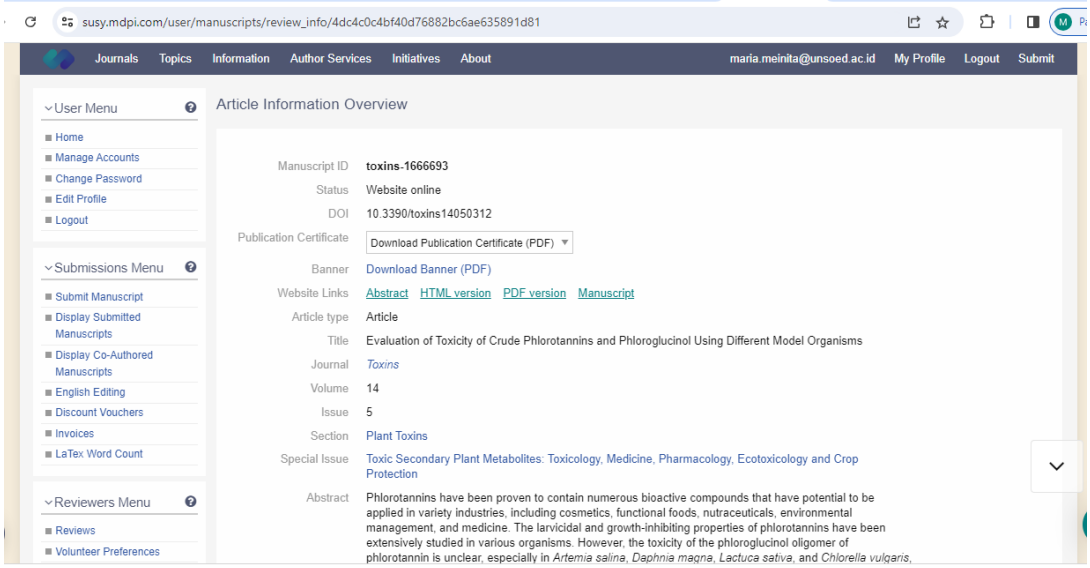

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Dear Dr. Meinita,

Thank you again for your manuscript submission:

Manuscript ID: toxins-1666693
Type of manuscript: Article
Title: Evaluation of toxicity of crude phlorotannins and phloroglucinol using different model organisms
Authors: Dicky Harwanto *, Bertoka Fajar Surya Perwira Negara, Gabriel Tirtawijaya, Maria Dyah Nur Meinita *, Jae-Suk Choi *
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Submitted to section: Plant Toxins, https://www.mdpi.com/journal/toxins/sections/plant_t
Toxic Secondary Plant Metabolites: Toxicology, Medicine, Pharmacology, Ecotoxicology and Crop Protection https://www.mdpi.com/journal/toxins/special_issues/plant_metabolites_toxicology_medicine

Your manuscript has now been reviewed by experts in the field. Please find your manuscript with the referee reports at this link:

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Please revise the manuscript according to the referees' comments and upload the revised file within *7 days*.

Please use the version of your manuscript found at the above link for your revisions.

(I) Any revisions to the manuscript should be marked up using the “Track Changes” function if you are using MS Word/LaTeX, such that any changes can be easily viewed by the editors and reviewers.
(II) Please provide a *cover letter* to explain, *point by point*, the details of the revisions to the manuscript and your responses to the referees’ comments.
(III) If you found it impossible to address certain comments in the review reports, please include an explanation in your rebuttal.
(IV) The revised version will be sent to the editors and reviewers.
(V) *Please add Key Contribution after Keywords.*

If one of the referees has suggested that your manuscript should undergo extensive English revisions, please address this issue during revision. We propose that you use one of the editing services listed at https://www.mdpi.com/authors/english or have your manuscript checked by a native English-speaking colleague.

Do not hesitate to contact us if you have any questions regarding the revision of your manuscript. We look forward to hearing from you soon.

Kind regards,
Ms. Mignon Yu
Assistant Editor
E-Mail: mignon.yu@mdpi.com

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MDPI Tianjin Office 170 North Road, Room 1804, Block A, Lujiazui Financial Plaza, Hongqiao District, China

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http://www.mdpi.com/journal/toxins

Date of submission: 20 March 2022

Prof. Dr. Jay Fox
Editor In Chief
Toxin

Dear Editor:

We would like to re-submit the manuscript titled "**Evaluation of toxicity of crude phlorotannins and phloroglucinol using different model organisms**". The manuscript ID is toxin-1628458.

We thank you and the reviewers for your thoughtful suggestions and insights. The manuscript has benefited from these insightful suggestions. I look forward to working with you and the reviewers to move this manuscript closer to publication in the *Toxin*.

