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The Effect of Ethyl Acetate Fraction of Bangle (*Zingiber Cassumunar Roxb.*) Rhizome Extracts on Interleukin-10 and Interleukin-14 Expression in Vitro

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***Abstract*—An unbalanced immune system results in reduced ability to protect the body from pathogens. Immune system imbalances can be restored with immunomodulators. One plant that has the potential to be immunomodulatory is the bangle rhizome (*Zingiber cassumunar Roxb.*) Which has curcumin as an immunomodulator. Its activity as an immunomodulator can be seen by increasing the expression of IL-10 and IL-14 which play an important role in controlling the immune response. This study aims to determine the effect of ethyl acetate fraction of bangle extract (*Zingiber cassumunar Roxb.*) on the expression of IL-10 and IL-14 in vitro. Macrophages were isolated from the peritoneum of male mice Balb / C strain, then cultured for 24 hours. The macrophages were treated with ethyl acetate fraction of bangle extract with concentrations of 25, 50, 100 µg/mL and the control group did not treated with ethyl acetate fraction of bangle extract. The method used to analyze the expression of IL-10 and IL-14 is immunocytochemistry. Examination of the presence of curcumin is carried out by Thin Layer Chromatography (TLC) method. The data obtained were analyzed using a one-way ANOVA statistical test with a confidence level of 95%. The results showed that the administration of ethyl acetate fraction of bangle extract to mice macrophages with a concentration of 25, 50, 100 µg/mL compared to the negative control group had a significant difference (p) <0.05) in IL-10 and IL-14 expression. In IL-10 there was an increase in percentage of expression in the control group, 25, 50, 100 µg/mL, respectively 55.83, 62.047, 66.593, and 74.387%. In the control group and 25, 50, and 100 µg/mL had significant differences between groups. In IL-14 there was an increase in the percentage of expression in the control group, 25, 50, 100 µg/mL, respectively 48.017, 70.649, 78.821, and 80.645%. In groups with a concentration of 50 µg/mL with 100 µg/mL did not show a significant difference with a significance value of 0.407. In conclusion, the administration of ethyl acetate fraction of bangle extract with a concentration of 25, 50, 100 µg/mL can be used as an immunomodulator by increased expression of IL-10 and IL-14.**

***Keywords*—zingiber cassumunar, immunomodulators, interleukin-10, interlukin-14**

I. INTRODUCTION

The immune system is divided into a specific immune system and a non-specific immune system [1]. Antigen exposure in the body will be responded first by the non-specific immune system that is macrophages [2]. Macrophages function to destroy foreign agents that enter the body [3]. The compound contained in bangle (*Zingiber cassumunar Roxb.*) can increase Antigen Precenting Cell (APC) to activate macrophages that stimulate helper T cells (hT) release and then release cytokines which function to send intracellular signals to stimulate immune system [1]-[4]. There are several types of cytokines in the body, in this study we conducted research on interleukin-10 (IL-10) and interleukin-14 (IL-14).

Interleukin-10 (IL-10) as a center in limiting host immune responses to pathogens, thus preventing damage to hosts and maintaining normal tissue homeostasis [4]. In addition, the action of IL-10 can trigger Natural Killer cells (NK) and IL-2 which are cytotoxic lymphocyte cells [5]. IL-10 regulates the growth and / or differentiation of B cells, helper T cells, mast cells, dendritic cells. While the role of IL-14 is one of the cytokines produced from the immune system. IL-14 is produced by activation of B cells and T cells. When antigens enter they will be detected by T cells [6]. IL-14 regulates the growth of B cells, B cells through plasma cells will secrete antibodies that will fight the antigen that causes infection [2]. Enhancement of the immune system can be enhanced with medicinal ingredients that can restore the balance of the immune system called immunomodulators [7].

The use of chemical compounds from plants can be used as an alternative to increase the activity of the immune system. Bangle (*Zingiber cassumunar Roxb.*) is a spice of the family Zingiberaceae and has been proven to be an immunomodulator. The ethyl acetate fraction of bangle (*Zingiber cassumunar Roxb.*) had highest phagocytised activity and capacity followed by Curcumae.mangga (temu mangga) and Kaempferia Angustifolia (key menir) [11]. Previous research proved that the compounds contained in bangle are curcumin, alkaloids, flavonoids and phenylbutanoid [4].

