Biosaintifika 8 (1) (2016) 12-17

Biosaintifika
Journal of Biology & Biology Education

http://journal.unnes.ac.id/nju/index.php/biosaintifika

Influence of Polysaccharide Krestin from *Coriolus versicolor* Extract on Nitrite and Malondialdehyde Concencentrations of *Mus musculus* Serum Exposed by *Mycobacterium tuberculosis*

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DOI: 10.15294/biosaintifika.v8i1.4969

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History Article

Received 30 January 2016
Approved 19 February 2016
Published 12 March 2016

Keywords:

Malondialdehyde; nitrite; polysaccharide krestin; tuberculosis

Abstract

Mycobacterium tuberculosis is a major infection agent of tuberculosis that is controlled by the response of cell-mediated immunity. It is macrophages and cytolytic T lymphocytes. Activated macrophages will produce free radicals. Excessive free radicals cause tissue damage. Polysaccharide krestin contains β-glucan. It is a scavenger of free radicals. This research aimed to identify the influence of polysaccharide krestin from *C. versicolor* on nitrite and malondialdehyde concentrations of mice serum exposed by *M. tuberculosis*. Nitrite concentration was determined by nitrite assay. Malondialdehyde concentration was determined by TBARS assay. The result showed that adding polysaccharide krestin before exposure (P1) and adding polysaccharide krestin before-after exposure (P3) had the best potential to decrease nitrite concentration. Nitrite concentrations of P1 and P3 were 1.364 ± 0.523 M and 1.456 ± 0.712 M respectively. Meanwhile, P1 group and adding polysaccharide krestin after exposure (P2) had the best potential to decrease malondialdehyde concentration. Malondialdehyde concentrations of P1 and P2 were 1125.86 ± 97.96 μM and 953.86 ± 328.16 μM respectively. Their nitrite and malondialdehyde concentrations decreased, compared to K and K- groups. The research conclusion was that adding polysaccharide krestin before exposure could decrease both nitrite and malondialdehyde concentrations.

How to Cite

Wahyuningsih, S., Pramudya, M., & Sugiharto, S. (2016). Influence of Polysaccharide Krestin from *Coriolus versicolor* Extract on Nitrite and Malondialdehyde Concencentrations of *Mus musculus* Serum Exposed by *Mycobacterium tuberculosis*. *Biosaintifika: Journal of Biology & Biology Education*, 8(1), 12-17.

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p-ISSN 2085-191X
e-ISSN 2338-7610

INTRODUCTION

Tuberculosis is infectious disease caused by *Mycobacterium tuberculosis.* In 2014, tuberculosis killed 1.5 million people worldwide. Tuberculosis ranks alongside HIV as leading cause of death worldwide (WHO, 2015). Individual who is infected by infectious disease, virulent, and pathogen will give immune responses, as both specific immune response and nonspecific immune response. Macrophage is one of the nonspecific immune system components. Macrophage will engulf and kill *Mycobacterium tuberculosis* by phagocytosis. During phagocytosis, reactive oxygen intermediates and nitric oxide in the form of peroxynitrite radicals has able to kill pathogen (Abbas et al., 2012). Reactive oxygen intermediates are needed to kill pathogen, however excessive reactive oxygen intermediates will cause DNA damage and induce lipid peroxidation. Excessive nitric oxide will increase amount of reactive nitrogen species. It is produced from reaction between nitric oxide and superoxide radical. Reactive nitrogen species are strong pro-inflammation molecule. Reactive nitrogen species will cause tissues damage. High concentration of nitric oxide kill pathogens, immune cells, and other cells in the vicinity (Zhang et al., 2011).

Free radical is difficult to measure directly, but it can be measured effectively using lipid peroxidation concentration that measured by malondialdehyde concentration (Dalle-Donne et al., 2006). Nitric oxide can be measured by nitrite concentration (Jose et al., 2003). In normal condition, antioxidant system will neutralize free radical. Low endogenous antioxidant will form more free radicals. Body needs exogenous antioxidant. *Coriolus versicolor* is medicinal mushroom used in Japan, China, Korea and other Asian countries. Polysaccharide krestin is extraction product of *Coriolus versicolor.*

Polysaccharide krestin imitates activity of superoxide dismutase. Superoxide dismutase will change superoxide into hydrogen peroxide (Ooi and Liu, 2000). Powdered polysaccharide krestin contain 34 – 35 soluble carbohydrate (91 – 93% β–glucan), 28 – 35% protein, 7% moisture, 6 – 7% ash, and the rest are free sugars and amino acids (Cui and Chisti, 2003). β–Glucan can be used as antioxidant and scavenger for free radicals (Chen and Seviour, 2007). The objective of this research was to know the influence of polysaccharide krestin from *Coriolus versicolor* extract on nitrite and malondialdehyde concentrations of mice serum exposed by *Mycobacterium tuberculosis.*

METHODS

Research site

The research was conducted in the Molecular Genetics Laboratory, Departement of Biology, Faculty of Science and Technology, Universitas Airlangga, Jalan Mulyorejo Kampus C, Surabaya, East Java, Indonesia.

