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Immunomodulatory activity of yogurt fortified with roselle (*Hibiscus sabdariffa* L.) extract

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Abstract

Yogurt is a probiotic food that can boost the immune system even when added with fruit extract, such as roselle (*Hibiscus sabdariffa*). An *in vivo* study was carried out to prove the potency of yogurt fortified with roselle extract in enhancing the immune system using 25 male BALB/c mice. The test animals were divided into five groups namely (I) normal group, (II) plain yogurt group, and groups of yogurt fortified with (III) 2%, (IV) 4%, and (V) 8% of roselle extract, respectively. The effects of these treatments were evaluated from macrophage activity using the combination of latex beads and Giemsa staining. The amounts of actively phagocytic macrophages in groups III, IV, and V were 89, 97, and 45%, respectively, while the MTT assays showed that their lymphocyte proliferation activities, represented by absorbance values, were 0.50, 0.79, and 0.68%, respectively. Immunocytochemistry observation found that the secretions of interleukin-10 and interleukin-14 increased. Based on the statistical analysis, there was a significant increase in the phagocytic activity of macrophages, lymphocyte proliferation, and secretion of IL-10 and IL-14. Overall, yogurt fortified with 2, 4, and 8% of roselle extract can be used as immunomodulators.

Introduction

Yogurt is the fermentation result of dairy products that most commonly involves lactic acid bacteria (LAB) such as *Lactobacillus bulgaricus*, and *Streptococcus thermophilus*. *S. thermophilus* is a lactic acid-producing bacterium, while *L. bulgaricus* has proteolytic activity and peptidase, both of which play an essential role in forming the texture and taste of yogurt. They produce acid and can reduce the pH to lower than 4.6, thus contributing to the sour taste of yogurt (Baglio, 2014).

The LAB produce compounds that can kill pathogenic bacteria (Klaenhammer *et al.,* 2005; Parada *et al.,* 2007). Prior studies reported that consuming LAB can improve cellular and humoral immune systems by enhancing the lymphocyte proliferation and the secretions of interferon-γ (IFN-γ), interleukin-12 (IL-12), IL-10, immunoglobulins (Ig) A, IgE, IgG, and IgM (Gackowska *et al.,* 2006), as well as T cells and B cells that produce IL-14 (Galdeano and Perdigón, 2006; Rungsri *et al.,* 2017).

Roselle (*Hibiscus sabdariffa* L.) has been widely used to prevent various diseases because it is rich in antioxidants (Nurkhasanah *et al.,* 2017b; 2018). Roselle extract reportedly increases the secretions of IL-10 and IL-14 (Nurkhasanah, 2015), and this antioxidant activity is attributable to the high content of anthocyanins. Anthocyanin can stimulate the immune system by increasing the cytokine production (Zafra-Stone *et al.,* 2007). Because it is stable in acidic or low pH environments like yogurts (Oancea and Drăghici, 2013), the addition of roselle extract to yogurt is expected to stabilise and accentuate the activity of anthocyanin, which, in the present work, was observed from the increased phagocytic activity of macrophages, lymphocyte proliferation, and cytokine production.

Materials and methods

Materials

The roselle plants used in the present work were cultivated in Kulon Progo, Yogyakarta, Indonesia, and their calyxes were picked and tested for authenticity at the Laboratory of Biology, Universitas Ahmad Dahlan. The test animals were BALB/c mice, which were procured from the Integrated Research Laboratory, Universitas Gadjah Mada. The research design and the use of test animals in the present work have received ethical approval from the Research Ethics Committee of Universitas Ahmad Dahlan (No. 011710141).

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Preparation of extract

Firstly, 100 g of roselle calyx powder was added with 200 mL of water, and heated at 90°C for 15 min. Then, the resulting extract was filtered, and its volume was added with water up to 100 mL. The concentration of the stock was 100%.

Preparation of yogurt

Firstly, 13 g of Dancow® full cream milk was added with water up to 100 mL (concentration 13%), and continuously stirred while heated until the temperature reached 60°C. This temperature was maintained for 30 min, and then left to cool to 43°C. Next, 3 mL of starter culture containing *L. bulgaricus* and *S. thermophilus* (at a volume ratio of 1:1) was added to the milk up to 100 mL. This mixture was then incubated at 37°C for 16 h.

