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Jurnal RJCE
Research Journal of Chemistry and Environment Vol. 23 (8) August (2019)
Res. J. Chem. Environ.

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

Nurkhasanah*, Sulistyani Nanik and Noorlina

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19 Des 2019 : Pemberian LoA dari panitia

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17 Januari : Pengiriman perbaikan naskah setelah pemberitahuan permintaan perbaikan naskah oleh panitia pada 8 Januari 2019.
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9 Maret 2019 : Pemberitahuan hasil review artikel dan permintaan perbaikan naskah, dilampiri naskah hasil review

Lampiran 4
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Yogyakarta, 18 Desember 2018

Assalamualaikum Wr. Wb.

Dear **Noorlina, S.Farm., Apt**

Atas nama panitia seminar ***"The 1st UAD-UNPAD Post Graduate's Student Seminar"***, kami menginformasikan bahwa abstrak Anda yang berjudul:

"AKTIVITAS IMUNOMODULATOR FRAKSI N-HEKSAN EKSTRAK ETANOL BANGLE (Zingiber cassumunar) SECARA IN VITRO"

DITERIMA

Untuk

Presentasi Oral

Kami mengundang Anda untuk mempresentasikan hasil penelitian anda dalam kegiatan yang akan diselenggarakan pada:

Tanggal : 22 Desember 2018

Tempat : Auditorum Kampus IV Universitas Ahmad Dahlan

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Kami juga mengharapkan partisipasi Anda untuk melengkapi fullpaper yang akan dikirimkan ke jurnal Pharmaciana, Media Farmasi, IJCP dan selambat-lambatnya 15 Desember 2018. Keputusan diterima pada jurnal yang dituju adalah hak editor jurnal. Untuk jadwal dan rundown acara dapat anda lihat mulai tanggal 20 Desember 2018 pada situs web https://pasca-farmasi.uad.ac.id

Jika Anda memiliki pertanyaan, kami mempersilakan untuk menghubungi Susan (+6282175357584)

Sincerely

Dr.Hari Susanti, M.Si., Apt

Lampiran 2. Pengiriman perbaikan naskah setelah pemberitahuan permintaan perbaikan naskah oleh panitia pada 8 Januari 2019.
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IMMUNOMODULATOR ACTIVITIES OF N-HEXANE FRACTION OF ETHANOL EXTRACT OF BANGLE (*Zingiber cassumunar* Roxb.) IN VITRO

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Abstract

Zingiber cassumunar potentially an immunomodulator. The study aim to determine the immunomodulatory activity of n-hexane fraction of ethanol extracts of Z.cassumunar in vitro with a measurement of phagocytic activity and lymphocyte proliferation.

Macrophages are isolated from the peritoneum. Cells were planted and added latex and n-hexane fraction of ethanol extract of Z.cassumunar. Then painted using 20% giemsa. While lymphocyte cells are isolated from lymph. Cells were planted and given n-hexane fraction of ethanol extract bangle. Incubation for 3 days using a 5% CO2 incubator at 37°C. Then given MTT and SDS 10%, read using ELISA with a wavelength of 595 nm. Data were analyzed using SPSS with a confidence level of 95%.

The results of the average percentage of phagocytic activity and phagocytic index of hexane fraction of ethanol extract of Z.cassumunar of 25 ppm, 50 ppm, 100 ppm 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 of 25 ppm, 50 ppm, 100 ppm and controls respectively are 0.107, 0.127, 0.126 and 0.149.

The N-hexane fraction of ethanol extract of Z.cassumunar can reduce the phagocytic activity of macrophages and lymphocyte proliferation in mice in vitro.

