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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 mices 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 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 (Baratawidjaya, 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 antihyper-cholesterolemic 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).

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

Materials	I	II	III	IV
Rosella Extract	-	2mL	4mL	8mL
Honey	-	8mL	8mL	8mL
Yogurt	100mL	90mL	90mL	90mL

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. thermophylus* 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 infundation 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.

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 diaminehydrochloride 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 microtitter 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 NADH → NAD^++ H^+. The microscopic view of ROI can be seen in Figure 1.

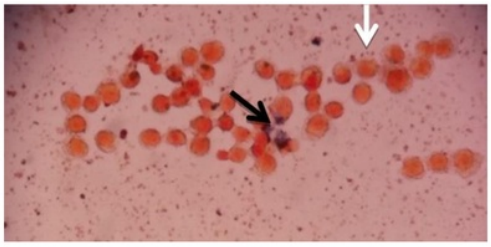

Figure 1. Microscopic results of the peritoneal macrophages with ROI assay, positive (black arrow) and negative secretion of ROI (white arrow)

The more formazan is formed, the higher the percentage of macrophages that secrete ROI is, this indicates a higher activity of ROI secretion by macrophages. The provision of LPS in mice makes it possible to cause mice to become stressed and feverish so that there is a decrease in NBT activity which is characterized by reduced formation of insoluble formazan.

Table I. The percentage of ROI secretion by peritoneal macrophages of Balb/c mice which were treated by rosella fortified yogurt

Group	Average % ROI ± SD secretion
Normal	20.67±2.45
Plain yoghurt	37.33±3.25[(a)]
Fortified yogurt honey & rosella 2%	47±4.45[(a)]
Fortified yogurt honey & rosella 4%	68.67±10.40[(b)]
Fortified yogurt honey & rosella 8%	52.67±8.81[(b)]

a: significant difference compared to the normal group ($p<0.05$); b: significant difference compared to the plain yogurt group ($P<0.05$)

Table II. The results of NO secretion levels on macrophages of balb / c mice given fortified yogurt with rosella and honey

Group	Average% NO ± SD secretion
Normal	0.4658±0.1054
Yoghurt	2.1732±0.7544[(a)]
Fortified yogurt honey & rosella 2%	5.3593±0.2868[(b)]
Fortified yogurt honey & rosella 4%	7.9106±1.5457[(b)]
Fortified yogurt honey & rosella 8%	3.5469±0.9978[(b)]

a: significant difference compared to the normal group ($p <0.05$); b: significant difference compared to the yogurt group ($P <0.05$)

Administration of fortified rosella yogurt as an immunomodulator could stimulate the immune system by markedly increasing ROI secretion (Table II).

This research found that treatment with rosella 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 rosella 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 rosella 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 rosella fortified yogurt group of 2%, 4%, and 8% were higher than the yogurt group. This indicates that the addition of rosella to yogurt can improve the activity of rosella in increasing the immune system response. The content of anthocyanin compounds contained in rosella calyx plays an important role in this case. The previous research also found that administration of ethanol extract of rosella calyx could be used as a preventive therapy that could increase NO secretion in DMBA-induced rats. (Nurkhasanah and Zulkarmen, 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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REFERENCES

Abbas A., Lichtman AH. and Pilla S. 2017. *Cellular and Molecular Immunology* (9th ed.). Elsevier.

Akrom A., Widjaya A. and Armansyah T. 2015. Extract of Black Cumin (Nigella sativa) Seed Increases Macrophage Phagocytic Activity of Swiss Mice Infected with Lysteria monocytogenes. *Jurnal Kedokteran Hewan*, *9*(2), 94–100.

Astawan M., Wresdiyati T., Arief II. and Febiyanti D. 2011. Potency of Indigenous Probiotic Lactic Acid Bacteria as Antidiarrheal Agent and Immunomodulator. *Jurnal Teknologi Dan Industri Pangan*, *XXII*(1), 11–16.

Baratawidjaya. 2006. *Imunologi Dasar*. Jakarta: fakultas Kedokteran Universitas Indonesia.

Chiu C., Chen J., Chou F. and Lin H. 2015. Hibiscus sabdariffa Leaf Extract Inhibits Human Prostate Cancer Cell Invasion via Down-Regulation of Akt/NF-(B/MMP-9 Pathway. *Nutrients*, *7*, 5065–5087. https://doi.org/10.3390/nu7075065

Fakeye T. 2008. Toxicity and immunomodulatory activity of fractions of Hibiscus sabdariffa Linn (family Malvaceae) in animal models. *African J. Trad. Complementary and Alternative Medicine*.

Galdeano CM. and Perdigo G. 2006. The Probiotic Bacterium Lactobacillus casei Induces Activation of the Gut Mucosal Immune System through Innate Immunity. *Clinical and Vaccine Immunology*, *13*(2), 219–226. https://doi.org/10.1128/CVI.13.2.219

Herrera-Arellano A., Miranda-Sánchez J., Ávila-Castro P., Herrera-Álvarez S *et al.,* 2007. Clinical effects produced by a standardized herbal medicinal product of Hibiscus sabdariffa on patients with hypertension. A randomized, double-blind, lisinopril-controlled clinical trial. *Planta Medica*, *73*(1), 6–12. https://doi.org/10.1055/s-2006-957065

Hikmah N. and Dewanti IDAR. 2011. Peran Toll-like receptors (TLRs) pada innate immunity. *Stomatognatic (JKG Unej)*, *8*(1), 21–26.

Hirunpanich V., Utaipat A., Morales NP., Bunyapraphatsara N., Sato H., Herunsalee A., and Suthisisang C. 2005. Antioxidant effects of aqueous extracts from dried calyx of Hibiscus sabdariffa Linn. (Roselle) in vitro using rat low-density lipoprotein (LDL). *Biological & Pharmaceutical Bulletin*, *28*(3), 481–484. https://doi.org/10.1248/bpb.28.481

Lima ADJB., Corrêa AD., Saczk AA., Martins MP. and Castilho RO. 2011. Anthocyanins , pigment stability and antioxidant activity in jabuticaba [myrciaria cauliflora (mart .) O . Berg]. *Rev. Bras. Fruit*, *33*(3), 877–887.

Nurkhasanah and Santoso DR., Fauziah R. 2017. The immunomodulatory effect of Zingiber cassumunar ethanolic extract on phagocytic activity , nitrit oxide and reaxtive oxygen intermediate secretions of macrophage in mice. *IOP Conf. Series: Materials Science and Engineering*, *259*(2017), 1–10. https://doi.org/10.1088/1757-899X/259/1/012007

Nurkhasanah and Zulkarmen LR. 2014. Efek ekstrak etanol kelopak rosella (Hibiscus sabdariffa, L.) terhadap sekresi nitrit oksida (NO) makrofag peritoneum tikus yang diinduksi 7,12-dimethylbenz(a)antracene

(DMBA). *Media Farmasi*, *11*(2), 155–166.

Uematsu S. and Akira S. 2008. Toll-Like Receptors (TLRs) and Their Ligands. In *Handbook of Experimental Pharmacology* (pp. 1–21).

Usoh IF., Akpan EJ., Etim EO., and Farombi EO. 2005. Antioxidant Actions of Dried Flower Extracts of Hibiscus sabdariffa L. On Sodium Arsenite - Induced Oxidative Stress in Rats, *4*(3), 135–141.

Widodo W. 2002. *Bioteknologi fermentasi susu*. Malang: Pusat Pengembangan Bioteknologi, Universitas Muhammadiyyah Malang.

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