**BUKTI KORESPONDENSI ARTIKEL**

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

**NAMA JURNAL** : International Food Research Journal 28(2): 255 - 261 (April 2021) (Q3)

**RANGKUMAN KORESPONDENSI**

| TANGGAL | EDITOR | AUTHOR |
|---|---|---|
| 11-2-2020 | Penerimaan submission artikel dari Editor, dan konfirmasi dari Editor kalau artikel diterima akan dikenakan APC USD 250. | |
| 12-2-2020 | | Persetujuan author untuk membayar 250 USD, jika artikel diterima |
| 27-6-2020 | | Pertanyaan status artikel |
| 10-7-2020 | Respon, bahwa artikel masih menunggu hasil review | |
| 20-7-2020 | Respon, bahwa artikel masih menunggu hasil review, dan akan dikirim segera | |
| 22-7-2020 | Pengiriman hasil review dengan status MAJOR REVISION | |
| 9-9-2020 | | Author mengirimkan perbaikan terhadap masukan reviewer dan mengirimkan sertifikat proof reading |
| 14-10-2020 | Pernyataan diterima (accepted) dari editor | |
| 16-10-2020 | | Pengiriman copyright agreement |
| 4-12-2020 | | Pertanyaan author manuskrip akan dipublikasikan di volume dan issu berapa? |
| 29-12-2020 | Respon editor, bahwa manuskrip akan dipublikasikan pada April 2021 | |

**Korespondensi 11-02-2020**

IFRJ19540 - International Food Research Journal Inbox ×

**International Food Research Journal** <onbehalfof@manuscriptcentral.com> 11 Feb 2020, 16:03
to me, ibufathan

11-Feb-2020

IFRJ19540 - The activity of yoghurt fortified with rosella (Hibiscus sabdariffa L) extract as immunomodulator

Dear Dr. Mahfudh:

Thank you very much for submitting your manuscript to International Food Research Journal (IFRJ).

We confirm that, if accepted for publication, Article Processing Charges (APC) will apply to your article with the amount of USD250.

Please confirm that you support open access publishing and that you will pay the APC if your manuscript is accepted.
Thank you in advance for your cooperation. I look forward to hearing from you.

Sincerely,
Ms. Norhafizah Mohamad Noh
Editorial Assistant
International Food Research Journal

**Korespondensi 12-2-2020**

**Nurkhasanah Mahfudh** <nurkhas@gmail.com> 12 Feb 2020, 11:24
to International

we agree to pay the article processing charge (250 USD) if the manuscript is accepted.

Regards

•••

--
Dr, Nurkhasanah, M.Si., Apt
Fakultas Farmasi
Universitas Ahmad Dahlan
Yogyakarta
Jl. Prof Soepomo, Janturan, Yogyakarta

**Korespondensi 27-6-2020**

**Nurkhasanah Mahfudh** <nurkhas@gmail.com> 27 Jun 2020, 13:38
to International

Dear Editor of IFRJ

Could we find the information about the progress of manuscript with ID IFRJ19540 - The activity of yoghurt fortified with rosella (Hibiscus sabdariffa L) extract as immunomodulator?
The status in the system is still under review.

We have submitted stince Nov 2019.

regards

•••

**Korespondensi 10-7-2020**

**International Food Research Journal / UPM** <ifrj@upm.edu.my> 10 Jul 2020, 08:28
to me

Dear Dr. Mahfudh,
Apologies for the late reply and inconvenience caused.
Upon checking, your paper is waiting for another review before a decision can be made.
I will remind the editor to take necessary action accordingly.

Apologies again.

Regards,

Ms. Norhafizah Mohamad Noh
Editorial Assistant
International Food Research Journal Editorial Office

**Korespondensi 20-7-2020**

**International Food Research Journal / UPM** <ifrj@upm.edu.my> Mon, 20 Jul 2020, 13:33
to me

Dear Dr. Mahfudh,
Apologies for the late response.
Upon checking in the system, your paper received one review and I already urge the editor to decide on this paper as it has been a while.
Hopefully you will receive the feedback from the editor as soon as possible.

Thank you.

Regards,

Ms. Norhafizah Mohamad Noh
Editorial Assistant
International Food Research Journal Editorial Office

**Korespondensi 22-7-2020**

International Food Research Journal - Decision on Manuscript ID IFRJ19540

Inbox ×

**International Food Research Journal** <onbehalfof@manuscriptcentral.com> 22 Jul 2020, 10:42
to me, ibufathan

22-Jul-2020

Dear Dr. Mahfudh

Manuscript IFRJ19540, entitled 'The activity of yoghurt fortified with rosella (Hibiscus sabdariffa L) extract as immunomodulator', which you submitted to International Food Research Journal, has been reviewed. The comments of the reviewers appear below.

Based on the advice received, your manuscript could be reconsidered for publication should you be prepared to incorporate MAJOR REVISIONS.

When preparing and submitting your revised manuscript (which should be labelled as such), you will be able to respond to the comments made by the reviewers, the Associate Editor and the Editor in the space provided. You can use this space to document the changes you make to the original manuscript and submit a list of responses to the comments and suggestions, item by item.

Your list of responses should also be uploaded as a separate file (i.e. Word) in addition to your comments in the system. For the separate uploaded file, please explicitly and clearly state where and how you have addressed the referees' criticisms or provide your reasons for rebuttal of the criticisms. Please submit 'Responses to Reviewers' Comments' for each of the point raised by each referee with numbering system (include line and page numbers), and to cross reference it with the revised manuscript. Please also highlight the changes to your manuscript within the document by highlighting the text or using colored text.

Please note that there is/are comments on the English grammar. Therefore, please make sure to send the manuscript to English Editing Services, and attach the certificate or evidence that you are doing so.

Please download a copy of your manuscript from the system at STEP 3: File Upload after clicking 'CREATE REVISION' and use the file to do the revision. This is because the file has been edited according to the Journal's format.

The manuscript must be submitted electronically within 60 days, or it may be considered withdrawn.

Thank you for submitting your manuscript to International Food Research Journal; I look forward to receiving your revision. Instructions on how to revise your manuscript are included at the end of this letter.

Sincerely,
Dr. Chin Ping Tan
Editor, International Food Research Journal

**********

Reviewers' Comments to Author:

Reviewer: 1

Comments to the Author
Dear author,
Please revise the article according to the highlighted comments. Need to do major correction on the article before it can be accept for publication.

**********

HOW TO REVISE YOUR MANUSCRIPT

To revise your manuscript, log in to https://mc.manuscriptcentral.com/upm-ifrj and enter your Author Centre, where you will find this manuscript listed under 'Manuscripts with Decisions'. Under 'Actions', click on 'Create a Revision'.

You can also click the link below to start the revision process (or continue the process if you have already started your revision) for your manuscript. If you use the link below you will not be required to log in to ScholarOne Manuscripts.

*** PLEASE NOTE: This is a two-step process. After clicking on the link, you will be directed to a webpage to confirm. ***

https://mc.manuscriptcentral.com/upm-ifrj?URL_MASK=5f4a46f25f254e61baa0d37daded96eb

You will be unable to revise the files that you submitted for the original version of the manuscript. Instead, revise your manuscript using a word-processing program and save it on your computer. Once the revised manuscript is prepared, you can upload it and submit it through your Author Centre.

