Dokumen Korespondensi

Jurnal
BIOTROPIA Vol. 28 No. 2, 2021: 109 - 116

Judul artikel
INTERLEUKIN LEVELS IN THE Zingiber cassumunar-TREATED MICE

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

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Nurkhasanah
Faculty of Pharmacy
Universitas Ahmad Dahlan
Jl. Prof. Soepomo, Janturan, Yogyakarta, Indonesia
Email: nurkhas@gmail.com

Lampiran 1 :
19 Februari 2019 : ucapan terimakasih dari editor atas pengiriman artikel
25 April 2019 : pemberitahuan dari editor tentang status under review

Lampiran 2 :
13 Januari 2020 : Pemberitahuan dari editor tentang hasil review artikel, disertai lampiran naskah dan form review

Lampiran 3 :
2 Februari 2020 : Penyampaian artikel yang sudah diperbaiki author, disertai lampiran naskah dan respon sheet

Lampiran 4 :
6 Mei 2020 : Pemberitahuan dari editor tentang in press version

Lampiran 5 :
6 Juli 2021 : Permintaan dari editor tentang penyajian foto hasil penelitian di naskah
22 Juli 2022 : Penyampaian gambar hasil penelitian sesuai permintaan editor

Lampiran 6 :
27 Juli 2022 : Penyampaian Galleyproof oleh editor dan info akan publikasi Agustus 2021, dilampiri naskah

Lampiran 1.

Gmail **Nurkhasanah Mahfudh <nurkhas@gmail.com>**

[BIOTROPIA] #1162 Acknowledgement Letter

publication@biotrop.org <publication@biotrop.org> 14 February 2019 at 10:32
To: Nurkhasanah Mahfudh <nurkhas@gmail.com>

Dear Authors,

Thank you very much for your interest in submitting your manuscript to our BIOTROPIA journal, titled:

"The increasing level of interleukin in the Zingiber cassumunar treated rat"

For your information, starting from 2012, BIOTROPIA has been successfully indexed in SCOPUS. The publication process covers initial check, plagiarism check, internal review and external review by the expert reviewers.

In order to ease the process, it will be a great help if you can inform us the name, email address and the institution of three possible external reviewers who are experts/reputable scientist in your paper's subject from your country and abroad not later than 21 February 2019.

Thank you very much for your kind attention.

Sincerely,

Rima Febriana
Managing Editor

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Nurkhasanah Mahfudh <nurkhas@gmail.com>

[BIOTROPIA] # 1162 Requesting confirmation on your manuscript titled "the Increasing Level of Interleukin in the Zingiber cassumunar Treated Rat"

biotropia@biotrop.org <biotropia@biotrop.org> 25 April 2019 at 16:08
To: nurkhas@gmail.com

Dear Authors,

Thank you for submitting your manuscript to BIOTROPIA Journal.

We would like to inform you that currently your manuscript is still in review process.

For your information, the soonest publication of your manuscript will likely be in 2020, without any guarantees. It will be a great help if you could assist us by proposing other experts to be the reviewers.

We look forward to receiving your confirmation as soon as possible.
Should you need further information, please feel free to contact us at this email address.

Thank you very much for your kind cooperation.

Sincerely,

Sri I. Soerianegara
Publication Assistant

Lampiran 2. Hasil review artikel

Gmail Nurkhasanah Mahfudh <nurkhas@gmail.com>

[BIOTROPIA] #1162 external review results

biotropia@biotrop.org <biotropia@biotrop.org> 13 January 2020 at 09:19
To: nurkhas@gmail.com

Dear Authors,

We are glad to inform you that we have received the external review results of your manuscript from well-known experts. Please check the review form and the reviewed manuscript (if available) and note the comments/suggestions/answers to questions of the reviewers.

As the journal will have to be published soon, we request you to incorporate the reviewers' comments/suggestions/answers to questions in the manuscript as soon as possible, not later than 31 January 2020.

We also attach the table of responses to the reviewers' comments. Please list the corrections and mention the line numbers where the changes were made. If you disagree with comments or suggestions from the reviewers, please fill the column responses by stating the reason as a clarification to the reviewer's comments.

We look forward to hearing from you. Thank you very much for your kind attention and cooperation.

Best regards,

Rima Febriana
Managing Editor

On 2019-05-21 09:59, biotropia@biotrop.org wrote:
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>
> We would like to inform you that your manuscript has been reviewed by one external reviewer and the recommendation is to MODIFY. We are still waiting for the result from the second external reviewer. We will inform you again after receiving the second result. Thank you very much for your kind attention.
>
> Sincerely yours,
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> Zanne Sandriati Putri
> Publication Assistant
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> On 2019-05-10 13:37, biotropia@biotrop.org wrote:
> > Dear Authors,
> >
> > We would like to inform you that your manuscript has been through the internal review process. We will inform you again after receiving the review results from the external reviewers. Thank you very much for your kind attention.
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> > Best regards,
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> > Zanne Sandriati Putri
> > Publication Assistant
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> > > Thank you for submitting your manuscript to BIOTROPIA Journal.
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review process.

For your information, the soonest publication of your manuscript will likely be in 2020, without any guarantees. It will be a great help if you could assist us by proposing other experts to be the reviewers.

We look forward to receiving your confirmation as soon as possible. Should you need further information, please feel free to contact us at this email address.

Thank you very much for your kind cooperation.

Sincerely,

Sri I. Soerianegara
Publication Assistant

4 attachments

Table of Responses to the Reviewers' Comments.docx
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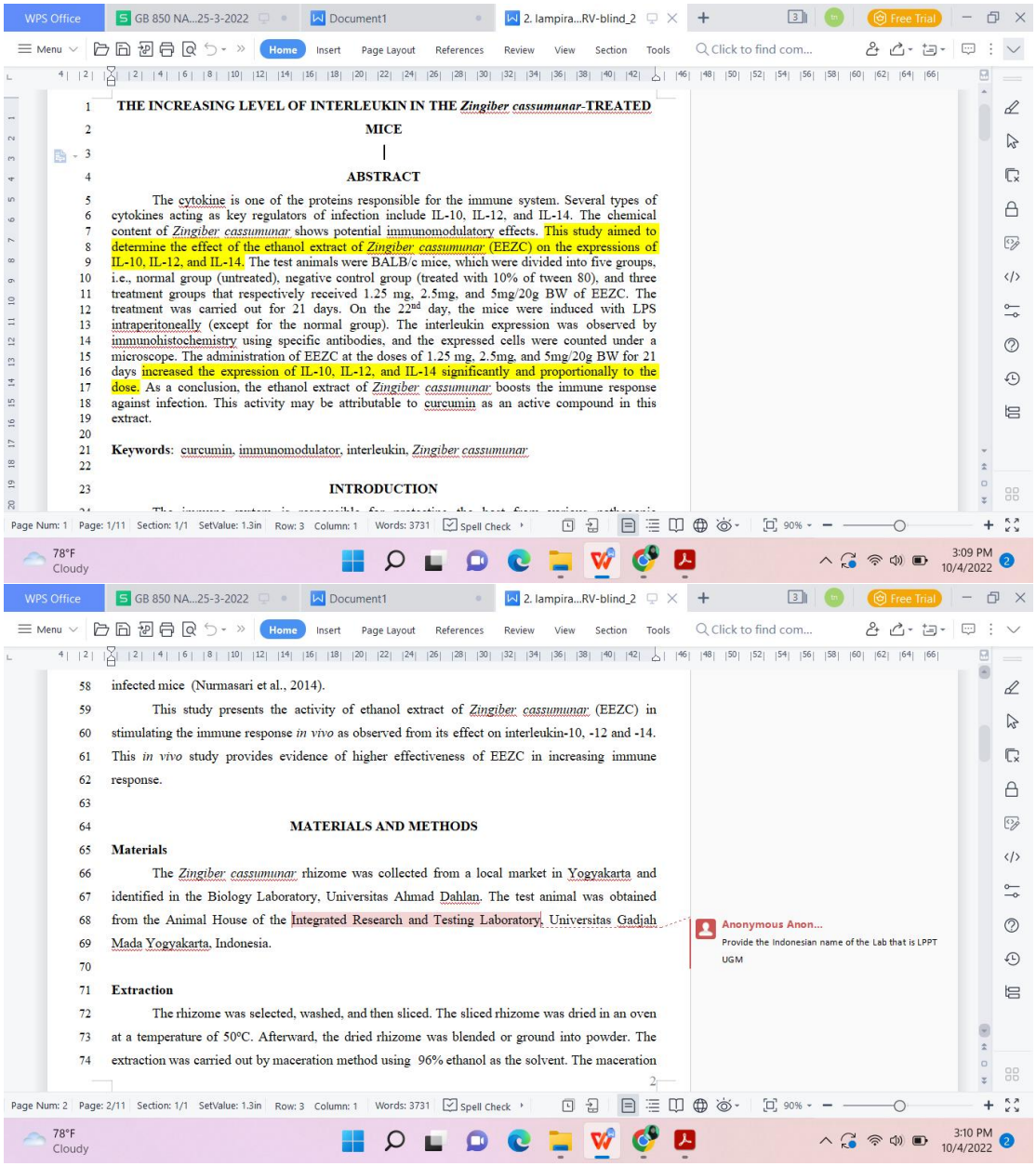
Lampiran beberapa catatan untuk revisi artikel :



