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THE INCREASING LEVEL OF INTERLEUKIN IN THE *Zingiber cassumunar*-TREATED MICE

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ABSTRACT

The cytokine is one of the proteins responsible for the immune system. Several types of cytokines acting as key regulators of infection include IL-10, IL-12, and IL-14. The chemical content of *Zingiber cassumunar* shows potential immunomodulatory effects. This study aimed to determine the effect of the ethanol extract of *Zingiber cassumunar* (EEZC) on the expressions of IL-10, IL-12, and IL-14. The test animals were BALB/c mice, which were divided into five groups, i.e., normal group (untreated), negative control group (treated with 10% of tween 80), and three treatment groups that respectively received 1.25 mg, 2.5mg, and 5mg/20g BW of EEZC. The treatment was carried out for 21 days. On the 22nd day, the mice were induced with LPS intraperitoneally (except for the normal group). The interleukin expression was observed by immunohistochemistry using specific antibodies, and the expressed cells were counted under a microscope. The administration of EEZC at the doses of 1.25 mg, 2.5mg, and 5mg/20g BW for 21 days increased the expression of IL-10, IL-12, and IL-14 significantly and proportionally to the dose. and suggested the potency of extract to induce both innate and adaptive immunity. This activity may be attributable to curcumin as an active compound in this extract.

Keywords: curcumin, immunomodulator, interleukin, *Zingiber cassumunar*

INTRODUCTION

The immune system is responsible for protecting the host from various pathogenic microorganisms. At the same time, it functions as a control of immune responses and prevents over-reaction to the body's own cells (Saraiva and O'Garra, 2010). A decrease in the immune system can affect the body's strength to fight infections or other diseases. Therefore, the presence of immunomodulator compound that can improve the immune response to disease or infection is necessary.

Immunomodulator could be defined as a substance, which can stimulate, suppress or modulate any of the components of the immuno system including both specific and nonspecific immune system (Das et al., 2014). Modulation of the immune system was remarked by induction, expression, amplification or inhibition of certain part in the immune signaling and response. Thus, immunomodulator is a substance used for its effect on the immune system.

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that has a crucial role in preventing inflammatory and autoimmune pathologies. It can both impede pathogen clearance and ameliorate immunopathology. Many different types of cells can produce IL-10, with the major source of IL-10

varying in different tissues or during acute or chronic stages of the same infection (Couper et al., 2008). IL-10 has a central role in infection by limiting the immune response to pathogens and thereby preventing damage to the host.The production of IL-10 was associated with regulatory T (Treg) cells. (Saraiva and O'Garra, 2010).

Interleukin-12 is an important cytokine in immunoregulation produced mainly by antigen-presenting cells (APC), including macrophages and dendritic cells that respond to microbes (Abbas et al., 2017). During the immune response, IL-12 is produced as a reaction to stimuli of various compounds (including lipopolysaccharide/LPS).

Interleukin-14 (IL-14) is one of the cytokines produced by the immune system. It is produced by activated B cells and T cells (Leca et al., 2008). The role of IL-14 is to regulate B-cell proliferation. IL-14 can increase antibody responses to vaccinations causing autoimmunity and contribute to B-cell lymphoma formation (Shen et al., 2006).

Zingiber cassumunar, known locally as *bengle* (Javanese, Indonesia), has been used for treating various diseases traditionally. *Z. cassumunar* belongs to Zingiberaceae family and contains terpenoids, essential oil, and curcuminoids. Several studies on *Z.cassumunar* reported some activities including anticancer (Varalakshmi et al., 2008), antioxidant (Vankar et al., 2006) (Bua-in and Paisooksantivatana, 2009) and immunomodulator (Nurkhasanah et al., 2017; Rahmawati, 2013). This plant has been reported to exhibit an immunomodulatory activity by increasing phagocytic activity *in vitro* (Chairul et al., 2009), increasing of nitric oxide and reactive oxygen species (Nurkhasanah et al., 2017) and decreasing of malondialdehyde product in *Plasmodium berghei*-infected mice (Nurmasari et al., 2014).

This study presents the activity of ethanol extract of *Zingiber cassumunar* (EEZC) in stimulating the immune response *in vivo* as observed from its effect on interleukin-10, -12 and -14. The present study will focus on these cytokines due to the important function of these cytokine in the immune respons, either innate and adaptive immunity. The expression IL-10 and IL-14 are close related to activation of adaptive immunity while the IL-12 is important in cell communication between the macrophage and T cells. The study was carried on Balb/c male mice for 28 days treatment and through oral administration. This *in vivo* study provides evidence of higher effectiveness of EEZC in increasing immune response. This evidence was important information for development of *Z. cassumunar* to be an immunomodulatory product.