Keywords : N-hexane fraction, ethanol extract of *Zingiber cassumunar*, immunomodulator, phytocytic 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[8]. Rhizome of bangle (*Z.cassumunar*) is one of the plants that can potentially an immunomodulator. Rhizome of bangle (*Z.cassumunar*) contains active compounds of phenylbutanoid which are known to have phagocytic activity[2]. Rhizome of bangle (*Z.cassumunar*) also contains fat, tannins, alkaloids, steroids, terpenoids and flavonoids[7]. Chemical compounds contained in immune regulation namely tannins, alkaloids, terpenoids and flavonoids[10]. Rhizome of bangle (*Z.cassumunar*) also contains curcuminoid compounds[6]. Curcumin can inhibit cell proliferation and cytokine production by inhibiting the target NF-kB gene involved in the immune process[11]. Ethanol extract of bangle (*Z.cassumunar*) can increase phagocytic activity, NO secretion and ROI by macrophages[4]. This study aims to determine the immunomodulatory activity of n-hexane fraction of ethanol extract of bangle (*Z.cassumunar*) through phagocytic activity and lymphocyte proliferation.

Materials and Methods

Material

The main material used in this research is the rhizome of bangle (*Z.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 dose of fraction of hexane used are 25 ppm, 50 ppm and 100 ppm.

Animal

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

Procedure

1. Phagocytic Activity Test

Before the test, the macrophage cells isolation from the mice 8-week old Balb was carried out. The mice are narkosed with chloroform and disinfected with 70% ethanol and placed in the supine position. Open the peritoneal cavity and disinfected using 70% ethanol. Injected 10 ml of cold RPMI into the peritoneal cavity and carried out a massage for 3 minutes. Remove the peritoneal cavity by pressing on the cavity using 2 fingers. Liquid aspiration using a syringe and centrifugation at 4°C, 1200 rpm for 10 minutes. Discard the supernatant and take the precipitate formed. Resuspensation of deposits with a complete medium and counted number of cells using haemocytometers[1].

A total of 100 µl of cells suspension was inserted into a microplate of 6 wells and each well had been given a coverslip. Incubation 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 again for 24 hours until the cells were attached. The complete medium in the each well is discarded. Added with test compounds in each well. Incubate the 5% CO_2 incubator at 37°C for 24 hours. Inserted latex in the PBS in the each well. Incubate at 5% CO2 incubator at 37°C for 1 hour. Dispose of liquid from the wells. Dry it at room temperature. Fixed with PBS 1x in the each well as much as 1 ml. Dry it again. Fixation with methanol 1x for 30 seconds as much as 300 µl in the each well. Dry it at room temperature. Perform painting by adding 20% giemsa solution for 20 minutes as much as 400 µl in the each well. Discard the remaining giemsa and rinse with distilled water until the soaking water in the each well is clear. Dry it at room temperature and calculate the percentage of macrophage cells that phagocytosis of latex particles calculated from about 100 cells examined by a 400x magnification light microscope[1].

2. Lymphocyte Proliferation Test

Before the test, lymphocyte cells isolation from the lymph part of the mice. Put the spleen 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 minutes. The precipitate is separated from the supernatant, then added with a complete medium. Lymphocyte cells are calculated by haemocymeter[5].

Inserted to 50 µl of cell suspension in the 96 wells microplates. The 50 µl of n-hexane fraction of ethanol extract of bangle (*Z.cassumunar*) were included in the each well. Incubate with 5% CO_2 incubator at 37°C for 3 days. Take the plate in the 5% CO_2 incubator. Added MTT concentration of 5 mg / ml as much as 50 µl in the each well. Incubate with 5% CO_2 incubator at 37°C for 4 hours. Take the plate in the 5% CO_2 incubator at 37°C. Added SDS 10% as much as 50 µl in the each well. Store the mikroplate at room temperature in dark conditions for 12 hours and read the results using ELISA with a wavelength of 595 nm.

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

Result and Discussion

1. 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 the Figure 1.