THE INCREASING LEVEL OF INTERLEUKIN IN THE *Zingiber cassumunar*-TREATED MICE

ABSTRACT

The cytokine is one of the proteins responsible for the immune system. Several types of 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 ethanol extract of *Zingiber cassumunar* (EEZC) on the expressions of IL-10, IL-12, and IL-14. The test animals were BALB/c mice, which 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. The treatment was carried out for 21 days. On the 22nd day, the mice were induced with 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 administration of EEZC at the doses of 1.25 mg, 2.5mg, and 5mg/20g BW for 21 days increased the expression of IL-10, IL-12, and IL-14 significantly and proportionally to the dose. As a conclusion, the ethanol extract of *Zingiber cassumunar* boosts the immune response against infection. This activity may be attributable to curcumin as an active compound in this extract.

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

INTRODUCTION

infected mice (Nurmasari et al., 2014).

This study presents the activity of ethanol extract of *Zingiber cassumunar* (EEZC) in stimulating the immune response *in vivo* as observed from its effect on interleukin-10, -12 and -14. This *in vivo* study provides evidence of higher effectiveness of EEZC in increasing immune response.

MATERIALS AND METHODS

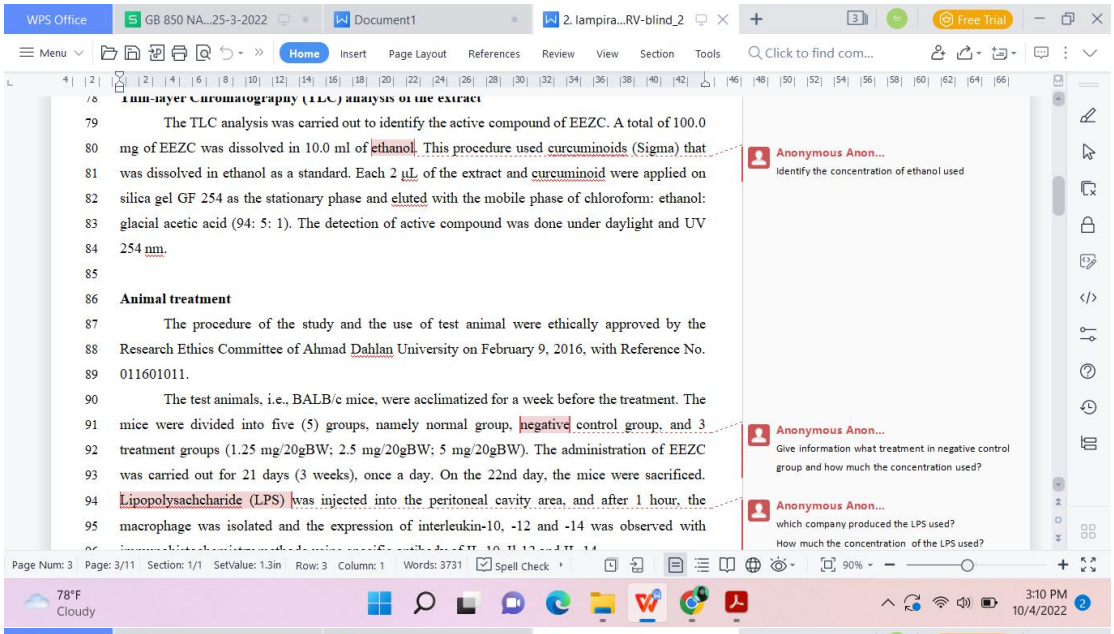
Materials

The *Zingiber cassumunar* rhizome was collected from a local market in Yogyakarta and identified in 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 Yogyakarta, Indonesia.

Anonymous Anon...
Provide the Indonesian name of the Lab that is LPPT UGM

Extraction

The rhizome was selected, washed, and then sliced. The sliced rhizome was dried in an oven at a temperature of 50°C. Afterward, the dried rhizome was blended or ground into powder. The extraction was carried out by maceration method using 96% ethanol as the solvent. The maceration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Thin-layer Chromatography (TLC) analysis of the extract
The TLC analysis was carried out to identify the active compound of EEZC. A total of 100.0 mg of EEZC was dissolved in 10.0 ml of 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 active compound 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, i.e., BALB/c mice, were acclimatized for a week before the treatment. The mice were divided into five (5) groups, namely normal group, negative control group, and 3 treatment groups (1.25 mg/20gBW; 2.5 mg/20gBW; 5 mg/20gBW). The administration of EEZC was carried out for 21 days (3 weeks), once a day. On the 22nd day, the mice were sacrificed. Lipopolysachcharide (LPS) was injected into the peritoneal cavity area, and after 1 hour, the macrophage was isolated and the expression of interleukin-10, -12 and -14 was observed with
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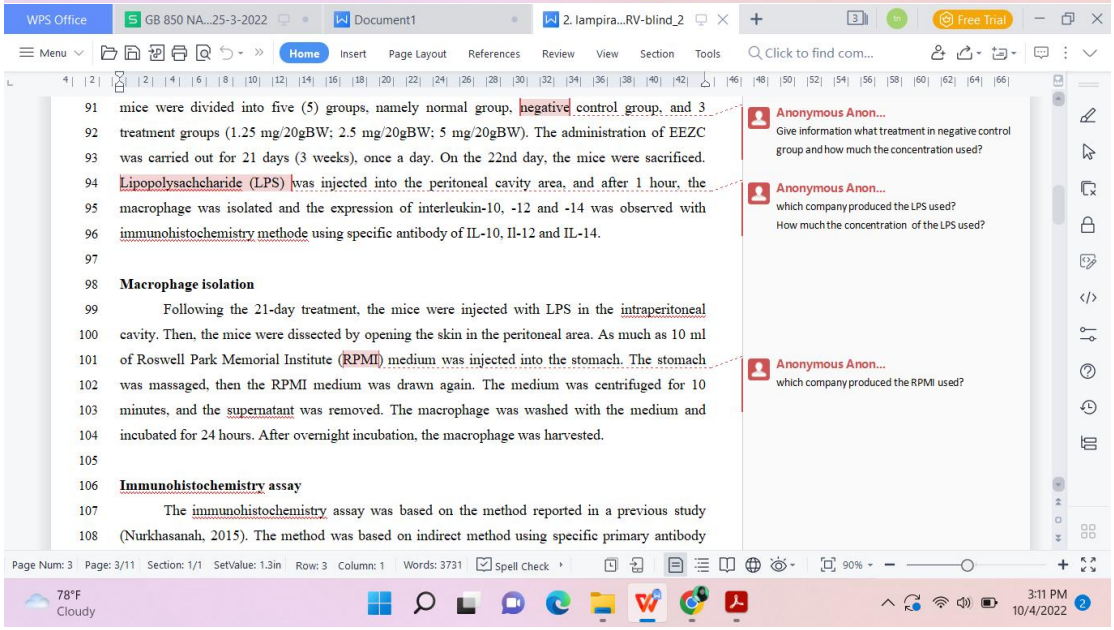
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mice were divided into five (5) groups, namely normal group, negative control group, and 3 treatment groups (1.25 mg/20gBW; 2.5 mg/20gBW; 5 mg/20gBW). The administration of EEZC was carried out for 21 days (3 weeks), once a day. On the 22nd day, the mice were sacrificed. Lipopolysachcharide (LPS) was injected into the peritoneal cavity area, and after 1 hour, the macrophage was isolated and the expression of interleukin-10, -12 and -14 was observed with immunohistochemistry methode using specific antibody 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. Then, the mice were dissected by opening the skin in the peritoneal area. As much as 10 ml of Roswell Park Memorial Institute (RPMI) 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 a previous study (Nurkhasanah, 2015). The method was based on indirect method using specific primary antibody
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running water and then with PBS. A total of 50µl of blocking serum was added to the preparation and then incubated in a humid temperature for 10-15 minutes. Then, 100µl of specific antibody (anti-IL-10, anti-IL-12, and anti-IL-14) was 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 of secondary antibody (biotin) was added to each preparation, which was incubated at a humid temperature for 20 minutes and then washed with PBS.