**One attachment** • Scanned by Gmail


**HASIL REVIEW DARI REVIEWER**

International FOOD RESEARCH Journal

# The activity of yoghurt fortified with rosella (Hibiscus sabdariffa L) extract as immunomodulator



| | |
|---|---|
| Journal: | *International Food Research Journal* |
| Manuscript ID | IFRJ19540 |
| Manuscript Type: | Original Article |
| Keyword: | yoghurt, rosella, immunomodulator |
| | |

# The activity of yoghurt fortified with rosella (*Hibiscus sabdariffa* L) extract as immunomodulator


## Abstract

Yoghurt is probiotic food which can use as functional food to increase the immune system. The addition of some fruit extract could increase the activity. Rosella (*Hibiscus sabdariffa* L) was reported to have immunomodultaory activity. The *in vivo* study was carried out to prove the potency of yoghurt fortified by rosella ectract in increasing of immune system. This research was carried out *in vivo* using 25 male Balb./C mice which were divided into 5 groups consisting of normal groups, yogurt treatment group, rosella-fortified yogurt treatment group consisting of 2%, 4% and 8% of rosella extract. The results showed that there was a significantly increase in the macrophage phagocytic activity and lymphocyte proliferation ($p<0.05$). The percentage data on phagocytic activity of macrophages in yoghurt fortified rosella 2%, 4% and 8% respectively were 89%, 97% and 45%. While the lymphocyte proliferation in yoghurt fortified with rosella 2%, 4% and 8% respectively 0,50%, 0,79% and 0,68%. The observation on Interleukin-10 and interleukin-14 were also found to increase significantly ($p<0.05$). It can be concluded that the administration of fortified yoghurt rosella extract 2%; 4%; 8% can be used as an immunomodulatory


**Keywords:** yoghurt, rosella, immunomodulator

## Introduction

Yogurt is a fermented product from dairy products which is involved the lactic acid bacteria (LAB) in the process. Some bacteria were frequently used including *Lactobacillus bulgaricus* and *Streptococcus thermophiles*. *S. thermophilus* is a bacterium that produces lactic acid while *L. bulgaricus* has proteolytic activity and peptidase, these two bacteria play an important role in the formation of texture and taste in yogurt. The bacteria *L. bulgaricus* and *S. thermophilus* will produce more acid and can reduce the pH of 4.6 or lower so that the taste of yogurt becomes acidic (Baglio, 2014).

Lactic acid bacteria (LAB) have the ability to produce compounds that can kill pathogenic bacteria (Azcárate-peril *et al*., 2005; Parada *et al*., 2007). Some researchers report that consuming Lactobacillus BAL can improve cellular and humoral immune systems including increased population and proliferation of lymphocyte cells, production of interferon-γ (IFN- γ) cytokines, interleukin-12 (IL-12), IL-10, Th immune cells and immunoglobulins (Ig) A, IgE, IgG, and IgM (Gackowska *et al.,* 2006) and T cells and B cells that produce IL-14 (Galdeano and Perdigo, 2006; Rungsri *et al*., 2017).

Rosella (*Hibiscus sabdariffa* L) has been widely used to prevent disease because of the high content of antioxidants (Nurkhasanah *et al.,* 2017; Nurkhasanah *et al*., 2018). Rosella extract were also reported to increase the secretion of IL-10 and IL-14 (Nurkhasanah, 2015). The high content of antocyanins in the rosella extract is closely related to this

antioxidant activity. Anthocyanin can stimulate the immune system by increasing cytokine production (Zafra-stone *et al*. 2007). Antocyanin compounds are more stable in acidic or low pH conditions (Oancea and Drăghici, 2013). Addition of roselle extract to yogurt will increase the stability of the anthocyanin and consider to increase the activity.

## Materials and methods

### *Materials*

The rosella calyx was found from Kulon Progo, Yogyakarta, Indonesia and identified its authenticity in the Laboratory of Biology of Universitas Ahmad Dahlan. The animal test used were Balb/C mice was found from Integrated Research Laboratory Universitas Gadjah Mada.

### *Preparation of extract*

The rosella calyx was extracted with water. The 100 g of rosella calyx powder was added with 200 mL of water and heated for 15 minutes on 90$^{o}$C. After heating, the extract was filtered and the volume of extract was added with water up to 100 mL. The concentration of the stock is 100%.

### *Yoghurt preparation*

The 13 grams of Dancow® full cream milk was mixed with water to 100 mL (concentration 13%) are heated until the temperature of 60°C while stirring and maintaining temperature for 30 minutes, then cooled it to 43°C.

Starter inoculation contain culture of *L. bulgaricus* and *S. thermophilus*) with a volume ratio of 1:1. The 3 mL of inoculation starter was added to the milk, until the volume of 100mL. The mixture was then incubated at 37°C for 16 hours.

*Fortification of rosella extract into yoghurt*

The yogurt was added with rosella extract with variations in concentrations of 2%, 4%, and 8% v/v. Beside the rosella extract, yoghurt was also added with honey to improve the taste and reduce the acid taste. The formula of fortification was presented in Table 1.

*Animal treatment*

The research design and use of test animals in this study have received ethical approval from the Ahmad Dahlan University Ethics Research Committee by number of 011710141. The test animal (25 mice) was divided into 5 groups, each group consist of 5 mice. All mice were adapted for 1 week. Group I is a normal group of mice that are only given food and drink. Group II is a group was given plain yogurt. Group III, IV and V were given fortified yogurt with honey and rosella 2%, 4% and 8% respectively. Each group was treated for 21 days orally with dose of 2mL/kgBW. On day 22 mice were induced by using lipopolysachcharide (LPS).

*The isolation of peritoneal macrophage*

Mice that have been treated for 21 days, then killed using narcose with chloroform. The mice are placed on their backs, then the skin of the abdomen is opened and cleaned in the peritoneal sheath with 70% alcohol. The 10 ml of cold RPMI was injected into the peritoneal cavity and wait for 3 minutes while slowly massaging. Peritoneal fluid is removed from the peritoneal cavity by pressing the organ with 2 fingers, then the fluid was aspirated with syringe injection tube. The liquid obtained was then centrifuged at 1,200 rpm for 10 minutes. The supernatant was removed and added 3 ml of RPMI medium. The number of cells was counted with a hemocytometer and was resuspended to make density of $2,5x10^6$ cell/ ml). The isolated macrophage was cultured by RPMI medium in 5% $CO_2$ incubator.

*Immunocytochemistry assay for detection of IL-10 and IL-14*

The immunochemistry of IL-10 and IL-14 was carried out using method as reported previously (Nurkhasanah, 2015). The specific antobody anti !L-10 (Biovision) and anti IL-14 was used for the study. The immunocytochemistry was carried out indirectly by secondary antibody and labelled with dimethyl amino benzidyne (DAB) as chromogene. Mayer-Hematocsylin counter stain was added to make clear observation. The cell with positive expression of IL-10 or IL-14 will show brown colour while the cells with negative expression of IL-10 or IL-14 will show blue colour.

*Phagocytosis assay*

Following isolation of peritoneal macrophage, the cells were cultured at the cover slips and placed on 6 wells microplate and then incubated for 24 hours. After incubation, the cells were washed twice with RPMI-1640. The suspension of latex with density of $5x10^6$ (200 microliter/wells) was added in each well and incubated at 5% $CO_2$ incubator, 37°C for 60 minutes. The cells were then washed with PBS 3 times to remove excess latex beads. The cells was dried at room temperature and fixed with methanol for 30 seconds. The cells was then stained with giemsa for 10 minutes and then washed with distilled water.

The number of macrophages that phagocyte latex beads and the number of latex beads phacytosed by macrophages is calculated under a light microscope with magnification 400x to calculate the active phagocytic cells and phagocytosis index (Nurkhasanah *et al*., 2017).

*Lymphocyte proliferation assay*

The lymphocyte proliferation assay was carried out using cells which isolated from the spleen of treated mice. The spleen was placed in a petri dish containing 5 mL of RPMI medium. The RPMI was inserted to the spleen to isolate the lymphatic fluid nad limphocyte cells.

The cell suspension was centrifuged at 1200 rpm for 10 minutes to get pellets. The supernatant was discarded, the pellet was then suspended in 1 mL of ammonium chloride buffer to lyse erythrocytes. Cells were then mixed using a pipette and left at room temperature for 5 minutes. The pellets were washed twice using RPMI and centrifuged at 1200 rpm, 4°C for

10 minutes. The lymphocyte cells were counted using haemocytometer and added with RPMI to get the density 1.5 x $10^6$ cells/mL. The cells were then divided into 96 wells microplate (100 µl per well) and incubated in a 5% $C0_2$ incubator at 37˚C for 72 hours.

Following incubation, each well was added 50 µl MTT 0.1 mg/ml and then incubated at 37˚C in the 5%$C0_2$ incubator for 4 hours. Living cells will react with MTT to form purple formazan. The MTT reaction was stopped by adding 100 µl of 10% SDS solution in 0.01 N HCl in each well. Then, the microplate is stored at room temperature for 12 hours in dark conditions. Then the absorbance was determined using ELISA reader with a wavelength of 595 nm.