MATERIALS AND METHODS

Materials

The *Zingiber cassumunar* rhizome was collected from a local market in Yogyakarta and identified in the Biology Laboratory, Universitas Ahmad Dahlan. The test animal was obtained from

the Animal House of the Integrated Research and Testing Laboratory, Universitas Gadjah Mada (LPPT UGM) Yogyakarta, Indonesia.

Extraction

The rhizome was selected, washed, and then sliced. The sliced rhizome was dried in an oven at a temperature of 50ºC. Afterward, the dried rhizome was blended or ground into powder. The extraction was carried out by maceration method using 96% ethanol as the solvent. The maceration lasted for 24 hours, and the yield were evaporated in a vacuum rotary evaporator to obtain a concentrated extract. The concentrated extract was used for treatment and designated as EEZC.

Thin-layer Chromatography (TLC) analysis of the extract

The TLC analysis was carried out to identify the active compound of EEZC. A total of 100.0 mg of EEZC was dissolved in 10.0 ml of absolute ethanol. This procedure used curcuminoids (Sigma) that was dissolved in ethanol as a standard. Each 2 µL of the extract and curcuminoid were applied on silica gel GF 254 as the stationary phase and eluted with the mobile phase of chloroform: ethanol: glacial acetic acid (94: 5: 1). The detection of active compound was done under daylight and UV 254 nm.

Animal treatment

The procedure of the study and the use of test animal were ethically approved by the Research Ethics Committee of Ahmad Dahlan University on February 9, 2016, with Reference No. 011601011.

The test animals, i.e., BALB/c mice (8 weeks), were acclimatized for a week before the treatment. The mice were divided into five (5) groups, namely normal group, negative control group which treated with the solven (solution of tween 80 10%), and 3 treatment groups (1.25 mg/20gBW; 2.5 mg/20gBW; 5 mg/20gBW). The administration of EEZC was carried out orally for 21 days (3 weeks), once a day. On the 22nd day, the mice were sacrificed using CO_2 gas. Following sacrificing, lipopolysachcharide (LPS) (Sigma) with dose 0.01 mg/20 g BW was injected into the peritoneal cavity area, and after 1 hour, the macrophage was isolated and the expression of interleukin-10, -12 and -14 was observed with immunohistochemistry methode using specific antibody of IL-10, IL-12 and IL-14.

Macrophage isolation

Following the 21-day treatment, the mice were injected with LPS in the intraperitoneal cavity. Then, the mice were dissected by opening the skin in the peritoneal area. As much as 10 ml of Roswell Park Memorial Institute (RPMI) (Sigma) medium was injected into the stomach. The stomach was

massaged, then the RPMI medium was drawn again. The medium was centrifuged for 10 minutes, and the supernatant was removed. The macrophage was washed with the medium and incubated for 24 hours. After overnight incubation, the macrophage was harvested.

Immunohistochemistry assay

The immunohistochemistry assay was based on the method reported in a previous study (Nurkhasanah, 2015). The method was based on indirect method using specific primary antibody and was conjugated with secondary antibody and chromogen. The interleukin expressed was observed as brown colour as product of DAB (dimethyl amino benzidine) chromogen and detected under light microscope.

The cultured macrophage was fixed with 1 ml of methanol. The methanol was removed, and the macrophage was washed in PBS (phosphate buffer saline) for 5 minutes. The preparation was then immersed in peroxidase blocking solution for 10 minutes at room temperature. Afterward, it was washed under running water and then with PBS. A total of 50µl of blocking serum was added to the preparation and then incubated in a humid temperature for 10-15 minutes. Then, 100µl (with dilution 1:100) of specific antibody (anti-IL-10, anti-IL-12, and anti-IL-14, murine recombinant, Biovision) was added to the preparation and incubated on a moist tray at room temperature for 1 hour. After the incubation, the preparations were washed with 1 mL of PBS. A total of 50 µl (with dilution 1:100) of antimouse biotin secondary antibody (Biovision) was added to each preparation, which was incubated at a humid temperature for 20 minutes and then washed with PBS.