The preparations were incubated with 50 µl of the streptavidin-peroxidase enzyme for 10 minutes, washed with PBS, and incubated with 50 µl of DAB chromogen (peroxidase substrate solution). Afterward, the preparations were washed with PBS and incubated with Mayer's hematoxylin as the counterstain. The process was followed by washing with PBS. The macrophage was ready for microscopic observation at 400x magnification. Macrophages that expressed interleukin would show as brown stains.

Anonymous Anon...
Give detailed information of the antibodies used (the company that produced, the host of the antibody, the dilution of the antibody)

Anonymous Anon...
Give detailed information of the antibodies used (the company that produced, the host of the antibody, the dilution of the antibody)

RESULTS AND DISCUSSION

Extraction

The ethanol extract of *Zingiber cassumunar* rhizome was dark brown with a specific odor, thick consistency, and slightly bitter taste. The extraction process produced 25.55% yield. This result has met the standard of the Indonesian Herbal Pharmacopoeia (Depkes RI, 2008). The

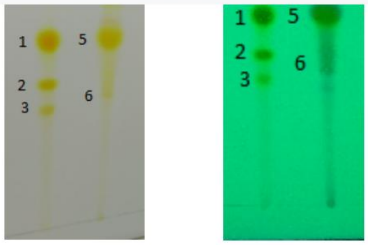

Figure 1. The TLC profile of the ethanol extract of *Zingiber cassumunar* (b) and curcuminoids standard (a), detected in daylight (D) and UV 254 nm (U).

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Please revise the caption.
Describe briefly the TLC system.
Mention sequentialy from a to b.
Arrange the two figures at the same height and same font size
Indicate with A and B instead of D and U and describe the meaning in the figure caption

Expression of IL-10

Indirect immunocytochemistry was employed to detect the expression of interleukin in the macrophage. The specific antibody of IL-10 interacted with interleukin-10 in the cells and attached to the secondary antibody. During the detection process, the secondary antibody attached to DAB as the chromogen, and the expression appeared as brown stains on the cytoplasm area. Meanwhile, the

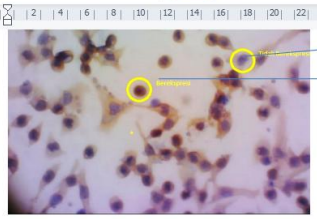

Figure 2 The immunocytochemistry of IL-10 expression in macrophage cells after treatment with ethanol extract of *Zingiber cassumunar*: (A) macrophage with no expression of interleukin (B), macrophage with positive expression of interleukin

Table 1 The percentage of IL-10 expression on the macrophage cells of BALB/c mice treated with ethanol extract of *Zingiber cassumunar*.

Groups	Mean ± SD
Normal	45.650% ± 1.918*
Negative control	51.865% ± 1.422
Treatment Dose of 1.25mg/20gBW	55.908% ± 3.067
Treatment Dose of 2.5mg/20gBW	63.680% ± 2.932*
Treatment Dose of 5mg/20gBW	68.653% ± 4.421*

*significant difference with negative control (p<0.05)

Anonymous Anon...
There is an information in the figure that is written in bahasa Indonesia
the indication of a nd b should be consistent between the figure and the caprion (in capital or not capital letter).
Is there larger picture
Give information of the magnification (200x)?

Anonymous Anon...
The number should be two decimal number only

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the expression of IL-12 after the administration of EEZC is shown in Table 2.

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

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*

*) significant difference with negative control (p<0.05)

The study found that the IL-12 expression in the negative control group was not significantly different from the normal group, indicating that the solvent (tween 80) does not affect the immune response. The treatment of EEZC at a dose of 1.25mg/20g BW induced lower IL-12 expression than the negative control. In other words, the lower the dose, the less effective the active compound of EEZC in increasing the IL-12 expression. When the dose increased, the IL-12 expression was also found to be elevated.

Anonymous Anon...
Please discuss your finding with this Unfortunately, the use of polysorbate 80 in formulation can lead to formation of micelles that may cause aggregation and enhance immunogenicity (Villalobos et al., 2005)
Maybe thre is a differnce in the concentration.

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Expression of Interleukin-14

IL-14 was first known as a high-molecular-weight B-cell growth factor and originally identified as a B cell growth factor (Shen et al., 2006). It is produced by T cells and B-cells. IL-14 binds and signals through a 90-kDa receptor expressed on activated B cells to promote B-cell proliferation (Akdis et al., 2016). High level of IL-14 can enhance B-cell proliferation and expand a subpopulation of memory B cells (Leca et al., 2008), and if followed by the secretion of antibody, it can eliminate the invader. The expression of IL-14 in EEZC-treated mice is shown in Table 3.

Table 3 The expressions of Interleukin-14 in mice treated with ethanol extract of *Zingiber cassumunar*

Groups	Expressions (X ± SD)
Normal	59.193 ± 3.073
Negative control	61.243 ± 1.513
Treatment Dose of 1.25 mg/20g BW	57.025 ± 1.940*
Treatment Dose of 2.5 mg/20g BW	67.410 ± 6.598
Treatment Dose of 5 mg/20g BW	71.068 ± 1.360*

(*) showed significant difference with negative control ($p<0.05$)

Anonymous Anon...
The number should be two decimal number only

curcumin and essential oil that contains phenyl butanoic compound. Furthermore, a toxicity study states that *Zingiber cassumunar* extract has no observable adverse effect and it is well-tolerated for both acute and chronic studies (Koontongkaew et al., 2014).

Anonymous Anon...
Please give further discussion related the structure and the activities. What does this sentence mean?

CONCLUSION

The ethanol extract of *Zingiber cassumunar* has immunomodulatory activity through increasing level of IL-10, IL-12 and IL-14 cytokines and suggested the potency of extract to induce both innate and adaptive immunity.

ACKNOWLEDGMENT

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REFERENCES

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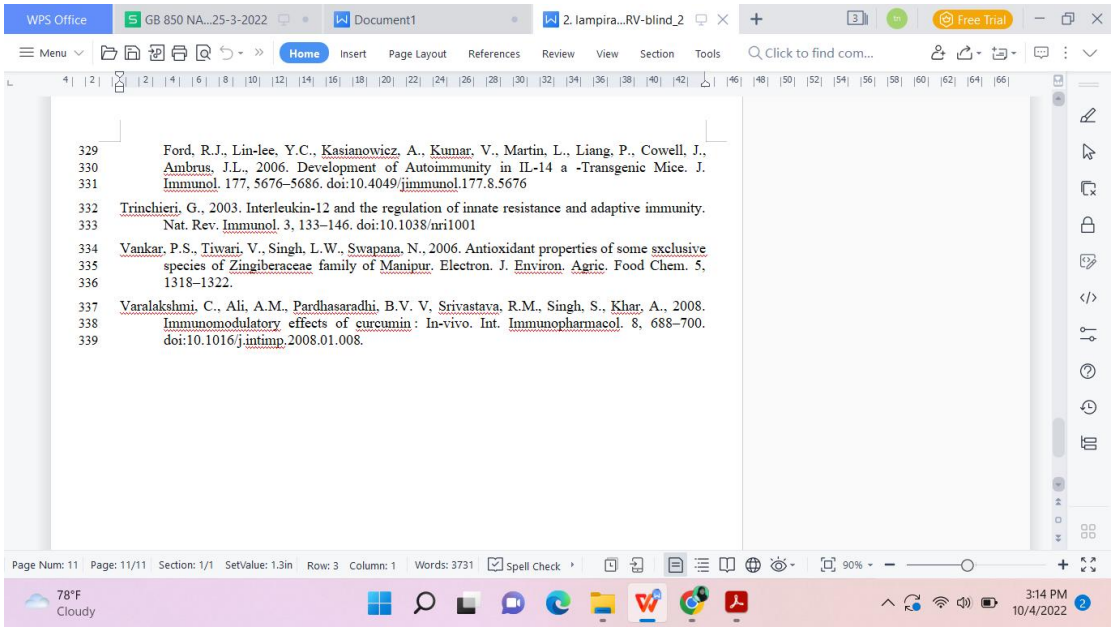
Akdis, M., Altunbulakli, C., Azkur, K., Costa, R.A., Crameri, R., Duan, S., Eiwegger, T.,