Material

Polysaccharide krestin was obtained from *Coriolus versicolor* extract method, adapted from Wahyuningsih (2014), *Mycobacterium tuberculosis* (0.25 Mc. Farland) was got from Balai Besar Laboratorium Kesehatan, Surabaya, East Java, Indonesia. Malondialdehyde assay kits were purchased from Northwest Life Science (Washington DC). Nitrite assay used Griess reagent. 10mL Griess reagent I was made from 0.3% sulfanilamide in 2.5% phosphoric acid. 10mL Griess reagent II was made from 0.3% naphtylendiamine in 2.5% phosphoric acid.

Animal and experimental design

This research used 30 adult female mice (*Mus musculus*) strain Balb/C, age between 8 – 10 weeks, weight between 30 – 40 g. Mice were obtained from Faculty of Pharmacy, Airlangga University (Surabaya, Indonesia). Mice went to acclimatization for a week. Completely randomized design was used with six groups and five repetition, namely control (K), positive control (K+), negative control (K-). It is treated by polysaccharide krestin before being exposed by *M. tuberculosis* (P1), by polysaccharide krestin after being exposed by *M. tuberculosis* (P2) and and by polysaccharide krestin before-after being exposed by *M. tuberculosis* (P3). Mice were exposed to *M. tuberculosis* (10^6 cell/mL) twice through intraperitoneally with two weeks gap from the first to the second exposure. 50 mg/kg BW of polysaccharide krestin was added by gavage in seven days.

Serum isolation

One week after polysaccharide krestin adding, 1mL intra-cardiac blood was taken using 23G injection needle and collected to 1.5mL micro tube. Blood was incubated for 2 hours. Blood was centrifuged at 3000 rpm for 10 min. Supernatant layer was taken for nitrite and malondialdehyde assays.

Nitrite assay

After creating the dilution (0 M, 0.2 M, 0.4 M, 0.6 M, 0.8 M, 1.0 M) from nitrite standard (0.69 mg $NaNO_2$ in 100mL aquadest), standard

nitrite regression equation y = ax + b was made. y is optical density value, and b is nitrite concentration. 50 µL serum was prepared in 1.5mL micro tube. 100 µL Griess reagent I and 100 µL Griess Reagent II were added to serum. After 10 – 15 min incubation, optical density values were read at 540 nm. Nitrite concentrations (M) were known after putting optical density values in standard nitrite regression equation.

Thiobarbituric Acid Reactive Substance (TBARS) assay

For serum preparation, 200 µL TCA 10% was added to 100 µL of each serum. After 5 min incubation on ice, serum was centrifuged at 14.000 rpm for 5 min. 200 µL of each serum was transferred to 1.5mL micro tubes. To each of the serum, 200 µL TBA was added. Serum was homogenized and incubated at 100^0C for 60 min. After cooling down, serum was homogenized and centrifuged briefly. Optical density values were read at 540 nm. Malondialdehyde concentrations (µM) were known after put optical density values in standard malondialdehyde regression equation Y = 0.0007X – 0.0003; y is optical density value and b is malondialdehyde concentration.

Data Analysis

All values were analyzed statistically by one way analysis of variances (ANOVA) to determine whether there were differences in the average of variable among treatments. If there were significant differences, Duncan test (α = 0.05) was taken to determine the best treatment.

RESULT AND DISCUSSION

Nitrite Concentration

Free radical is difficult to be directly measured. Free radical is measured by other substance indirectly like nitrite (NO_2^-). Nitrite is product of nitric oxide, O_2 and H_2O reaction (Gharavi and El-Kadi, 2003). According to the result we obtained (Figure 1), the highest nitrite concentration was shown by treatment K- (2.938 M) and the lowest nitrite concentration was shown by P1 group (1.364 M). ANOVA Statistical analysis showed that there was significant difference in the average of variable among treatments (0.036 < 0.05). According to Duncan test resulted in Figure 1 (different characters showed significant difference), the best treatment was identified. No significant difference was observed in groups of K, K+ and P2. Groups of P1 and P3 showed significant differences compared to K- group. Figure 1 showed that the present of polysaccharide krestin before exposure (P1) and before-after exposure (P3) decreased nitrite concentration significantly.