**Result and discussion**

The treatment of yoghurt+rosella extract was carried out for 21 days. Following the complete of treatment, the mice was injected by lipopolysachcharide (LPS). The treatment of LPS will induce the immune respon through the activation of macrophage and cytokine secretion as LPS recognise as antigen. The different respon will occure between the groups.

*Increasing of Interleukin-10 and interleukin-14 after treatment with yoghurt+rosella exract*

Yogurt is prepared by adding lactic acid bacteria (LAB). This bacteria will convert natural milk sugar into lactic acid and cause the

increasing of acidity of milk into pH 4-5. It also change the consistency of milk from liquid become pasta. Generally yogurt cultures involve two or more different bacteria for the fermentation process, usually *Streptococcus salivarius and thermophilus and the genus Lactobacillus, such as L.acidophilus, bulgaricus, casei and bifidus*

The potency of yoghurt as immunomodulator has been reported in several studies (Astawan *et al*., 2011; Santagati *et al.,* 2012; Rungsri *et al*., 2017). The fortified of yoghurt with rosella extract was expected to increase the effect of yoghurt as well as rosella extract as immunomodulatory agent.

Interleukin-10 (IL-10) is expressed by myeloid, dendritic (DC) cells, and macrophages to respond microbes invading through extra cellular signal regulated kinase 1 (ERK1) and ERK2 pathways. Through this pathway the signaling escalation is activated in the cells resulting in the expression of IL-10 (Saraiva and O'Garra, 2010). The present study found that treatment with yoghurt fortified with rosella extract increase the IL-10 expression in macrophage cells as showed in Table 2.

The IL-10 level was found to increase in yoghurt+rosella extract group compare to plain yoghurt group as well as normal group. The finding was met to (Fakeye, 2008; Nurkhasanah, 2015) which reported that treatment with rosella extract and fraction increase the IL-10 expression. Increasing of IL-10 was followed by decreasing of pro inflammatory cytokine TNF-α and affected the B cell maturation and antibody production (Fakeye, 2008).

Treatment with plain yoghurt was also found to increase the expression of IL-10, but the treatment with yoghurt+rosella extract give higher increasing. The probiotic yoghurt was also found to stimulate the immune respon. It was reported that yoghurt probiotic could increase the IL-6 and IL-10 in patient with inflammatory bowel disease (Shadnoush *et al*., 2013). Probiotic can induce the dendritic cells and increase the production of IL-10 (Becker *et al*., 2004).

The increasing of IL-10 was followed by decreasing of TNF-α expression. IL-10 was reported as potent antiinflammatory cytokine in several studies (Couper *et al*., 2008; Saraiva and O'Garra, 2010). The increasing of IL-10 enhances the differentiation of IL-10-secreting Treg 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. So, IL-10 is a cytokine with important effects on the development of an immune response (Saraiva and O'Garra, 2010).

The current study also was found that treatment of yoghurt+rosella extract increase the IL-14 cytokines. The Table 1 showed that treatment with yoghurt+rosella extract give higher increasing compare to treatment with plain yoghurt as well as normal group.. This result showed that the highest level of IL-14 was showed in concentration treatment of yoghurt+ rosella extract 4%. The current study was met to the previous study (Nurkhasanah, 2015) which also reported the increasing of IL-14 after treatment with rosella extract. The antocyanin which found abundantly in rosella extract are potential antioxidant compound (Zafra-stone *et al*., 2007)

which can induce some cytokines production. Antioidants Interleukin-14 is a cytokine which has important role in immune system. Il-14 can produce following activation of B cell and T cell (Leca *et al*., 2008).

*Increasing of phagocytic activity*

The phagocytosis activity was evaluated using latex beads Macrophage activity can be stimulated by the presence of antigens in the form of macromolecules or pathogens. Latex is a macromolecule and considered as nonself which is used as a model to stimulate phagocytic activity of macrophages. Phagocytosis is a process of eliminating of bacteria and other non nonself.

The parameter for evaluating macrophage phagocytosis assay were the active phagocitic cell (APC), phagocytic capacity and Phagocytosis index (IP). The APC parameter represent the number of macrophage cells that phagocytes latex cells in 100 macrophage cells. The phagocytic capacity represented the amount of latex which is phagocytosied in 100 macrophage cells and the Phagocytosis Index (PI) represent the average of latex which is phagocytosed by active phagocytic cells. The result of phagocytic activity after treatment with yoghurt+rosella extract were presented in Table 3.

The result showed that there are significant differences between the treatment groups with the normal group as well as the yogurt group. In the comparison of normal groups with yogurt, it can be seen that there are significant differences in SFA and phagocytic capacity but there is no

significant difference in the phagocytosis index. Treatment with plain yoghurt has an effect in increasing the SFA value and phagocytic capacity. The previous study also reported that probiotic could increase the immune system through the activation of phagocytosis of macrophage to eliminat the invader (Toma and Pokrotnieks, 2006).

Rosella contains antioxidant compounds which have important role in immune system stimulaion through preventing of cell damage from free radicals. Macrophages are known to be able to produce free radicals and H2O2 which play an important role in defense against microbes or other nonself objects (Puertollano *et al*., 2011). The excessive of free radicals can cause damage to immune system. The provision of appropriate antioxidants is important to avoid damage of free radicals to immune cells.

The current study showed that yogurt fortified with rosella extract can increase the phagocytic activity and the highest activity with parameter of active phagocytic cells (APC), phagocytosis capacity and phagocytosis index was shown by the group of yoghurt which fortified by 4% rosella extract.

The several studies on yogurt was also revealed the effect of probiotics on increasing the immunity through. The lactic acid bacteria has been showed increase the activity of immune respon by increase the activity of NK cell (Gill *et al*., 2001). Antocyanin compounds, which is found abundantly in rosella extract was also reported to increase the the immune system through increasing of phagocytic activity of macrophage.

*The increasing of lymphocyte proliferation*

Lymphocyte cell has important role in immune respon both in the innate and adaptive immunity. The increasing of lymphocyte proliferation is one of parameter of good immune respon. In this study, the lymphocyte proliferation activity was observed using the MTT assay method. In this method, MTT (3- (4,5-dimethyllthiasil-2-yl) -2,5 diphenyl-tetrazolium bromide) tetrazolium salt will convert into a color product with a succulent tetrazolium reductase system in the living cell, through mitochondrial cell respiration. So that it will form formazan crystals. The formation of formazan crystals was correlated with the number of living cells. Then it can be said when the absorbance value is increased, it showed the more number of lymphocyte cells. The increasing number of lymphocyte cells after treatment with yoghurt fortified with rosella extract was presented on Table 4.

The results showed that the proliferation in plain yoghurt treated group was higher tnan normal group ($p<0.05$). Furthermore, the results lymphocyte proliferation was higher in the yoghurt fortified with rosella extract group. The higher concentration of rosella extract the higher of lymphocyte proliferation. This result showed that activity of increasing lymphocyte proliferation was cummulative effect of yoghurt and rosella extract. The activity of yoghurt fortified with rosella extract was much higher in increasing of lymphocyte proliferation could be caused by the antocyanin. Antocyanin will more stable in the lower pH and give higher activity (Puspita and Kumalasari, 2018).

In the recent study, the LPS induction will promote the activation of lymphocyte. Lymphocytes will play an important role in stimulating of humoral and cellular immunological responses. When T lymphocytes are stimulated it will release a lymphokine. Lymphokine has a role in stimulating and activating macrophages in carrying out their phagocytic functions. Active lymphocyte cells will produce lymphokine and IL-2 which trigger lymphocyte proliferation (Pincrok, 2002).

Lymphocyte cells as specific immune responses associated with T cells. T cell lymphocytes also play a role in B cell activation and proliferation in producing antibodies and activation of macrophages in phagocytosis (Baratawidjaya, 2006). The lymphocyte proliferation was induced by binding of antigens to the surface receptors of T cells with IL-1 from APC which activates G-protein to produce phospholipase C. The phospholipase C enzyme will hydrolyze phosphatidyl inositol biphosphate (PIP2) to produce glycerol (DAG) and inositol triphosphate (IP3) as a reactiv product. Furthermore, the release of Ca2 + increases due to stimulation by IP3 to the cytoplasm. Because an increase in Ca2 + will stimulate the protein kinase C and 5-lipoxygenase enzymes. As a result of this stimulation IL-2 will be produced which activates B cells/ T cells to proliferate (Otsuka *et al.*, 2006).

