spleen weight and B-lymphocytes proliferation in mice infected by *Staphylococcus aureus*. Okra polysaccharides were obtained by water extraction and ethanol precipitation. Okra polysaccharides with doses of 25, 50, 75, and 100 mg/kg BW were orally administrated to mice with or without *Staphylococcus aureus* infection. Spleen weight was evaluated in both treatment group and control group. B-lymphocytes proliferation was evaluated by MTT assay using LPS induction. Results showed that okra polysaccharide at the doses of 50, 75, and 100 mg/kg increased spleen weight ($p < 0.05$) significantly. While at the dose of 75 and 100 mg/kg, it increased the B-lymphocytes proliferation ($p < 0.05$) significantly. There is positive correlation between the spleen weight and B-lymphocytes proliferation by 73.3%. These result reveal that okra polysaccharide could improve the immune response and be utilized as a novel candidate of neutraceutical.

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INTRODUCTION

High demand of nutritional food is as high as the increase of knowledge about food for health. Nutraceutical product is any product derived from food sources that provides health and medical benefits. Nutraceutical product shoud act as an antioxidant, anti-inflammation, be able to improve immune system and prevent cancer (Bhowmik *et al.,* 2013). On the other hand, human body is surrounded by environment contained microbes including extracellular bacteria such as *Staphylococcus aureus.* The bacteria is one of the main causes of nosocomial infections which can result in serious infections such as bacteremia, pneumonia and endocarditis (Diekema *et al.,* 2001; Schito, 2006). From all infection cases in Indonesia, 7.1% was identified as nosocomial cases (World Health Organization, 2011)

In recent years, using immunomodulator to enhance the immune response has proven to be an effective way to increase resistance to disease (Zhao *et al.,* 2014). Study conducted by Wahyuningsih *et al.* (2016) showed a significant increase of phagocytic activity in treatment group treated by polysaccharides from *Coriolus versicolor.* Polysaccharides from plants have been known as an important immunostimulatory agent with broad spectrum, low toxicity and few side effects (Cho *et al.,* 2014). One of promising polysaccharides is the one derived from okra. Okra (*Abelmoschus esculentus*) is a flowering plant from Malvaceae family cultivated in tropical, subtropical and warm temperate regions around the world (Khatun *et al.,* 2011; Naveed *et al.,* 2009).Okra pods often were used as a traditional medicine for the treatment of gastric irritation and inflammation (Lim, 2012). The active compounds of okra are flavonoids and vitamine C as antioxidant (Arapitsas, 2008; Lim, 2012). Meanwhile, okra polysaccharides acts were used as immunomodulator (Chen *et al.,* 2016).

A study by Chen *et al.* (2012) showed that okra polysaccharides increased spleen index, splenocytes proliferation, Interleukin (IL)-10 level, Tumor Necrosis Factor (TNF)-α level, and Interferon (IFN)-γ level with cyclophosphamide as an antigen. Okra extract also modulated immune response by increasing IL-12 level (Sheu and Lai, 2012). According to Zheng *et al.* (2016), okra polysaccharides induced NO and cytokine production of mouse macrophage cell line RAW264.7. Okra fruit extract was also reported to have anti-adhesive activity against *Helicobacter pylori,* made rational basis for its use against gastric irritative and inflammatory diseases (Lengsfeld *et al.,* 2007).

However, there was little report on okra consumption as immunomodulator for combating *S. aureus* infection. Hence the objective of the present study is to examine the immunomodulatory activities of okra after *S. aureus* infection based on spleen weight and B-lymphocytes proliferation. This research is conducted to reveal okra polysaccharides potention as alternative food that provides health and medical benefits in addition to the basic nutritional value found in foods.

METHODS

For preparation of okra polysaccharides, fresh okra pods (500 g) were mashed and macerated with 500 ml of distilled water overnight three times. Supernatants were collected and centrifuged at 4300 rpm for 5 min. The supernatant was precipitated in absolute ethanol 1X sample volume, incubated for 24 h at 4^{o}C and centrifuged. The pellet was dissolved in distilled water and centrifuged. The supernatant was collected and lyophilized (Chen *et al.,* 2016).