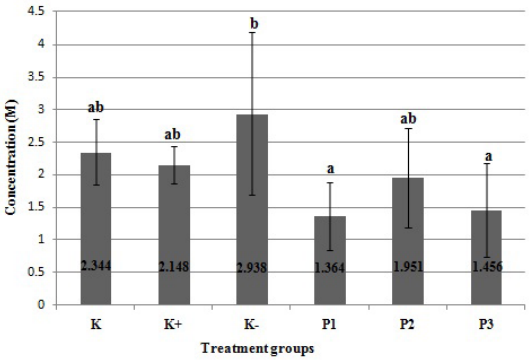

Figure 1. Nitrite Concentration among Research Groups. Note: different letters in each section show significant difference.

Reactive nitrogen intermediates are free radicals that able to kill pathogen. In macrophage, reactive nitrogen intermediates are formed by inducible nitric oxide synthase (iNOS). iNOS catalyzes arginine conversion to citrullin and produced nitric oxide. In phagolysosome, nitric oxide, hydrogen peroxide and superoxide form peroxynitrite radicals to kill pathogen (Abbas et al., 2012). In cells/tissues, nitric oxide is not stable and has very short half life (Zhang et al., 2011). Nitric oxide can make reaction with O_2 and H_2O to form stable metabolites including nitrite and nitrate. Generally, Nitric oxide is neutralized by endogenous antioxidant but exogenous antioxidant sometimes is needed to utilize body system against excessive free radicals. Polysaccharide krestin are exogenous that potential antioxidant in term of combating the free radicals. Chen and Seviour (2007) stated that polysaccharide krestin containing β-glucan could be used as free radicals scavenger.

In this research, adding polysaccharide krestin showed significantly different nitrite concentration among treatment groups. K- group that was exposed by *Mycobacterium tuberculosis* without adding polysaccharide krestin showed highest nitrite concentration. Excessive free radicals cannot be neutralized when free radicals were increased and amount of endogen antioxidant was constant. It is important to remember that nitric oxide made by iNOS is beneficial to the host defense reaction by contributing to microbial killing. In this condition, nitric oxide has cyto-protective characteristic. When large amounts of nitric oxide formed by iNOS surpass the physiological amounts of nitric oxide, nitric oxide has cytotoxic characteristic. Nitric oxide action in immune regulation depends on con-

centrations, cellular environment and whether cells have undergone prior activation (Guzik and Guzik, 2003).

According to Figure 1, group of K+ added by polysaccharide krestin without *M. tuberculosis* exposure had similar average nitrite concentration with K group although the concentration was lower than K- group. The presence of nitrite concentration of K group showed that body still produced free radical although it did not exposed by pathogen. As stated by Caramori and Papi (2004), oxidant generation was part of the normal metabolism of many types of cells and is critical for cell homeostasis.

Significant decline of nitrite concentration was showed by P1 and P3 groups. In P1 group, adding polysaccharide krestin before exposure could stimulate enzyme formation related to suppression of free radical. Cui and Chisti (2007) stated that polysaccharide of *C. versicolor* might give benefit in general healthy by inducing enzymes that mop up free radicals and mitigate oxidative damage. Adding polysaccharide krestin before exposure can prevent free radical formation. This evidence was supported by Pham-Huy et al. (2008) that classify antioxidant based on their mode on action including preventive antioxidant. Modes of preventive antioxidant actions are non-radical decomposition of LOOH and H_2O_2, sequestration of metal by chelating and quenching of active O_2.

Polysaccharide krestin induces antioxidant formation that breaks free radical chain including superoxide dismutase, catalase and glutathione peroxidase. As stated by Ooi and liu (2000), polysaccharide krestin has SOD-mimicking activity. Polysaccharide krestin induces glutathione peroxidase in mouse peritoneal macrophage (Pang et al., 2000). Enzyme that related to nitric oxide is superoxide dismutase that changed into superoxide radical from reduction of univalent oxygen molecule into H2O2. It was changed to H2O and O2 by catalase. Reactive nitrogen species would not be formed because nitric oxide did not react with superoxide. There was no structural change of polysaccharide krestin which added before exposure because β-glucan of polysaccharide krestin was resistant from enzymatic proteolysis of gastric. In P3 group, polysaccharide krestin which was added before exposure induced antioxidant forming so that antioxidant was formed and could bind with free radical.