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Review Form 1

SEAMEO BIOTROP		Nomor Dokumen	BIO / F / KMD 07		
		Mulai Berlaku	1 Agustus 2008		
Southeast Asian Regional Centre for Tropical Biology		Revisi	01/00		
REVIEW FORM		Tanggal Revisi	13 Juli 2018		
		Halaman	2	dari	3

TITLE OF ARTICLE _____

GENERAL COMMENTS
(statements of your general impression of the article, summary of results of the study, major shortcomings of the article).

THE INCREASING LEVEL OF INTERLEUKIN IN THE *Zingiber cassumunar*-TREATED MICE

SPECIFIC COMMENTS ON

1. **Significance of study**
The study provides continued information related to previous study done by Nurkhasanah. The study gives information about the effect of Z. *cassumunar* to increase immune response (innate and adaptive)

2. **Validity of methodology/experimental design OK**

3. **Soundness of interpretation/conclusion. The interpretation sounds scientifically related**
4. **Relevance of discussion. Some detailed or further explanation is needed**
5. **Adequacy of title and abstract OK**
6. **Appropriateness of figures and tables I have put some suggestions for the figures and tables**
7. **Other specific comments. I have put it in the file (as comments)**
8. **Please provide necessary corrections on the manuscript. I have put it in the file (as comments)**
9.

RECOMMENDATION
(Please check one)

☐ Accept ☐ Reject V Modify

Review Form 2

SEAMEO BIOTROP		Nomor Dokumen	BIO / F / KMD 07		
		Mulai Berlaku	1 Agustus 2008		
Southeast Asian Regional Centre for Tropical Biology		Revisi	01/00		
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TITLE OF ARTICLE

THE INCREASING LEVEL OF INTERLEUKIN IN THE *Zingiber cassumunar*-TREATED MICE

GENERAL COMMENTS

Thank you for inviting me to review this research article, which addressing an interesting topic.

The manuscript describes the effect of ethanol extract of Z. cassumunar (EEZC) on the cytokine expression level, particularly the expressions of IL-10, IL-12, and IL-14 in lipopolysaccharide (LPS)-treated Balb/c mice.

The introduction is relevant and sufficient information about the previous reports is mentioned for readers to follow the present study rationale and procedures. However, some of the procedures are not well described. For example, the sex and age of the mice used are not stated. The route of administration of EEZC for the treatment also is not mentioned.

The main findings, based on the data obtained, are that EEZC (at doses of 1.25 mg, 2.5mg, and 5mg/20g BW) was able to increase the expression level of IL-10, IL-12, and IL-14 in LPS-treated Balb/c mice in a dose dependent manner.

The analysis is mostly convincing, though there are several technical issues that need to be addressed. In particular, the statistical analysis done to the data need to be clarified. In addition, the method / procedure used to count and to calculate the percentage of stained macrophages should be explained.

Under the topic Results and Discussion, I suggest splitting the two components for better and clearer understanding. The labels on the figures should be made clear and related to their legend. In addition, results from the TLC analysis didn't mention about the Rf values of both EEZC and the cucurminoids standards. TLC profiles of EEZC should be properly presented as the evidence that curcumin is the major compound in EEZC, and the findings should be elaborated in such a way that could convince the reader that curcumin may contribute to the immunomodulatory effect of EEZC.

I recommend this manuscript for publication after the questions are addressed and some adjustments have been taken into account, because new data on the effect of ethanol extract from Z. cassumunar are presented.

SPECIFIC COMMENTS ON

1. **Significance of study**
   The significance of the study was not clearly mentioned anywhere in the manuscript. _____
2. **Validity of methodology/experimental design** _____
   The methods are generally appropriate and adequately described. However, the methods section does not specify a few details, in particular, the type of statistical analysis done on the data obtained. The rationale for the use of immunohistochemistry assay in measuring the expression level of IL-10, IL-12 and IL-14 should also be provided.

The experimental design is adequate to answer this type of research question.

3. **Soundness of interpretation/conclusion**
The conclusion in the abstract (on page 1) should be consistent with the conclusion on page 9, line 258-260.

4. **Relevance of discussion**
The discussion is well based on results obtained from the study. However, a more extensive discussion on the possible underlying mechanism of actions of EEZC in increasing the expression level of IL-10, IL-12 and IL-14 should be provided.

5. **Adequacy of title and abstract**
The title and abstract are relatively adequate

6. **Appropriateness of figures and tables**
All tables are clear but the labels and the legend in Figure 1 should be clarified/modified for better understanding.

7. **Other specific comments**
1) Under 'Animal Treatment', the age and sex of the animals used should be stated. The route of administration of EEZC for the treatment also should be mentioned.
2) Please clarify why only interleukin-10, -12 and -14 were chosen for the study?
3) How do you decide the doses of EEZC used in the *in vivo* study?

8. **Please provide necessary corrections on the manuscript**
Specific corrections on the manuscript are as follows:

1. Page 1, line 16-17: Consider rephrasing the phrase "against" infection to "against LPS-treated inflammation"

2. Page 2, line 55: A typo error: "nitrit" oxide. Did you mean "nitric oxide"?

3. Page 2, line 56: consider putting a hyphen, - in between "berghei" and "infected"

4. Page 2, line 60: Consider rephrasing the sentence because when mentioned the word "higher" effectiveness, it indicates comparing the effectiveness of EEZC with something.

5. Page 2, line 73: consider changing "the results" to "the yield"

6. Page 2, line 74: consider changing "marked" to "designated"

7. Page 3, under "Animal Treatment", the sex, age and the route of administration of EEZC were not mentioned. What is the method use to sacrifice the animals? Please provide the necessary.

Page, line 89-92, the author(s) mentioned that "The administration of EEZC was carried out for 21 days (3 weeks), once a day. On the 22nd day, the mice were sacrificed. Lipopolysachcharide (LPS) was injected into the peritoneal cavity area, and after 1 hour, the macrophage was isolated.."
The written procedure is somewhat confusing. To my understanding, on day 22, LPS was injected to the intraperitoneal cavity after the mice were sacrificed. Please clarified.

Page 3, line 107: the full name for DAB should be mentioned first before using the abbreviation.

Page 4, line 111: the full name for PBS should be mentioned first before using the abbreviation.

Page 4, line 128-131: The author(s) stated that "The curcumin content was detected in the extract, as shown in Figure 1. While curcumin was found to be a major content in the extract, the other curcumin derivates (i.e., demethoxycurcumin and bisdemethoxycurcumin) were not detected."

On what basis do the author(s) come to an agreement that the curcumin was detected in the extract and found to be a major content in the extract? As far I understand, the standard used for the TLC analysis is only curcuminoids. Figure 1 also was poorly labelled and thus, didn't help in indicating that curcumin is present in the extract. What are the Rf values for the spots/bands produced from the TLC?

Page 5, Figure 1: The picture in Figure 1 should be labelled clearly especially the numbering at the TLC spots/bands.

Page 5, Figure 2: the use of lowercase/uppercase of "a" and "b" for the picture and the legend should be consistent.

Page 6, Table 1: In Table 1, the % of expression level of IL-10 was significantly higher in the negative control group compared to the normal group. Please explain why.

Page 8, line 244: Please italicize the word "in vitro"

Page 8, line 246-247: Please remove/delete the unnecessary symbols, °

Page 9, line 258-260: The conclusion should be consistent with the conclusion in the abstract on page 1.

