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Zingiber cassumunar roxb. Extract increase the reactive oxidant level and interleukins expression *in vitro*

Nurkhasanah Mahfudh, Nanik Sulistyani, Ghina Adhila

ABSTRACT

Zingiber cassumunar Roxb. (bangle) has a variety of active compounds, including curcumin and phenylbutenoid. Bangle rhizoma reported exhibiting immunomodulatory activities. This research aims to determine the mechanism of bangle extract as an immunomodulator by the secretion of Reactive Oxygen Intermediate (ROI), Nitric Oxide (NO), and interleukin (IL-10 and IL-14) expression level. Bangle extract (*Zingiber cassumunar* Roxb.) was made by the maceration method using 96% ethanol solvent. This research was administered *in vitro* using macrophage cells from male mice with Balb/C strain divided into 2 groups: normal control and treatment group (receiving 25, 50, and 100 ppm of extract). The administration of bangle extract can function as an immunomodulator by an increase of ROI in 25 and 50 ppm of the extract significantly than the control group (p <0.05), the treatment groups decrease NO level (p <0.05), it also was found to increase expression of IL-10 and IL-14 expression levels (p <0.05). *Zingiber cassumunar Roxb*. extract was potentially to be developed as an immunomodulator.

Keywords: Immunomodulator; *Zingiber cassumunar* Roxb.; ROI; NO; IL-10; IL-14

INTRODUCTION

The immune system defends our body against invaders, such as viruses, bacteria, and foreign bodies which are the cause of various diseases. It consists of a natural immune system (innate immunity/non-specific) and an adaptive immune system (adaptive immunity/specific) **(Akrom, 2017; Baratawidjaja and Rengganis, 2014)**. Immunomodulators are pharmacological agents that can modulate a partial immune response that is spurred by an immune response, on the other hand, it inhibits some of the other immune system. Immunomodulators are restoring the imbalance of the disrupted immune system **(Akrom, 2017)**.

Macrophages are professional phagocytes that act as APC and the main effectors in cellular innate and adaptive immune response **(Murphy, 2012)**. In the body's defense mechanism against invaders, macrophages become the leading component of immune blocking. Macrophages express many surface receptors that can catch and swallow (degrade) microbes, in a process called phagocytosis **(Baratawidjaja and Rengganis, 2014)**. Phagocytosis and *reactive oxygen intermediates* (ROI) are the macrophages main mechanism in destroying infected cells **(Akrom et al., 2015)**.

Activate macrophages can stimulate the proliferation and activity of T and B lymphocyte cells. Macrophage cells act as antigen-presenting cells (APC) that will activate Th-0 lymphocytes. Activated CD4 Th-0 lymphocytes will proliferate towards Th-1 or Th-2 depending on the cytokine environment and the location of the antigen. Activated T lymphocytes will release various mediators, differentiation towards Th-1 will produce proinflammatory cytokines while differentiation towards Th-2 produces anti-inflammatory cytokines **(Thiery et al., 2003; Bastos et al., 2004; Akrom and Mustofa, 2017)**.

One of the potential plants in Indonesia is bangle (*Zingiber cassumunar* Roxb.) has been proven to have scientific activity as an immunomodulator. Bangle ethanol extract has been shown to have an immunomodulatory effect, indicated by an increase in the activity of macrophage cell phagocytosis, ROI secretion, and IL-10 expression, decreased NO secretion, and TNF-α production *in vivo* **(Arini et al., 2014; Nurkhasanah et al., 2017; Fitriana et al., 2018)**. *In vitro* research, phenylbutanoid compounds from bangle rhizomes can increase phagocytic activity of macrophage cells, and inhibit NO production **(Chairul et al., 2009; Nakamura et al., 2009; Kaewchoothong et al., 2012)**. Besides, the n-hexane bangle fraction can reduce the phagocytic activity of macrophage cells, and decrease lymphocyte proliferation **(Nurkhasanah et al., 2019b)**. In this study, we want to clarify the activity of bangle ethanol 96% extract as an immunomodulator by analyzing the secretion of ROI, NO, IL-10, and IL-14 by the macrophage.

Scientific hypothesis

Bangle has immunomodulatory activities with a mechanism of increasing ROI and NO secretion level, increasing IL-10 and IL-14 expression level, *in vitro*.

MATERIAL AND METHODOLOGY

Material and subject

Fresh *Z. cassumunar* rhizome purchased from the local market, Yogyakarta, Indonesia. The sample was verified and identified in the Biology Laboratory of Universitas Ahmad Dahlan. Macrophages were obtained from the peritoneal cavity of mice Balb/c strain aged eight weeks old (20 – 30 g) from the Integrated Research Laboratory of Universitas Gadjah Mada (Laboratorium Penelitian dan Pengujian Terpadu, LPPT UGM).

Research Procedure

Preparation of Bangle Extract

Z. cassumunar extraction was carried out using the maceration method and 96% ethanol as the solvent. The maceration was done for 3 x 24 hours. The macerate was filtered, and then evaporated (rotary evaporator) for 2 hours per day in a week, and used a water bath until a thick extract was obtained.

Preparation of Test Animals

The use of test animals in this research had received ethical approval from the Commission for Research Ethics of Universitas Ahmad Dahlan with Number: 011804063. The test animals were male mice Balb/c strain aged eight weeks old.

Isolation of Macrophages

Mice were narcotized with chloroform after being fasted for 10 – 12 hours. Then, mice were placed in the supine position. The mice's abdomen skin cleaned using disinfectant (70% alcohol) and dissected. The peritoneal sheath cleaned with 70% alcohol. Then, 10 mL of cold RPMI injected into the peritoneal cavity and shaken slowly for three minutes. The inner cavity pressed with two fingers and the fluid from the peritoneal cavity (the non-fatty part) drawn using an injection syringe to obtain an aspirate.