## Conclusion

Yoghurt fortified with rosella extract could increase the immune respon through the increasing of interleukin-10 and interleukin-14 expression, macrophage phagocytosis activity and lymphocyte proliferation.

**Acknowledgment**

The authors thank to Institute of Research, Development and Community Service of Universitas Ahmad Dahlan for funding this research through the Innovative Research Scheme.

**References**

Astawan, M., Wresdiyati, T., Arief, I.I. 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.

Azcárate-peril, M.A., Klaenhammer, T.R., Barrangou, R., Buck, B.L., Azcarate-peril, M.A. and Altermann, E. 2005. Genomic features of lactic acid bacteria effecting bioprocessing and health. FEMS Microbiology Reviews 29: 393–409.

Baglio, E. 2014. Chemistry and Technology of Yoghurt Fermentation. Spain: Springer.

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

Becker, C., Fantini, M. C., Schramm, C., Lehr, H.A., Wirtz, S., Nikolaev, A., … and Neurath, M. F. 2004. TGF-beta suppresses tumor progression

in colon cancer by inhibition of il-6 transsignaling. Immunity 21: 491-501.

Couper, K. N., Blount, D.G. and Riley, E.M. 2008. IL-10: The master regulator of immunity to infection. Journal of Immunologgy 180: 5771–77.

Fakeye, T. 2008. Toxicity and immunomodulatory activity of fractions of Hibiscus sabdariffa Linn (Family Malvaceae) in animal models. African Journal of Traditional Complementary and Alternative Medicine 5(4): 394–98.

Gackowska, L., Helmin-basa, A. and Kubiszewska, I. 2006. Combined effect of different lactic acid bacteria strains on the mode of cytokines pattern expression in human peripheral blood mononuclear cells. Journal of Physiology and Pharmacology 57(Suppl 9): 13–21.

Galdeano, C. M. and Perdigo, G. 2006. The probiotic bacterium L*actobacillus casei* induces activation of the gut mucosal immune system through innate immunity. Clinical and Vaccine Immunology 13(2): 219–26.

Gill, H.S., Rutherfurd, K.J. and Cross, M.L. 2001. Dietary probiotic supplementation enhances natural killer cell activity in the elderly: an investigation of age-related immunological changes. Journal of Clinical Immunology 21(4): 264–71.

Leca, N., Laftavi, M., Shen, L., Matteson, K., Jr Ambrus, J. and Pankewycz, O. 2008. Regulation of human interleukin 14 transcription in vitro and in vivo after renal transplantation. Transplantation 86(2): 336–41.

Nurkhasanah. 2015. The effect of rosella (Hibiscus sabdariffa L) treatment on IL-10 and IL-14 secretion on dimethylbenz (a) anthracene (DMBA) induced rat. International Journal of Pharmacy and Pharmaceutical Sciences 7(4): 402–4.

Nurkhasanah, Nurani, L.H. and Hakim, Z.R. 2017. Effect of rosella ( Hibiscus sabdariffa L) extract on glutathione-S-transferase activity in rats. Tropical Journal of Pharmaceutical Research 16(October): 2411–16.

Nurkhasanah, Santoso, D.R. and 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.

Nurkhasanah, Sulistyani, N. and Fatmawati, E., 2018. The increasing of catalase activity in dimethylbenz- α -anthracene (DMBA ) induced rat treated by Hibiscus sabdariffa L. extract. Pakistan Journal of Pharmaceutical Science 31(3): 851–56.

Oancea, S. and Drăghici, O., 2013, PH and thermal stability of anthocyanin-based optimised extracts of romanian red onion cultivars. Czech J Food Sci 31(3): 283–91.

Otsuka, H., Okano, T. and Kataoka, K. 2006. Activation of lymphocyte proliferation by boronate-containing polymer immobilised on substrate: the effect of boron content on lymphocyte proliferation, European Cells and Materials 12: 36–43.

382 Parada, J.L., Caron, C.R., Bianchi, A., Medeiros, P. and Ricardo, C. 2007.
383 Bacteriocins from lactic acid bacteria : purification , properties and use
384 as biopreservatives. Brazilian Archives of Biology and Technology
385 50(3): 521–42.
386 Pincrok, G. 2002. Schaum's Outline Immunology. United States: Mc-Graw
387 Hill.
388 Puertollano, M.A., Puertollano, E., de Cienfuegos, G.A. and de Pablo, M.A.
389 2011. Dietary antioxidants: immunity and host defense. Current Topics
390 in Medicinal Chemistry 11(14): 1752–66.
391 Puspita, W., Nurkhasanah and Kumalasari, I.D. 2018. Yoghurt fortified
392 formulation of lakum fruit (*Cayratia trifolia* ( L .) Domin ) extract as an
393 antioxidant. Traditional Medicine Journal 23(3): 91–96.
394 Rungsri, P., Akkarachaneeyakorn, N., Wongsuwanlert, M., Piwat, S. and
395 Nantarakchaikul, P. 2017. Effect of fermented milk containing
396 *Lactobacillus rhamnosus* SD11 on oral microbiota of healthy
397 volunteers : a randomized clinical trial. Journal of Dairy Science
398 100(10): 7780–87.
399 Santagati, M., Scillato, M., Patane, F., Aiello, C. and Stefani, S. 2012.
400 Bacteriocin-producing oral streptococci and inhibition of respiratory
401 pathogens, FEMS Immunology and Medical Microbiology 65: 23–41.
402 Saraiva, M. and O'Garra, A. 2010. The regulation of IL - 10 production by
403 immune cells." Nature Reviews Immunology: 1–13.
404 Shadnoush, M., Hosseini, R. S., Mehrabi, Y., Delpisheh, A., Alipoor, E.,
405 Faghfoori, Z. and Moghadam, J.Z. 2013. Probiotic Yogurt Affects Pro-

406 and Anti-Inflammatory Factors in Patients with Inflammatory Bowel
407 Disease. Iranian Journal of Pharmaceutical Research 12(4): 929–36.
408 Toma, M.M. and Pokrotnieks, J. 2006. Probiotics as functional food:
409 microbiological and medical aspects. Acta Universitatis Latviensis 710
410 (710): 117–29.
411 Zafra-stone, S., Yasmin, T., Bagchi, M. and Chatterjee, A. 2007. Berry
412 anthocyanins as novel antioxidants in human health and disease
413 prevention research article berry anthocyanins as novel antioxidants in
414 human health and disease prevention. Molecular Nutrition & Food
415 Research 51:675–83.
416
417
418
419
420
421
422
423
424
425
426
427
428
429

430 **Table 1:** The formula of yoghurt fortified by rosella extract

| Materials | I | II (2%0 | III (4%) | IV (8%) |
|---|---|---|---|---|
| Rosella Extract | - | 2mL | 4mL | 8mL |
| Honey | - | 8mL | 8mL | 8mL |
| Yogurt | 100mL | 90mL | 90mL | 90mL |

431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449

**Table 2.** The expression of Interleukin-10 and interleukin-14 of mice which is treated by yoghurt fortified with rosella extract

| Group | IL-10 expression | IL-14 expression |
|---|---|---|
| Normal | 47,67 ± 4,07[a] | 62,07 ± 6,83 |
| Plain Yoghurt | 66,26 ± 0,81[b] | 66,08 ± 7,38 |
| Yoghurt+Rosella 2 % | 80,24 ± 0,41[a] | 71,14 ± 1,86[b] |
| Yoghurt+Rosella 4 % | 90,25 ± 1,55[a] | 88,47 ± 0,75[ab] |
| Yoghurt+Rosella 8 % | 73,86 ± 1,99[a] | 75,93 ± 7,13[b] |

[a] Significantly different with plain yoghurt group ($p<0.05$)

[b] Significantly different with normal group ($p<0.05$)

