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INTERLEUKIN LEVELS IN THE *Zingiber cassumunar*-TREATED MICE

NURKHASANAH*, NANIK SULISTYANI, YUNI ARUM HANDAYANI, QANITA KAMILA AND ANNISA CANDRA NUR ISNAINI

Faculty of Pharmacy, Universitas Ahmad Dahlan, Yogyakarta 55164, Indonesia

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ABSTRACT

The protein compound, cytokine, is responsible for the body's immune system. Several 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 *Zingiber cassumunar* ethanol extract (EEZC) on the expressions of IL-10, IL-12, and IL-14. The test animals, BALB/c mice which were treated for 21 days, 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. On the 22nd day, the mice were induced with Lipopolysaccharide (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 21-day administration of EEZC at doses of 1.25 mg, 2.5mg, and 5mg/20g BW significantly increased the expression of IL-10, IL-12, and IL-14 in proportion to the dose thereby suggesting the potency of the extract to induce both innate and adaptive immunity. This activity may be attributable to curcumin as the active compound of the extract.

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

INTRODUCTION

The immune system which is responsible for protecting the host from various pathogenic microorganisms also controls the immune responses and prevents over-reaction of the body's own cells (Saraiva & 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 diseases or infections is a vital component of the immune system.

Immunomodulator is a substance, which can stimulate, suppress or modulate any of the components of the immune system including both the specific and nonspecific immune system (Das *et al.* 2014). Modulation of the immune system is marked by induction, expression, amplification or inhibition of certain parts in the immune signaling and response mechanism. Thus, the immunomodulator substance is used as immune stimulant for its effect on the immune system.

The anti-inflammatory cytokine Interleukin-10 (IL-10) plays a crucial role in preventing inflammatory and autoimmune pathogens. It can both impede pathogen clearance and ameliorate the immunopathology process. Several 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 plays a central role during infection by limiting the immune response to pathogens and thereby preventing damage to the host. The production of IL-10 was associated with the regulatory T (Treg) cells. (Saraiva & O'Garra 2010).

Interleukin-12 cytokine, produced mainly by the antigen-presenting cells (APC) which include the macrophages and dendritic cells that respond to microbes, is also vital in the immunoregulation process (Abbas *et al.* 2017).

*Corresponding author, email: nurkhas@gmail.com

During the immune response, IL-12 is produced as a reaction to stimuli of various compounds (including lipopolysaccharide/LPS).

Interleukin-14 (IL-14) cytokines, produced by the immune system, particularly by the activated B cells and T cells, regulates the B-cell proliferation (Leca *et al.* 2008). IL-14 can increase antibody responses to vaccinations causing autoimmunity and contribute to B-cell lymphoma formation (Shen *et al.* 2006).

Zingiber cassumunar of the Zingiberaceae family, known locally as *bengle* (Javanese, Indonesia), has been traditionally used for treating various diseases. *Z. cassumunar* contains terpenoids, essential oil, and curcuminoids. Several studies on *Z. cassumunar* include its performance as anticancer (Varalakshmi *et al.* 2008), antioxidant (Vankar *et al.* 2006) (Bua-in & Paisooksantivatana 2009) and immunomodulator (Nurkhasanah *et al.* 2017; Rahmawati 2013). This plant exhibited an immunomodulatory activity by increasing phagocytic activity *in vitro* (Chairul *et al.* 2009), increasing nitric oxide (NO) and reactive oxygen species (ROS) (Nurkhasanah *et al.* 2017) and decreasing malondialdehyde products in *Plasmodium berghei*-infected mice (Nurmasari *et al.* 2014).

This study documents the activity of *Zingiber cassumunar* ethanol extract (EEZC) in stimulating the immune response *in vivo* as observed from its effect on interleukin-10, -12 and -14. It focuses on the immune responses of these cytokines on both the innate and adaptive body immunity. The expression IL-10 and IL-14 are closely related to activation of adaptive immunity, while the IL-12 is important in cell communication between the macrophage and T cells. This study was conducted for 28 days through oral administration on Balb/c male mice. This *in vivo* experiment provides evidence for the higher effectiveness of EEZC in increasing the immune responses, a very vital information for the development of *Z. cassumunar* as an immunomodulatory product.

