The immunomodulatory effect of *Zingiber cassumunar* ethanolic extract on phagocytic activity, nitrite oxide and reactive oxygen intermediate secretions of macrophage in mice

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**Abstract.** Immunomodulators could protect the body from a variety of infectious agents and boost immunity. *Zingiber cassumunar* rhizome or bangle potentially showed as an immunomodulator through increasing of macrophage activity *in vitro*. The objective of the study was to determine the effect of *Z. cassumunar* rhizome ethanolic extract on phagocytic activity, nitrite oxide (NO) and reactive oxygen intermediate (ROI) secretions in macrophages *in vivo*. A total of 200 g of *Z. cassumunar* rhizome was powdered, macerated in 96% ethanol and evaporated to get concentrated extract. Mice were divided into 5 groups as follow: the normal group was given by water only, the negative control group was given by a 0.94% CMC-Na suspension, the treatment groups were given by 250, 500 and 1000 mg/kgBW, respectively, of *Z. cassumunar* ethanolic extract. The extract was administered orally for 7 days. On the 8th day the mice were injected intraperitoneally 0.7 mg/kg BW of lipopolysaccharide. Four hours later macrophage was isolated. Furthermore, the determination of the phagocytic activity, NO and ROI secretions levels of macrophage were performed. The treatments of 250, 500 and 1000 mg/kg BW of *Z. cassumunar* ethanolic extract significantly increase the ROI and NO secretions levels (p<0.05), but did not increase the phagocytic activity (p>0.05) of macrophage. *Z. cassumunar* ethanolic extract have immunomodulatory effect *in vivo*.

**Keywords:** *Z. cassumunar*, immunomodulator, phagocytic activity, ROI, NO

1. Introduction
The environment around humans contains various types of pathogenic elements, such as bacteria, viruses, fungi, protozoa and parasites which can cause disease in human body. Infections that occur in normal people are generally short and rarely leave permanent damage. This is because the human body has a system called the immune system that responds and protects the body against the pathogenic elements. The immune response is depended on the ability of the immune system to recognize foreign molecules (antigens) which presented in pathogens and then generate appropriate reactions to exclude antigenic sources [1].

Increasing of immune response could be done by improving the function of the immune system using immunostimulant. Immunostimulants can increase the body’s resistance in fighting against various infections or to assist in the treatment of diseases associated with suppression of the immune system. Immunostimulants work by stimulating the main factors of the immune system, through
phagocytosis either through direct phagocytic mechanisms or by indirect mechanisms by releasing reactive oxygen compounds such as NO and ROI [2]. NO and ROI play a role in cooperating with macrophages destroy pathogens with dependent oxygen on the immune system.

Traditionally, Z. cassumunar has been used for treatment various deseases including antidiarrheal, fever, stomach, gout, asthma, and jaundice. Previous studies reported that Z. cassumunar Roxb have potential antioxidant activity [3–6]. Z. cassumunar also was reported to be an immunomodulator by increasing phagocytic activity in vitro [7,8]. The main components of the Z. cassumunar rhizome are essential oils [9] containing of chemical compounds such as sabinene, pinen, caryofillen oxide and karyofillen [10].

Understanding of mechanisms is an important part of developing extracts to herbal remedies which can be used in formal health services. Z. cassumunar has been used traditionally to treat various diseases but the mechanism of its action is still very limited. This study will examine the potential of ethanol extract of Z. cassumunar as an immunomodulator and assess the mechanism. This research is expected to provide benefits for the development and utilization of Z. cassumunar in health services.

2. Materials and Methods
2.1. Plant material
Z. cassumunar rhizome was purchased from local market of Pasar Beringharjo, Yogyakarta, Indonesia. The rhizome was macerated in 96% ethanol and evaporated to get a concentrated extract.

2.2. Animal treatment
The animal handling procedures in this study was approved by the Research Ethics Committee, Universitas Ahmad Dahlan, with approval number 011601011. The test animal used in this study was Swiss male mice, 8 weeks of age. The mice were divided into 5 groups: the normal group was given water only, the negative control group was given a 0.94% CMC-Na suspension only, the treatment groups were given with 250, 500 and 1000 mg/kg bw of Z. cassumunar ethanol extract. The extract was administered orally for 7 days. On the 8th day the mice were injected intraperitoneally of 0.7 mg/kgBW lipopolysaccharide, 4 hours later macrophages were isolated.