466 **Table 3.** The effect of yoghurt fortified by rosella extract treatmen on
467 phagocytic activity of macrophage

| Group | Active phagocytic cells | Phagocytic capacity | Phagocytosis index (PI) |
|---|---|---|---|
| Normal | 73 ± 7,18 | 155 ± 18,86[ab] | 2,12 ± 0,09[a] |
| Plain Yoghurt | 87 ± 7,13[b] | 187 ± 9,88[b] | 2,151 ± 0,10 |
| Yoghurt+Rosella 2 % | 89 ± 8,04[b] | 248 ± 21,14[ab] | 2,740 ± 0,23[ab] |
| Yoghurt+Rosella 4 % | 97 ± 1,00[b] | 306 ± 5,03[ab] | 3,158 ± 0,02[ab] |
| Yoghurt+Rosella 8 % | 45 ± 16,09[ab] | 98 ± 33,02[ab] | 2,207 ± 0,15[a] |

468 [a] Significantly different with plain yoghurt (p<0,05)
469 [b] Significantly different with normal group (p<0,05)
470
471
472
473
474
475
476
477
478
479
480
481

482 **Table 4.** The absorbance of MTT in the proliferation assay on tthe yoghurt
483 fortified with rosella extract treated mice

| Group | absorbance |
|---|---|
| Normal | 0,27 ± 0,01[a] |
| Plain Yoghurt | 0,40 ± 0,02[b] |
| Yoghurt+Rosella 2% | 0,50 ± 0,03[ab] |
| Yoghurt+Rosella 4% | 0,79 ± 0,06[ab] |
| Yoghurt+Rosella 8% | 0,68 ± 0,04[ab] |

484 [a] Significantly different with plain yoghurt (p<0,05)

485 [b] Significantly different with normal group (p<0,05)

486

**Korespondensi 9-Sept 2020**

**Nurkhasanah Mahfudh** <nurkhas@gmail.com>
to International

9 Sept 2020, 12:59

Dear editorial team of IFRJ

Here we submitted the revised version of our manuscript entitled "**Immunomodulatory activity of yogurt fortified with rosella (*Hibiscus sabdariffa* L) extract" (IFRJ 19540).** We have revised as suggested by reviewer. We changedt he revised section to blue color. We also sent this manuscript to proofreading consultant to increase the quality of language and minimize the grammatical error. The certificate of proofreading was also provided.

Hopefully, the manuscript will fulfill the requirement to be published in your esteemed journal (International Food Research Journal (IFRJ). We herewith conform that the work described has not been published before, that is not under consideration for publication elsewhere.

We also have uploaded to your system.

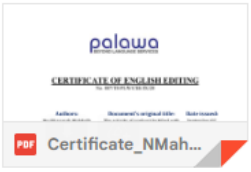
Thank you

Sincerely yours,

--
Dr, Nurkhasanah, M.Si., Apt
Fakultas Farmasi
Universitas Ahmad Dahlan
Yogyakarta
Jl. Prof Soepomo, Janturan, Yogyakarta

**3 Attachments** • Scanned by Gmail


respon to reviewe...



Certificate_NMah...


IFRJ19540_proof_...

**RESPON PERBAIKAN DARI AUTHOR**

**RESPON TO REVIEWER COMMENT**

**Tittle** : **Immunomodulatory activity of yogurt fortified with rosella (*Hibiscus sabdariffa* L) extract**

| LINE | REVIEWER COMMENT | RESPON |
|---|---|---|
| 5 | change to boost | done |
| 6-8 | Please rearrange both sentences. | Done |
| 9 | change to enhancing | done |
| 14-20 | Need to include how macrophage phagocytic activity, lymphocyte proliferation, IL-10 and IL-4 were measured. | The amounts of actively phagocytic macrophages Groups III, IV, and V were 89%, 97%, and 45%, while the MTT assays showed that their lymphocyte proliferation activities, represented by absorbance values, were 0.50%, 0.79%, and 0.68%, respectively. Also, 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 ($p<0.05$). |
| 28 | Please merge with previous sentence. Include reference | done |
| 52 | add one more paragraph of justification of study. | the addition of rosella extract to yogurt is expected to stabilize and accentuate the activity of anthocyanin, which, in this study, was observed from the increased phagocytic activity of macrophages, lymphocyte proliferation, and cytokine production |
| 60 | add statement of approval by animal ethic committee and please include approval number | . The research design and use of test animals in this study have received ethical approval from the Research Ethics Committee of Universitas Ahmad Dahlan (No. 011710141). |
| 84 | remove to materials section | done |
| 92 | please specify daily or how many times in 21 days | These oral treatments lasted for 21 days and were administered once a day, with a dose of 2 mL/kg BW |
| 107 | complete RPMI or RPMI only..please specify | The isolated macrophage was cultured in the RPMI medium supplemented with FBS 10% |
| 108 | for how long | for 24 hours before receiving further treatment. |

| | | |
|---|---|---|
| 111-118 | Please rearrange the sentences...Please include what tissue use and briefly describe the method. Please alert on grammar mistake | Macrophage isolated from the treated mice was cultured using coverslips in a 6-well microplate for 24 hours 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 that had been labeled with a chromogen, i.e., dimethylamino benzidine (DAB). Finally, the Mayer-Hematocsylin counterstain was added to facilitate clear observation. Brown marks the cells with positive expression of IL-10 or IL-14, while blue shows the cells with negative expression of IL-10 or IL-14 |
| 121-122 | Please rearrange:<br>the cells were culture on the cover slips that placed inside the 6 wells microplate | , the cells were cultured using coverslips that were placed inside a 6-well microplate |
| 138-139 | please rearrange this sentence and use proper words | Then, the complete RPMI medium was injected into the spleen tissue to isolate the lymphocytes |
| 158-163 | Please remove...just repetition | The authors argue that this statement is important to introduce the readers before they read the result of study |
| 167-173 | Please remove...another repetition | done |
| 174-177 | Please remove...another repetition | The authors argue that this statement is important to confirm the result of study |
| 178-182 | please rearrange the sentence | The IL-10 level also increased more significantly in the Groups III, IV, and V (yogurt+rosella extract treatment treatment 2%, 4% and 8% respectively) than Groups I (normal) and II (plain yogurt). This finding is in agreement with (Fakeye, 2008; Nurkhasanah, 2015), which also reported that rosella extract and fraction increase IL-10 expression |
| 183 | need mention 2%, 4% and 8% | The IL-10 level also increased more significantly in the Groups III, IV, and V (yogurt+rosella extract treatment 2%, 4% and 8% respectively |
| 192 | rearrange the sentence | Line 182<br>The same case applies to plain yogurt (Group II), which also increased such expression higher than Group I (normal) and stimulated the immune response |

| | | |
|---|---|---|
| 199 | Remove..another repetition | done |
| 204 | reference | (Couper *et al.*, 2008; Saraiva and O'Garra, 2010) |
| 208 | 2%, 4% and 8% | Line 200<br>2%, 4%, and 8% |
| 208 | Table 2 | Table 2 |
| 224 | reference | (Molina-Bolívar and Galisteo-González 2005) |
| 236-241 | need to rephrase this results...confusing | The macrophage phagocytic activities decreased as the rosella extract concentration increased. When applied In high concentrations, the antioxidant effects of rosella extract become the major mechanism. Antioxidants eliminate 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 macrophage (Wang et al. 2019). |
| 245-246 | please rephrase the sentence | Rosella contains antioxidant compounds that stimulate the immune system by preventing free radicals from causing cellular damage |
| 249-251 | rephrase the sentences and describe how h2o2 cause damage to immune system | Excess ROS can lower the immune system, damage macrophage, and induce the aging process of macrophage (Fresta et al. 2020; Wang et al. 2019) |
| 252-256 | please rephrase this paragrapgh...please describe your results clearly based on the table.. | Based on the analysis results, yogurt fortified with rosella extract could increase the phagocytic activity. Group IV (yougurt+rosella extract 4%) exhibited the highest activity, as observed from active phagocytic cells (APC), phagocytosis capacity, and phagocytosis index |
| 257-258 | please rephrase | Previous studies on yogurt have revealed the significance of probiotics in boosting the immune system |
| 266-267 | please describe further | 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 |

| | | |
|---|---|---|
| 269-275 | another repetition | It has been simplified |
| 282 | rephrase..confusing | , the enhanced lymphocyte proliferation in the treatment groups was the cumulative effect of yogurt and rosella extract, which may be attributable to anthocyanins—a compound that is more stable in environments with lower pH |
| 288-294 | Rephrase.....not the correct statement | 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 (Pincrok, 2002). |
| 295-307 | Rephrase.....not the correct statement | 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 hydrolyze phosphatidylinositol bisphosphate (PIP2) to produce glycerol (DAG) and inositol triphosphate (IP3) as a reactive product. Furthermore, IP3 stimulates cytoplasm and increase 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). |

palawa
BEYOND LANGUAGE SERVICES

# CERTIFICATE OF ENGLISH EDITING

No. 007/TS-PLW/CEE/IX/20

| Authors: | Document's original title: | Date issued: |
|---|---|---|
| Nurkhasanah Mahfudh<br>Afandi Hadi<br>Roichana Aifa Zakia Solechan | The activity of yoghurt fortified with rosella (*Hibiscus sabdariffa* L) extract as immunomodulator | September 07, 2020 |

To whom it may concern,

This is to certify that the above manuscript has been proofread and edited by the Department of Language and Interpreting Consultancy of CV. Palawa Karya. The document has been reviewed for proper English language, including structure, grammar, spelling, punctuation, and style by one or more of our academic editors. The editor team ensures that the process did not alter the author(s)'s intended meaning. All amendments are visible with Microsoft Word's Track Changes feature, providing authors with option to reject or accept each change.