MATERIALS AND METHODS

Materials

The *Zingiber cassumunar* rhizome, collected from a local Yogyakarta market, was identified at 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, sliced and finally oven-dried at a temperature of 50 °C. The dried rhizome was then 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 the concentrated extract (EEZC) which was used as the treatment.

Thin-layer Chromatography (TLC) analysis of the extract

The Thin-layer Chromatography (TLC) analysis was used to identify the active EEZC compound. 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 EEZC 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, 8 week-old BALB/c mice, were acclimatized for a week before the treatment. The mice were divided into five (5) groups, namely; the normal group, negative control group which were treated with the solvent Tween 80 at 10% concentration, and the 3 treatment groups (1.25 mg/20g BW; 2.5 mg/20g BW; 5 mg/20g BW; BW is abbreviation of Body Weight). The administration of EEZC was carried out once a day, orally for 21 days (3 weeks). On the 22nd day, the mice were sacrificed using CO_2 gas. Following sacrificing, lipopolysachcharide (LPS) (Sigma) with dose of

0.01 mg/20 g BW was injected into the peritoneal cavity area. After 1 hour, the macrophage was isolated and the expressions of interleukin-10, -12 and -14 were observed using the immunohistochemistry method with the specific antibodies 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. The mice were then 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 Nurkhasanah (2015), an indirect method using specific primary antibody that was conjugated with secondary antibody and chromogen. The expressed brown-colored interleukin was the product of Dimethyl Amino Benzidine (DAB) chromogen detected under the light microscope.

The cultured macrophage, which was previously fixed with 1 mL of methanol that was later removed, was washed in PBS (phosphate buffer saline) for 5 minutes. The fixed macrophage was then immersed in peroxidase blocking solution at room temperature for 10 minutes. The macrophage was then washed with running water and then re-washed with PBS. A total of 50 µL of blocking serum was added to the preparation which was then incubated in a humid temperature for 10-15 minutes. The 100 µL (with dilution 1:100) of specific antibodies (anti-IL-10, anti-IL-12, and anti-IL-14, murine recombinant, Biovision) was then 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 anti-mouse biotin secondary antibody (Biovision) was added to each preparation, which was incubated at a humid temperature for 20 minutes and then re-washed with PBS.

The preparations were incubated with 50 µL of the streptavidin-peroxidase enzyme for 10 minutes, washed with PBS, and re-incubated with 50 µL of Dimethyl Amino Benzidine (DAB) chromogen (peroxidase substrate solution). The preparations were then washed with PBS and incubated with Mayer's hematoxylin as the counterstain and then re-washed with PBS in preparation for the microscopic observation at 400x magnification. The macrophages that expressed interleukin manifested brown stains.

The observation was carried out from several Fields of View (FOV) of the microscope. The number of expressed positive cell was compared with the total number of observed cell and presented as percentage value. The results of the treated groups were statistically compared with that of the control group to analyze the effect of treatment.

Statistical analysis

The quantitative percentages of IL-10, IL-12 and IL-14 expressions were analyzed statistically for normality and homogeneity and then further analyzed using ANOVA and followed by LSD analysis among the treated group.

RESULTS AND DISCUSSION

Extraction

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

Curcumin reportedly showed immuno-modulatory activities (Varalakhmi *et al.* 2008). After the treatment, significant increases of IL-12 levels were observed among the curcumin-treated animals on day 10 and 20. Curcumin was

also found to induce generation of Reactive Oxygen Species (ROS) which are important in the immune responses (Varalakhmi *et al.* 2008). Curcuminoids (cassumunin A and cassumunin B) isolated from *Z. cassumunar* were observed to have a protective effect on living cells suffering from oxidative stress (Nagano *et al.* 1997).

Besides curcumin, essential oil was also reported as one main compound in *Zingiber cassumunar* rhizome. The high essential oil content was responsible for the specific odor of *Z. cassumunar* rhizome and extract. Several studies on the phytochemical compounds and biological activities of *Z. cassumunar* Roxb had reported the main component of *Z. cassumunar* rhizome essential oil as triquinacene 1,4-bis (methoxy), (Z)-ocimene and terpinen-4-ol (Buain & Paisooksantivatana 2009). Previous studies on its rhizome also found several phenylbutenoid compounds, curcuminoid, and sesquiterpene (zerumbon) (Nakamura *et al.* 2009).