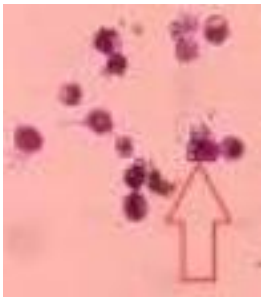

Figure 1. Examples of macrophage cells that eat latex particles

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 the table 1.

Table 1. Phagocytic activity

Group	% Phagocytic Activity	% Phagocytic Index
Dose 25 ppm	5,247±0,452*	5,311±0,575*
Dose 50 ppm	7,475±0,295*	7,720±0,856*
Dose 100 ppm	10,593±1,412*	11,393±1,799*
Normal Control	15,989±2,161	19,317±3,339

* 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 100 ppm with values of 10,593% and 11,393%, respectively. While the results of the average percentage of phagocytic activity and the lowest index were shown in the n-hexane fraction dose of 25 ppm with values of 5.247% and 5.311%, respectively. The three fractions showed significantly different results from the normal control group. The results showed that the n-hexane fraction can reduced phagocytic activity compared to the normal control group.

The n-hexane fraction of bangle (*Z.cassumunar*) contains phenylbutanoid compounds which show anti-inflammatory activity[9]. 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 activated macrophage when inflammation occurs. The anti-inflammatory activity by n-hexane fraction of bangle (*Z.cassumunar*) causes inhibition of prostaglandin and macrophage becomes inactive and does not occur phagocytosis process, so that it can cause phagocytic activity is smaller[3].

2. Lymphocyte Proliferation Test

Lymphocyte proliferation test is performed to determine the response of regulatory components in the immune system. The results of lymphocyte proliferation absorbance can be seen in the table 2.

Table 2. Lymphocyte Proliferation Absorbance

Group	Average Absorbance
Dose 25 ppm	0.107 ± 0,0058*
Dose 50 ppm	0.127 ± 0,007*
Dose 100 ppm	0.126 ± 0,008*
Normal Control	0.149 ± 0,006

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

The average results of absorbance of the highest lymphocyte proliferation were shown in the n-hexane fraction dose of 50 ppm with a value of 0.127. While the average yield of the lowest proliferation absorbance is indicated by the n-hexane fraction dose of 25 ppm with a value of 0.107. The three fractions showed significantly different results for the control group. The results show that the n-hexane fraction can reduced the lymphocyte proliferation process compared to the control group.

Conclusion

Based on research conducted by giving n-hexane fraction of ethanol extract of bangle (*Zingiber cassumunar*) can reduced phagocytic activity and reduced lymphocytes proliferation.

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Assalamualaikum,

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Di tempat

Berikut ini kami sampaikan hasil review terhadap artikel yang Bapak/Ibu kirimkan ke panitia Seminar UAD UNPAD. Hasil revisi maksimal dikirmkan kembali ke panitia pada **hari Rabu, tanggal 13 Maret 2019** dengan **me-reply** email ini. File artikel hasil revisi mohon dapar diberi nama file sbb: RJCE-nama author-revisi2.