Fortification of yogurt with roselle extract

The yogurt was added with roselle extract at different concentrations; 2, 4, and 8% (v/v). It was also added with honey to reduce the sourness and improve the overall taste. The formula of fortification is presented in Table 1.

Table 1. The formula of yogurt fortification with roselle extract.

Ingredient	I	II (2%)	III (4%)	IV (8%)
Roselle extract	-	2 mL	4 mL	8 mL
Honey	-	8 mL	8 mL	8 mL
Yogurt	100 mL	90 mL	90 mL	90 mL

Animal treatment

The test animal (25 BALB/c mice) was first divided into five groups, each consisting of five mice, and then allowed to acclimatise for 1 w. Group I (normal) only received food (BR2) and drink, while Group II was given plain yogurt. Groups III, IV, and V were given yogurt that had been fortified with honey and roselle extract at concentrations of 2, 4, and 8%, respectively. These oral treatments lasted for 21 d, and were administered once a day, with a dose of 2 mL/kg BW. On day 22, the test mice were given lipopolysaccharide (LPS) to activate the immune system.

The isolation of peritoneal macrophage

After 21 d of treatment, the test mice were sacrificed by chloroform narcosis. Then, they were placed on their backs, the skin of the abdomen was cut open, and the peritoneal sheath was cleaned with 70% alcohol. Next, 10 mL of cold RPMI medium was injected into the peritoneal cavity, and it slowly massaged for 3 min. Peritoneal fluid was removed from the peritoneal cavity by pressing the organ with two fingers, aspirated with a syringe injection tube, and then centrifuged at 1,200 rpm for 10 min. The supernatant was removed, and the remaining pellet was added with 3 mL of RPMI medium. The number of cells was counted with a haemocytometer, and resuspended to achieve a density of 2.5×10^6 cells/mL. The isolated macrophage was cultured in the RPMI medium supplemented with FBS 10% in a 5% CO_2 incubator for 24 h before receiving further treatment.

Immunocytochemistry assay for IL-10 and IL-14 detection

The immunochemistry assays of IL-10 and IL-14 was performed following the methods previously described (Nurkhasanah, 2015). The macrophage isolated from the treated mice was cultured using coverslips in a 6-well microplate for 24 h in a 5% CO_2 incubator. Afterward, the medium was removed, and the macrophage was washed using PBS. The assay used two specific antibodies, namely anti-IL-10 and anti-IL-14 (Biovision), and was carried out indirectly using a secondary antibody labelled with a chromogen, *i.e.,* dimethylamino benzidine (DAB). Finally, the Mayer-Hematocsylin counterstain was added to achieve clearer visualisation. Brown colour marked the cells with positive expression of IL-10 or IL-14, while blue colour marked the cells with negative expression of IL-10 or IL-14.

Phagocytosis assay

After the isolation of peritoneal macrophage, the cells were cultured using coverslips that were placed inside a 6-well microplate, and then incubated for 24 h. Afterward, the cells were washed twice with RPMI-1640. Each well was added with the suspension of latex, with a density of 5×10^6 (200 µL/well) and incubated in a 5% CO_2 incubator (37°C, 60 min). The cells were then washed with PBS three times to remove excess latex beads, dried at room temperature, fixed with methanol for 30 s, stained with Giemsa for 10 min, and then washed with distilled water.

The number of macrophages phagocytosing latex beads and the number of latex beads phagocytosed by macrophages were counted under a light microscope with 400× magnification; this yielded the number of active phagocytic cells and phagocytosis index (Nurkhasanah *et al.,* 2017a).

Lymphocyte proliferation assay

In the present work, the lymphocyte proliferation assay used the cells isolated from the spleen of the treated mice. The spleen was placed in a Petri dish containing 5 mL of RPMI medium. Then,

the complete RPMI medium was injected into the spleen tissue to isolate the lymphocytes.