Expression of IL-10

Indirect immunocytochemistry was used to detect the interleukin expression in the macrophage (Fig. 2). The specific antibody of IL-10 interacted with interleukin-10 in the cells and attached itself to the secondary antibody. During the detection process, the secondary antibody attached itself to Dimethyl Amino Benzidine (DAB) as the chromogen, and the expression appeared as brown stains on the cytoplasm area, while the cells with negative expression appeared as blue stains as the result of counterstaining. The percentage of the IL-10 expression is shown in Table 1.

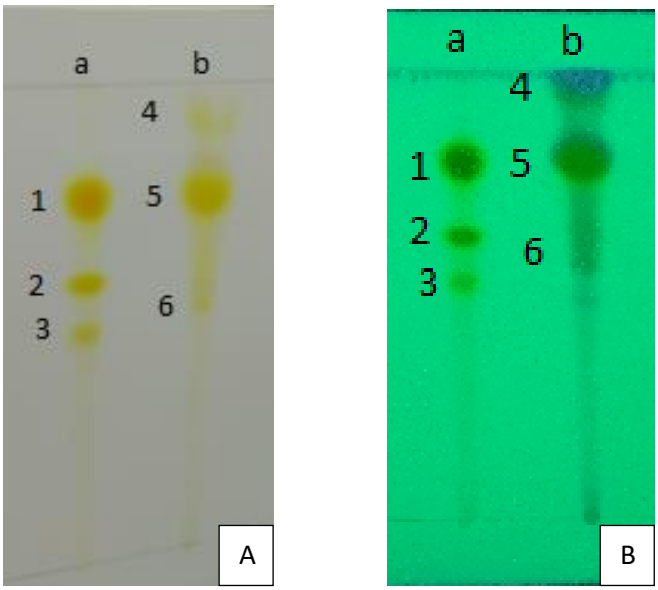

Figure 1 The TLC profile of curcuminoids standard (a) and *Zingiber cassumunar* ethanolic extract (b), detected in daylight (A) and UV 254 nm (B).

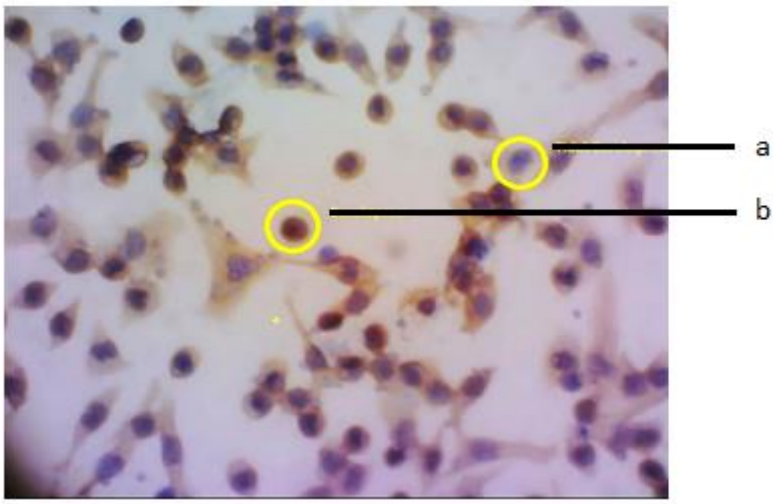

Figure 2 Immunocytochemistry of IL-10 expression in macrophage cells after beign treated with ethanol extract of *Zingiber cassumunar* which was observed with 400x magnification: (a) macrophage with no expression of interleukin; and (b) macrophage with positive expression of interleukin

Table 1 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%*

Notes: * = significant difference with negative control ($p<0.05$); BW = body weight.

The treatment of *Zingiber cassumunar* ethanol extract (EEZC) has increased the expression of IL-10, affirming its potential 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 & 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 the negative control group significantly increased as compared to the normal group, which could be caused by the Tween 80 effect. Another 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. However, an extremely high level of IL-10 can inhibit chemokine production and prevent its role in directing lymphocytes to the lymph nodes, as manifested 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).