Best regards,

palawa

Erik Febriarta
Director of CV. Palawa Karya

CV. PALAWA KARYA

Head Office
Jl. Damai No. 7, Prujakan, Sinduharjo,
Sleman, DI Yogyakarta 55581 – Indonesia
e-mail: palawakarya@gmail.com phone: +62 813 92 055 897

**MANUSKRIP PERBAIKAN (PERBAIKAN DITUNJUKKAN DENGAN WARNA BIRU)**

# Immunomodulatory activity of yogurt fortified with rosella (*Hibiscus sabdariffa* L) extract


## Abstract

Yogurt is a probiotic food that can boost the immune system, even when added with some fruit extract, such as rosella (*Hibiscus sabdariffa*). An in vivo study was carried out to prove the potency of yogurt fortified with rosella 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) yogurt treatment group, and groups receiving yogurt fortified with (III) 2%, (IV) 4%, and (V) 8% of rosella extract. 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 Groups III, IV, and V were 89%, 97%, and 45%, while the MTT assays showed that their lymphocyte proliferation activities, represented by absorbance values, were 0.50%, 0.79%, and 0.68%, respectively. Also, 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 ($p<0.05$). Overall, yogurt fortified with 2%, 4%, and 8% of rosella extract can be used as immunomodulators.



**Keywords:** yogurt, rosella, immunomodulator


## Introduction

Yogurt is the fermentation result of dairy products that most commonly involves lactic acid bacteria (LAB), *Lactobacillus bulgaricus,* and *Streptococcus thermophiles* in the preparation. *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 more acid and can reduce the pH to lower than 4.6, contributing to the sour taste of yogurt (Baglio, 2014).

Lactic acid bacteria (LAB) produce compounds that can kill pathogenic bacteria (Azcárate-peril *et al*., 2005; Parada *et al*., 2007). Prior scholars reported that consuming Lactobacillus (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 Perdigo, 2006; Rungsri *et al*., 2017).

Rosella (*Hibiscus sabdariffa* L) has been widely used to prevent various diseases because it is rich in antioxidants (Nurkhasanah *et al.,* 2017; Nurkhasanah *et al*., 2018). Rosella 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 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 rosella extract to yogurt is expected to stabilize and accentuate the activity of anthocyanin, which, in this study, was observed from the increased phagocytic activity of macrophages, lymphocyte proliferation, and cytokine production.

## Materials and methods

### *Materials*

The rosella plants chosen in this research were grown 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 procured from the Integrated Research Laboratory, Universitas Gadjah Mada. The research design and use of test animals in this study have received ethical approval from the Research Ethics Committee of Universitas Ahmad Dahlan (No. 011710141).

*Extract preparation*

One-hundred g of rosella calyx powder was added with 200 mL of water and heated at 90ºC for 15 minutes. Then, the resulting extract was filtered, and its volume was added with water up to 100 mL. The concentration of the stock was 100%.

*Yogurt preparation*

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

*Fortification of yogurt with rosella extract*

The yogurt was added with rosella extract with different concentrations, namely 2%, 4%, and 8% v/v. Also, it was added with honey to reduce the sourness and improve the overall taste. The formula of fortification is presented in Table 1.

*Animal treatment*

The test animal (25 BALB/c mice) was first divided into five groups, each consisting of five mice, and then allowed to acclimatize for one week. 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 rosella extract at concentrations of 2%, 4%, and 8%, respectively. These oral treatments lasted for 21 days 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 days 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. Ten ml of cold RPMI was injected into the peritoneal cavity and slowly massaged for 3 minutes. 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 minutes. The supernatant was removed and the remaining pellet was added with 3 ml of RPMI medium. The number of cells was counted with a hemocytometer and resuspended to achieve a density of $2{,}5x10^6$ cells/mL. The isolated macrophage was cultured in the RPMI medium supplemented with FBS 10% in a 5% $CO_2$ incubator for 24 hours before receiving further treatment.

*Immunocytochemistry assay for IL-10 and IL-14 detection*

The immunochemistry assays of IL-10 and IL-14 used a method as proposed by a prior scholar (Nurkhasanah, 2015). Macrophage isolated from the treated mice was cultured using coverslips in a 6-well microplate for 24 hours 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 that had been labeled with a chromogen, i.e., dimethylamino benzidine (DAB). Finally, the Mayer-Hematocsylin counterstain was added to facilitate clear observation. Brown marks the

cells with positive expression of IL-10 or IL-14, while blue shows 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 hours. Afterward, the cells were washed twice with RPMI-1640. Each well was added with the suspension of latex, with a density of $5x10^6$ (200 microliters/wells), and incubated in a 5% $CO_2$ incubator (37$^o$C, 60 minutes). The cells were then washed with PBS 3 times to remove excess latex beads, dried at room temperature, fixed with methanol for 30 seconds, stained with Giemsa for 10 minutes, 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 400x magnification; this process also the number of active phagocytic cells and phagocytosis index (Nurkhasanah *et al*., 2017).

*Lymphocyte proliferation assay*

In this research, the lymphocyte proliferation assay used 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 1200 rpm for 10 minutes to obtain pellets. After the supernatant was discarded, the pellet was suspended in 1 mL of ammonium chloride buffer to lyse erythrocytes. Cells were then mixed using a pipette and left at room temperature for 5 minutes. The pellets were washed twice using RPMI and centrifuged at 1200 rpm (4$^o$C, 10 minutes). Afterward, the lymphocyte cells were counted using a hemocytometer, added with RPMI

to obtain a density of 1.5 x $10^6$ cells/m, divided and placed in a 96-well microplate (100 µl per well), and then incubated in a 5% $C0_2$ incubator at 37˚C for 72 hours.

Following the incubation, each well was added with 50 µl of 0.1 mg/ml MTT and then incubated in a 5% $C0_2$ incubator (37˚C, 4 hours). 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 hours in dark conditions, and the absorbance was measured using an ELISA reader at a wavelength of 595 nm.

**Results and discussion**

The present study used healthy animals that, after 21 days 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, and the results are discussed in this section.

*Increased Interleukin-10 and interleukin-14 after the yogurt+rosella 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 rosella extract is expected to amplify the immunomodulatory effects of both yogurt and rosella 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 (DC) cells, and macrophages to respond to microbes invading through extracellular signal-regulated kinase 1 (ERK1) and ERK2 pathways. Through these pathways, the signaling escalation is activated in the cells, resulting in the expression of IL-10 (Saraiva and O'Garra, 2010). The present study found that treatment with

yogurt that had been fortified with rosella 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+rosella extract treatment 2%, 4% and 8% respectively) than Groups I (normal) and II (plain yogurt). This finding is in agreement with (Fakeye, 2008; Nurkhasanah, 2015), which also reported that rosella 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 scholars 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 dendritic cells 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 antiinflammatory cytokine (Couper *et al.*, 2008; Saraiva and O'Garra, 2010). Its level elevation enhances the differentiation of IL-10-secreting Treg 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).

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 rosella 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 rosella extract. This finding corresponds to (Nurkhasanah, 2015), which also detected an increase in IL-14 after the administration of rosella extract. Anthocyanin, a compound found abundantly in rosella extract, is a 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 this research, 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 material is digested in phagosomes.