The cell suspension was centrifuged at 1,200 rpm for 10 min to obtain the pellets. After the supernatant was discarded, the pellet was suspended in 1 mL of ammonium chloride buffer to lyse the erythrocytes. The cells were then mixed using a pipette, and left at room temperature for 5 min. The pellets were washed twice using RPMI, and centrifuged at 1,200 rpm (4°C, 10 min). Afterward, the lymphocyte cells were counted using a haemocytometer, added with RPMI to obtain a density of 1.5×10^6 cells/mL, divided, placed in a 96-well microplate (100 µL/well), and then incubated in a 5% CO_2 incubator at 37°C for 72 h.

Following the incubation, each well was added with 50 µL of 0.1 mg/mL MTT, and then incubated in a 5% CO_2 incubator (37°C, 4 h). Living cells would react with MTT and form purple formazan. The MTT reaction was stopped by adding 100 µL of 10% SDS solution in 0.01 N HCl to each well. Then, the microplate was stored at room temperature for 12 h in the dark, and the absorbance was measured using an ELISA reader at 595 nm.

Results and discussion

The present work used healthy animals, which after 21 d of treatment, were injected by lipopolysaccharide (LPS), an antigen to induce the immune response through the activation of macrophage and cytokine secretion. Different responses between the groups were observed.

Increased interleukin-10 and interleukin-14 after the yogurt + roselle extract treatment

The potency of yogurt as an immunomodulator has been reported in several studies (Astawan *et al.,* 2011; Santagati *et al.,* 2012; Rungsri *et al.,* 2017); hence, the fortification of yogurt with roselle extract is expected to amplify the immunomodulatory effects of both yogurt and roselle extract.

Cytokines, including IL-10 and IL-14, play a crucial role in the regulation of immune response. Interleukin-10 (IL-10) is expressed by myeloid, dendritic cells (DC), and macrophages to respond to microorganisms invading through extracellular signal-regulated kinase 1 (ERK1) and ERK2 pathways. Through these pathways, the signalling escalation is activated in the cells, resulting in the expression of IL-10 (Saraiva and O'Garra, 2010). The present work found that treatment with yogurt that had been fortified with roselle extract 2, 4, and 8% increased the IL-10 expression in macrophages, as shown in Table 2.

The IL-10 level also increased more significantly in the Groups III, IV, and V (yogurt + roselle extract treatment 2, 4, and 8%, respectively) than Groups I (normal) and II (plain yogurt). This finding is in agreement with Fakeye (2008) and Nurkhasanah (2015) who also reported that roselle extract and fraction increase IL-10 expression. The same case applies to plain yogurt (Group II) which also increased such expression higher than Group I (normal), and stimulated the immune response. Prior study have also correlated yogurt consumption with the increased secretions of IL-6 and IL-10 in patients with inflammatory bowel disease (Shadnoush *et al.,* 2013). The release of probiotic from this food product can trigger DC to secrete IL-10 (Becker *et al.,* 2004).

An increase in IL-10 expression not only leads to lowered TNF-α, a pro-inflammatory cytokine, but it also influences the B cell maturation and antibody production (Fakeye, 2008). In several studies, IL-10 has been proven as a potent anti-inflammatory cytokine (Couper *et al.,* 2008; Saraiva and O'Garra, 2010). Its elevation enhances the differentiation of IL-10-secreting T_{reg} cells, thus providing a positive regulatory loop for its induction. IL-10 also activates mast cells and enhances the functions of CD8+ T cells, NK cells, and B cells. Therefore, IL-10 is a cytokine that significantly shapes the development of an immune response (Saraiva and O'Garra, 2010).

Table 2. The expression of interleukin-10 and interleukin-14 in different groups of test mice.

Group	IL-10 expression	IL-14 expression
I (normal)	47.67 ± 4.07^a	62.07 ± 6.83
II (plain yogurt)	66.26 ± 0.81^b	66.08 ± 7.38
III (yogurt + roselle 2%)	80.24 ± 0.41^a	71.14 ± 1.86^b
IV (yogurt + roselle 4%)	90.25 ± 1.55^a	88.47 ± 0.75^{ab}
V (yogurt + roselle 8%)	73.86 ± 1.99^a	75.93 ± 7.13^b

[a]Significantly different from Group II (plain yogurt) ($p < 0.05$), and
[b]Significantly different from Group I (normal) ($p < 0.05$).