Okra polysaccharides were administrated by gavage daily for 14 consecutive days. Male BALB/c mice were randomly divided into six groups (5 for each group). Group KN was control, group K- was negative control which being exposed to *S. aureus* without okra polysaccharides administration, group P1, P2, P3 and P4 were administrated with okra polysaccharides at dose of 25 mg/kg BW, 50 mg/kg BW, 75 mg/kg BW, and 100 mg/kg BW respectively before being exposed to *S. aureus*. Mice were exposed to *S. aureus* (0.5 Mc. Farland) by means of intraperitoneal injection once, at 15th day after okra administration. Two weeks after *S. aureus* infection, the mice were sacrificed by ketamine anesthesia (0.1 ml for each). The spleens were taken under aseptic condition. Spleens were cleaned from blood cloth and contaminating tissue. Each spleen weight was measured to evaluate the effect of polysaccharides in all groups. After that, spleens were placed in PBS-Penicillin-Streptomycin on ice.

To examine B-lymphocytes proliferation, spleen was placed in 10 cm of petridish containing 5 ml of PBS, cut in small pieces and crushed using two sterile glass slides. The lymphocytes suspension was filtered through 200-mesh to collect a single suspension and centrifuged at 1000 rpm for 5 min. Pellet was resuspended in Tris-buffered NH_4Cl, pH 7.2 and centrifuged to lyse red blood cells. Previous step can be repeated again until the white pellet was obtained. Lymphocytes were washed with 5 ml of PBS-Penicil-

lin Streptomycin and resuspended in RPMI-1640 medium supplemented with FBS 10%. Cells numbers were counted by haemocytometer. 195 µl of splenocytes ($3x10^5$ cells/well) and 5 µl of LPS (200 µg/ml) were cultured in 96 well plates to induce B-lymphocytes proliferation. Plates were incubated at 37°C in incubator with 5% CO_2 for 48 h. 50 µl of MTT solution was added and incubated for 4 h. The DMSO solution was added and incubated in incubator with 5% CO_2 for 1 h. Absorbance was measured in microplate reader at 560 nm.

Statistical analysis for spleen weight was performed by One-Way ANOVA test followed by Duncan test. Statistical analysis for B-lymphocytes proliferation was performed by Brown-Forsythe test followed by Games-Howell test. The results were presented as mean ± SD. *P*-values less than 0.05 considered statistically significant.

RESULTS AND DISCUSSION

Immune system protects organism against bacteria infection. Immune system is divided into innate immunity, which provides immediate protection against microbial invasion and adaptive immunity, which more slowly developed but provides more specialized defense against infections (Abbas *et al.,* 2010). Immune system is a complex and important physiological system, which can cause various serious diseases when it out of balance. In the last few decades, many polysaccharide-protein complexes with immunomodulatory activity from medicinal herbs have become primary concern of researchers to prevent the disease (Schepetkin & Quinn, 2006). Okra pods have long been used both as a food source and as traditional medicine for many disease (Chen *et al.,* 2016). Okra (*Abelmoschus esculentus*) is a vegetable plant known as bendi in Malaysia, kra jeab khiew in Thailand, lender in Riau, or Arab nuts in West Kalimantan (Nadira *et al.,* 2009). The active compound presented in okra are vitamin C and flavonoid in the form of oligomer catechin and quercetin (Arapitsas, 2008; Lim, 2012). Phenol compounds and vitamin C have scavenger activity against free radicals (Battino *et al.,* 1999). It was also reported that okra pods contain many polysaccharides (Arapitsas, 2008; Movin-Jesu, 2007). Polysaccharides from okra pods are receiving attention in the field of functional food and can be a novel nutraceutical in the future

Host defense gives different immune response due to an infection of intracellular and extracellular bacteria. *Staphyloccoccus aureus* is extracellular bacteria. Based on D'Elios *et al.* (2011), *S. aureus* will be recognized first by phagocytes such as macrophages and dendritic cell. Beside doing phagocytosis, activated phagocytes play a key role in presenting *S. aureus* to Helper T-lymphocytes (Th) naïve. The presence of presented *S. aureus* and cytokines produced by phagocytes induce the activation Th-17 cell. Then, Th-17 cells induce the differentiation of B-lymphocyte followed with antibody secretion to lyse *S. aureus*. Normally, immune system components such as macrophage, lymphocytes, natural killer cells, cytokines and complement will inhibit the transmission of *S. aureus* but these bacteria also release components against the immune system (Lin & Peterson, 2010). Human body needs compound to enhance immune response.

