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In vitro immunomodulatory activity test of Bengle rhizoma extract (*Zingiber cassumunar* Roxb.): phagocytic activity of macrophages and lymphocyte proliferation in mice

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ABSTRACT

Immunomodulators are pharmacological agents that affect the immune system at different levels. Aside from modulating, some immunomodulators stimulate, while some others inhibit immune responses. *Zingiber cassumunar* Roxb. or Bengle rhizoma has been reported to exhibit immunomodulatory activities. This research was intended to determine the pharmacological effects of its extract on the phagocytic activity of macrophages and lymphocyte proliferation in vitro. It used the macrophages and lymphocytes of male BALB/c mice, which were divided into normal control and treatment group (receiving 25, 50, and 100 ppm of extract). The immunomodulatory activity test results showed that 100 ppm of Bengle rhizoma extract reduced phagocytosis in macrophages much significantly than the control group and that the treatment groups suppressed the proliferation of lymphocytes more substantially than the control group. The extract decreased the phagocytic activity of macrophages and the proliferation capacity of lymphocytes when administered at a concentration of 100 ppm.

Keywords: *Zingiber cassumunar*, immunomodulator, phagocytic activity of macrophages, lymphocyte proliferation

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INTRODUCTION

The environment is a source of various pathogenic elements, such as bacteria, viruses, fungi, and protozoa, that can cause health problems in humans. Although the immune system functions to protect the body from them (Kresno, 2001), the body sometimes needs substances to help the immune system to work. Immunomodulators are substances that can restore the imbalance in disturbed immune systems by stimulating and improving their function (Bratawidjaya, 2014). There are two types of immunomodulators, namely immunostimulants (stimulating immune responses) and immunosuppressants (inhibiting immune responses) (Akrom, 2013), which work by influencing the main factors of the immune system directly (phagocytosis) and indirectly (the release of NO and ROI) (Bratawidjaya, 2014).

Zingiber cassumunar Roxb. or Bengle rhizoma has an immunomodulatory effect. It is evident from increased ROI secretion and reduced NO secretion in mouse macrophages treated with 5 and 20 mg/20g BW of *Z. cassumunar* extract for several days (Nurkhasanah *et al*., 2017). *Z. cassumunar* is mainly composed of essential oils, namely triquinacene 1,4-bis (methoxy), (Z)-ocimene, and terpinene-4-ol, and also chemical components like sabinene, pinene, caryophyllene oxide, caryophyllene, sesquiphellandrene, cineole, vitamin C, vitamin E, carotenoid, phenylbutenoid, and curcuminoid (Nurkhasanah *et al*., 2017; Bhuiyan *et al*., 2008; Alam *et al*., 2012).

Although making use of *Z. cassumunar* at the level of formal health services requires an understanding of its precise mechanism of action, only a few studies have deeply analyzed how the immunomodulatory effects of this rhizome work. In vitro testing on samples of phenylbutenoid isolated from the methanolic extract of Bengle rhizoma has shown an increase in the phagocytic activity of macrophages (Chairul *et al*., 2009). This study was designed to determine the effects of Bengle rhizoma extract on the phagocytic activity of macrophages and lymphocyte proliferation in vitro to obtain more information about the mechanism of this extract as an immunomodulator.

MATERIALS AND METHODS

Materials

The research material was fresh *Z. cassumunar* rhizome purchased at Beringharjo Market, Yogyakarta. This rhizome was sliced and dried in an oven (40-50°C), then the dried rhizome was ground into a powder with a blender. Macrophages were obtained from the peritoneal cavity of BALB/c mice aged eight weeks old (20-30 g). The solvents and reagents used in the experiment were as follows: 96% ethanol, Roswell Park Memorial Institute (RPMI) medium, Phosphate Buffer Saline (PBS), complete media (composition: RPMI medium, Fetal Bovine Serum (FBS), Pen-Strep, and PBS), latex suspension, 20% Giemsa stain, 3-[4,5-dimethilthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and 10% Sodium Dodecyl Sulfate (SDS).

The research equipment included an oven, rotary evaporator, water bath, CO_2 incubator (temperature : 37°C, CO_2 concentration : 5%), autoclaves for sterilization, Laminar Air Flow (LAF), centrifuge, hemocytometer, and microscope.