2.3. Macrophage isolation
The mice were fasted for 10-12 hours, then narcosed with chloroform. After that, mice was sprayed with disinfectant solution and placed in the supine position. The abdominal skin was opened and cleaned the peritoneum sheath with 70% alcohol. The 10 ml of RPMI medium was injected into the peritoneum cavity and waited for 3 minutes.

The peritoneal cavity fluid was aspirated with an injection syringe from a non-fat and distant part of the intestine. The aspirate was centrifuged at 1200 rpm, 4°C, for 10 min. The supernatant was removed, and the obtained macrophages resuspended by 1000 μl of complete medium. The number of cells was calculated by the hemocytometer with 10 μl of macrophages suspension.

The cell suspension was grown by 1000 μl into a 24-well microplate well with a density of 1x10^5 cells/ml for NO secretion testing. For phagocytic activity test and ROI secretion, the cells were grown with density of 5x10^5 cells/well into 6-well microplate. The cells was then incubated for 30 min in 5% CO_2 incubator with 37°C temperature, then 800 μl complete medium added into the susceptibility. Cell suspension for NO or ROI secretion test was incubated in 5% CO_2 incubator, 37°C for 24 hours.

2.4. Phagocytic assay
The medium in the microplate well was discard from cultured cell. Each wells was then added with 50 μl latex suspension and incubated for 60 min in 5% CO_2 incubator at 37°C. Following incubation, latex suspension was discard and cell was let in the room temperature for drying. Cell was then fixed using methanol for 3 min and methanol was discarded. After that, cells was stained by 10% Giemsa for 30 min. The cell was then washed with distilled water and observed using microscope with 400x magnification.
2.5. *Greiss assay for detection of NO secretion*

Greiss reagent was prepared by mixing Greiss A and Greiss B in the same amount. Greiss A was composed by of 0.1 g N-[1-naphthyl] ethylene diaminehydrochloride (NED) (Sigma N, 5889) in 100 ml of distilled water. Greiss B was prepared by mixing 1 g of sulfanilamide (Sigma N 5589) in 100 ml of 5% orthophosphoric acid. Greiss A and Greiss B must be stored in the dark place for protecting from direct light.

The culture suspension macrophage of 50 ul was placed onto each well in 96 well microplate. The sample was added by 50 ul greiss reagent, and let in room temperature for 15 min until the colour was changed. The absorbance of nitric oxide was observed on wavelength of 550 nm. Nitrite standard solution was used for calculating the concentration.

2.6. *ROI secretion assay*

The 50 μl of NBT solution was added to each wells. The cells was then added by 1 ml of PBS containing 125 ng/ml of PMA in the middle of coverslip and incubated in 5% CO₂ incubator, 37°C for 60 min. The reagent was removed from the wells, and dried at room temperature. After cells were dry, cells were fixed with 1000 μl methanol for 30 seconds. After drying, cells were added by 200 μl neutral red spultion 2% for 15 minutes and dried at room temperature. After drying, cellswere rinsed with distilled water. The percentage of macrophage cells showing NBT reduction was calculated from about 100 cells examined with a 400 times magnification of light microscope.

3. Results and Discussion

The immune system is a mechanism by which the body maintains its integrity to protect against various pathogenic matter. The immune system consist of a specific and a nonspecific immune system. *Z. cassumunar* was reported to be a potential an immunomodulatory agent. One of chemical content was found in *Z. cassumunar* was curcumin. The dose treatment of extract in mice was equal to the treatment of 18.08 mg/20g BW of curcumin. The dose can increase the NO secretion compared to negative control [11]. The treatment of ethanol extract of *Z. cassumunar* for 14 days was also found to increase the phagocytic capacity of macrophages in mice induced by *Plasmodium berghei* [12].

In this research, lipopolysaccharide (LPS) was used as antigen for boosting the immune response. Injection of 1 mg/kg BW of LPS from *E. coli* O111B4 intraperitoneally could increase the C-reactive protein in mice serum as a part of acute inflammation reaction [13].