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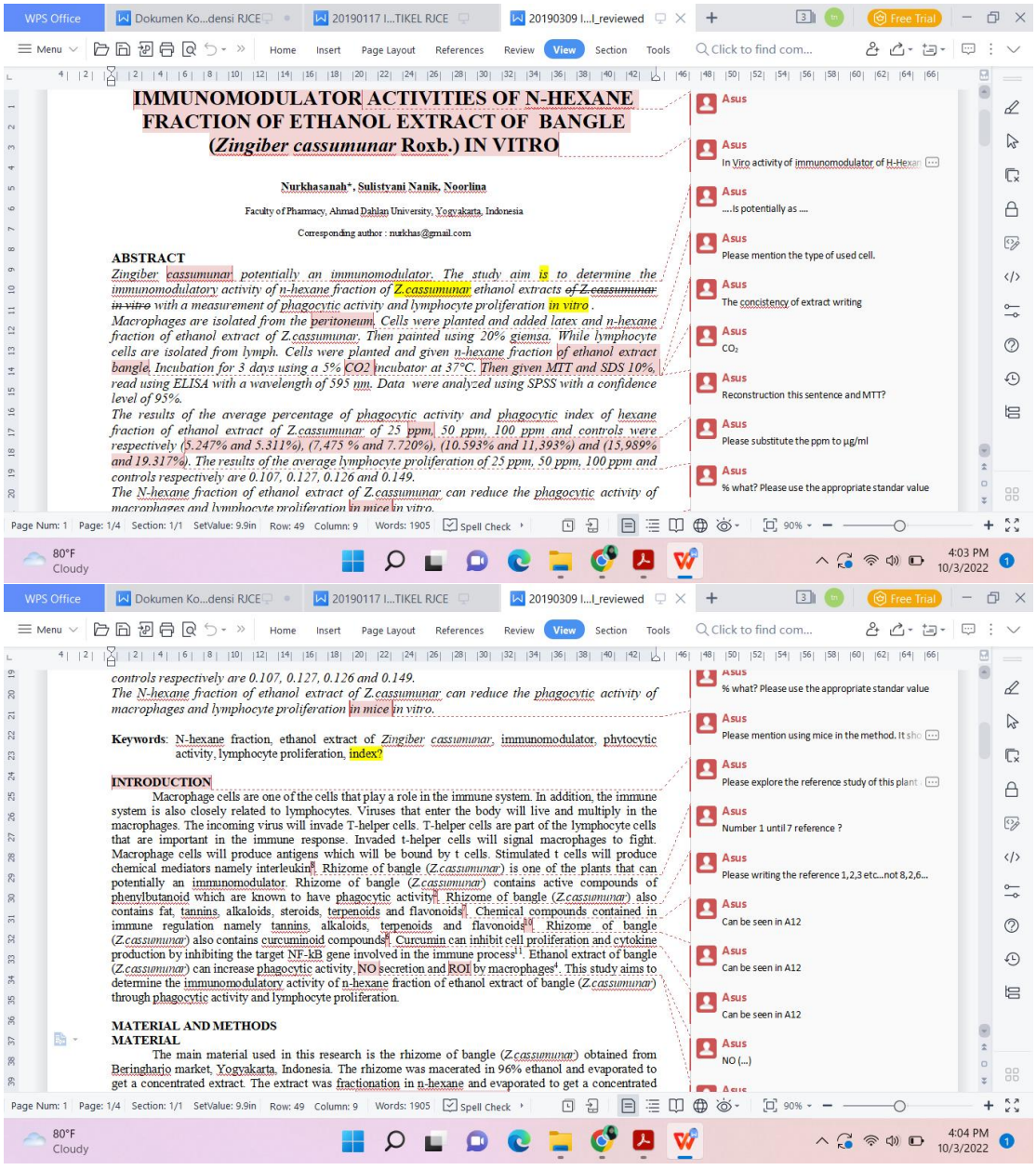

IMMUNOMODULATOR ACTIVITIES OF N-HEXANE FRACTION OF ETHANOL EXTRACT OF BANGLE (*Zingiber cassumunar* Roxb.) IN VITRO

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ABSTRACT

Zingiber cassumunar potentially an immunomodulator. The study aim is to determine the immunomodulatory activity of n-hexane fraction of Z.cassumunar ethanol extracts ~~of Z.cassumunar in vitro~~ with a measurement of phagocytic activity and lymphocyte proliferation in vitro .
Macrophages are isolated from the peritoneum. Cells were planted and added latex and n-hexane fraction of ethanol extract of Z.cassumunar. Then painted using 20% giemsa. While lymphocyte cells are isolated from lymph. Cells were planted and given n-hexane fraction of ethanol extract bangle. Incubation for 3 days using a 5% CO2 incubator at 37°C. Then given MTT and SDS 10%, read using ELISA with a wavelength of 595 nm. Data were analyzed using SPSS with a confidence level of 95%.
The results of the average percentage of phagocytic activity and phagocytic index of hexane fraction of ethanol extract of Z.cassumunar of 25 ppm, 50 ppm, 100 ppm 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 of 25 ppm, 50 ppm, 100 ppm and controls respectively are 0.107, 0.127, 0.126 and 0.149.
The N-hexane fraction of ethanol extract of Z.cassumunar can reduce the phagocytic activity of macrophages and lymphocyte proliferation in mice in vitro.