The content contained in bangle ethanol extract can activate macrophages [8]. Increased IL-14 can be caused by activation of macrophages which gives a signal to the T cells to proliferation and differentiation of B cells [9]. Curcumin found in bangle can increase the exsion of Cluster of

Differentiation 36 (CD36) to increase phagocytosis of macrophages [10]. Curcumin is able to increase Toll-like receptor 2 (TLR2) as a production receptor from (IL-10) on Antigen Precenting Cell (APC), regulating the shift in response from helper T1 cells (hT1) to helper T2 cells (hT2) [4].

Other studies on bangle that ethyl acetate fraction of bangle extract with variations in concentrations of 0.1, 1, 10, 100, 1000 µg (ppm) have a higher phagocytic activity compared to methanol extract and hexane fraction in vitro [11]. The selection of ethyl acetate fraction because ethyl acetate with ethanol 96% has different levels of polarity so it is expected that the separation of non-polar compounds from ethanol extract of rhizome bangle. Ethyl acetate has a polarity index of 4.4 [12], while ethanol 96% has a polarity index of 7.6 [13]. Based on the above descriptions, it is necessary to do research on the effect of ethyl acetate fraction on bangle extract by looking at the expression of IL-10 and IL-14 which are responsible for the immune system.

II. Material and Method

A. Research Design

This study was an experimental study with post-test control design by comparing four groups: the control group, the ethyl acetate fraction of bangle extract group with concentrations of 25, 50, and 100µg/mL.

B. Fractionation of Bangle Extract

In this study, the sample used was the rhizome bangle (*Zingiber cassumunar Roxb.*) taken from the Bringharjo Market in Yogyakarta. The 200 grams of rhizome bangle powder was macerated using 96% ethanol until thick extracts were obtained. The thick ethanol extract obtained was fractionated with n-hexane solvent in a ratio of 1: 4 (ethanol extract: ethyl acetate). The insoluble part of hexane is then fractionated using ethyl acetate solvents with a ratio of 1: 2.5 (ethanol extract: ethyl acetate). The ethyl acetate fraction was collected and then evaporated until a thick fraction was obtained.

C. TLC (Thin Layer Chromatography)

Test the presence of curcumin compounds using TLC with the stationary phase of silica gel GF254 and the mobile phase of chloroform: dichloromethan (3.25: 6.75) then seen on UV 366 nm and UV 254 nm.

D. Making Ethyl Acetate Fraction Solution of Bangle

The 50 mg of ethyl acetate fraction of bangle extract added Dimethyl sulfoxide (DMSO) as much as 200µL to dissolve the ethyl acetate fraction of bangle extract. The suspension of ethyl acetate fraction of bangle extract was made with concentrations of 25, 50 and 100 µg/mL.

E. Macrophage Isolation

In this study, using 2-3 months old male white mice Balb/C strain mice were obtained from Research Laboratory and Integrated Testing Gadjah Mada University Yogyakarta and has obtained ethical approval from the Ahmad Dahlan University Research Ethics Committee under the number 011804063. The mice were fasted 10-12 hours, then anesthetized with chloroform. After that, mice was sprayed with disinfectant solution and placed in the supine position. The skin of the abdomen is opened. Cleaned the peritoneum sheath with 70% alcohol. Injected 10 mL of cold RPMI into the peritoneal cavity. Peritoneal fluid is removed from the peritoneal cavity. The aspirate is centrifuged at 1200 rpm, 4°C for 10 minutes. Macrophages are resuspended with 1000 µL of complete medium. With 10 µL of suspension of macrophages, the number of cells is calculated by a hemocytometer. The cell suspension is grown in a 6-well microplate with a density of 5x105 cells / mL. The cells were incubated in a 5% CO2 incubator at 37oC for 24 hours. After that, added an ethyl acetate fraction of bangle suspension, which was incubated for 24 hours. The supernatant was removed from the wells. After that, cells were fixed with 1000 µl methanol for 10 minutes, then microcultures were frozen at -20°C.