Lymphocytes circulated in blood and lymph vessels also can be found in lymphoid tissue and lymphoid organ. One of the lymphoid organ is spleen. Spleen is a place for maintaining the mature naïve lymphocytes (Murphy & Weaver, 2017). The spleen plays multiple supporting roles in the body. It acts as a filter for blood as part of the immune system. Old red blood cells are recycled in the spleen, and platelets and white blood cells are stored there. The spleen also helps fight certain kinds of bacteria. If spleen index is lower than normal, this indicates a mildly compromised immune system. This is usually an indication that spleen is not producing an adequate amount of red and white blood cells causing immune system to become weak. The spleen may also be enlarged due to viral or bacterial infections (Siddiqui & Ali, 2015).

Spleen weight and spleen index may be changed in response to adaptive immunity. Spleen weight could be enhanced by the immune system activators. It has been reported that immunomodulator can enhance the spleen index in the mice exposed to cyclophosphamide (Zhao *et al.,* 2015). Spleen index is ratio of spleen weights (mg) and body weight (kg). In the research conducted by Zhao *et al.* (2015), polysaccharides from *Scissandra chinensis* with low, medium and high doses could enhance spleen index compare with normal control and negative control groups.

This present result of spleen weight is presented in Figure 1. The highest spleen weight was shown by P3 group (480±83.66 mg) and the lowest spleen weight was shown by KN group (260±54.77). P1 group (340±89.44 mg) showed an increase of spleen weight but the difference was not statistically significant. Compared with KN and K- group, P2 group at dose of 50 mg/kg BW, P3 group at dose of 75 mg/kg BW, and P4 group at dose of 100 mg/kg BW have signifi-

cantly increased the spleen weight (p<0.05). Oral administration of okra polysaccharides at dose of 50, 75, and 100 mg/kg BW increased spleen weight to 460±134.16 mg, 480±83.66 mg, and 460±89.44 mg respectively.

Similarly, study conducted by Chen *et al.* (2016) showed that highest dose of okra polysaccharides (100 mg/kg BW) increased the spleen index significantly with cyclophosphamide as an antigen. Our present result suggested that okra polysaccharides enhance spleen weight and induce the increase in number of splenocytes in spleen. Rising number of splenocytes will be followed by increasing spleen weight.

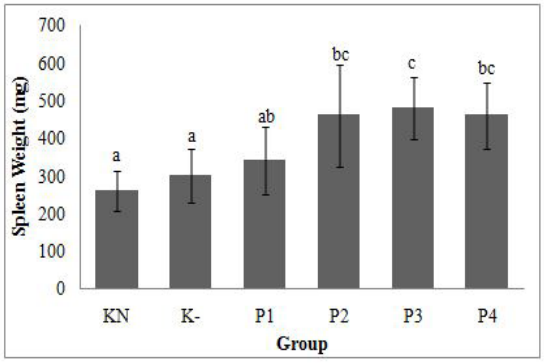

Figure 1. Effect of okra polysaccharides on spleen weight. Values are means ± SD (n = 5). Different superscripts indicate a significance difference (p<0.05).

The increase of spleen weight is a response to proliferation of spleen cell. Therefore, we investigated the effect of okra polysaccharides on B-lymphocytes proliferation placed in spleen. Splenocytes are immune cells found in spleen. Splenocytes consist of T-lymphocytes, B-lymphocytes, macrophages, and dendritic cells (Cho *et al.,* 2015). The primary function of B-lymphocytes is the production of antibodies that are specific for a given antigenic component of an invading pathogen (O'Gorman & Donnenberg, 2008). B-lymphocytes mediate humoral immunity. Soluble antigens and antigens on the surface of *S. aureus* will bind to these B-lymphocytes antigen receptors and leads to the secretion of soluble form of antibodies (Abbas *et al.,* 2010).

Polysaccharides regulate host immune system by activating immune-related cell such as lymphocytes, macrophages, and NK cells (Jiang *et al.,* 2010). Activating one of immune components will result in the activation of other immune components including B-lymphocytes in spleen. This result of B-lymphocytes proliferation is presented in Figure 2. The highest B-lymphocytes proliferation was shown by P3 group (0.453±0.028) and the lowest B-lymphocytes proliferation was shown by KN group (0.287±0.005). P1 group (0.301±0.015) and P2 group (0.321±0.033) showed slight increase of B-lymphocytes proliferation but did not statistically show difference. P3 group at dose of 75 mg/kg BW and P4 group at dose of 100 mg/kg BW significantly increased spleen weight (p<0.05) compared with KN group and K-group. Oral administration of okra polysaccharides at dose of 75 and 100 mg/kg BW increased B-lymphocytes proliferation with value of 0.453±0.028 and 0.444±0.014 respectively.