Research Procedure

Preparation of Bengle rhizoma extract

Z. cassumunar powder was macerated using 96% ethanol, stirred again for 3x24 hours, filtered, and then evaporated until a thick extract was obtained.

Preparation of test animals

The procedure by which the test animals were prepared had received approval from the research ethics committee of Universitas Ahmad Dahlan (No. 011804063). The test animals were male BALB/c mice aged eight weeks old.

Isolation of macrophages

After fasting for 10-12 hours, the mice were narcotized or placed in a state of narcosis with chloroform. Then, they were placed in the supine position, and the abdomen was cleaned using a disinfectant (70% alcohol). The stomach skin was dissected, and the peritoneal sheath was cleaned with 70% alcohol. Cold RPMI medium was injected into the peritoneal cavity and shaken slowly for three minutes. Afterward, the inner cavity was pressed with two fingers to remove fluid from the peritoneal cavity. Peritoneal fluid from the non-fatty part far from the intestines was drawn using an injection syringe to obtain an aspirate.

The aspirate was centrifuged at 1200 rpm, 4°C for 10 minutes. Then, the supernatant was removed, and the pellets (macrophages) were separated. The pellets were resuspended with 1,000 µL of complete medium. The number of cells was counted from 10 µL of macrophage suspension in a hemocytometer.

In the hemocytometer, four counting rooms were used because macrophages are cells with low population density. The formula used for the counting (number of cells per mL) is as follows:

$$\frac{\Sigma cells\ in\ room\ A + \Sigma cells\ in\ room\ B + \Sigma cells\ in\ room\ C + \Sigma cells\ in\ room\ D}{4}\ x\ 10^4$$

In the phagocytic activity test, the density of the cultured macrophages was $5x10^5$ cells/well, and a 6-well microplate equipped with a coverslip was used. The cells were first incubated for 15 minutes in a 5% CO_2 incubator at 37°C. Finally, 700 µL of complete medium was added to each well, and the microplate was re-incubated for 24 hours Ulfah *et al*., 2017; Nurkhasanah *et al*., 2017.

Phagocytic activity test

After 24-hour incubation, the 6-well microplate containing macrophages was removed. Since the subsequent procedure was carried out in LAF, the complete medium in each well was discarded and replaced with 700 µL of test compound per well. The microplate was re-incubated in a 5% CO_2 incubator at 37°C for 24 hours.

In the phagocytosis test, 200 µL of latex was added into each well ($2.5x10^6$ density per well), and the microplate was re-incubated in a 5% CO_2 incubator at a temperature of 37°C for one hour. The cells in each well were washed with 1,000 µL of PBS once, dried at room temperature, and fixated with 300 µL of absolute methanol for 30 seconds. After the cells were dried, 400 µL of 20% Giemsa stain was applied to each well, and the microplate was left at room temperature for 20 minutes. The remaining Giemsa was removed, and the well was washed with distilled water. After the wells were dried at room temperature, the coverslip of each well was removed. The subsequent observation involved counting the number of macrophages that had phagocyted latex particles in 100 cells (percentage) under a light microscope at 400x magnification Ulfah *et al*., 2017; Nurkhasanah *et al*., 2017.

Isolation of Lymphocytes

The spleen tissue was isolated aseptically from mice that had been narcotized with chloroform. Then, it was placed in a petri dish, washed with PBS 2-3 times, and added with 10 mL of RPMI medium to produce a lymphocyte suspension in the medium. The suspension was centrifuged at 3,200 rpm and 4°C for 10 minutes. The formed sediments were separated from the supernatant and resuspended in 3 mL of complete medium.

The lymphocyte count was performed by placing 10.0 µL of lymphocyte suspension in a hemocytometer. Living cells with a bright and clear appearance and round shape were counted Ulfah *et al*., 2017.

Lymphocyte Proliferation Test

A total of 100 µL of the isolation results and lymphocyte cultures (final concentration= $1.5x10^6$ cells per mL) were distributed into a 96-well microplate. Then, 50 µL of the test compound was added to each well, followed by 72 hours of incubation (for 3-day proliferation testing) in a 5% CO_2 incubator at 37°C. Afterward, 50 µL of MTT with a concentration of 5 mg/mL was added to each well, which was later incubated for 4 hours Azadmehr *et al*., 2016; Ulfah *et al*., 2017.