F. Immunocytochemical Test

Macrophages are washed with Phosphate Buffer Saline (PBS), then the microplates were soaked in peroxidase blocking solution washed under running water. Microplates were incubated with 20µL of serum blocking protein for 10 minutes. Added Interleukin-10 and Interleukin-14 as much as 30µL. Microplates were washed with PBS. Add 30µL of biotin incubated 20 minutes. Microplates were washed with PBS. Microplates were incubated with 30µL streptavidin-peroxidase enzyme for 10 minutes, then washed with PBS. Microplates are incubated with 30µL peroxidase substrate solution (DAB) every well. Washed using water flow. Mayer hematoxylin (counterstain) is added as much as 100µL incubated for 2 minutes, microplates were washed with water flow. The slides are then dipped in absolute alcohol and dried. then dipped in xylol mounting media then closed using a deckglasser. After drying, it is ready to be observed in a microscope to see the color. If it is brown, mark the cell as expressed, if it is blue then it is the opposite [14].

G. Data Analysis

1) Calculation of Total Expression Cells

Data analysis was calculated using % cells expression = (number of cells expressing x 100%)/(total cell number)

Calculation of cell numbers is done by counting the number of cells that appear on the microscope through 6 different fields of view for the samples tested.

2) Statistics Test

Normality test was carried out using this test Saphiro-Wilk, with a data amount of less than 50. Continued variance homogeneity test using the Levene test. If the normal distribution test and the variance homogeneity test are homogeneous and normally distributed the, test is continued with parametric one-way variance analysis, ANOVA and LSD test.

III. Results and Discussion

A. Qualitative Analysis of Ethyl Acetate Bangle Faction

The extraction method used in this study is maceration. Maseration is the process of extracting simplicia using a solvent with stirring at room temperature [15]. The selection of this technique is based on the advantages that are owned, including: very simple, does not require special tools and is

more affordable and based on the physical chemical properties contained in the bangle rhizome. Fractionation is the separation process with a low or non-polar level solvent to a polar solvent. Ethyl acetate with ethanol has a different level of polarity so it is expected that the separation of non-polar compounds from ethanol extract of rhizome bangle. Qualitative analysis using Thin Layer Chromatography (TLC) aims to determine the components of compounds present in the ethyl acetate fraction of bangle extract obtained. The results of TLC observed in UV 254 and 366 nm rays can be seen in Fig 1.

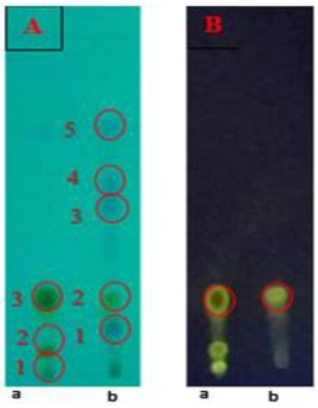


Fig. 1. Thin layer chromatogram profile with the mobile phase of chloroform: dichloromethane in UV 254 nm (1) dan 365 nm (2): a. curcumin standard, b. ethyl acetate fraction of bangle extract.

From the results of the TLC test obtained Rf spotting from the elution of the ethyl acetate fraction of bangle extract 1% parallel to Rf curcumin 0,1% as a comparison with a value of 0.225. The calculation results of Rf ethyl acetate fraction of bangle extract and curcumin standard can be seen in Table I. This shows that ethyl acetate fraction of bangle extrcat positive contains curcumin. Previous studies showed that there were 5 spots produced by elution of bangle rhizome extract with the same mobile phase composition. One of them has a spot with the same Rf value as the 0.1% curcumin standard, which is 0.37 [16].