The preparations were incubated with 50 µl of the streptavidin-peroxidase enzyme for 10 minutes, washed with PBS, and incubated with 50 µl of DAB chromogen (peroxidase substrate solution). Afterward, the preparations were washed with PBS and incubated with Mayer's hematoxylin as the counterstain. The process was followed by washing with PBS. The macrophage was ready for microscopic observation at 400x magnification. Macrophages that expressed interleukin would show as brown stains.

The observation was carried out in some of field of view. The number of positive cell expression was compared with the whole cell observed and presented as percentage value. The result of treated group was compared with control group with statistical analysis to analyze the effect of treatment.

Statistical analysis

The quantitative data of percentage expression of IL-10, IL-12 and IL-14 was analyze statistically for normality and homogeneity. The analysis was followed with variance analysis using ANOVA and followed by LSD for analysis between group of treated.

RESULTS AND DISCUSSION

Extraction

The ethanol extract of *Zingiber cassumunar* rhizome was dark brown with a specific odor, thick consistency, and slightly bitter taste. The extraction process produced 25.55% yield. This result has met the standard of the Indonesian Herbal Pharmacopoeia (Depkes RI, 2008). The curcumin content was detected in the extract, as shown in Figure 1. While curcumin was found to be a major content in the extract, the other curcumin derivates (i.e., demethoxycurcumin and bisdemethoxycurcumin) were not detected.

Curcumin is one of major chemical content in EEZC. Curcumin reported to have immunomodulatory activity (Varalakshmi et al., 2008). Previous study reported the significantly increase of IL-12 levels in curcumin-treated animals on day 10 and 20, after treatment. It is also found that curcumin induce generation of ROS which important in the immune respon (Varalakshmi et al., 2008). Curcuminoid (cassumunin A and cassumunin B) isolated from *Z. cassumunar* was found to have a protective effect on living cells suffering from oxidative stress (Nagano et al., 1997).

Beside curcumin, essential oil is also reported is one of main compound in *Zingiber cassumunar* rhizome. The highly essential oil content give specific odor of *Z. cassumunar* rhizome and extract. Several studies of phytochemical compounds and biological activity of *Z. cassumunar* Roxb had been reported the main component of *Z. cassumunar* rhizome essential oil were triquinacene 1,4-bis (methoxy), (Z)-ocimene and terpinen-4-ol (Bua-in and Paisooksantivatana, 2009). The previous studies on its rhizome also found several phenylbutenoid compounds, curcuminoid, and sesquiterpene (zerumbon) (Nakamura et al., 2009).

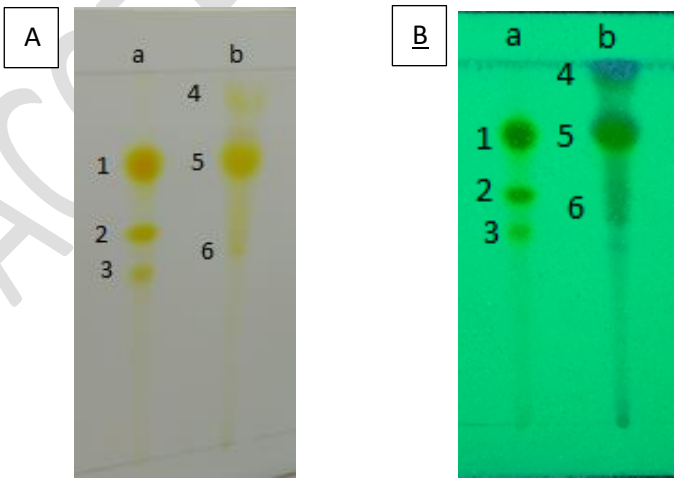

Figure 1. The TLC profile of curcuminoids standard (a) and *Zingiber cassumunar* ethanolic extract (b), detected in daylight (A) and UV 254 nm (B).

Expression of IL-10

Indirect immunocytochemistry was employed to detect the expression of interleukin in the macrophage. The specific antibody of IL-10 interacted with interleukin-10 in the cells and attached to the secondary antibody. During the detection process, the secondary antibody attached to DAB as the chromogen, and the expression appeared as brown stains on the cytoplasm area. Meanwhile, the cells with negative expression appeared in blue as the result of counterstaining. The immunocytochemistry of the macrophage is shown in Figure 2. The percentage of the IL-10 expression is shown in Table 1.