Living cells react to MTT by forming purple formazan crystals. A reagent stopper (10% SDS) was added to each well, and then the microplate was stored at room temperature in the dark for 12 hours. Afterward, an ELISA reader was used to measure the absorbance at a wavelength of 595 nm.

Data Analysis

The normality (Shapiro-Wilk) and homogeneity tests were performed on phagocytosis activity data, i.e., % phagocytic activity and index of macrophages, and lymphocyte proliferation data, namely absorbance (ELISA test results). These tests were followed by One-way ANOVA and LSD test (with a significance level of 0.05).

RESULTS AND DISCUSSION

The part of *Z. cassumunar* plant used in this study was rhizome. In the extract preparation, slices of *Z. cassumunar* rhizomes were dried in an oven to remove water content (up to less than 10%). This drying aims to minimize the possibility of fungus and mold growth on the dried specimen to prolong its utilization without changing the active ingredients in it (Rivai *et al*., 2014). As for oven-drying technique, this procedure can help avoid any damages to active components. The dried specimen was then ground into a powder and macerated using 96% ethanol. The macerated mixture was then filtered, evaporated in an evaporator, and incubated in a water bath until a dense extract was obtained. This dense extract was tested for its activities or effects on the immune system.

The immune system is a defense mechanism of the body to protect itself from pathogens that can cause health problems. There are specific and non-specific immune systems (Akrom, 2013). Previous studies have reported that *Z. cassumunar* Roxb. is an effective immunomodulator both in increasing the phagocytic activity of macrophages and ROI secretion and decreasing NO secretion (Nurkhasanah *et al*., 2017; Chairul *et al*., 2009).

The effects of Bengle rhizoma extract on the phagocytic activity of macrophages

Antigens from macromolecules and pathogens activate phagocytosis in macrophages, which in this study was measured based on the number of macrophages that phagocyted latex. The research parameters were % activity (i.e., the percentage of active macrophages from the total number of macrophages) and the phagocytosis index (i.e., the average number of latex particles stored in 100 macrophages, that is, the number of latex that the macrophages ingested multiplied by 100 and divided by the total number of macrophages) (Nurkhasanah *et al*., 2017).

The process by which macrophages engulf macromolecules and pathogens and create phagosome depends on their ability to activate themselves once a foreign body, e.g., latex, is identified. The Bengle rhizoma extracts were tested for their phagocytic activity, and the test results were compared with the normal control group. These data were processed statistically using ANOVA and LSD test. The results of the phagocytic activity of macrophages are summarized in Table I and II:

Table I. The percentage of the phagocytic activity of macrophages

Groups	(%) Phagocytic activity of macrophages
Normal Control	13.7647±1.6148
Bengle rhizoma extract, 25 ppm	11.8518±0.6621
Bengle rhizoma extract, 50 ppm	12.5652±0.6736
Bengle rhizoma extract, 100 ppm	10.1204±2.8215*

*denotes a significant difference with the normal control group ($p<0.05$)

Table II. The phagocytosis index of macrophages

Groups	Phagocytosis index of macrophages
Normal Control	1.4005±0.1522
Bengle rhizoma extract, 25 ppm	1.3819±0.0035
Bengle rhizoma extract, 50 ppm	1.2106±0.0731[a]
Bengle rhizoma extract, 100 ppm	1.1177±0.0356*[a]

*denotes a significant difference with the normal control group ($p<0.05$)
[a]denotes a significant difference with the administration of 25 ppm of Bengle rhizoma extract

Based on Tables I and II, Bengle rhizoma extracts with concentrations of 25 ppm and 50 ppm activated phagocytosis, although not significantly different from the normal control group. In contrast, at 100 ppm, the extract decreased the activity and index of phagocytosis much more substantially than the normal control group. The phagocytic activities of macrophages, as induced by different concentrations of Bengle rhizoma extracts, are presented in Figure 1.

