Immunomodulatory Activity of Yogurt Fortified with Honey and Hibiscus sabdariffa L. On Reactive Oxygen Intermediate and Nitric Oxide Secretion

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ABSTRACT
Yogurt is a food probiotic which are enriched with microbes that can raise the body's immune system. Rosella (Hibiscus sabdariffa Linn.) is known to have anthocyanin compounds that have antioxidant and immunomodulatory effects. The aim of this research was to determine the effect of immunomodulatory activity of yogurt which fortified by rosella (Hibiscus sabdariffa L.) extract to increase the secretion of Reactive Oxygen Intermediate (ROI) and Nitric Oxide (NO). This research was conducted in vivo using 25 male mice with Balb/C strain divided into 5 groups consisted of normal group, plain yogurt treated group and plain yogurt added with 2%, 4%, and 8% rosella yogurt treated group. Treatment was given for 21 days orally. On the 22nd day, the mice were sacrificed, the peritoneum macrophage cells were taken and then tested for the level of Reactive Oxygen Intermediate (ROI) and Nitrite Oxide (NO). The results showed that there was an increase of ROI and NO secretion in 2%, 4%, and 8% rosella yogurt treated groups compared to plain yogurt group. The fortification of yogurt with rosella extract and honey increased the potency of yogurt in increasing the immunomodulatory activity.

Keywords: Immunomodulator, Yogurt, Hibiscus sabdariffa L, ROI, NO epidermidis

INTRODUCTION
Immune system is the mechanism used by the body to maintain integrity in danger that can be caused by the environment. Macrophage is an effector in the immune system that acts as pathogen or germ that will damage the system in the body (Abbas, et al, 2017), either directly through intracellular phagocytosis or indirectly by releasing Nitric Oxide (NO), Reactive oxygen Intermediate (ROI) and cytokines (Baratwidjaya, 2006).

Immunity can be increased by administering immunomodulators. Immunomodulators are pharmacological agents that can modulate a partial immune response that is spurred by an immune response and on the other hand it inhibit some of the other immune systems (Akrom, et al, 2015). Yogurt is a product which is reported to have immunomodulatory activity. Yogurt is produced from milk fermentation by Lactic Acid bacteria (LAB) i.e. Lactobacillus bulgaricus and S thermophilus. Yogurt is also used as a symbiotic food ingredient which is a source of probiotics with prebiotic supplements et al, 2011). Previous studies reported that prebiotic treatment containing innate L. casei stimulated the immune response, with an increase in the specific markers of CD-206 and TLR-2 cells (Galdeano and Perdigo, 2006). TLR-2 is one of the receptors which recognize bacteria and will activate the innate immunity (Hikmah and Dewanti, 2011; Uematsu and Akira, 2008).

Rosella (Hibiscus Sabdariffa L) is one of medicinal plants with potential immunomodulator activity (Fakeye, 2008). Rosella calyx contains anthocyanins as natural pigments which give red color in rosella calyx. Anthocyanin compounds found in rosella flowers can be used as antioxidants, antihypertensive, and antihypercholesterolemic compounds (Chiu, et al, 2015; Herrera-Arellano et al, 2007; Hirunpanich et al., 2005; Usoh, et al, 2005). Anthocyanin compounds will be more stable in acidic environment (Lima, et al., 2011).
Therefore, the addition of rosella to yogurt will improve anthocyanin stability in yogurt which has low acidity. The addition of rosella extract to yogurt will also provide a refreshing sour taste, fragrant aroma and increase the antioxidant and immunomodulatory activity.

**MATERIAL AND METHODS**

**Materials and subjects**

Rosella calyx used in this research was obtained from local market of Yogyakarta, Indonesia. The sample was verified and identified in Biology Laboratory of Universitas Ahmad Dahlan. The honey used was pure honey which obtained from local market with brand of Madu Rambutan®. Milk which was used for yogurt preparation was Dancow® full cream milk. The *L. bulgaricus* and *S. thermophilus* were obtained from Food and Nutrition Laboratory of Universitas Gadjah Mada.

The animals used were mice of Balb/c strain with 1 month of age and were obtained from the Integrated Research Laboratory of Universitas Gadjah Mada (Laboratorium Penelitian dan Pengujian Terpadu, LPPT UGM). The use of test animal in this research had received the ethical approval from the Commission for Research Ethics of Universitas Ahmad Dahlan with Number: 011710141.

**Preparation of rosella extract**

Rosella extraction was carried out using infusion method and water as the solvent. A total of 100g of rosella calyx powder was added with extra water (2 times the weight of the ingredients) and 100mL of water were put into the infusion pan. The mixture was heated in a water bath for 15min, after the temperature in the pan reached 90°C then the mixture was stirred immediately. The infusion was filtered while hot, then adequate hot water was added to obtain an infusion volume of 100mL.