TABLE I. RF RESULTS OF ETHYL ACETATE FRACTION OF BANGLE EXTRACT AND CURCUMIN STANDARD

Spot	Ethyl Acetate Fraction		Rf	Curcumin Standard		Rf
	UV 254	*UV 366*		*UV 254*	*UV 366*	
1	Blue-Purple		0,15	Yellow-Brown	Yellow	0,063
2	Yellow-Brown	yellow	0,225	Yellow-Brown	Yellow	0,1
3	Blue-Purple		0,363	Yellow-Brown	Yellow	0,225
4	Blue-Purple		0,4			
5	Blue-Purple		0,75			

B. *Sampling and Isolation of Macrophages*

Surgery is performed by tearing abdominal skin of mice, but not to damage the peritoneal membrane, because macrophages taken from the peritoneal fluid. The macrophages in that section are located freely along the capillaries so that it is easy to catch incoming antigens. The process of isolating macrophages using the media of Roswell Park Memorial Institute 1640 (RPMI 1640) which contains nutrients to support cell growth. Complete medium (MC) containing RPMI, Foetal Bovine Serum 10% (FBS 10%), 2% penstrep, 0.5% fungision for fulfill the nutritional needs of cells to survive.

Ethyl acetate fraction of bangle extract was dissolved with Dimethyl Sulfoxide 1% (DMSO 1%), DMSO is an aprotic polarity solvent that effectively dissolves various organic and inorganic chemicals [17]. A high DMSO concentration can be a cause of cell death. DMSO with a concentration of 1.67% v/v did not affect T47D cell viability [18]. Suspense ethyl acetate fraction was diluted using media with a concentration of 25, 50, 100 µg/mL.

C. *Result of Immunocytochemistry Test*

Interleukin-10 (IL-10) has two opposing activities as an immunostimulator and as an immuno-suppressor. IL-10 activities as an immunostimulator include stimulating NK and IL-2 cells which were cytotoxic lymphocyte cells that induce cytokine production such as Interferon gamma (IFN-γ), Granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor alpha (TNF-α) [5]. While IL-10 activities as immunosuppressants include preventing the production of IL-12 and preventing costimulatory expression of major histocompatibility complex-II (MHC-II) molecules in macrophages and dendritic cells [2]. In addition, IL-10 had the ability to inhibit cytokine production by Th1 cells. [1]. The results of IL-10 staining in each group can be seen in Fig 2.

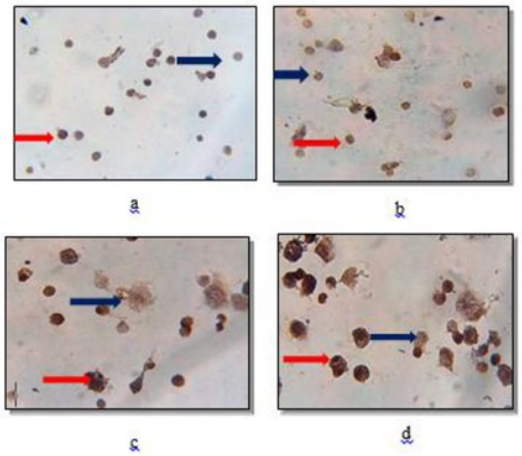


Fig. 2. Image of IL-10 expression on macrophages given variations in ethyl acetate fraction of bangle extrcat concentration. (a) negative control, (b) concentration of 25µg/mL, (c) concentration of 50µg / mL, (d) concentration of 100 µg / mL. Red arrow: expressed; Blue arrow: Not expressed.

The lowest average number of macrophages is in the 50µg/mL concentration group and the highest number of macrophages is in the 100µg / mL concentration group. From the results of the normality test obtain data that are normally distributed with a significance value> 0.05 with a confidence

level of 95%. The homogeneity test obtained a significance value of 0.538, concluded that the data of all groups were homogeneous. The data obtained were normal and homogeneous, then continued with ANOVA with a confidence level of 95%, the data showed that there were significant differences ($p < 0.05$) in each group, the results of the post hoc LSD test showed that the control group was negative and 3 concentration variations have significant differences between groups. The comparison graph of IL-10 expression between groups can be seen in Fig. 3.