Keywords: N-hexane fraction, ethanol extract of *Zingiber cassumunar*, immunomodulator, phytocytic activity, lymphocyte proliferation, index?

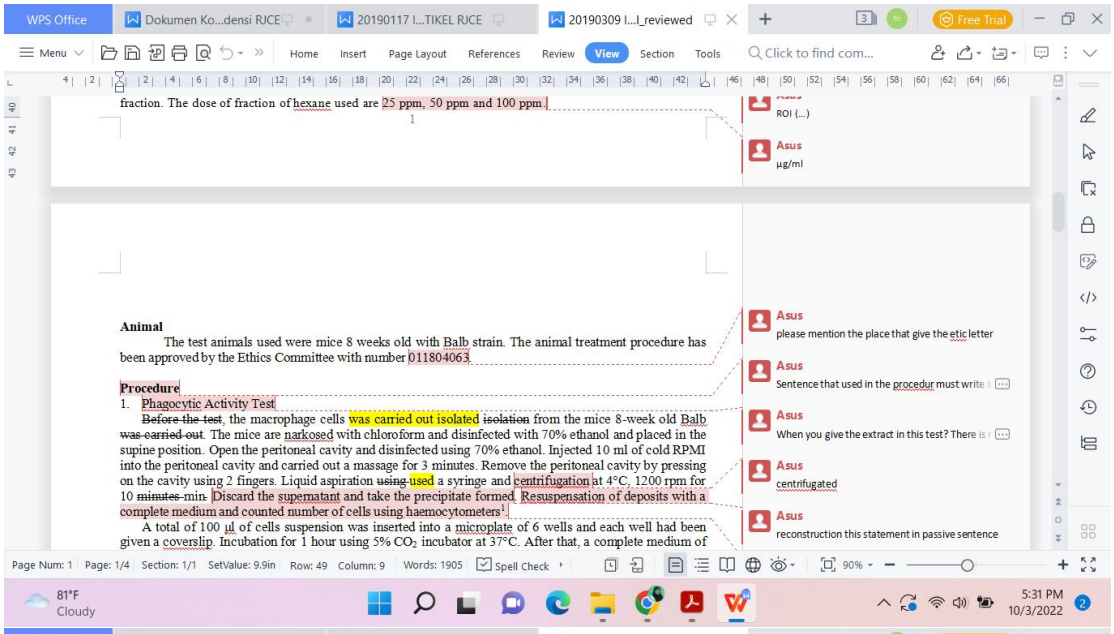
INTRODUCTION

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[8]. Rhizome of bangle (*Z.cassumunar*) is one of the plants that can potentially an immunomodulator. Rhizome of bangle (*Z.cassumunar*) contains active compounds of phenylbutanoid which are known to have phagocytic activity[3]. Rhizome of bangle (*Z.cassumunar*) also contains fat, tannins, alkaloids, steroids, terpenoids and flavonoids[9]. Chemical compounds contained in immune regulation namely tannins, alkaloids, terpenoids and flavonoids[10]. Rhizome of bangle (*Z.cassumunar*) also contains curcuminoid compounds[8]. Curcumin can inhibit cell proliferation and cytokine production by inhibiting the target NF-kB gene involved in the immune process[11]. Ethanol extract of bangle (*Z.cassumunar*) can increase phagocytic activity, NO secretion and ROI by macrophages[4]. This study aims to determine the immunomodulatory activity of n-hexane fraction of ethanol extract of bangle (*Z.cassumunar*) through phagocytic activity and lymphocyte proliferation.