**Preparation of yogurt**

The milk solution of 13% concentration was prepared and pasteurized with temperature of 60°C for 30min and then was cooled to 43°C. The culture of *L. bulgaricus* and *S. thermophilus* were added into the milk of 2% volume and (1:1) ratio. The mixture was then incubated at 37°C for 16 h. After the incubation, the yogurt was stirred and packed in a container.

**Preparation of fortified yogurt with honey and rosella**

The formula for preparing yogurt fortified by rosella extract (Table I). Honey was added to give a better taste on yogurt and to increase the immunomodulatory effect.

**Animal treatment**

The treatment animals (25 mice) were divided into 5 groups, each group consisted of 5 mice. All mice were adapted for 1 week. Group I was group of a normal mice that were only given with food and drink. Group II was a group that was given with plain yogurt. Group III, IV, and V were groups that were given fortified yogurt with honey and rosella of 2%, 4%, and 8% respectively. Each group was treated for 21 days orally with dose of 2mL/kgbw. On day 22, the mice were induced by using lipopolysaccharide (LPS).

After induction, mice in each group were dissected, macrophages were isolated and the secretion of Reactive Oxygen Intermediate (ROI) and Nitric Oxide (NO) were tested.

**Macrophage isolation and culture**

All mice were exposed to chloroform. Then, mice were placed in the supine position, the skin of the abdomen was opened, and the peritoneal sheath was cleaned using 70% alcohol, then 10 mL of cold RPMI was injected into the peritoneal cavity and it was waited for 3min while shaking it slowly. Peritoneal fluid was removed from the peritoneal cavity by pressing the inner cavity with 2 fingers, the liquid was aspirated with an injection syringe, selected on the non-fat portion and away from the intestine. The solution was then centrifuged at 1200rpm, 4°C for 10min. Supernatant was removed, the macrophages obtained were resuspended with 1000µL of complete medium.

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**Table I. The formula of yoghurt fortified by rosella extract and honey**

<table>
<thead>
<tr>
<th>Materials</th>
<th>I</th>
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<tbody>
<tr>
<td>Rosella Extract</td>
<td>-</td>
<td>2mL</td>
<td>4mL</td>
<td>8mL</td>
</tr>
<tr>
<td>Honey</td>
<td>-</td>
<td>8mL</td>
<td>8mL</td>
<td>8mL</td>
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<tr>
<td>Yogurt</td>
<td>100mL</td>
<td>90mL</td>
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<td>90mL</td>
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The cell suspension was grown into a 24-well microtiter plate well with a density of 1x10^5/mL for NO and ROI secretion assay.

**Nitric Oxide (NO) Secretion Assay**

The NO assay was carried out using Griess Reaction Assay. Griess A solution was prepared by dissolving 0.1g N-(1-naphthyl) ethylene diamine hydrochloride in 100 mL of distilled water. Griess B solution was prepared by dissolving 1g of sulfanilamide in 100mL of 5% (v/v) orthophosphoric acid, both were stored at 0-4°C and were protected from light. Standard nitrite was made by dissolving 69.0mg of sodium nitrate in 100mL of distilled water, was stored at a temperature of 0-4°C, protected from light, and standard solution of this stock between 0-50µM nitrite was made (Nurkhasanah, et al., 2017).

A total of 100µL of sample and standard nitrite were included in a 96-well microtiter plate, if less than 100µL of sample was used, the volume was increased to 100µL with water or medium. 100mL of griess solution was added and it was then incubated at room temperature for 15min until color changes occurred. After all the samples and standards were included in the microtiter plate, the absorbance was measured using an ELISA reader. The size of the absorbance for the sample and the standard was 540nm (Nurkhasanah et al., 2017).

**ROI Secretion Assay**

Macrophages were cultured in RPMI medium and were washed, then were added with 500µL of NBT solution, 1mg/mL of PBS containing 125µg/mL PMA and were incubated in a 5% CO2 incubator at 37°C for 60min. Cells were washed with PBS 3 times, were dried at room temperature and were fixed with absolute methanol for 30min. After it was dry, it was applied with 2% neutral red solution. The percentage of macrophage cells that showed NBT reduction was calculated from about 100 cells which were examined by a light microscope with 400 times magnification (Nurkhasanah et al., 2017).

**RESULTS AND DISCUSSIONS**

In the fermentation process with lactic acid bacteria (LAB), lactose is changed into lactic acid. The lactic acid produced can change the flavor of milk and increase its acidity (reduce its pH). The lower the pH or acidity of the milk after fermentation is the fewer the microbes that can survive (Widodo, 2002). Another organic acid produced during the fermentation process were lactic acid, acetic acid, citric acid, succinic acid, malic acid, acetaldehyde, diacetyl, and acetoin. These organic acids were reported to increase the effect of rosella as functional food with antibacterial effect (Astawan et al., 2011).