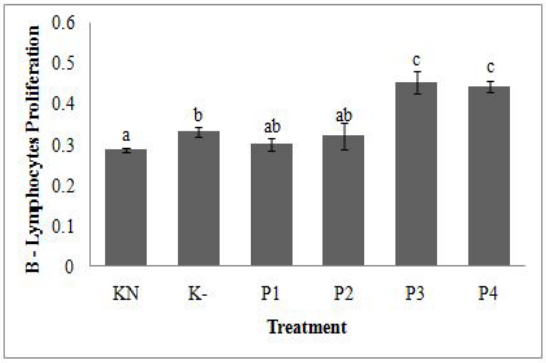

Figure 2. Effect of okra polysaccharides on B-lymphocytes proliferation. Values are means ± SD (n = 5). Different superscripts indicate a significance difference (p<0.05).

Similar with study conducted by Zha *et al.* (2014), *Dendrobium huoshananse* polysaccharides at doses of 50, 100, and 200 mg/kg BW increased splenocyte proliferation of 140%, 165%, and 196% respectively. Chen *et al.* (2016) also revealed that administration of okra polysaccharides at doses of 25, 50, and 100 mg/kg BW significantly increase splenocyte proliferation induced by LPS and okra polysaccharide at dose of 100 mg/kg BW showed highest rising of splenocyte proliferation. We can state that higher doses of polysaccharides help in rising the proliferation of B-lymphocytes. In line with our result, Wang *et al.* (2014) stated that splenocyte proliferation is related with improvement of T- or B-lymphocytes immunity which could be indicator of immune activation.

Increase proliferation of B-lymphocytes and spleen weight are affected by other immune-related cells. Zheng *et al.* (2014) stated that polysaccharides induces NO and cytokine production from the RAW264.7 cells through the activation of transcription factor NFκB. Transcription factor NFκB regulates many important biological and pathological processes and modu-

lating the transcription of a large number of genes involving iNOS, IL and TNF-α. Cytokines such as IL and TNF-α are produced by macrophages, T helper cells and NK cells (Detrick *et al.,* 2008). Cytokine activation can be indicator of immune-related cell activation. Immune-related cells such as macrophages, T-lymphocytes, and B-lymphocytes are also found in spleen. We can state that polysaccharides may induce cytokine production that activate immune-related cell in blood vessel and spleen. Activation of immune-related cells will be followed by proliferation of immune-related cells, including B-lymphocytes. Increasing of B-lymphocytes proliferation contributes on rising of spleen weight.

There is a positive correlation between spleen weight and B-lymphocytes by 73.3%. It showed that when the weight of the spleen was large, the spleen cells also actively divided. Polysaccharides are a type of macromolecules with complicated structure. This complicated structure can affect their biological activity in immune-related cells and their immunoregulatory activity (Zhao *et al.,* 2014). Immunomodulatory activities of polysaccharides may be due to direct or indirect interaction with immune system component. Complement proteins, monocytes, macrophages, dendritic cells, neutrophils, and lymphocytes have been reported as target responding to polysaccharides (Schepetkin & Quinn, 2006; Leung *et al.,* 2006, Ramesh *et al.,* 2002). A direct stimulatory effect on these immune cells involves specific recognition receptor. Upon binding of polysaccharides to specific recognition receptors on immune cells, diverse signaling pathway may be triggered, leading to response (Ferreira *et al.,* 2015).

This present study revealed that okra polysaccharides may act as immunomodulator by enhancing B-lymphocytes proliferation and spleen weight. Okra polysaccharide can be an alternative food that provides health and medical benefits in addition to the basic nutritional value found in foods. Okra polysaccharides will help body to defense against bacteria including *S. aureus.* By introducing okra consumption in Indonesia, it was expected to suppress prevalence of nosocomial infection.

CONCLUSION

Based on the result presented above, we could conclude that administration of polysaccharides from okra (*Abelmoschus esculentus*) significantly increased the spleen weight and B-lymphocytes proliferation. The optimum doses were 75 and 100 mg/kg BW. There was 73.3% positive correlation between spleen weight and B-lymphocytes proliferation. It proved that okra polysaccharide enhanced the immune response in spleen. These results suggest that okra polysaccharides could be utilized as a novel nutraceutical due to its effective immunomodulatory activity.

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