3.1. *Effect of Z. cassumunar rhizome extract on macrophage phagocytosis*

Phagocytic activity of macrophage is activated by the presence of antigens from macromolecules and pathogens. In this study the phagocytic activity of macrophage was observed by determining the macrophages which phagocytosed latex. The parameter used was Active Phagocytes Cells (SFA) refer to the number of macrophage cells that phagocytosed latex cells in 100 macrophage cells. The other parameter was the phagocytic capacity refer to the amount of latex which is phagocytosed in 100 cells macrophages and Phagocytosis Index (IF) refer to the average number of latex particles deposited in 100 macrophage cells.

Table 1 shows that the greater the dose of ethanol extract of *Z. cassumunar*, the greater the value of SFA, phagocytic capacity and phagocytosis index. This result indicate that the greatest activity of phagocytosis is present at a dose of 1000 mg/kgBW. This is probably due to the content of flavonoids and curcumin in the *Z. cassumunar* rhizome. Flavonoids potentially work against lymphokines produced by T cells which stimulate phagocyte cells to respond to phagocytosis [14].

The curcumin which is also found in the *Z. cassumunar* could enhance reactive oxygen species (ROS) which will further activate a signal involving peroxism proliferator activated receptor gamma (PPAR-γ) and NF-E2-related factor 2 (Nrf2). The activity of both signals on monocytes and macrophages results in increased expression of cluster deferensiation 36 (CD36) in order to increase macrophage phagocytosis. Activated macrophages receive signals from interferon alfa (INF-α) to produce inducible nitrite oxide synthase (iNOS). The enzyme catalyzes the conversion of L-arginine to L-citrulline which produces NO gas. Increasing of NO is associated with increasing of macrophage activity as phagocyte cells [11].
Phenylbutenoid which is found from the *Z. cassumunar* rhizome also show antioxidant activity through free radical scavenger and could increase phagocytic activity [7]. The highest value of SFA (%) among the groups treated by ethanol extract is 57.25% (in group which treated by 1000 mg/kgBW of extract). The previous study also reported that *Z. cassumunar* extract capable to increase the phagocytosis capacity in vitro study [15].

**Table 1.** The phagocytic activity of macrophage in mice treated with *Z. cassumunar* ethanol extract.

| Group                     | SFA ±SD (%) | Phagocytic Capacity ±SD | Phagocytosis index ± SD |
|---------------------------|-------------|-------------------------|-------------------------|
| Normal                    | 53.33±4.04  | 94.00±11.79             | 1.77±0.11               |
| Negative control          | 52.00±3.00  | 92.33±4.62              | 1.77±0.04               |
| Dose 250 mg/Kg bw         | 56.00±2.59  | 100.00±4.97             | 1.78±0.03               |
| Dose 500 mg/Kg bw         | 56.25±3.59  | 100.50±14.20            | 1.78±0.14               |
| Dose 1000 mg/Kg bw        | 57.25±3.86  | 110.75±19.65            | 1.92±0.22               |

The statistical analysis found that the number of active phagocyte cells (SFA), phagocytic capacity and phagocytosis index in this research did not show a significant difference (p>0.05).

3.2. The increasing of ROI secretion after treatment of *Z. cassumunar* extract

The measurement of peritoneal macrophage ability on secretion of ROI was measured by NBT Reduction assay containing PMA. PMA will stimulate the macrophages to secrete ROI. The presence of ROI (superoxide anion, O2-) secretion exhibits the increasing of respiration and causes reduction of NBT to form a non-dissolved formazan precipitate in blue colour.

![Figure 1](image)

**Figure 1.** The microscopic feature of peritoneal macrophage with ROI secretion. ROI secretion showed by blue colour

**Table 2.** The percentage of ROI secretion of macrophage in mice treated with *Z. cassumunar* extract.

| Groups               | Percentage of ROI ± SD |
|----------------------|-------------------------|
| Normal               | 19.00±2.00              |
| Negative control     | 18.33±0.57              |
| Dose 250 mg/kg bw    | 24.50±3.32<sup>a,b</sup>|
| Dose 500 mg/kg bw    | 22.75±3.59              |
| Dose1000 mg/kg bw    | 27.75±3.86<sup>a,b</sup>|

<sup>a</sup>: significant difference with normal group  
<sup>b</sup>: significant difference with negative control group

The research found that treatment of LPS 0.7 mg/kg bw did not give any significant difference in ROI secretion compare to normal. The treatment of extract with dose of 250 and 1000 mg/kg bw increase the ROI secretion significantly compared to the negative control and normal. But statistical analysis of increasing dose in this study resulted no significant difference in ROI secretion.
3.3. The increasing of NO secretion after treatment of Z. cassumunar extract