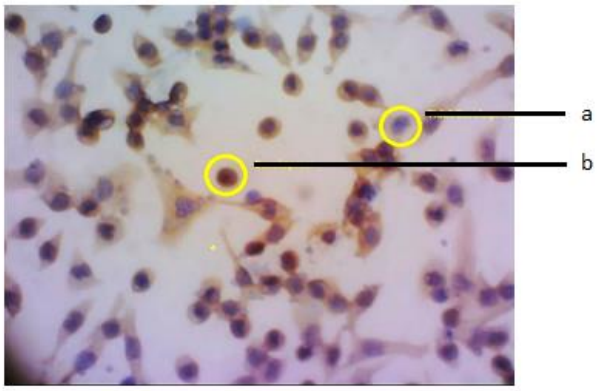

Figure 2 The immunocytochemistry of IL-10 expression in macrophage cells after treatment with ethanol extract of *Zingiber cassumunar* which observed with 400x magnification (a) macrophage with no expression of interleukin (b), macrophage with positive expression of interleukin

Table 1 The percentage of IL-10 expression on the macrophage cells of BALB/c mice treated with ethanol extract of *Zingiber cassumunar*.

Groups	Mean ± SD
Normal	45.65 ± 1.92%*
Negative control	51.86 ± 1.42%
Treatment Dose of 1.25mg/20gBW	55.91 ± 3.07%
Treatment Dose of 2.5mg/20gBW	63.68 ± 2.93%*
Treatment Dose of 5mg/20gBW	68.65 ± 4.42%*

*significant difference with negative control ($p<0.05$)

The treatment of ethanol extract of *Zingiber cassumunar* increased the expression of IL-10, affirming the potential of EEZC as an immunomodulator. IL-10 was expressed by macrophages and other dendritic cells (DC) as a response to microbial infection. The increase of IL-10 expression may be attributable to the activation of extracellular signal-regulated kinase 1 (ERK1) and ERK2 (Saraiva and O'Garra, 2010). Such an increase will activate the specific response of the immune system and

inhibit the nonspecific response. IL-10 has been identified as an inhibitor of the synthesis of inflammatory mediators and pro-inflammatory cytokines that play a role in modulating fever and sickness (Harden et al., 2013). The present study also found that the expression of IL-10 in negative control group increased significantly compared to normal, which could be caused by the tween 80 effect. The previous study also reported that polysorbate (tween) 80 could increase the immune response (Maggio, 2012).

The increased expression of IL-10 was proportional to the administered dose. The higher the treatment dose, the higher the expression of IL-10 was. However, an extremely high level of IL-10 can inhibit chemokine production and prevent its role in directing lymphocytes to the lymph nodes, as in mycobacterial infection, resulting in a failure to recruit and induce Th1 cell differentiation (Couper et al., 2008). Therefore, IL-10 has both immunosuppressive and immunostimulatory properties (Acuner-ozbabacan et al., 2014).

The regulation of IL-10 expression involves the enhancement or silencing of IL10 transcription and is regulated by certain transcription factors activated by discrete signal-transduction pathways. Following transcription, the post-transcriptional mechanisms exist and involeves many of the molecular events leading to IL-10 expression (Saraiva and O'Garra, 2010). Some molecule of *Zingiber cassumunar* could be involves and affect the transcriptional process of IL-10 and leading on increasing level of IL-10.

Expression of Interleukin-12

Interleukin-12 (IL-12) is a pro-inflammatory cytokine that induces the production of interferon-γ (IFN-γ), and leading to the differentiation of T helper 1 (TH1) cells and connected the link of innate immunity and adaptive immunity. Dendritic cells (DCs) and macrophages produce IL-12 in response to pathogens and infection (Trinchieri, 2003). Production of IL-12 is strongly regulated by positive and negative regulatory mechanisms. Microorganism products including bacteria, intracellular parasites, fungi, double-stranded RNA, bacterial DNA and oligonucleotides are strong inducers of IL-12 production by macrophages, monocytes, neutrophils and DCs. The LPS was used in this study to activate the macrophage to produce the IL-12. The quantitative analysis of the expression of IL-12 after the administration of EEZC is shown in Table 2.

Table 2 The expression of IL-12 in macrophage cells of BALB/c mice treated with ethanol extract of *Zingiber cassumunar*

Groups	Mean ± SD
Normal	64.63% ± 9.763
Negative Control	66.39% ± 1.603
Treatment Dose of 1.25mg/20g BW	51.56% ± 4.528*
Treatment Dose of 2.5mg/20g BW	70.62% ± 3.469

Treatment Dose of 5mg/20g BW	77.00% ± 5.110*

*) significant difference with negative control ($p<0.05$)

The study found that the IL-12 expression in the negative control group was not significantly different from the normal group, indicating that the solvent (tween 80) does not affect the immune response. Tween 80 could stimulate the immunogenicity (Maggio, 2012) as shown by the increasing of IL-10 in the present study. But the dose used in this study is not enough to increase the IL-12 expression. The treatment of EEZC at a dose of 1.25mg/20g BW induced lower IL-12 expression than the negative control. In other words, the lower the dose, the less effective the active compound of EEZC in increasing the IL-12 expression. When the dose increased, the IL-12 expression was also found to be elevated.