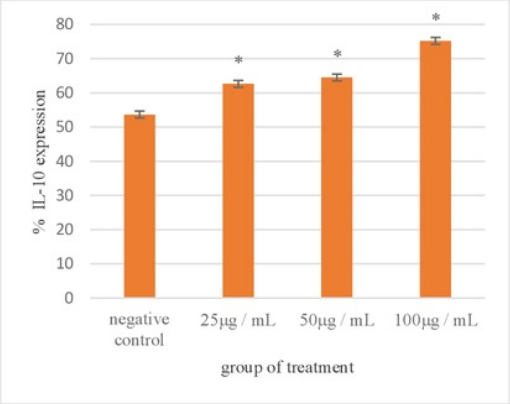


Fig. 3. Graph comparison of the percentage of IL-10 expression in mice macrophages treated with ethyl acetate fraction of bangle extract with a concentration of 25, 50, 100 µg/mL

Figure 3 shows percentage of IL-10 expression, there was an increase in percentage of expression in the control group, 25, 50, 100 µg/mL, respectively 55.83, 62.047, 66.593, and 74.387%. The higher the concentration given, the higher the percentage of IL-10 expression. Distribution ethyl acetate fraction of bangle extrcat in mice macrophages shows the efficacy as an immunomodulator by increasing the expression of IL-10. At the lowest concentration of 25µg/mL it can restore the balance of the immune system. Previous study reported, using the ethanol extract of rhizome bangle in vivo with healthy control groups; negative control; variation of dose 1.25; 2.5; 5 mg / 20 gBW of mice show potency as an immunomodulator through increased expression of IL-10 [19]. The previous study also reported that the effects of rosella treatment on the secretion of IL-10 and IL-14 were incubated by DMBA in rats. From the results of the study the percentage of negative control cell expression was lower than the group given the dose variation of roselle extract [20].

Curcumin in bangle can increase TLR2 as a production receptor from IL-10 on the APC then activate macrophages, regulate the shift in response from Th1 to Th2 [15]. The other studies reported that administration of bangle rhizome extract in acute inflammation of malaria can increase IL-10. The increase in IFN-γ will induce IL-10 in the Antigen Precenting Cell (APC) involved in efforts to form antibodies [21]. The production of TNF-α by monocytes / macrophages in inflammation of malaria will be excessive and then rapidly stimulate high counter-regulatory IL-10 production and affect B lymphocyte cells to produce antibodies [15].

Interleukin-14 (IL-14) is a High Molecular Weight B Cell Growth Factor (HMW-BCGF) cytokine. Interleukin-14 is secreted by activated B and T cells and dendritic follicular cells that promote the growth of B cells, memory B cells, and antibody production [6]. The results of IL-14 staining and comparison graph of the percentage of IL-14 expression in each group the can be seen in Figure 4 and 5.

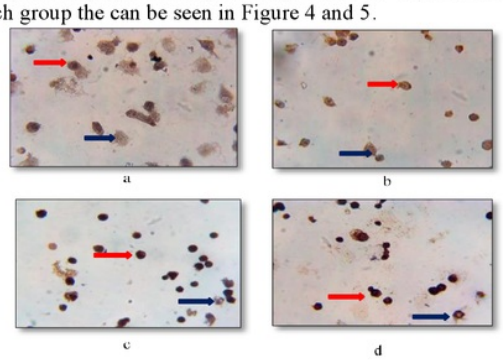
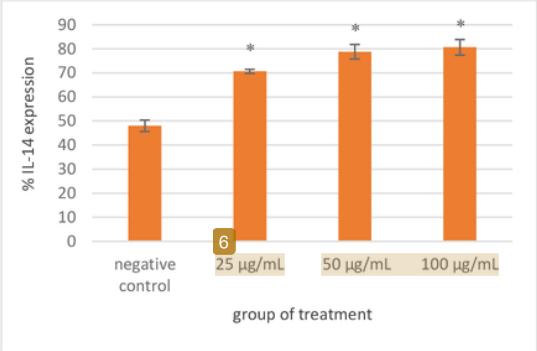


Fig. 4. Image of IL-14 expression on macrophages given variations in ethyl acetate fraction of bangle extrcat concentration. (a) negative control, (b) concentration of 25µg/mL, (c) concentration of 50µg / mL, (d) concentration of 100 µg / mL. Red arrow: expressed; Blue arrow: Not expressed.