Nitric oxide (NO) is an effective antibacterial effector in the immune system. The concentration of NO production secretion was found to increase after stimulating macrophages with IFN-γ and TNF-α. The IFN-γ is one of the cytokines which can induce NO secretion without stimulation by others, whereas TNF-α can not induce NO production without IFN-γ. NO is also used as an oxidative stress marker, which the accumulation of free radicals and inability of the antioxidant to eliminate the accumulation of free radicals cause imbalance production of reactive oxygen species [16].

NO is also recognized as an intercellular messenger that has been recognized as one of the most important players in the immune system. Cells of the innate immune system including macrophages, neutrophils and natural killer cells – use pattern recognition receptors to recognize the molecular patterns associated with pathogens. Activated macrophages then inhibit pathogen replication by releasing a variety of effector molecules, including NO. A large number of other immune-system cells produce and respond to NO. Thus, NO is important as a defense molecule against infectious microorganisms. It also regulates the functional activity, growth and death of many immune and inflammatory cell types including macrophages, T lymphocytes, antigen-presenting cells, mast cells, neutrophils and natural killer cells [17].

Previous study reported that the capability of curcumin, one of the active compound found in many Zingiber family, in increasing the reactive oxygen species (ROS) secretion by activating PPAR-γ and Nrf-2 of macrophage and caused the increasing of CD36 [11].

The present study found that LPS could significantly increase the NO secretion compared to the control. LPS was recognized as antigen by macrophage and followed by stimulating the NO secretion. Treatment with Z. cassumunar extract decreased the NO secretion significantly (Table 3).

| Tabel 3. The NO secretion level of macrophage in mice treated with Z. cassumunar extract |
|-----------------------------------------------|
| **Group** | NO secretion level |
|          | Median (Minimum-Maximum) |
| Normal   | 3.914 (3.914-23.629) |
| Negative control | 67.371 (34.514-111.09) |
| Dose of 250 mg/kg bw | 13.357 (9.629-27.629) |
| Dose of 500 mg/kg bw | 30.371 (8.914-47.657) |
| Dose of 1000 mg/kg bw | 7.657 (3.229-87.514) |

a : significant difference with normal group  
b : significant difference with negative control group

The NO secretion levels of groups treated with 250, 500 and 1000 mg/kg bw of extract was higher than that of the normal group, but lower than that of the group injected by 0.7 mg/kg bw of LPS. It means that the treatment of Z. cassumunar extract at all three doses decrease the levels of NO secretion of peritoneal macrophages peritoneum in mice induced by LPS 0.7 mg/kg bw. The previous study reported that intraperitoneal injection of LPS increased NO levels in serum macrophages [18]. The decrease of NO level after treatment with Z. cassumunar extract, could be resulted by the antioxidant compound of the extract. The curcumin compound reported to inhibit NO production in activated macrophages [19]. Curcumin also was reported to reduced the iNOS mRNA expression in LPS injected mice [20].

In vitro study confirmed the result, that Z. cassumunar could decrease the NO secretion in vitro in murine macrophage cell line RAW 264 [21]. Another active compound isolated from Z. cassumunar Roxb was also reported to inhibit NO production of peritoneal macrophage in LPS induce mice [22].

The high level of ROI and NO secretion indicate the good respon of immune system. Nevertheless excessive secretion of ROI and NO can result a reactive oxidant which involved the reactive oxygen compounds such as OH, OOH, O2, and H2O2. The presence of oxygen is important for the normal metabolism, but oxygen can increase the number of free radicals. Free radicals are highly reactive.
which can damage cells and can cause oxidative stress. Previous research reported the capability of cassumunin A and B which isolated from *Z. cassumunar* in prevented the hydrogen peroxide (H$_2$O$_2$)-induced decrease in cell viability caused by oxidative stress [23].

4. Conclusions
The administration of ethanol extract of *Z. cassumunar* rhizome for 7 days could increase ROI and decrease NO secretion levels but could not increase phagocytic activity (p> 0.05) of peritoneal macrophage in Swiss mice injected by LPS. These findings indicate that *Z. cassumunar* rhizome have immunomodulatory effect in vivo.

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