The aspirate is centrifuged at 1200 rpm, 4 °C for 10 minutes. The supernatant was removed, and the pellets (macrophages) resuspended with 1,000 µL complete medium. The number of cells counted from 10 µL macrophage suspension in a hemocytometer. The macrophage cell suspension grew in a 6-well microtiter plate (coverslip) with a density of 5 x 10^5 cells/well for the ROI and interleukin assay. And a 6-well microtiter plate with a density of 1 x 10^5 cells/well for the NO assay **(Ulfah et al., 2017; Nurkhasanah et al., 2017)**.

Reactive Oxygen Intermediate (ROI) Secretion Assay

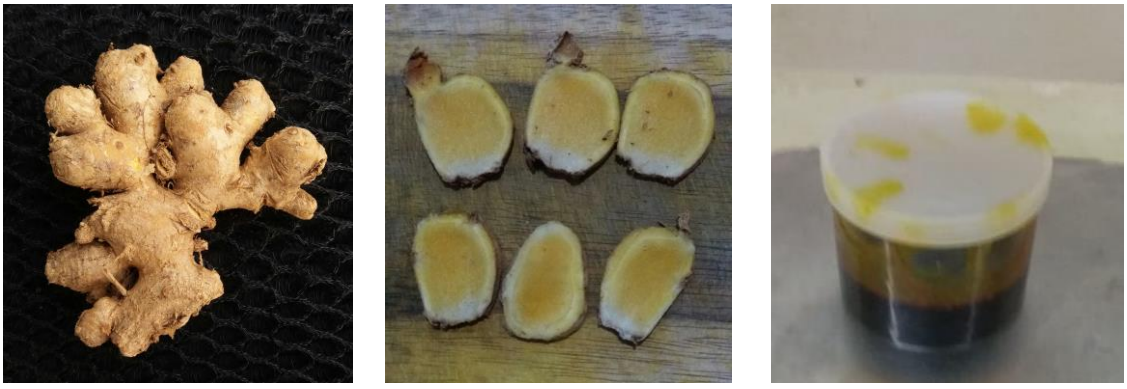
The 100 µL of macrophage cell suspension grew in a 6-well microtiter plate (coverslip) with a density of 5 x 10^5 cells/well. The cells incubated in a 5% CO_2 incubator at 37 °C for 15 minutes. 800 µL of complete RPMI medium added to each well, then microplates incubated overnight. The medium was removed and the sample added each well. The microplates were incubated in a 5% CO_2 incubator at 37 °C overnight.

The 50 – 100 µL of NBT solution and 1 mL of PBS (containing 125 PMA) added to each well, then incubated in 5% CO_2 incubator at 37 °C for 60 minutes. The reagent was removed, dried at room temperature, and fixed with absolute methanol. After dried, applied a 2% neutral red solution to the coverslip. The percentage NBT reduction of macrophage cells, it calculated from 100 cells examined by a binocular microscope (XSZ 107 BN, Novel) and Optilab with 400x magnification **(Nurkhasanah et al., 2017)**.

Nitric Oxide (NO) *Secretion Assay*

Griess Reaction Assay used for NO levels testing. Griess A solution prepared by dissolving 0.1 gram of N-(1-naphthyl) ethylene diamine hydrochloride (Sigma N, 5889) in 100 mL of distilled water. Griess B solution prepared by dissolving 1 gram of sulfanilamide (Sigma N 5589) in 100 mL of 5% orthiohisohoric acid (v/v). Both solutions stored at 0 – 4 °C protected from light. Standard nitrite prepared by dissolving 69.0 mg of sodium nitrite (Merck) in 100 mL of distilled water and stored at 0 – 4 °C protected from light. Stock standard solutions prepared using standard nitrite solutions in concentrations between 1.5625 – 100 µM **(Nurkhasanah et al., 2017)**.

100 µL macrophage cell suspension grew in 96-well microtiter plate-wells with a density of 1 x 10^5.mL^{-1}. The samples were added to each well, and the microplates were incubated overnight. The standard nitrite was inserted into the blank section of the 96-well microtiter plate, to determine the standard curve. Each well added 50.0 µL



a **b** **c**

Figure 1 *Z. cassumunar* (a. rhizome; b. pieces of rhizome; c. extract).

Griess reagent, allow to stand for 5 – 10 minutes at room temperature and protected from direct light, until the color changes. The absorbance measured using an ELISA reader at a wavelength of 550 nm **(Nurkhasanah and Zulkarmen, 2014; Nurkhasanah et al., 2017)**.

Interleukin-10 and Interleukin-14 Expression Assay

Previously, the preparation of cell culture is the same as in ROI assay. Macrophage cells culture fixed using methanol and then washed with Phosphate Buffer Saline (PBS). The microplates soaked in 300 µL peroxidase blocking solution and washed with distilled water.

Then the microplates added 20 µL protein blocking serum, incubated at humid temperature for 10 – 15 minutes. Added 30 µL Interleukin-10 and Interleukin-14, incubated at room temperature then washed with PBS. Added with 30 µL of biotin, incubated at room temperature then washed with PBS. Added with 30 µL of the enzyme streptavidin-peroxidase, incubated at room temperature then washed with PBS. Added with 30 µL peroxidase substrate solution (DAB), incubated at room temperature, and washed with distilled water. Added with 100.0 µL of Mayer Hematoxylin (counterstain), incubated at room temperature then washed with distilled water. Then the microplates soaked in absolute alcohol, cleaned, and dried. The microplates dipped in xylol and dried. Then the microplates dropped with mounting media and covered using a deckglasser. After dried, observed in a binocular microscope (XSZ 107 BN, Novel) and Optilab with 400x magnification to examine the color of the cells, expression of IL-10 and IL-14 has intense brown **(Javois, 1999; Nurkhasanah et al., 2019a)**.