RECOMMENDATION

☐ Accept ☐ Reject ☑ Modify

Lampiran 3. Penyampaian artikel yang sudah diperbaiki author, disertai lampiran naskah dan respon sheet

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To: biotropia@biotrop.org

Dear Editor

Please find in the attachment, the revised version of the manuscript and table of respond to the reviewer suggestion.

Regards
[Quoted text hidden]
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2 attachments

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Artikel :

THE INCREASING LEVEL OF INTERLEUKIN IN THE *Zingiber cassumunar*-TREATED MICE

ABSTRACT

The cytokine is one of the proteins responsible for the immune system. Several types of 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 ethanol extract of *Zingiber cassumunar* (EEZC) on the expressions of IL-10, IL-12, and IL-14. The test animals were BALB/c mice, which 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. The treatment was carried out for 21 days. On the 22nd day, the mice were induced with 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 administration of EEZC at the doses of 1.25 mg, 2.5mg, and 5mg/20g BW for 21 days increased the expression of IL-10, IL-12, and IL-14 significantly and proportionally to the dose. and suggested the potency of extract to induce both innate and adaptive immunity. This activity may be attributable to curcumin as an active compound in this extract.

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

INTRODUCTION

The immune system is responsible for protecting the host from various pathogenic microorganisms. At the same time, it functions as a control of immune responses and prevents over-reaction to the body's own cells (Saraiva and 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 disease or infection is necessary.

Immunomodulator could be defined as a substance, which can stimulate, suppress or modulate any of the components of the immuno system including both specific and nonspecific immune system (Das et al., 2014). Modulation of the immune system was remarked by induction, expression, amplification or inhibition of certain part in the immune signaling and response. Thus, immunomodulator is a substance used for its effect on the immune system.

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

Interleukin-12 is an important cytokine in immunoregulation produced mainly by antigen-presenting cells (APC), including macrophages and dendritic cells that respond to microbes (Abbas et al., 2017). During the immune response, IL-12 is produced as a reaction to stimuli of various compounds (including lipopolysaccharide/LPS).

Interleukin-14 (IL-14) is one of the cytokines produced by the immune system. It is produced by activated B cells and T cells (Leca et al., 2008). The role of IL-14 is to regulate B-cell proliferation. IL-14 can increase antibody responses to vaccinations causing autoimmunity and contribute to B-cell lymphoma formation (Shen et al., 2006).

Zingiber cassumunar, known locally as *bengle* (Javanese, Indonesia), has been used for treating various diseases traditionally. *Z. cassumunar* belongs to Zingiberaceae family and contains terpenoids, essential oil, and curcuminoids. Several studies on *Z.cassumunar* reported some activities including anticancer (Varalakshmi et al., 2008), antioxidant (Vankar et al., 2006) (Bua-in and Paisooksantivatana, 2009) and immunomodulator (Nurkhasanah et al., 2017; Rahmawati, 2013). This plant has been reported to exhibit an immunomodulatory activity by increasing phagocytic activity *in vitro* (Chairul et al., 2009), increasing of nitric oxide and reactive oxygen species (Nurkhasanah et al., 2017) and decreasing of malondialdehyde product in *Plasmodium berghei*-infected mice (Nurmasari et al., 2014).

This study presents the activity of ethanol extract of *Zingiber cassumunar* (EEZC) in stimulating the immune response *in vivo* as observed from its effect on interleukin-10, -12 and -14. The present study will focus on these cytokines due to the important function of these cytokine in the immune respons, either innate and adaptive immunity. The expression IL-10 and IL-14 are close related to activation of adaptive immunity while the IL-12 is important in cell communication between the macrophage and T cells. The study was carried on Balb/c male mice for 28 days treatment and through oral administration. This *in vivo* study provides evidence of higher effectiveness of EEZC in increasing immune response. This evidence was

important information for development of *Z. cassumunar* to be an immunomodulatory product.

MATERIALS AND METHODS

Materials

The *Zingiber cassumunar* rhizome was collected from a local market in Yogyakarta and identified in 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, and then sliced. The sliced rhizome was dried in an oven at a temperature of 50^{o}C. Afterward, the dried rhizome was 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 a concentrated extract. The concentrated extract was used for treatment and designated as EEZC.

Thin-layer Chromatography (TLC) analysis of the extract

The TLC analysis was carried out to identify the active compound of EEZC. 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 active compound 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, i.e., BALB/c mice (8 weeks), were acclimatized for a week before the treatment. The mice were divided into five (5) groups, namely normal group, negative control group which treated with the solven (solution of tween 80

10%), and 3 treatment groups (1.25 mg/20gBW; 2.5 mg/20gBW; 5 mg/20gBW). The administration of EEZC was carried out orally for 21 days (3 weeks), once a day. On the 22nd day, the mice were sacrificed using CO_2 gas. Following sacrificing, lipopolysachcharide (LPS) (Sigma) with dose 0.01 mg/20 g BW was injected into the peritoneal cavity area, and after 1 hour, the macrophage was isolated and the expression of interleukin-10, -12 and -14 was observed with immunohistochemistry methode using specific antibody 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. Then, the mice were 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 a previous study (Nurkhasanah, 2015). The method was based on indirect method using specific primary antibody and was conjugated with secondary antibody and chromogen. The interleukin expressed was observed as brown colour as product of DAB (dimethyl amino benzidine) chromogen and detected under light microscope.

The cultured macrophage was fixed with 1 ml of methanol. The methanol was removed, and the macrophage was washed in PBS (phosphate buffer saline) for 5 minutes. The preparation was then immersed in peroxidase blocking solution for 10 minutes at room temperature. Afterward, it was washed under running water and then with PBS. A total of 50µl of blocking serum was added to the preparation and then incubated in a humid temperature for 10-15 minutes. Then, 100µl (with dilution 1:100) of specific antibody (anti-IL-10, anti-IL-12, and anti-IL-14, murine recombinant, Biovision) was 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 antimouse biotin secondary antibody

(Biovision) was added to each preparation, which was incubated at a humid temperature for 20 minutes and then washed with PBS.

The preparations were incubated with 50 µl of the streptavidin-peroxidase enzyme for 10 minutes, washed with PBS, and incubated with 50 µl of DAB chromogen (peroxidase substrate solution). Afterward, the preparations were washed with PBS and incubated with Mayer's hematoxylin as the counterstain. The process was followed by washing with PBS. The macrophage was ready for microscopic observation at 400x magnification. Macrophages that expressed interleukin would show as brown stains.

The observation was carried out in some of field of view. The number of positive cell expression was compared with the whole cell observed and presented as percentage value. The result of treated group was compared with control group with statistical analysis to analyze the effect of treatment.

Statistical analysis

The quantitative data of percentage expression of IL-10, IL-12 and IL-14 was analyze statistically for normality and homogeneity. The analysis was followed with variance analysis using ANOVA and followed by LSD for analysis between group of treated.

RESULTS AND DISCUSSION

Extraction

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

Curcumin is one of major chemical content in EEZC. Curcumin reported to have immunomodulatory activity (Varalakshmi et al., 2008). Previous study reported the significantly increase of IL-12 levels in curcumin-treated animals on day 10 and 20, after treatment. It is also found that curcumin induce generation of ROS which important in the immune respon (Varalakshmi et al., 2008). Curcuminoid

(cassumunin A and cassumunin B) isolated from *Z. cassumunar* was found to have a protective effect on living cells suffering from oxidative stress (Nagano et al., 1997).

Beside curcumin, essential oil is also reported is one of main compound in *Zingiber cassumunar* rhizome. The highly essential oil content give specific odor of *Z. cassumunar* rhizome and extract. Several studies of phytochemical compounds and biological activity of *Z. cassumunar* Roxb had been reported the main component of *Z. cassumunar* rhizome essential oil were triquinacene 1,4-bis (methoxy), (Z)-ocimene and terpinen-4-ol (Bua-in and Paisooksantivatana, 2009). The previous studies on its rhizome also found several phenylbutenoid compounds, curcuminoid, and sesquiterpene (zerumbon) (Nakamura et al., 2009).