These findings are different from previous studies. Chairul *et al.* (2009) have reported that the in vitro testing of the isolated phenylbutenoid derivatives from Bengle rhizoma increase the phagocytic activity of macrophages, proving their immunostimulatory effects. There are three most active components in the *Z. cassumunar* rhizome fraction that have immunostimulant activity, namely **(1)** (E)-4-(3',4'-dimethoxy phenyl)but-3en-1-ol, **(2)** (E)-4-(2',4',5'-tri-methoxy phenyl)but-3en-1-ol, and **(3)** (E)-4-(3',4',1-tri-methoxyphenyl) ut-3en-1-ol. Number **(1)** is the main component and causes the largest phagocytic activity, which is in contrast to components (2) and (3) whose induced phagocytic activities are not significantly different from that of the negative control (PBS). The difference in double bonds (outside the aromatic ring of phenolic compounds) between the component **(1)** and both components **(2)** and **(3)** (containing a group of methoxy substituents) potentially determine the produced reactivity and phagocytic activity-capacity to decrease immunostimulants (Chairul *et al.*, 2009).

Another in vivo study has proven that the administration of Bengle rhizoma extract at a dose of 5 mg/20 mg BW in mice for seven days optimally increases NO and ROI secretion although it does not significantly influence the phagocytic activity of macrophages (Nurkhasanah *et al.*, 2017). Despite the successful dose optimization in the study, the best rule of administration has not been analyzed yet.

The immunostimulant properties of the test compound is a factor that can influence the effectiveness of a compound in shaping the immune response. Based on immunostimulant properties, chemical compounds are classified into two groups, namely low molecular weight (phenols, alkaloid, saponin, and quinone) and high molecular weight (Wagner, 1999). Any substance that can induce an immune response is called an immunogen, and the molecular weight of the most potent immunogen, i.e., protein macromolecule, is 100,000 daltons. Accordingly, immunogens weighing <100,000 daltons are deemed weak; hence, tiny molecules like amino acids are not immunogenic. Phenylbutenoid is a component of Bengle rhizoma (Chairul *et al.*, 2009). Since it is a phenolic compound, it is an immunostimulant with low molecular weight and weak immunogenic nature (Bhuiyan *et al.*, 2008). In addition to these properties, the immunogenicity of a substance also depends on the amount of

administered immunogen, as well as the way and the amount of which the substance enters into the body. The immune response can be optimized by determining the dose of immunogen, method of administration, and time of administration carefully (Bratawidjaya, 2014).

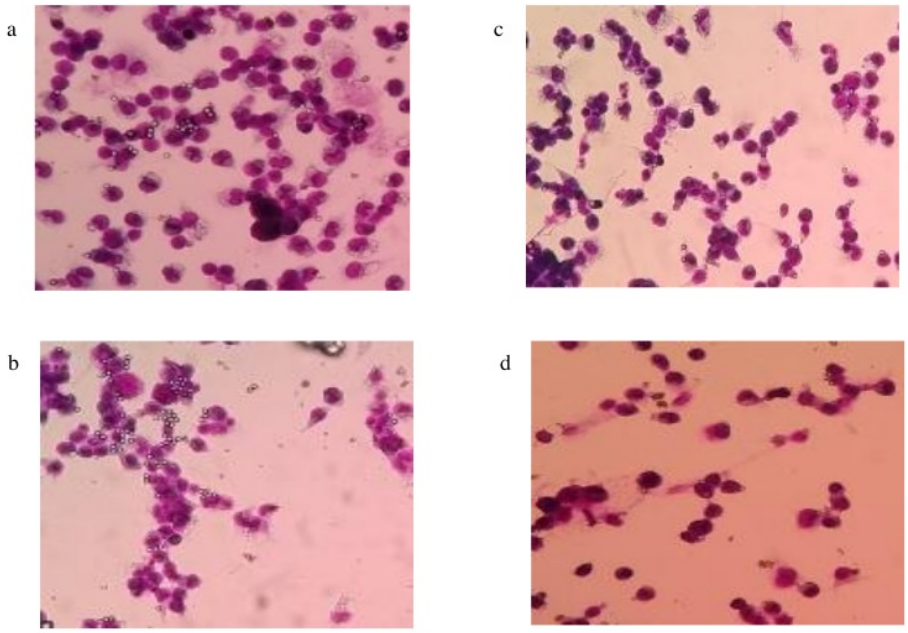

Figure 1. The phagocytic activity of mouse's macrophages in (a) normal control and treatment groups receiving (b) 25 ppm, (c) 50 ppm, and (d) 100 ppm of Bengle rhizoma extract