Statistic analysis

All statistical analyzes performed using the SPSS version 22 program. The normality test and homogeneity test performed about the data ROI levels, NO levels, and the expression of Interleukin-10 and Interleukin-14. Then proceed with the One-way ANOVA and LSD tests (with a significance level of 0.05).

The normality test performed using the Shapiro-Wilk‘s test, with total data of less than 50. The variant homogeneity test performed using the Levene‘s test. If the results of the normality test and homogeneity test are homogeneous variance and normally distributed, then the test continued with the analysis of one way ANOVA variants, and LSD test.

RESULTS AND DISCUSSION

Result of ROI Secretion Assay

The NBT reduction test (tetrazolium nitro blue reduction test, containing PMA (phorbol 12-myristate, 13-acetate)) was used to measure the ability of peritoneal macrophage cells to secrete ROI. NBT (formazan salt) will diffuse into cells, then tetrazolium succinate reductase enzyme will divide into formazan. ROI cause increased respiration and reduction of NBT by forming black formazan deposits **(Leijh et al., 1986)**. It can be seen in Figure 2, macrophage cells are black show secrete ROI due to formazan deposition.

In contrast to macrophage cells that do not secrete ROI, it looks only brown without any formazan deposits.

Table 1 shows the average levels of ROI secretion in the normal control group and the treatment group concentrations of 25, 50, 100 ppm.

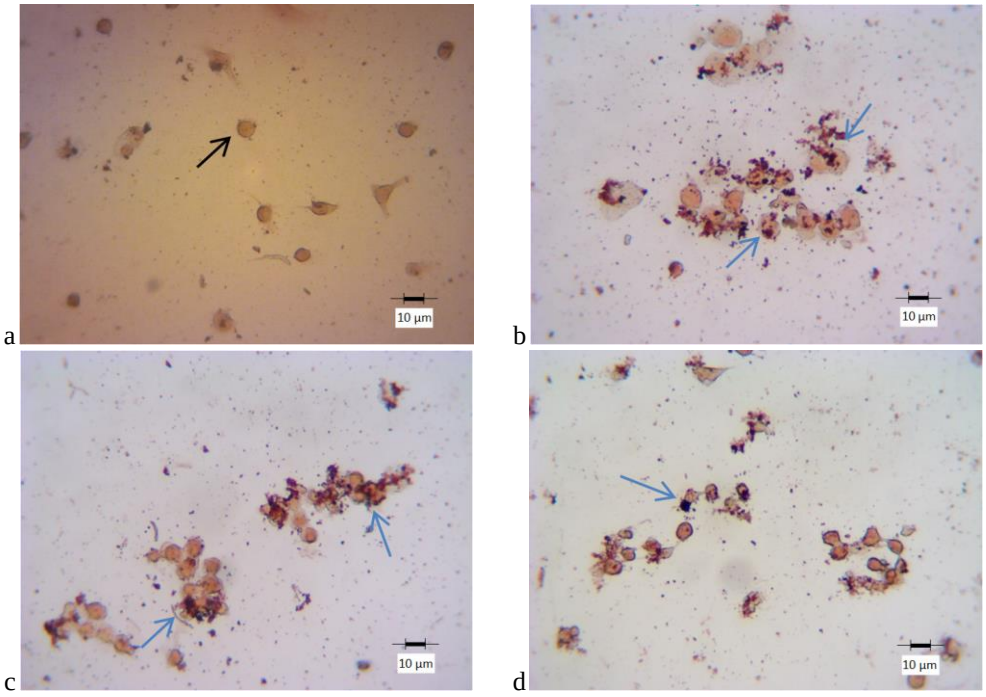


Figure 2 ROI secretion in macrophage cells after treated with bangle (Z. *cassumunar*) extract: a. normal control, b. bangle extract concentration of 25 ppm, c. bangle extract concentration of 50 ppm, d. bangle extract concentration of 100 ppm. (blue arrow: macrophage cells secrete ROI; black arrow: macrophage cells do not secrete ROI). Note: (400x magnification).

Table 1 ROI level by the administration of bangle (Z. *cassumunar*) extract.

Group	ROI secretion level (%) ±*SD*
Normal control	48.68 ±5.76
25 ppm	67.98 ±1.25*
50 ppm	70.38 ±8.35*
100 ppm	49.80 ±8.71[a,b]

Note: *significantly different compared to normal control group (p <0.05); [a]significantly different compared to concentration 25 ppm group (p <0.05); [b]significantly different compared to concentration 50 ppm group (p <0.05).

Table 2 NO level by the administration of bangle (Z. *cassumunar*) extract.

Group	NO secretion level (%) ±*SD*
Normal control	9.262 ±0.360
25 ppm	0.357 ±0.226*
50 ppm	0.762 ±0.840*
100 ppm	0.471 ±0.310*

Note: *significantly different compared to the normal control group (p <0.05).

The normal control group had the lowest ROI secretion (48.68%), not significantly different (p >0.05) with the treatment group concentration of 100 ppm (49.80%). The treatment groups concentrations of 25 and 50 ppm had ROI levels of 67.98% and 70.38%, significantly different (p <0.05) with normal controls, and the treatment group concentrations of 100 ppm.

There was an increase in ROI levels of the treatment group concentrations of 25 ppm and 50 ppm, but a decrease in ROI levels in the treatment group concentration of 100 ppm.