Previous research reported that rosella calyx was found to increase the NO and ROI secretion (Nurkhasanah and Zulkarmen, 2014). The addition of rosella extract into yogurt is expected to increase the immunomodulatory effect as the anthocyanin will be more stable in lower pH.

**Increase of NO Secretion**

Macrophage activity in secreting ROI was observed using the NBT (Nitro Blue Tetrazolium) reduction test. Nitro Blue Tetrazolium is formazan salt which diffuses into cells and breaks down into formazan by tetrazolium succinate reductase, a system that is included in the chain of mitochondrial respiration and is active in living cells. Nitro Blue Tetrazolium reduction shows an increase in respiration followed by the formation of superoxide (O2-) which would reduce NBT to formazan reaction products which are insoluble in blackish blue. Formazan is formed from NBT due to the rupture of N-N bonds between molecules and the N groups capture H+ which acts as an intermediate electron acceptor. H+ is produced from NADH which is then oxidized by the reaction of NADH → NAD^+ + H+.

The microscopic view of ROI can be seen in Figure 1.

![Microscopic results of the peritoneal macrophages with ROI assay, positive (black arrow) and negative secretion of ROI (white arrow)](attachment)

Figure 1. Microscopic results of the peritoneal macrophages with ROI assay, positive (black arrow) and negative secretion of ROI (white arrow)
Administration of fortified rosetta yogurt as an immunomodulator could stimulate the immune system by markedly increasing ROI secretion (Table I).

This research found that treatment with rosetta fortified yogurt increased the ROI secretion of animal compared with normal and plain yogurt group. This finding agreed with Verdiana (2014) which found that the administration of rosetta calyx ethanol extract could stimulate phagocytic activity of macrophages and increased reactive oxygen intermediate and nitric oxide.

The increased secretion of ROI could be caused by anthocyanin compound. Anthocyanin compounds contained in rosetta will bind to TLR cell surface receptors that are owned by macrophage cells. This can stimulate the activity of macrophages so that it can increase the secretion of Reactive Oxygen Intermediate (ROI) and Nitric Oxide (NO) which are the components of the nonspecific immune system. Anthocyanins with proteins can also increase the specific immune system by mediating ROI secretion of macrophages. ROI will increase the activity of T lymphocytes in secreting IFN-γ so that it will cause an increase in the number and activity of lymphocytes so that the specific cellular immune responses will increase (Akrom et al., 2015).

**Increase of NO Secretion**

The ability of peritoneal macrophages in mice to secrete NO was tested by using a Griess Reaction Assay which produced a pink color which could be measured by using the ELISA Reader. In this method nitrite is reacted with the diazotation reagent, sulfanilamide in acidic environment to form the diazonium salt. The diazonium salt then reacts with the coupling reagent that is N-naphthyl-ethylenediamine into a stable azo form. Griess Reaction Assay will produce an intense pink color. Nitrite absorbance was read at a wavelength of 540 nm. The increase of NO secretion (Table III).

Table III shows the NO levels in the rosetta fortified yogurt group of 2%, 4%, and 8% were higher than the yogurt group. This indicates that the addition of rosetta to yogurt can improve the activity of rosetta in increasing the immune system response. The content of anthocyanin compounds contained in rosetta calyx plays an important role in this case. The previous research also found that administration of ethanol extract of rosetta calyx could be used as a preventive therapy that could increase NO secretion in DMBA-induced rats. (Nurkhasanah and Zulkarnen, 2014). Anthocyanin also play as antioxidant and reduces the toxicity of free radical species produced by DMBA.
Some bacterial components including phenol soluble modulin are active ligand which will activate the TLR (Hikmah and Dewanti, 2011; Uematsu and Akira, 2008). Some of rosella components including anthocyanin have a part which meets with TLR. It will bind to macrophage cell surface receptor (TLR). This can stimulate macrophage activity so that it can increase the secretion of Nitric Oxide and Reactive Oxygen Intermediate which are the components of the nonspecific immune system. Phenol complexed with proteins can also enhance the specific immune system because of the NO released by macrophages. It can diffuse into T lymphocytes and increase T lymphocyte proliferation (Batteli et al., 2005; Van der veen et al., 2000).

The NO level of rosella fortified yogurt group of 4% was highest among the other groups. It suggested that optimum concentration of rosella was 4%.

CONCLUSION
Fortification of yogurt with rosella extract could increase the activity of yogurt in increasing ROI and NO secretion. This could be caused by the increase of stability of anthocyanin in acidic environment of yogurt.

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