According to Figure 2, P2 group that added polysaccharide krestin showed no significant difference. In this treatment, polysaccharide krestin did not actively work because polysaccharide krestin that was used had variation in molecular weight and branches. As stated by Chan et al. (2009), most of the β-glucan based on extract had variation in molecular weight and branches. β-Glucan with different molecular weight and branches possibly have variation in immune potency. Chan et al. (2009) also stated that variation in molecular size, branching frequency and solution conformation had an impact on the elimination half life, volume of distribution and clearance.

Malondialdehyde Concentration

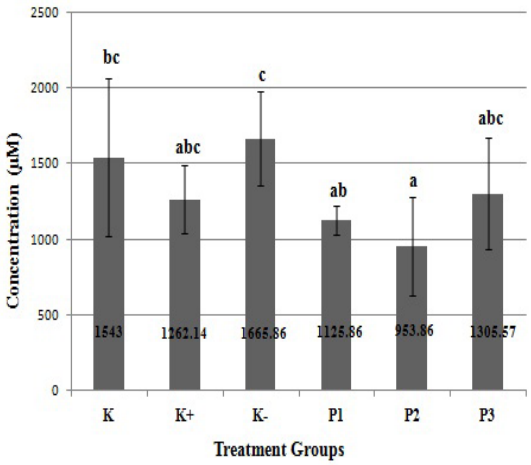

Figure 2. Malondialdehyde Concentration among Research Groups. Note: different letters in each section show significant difference.

Reactive oxygen species or nitrogen oxygen species are generally too reactive and have short half life to allow direct measurement in cells/tissues or body fluid. Molecular products that were formed from the reaction of reactive oxygen species or reactive nitrogen species with bio-molecules are more stable than reactive oxygen species or reactive nitrogen species themselves. Commonly, reactive oxygen species or reactive nitrogen species have been tracked by measuring stable metabolites and/or concentrations of their oxidation target products including malondialdehyde, lipid peroxidation left product (Dalle-Donne et al., 2006). According to the result in Figure 2, the highest malondialdehyde concentration was shown by K- group (1665.86 µM) and the lowest malondialdehyde concentration was shown by P2 group (953.86 µM). ANOVA statistical analysis showed that there was significant difference in the average of variable among treatments ($0.028 < 0.05$). Analysis was continued to Duncan test to determine the best treatment. According to Dun-

can test result in Figure 2 (different characters showed significant difference), no significant difference was shown by K+ and P3 groups. Group of K showed significant difference compared to P2 group. Significant difference was also shown by P1 and P2 groups compared to K- group. Figure 2 showed that the presence of polysaccharide krestin before exposure (P1) and after exposure (P2) decreased malondialdehyde concentration significantly.

Reactive oxygen intermediates are formed by activated macrophage. Reactive oxygen intermediates such as superoxide radical, hydroxyl radical, and single oxygen are needed to kill pathogen along with nitric oxide (Abbas et al., 2012) but excessive hydroxyl radical can make reaction with poly unsaturated fatty acid to form malondialdehyde. In this research, adding polysaccharide krestin showed difference on nitrite concentration among groups. According to Figure 2, K- group that was exposed by *M. tuberculosis* without adding polysaccharide krestin showed higher malondialdehyde concentration than K group. Excessive free radical without sufficient antioxidant increased malondialdehyde production. This evidence is supported by Dagli et al. (2003), who stated that excess malondialdehyde, as a by-product of free radicals can be related to decreased blood and tissue antioxidant levels.

Group of K+, added by polysaccharide krestin without *Mycobacterium tuberculosis* exposure showed similar average of malondialdehyde concentration with K group, although the concentration was lower than K- group. Under normal circumstances, the major sources of oxidants and free radicals produced in the body occur by mitochondrial activity, electron transport chains, phagocyte cells and the endogenous enzyme system (Dagli et al., 2004).

Significant decrease of malondialdehyde concentrations were observed in P1 and P2 groups. In P1 group, that was added polysaccharide krestin before *M. tuberculosis* exposure, could stimulate enzyme formation to bind with free radical. Polysaccharide krestin induced glutathione forming.