The content of curcumin compounds in bangle extract can cause increased levels of ROI. Curcumin can increase reactive oxygen species (ROS). This is related to macrophage activation and phagocytic activity of macrophages **(Mimche et al., 2011)**. Increased ROS can activate cellular signal pathways to form ROI **(Nathan and Ding, 2010)**. Bangle chloroform extract concentrations of 25, 50, and 100 $\mu g.mL^{-1}$ in vitro showed significantly increased ROI secretion compared to normal controls **(Nurkhasanah et al., 2019b)**. In vivo study, administration of bangle (5 $mg.20g^{-1}$ BW) by seven days duration can increase ROI secretion in mice induced by LPS (0.7 $mg.kg^{-1}$ BW) **(Nurkhasanah et al., 2017)**.

Phenylbutenoid is another component of the bangle extract and has anti-inflammatory activity by inhibiting enzyme cyclooxygenase-2 (COX-2) **(Jeenapongsa et al., 2003; Han et al., 2005; Leelarungrayub et al., 2017)**. The anti-inflammatory mechanism of this compound can be related to the ability of bangle to increase the expression of IL-10 **(Fitriana et al., 2018)**. Increased IL-10 expression will inhibit the production of IL-12, IL-1, and TNF-α. Inhibition of IL-1 and TNF-α production can affect T-cell activation to inhibit the inflammatory reaction. IL-12 has an important role in differentiating CD4 + into Th1 cells, then Th1 cells will secrete IFN-γ to activate macrophage cells to produce ROI. The inhibition of IL-12 production will indirectly inhibit the secretion of IFN-γ in case ROI production will decrease **(Bratawidjaja, 2014)**.

Result of NO Secretion Assay

NO is an effective antibacterial effector in the immune system. NO is a free radical synthesized by the enzyme nitric oxide synthase (NOS) through complex reactions. The main isoform expressed by macrophages is iNOS, this isoform will induce NO expression **(Kil et al., 2012)**. In this study, NO levels were measured using a Griess Reaction Assay (colorimetric method).

The concentration of NO secreted by macrophages will be calculated in the form of nitrites. Sulfanilamide (diazotization reagent) react with nitrite (in alkaline) will form to diazonium salt, then react with N-(1-naphthyl) ethylene diamine hydrochloride (coupling reagent) to be a stable form. The final result is intensive pink color and absorbance can be measured at wavelength 550 nm using Elisa Reader **(Nurkhasanah et al., 2017)**.

In Table 2 the average levels of NO secretion in the treatment group concentrations of 25, 50, and 100 ppm were 0.36; 0.76; and 0.47 μM. This result is significantly different (p <0.05) compared to the average NO level in the normal control group (9.26 μM). The decrease NO levels in the treatment group concentrations of 25, 50, and 100 ppm can be explained because of the results of IL-10 expression parameters. The results of IL-10 expression parameters are the treatment group 25, 50, 100 ppm has higher levels of IL-10 expression, and significantly different (p <0.05) compared to the normal control group.

The decrease NO levels were related to the results of the IL-10 parameter. In this study, the treatment group has a higher IL-10 expression than the normal group. iNOS gene expression is dependent on numerous proinflammatory cytokines in the cellular microenvironment of the macrophage, two of which include interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) **(Salim et al., 2016)**. IL-10 (macrophage inhibitor) acts to inhibit proinflammatory cytokine production included TNF-α, IL-1, and IL-12. IL-12 has a role to stimulate IFN-γ production. Over this explanation, the active component in bangle can act as an immunomodulator by reducing NO levels **(Goodyear-bruch and Pierce, 2002; Akrom, 2017)**.

Table 3 IL-10 expression of mice macrophages by the administration of bangle (Z. *cassumunar*) extract.

Group	IL-10 expression level (%) ±*SD*
Normal control	9.61 ±1.44
25 ppm	69.96 ±3.46*
50 ppm	69.26 ±2.98*
100 ppm	53.29 ±8.39*[a]

Note: *significantly different compared to normal control group (p <0.05); [a]significantly different compared to concentration 25 ppm group (p <0.05).

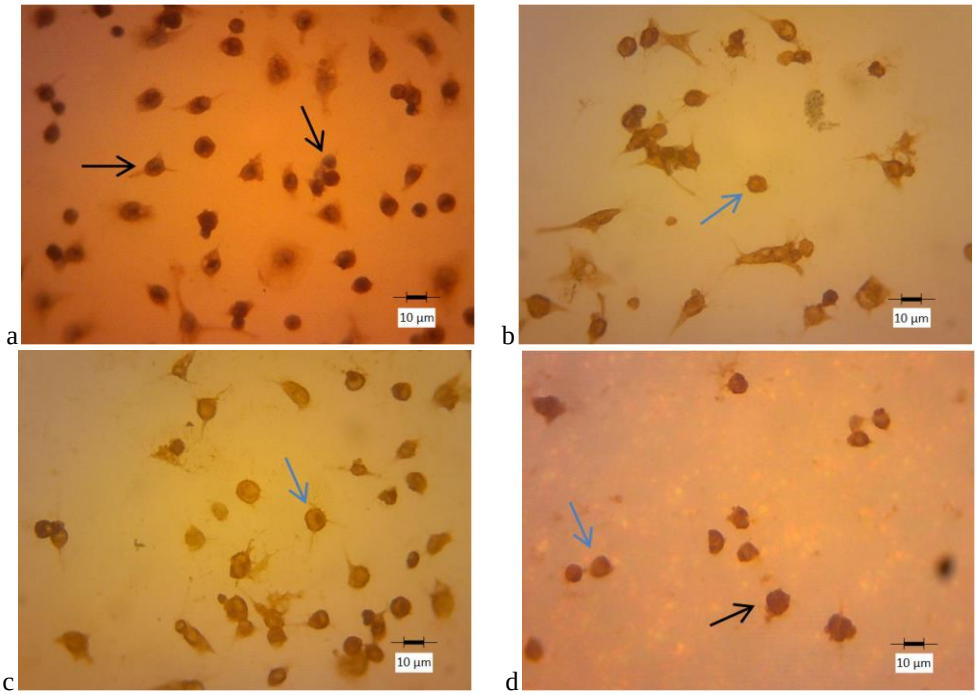


Figure 3 IL-10 expression on macrophage cells after being treated with bangle (Z. *cassumunar*) extract: a. normal control, b. bangle extract concentration of 25 ppm, c. bangle extract concentration of 50 ppm, d. bangle extract concentration of 100 ppm. (blue arrows: macrophage cells express IL-10; black arrows: macrophage cells do not express IL-10). Note: (400x magnification).