The effects of Bengle rhizoma extract on lymphocyte proliferation

The test of lymphocyte proliferation activity used the MTT assay and was based on the ability of the living cells to reduce yellow MTT (water-soluble) into formazan salt (water-insoluble). The produced formazan salts are purple, and this has a direct correlation with cell count. The microplate reader shows the absorbance that indicates the number of living cells (Mosmann, 1983; Ulfah *et al*., 2017).

In this study, the MTT assay was prepared for two sets of tests, namely the MTT assay for three days (MTT-3) and five days (MTT-5). It aimed to identify any differences in lymphocyte proliferation in three and five days. The ability of lymphocyte to proliferate after the addition of Bengle rhizoma extract into the wells was visible by comparing the absorbances of the test groups and the normal control group.

In the test with MTT-3 assay, the addition of various concentrations of Bengle rhizoma extract (test groups) to the cultures turned out to decrease the number of lymphocytes, while it was not the case with the normal control group. This effect was apparent from the absorbance of the cultures. The microplate reader detects absorbance values. In this case, these values are the measure of light absorption by formazan crystals—formed as the product of MTT reduction to its insoluble formazan due to succinate dehydrogenase enzyme. This enzyme is produced from the mitochondria of living cells, meaning that formazan crystals can only form in living cells. The number of living cells

correlates positively with that of formazan crystal and, by extension, absorbance (Mosmann, 1983; Ulfah *et al*., 2017). In the MTT-5 assay, the test showed the same results as the MTT-3 assay. Contrary to the normal control group, all concentrations of Bengle rhizoma extract reduced the number of cells in the culture.

Table III. The absorbances of cultures added with various concentrations of Bengle rhizoma extract during the lymphocyte proliferation test in mice

Groups	Absorbances
Normal control	0,149±0,006
Bengle rhizoma extract, 25 ppm	0,129±0,010*
Bengle rhizoma extract, 50 ppm	0,129±0,006*
Bengle rhizoma extract, 100 ppm	0,113±0,013*

*denotes a significant difference with the normal control group (p<0.05).

The LSD test results, as seen in Table III, showed a significant difference between the normal control group (added with lymphocytes and the complete medium) and the test groups. It indicates that Bengle rhizoma extract at concentrations of 25, 50, and 100 ppm produces an effect, that is, the reduction and suppression of lymphocyte proliferation. In other words, this test compound can lower the body's immune system.

Curcumin is known to be an ingredient of *Z. cassumunar*. It can explain the decrease in lymphocyte proliferation in this study. Previous studies have claimed that curcumin in *Curcuma xanthorrhiza* has an inhibitory effect on lymphocyte proliferation in rabbit (Huang *et al*., 1992) and humans—where lymphocyte proliferation was induced by 0.01 and 0.05 mg/ml of PHA (Yadav *et al*., 2005). In another research, splenic cell antiproliferation is associated with the effects of curcumin on myelin basic protein (MBP)-specific lymphocyte T that leads to the inhibition of allergic reactions (Natrajan and Bright, 2002), confirming the results of this study.

Although the exact mechanism of the anti-proliferative effect of curcumin is not certainly known, there is a possibility that curcumin impedes proliferation by inhibiting ribonucleotide reductase and DNA polymerase activation, which are the two main enzymes in DNA synthesis or the essential processes in cell duplication in the S phase of the cell cycle (Fontecave, 1997; Sun *et al*., 1998).

CONCLUSION

In vitro administration of Bengle rhizoma extract (*Z. cassumunar*) to macrophages and lymphocytes can result in reductive effects on the body's immune system, as evident in the phagocytic activity of macrophages and the ability of lymphocyte to proliferate. At concentrations of 25 and 50 ppm, the extract can reduce the phagocytic activity of macrophages, although less significantly different than the control group. On the contrary, phagocytosis in macrophages added with 100 ppm of Bengle rhizoma extract is reduced much significantly than the control group. Also, these three concentrations successfully suppress lymphocyte proliferation.

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