In P2 group that was added polysaccharide krestin after *M. tuberculosis* exposure, stimulated forming of macrophage as phagocytes cell, therefore, the immune system was raised. This evidence was supported by Cui and Chisti (2003) who stated that main effects of using polysaccharide krestin are inducing production of interleukin-6, interferon, immunoglobulin-G, macrophages and T-lymphocytes. The increasing of activated macrophages helped raising a better condition of mice, therefore, production of endogenous antioxidant increased indirectly. Polysaccharide krestin could bind with receptors on immune cell membrane, particularly macrophage because it had β-glucan. Multicellular organisms possess receptors, called pattern recognition receptors (PRRS), to detect innately non-self structures (including pathogen-associated molecular patterns or PAMPs) (Brown and Gordon, 2005). Chen and Seviour (2007) reported that β-glucan probably act as PAMPs and are recognized by appropriate cell surface receptors, initiating immune responses. Specific receptor to recognize β-glucan is Dectin-1. Malondialdehyde forming could be prevented by glutathione. Based on Pham-Huy et al. (2008), glutathione is classified in preventive antioxidant.

Polysaccharide krestin stimulated glutathione forming. Polysaccharide krestin induced glutathione peroxides activity in mouse peritoneal macrophages (Pang et al., 2000). Ozkan et al. (2010) reported that significant increased of glutathione peroxides and decreased of malondialdehyde were observed in acetylsalicylic acid and β-glucan treatment. β-Glucan appeared to attenuate the gastric damage. Glutathione peroxidase in erythrocytes oxidized will reducte glutathione into glutathione sulphide. Glutathione sulphide protected lipid membrane and haemoglobine from oxidation of peroxide. Glutathione is formed by glutamic acid, serine and glycine (Eide, 2011). Polysaccharide krestin contain large amounts of aspartic acid and glutamic acid (Ooi and Liu, 2000). Acidic and neutral amino acids such as aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, and leucine (Cui and Chisti, 2003). Polysaccaride krestin could induce glutathione forming because it contained the same amino acid as glutathione.

Group of P3 showed lower malondialdehyde concentration than K- group but did not show significant difference. In P3 group, polysaccharide krestin was added twice, before and after *M. tuberculosis* exposure. Therefore immunostimulator capability of polysaccharide krestin increased. This evidence was supported by research conducted by Nurdini (2015) that reported lowest interleukin-10 concentration found in mice that were added polysaccharide krestin before and after *M, tuberculosis* exposure. While interleukin-10 was in low concentration, production of Th1 increased. Raising amount of Th1 increased amount of activated macrophages so macrophages could do phagocytosis. Phagocytosis produced reactive oxygen intermediates and nitric oxide. While more of reactive oxygen intermediates and nitric

oxide were produced, possibilities of nitrite and malondialdehyde forming were higher. The capability of polysaccharide krestin to mop up free radical would decrease because the amount of free radicals exceeds the capacity of polysaccharide krestin.

There was different result in P3 nitrite group and P3 malondialdehyde group. P3 nitrite group showed significant difference compared to K- group but P3 malondialdehyde group did not. The difference could happen because immune response of each individual could be different toward nitrite and malondialdehyde formation. Fungal β-glucan-induced immune responses are different in their actions based on individual immune system (Chen and Seviour, 2007).

Research results useful to give the immune system so as to prevent the transmission of pulmonary TB disease due to infection with *M. tuberculosis*. It is given that TB is a tropical disease that desperately need attention to be tackled. *Mycobacterium tuberculosis* is easily transmitted through the air when the patient coughs or sneezes TB, even when spitting and talking. One patient can spread the TB bacteria in 10-15 people in one year. In general, the reaction of the immune system can stop the progression of TB germs. If the immune system decreases the bacteria will grow, and consequently in a few months in question would be tuberculosis.

CONCLUSION

It was concluded that adding polysaccharide krestin could influence nitrite and malondialdehyde concentration of mice serum exposed by *Mycobacterium tuberculosis*. Adding polysaccharide krestin before exposure (P1) and before-after exposure (P3) could decrease nitrite concentration. Adding polysaccharide krestin before exposure (P1) and after exposure (P2) could decrease malondialdehyde concentration. Based on the evidences, adding polysaccharide krestin before *Mycobacterium tuberculosis* exposure could decrease both of nitrite and malondialdehyde concentrations.

ACKNOWLEDGEMENT

The authors are grateful for the financial support provided by the Center through Annual Research Project DIPA PUPT 2014, Airlangga University.

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