Phenylbutanoid was isolated from bangle (*Zingiber cassumunar* Roxb.) has an inhibitory effect of NO production on lipopolysaccharide-induced mouse macrophage cells (LPS) **(Nakamura et al., 2009)**. In other studies, *in vitro*, bangle can reduce NO secretion in murine macrophage RAW 264.7 cell lines **(Kaewchoothong et al., 2012)**. *In vivo* study, administration of bangle (5 mg/20g BW) can significantly reduce NO secretion in mice induced by LPS (0.7 $mg.kg^{-1}$ BW) **(Nurkhasanah et al., 2017)**. LPS causes an increase in NO levels of serum macrophages **(Tunctan et al., 1998)**. NO levels decrease after the administration of bangle extract could be due to the antioxidant content in the extract. Also, curcumin is another active component of bangle reported to inhibit NO production in macrophage activity **(Brouet and Ohshima, 1995)**.

Result of Interleukin Expression Assay

Interleukin-10

Observation of interleukin (IL) expression was carried out by an immunocytochemical method that uses specific antibodies to detect the expression of specific proteins (antigens) in cells. This research uses indirect immunocytochemical methods, the advantage is the results obtained have a more intense color, but it requires more time in the process **(Meshcer, 2017)**. The antigen will be bound indirectly to the primary antibody (IL-10 and IL-14) which has a role to recognize the antigen (first layer), then add a secondary antibody (biotin which binds to the enzyme streptavidin peroxidase) being the second layer.

The addition of secondary antibodies is also followed by the addition of chromogen substrate (DAB or 3,3-diaminobenzidine tetrahydrochloride), this substrate will be changed by enzymes so that it will form color deposits (pigments) in cells. To differentiate cells that are expressed IL-10 will have a brown color by DAB, while cells that are not expressed have a blue or purple color by Mayer hematoxylin (counterstain). Figure 3 shows the expression of IL-10 in macrophage cells treated with extract concentrations of 25, 50, and 100 ppm.

In Table 3 the average levels of IL-10 expression in the treatment group concentrations of 25, 50, and 100 ppm were 69.96%; 69.26%; and 53.29%. This result was higher and significantly different (p <0.05) compared to the average level of IL-10 expression in the normal control group (9.61%). The results obtained are suitable to those reported by other researchers that *Zingiber cassumunar* has the immunomodulatory activities one of activity by increase IL-10. This activity may be attributable to curcumin and phenylbutanoic as an active compound in this extract **(Fitriana et al., 2018; Nurkhasanah et al., 2020)**.

Table 4 IL-14 expression of mice macrophages by the administration of bangle (Z. *cassumunar*) extract.

Group	IL-14 expression level (%) ±SD
Normal control	2.16 ±0.30
25 ppm	87.44 ±7.35*
50 ppm	70.13 ±3.92*[a]
100 ppm	61.15 ±1.52*[ab]

Note: *significantly different compared to normal control group (p <0.05); [a]significantly different compared to concentration 25 ppm group (p <0.05); [b]significantly different compared to concentration 50 ppm group (p <0.05).

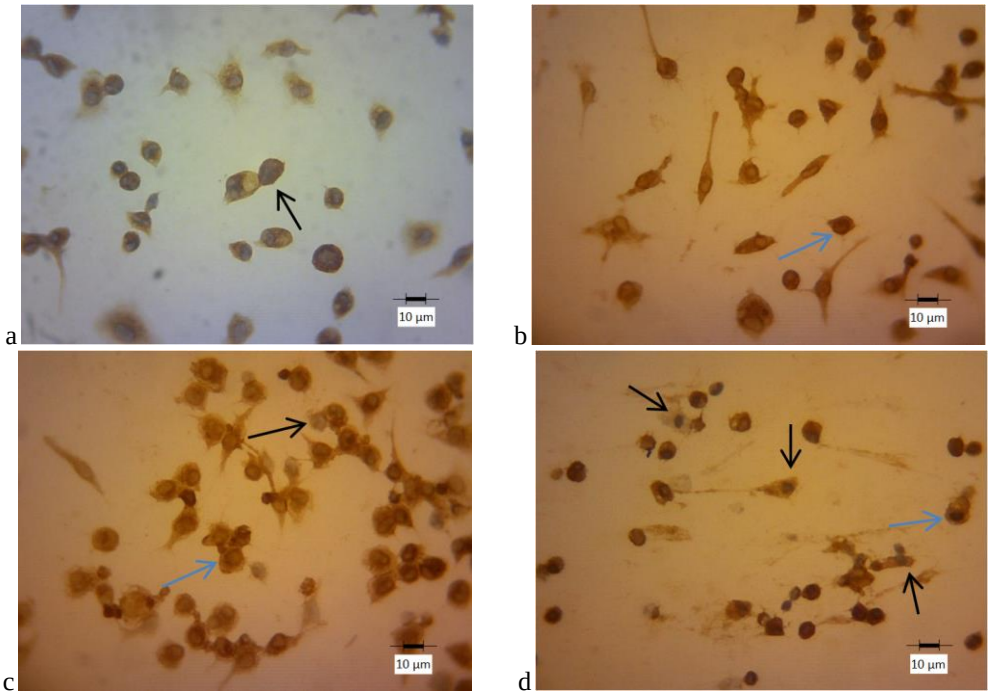


Figure 4 IL-14 expression on macrophage cells after being treated with bangle (Z. *cassumunar*) extract: a. normal control, b. bangle extract concentration of 25 ppm, c. bangle extract concentration of 50 ppm, d. bangle extract concentration of 100 ppm. (blue arrows: macrophage cells express IL-14; black arrows: macrophage cells do not express IL-14). Note: (400x magnification).