The phagocytosis assay observed several parameters, namely active phagocytic cell (APC), phagocytic capacity, and phagocytosis index (PI). 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 Phagocytosis Index (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+rosella extract) are presented in Table 3.

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

The macrophage phagocytic activities decreased as the rosella extract concentration increased. When applied In high concentrations, the antioxidant effects of rosella extract become the major mechanism. Antioxidants eliminate 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 macrophage (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).

Rosella 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 reactive oxygen species (ROS), which determines defense against microbes or other non-self antigens (Puertollano *et al*., 2011). Excess ROS can lower the immune system, damage macrophage, and induce the aging process of macrophage (Fresta et al. 2020; Wang et al. 2019). 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 rosella extract could increase the phagocytic activity. Group IV (yougurt+rosella extract 4%) exhibited the highest activity, as observed from active phagocytic cells (APC), phagocytosis capacity, and phagocytosis index. Previous studies on yogurt have revealed the significance of probiotics in boosting the immune system. Lactic acid bacteria reportedly enhance immune response activity by increasing the activity of NK cells (Gill *et al*., 2001). Anthocyanins, which are found abundantly in rosella 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 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 this study, the lymphocyte proliferation activity was observed using the MTT assay. The increased number of lymphocyte cells after the administration of yogurt fortified with rosella extract is presented in Table 4.

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+rosella extract). The higher the concentration of rosella 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 rosella extract, which may be attributable to anthocyanins—a compound that is more stable in environments with lower pH (Puspita and Kumalasari, 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 (Pincrok, 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 hydrolyze phosphatidylinositol bisphosphate (PIP2) to produce glycerol (DAG) and inositol triphosphate

(IP3) as a reactive product. Furthermore, IP3 stimulates cytoplasm and increase 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 rosella extract can increase the immune response by enhancing expressions of interleukin-10 and interleukin-14, macrophage phagocytosis activity, and lymphocyte proliferation.


## Acknowledgment

The authors would like to thank the Institute of Research, Development, and Community Service of Universitas Ahmad Dahlan for funding this research under the Innovative Research Grant Scheme.


## References


Astawan, Made, Tutik Wresdiyati, Irma Isnafia Arief, and Dwi Febiyanti. 2011. "Potency of Indigenous Probiotic Lactic Acid Bacteria as Antidiarrheal Agent and Immunomodulator." *Jurnal Teknologi Dan Industri Pangan* XXII(1):11–16.

Azcárate-peril, Maria Andrea, Todd R. Klaenhammer, Rodolphe Barrangou, B. Logan Buck, M. Andrea Azcarate-peril, and Eric Altermann. 2005. "Genomic Features of Lactic Acid Bacteria Effecting Bioprocessing and Health." *FEMS Microbiology Reviews* 29:393–409.

Baglio, Ettore. 2014. *Chemistry and Technology of Yoghurt Fermentation*. Spain: Springer.

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

Becker, C., M. C. Fantini, C. Schramm, H. A. Lehr, S. Wirtz, A. Nikolaev, J. Burg, S. Strand, R.

Kiesslich, S. Huber, H. Ito, N. Nishimoto, K. Yoshizaki, T. Kishimoto, P. R. Galle, M. Blessing, S. Rose-John, and M. F. Neurath. 2004. “TGF-Beta Suppresses Tumor Progression in Colon Cancer by Inhibition of IL-6 Transsignaling.” *Immunity* 21:491=501.

Couper, Kevin N., Daniel G. Blount, and Eleanor M. Riley. 2008. “IL-10 : The Master Regulator of Immunity to Infection.” *Journal of Immunology* 180:5771–77.

Fakeye, Titilayo. 2008. “Toxicity and Immunomodulatory Activity of Fractions of Hibiscus Sabdariffa Linn (Family Malvaceae) in Animal Models.” *African Journal of Traditional Complementary and Alternative Medicine* 5(4):394–98.

Fresta, Claudia G., Annamaria Fidilio, Giacomo Lazzarino, Nicol Musso, Barbara Tavazzi, Giuseppe Lazzarino, Susan M. Lunte, and Filippo Caraci. 2020. “Modulation of Pro-Oxidant and Pro-Inflammatory Activities of M1 Macrophages by the Natural Dipeptide Carnosine.” *International Journal of Molecular Sciences* 21(776):1–22.

Gackowska, Lidia, Anna Helmin-basa, and Izabela Kubiszewska. 2006. “Combined Effect of Different Lactic Acid Bacteria Strains on the Mode of Cytokines Pattern Expression in Human Peripheral Blood Mononuclear Cells.” *Journal of Physiology and Pharmacology* 57(Suppl 9):13–21.

Galdeano, C. Maldonado and G. Perdigo. 2006. “The Probiotic Bacterium Lactobacillus Casei Induces Activation of the Gut Mucosal Immune System through Innate Immunity.” *Clinical and Vaccine Immunology* 13(2):219–26.

Gill, HS, KJ Rutherfurd, and ML Cross. 2001. “Dietary Probiotic Supplementation Enhances Natural Killer Cell Activity in the Elderly: An Investigation of Age-Related Immunological Changes.” *Journal of Clinical Immunology* 21(4):264–71.

Leca, N., M. Laftavi, L. Shen, K. Matteson, J. Jr Ambrus, and O. Pankewycz. 2008. “Regulation

of Human Interleukin 14 Transcription in Vitro and in Vivo after Renal Transplantation." *Transplantation* 86(2):336–41.

Molina-Bolívar, Jose A. and Francisco Galisteo-González. 2005. "Latex Immunoagglutination Assays." *Journal of Macromolecular Science, Part C: Polymer Reviews* 45(1):59–98.

Nurkhasanah. 2015. "The Effect of Rosella (Hibiscus Sabdariffa L) Treatment on lL-10 and IL-14 Secretion on Dimethylbenz (A) Anthracene (DMBA) Induced Rat." *International Journal of Pharmacy and Pharmaceutical Sciences* 7(4):402–4.

Nurkhasanah, Laela Hayu Nurani, and Zainur Rahman Hakim. 2017. "Effect of Rosella ( Hibiscus Sabdariffa L ) Extract on Glutathione-S-Transferase Activity in Rats." *Tropical Journal of Pharmaceutical Research February* 16(10):2411–16.

Nurkhasanah, Dewi R. Santoso, and Rahmi Fauziah. 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* 259:1–10.

Nurkhasanah, Nanik Sulistyani, and Ery Fatmawati. 2018. "The Increasing of Catalase Activity in Dimethylbenz- α -Anthracene (DMBA ) Induced Rat Treated by Hibiscus Sabdariffa L Extract." *Pakistan Journal of Pharmaceutical Science* 31(3):851–56.

Oancea, Simona and Olga Drăghici. 2013. "PH and Thermal Stability of Anthocyanin-Based Optimised Extracts of Romanian Red Onion Cultivars." *Czech J Food Sci* 31(3):283–91.

Otsuka, Hidenori, Teruo Okano, and Kazunori Kataoka. 2006. "Activation of Lymphocyte Proliferation by Boronate-Containing Polymer Immobilised on Substrate : The Effect of Boron Content on Lymphocyte Proliferation." *European Cells and Materials* 12(2):36–43.

Parada, José Luis, Carolina Ricoy Caron, Adriane Bianchi P. Medeiros, and Carlos Ricardo. 2007.

"Bacteriocins from Lactic Acid Bacteria : Purification, Properties and Use as Biopreservatives." *Brazilian Archives of Biology and Technology* 50(3):521–42.

Pincrok, George. 2002. *Schaum's Outline Immunology*.

Puertollano, Maria A., Elena Puertollano, Gerardo Alvarez de Cienfuegos, and Manuel A. de Pablo. 2011. "Dietary Antioxidants: Immunity and Host Defense." *Current Topics in Medicinal Chemistry* 11(14):1752–66.

Puspita, Weni and Ika Dyah Kumalasari. 2018. "Yoghurt Fortified Formulation of Lakum Fruit ( Cayratia Trifolia ( L .) Domin ) Extract as an Antioxidant." *Traditional Medicine Journal* 23(3):91–96.