Similar to IL-10, Groups III, IV, and V showed a more significant increase in the secretion of IL-14 than Groups I and II, as seen in Table 2. Among the concentrations of the added roselle extract (*i.e.,* 2, 4, and 8%), the highest level of IL-14 was produced in Group IV that received yogurt fortified with 4% of roselle extract. This finding corresponds to Nurkhasanah (2015) who also detected an increase in IL-14 after the administration of roselle extract. Anthocyanin, a compound found abundantly in roselle extract, is the prospective antioxidant (Zafra-Stone *et al.,* 2007) that can induce the production of some cytokines. Interleukin-14 is a cytokine that has a vital role in the immune system as it can activate B cells and T cells (Leca *et al.,* 2008).

Increased phagocytic activity

The phagocytic activity of macrophages can be stimulated by the presence of antigens in the form of macromolecules or pathogens. In the present work, it was evaluated using latex beads; latex is a non-self macromolecule, which is widely used to stimulate phagocytic activities of macrophages in a model (Molina-Bolívar and Galisteo-González, 2005). Phagocytosis is a process of eliminating pathogens, including bacteria and cell debris, then the ingested materials are digested in the phagosomes.

The phagocytosis assay observes several parameters, namely active phagocytic cell (APC), phagocytic capacity, and phagocytosis index (PI). The APC represents the number of macrophage cells that phagocytoses latex cells (per 100 macrophage cells). Phagocytic capacity is the number of latex beads that are phagocytosed in 100 macrophage cells, and PI is the average number of latex beads that are phagocytosed by active phagocytic cells. The phagocytic activities of Groups III, IV, and V (yogurt + roselle extract) are presented in Table 3.

The results showed that treatments with yogurt fortified with 2 and 4% of roselle extract increased the phagocytic activity of macrophages. SFA, phagocytic capacity, and PI of Groups III and IV were significantly enhanced than those of Group I (normal). On the contrary, Group V (yogurt + 8% roselle extract) showed a more noticeable decrease in SFA, phagocytic capacity, and PI than Groups I (normal) and II (plain yogurt) (Table 3).

The macrophage phagocytic activities decreased as the roselle extract concentration increased. When applied at high concentrations, the antioxidant effects of roselle extract become the major mechanism. Antioxidants eliminate reactive oxygen species (ROS) production, and cause further oxidative damage to cells. ROS are also known to activate macrophages that engulf harmful microorganisms and destroy them in phagosomes. In other words, a decrease in ROS lowers the phagocytic activity of macrophages (Wang *et al.,* 2019). Plain yogurt elevates SFA value and phagocytic capacity. In a previous study, probiotic food has been proven to boost the immune system through the activation of phagocytosis of macrophage to eliminate the invader (Toma and Pokrotnieks, 2006).

Roselle contains antioxidant compounds that stimulate the immune system by preventing free radicals from causing cellular damage. Macrophages are known to be able to produce free radicals and ROS which determine defence against microbial invaders or other non-self antigens (Puertollano *et al.,* 2011). Excess ROS can lower the immune system, damage macrophages, and induce the aging process of macrophages (Wang *et al.,* 2019; Fresta *et al.,* 2020). Therefore, in an attempt to maintain the immune system, the generation and elimination of ROS should be in balance. The provision of appropriate antioxidants can help avoid the damage caused by free radicals to immune cells.

Based on the analysis results, yogurt fortified with roselle extract could increase the phagocytic activity. Group IV (yogurt + roselle extract 4%) exhibited the highest activity, as observed from APC, phagocytosis capacity, and PI. Previous studies on

Table 3. The effects of different treatments on the phagocytic activity of macrophage based on active phagocytic cells, phagocytic capacity, and phagocytosis index in different groups of test mice.