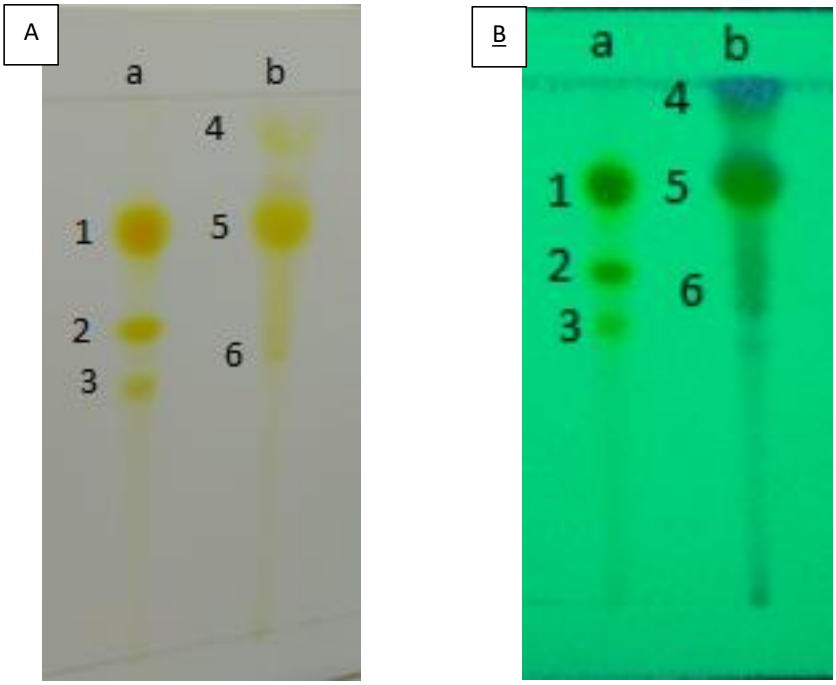

Figure 1. The TLC profile of curcuminoids standard (a) and *Zingiber cassumunar* ethanolic extract (b), detected in daylight (A) and UV 254 nm (B).

Expression of IL-10

Indirect immunocytochemistry was employed to detect the expression of interleukin in the macrophage. The specific antibody of IL-10 interacted with interleukin-10 in the cells and attached to the secondary antibody. During the detection process, the secondary antibody attached to DAB as the chromogen, and the expression appeared as brown stains on the cytoplasm area. Meanwhile, the cells with negative expression appeared in blue as the result of counterstaining. The

immunocytochemistry of the macrophage is shown in Figure 2. The percentage of the IL-10 expression is shown in Table 1.

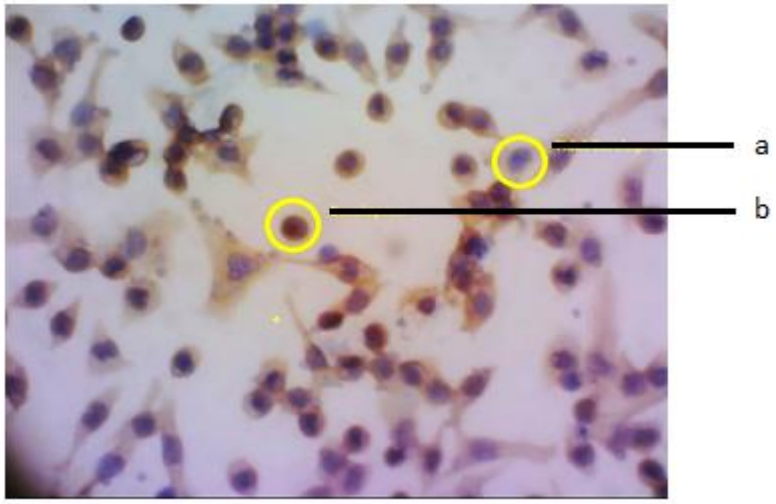

Figure 2 The immunocytochemistry of IL-10 expression in macrophage cells after treatment with ethanol extract of *Zingiber cassumunar* which observed with 400x magnification (a) macrophage with no expression of interleukin (b), macrophage with positive expression of interleukin

Table 1 The 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%*

*significant difference with negative control ($p<0.05$)

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

The regulation of IL-10 expression involves the enhancement or silencing of IL10 transcription and is regulated by certain transcription factors activated by discrete signal-transduction pathways. Following transcription, the post-transcriptional mechanisms exist and involeves many of the molecular events leading to IL-10 expression (Saraiva and O'Garra, 2010). Some molecule of *Zingiber cassumunar* could be involves and affect the transcriptional process of IL-10 and leading on increasing level of IL-10.

Expression of Interleukin-12

Interleukin-12 (IL-12) is a pro-inflammatory cytokine that induces the production of interferon-γ (IFN-γ), and leading to the differentiation of T helper 1 (TH1) cells and connected the link of innate immunity 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. The LPS was used in this study to activate the macrophage to produce the IL-12. The quantitative analysis of the expression of IL-12 after the administration of EEZC is shown in Table 2.

Table 2 The expression of IL-12 in macrophage cells of BALB/c mice treated with

ethanol extract of *Zingiber cassumunar*

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*

*) significant difference with negative control ($p<0.05$)

The study found that the IL-12 expression in the negative control group was not significantly different from the normal group, indicating that the solvent (tween 80) does not affect the immune response. Tween 80 could stimulate the immunogenicity (Maggio, 2012) as shown by the increasing of IL-10 in the present study. But the dose used in this study is not enough to increase the IL-12 expression. The treatment of EEZC at a dose of 1.25mg/20g BW induced lower IL-12 expression than the negative control. In other words, the lower the dose, the less effective the active compound of EEZC in increasing the IL-12 expression. When the dose increased, the IL-12 expression was also found to be elevated.

The immunomodulatory effects of EEZC might be caused by the presence of curcumin as an active compound. Curcumin has been reported to increase the immune response of the cells (Nagano et al., 1997; Nurkhasanah et al., 2017). The previous study also found that curcumin treatment elevated the IL-12 level in mice (Varalakshmi et al., 2008). The increasing level of IL-12 in the treatment could be caused by the capacity of curcumin in increasing of ROS and NO (Nurmasari et al., 2014; Rahmawati, 2013). ROS is known to regulate the IL-12 generation. Another studies have revealed that curcumin stimulates T cell, B cell, neutrophil, NK cell, and dendritic cell (Nurmasari et al., 2014).

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

The treatment of EEZC increased IL-12 expression, activating T cells and stimulating the production of IFN-γ, which lead to macrophage activation and the secretion of reactive oxygen species (ROS) that eliminate infections (Abbas et al.,

2017). Furthermore, this treatment can intensify the phagocytic activity of macrophage (Nurkhasanah et al., 2017).

Expression of Interleukin-14

IL-14 was first known as a high-molecular-weight B-cell growth factor and originally identified as a B cell growth factor (Shen et al., 2006). It is produced by T cells and B-cells. IL-14 binds and signals through a 90-kDa receptor expressed on activated B cells to promote B-cell proliferation (Akdis et al., 2016). High level of IL-14 can enhance B-cell proliferation and expand a subpopulation of memory B cells (Leca et al., 2008), and if followed by the secretion of antibody, it can eliminate the invader. The expression of IL-14 in EEZC-treated mice is shown in Table 3.

Table 3 The expressions of Interleukin-14 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%*

(*) showed significant difference with negative control ($p<0.05$)

The previous research proposes increasing the expression of IL-14 by the treatment of some herbal medicine extract (Nurkhasanah, 2015). The treatment of anthocyanin-rich rosella extract increases 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 because LPS was recognized as endotoxin consisting of a lipid and a polysaccaride found on the outer membrane of gram-negative bacteria. The activity of EEZC in increasing of IL-10, IL-12 and IL-14 suggested the potency of this extract in inducing the immune system in both innate and adaptive immunity.

A previous study on Zingiberaceae family, including *Curcuma mangga*, *Kaempferia angustifolia,* and *Zingiber cassumunar,* highlights that *Zingiber cassumunar* has the highest immunomodulatory activity (Chairul et al., 2009), which may be caused by the active compound of curcumin and essential oil. The major compound found in *Z. cassumunar* essential oil was phenyl butanoic compound.

Furthermore, a toxicity study states that *Zingiber cassumunar* extract has no observable adverse effect and it is well-tolerated for both acute and chronic studies (Koontongkaew et al., 2014).

