In vitro activity of immunomodulator of N-Hexane fraction of bangle (*Zingiber cassumunar* Roxb.) Ethanol extract

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Abstract

Zingiber cassumunar is potentially an immunomodulator. The aim of study is to determine the immunomodulatory activity of n-hexane fraction of Zingiber cassumunar ethanol extracts with a measurement of phagocytic activity and lymphocyte proliferation in vitro. Macrophage cells are isolated from peritoneum of mice. Cells were planted and added to latex and n-hexane fraction of Zingiber cassumunar ethanol extract and then painted using 20% giemsa. Lymphocyte cells are isolated from lymph. Cells were planted and given n-hexane fraction of Zingiber cassumunar ethanol extract. Incubate for 3 days using 5% CO_2 incubator at 37°C. Data were analysed using SPSS with a confidence level of 95%.

The results of the average percentage of phagocytic activity and phagocytic index of n-hexane fraction of Zingiber cassumunar ethanol extract of 0.025 µg/ml, 0.050 µg/ml, 0.100 µg/ml and controls were respectively 5.247% and 5.311%, 7.475% and 7.720%, 10.593% and 11.393% and 15.989% and 19.317%. The results of the average lymphocyte proliferation absorbance of 0.025 µg/ml, 0.050 µg/ml, 0.100 µg/ml and controls respectively are 0.107, 0.127, 0.126 and 0.149. The n-hexane fraction of Zingiber cassumunar ethanol extract can reduce the phagocytic activity of macrophages ($p<0.05$) and lymphocyte proliferation ($p<0.05$) in mice in vitro.

Keywords: N-hexane fraction, *Zingiber cassumunar* ethanol extract, immunomodulator, phagocytic activity, lymphocyte proliferation.

Introduction

Macrophage cells are one of the cells that play a role in the immune system. In addition, the immune system is also closely related to lymphocytes. Viruses that enter the body will live and multiply in the macrophages. The incoming virus will invade T-helper cells. T-helper cells are part of the lymphocyte cells that are important in the immune response. Invaded t-helper cells will signal macrophages to fight. Macrophage cells will produce antigens which will be bound by t cells. Stimulated t cells will produce chemical mediators namely interleukin[1]. Rhizome of bangle (*Zingiber cassumunar*) is one of the plants that can act potentially as immunomodulator. Rhizome of bangle (*Zingiber cassumunar*) contains active compounds of phenylbutanoid which are known to have phagocytic activity[2].

Rhizome of bangle (*Zingber cassumunar*) also contains fat, tannins, alkaloids, steroids, terpenoids and flavonoids[3]. Chemical compounds are contained in immune regulation namely tannins, alkaloids, terpenoids and flavonoids[4]. Rhizome of bangle (*Zingiber cassumunar*) also contains curcuminoid compounds[5]. Curcumin can inhibit cell proliferation and cytokine production by inhibiting the target NF-kB gene involved in the immune process[6]. Ethanol extract of bangle (*Zingiber cassumunar*) can increase phagocytic activity, NO (nitrit oxide) secretion and ROI (reaxtive oxygen intermediate) by macrophages[7]. This study aims to determine the immunomodulatory activity of n-hexane fraction of *Zingiber cassumunar* ethanol extract through phagocytic activity and lymphocyte proliferation *in vitro*.

Material and Methods

Material: The main material used in this research is the rhizome of bangle (*Zingiber cassumunar*) obtained from Beringharjo market, Yogyakarta, Indonesia. The rhizome was macerated in 96% ethanol and evaporated to get a concentrated extract. The extract was fractionation in n-hexane and evaporated to get a concentrated fraction. The doses of fraction of n-hexane used are 0.025 µg/ml, 0.050 µg/ml *and* 0.100 µg/ml.

Animal: The test animals used were mice 8 weeks old with Balb strain. The animal treatment procedure has been approved by the Ethics Committee of Ahmad Dahlan University with number 011804063.

Methods:

Preparation of animal: The macrophage cells and lymphocytes cell were isolated from the healthy mice 8-week old with Balb strain. The mice are narkosed with chloroform and disinfected with 70% ethanol and placed in the supine position. The mice perioneal cavity was opened and disinfected used 70% ethanol.

The macrophage cells were taken by injected cold RPMI 10 ml into peritoneal cavity and a massage was carried out for 3 min. The peritoneal cavity of mice was removed. Liquid aspiration used a syringe and was centrifugated at 4°C, 1200 rpm for 10 min. The supernatant was discarded. The pellet was re-suspended with a complete medium and cell was counted using haemocytometers[8].

The Lymphocyte cells were isolated from the lymph part of the mice. The spleen was put in the Petri disks and washed with PBS 2-3 times. RPMI was added to 10 ml of suspension and centrifuged at 3.200 rpm 4ºC for 10 min. The supernatant was discarded and pellet was added with a complete medium. Lymphocyte cells are calculated by haemocymeter[9].

Phagocytic Activity: A total of 100 µl of cells suspension were inserted into a microplate of 6 wells and each well had been given a coverslip. The cells were incubated for 1 hour using 5% CO_2 incubator at 37°C. After that, a complete medium of 700 µl was added in each well and incubated for 24 hours until the cells were attached. The complete medium in the each well was discarded. The samples (0.025 µg/ml, 0.050 µg/ml, 0.100 µg/ml and normal control) was added in the each well and incubated with 5% CO_2 incubator at 37°C for 1 hour. Then, latex was inserted in PBS (phosphate buffer saline) in each well and incubated with 5% CO_2 incubator at 37°C for 1 hour.