Rungsri, P., N. Akkarachaneeyakorn, M. Wongsuwanlert, S. Piwat, and P. Nantarakchaikul. 2017. "Effect of Fermented Milk Containing Lactobacillus Rhamnosus SD11 on Oral Microbiota of Healthy Volunteers : A Randomized Clinical Trial." *Journal of Dairy Science* 100(10):7780–87.

Santagati, Maria, Marina Scillato, Francesco Patane, Caterina Aiello, and Stefania Stefani. 2012. "Bacteriocin-Producing Oral Streptococci and Inhibition of Respiratory Pathogens." *FEMS Immunology and Medical Microbiology* 65:23–41.

Saraiva, Margarida and Anne O'Garra. 2010. "The Regulation of IL - 10 Production by Immune Cells." *Nature Reviews Immunology* 1–13.

Shadnoush, M., R. S. Hosseini, Y. Mehrabi, A. Delpisheh, E. Alipoor, Z. Faghfoori, and J. Z. Moghadam. 2013. "Probiotic Yogurt Affects Pro-and Anti-Inflammatory Factors in Patients with Inflammatory Bowel Disease." *Iranian Journal of Pharmaceutical Research* 12(4):929–36.

Toma, Malda Maija and Juris Pokrotnieks. 2006. "Probiotics as Functional Food : Microbiological

and Medical Aspects.” *Acta Universitatis Latviensis* 710(710):117–29.

Wang, Ping, Jing Geng, Jiahui Gao, Hao Zhao, Junhong Li, Yiran Shi, Bingying Yang, Chen Xiao, Yueyue Linghu, Xiufeng Sun, Xin Chen, Lixin Hong, Funiu Qin, Xun Li, Jau-Song Yu, Han You, Zengqiang Yuan, Dawang Zhou, Randy L. Johnson, and Lanfen Chen. 2019. “Macrophage Achieves Self-Protection against Oxidative Stress-Induced Ageing through the Mst-Nrf2 Axis.” *Nature Communications* 10(1):755.

Zafra-stone, Shirley, Taharat Yasmin, Manashi Bagchi, and Archana Chatterjee. 2007. “Berry Anthocyanins as Novel Antioxidants in Human Health and Disease Prevention Research Article Berry Anthocyanins as Novel Antioxidants in Human Health and Disease Prevention.” *Molecular Nutrition & Food Research* 51:675–83.

**Table 1:** The formula of yogurt fortification with rosella extract

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

**Table 2.** The expression of Interleukin-10 and interleukin-14 in the test mice

| Groups | 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+Rosella 2 %) | 80.24 ± 0.41[a] | 71.14 ± 1.86[b] |
| IV (Yogurt+Rosella 4 %) | 90.25 ± 1.55[a] | 88.47 ± 0.75[ab] |
| V (Yogurt+Rosella 8 %) | 73.86 ± 1.99[a] | 75.93 ± 7.13[b] |

[a] Significantly different from Group II (plain yogurt) ($p<0.05$)

[b] Significantly different from Group I (normal) ($p<0.05$)

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

| Groups | Active phagocytic cells | Phagocytic capacity | Phagocytosis index (PI) |
|---|---|---|---|
| I (Normal) | 73 ± 7.18 | 155 ± 18.86[ab] | 2.12 ± 0.09[a] |
| II (Plain Yogurt) | 87 ± 7.13[b] | 187 ± 9.88[b] | 2.151 ± 0.10 |
| III (Yogurt+Rosella 2 %) | 89 ± 8.04[b] | 248 ± 21.14[ab] | 2.740 ± 0.23[ab] |
| IV (Yogurt+Rosella 4 %) | 97 ± 1.00[b] | 306 ± 5.03[ab] | 3.158 ± 0.02[ab] |
| V (Yogurt+Rosella 8 %) | 45 ± 16.09[ab] | 98 ± 33.02[ab] | 2.207 ± 0.15[a] |

[a] Significantly different from Group II (plain yogurt) ($p<0.05$)

[b] Significantly different from Group I (normal) ($p<0.05$)

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

| Groups | Absorbance |
|---|---|
| I (Normal) | 0.27 ± 0.01[a] |
| II (Plain Yogurt) | 0.40 ± 0.02[b] |
| III (Yogurt+Rosella 2%) | 0.50 ± 0.03[ab] |
| IV (Yogurt+Rosella 4%) | 0.79 ± 0.06[ab] |
| V (Yogurt+Rosella 8%) | 0.68 ± 0.04[ab] |

[a] Significantly different from Group II (plain yogurt) ($p<0.05$)

[b] Significantly different from Group I (normal) ($p<0.05$)

**Korespondensi 14-10-2020, acceptance letter**

International Food Research Journal - Decision on Manuscript IFRJ19540.R1

Inbox ×

**International Food Research Journal** <onbehalfof@manuscriptcentral.com> 14 Oct 2020, 13:06

to me, ibufathan

14-Oct-2020

Dear Dr. Mahfudh

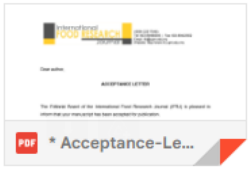
Thank you for sending the revised version of this paper ('The activity of yoghurt fortified with rosella (Hibiscus sabdariffa L) extract as immunomodulator'). I have now had the opportunity to examine your revised manuscript and I am pleased to accept it for publication in International Food Research Journal. Kindly refer to the attachment for the acceptance letter.

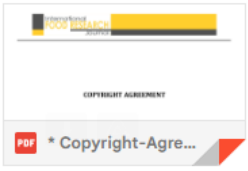
Please note that all manuscripts must be accompanied by a signed copyright agreement form to enable the manuscript to be processed for publication. You are requested to send the copyright agreement form within three (3) working days from the date of the email. Please print, fill and sign the attached copyright agreement form and send the scanned copy to ifrj@upm.edu.my

Thank you again for your contribution to the Journal.

Sincerely,
Dr. Chin Ping Tan
Editor, International Food Research Journal

**2 Attachments** • Scanned by Gmail



International FOOD RESEARCH Journal
(ISSN 22317546)
Tel: 603-89468395 | Fax: 603-89423552
Email: ifrj@upm.edu.my
Website: http://www.ifrj.upm.edu.my

Dear author,

**ACCEPTANCE LETTER**

The Editorial Board of the International Food Research Journal (IFRJ) is pleased to inform that your manuscript has been accepted for publication.

We thank you for your contribution to IFRJ, and strongly encourage you to submit other articles to IFRJ in the near future.

Please note that accepted manuscript(s) must be accompanied by a signed Copyright Agreement Form to enable the manuscript(s) to be processed for publication. You are therefore requested to print, fill up and sign the attached Copyright Agreement Form, and return the scanned copy to ifrj@upm.edu.my within three (3) working days from the date of the acceptance notification email.

Thank you.

Yours sincerely,

**Prof. Dr. Tan Chin Ping**
Editor-in-Chief
International Food Research Journal

## Korespondensi 16-10-2020

**Nurkhasanah Mahfudh** <nurkhas@gmail.com>
to International

16 Oct 2020, 11:17

Dear Editor of IFRJ

Here we submitted the signed of copyright agreement.

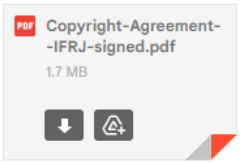
Thank you

•••

--

Dr, Nurkhasanah, M.Si., Apt
Fakultas Farmasi
Universitas Ahmad Dahlan
Yogyakarta
Jl. Prof Soepomo, Janturan, Yogyakarta

**One attachment** • Scanned by Gmail ⓘ




## Korespondensi 4-12-2020

**Nurkhasanah Mahfudh** <nurkhas@gmail.com>
to International

4 Dec 2020, 13:01

Dear Editor IFRJ

Could we find some information about our manuscript entitled "The activity of yoghurt fortified with rosella (Hibiscus sabdariffa L) extract as immunomodulator". When it will be published? In which issue of journal?

Thank you

•••

## Korespondensi 29-12-2020

**International Food Research Journal / UPM** <ifrj@upm.edu.my>
to me

29 Dec 2020, 11:12

Dear Dr. Nurkhasanah Mahfudh,
Apologies for the late reply and inconvenience caused.
Upon checking, your paper is scheduled for publication in IFRJ Vol 28 Issue 2, 2021 (April).

Thank you.

Regards,

Ms. Norhafizah Mohamad Noh
Editorial Assistant
International Food Research Journal Editorial Office