The treatment of bangle methanol fraction as a complementary therapy in mice infected with *P. berghei* can increase IL-10 levels **(Fitriana et al., 2018)**. It is known that the administration of bangle ethanol extract can inhibit the production of TNF-α which is a proinflammatory cytokine from Th1 cells, a decrease in TNF-α levels indicates an increase in IL-10 expression, where IL-10 (anti-inflammatory cytokines from Th cells - 2) can inhibit TNF-α production **(Perera et al., 2013; Arini et al., 2014)**. Also, there is a level of *in vivo* research with a length of 21 days and LPS stimulation of E. coli, 2,5 and 5 mg/20g BW of ethanol extract of bangle rhizome in mice can increase the expression of IL-10 **(Nurkhasanah et al., 2020)**.

Increasing the concentration of the test compound does not accord with an increased level of IL-10 expression because IL-10 is produced by active macrophages and Th-2 cells **(Akrom, 2017)**.

The results of the phagocytic activity parameters of macrophages also showed a decrease (%) of active phagocytic cells and phagocytosis index with an increase in the concentration of the test compound **(Adhila et al., 2019)**. The decrease in active macrophages will reduce the expression of IL-10 produced.

Interleukin-14

Similar to IL-10, the way to differentiate between cells expressed IL-14 will have a brown color by DAB, while cells that are not expressed have a blue or purple color by Mayer hematoxylin (counterstain). Figure 4 shows the expression of IL-14 in macrophage cells treated with extract concentrations of 25, 50, and 100 ppm.

In Table 4 the average levels of IL-14 expression in the treatment group concentrations of 25, 50, and 100 ppm were 87.44%; 70.13%; and 61.15%. This result was higher and significantly different (p <0.05) compared to the average level of IL-14 expression in the normal control group (2.16%). The administration of bangle ethanol extract showed the effect was to increase IL-14 expression. Although a decrease in IL-14 levels was seen with the administration the higher concentration of the extract. From this explanation, it can be seen that the active component of the bangle can act as an immunomodulator by improved IL-14 levels.

A previous study highlights that *Zingiber cassumunar* has the immunomodulatory activity which may be caused by curcumin and phenylbutanoic compound **(Chairul et al., 2009; Nurkhasanah et al., 2019b; Nurkhasanah et al., 2020)**. The ethyl acetate fraction of bangle extract concentration of 25, 50 and, 100 µg.mL^{-1} *in vitro* had a

role as an immunomodulator through increased IL-14 expression and the higher extract concentration showed the higher IL-14 expression produced **(Nurkhasanah et al., 2019b)**. Also, there is a level of *in vivo* research with a length of 21 days and LPS stimulation of E. coli, 5 mg.20g^{-1} BW of ethanol extract of bangle rhizome in mice can increase the expression of IL-14 **(Nurkhasanah et al., 2020)**.

CONCLUSION

Bangle ethanol 96% extract decreased NO levels (in all variations of extract concentration), and increased ROI levels compared to normal control groups (at extract concentrations of 25 and 50 ppm) with a significant effect (p <0.05). Also, bangle ethanol 96% extract increased in IL-10 and IL-14 levels compared to the normal control group (in all variations of the extract concentration) with a significant effect (p <0.05). These results indicate that bangle ethanol 96% extract has an immunomodulatory effect *in vitro*.

REFERENCES

Adhila, G., Nurkhasanah, N., Sulistyani, N. 2019. In vitro immunomodulatory activity test of bengle rhizoma extract (*Zingiber cassumunar* Roxb.): phagocytic activity of macrophages and lymphocyte proliferation in mice. *Pharmaciana*, vol. 9, no. 2, p. 211-218. https://doi.org/10.12928/pharmaciana.v9i2.12881

Akrom, A. 2017. *Pengantar imunologi untuk farmasi* (*Introduction of immunology for pharmacy*). Yogyakarta : Pustaka Imany. (In Indonesian)

Akrom, A., Mustofa, M. 2017. Black cumin seed oil increases phagocytic activity and secretion of IL-12 by macrophages. *Biomedical Research*, vol. 28, p. 5241-5246.

Akrom, A., Widjaya, A., Armansyah, T. 2015. Ekstrak etanol biji jintan hitam (*Nigella sativa*) meningkatkan aktivitas fagositosis makrofag mencit swiss yang diinfeksi *Lysteria monocytogenes* (Ethanolic extract of black cumin (*Nigela sativa*) seed increases macrophage phagocytic activity of swiss mice infected with *Lysteria monocytogenes*). *Jurnal Kedokteran Hewan*, vol. 9, no. 2, p. 94-100. https://doi.org/10.21157/j.ked.hewan.v9i2.2807

Arini, P. S., Utami, W. S., Sulistyaningsih, E. 2014. Pengaruh ekstrak bangle (*Zingiber cassumunar* Roxb.) terhadap kadar TNF-α pada mencit yang diinfeksi *Plasmodium berghei* (The effect of bangle extract (*Zingiber cassumunar* Roxb.) on TNF-α in mice infected with *Plasmodium berghei*). *Pustaka Kesehatan*, vol. 2, no. 2, p. 230-234.