MATERIAL AND METHODS

MATERIAL

The main material used in this research is the rhizome of bangle (*Z.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 dose of fraction of hexane used are 25 ppm, 50 ppm and 100 ppm.
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Animal
The test animals used were mice 8 weeks old with Balb strain. The animal treatment procedure has been approved by the Ethics Committee with number 011804063.
Procedure
1. Phagocytic Activity Test
Before the test, the macrophage cells was carried out isolated isolation from the mice 8-week old Balb was carried out. The mice are narkosed with chloroform and disinfected with 70% ethanol and placed in the supine position. Open the peritoneal cavity and disinfected using 70% ethanol. Injected 10 ml of cold RPMI into the peritoneal cavity and carried out a massage for 3 minutes. Remove the peritoneal cavity by pressing on the cavity using 2 fingers. Liquid aspiration using used a syringe and centrifugation at 4°C, 1200 rpm for 10 minutes min. Discard the supernatant and take the precipitate formed. Resuspension of deposits with a complete medium and counted number of cells using haemocytometers[1].
A total of 100 µl of cells suspension was inserted into a microplate of 6 wells and each well had been given a coverslip. Incubation for 1 hour using 5% CO_2 incubator at 37°C. After that, a complete medium of
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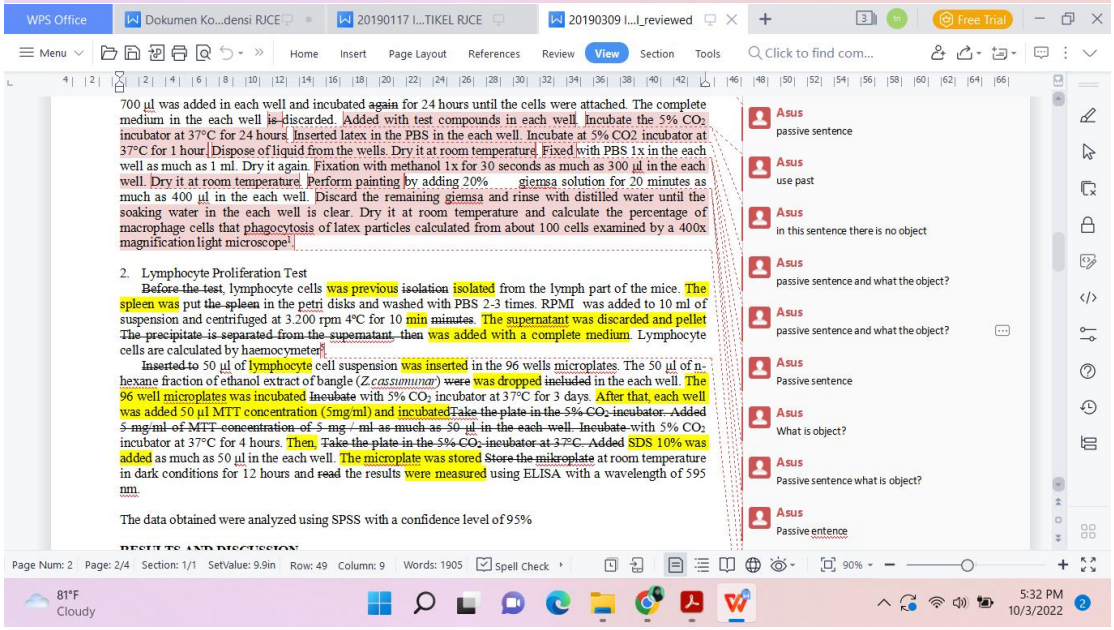
700 µl was added in each well and incubated again for 24 hours until the cells were attached. The complete medium in the each well is discarded. Added with test compounds in each well. Incubate the 5% CO_2 incubator at 37°C for 24 hours. Inserted latex in the PBS in the each well. Incubate at 5% CO2 incubator at 37°C for 1 hour. Dispose of liquid from the wells. Dry it at room temperature. Fixed with PBS 1x in the each well as much as 1 ml. Dry it again. Fixation with methanol 1x for 30 seconds as much as 300 µl in the each well. Dry it at room temperature. Perform painting by adding 20% giemsa solution for 20 minutes as much as 400 µl in the each well. Discard the remaining giemsa and rinse with distilled water until the soaking water in the each well is clear. Dry it at room temperature and calculate the percentage of macrophage cells that phagocytosis of latex particles calculated from about 100 cells examined by a 400x magnification light microscope[1].
2. Lymphocyte Proliferation Test
Before the test, lymphocyte cells was previous isolation isolated from the lymph part of the mice. The spleen was put the spleen 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 minutes. The supernatant was discarded and pellet The precipitate is separated from the supernatant, then was added with a complete medium. Lymphocyte cells are calculated by haemocymeter[1].
Inserted to 50 µl of lymphocyte cell suspension was inserted in the 96 wells microplates. The 50 µl of n-hexane fraction of ethanol extract of bangle (*Z.cassumunar*) were was dropped included in the each well. The 96 well microplates was incubated Incubate with 5% CO_2 incubator at 37°C for 3 days. After that, each well was added 50 µl MTT concentration (5mg/ml) and incubatedTake the plate in the 5% CO_2 incubator. Added 5 mg/ml of MTT concentration of 5 mg / ml as much as 50 µl in the each well. Incubate with 5% CO_2 incubator at 37°C for 4 hours. Then, Take the plate in the 5% CO_2 incubator at 37°C. Added SDS 10% was added as much as 50 µl in the each well. The microplate was stored Store the mikroplate at room temperature in dark conditions for 12 hours and read the results were measured using ELISA with a wavelength of 595 nm.
The data obtained were analyzed using SPSS with a confidence level of 95%
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RESULTS AND DISCUSSION