Group	Active phagocytic cell	Phagocytic capacity	Phagocytosis index (PI)
I (normal)	73 ± 7.18	155 ± 18.86^{ab}	2.120 ± 0.09^{a}
II (plain yogurt)	87 ± 7.13^{b}	187 ± 9.88^{b}	2.151 ± 0.10
III (yogurt + roselle 2%)	89 ± 8.04^{b}	248 ± 21.14^{ab}	2.740 ± 0.23^{ab}
IV (yogurt + roselle 4%)	97 ± 1.00^{b}	306 ± 5.03^{ab}	3.158 ± 0.02^{ab}
V (yogurt + roselle 8%)	45 ± 16.09^{ab}	98 ± 33.02^{ab}	2.207 ± 0.15^{a}

^{a}Significantly different from Group II (plain yogurt) ($p < 0.05$), and ^{b}Significantly different from Group I (normal) ($p < 0.05$).

yogurt have revealed the significance of probiotics in boosting the immune system. LAB reportedly enhance immune response activity by increasing the activity of NK cells (Gill *et al.,* 2001). Anthocyanins, which are found abundantly in roselle extract, also boost the immune system through increasing the phagocytic activity of macrophage.

Enhanced lymphocyte proliferation

Lymphocytes play an essential part in immune response, both in innate and adaptive immunity, and an enhanced lymphocyte proliferation is a parameter of a good immune response. The proliferation of lymphocytes is the first phase in a proper immune response as it produces effector lymphocytes that help remove a present antigen or memory lymphocytes that eliminate the same antigen in the future. In the present work, the lymphocyte proliferation activity was observed using the MTT assay. The increased number of lymphocyte cells after the administration of yogurt fortified with roselle extract is presented in Table 4.

Table 4. The absorbance values of MTT in the lymphocyte proliferation assay of different groups of test mice.

Group	Absorbance
I (normal)	0.27 ± 0.01^{a}
II (plain yogurt)	0.40 ± 0.02^{b}
III (yogurt + roselle 2%)	0.50 ± 0.03^{ab}
IV (yogurt + roselle 4%)	0.79 ± 0.06^{ab}
V (yogurt + roselle 8%)	0.68 ± 0.04^{ab}

[a]Significantly different from Group II (plain yogurt) ($p < 0.05$), and [b]Significantly different from Group I (normal) ($p < 0.05$).

The results showed that the proliferation in Group II (plain yogurt) was higher than Group I (normal) ($p < 0.05$), but the most enhanced multiplication activities were identified in Groups III, IV, and V (yogurt + roselle extract). The higher the concentration of roselle extract added to yogurt, the larger the lymphocyte proliferation. Also, the enhanced lymphocyte proliferation in the treatment groups was the cumulative effect of yogurt and roselle extract, which may be attributable to anthocyanins—a compound that is more stable in environments with lower pH (Puspita *et al.,* 2018).

Based on the analysis results, LPS induced the activation of lymphocytes, *i.e.,* cells that trigger humoral and cellular immunological responses. T lymphocytes, when stimulated, will release lymphokines, which function to activate macrophages in phagocytosis. Lymphokine and IL-2 are released by active lymphocyte cells, inducing lymphocyte proliferation (Pinchuk, 2002). T lymphocytes regulate specific immune responses associated with T cells, and play a central role in the activation and proliferation of B cells to generate antibodies and activate macrophage in phagocytosis (Baratawidjaya, 2006). Lymphocyte proliferation starts with binding antigens with the surface of T cells receptors, which will induce the secretion of IL-1, and subsequently, activate G-protein to produce phospholipase C. Phospholipase C enzyme will hydrolyse phosphatidylinositol bisphosphate (PIP2) to produce glycerol (DAG) and inositol triphosphate (IP3) as a reactive product. Furthermore, IP3 stimulates cytoplasm, and increases the release of Ca^{2+}, which will trigger the production of protein kinase C and 5-lipoxygenase enzymes. The result of IL-2 stimulation will induce the proliferation of B cells or T cells (Otsuka *et al.,* 2006).

Conclusion

Yogurt fortified with roselle extract can increase the immune response by enhancing expressions of interleukin-10 and interleukin-14, macrophage phagocytosis activity, and lymphocyte proliferation.

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