CONCLUSION

The ethanol extract of *Zingiber cassumunar* has immunomodulatory activity through increasing level of IL-10, IL-12 and IL-14 cytokines and suggested the potency of extract to induce both innate and adaptive immunity.

ACKNOWLEDGMENT

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Title of manuscript : **THE INCREASING LEVEL OF INTERLEUKIN IN THE *Zingiber cassumunar*-TREATED MICE**

Authors :

A TABLE OF RESPONSES TO THE REVIEWERS' COMMENTS

Please provide responses to each reviewer's comments:

Reviewer 1:

No	Reviewer's Comments	Responses to Comments
1	The introduction is relevant and sufficient information about the previous reports is mentioned for readers to follow the present study rationale and procedures. However, some of the procedures are not well described. For example, the sex and age of the mice used are not stated. The route of administration of EEZC for the treatment also is not mentioned	We added at the end of introduction (p2, l 62) The study was carried on Balb/c male mice for 28 days orally
2	The analysis is mostly convincing, though there are several technical issues that need to be addressed. In particular, the statistical analysis done to the data need to be clarified. In addition, the method / procedure used to count and to calculate the percentage of stained macrophages should be explained.	We have added at the end of the method (l 138-141)
3	Under the topic Results and Discussion, I suggest splitting the two components for better and clearer understanding. The labels on the figures should be made clear and related to their legend. In addition, results from the TLC analysis didn't mention about the Rf values of both EEZC and the cucurminoids standards. TLC profiles of EEZC should be properly presented as the evidence that curcumin is the major compound in EEZC, and the findings should be elaborated in such a way that could convince the reader that curcumin may contribute to the immunomodulatory effect of EEZC	We do not split the Result and discussion in two part, as the style of this journal, this two part was not separated. We have discussed about curcumin in 3 paragraphs of early step of discussion. Hopefully the presentation about curcumin could invite the reader to understand about the importance of curcumin in this study. The TLC profile of EEZC has been revised (l 164)
4	The significance of the study was not clearly mentioned anywhere in the manuscript	We have stated at the end of introduction "This *in vivo* study provides evidence of higher effectiveness of EEZC in increasing immune response. This evidence was important information for development of *Z. cassumunar* to be an immunomodulatory product". (l 64)
5	The methods are generally appropriate and adequately described. However, the methods section does not specify a few	The detail information about immunohistochemistry has been added in the method (L 117-136).

	details, in particular, the type of statistical analysis done on the data obtained. The rationale for the use of immunohistochemistry assay in measuring the expression level of IL-10, IL-12 and IL-14 should also be provided	
6	The conclusion in the abstract (on page 1) should be consistent with the conclusion on page 9, line 258-260.	The sentence has been reformated
7	The discussion is well based on results obtained from the study. However, a more extensive discussion on the possible underlying mechanism of actions of EEZC in increasing the expression level of IL-10, IL-12 and IL-14 should be provided.	The suggestion has been done
8	All tables are clear but the labels and the legend in Figure 1 should be clarified/modified for better understanding	The suggestion has been done
9	1) Under 'Animal Treatment', the age and sex of the animals used should be stated. The route of administration of EEZC for the treatment also should be mentioned. 2) Please clarify why only interleukin-10, -12 and -14 were chosen for the study? 3) How do you decide the doses of EEZC used in the *in vivo* study	1. The suggestion has been done 2. The present study will focus on these cytokines due to the important function of these cytokine in the immune respons, either innate and adaptive immunity. The expression IL-10 and IL-14 are close related to activation of adaptive immunity while the IL-12 is important in cell communication between the macrophage and T cells (l 60-63)
10	1. Page 1, line 16-17: Consider rephrasing the phrase "against" infection to "against LPS-treated inflammation" 2. Page 2, line 55: A typo error: "nitrit" oxide. Did you mean "nitric oxide"? 3. Page 2, line 56: consider putting a hyphen, - in between "berghei" and "infected" 4. Page 2, line 60: Consider rephrasing the sentence because when mentioned the word "higher" effectiveness, it indicates comparing the effectiveness of EEZC with something. 5. Page 2, line 73: consider changing "the results" to "the yield" 6. Page 2, line 74: consider changing "marked" to "designated" 7. Page 3, under "Animal Treatment", the sex, age and the route of administration of EEZC were not mentioned. What is the method use to sacrifice the animals? Please provide the necessary.	1. It has been changed 2. It has been changed 3. It has been changed 4. It has been changed 5. It has been changed 6. It has been changed 7. It has been changed
11	Page, line 89-92, the author(s) mentioned that "The administration of EEZC was carried out for 21 days (3 weeks), once a day. On the	It has been changed

	22nd day, the mice were sacrificed. Lipopolysachcharide (LPS) was injected into the peritoneal cavity area, and after 1 hour, the macrophage was isolated..” The written procedure is somewhat confusing. To my understanding, on day 22, LPS was injected to the intraperitoneal cavity after the mice were sacrificed. Please clarified. Page 3, line 107: the full name for DAB should be mentioned first before using the abbreviation. Page 4, line 111: the full name for PBS should be mentioned first before using the abbreviation. Page 4, line 128-131: The author(s) stated that “The curcumin content was detected in the extract, as shown in Figure 1. While curcumin was found to be a major content in the extract, the other curcumin derivates (i.e., demethoxycurcumin and bisdemethoxycurcumin) were not detected.” On what basis do the author(s) come to an agreement that the curcumin was detected in the extract and found to be a major content in the extract? As far I understand, the standard used for the TLC analysis is only curcuminoids. Figure 1 also was poorly labelled and thus, didn’t help in indicating that curcumin is present in the extract. What are the Rf values for the spots/bands produced from the TLC? Page 5, Figure 1: The picture in Figure 1 should be labelled clearly especially the numbering at the TLC spots/bands. Page 5, Figure 2: the use of lowercase/uppercase of “a” and “b” for the picture and the legend should be consistent. Page 6, Table 1: In Table 1, the % of expression level of IL-10 was significantly higher in the negative control group compared to the normal group. Please explain why. Page 8, line 244: Please italicize the word “in vitro” Page 8, line 246-247: Please remove/delete the unnecessary symbols, ° Page 9, line 258-260: The conclusion should be consistent with the conclusion in the abstract on page 1	It has been changed It has been changed It has been changed The standard used was curcuminoids which contain its 3 derivates, from the structure we can predict the Rf value of curcumin was higher than other derivates, so authors can conclude that spot which is found in the sample is curcumin. It has been changed It has been changed It has been changed The present study also found that the expression of IL-10 in negative control group increased significantly compared to normal, which could be caused by the tween 80 effect. The previous study also reported that polysorbate (tween) 80 could increase the immune response (Maggio, 2012). It has been changed

Reviewer 2:

No	Reviewer's Comments	Responses to Comments
1	**Relevance of discussion. Some detailed or further explanation is needed**	It has been followed
2	**Appropriateness of figures and tables I have put some suggestions for the figures and tables**	It has been followed
3	**Other specific comments. I have put it in the file**	It has been followed
4	**Please provide necessary corrections on the manuscript. I have put it in the file (as comments**	It has been followed

Lampiran 4. Pemberitahuan dari editor tentang in press version

Gmail Nurkhasanah Mahfudh <nurkhas@gmail.com>

[BIOTROPIA] #1162 in-press version

biotropia@biotrop.org <biotropia@biotrop.org> 6 May 2020 at 15:33
To: Nurkhasanah Mahfudh <nurkhas@gmail.com>

Dear Authors,

Greetings from BIOTROPIA!

Hereby we inform you that your manuscript is under queue to be final edited by our editor. In order to speed up the publication process of your manuscript, we have published the in-press version of your manuscript at BIOTROPIA OJS. You can access it at https://journal.biotrop.org/index.php/biotropia/article/view/1162. Please be noted that this in-press version is unedited; thus, it will undergo the final copyediting and proofreading process before being published in its final form. We will let you know about further progress later. Thank you for your cooperation.