1. 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 the Figure 1.

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Figure 1. Examples of macrophage cells that eat latex particles

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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 the table 1.

Table 1. Phagocytic activity

Group	% Phagocytic Activity	% Phagocytic Index
Dose 25 ppm	5,247±0,452*	5,311±0,575*
Dose 50 ppm	7,475±0,295*	7,720±0,856*
Dose 100 ppm	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 100 ppm with values of 10,593% and 11,393%, respectively. While the results of the average percentage of phagocytic activity and the lowest index were shown in the n-hexane fraction dose of 25 ppm with values of 5.247% and 5.311%, respectively. The three fractions showed significantly different results from the normal control group. The results showed that the n-hexane fraction can reduced phagocytic activity compared to the normal control group.

The n-hexane fraction of bangle (*Z. cassumunar*) contains phenylbutanoid compounds which show anti-inflammatory activity[9]. 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 activated macrophage when inflammation occurs. The anti-inflammatory activity by n-hexane fraction of bangle (*Z. cassumunar*) causes inhibition of prostaglandin and macrophage becomes inactive and does not occur phagocytosis process, so that it can cause phagocytic activity is smaller[3].

2. Lymphocyte Proliferation Test

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The n-hexane fraction of bangle (*Z. cassumunar*) contains phenylbutanoid compounds which show anti-inflammatory activity[9]. 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 activated macrophage when inflammation occurs. The anti-inflammatory activity by n-hexane fraction of bangle (*Z. cassumunar*) causes inhibition of prostaglandin and macrophage becomes inactive and does not occur phagocytosis process, so that it can cause phagocytic activity is smaller[3].