Regulation of the IL-10 expression involved the enhancement or silencing of IL10 transcription and is performed by certain transcription factors activated by discrete signal-transduction pathways. Following transcription, the post-transcriptional mechanisms existed and involved many of the molecular events leading to IL-10 expression (Saraiva & O'Garra 2010). Some molecules of *Zingiber cassumunar* were involved and had affected the transcriptional

process of IL-10 and resulted in the increasing IL-10 levels.

Expression of Interleukin-12

Interleukin-12 (IL-12) is a pro-inflammatory cytokine that induces the production of interferon-γ (IFN-γ), leading to the differentiation of T helper 1 (TH1) cells and connecting the link between innate 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. In this study, the LPS was used to activate the macrophage production of the IL-12 expression after the administration of EEZC and was analyzed quantitatively (Table 2).

The study found out that the IL-12 expression in the negative control group was not significantly different from the normal group, indicating that the solvent (Tween 80) did not affect the immune response. Tween 80 can stimulate the immunogenicity (Maggio 2012) as also shown by the increased IL-10 levels in the present study. However, the dosage applied in this study was not enough to increase the IL-12 expression. EEZC treatment at a dose of 1.25mg/20g BW resulted in an IL-12 expression lower than the negative control. Hence, the lower the dose, the less effective is the EEZC active compound in increasing the IL-12 expression. When the dose was increased, the IL-12 expression was also heightened.

The EEZC immunomodulatory effects might have been due to the presence of curcumin, the active compound known to increase the immune response of the cells (Nagano *et al.* 1997; Nurkhasanah *et al.* 2017). Curcumin treatment also elevated the IL-12 expressions in mice (Varalakhmi *et al.* 2008). The increasing level of IL-12 in the treatment was caused by the capacity of curcumin in increasing Reactive Oxygen Species (ROS) and Nitric Oxide (NO) (Nurmasari *et al.* 2014; Rahmawati 2013). ROS is known to regulate the IL-12 generation. Curcumin was also found to stimulate the T cells, B cells, neutrophil, NK cell, and dendritic cell (Nurmasari *et al.* 2014).

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

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

Expression of Interleukin-14

EEZC treatment also increased the IL-14 expressions after LPS induction (Table 3). IL-14 was the first known high-molecular-weight B-cell growth factor, originally identified as a B cell growth factor (Shen *et al.* 2006). As produced by T cells and B-cells, the IL-14 binds and signals through a 90-kDa receptor that promotes B-cell proliferation (Akdis *et al.* 2016). High levels of IL-14 can enhance B-cell proliferation and can expand a subpopulation of memory B cells (Leca *et al.* 2008), and if followed by the secretion of antibody, it can also eliminate the invader.

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

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*

Note: * = showed significant difference from the negative control ($P<0.05$).

Table 3 Interleukin-14 expressions 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%*

Note: * = showed significant difference with the negative control (P<0.05).

Previous researches studied the increase of IL-14 expression by using some medicinal herbal extracts (Nurkhasanah 2015). The treatment of anthocyanin-rich rosella extract increased both 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 as LPS was recognized as an endotoxin, consisted of a lipid and a polysaccaride, found on the outer membrane of gram-negative bacteria. EEZC's active role in increasing the IL-10, IL-12 and IL-14 exhibited its potency in inducing both the innate and adaptive body immunity.

Studies on Zingiberaceae family, including *Curcuma mangga*, *Kaempferia angustifolia,* and *Zingiber cassumunar* recorded that *Zingiber cassumunar* displayed the highest immunomodulatory activity (Chairul *et al.* 2009). This is probably due to the active curcumin compound and its essential oil which has the phenyl butanoic substance. Furthermore, a toxicity study confirmed that *Z. cassumunar* extract has no observable adverse effect and it is well-tolerated for both acute and chronic toxicity studies (Koontongkaew *et al.* 2014).

CONCLUSION

The *Zingiber cassumunar* ethanol extract (EEZC) exhibited immunomodulatory activities by increasing the levels of IL-10, IL-12 and IL-14 cytokines in the treated mice. This study also suggested the extract's potency to induce both the innate and adaptive body immunity.

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