Baratawidjaja, K. G., Rengganis, I. 2014. *Immunologi dasar* (*Basic immunology*). 11th ed. Jakarta : Badan Penerbit Fakultas Kedokteran Universitas Indonesia. ISBN 978-979-496-819-2. (In Indonesian)

Bastos, K. R. B., Marinho, C. R. F., Barboza, R., Russo, M., Alvarez, J. M., Lima, M. R. D. 2004. What kind of message does IL-12/IL-23 bring to macrophages and dendritic cells. *Microbes Infect*, vol. 6, p. 630-636. https://doi.org/10.1016/j.micinf.2004.02.012

Brouet, I., Ohshima, H. 1995. Curcumin, an anti-tumour promoter and anti-inflammatory agent, inhibits induction of nitric oxide synthase in activated macrophages. *Biochemical and Biophysical Research Commun*ication, vol. 206, p. 533-540. https://doi.org/10.1006/bbrc.1995.1076

Fitriana, A., Hermansyah, B., Jauhani, M. A., Utami, W. S. 2018. Kadar IL-10 mencit Balb/c terinfeksi *Plasmodium berghei* dengan pemberian fraksi metanol bangle (*Zingiber cassumunar* Roxb.) sebagai terapi komplementer (The IL-10 level of *Plasmodium berghei*-infected Balb/C mice after methanolic fraction of bangle (*Zingiber cassumunar* Roxb.) administration as a complementary therapy). *Pustaka Kesehatan*, vol. 6, no. 1, p. 92-96. https://doi.org/10.19184/pk.v6i1.6791

Goodyear-bruch, B. C., Pierce, J. D. 2002. Oxidative stress in critical ill patients. *American Journal of Critical Care*, vol. 11, p. 543-551. https://doi.org/10.4037/ajcc2002.11.6.543

Han, A. R., Kim, M. S., Jeong, Y. H., Lee, S. K., Seo, E. K. 2005. Cyclooxygenase-2 inhibitory phenylbutenoids from the rhizomes of *Zingiber cassumunar* Roxb. *Chemical Pharmaceutical Bulletin*, vol. 53, p. 1466-1468. https://doi.org/10.1248/cpb.53.1466

Chairul, C., Praptiwi, P., Chairul, S. M. 2009. Phagocytosis effectivity test of phenylbutenoid compounds isolated from bangle (*Zingiber cassumunar* Roxb.) rhizome. *Biodiversitas Journal of Biological Diversity*, vol. 10, no. 1, p. 40-43. https://doi.org/10.13057/biodiv/d100108

Javois, L. C. 1999. Immunocytochemical methods and protocols. Totowa, New Jersey, USA : Humana Press. ISBN 978-1-59259-213-5.

Jeenapongsa, R., Yoovatharworn, K., Sriwatanakul, K. M., Pongprayoon, U., Sriwatanakul, K. 2003. Anti-inflamatory activity of (*E*)-1-(3,4-dimethoxyphenyl) butadiene from *Zingiber cassumunar* Roxb. *Journal of Ethnopharmacology*, vol. 87, p. 143-148. https://doi.org/10.1016/S0378-8741(03)00098-9

Kaewchoothong, A., Tewtrakul, S., Panichayupakaranant, P. 2012. Inhibitory effect of phenylbutanoid-rich *Zingiber cassumunar* extracts on nitric oxide production by murine macrophage-like RAW 264.7 cells. *Phytotherapy Research*, vol. 26, p. 1789-1792. https://doi.org/10.1002/ptr.4661

Kil, J. S., Son, Y., Cheong, Y. K., Kim, N. H., Jeong, H. J., Kwon, J. W., Lee, E. J., Kwon, T. O., Chung, H. T., Pae, H. O. 2012. Okanin, a chalcone found in the genus bidens, and 3-penten-2-one inhibit inducible nitric oxide synthase expression via heme oxygenase-1 induction in RAW 264.7 macrophages activated with lipopolysaccharide. *Journal of Clinical Biochemistry and Nutrition*, vol. 50, p. 53-58. https://doi.org/10.3164/jcbn.11-30

Leelarungrayub, J., Manorsoi, J., Manorsoi, A. 2017. Anti-inflamatory activity of niosomes entrapped with plai oil (*Zingiber cassumunar* Roxb.) by therapeutic ultrasound in rat model. *International Journal of Nanomedicine*, vol. 12, p. 2469-2476. https://doi.org/10.2147/IJN.S129131

Leijh, P. C. J., van Furth, R., van Zwet, T. L. 1986. *In vitro* determination of phagocytosis and intracellular killing by polymorphonuclear and mononuclear phagocytes. In: Weir, M. D., Herzenberg, L. A., Blackwell, C. *Handbook of experimental immunology*. Oxford : Blackwell Scientific Publication, 813 p. ISBN 0-632-01499-7.

Meshcer, A. L. 2017. *Histologi dasar junqueira teks and atlas* (*Junqueira's basic histology tekxt and atlas*). 14th ed. Jakarta : EGC, 626 p. ISBN 978-979-044-813-1. (In Indonesian)

Mimche, P. N., Taramelli, D., Vivas, L. 2011. The plant-based immunomodulator curcumin as a potential candidate for the development of an adjunctive therapy for cerebral malaria. *Malaria Journal*, vol. 10, p. 1-9. https://doi.org/10.1186/1475-2875-10-S1-S10

Murphy, K. P. 2012. *Janeway's Immunobiology*. New York, USA : Garland Science. ISBN 978-0815342434.