The immunomodulatory effects of EEZC might be caused by the presence of curcumin as an active compound. Curcumin has been reported to increase the immune response of the cells (Nagano et al., 1997; Nurkhasanah et al., 2017). The previous study also found that curcumin treatment elevated the IL-12 level in mice (Varalakshmi et al., 2008). The increasing level of IL-12 in the treatment could be caused by the capacity of curcumin in increasing of ROS and NO (Nurmasari et al., 2014; Rahmawati, 2013). ROS is known to regulate the IL-12 generation. Another studies have revealed that curcumin stimulates T cell, B cell, neutrophil, NK cell, and dendritic cell (Nurmasari et al., 2014).

The essential oil, which emitted a special odor, was also identified in EEZC (Bhuiyan et al., 2008). The presence of essential oils in EEZC has also been reported to boost the immune response, including the phagocytic activity of macrophages (Chairul et al., 2009; Nakamura et al., 2009). The active compound from volatile oil which successfully identified as immunomodulatory compound are phenilbutenoids compound (Chairul et al., 2009).

The treatment of EEZC increased IL-12 expression, activating T cells and stimulating the production of IFN-γ, which lead to macrophage activation and the secretion of reactive oxygen species (ROS) that eliminate infections (Abbas et al., 2017). Furthermore, this treatment can intensify the phagocytic activity of macrophage (Nurkhasanah et al., 2017).

Expression of Interleukin-14

IL-14 was first known as a high-molecular-weight B-cell growth factor and originally identified as a B cell growth factor (Shen et al., 2006). It is produced by T cells and B-cells. IL-14 binds and signals through a 90-kDa receptor expressed on activated B cells to promote B-cell proliferation (Akdis et al., 2016). High level of IL-14 can enhance B-cell proliferation and expand a

subpopulation of memory B cells (Leca et al., 2008), and if followed by the secretion of antibody, it can eliminate the invader. The expression of IL-14 in EEZC-treated mice is shown in Table 3.

Table 3 The expressions of Interleukin-14 in mice treated with ethanol extract of *Zingiber cassumunar*

Groups	Expressions (X ± SD)
Normal	59.19 ± 3.07%
Negative control	61.24 ± 1.51%
Treatment Dose of 1.25 mg/20g BW	57.02 ± 1.94%*
Treatment Dose of 2.5 mg/20g BW	67.41 ± 6.60%
Treatment Dose of 5 mg/20g BW	71.07 ± 1.30%*

(*) showed significant difference with negative control ($p<0.05$)

The previous research proposes increasing the expression of IL-14 by the treatment of some herbal medicine extract (Nurkhasanah, 2015). The treatment of anthocyanin-rich rosella extract increases the IL-10 and IL-14 expressions in vitro. The present research also found that the treatment of EEZC increased the IL-10, IL-12, and IL-14 expressions after LPS induction. This induction stimulated the immune response because LPS was recognized as endotoxin consisting of a lipid and a polysaccaride found on the outer membrane of gram-negative bacteria. The activity of EEZC in increasing of IL-10, IL-12 and IL-14 suggested the potency of this extract in inducing the immune system in both innate and adaptive immunity.

A previous study on Zingiberaceae family, including *Curcuma mangga*, *Kaempferia angustifolia*, and *Zingiber cassumunar*, highlights that *Zingiber cassumunar* has the highest immunomodulatory activity (Chairul et al., 2009), which may be caused by the active compound of curcumin and essential oil. The major compound found in *Z. cassumunar* essential oil was phenyl butanoic compound. Furthermore, a toxicity study states that *Zingiber cassumunar* extract has no observable adverse effect and it is well-tolerated for both acute and chronic studies (Koontongkaew et al., 2014).

CONCLUSION

The ethanol extract of *Zingiber cassumunar* has immunomodulatory activity through increasing level of IL-10, IL-12 and IL-14 cytokines and suggested the potency of extract to induce both innate and adaptive immunity.

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