2. Lymphocyte Proliferation Test

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

Table 2. Lymphocyte Proliferation Absorbance

Group	Average Absorbance
Dose 25 ppm	0.107 ± 0,0058*
Dose 50 ppm	0.127 ± 0,007*
Dose 100 ppm	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 were shown in the n-hexane fraction dose of 50 ppm with a value of 0.127. While the average yield of the lowest proliferation absorbance is indicated by the n-hexane fraction dose of 25 ppm with a value of 0.107. The three fractions showed significantly different results for the control group. The results show that the n-hexane fraction can reduced the lymphocyte proliferation process compared to the control group.

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CONCLUSION

Based on research conducted by giving n-hexane fraction of ethanol extract of bangle (*Zingiber cassumunar*) can reduced phagocytic activity and reduced lymphocytes proliferation.

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IN VITRO ACTIVITY OF IMMUNOMODULATOR OF N-HEXANE FRACTION OF BANGLE (*Zingiber cassumunar* Roxb.) ETHANOL EXTRACT

Nurkhasanah*, Sulistyani Nanik, Noorlina

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ABSTRACT

Zingiber cassumunar is potentially as immunomodulator. The study aim 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 latex and n-hexane fraction of Zingiber cassumunar ethanol extract. Then painted using 20% giemsa. While lymphocyte cells are isolated from lymph. Cells were planted and given n-hexane fraction of Zingiber cassumunar ethanol extract. ncubation for 3 days using a 5% CO_2 incubator at 37°C. The lymphocyte cells added MTT ([3-(4,5-dimetiltiazol-2-yl)-2,5-dipeniltetrazoliumbromida]) and SDS 10% read using ELISA with a wavelength of 595 nm. Data were analyzed 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 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 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 dose 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

1. Preparation of animal

The macrophage cells and lymphocytes cell was carried out isolated from the health 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 was taken by injected cold RPMI 10 ml into peritoneal cavity and carried out a massage for 3 min. The peritoneal cavity of mice was removed with pressed used 2 fingers. Liquid aspiration used a syringe and centrifugated at 4°C, 1200 rpm for 10 min. The supernatant was discharded and taken pellet. The pellet resuspended with a complete medium and cell was counted used haemocytometers[8].

The Lymphocyte cells was previous 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].

2. Phagocytic Activity

A total of 100 µl of cells suspension was inserted into a microplate of 6 wells and each well had been given a coverslip. The cells was incubated for 1 hour used 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 (phosphat buffer saline) in the 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 fixation 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 the each well was discarded and was washed with distilled water until in the each well clear. The each well was drained at room temperature and was calculated percentage of macrophage cells that phagocytosis of latex particles from 100 cells with 400x magnification light microscope[8].

3. Lymphocyte Proliferation

The 50 µl of lymphocyte cell suspension was inserted in the 96 wells microplates. The 50 µl of n-hexane fraction of *Zingiber cassumunar* ethanol extract was dropped in the each well. The 96 wells microplates was incubated with 5% CO_2 incubator at 37°C for 3 days. After that, each well was added 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 was 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

3. 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 the Figure 1.

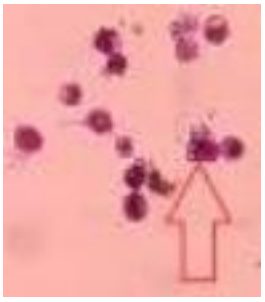

Figure 1. Examples of macrophage cells that eat latex particles

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 the table 1.

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 the contain of 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 different to researched by Nurkhasanah the show 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 reduced 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 activated 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 does not occur phagocytosis process, so that it can cause phagocytic activity is smaller[11].

4. 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 the 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 were 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 reduced the lymphocyte proliferation process compared to the control group.

CONCLUSION

Based on research conducted by giving n-hexane fraction of *Zingiber cassumunar* ethanol extract can reduced phagocytic activity and reduced lymphocyte proliferation with $p<0,05$. These result indicate that n-hexane fraction of *Zingiber cassumunar* ethanol extract have immunomodulatory effect in vitro.

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