The supernatant was discarded and drained at room temperature. Each well was fixed with methanol 300 µl 1x for 30 seconds. Each well was drained at room temperature. Each well was painted with 20% giemsa solution 400 µl for 20 min. The Giemsa in each well was discarded and was washed with distilled water until clear in the each well. Each well was drained at room temperature and percentage of macrophage cells was calculated by phagocytosis of latex particles from 100 cells with 400x magnification light microscope[8].

Lymphocyte Proliferation: 50 µl of lymphocyte cell suspension was inserted in the 96 wells microplates. 50 µl of n-hexane fraction of *Zingiber cassumunar* ethanol extract was dropped in the each well. 96 wells microplates were incubated with 5% CO_2 incubator at 37 °C for 3 days. After that, each well was added with 50 µl MTT concentration (5mg/ml) and incubated with 5% CO_2 incubator at 37 °C for 4 hours. Then, SDS 10% was added 50 µl in the each well. The microplates were stored at room temperature in dark conditions for 12 hours and the results were measured used ELISA with a wavelength of 595 nm.

The data obtained were analyzed using SPSS with a confidence level of 95%.

Results and Discussion

Phagocytic Activity Test: The phagocytic activity of macrophages was measured by looking at the ability of macrophages to eat latex particles that were assisted by using a microscope. Examples of the results of phagocytic activity of macrophages can be seen in figure 1. Measuring the percentage of phagocytic activity is calculated by comparing macrophage cells that eat latex particles with macrophage cells that do not eat latex particles. The results of phagocytic activity can be seen in table 1.

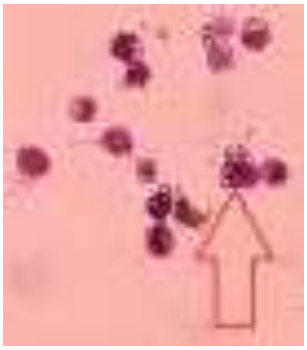

Figure 1: Examples of macrophage cells that eat latex particles

Table 1
Phagocytic activity of macrophage in mice treated with *Zingiber cassumunar* ethanol extract *in vitro*

Group	% Phagocytic Activity	% Phagocytic Index
Dose 0.025 µg/ml	5,247±0,452*	5,311±0,575*
Dose 0.050 µg/ml	7,475±0,295*	7,720±0,856*
Dose 0.100 µg/ml	10,593±1,412*	11,393±1,799*
Normal Control	15,989±2,161	19,317±3,339

* data showed that there are significant differences with the control group ($p<0.05$)

The highest results of the percentage of phagocytic activity and the highest phagocytic index were shown in the n-hexane fraction of 0.100 µg/ml with values of 10,593% and 11,393% respectively. This is probably due to phenylbutanoids in the *Zingiber cassumunar*. Phenylbutanoids was known to have phagocytic activity[2] while the results of the average percentage of phagocytic activity and the lowest index were shown in the n-hexane fraction dose of 0.025 µg/ml with values of 5.247% and 5.311% respectively. The three fractions showed significantly different results from the normal control group with $p<0.05$. This result shows that phagocytic activity and phagocytic index *Zingiber cassumunar* extract ethanol did not show a significant difference with $p>0.05$[7]. The results showed that the n-hexane fraction can reduce phagocytic activity compared to the normal control group.

The n-hexane fraction of *Zingiber cassumunar* contains phenylbutanoid compounds which show anti-inflammatory activity[10]. One of mechanism for anti-inflammatory is through inhibition of prostaglandin. PGE2 (prostaglandin E2) is a molecule that is included in regulating inflammation and various immune cell functions. PGE2 can activate macrophage when inflammation occurs. The anti-inflammatory activity by n-hexane fraction of *Zingiber cassumunar* or bangle causes inhibition of prostaglandin and macrophage becomes inactive and phagocytosis process does not occur so that it can cause phagocytic activity[11].

Lymphocyte Proliferation Test: Lymphocyte proliferation test was carried out to determine the response of regulatory components in the immune system. The results of lymphocyte proliferation absorbance can be seen in table 2.

Table 2
Lymphocyte proliferation Absorbance in mice treated with *Zingiber cassumunar* ethanol extract *in vitro*

Group	Average Absorbance
Dose 0.025 µg/ml	0.107 ± 0,0058*
Dose 0.050 µg/ml	0.127 ± 0,007*
Dose 0.100 µg/ml	0.126 ± 0,008*
Normal Control	0.149 ± 0,006

* data showed that there are significant differences with the control group ($p<0.05$)

The average results of absorbance of the highest lymphocyte proliferation are shown in the n-hexane fraction dose of 0.050 µg/ml with a value of 0.127 while the average yield of the lowest proliferation absorbance is indicated by the n-hexane fraction dose of 0.025 µg/ml with a value of 0.107. The three fractions showed significantly different results for the normal control group with $p<0.05$. The results showed that the n-hexane fraction of *Zingiber cassumunar* ethanol extract can reduce the lymphocyte proliferation process compared to the control group.

Conclusion

N-hexane fraction of *Zingiber cassumunar* ethanol extract can reduce phagocytic activity and can reduce lymphocyte proliferation with $p<0,05$. These results indicate that n-hexane fraction of *Zingiber cassumunar* ethanol extract has immunomodulatory effect *in vitro*.

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