Best regards,

Zanne Sandriati Putri
Publication Assistant

Lampiran 5. Permintaan dari editor tentang penyajian foto hasil penelitian di naskah

Gmail Nurkhasanah Mahfudh <nurkhas@gmail.com>

Re: [BIOTROPIA] #1162 Editing results and request for picture/photos for manuscripts published in August 2021

biotropia@biotrop.org <biotropia@biotrop.org> 6 July 2021 at 18:01
To: Nurkhasanah Mahfudh <nurkhas@gmail.com>

Dear Dr. Nurkhasanah,

I am happy to inform you that your manuscript # 1162 titled "THE INCREASING LEVEL OF INTERLEUKIN IN THE Zingiber cassumunar-TREATED MICE" is being prepared for publication in the August 2021 edition of BIOTROPIA Journal.

For publishing purposes, we have edited the language and several aspects of the manuscript.

Therefore, please carefully check this edited manuscript and please let me know if you agree for this edited manuscript to be published in BIOTROPIA.

There are also many things in question, which need to be answered and revised.

If you agree with the suggestions, please revise the manuscript accordingly.

Also, please double-check whether the citation written in the manuscript is already listed in the reference list.

Please email back the revised version of your manuscript no later than 13 July 2021, since we are in the preparation stage of publishing the August 2021 edition of BIOTROPIA Journal.

Also, we would like to request original photos of your research or the commodity/subject of your research/study to be a candidate for the front cover of BIOTROPIA.

Please submit original photos with minimum size of no less than 3 MB in JPEG format, so that the photos will not be blurred when going through printing process.

The Editorial Board will choose the best photo from several photos submitted by the authors.

Thank you.

Sri Ismawati Soerianegara
Internal Editor

On 2020-05-07 08:07, Nurkhasanah Mahfudh wrote:

> Thank you for the information.
> I agreed to be published.
>
> Thank you
>
> On Wed, May 6, 2020 at 3:33 PM <biotropia@biotrop.org> wrote:
>
> > Dear Authors,
> >
> > Greetings from BIOTROPIA!

Gmail Nurkhasanah Mahfudh <nurkhas@gmail.com>

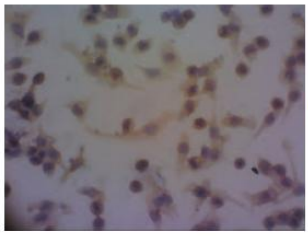
Re: [BIOTROPIA] #1162 Editing results and request for picture/photos for manuscripts published in August 2021

Nurkhasanah Mahfudh <nurkhas@gmail.com> 22 July 2021 at 16:27
To: biotropia@biotrop.org

Here we send the edited manuscript.
We also send some photo of cells

[Quoted text hidden]

7 attachments



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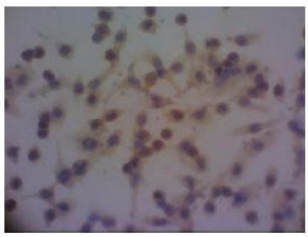

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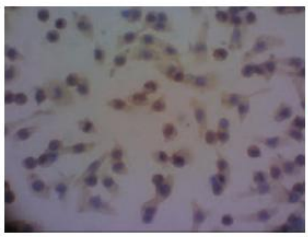

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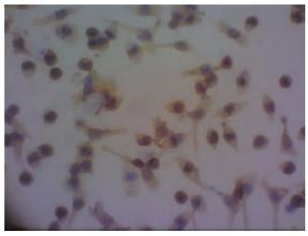

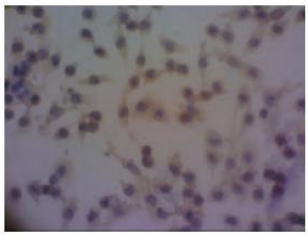

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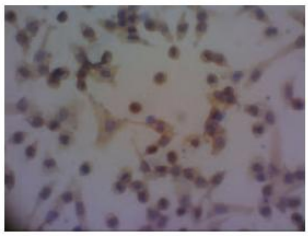

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Lampiran 6 : Galleyproof

Gmail Nurkhasanah Mahfudh <nurkhas@gmail.com>

Re: [BIOTROPIA] #1162 Galley proof of your manuscript to be published in BIOTROPIA August 2021

biotropia@biotrop.org <biotropia@biotrop.org> 27 July 2021 at 16:42
To: Nurkhasanah Mahfudh <nurkhas@gmail.com>

Dear Dr Nurkhasanah,

Thank you for your revisions and images.

Please find attached the final galley proof of your manuscript # 1162 to be published in BIOTROPIA Vol. 28 No. 2 August 2021.

Would you be so kind as to carefully read the galley proof and please make necessary minor corrections, if there are still any mistyped words?

The corrected manuscript should be returned to us not later than 31 July 2021.

If we do not hear any comments after the said date, we will process it for final printing.

Thank you for your attention and cooperation.

Best regards,

Sri Ismawati Soerianegara
Internal Editor

On 2021-07-22 16:27, Nurkhasanah Mahfudh wrote:

> Here we send the edited manuscript.
> We also send some photo of cells
>
> On Tue, 6 Jul 2021 at 18:01, <biotropia@biotrop.org> wrote:
>
> > Dear Dr. Nurkhasanah,
> >
> > I am happy to inform you that your manuscript # 1162 titled "THE
> > INCREASING LEVEL OF INTERLEUKIN IN THE Zingiber cassumunar-TREATED
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> > Therefore, please carefully check this edited manuscript and please
> > let
> > me know if you agree for this edited manuscript to be published in
> > BIOTROPIA.
> >
> > There are also many things in question, which need to be answered
> > and
> > revised.

BIOTROPIA Vol. 28 No. 2, 2021: 1 - 8 DOI: 10.11598/btb.2021.28.2.1162

INTERLEUKIN LEVELS IN THE *Zingiber cassumunar*-TREATED MICE

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Received 6 December 2018/Accepted 12 January 2020

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

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immunoregulation process (Abbas *et al.* 2017). 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,

lipopolysachchariде (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 immunomodulatory 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.

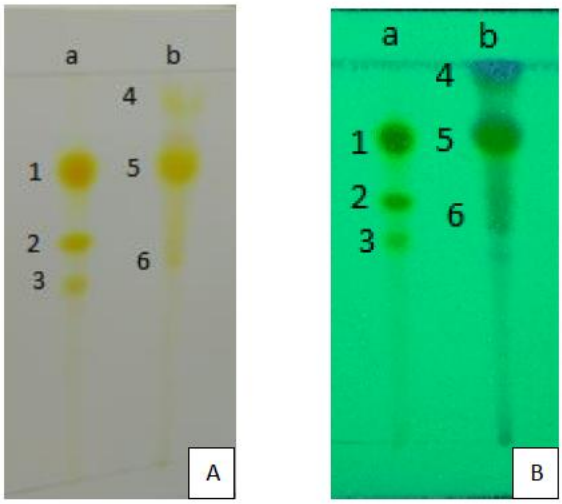

Figure 1 The TLC profile of curcuminoids standard (a) and *Zingiber cassumunar* ethanolic extract (b), detected in daylight (A) and UV 254 nm (B).

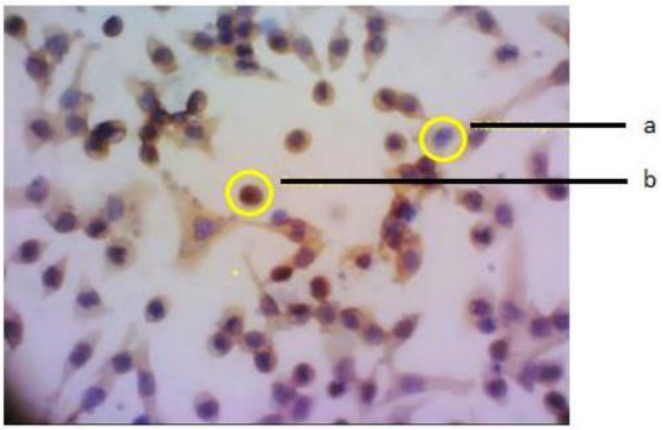

Figure 2 Immunocytochemistry of IL-10 expression in macrophage cells after beign treated with ethanol extract of *Zingiber cassumunar* which was observed with 400x magnification: (a) macrophage with no expression of interleukin; and (b) macrophage with positive expression of interleukin

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.

ACKNOWLEDGMENT

The authors would like to thank the Indonesian Ministry of Research, Technology, and Higher Education for funding this research through the Postgraduate Research Team Scheme with Ref number 109/SP2H/LT/DRPM/2018.

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