Increasing the concentration of ethyl acetate fraction causes an increase in the number of macrophages. The lowest average number of macrophages is in the 25µg / mL concentration group and the highest average number of macrophages is in the 100µg / mL concentration group. From the results of the normality test obtain data that are normally distributed with a significance value > 0.05 with a confidence level of 95%. The homogeneity test obtained a significance value of 0.275, it was concluded that the data of all groups were homogeneous. From the ANOVA test results showed a negative control group had a significant difference with all groups given the ethyl acetate fraction of bangle extract with a significance value ($p < 0.05$) with a confidence level of 95%. This showed that the ethyl acetate fraction of bangle bangle could have an effect as an immunomodulator with a mechanism for increasing IL-14 expression. The LSD test results showed that the concentration group of 50 µg / mL with 100 µg / mL did not have a significant difference. This can be seen from the significance value of 0.412. While in the variation group concentration of 25 µg / mL with 50 µg / mL there were significant differences. This is seen from the significance value of 0.407.



* = significantly different with negative controls group

Fig. 5. Graph comparison of the percentage of IL-14 expression in mice macrophages treated with ethyl acetate fraction of bangle extrcat with a concentration of 25, 50, 100 µg/mL.

Figure 5 shows percentage of IL-14 expression, there was an increase in percentage of expression in the control group, 25, 50, 100 µg/mL, respectively 48.017, 70.649, 78.821, and 80.645%. From the results percentage of IL-14 expression in each group has different prices. The results of the percentage of negative control group expression showed a significant difference with the group given the ethyl acetate fraction of bangle extrcat. Where the percentage of negative control expressions is smaller than the percentage expression of groups given ethyl acetate fraction of bangle extract. The results showed an increase in the percentage of IL-14 expression in 3 variations of concentration. Increased concentration is a factor that influences the percentage of expressed cells. The higher the concentration, the higher the percentage of IL-14 expression. The variation of the concentration of ethyl acetate fraction of bangle extract on male mice macrophages showed efficacy as an immunomodulator by increasing the expression of IL-14 at the lowest concentration of 25 µg / mL.

Curcumin could modulate T cell activity, B cells, macrophages, neutrophils, NK cells, and dendritic cells in the immune system [22]. The content in bangle ethanol extract could activate macrophages [8]. Increased IL-14 can be caused by activation of macrophages which signal T cells to proliferation and differentiation of B cells to produce antibodies. Used of rhizome bangle ethanol extract in vivo with normal control groups; negative control; variation of dose 1.25; 2.5; 5mg/20gBW of mice at the lowest dose of 1.25mg/20gBW of mice could'nt increase the percentage of cell expression. With a dose of 2.5; 5mg/20gBW shows potency as an immunomodulator through increased expression of IL-14 [8]. The previous study also reported that rosella extract which was incubated by DMBA in rat can increase IL-14 expression in the lowest group of doses of 10 mg/kgBW. Increased dosage does not cause a significant increase or decrease in percentage of cell expression. The results of this study show that low doses of 10 mg/kgBW are most effective in increasing IL-14 secretion [20]. IL-14 is secreted by activated B and T cells [6]. Increased IL-14 can increase lymphocyte proliferation. IL-14 produced by lymphocytes can decrease due to a decrease in lymphocytes. This increase lymphocyte proliferation was proven from provious studies, administration of Ethanol Bangle Extract with doses of 1.25 and 5mg/20gramBW significantly increased the proliferation of Lipopolysaccharide (LPS) stimulated mice [23]. The other previous studies, administration of ethyl acetate fraction of bangle extract at doses of 25, 50, 100 µg/mL to increase lymphocyte proliferation [24].

IV. Conclusion

The administration of ethyl acetate fraction of bangle extract with a concentration of 25, 50, 100 µg/mL could increase the expression of IL-10 and IL-14. Increasing the concentration of ethyl acetate fraction of bangle extract caused a higher percentage of IL-10 and IL-14 expression. The percentage of IL-10 expression in the control group, 25, 50, 100 µg/mL, respectively 55.83, 62.047, 66.593, and 74.387%. The percentage of IL-14 expression in the control group, 25, 50, 100 µg/mL, respectively 48.017, 70.649, 78.821, and 80.645%. It was concluded that bangle (*Zingiber cassumunar Rox.b*) extract was potentially an immunomodulator.

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