Nakamura, S., Junko, I., Matsuda, H., Wakayama, H., Pongpiriyadacha, Y., Yoshikawa, M. 2009. Structures of new

phenylbutanoids and nitric oxide production inhibitors from the rhizomes of Zingiber cassumunar. *Chemical Pharmaceutical Bulletin*, vol. 57, p. 1267-1272. https://doi.org/10.1248/cpb.57.1267

Nathan, C., Ding, A. 2010. Nonresolving inflamation. *Cell*, vol. 140, p. 871-882. https://doi.org/10.1016/j.cell.2010.02.029

Nurkhasanah, M., Sulistyani, N., Handayani, Y. A., Kamila, Q., Isnaini, A. C. N. 2020. The increasing level of interleukin in the *Zingiber cassumunar*-treated mice. *Biotropia Journal*, in press.

Nurkhasanah, N., Santoso, R. D., 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 Conference Series: Materials Science and Engineering*, vol. 259, p. 1-7. https://doi.org/10.1088/1757-899X/259/1/012007

Nurkhasanah, N., Sulistyani, N., Ghifarizi, M. A. 2019a. The effect of bengle (*Zingiber cassumunar* Roxb.) rhizome chloroform extract on nitric oxide and reactive oxygen intermediate secretions in vitro. *In Ahmad Dahlan International Conference Series on Pharmacy and Health Science*, vol. 18. Yogyakarta, Indonesia : Atlantis Press, p. 43-47. ISBN 978-94-6252-845-1. https://doi.org/10.2991/adics-phs-19.2019.9

Nurkhasanah, N., Sulistyani, N., Noorlina, N. 2019b. In vitro activity of immunomodulator of n-hexane fraction of bangle (*Zingiber cassumunar* Roxb.) ethanol extract. *Research Journal of Chemistry and Environment*, vol. 23, p. 62-64.

Nurkhasanah, N., Sulistyani, N., Sofyan, A. D. 2019. The effect of ethyl acetate fraction of bangle (*Zingiber cassumunar Roxb*.) rhizome extracts on Interleukin-10 and Interleukin-14 expression in vitro. *In Ahmad Dahlan International Conference Series on Pharmacy and Health Science*, vol. 18. Yogyakarta, Indonesia : Ahmad Dahlan University, Atlantis Press, p. 38-42. ISBN 978-94-6252-845-1. https://doi.org/10.2991/adics-phs-19.2019.8

Nurkhasanah, N., Zulkarmen, L. R. 2014. Efek ekstrak etanol kelopak rosella (*Hibiscus sabdariffa* L.) terhadap sekresi nitrit oxida (NO) makrofag peritoneum tikus yang diinduksi 7,12-*dimethylbenz(a)antracene* (DMBA) (The effect of roselle (*Hibiscus sabdariffa* L.) calyx ethanolic extract on the secretion of nitit oxide (NO) of peritoneal macrophage of *7,12-dimethylbenz(a)antracene* (DMBA) induced rats). *Media Farmasi*, vol. 11, no. 2, p. 155-166.

Perera, M. K., Herath, N. P., Pathirana, S. L., Phone-kyaw, M., Alles, H. K., Mendis, K. N., Premawansa, S., Handunnetti, S. M. 2013. Association of high plasma TNF-alpha levels and TNF-alpha/IL-10 ratios with TNF2 allele in severe P. *falciparum* malaria patients in Sri Lanka. *Pathogen and Global Health*, vol. 107, no. 1, p. 21-29. https://doi.org/10.1179/2047773212Y.0000000069

Salim, T., Sershen, C. L., May, E. E. 2016. Investigating the role of TNF-α and IFN-γ activation on the dynamics of iNOS gene expression in LPS stimulated macrophages. *Plos One*, vol. 11, no. 6, p. 1-35. https://doi.org/10.1371/journal.pone.0153289

Thiery, J., Dorothee, G., Haddada, H., Echchakir, H., Richon, C., Stancou, R., Vergnon, I., Benard, J., Mami-Chouaib, F., Chouaib, S. 2003. Potentiation of a tumor cell susceptibility to autologous CTL killing by restoration of wild-type p53 function. *Journal Immunology*, vol. 170, p. 5919-5926. https://doi.org/10.4049/jimmunol.170.12.5919

Tunctan, B., Uludag, O., Altug, S., Abacioglu, N. 1998. Effects of nitric oxide synthase inhibition in lipopolysaccharide-induced sepsis in mice. *Pharmacological Research*, vol. 38, p. 405-411. https://doi.org/10.1006/phrs.1998.0381

Ulfah, M., Cahyani, V. S. N., Kinasih, I. 2017. Pengaruh pemberian seduhan teh daun sirsak (*Annona muricata* L.) terhadap aktivitas fagositosis sel makrofag dan proliferasi sel limfosit mencit galur Babl/C yang diinduksi vaksin hepatitis B (The effect of treatment soursop leaf tea (*Annona muricata* L.) on phagocytic activity of macrophage cells and lymphocyte cell proliferation of mice of Babl / C strain induced by hepatitis B vaccine). *Majalah Ilmiah Momentum*, vol. 13, no. 2, p. 63-71.

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Contact address:

Nurkhasanah Mahfudh, Universitas Ahmad Dahlan, Faculty of Pharmacy, Jalan Prof. Soepomo, Yogyakarta 55164, Indonesia, Tel.: (0274) 563515,
E-mail: nurkhas@gmail.com
ORCID: https://orcid.org/0000-0001-6156-4318

*Nanik Sulistyani, Universitas Ahmad Dahlan, Faculty of Pharmacy, Jalan Prof. Soepomo, Yogyakarta 55164, Indonesia, Tel.: (0274) 563515,
E-mail: naniksulistyani@gmail.com
ORCID: https://orcid.org/0000-0002-6541-8198

Ghina Adhila, Universitas Ahmad Dahlan, Faculty of Pharmacy, Jalan Prof. Soepomo, Yogyakarta 55164, Indonesia, Tel.: (0274) 563515,
E-mail: adhilaaghinaa@gmail.com
ORCID: https://orcid.org/0000-0002-7147-8859

Corresponding author: *