The author's revisions are indicated with highlighted using red color font. The responses to all comments have been prepared and attached herewith/given below. Please check the attachment below.

Thank you for your consideration. Looking forward to hearing from you.

Sincerely,

Maria Dyah Nur Meinita

Faculty of Fisheries and Marine Science, Jenderal Soedirman University, Purwokerto 53123, Indonesia
E-mail: maria.meinita@unsoed.ac.id (M.D.N.M)

A. RESPONSE TO THE COMMENTS OF REVIEWER 1

REVIEWER 1

This study revealed the effects of phlorotannins and phloroglucinol on four representative organisms. The results obtained showed potential used of Ecklonia cava. The MS should be edited and language should be polished for better readability. Also, if bar-chart or column chart could be used, data presented in tables should be omitted.

L6: As mentioned in the abstract, the macro algae, *Ulva lactuca* should be included in the study, instead, Lactuca sativa was used.

Response: We thank the reviewer for correcting the macroalgae species name. It should be *Lactuca sativa*. We have corrected it. Please check line 28 and 32.

L11-13: Too much description of experimental details in Abstracts part

Response: We thank the reviewer for the suggestion. We have reduced experimental details in Abstract part.

L454: Define time (at least seasonal) of sample collection.

Response: We thank the reviewer for the suggestion. We have defined time of sample collection. Please check line 501.

L456: specify room Temp and etc. in other parts of the whole MS

Response: We thank the reviewer for the suggestion. We have specified room temperature and etc thoroughly.

L458: 21,24,26,64 Too many Refs than needed

Response: We thank the reviewer for the suggestion. We have reduced references.

L540: the use of fresh water C. vulgaris and the salinity increase are confusing, since there is marine Chlorella species. Why use fresh water species?

Response: We thank the reviewer for the correction. We have corrected it. Please check line 525-529.

L543:Too many Refs than needed

Response: We thank the reviewer for the suggestion. We have reduced references.

L545: by ‘changed’, does this mean diluted, or fed-batch cultivation? As ‘changed’ means collected the cells and re-add new medium.

Response: We thank the reviewer for the correction. We have corrected it. Please check line 533-534.

L548: light/dark cycle

Response: We thank the reviewer for the correction. We have corrected it. Please check line 536.

L467: and Chlorella

Response: We thank the reviewer for the correction. We have added it. Please check line 551.

All materials used, especially the four organisms, should be included in one separated part in section ‘materials and methods’

Response: We thank the reviewer for the suggestion. We have included all materials used, including the four organisms we used in one separated part in section ‘materials and methods’. Please check line 499-536.

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B. RESPONSE TO THE COMMENTS OF REVIEWER 2

REVIEWER 2

Major revisions:

Ad Introduction:

The introduction is quite poor. It is rather a list of work which had been done and work which had not been done yet. The aim of the present study is not defined. For instance, the authors write (L 49 – 52): *Knowledge of the toxic effects of phlorotannin extracted from E. cava and phloroglucinol on D. magna, A. salina, L. sativa, and C. vulgaris is important. The purpose of this study was to investigate the toxic effects of various concentrations of phlorotannins and phloroglucinol on invertebrate larvae, plants, and microalgae.* But the reason why the knowledge of the toxic effects of phlorotannin extracted from *E. cava* is important is not described.

Response: We thank the reviewer for the insightful suggestion. We have revised the introduction part based on your suggestion. Please check line L80-82

Results are too detailed. Some of the details should be omitted, such as
L 203-204: „*Freshwater C. vulgaris in the control group and groups treated with various concentrations of phloroglucinol displayed a decrease in CD within 24 h (Figure 4a).*“

Response: We thank the reviewer for the suggestion. We have improved the result part.

For algal tests, there are too many details included and the results seem confusing to the reader. I suppose, that reduction of the graphs and/or tables would be helpful. Growth curves (such as Figure 3a) are not necessary, when inhibition curves (such as Figure 3b) are presented etc. That is only a mathematical operation which brings no further information.
Moreover, the same could be said about CD and DCW values. Both of them are only calculated based on the same original measured data (optical density), using a formula. Thus, the second endpoint brings no further information.
Using so many graphs and charts with data derived from only one measurement only artificially enlarges the article volume and makes it less intelligible.

Response: We thank the reviewer for the suggestion. To avoid duplication, we decided to replace the Cell Density (CD) graph with a table and delete Dry Cell Weight (DCW) graph.

Ad Discussion:
General texts on methods such as L 273-283, L 313-323 etc. should be omitted in the discussion.
L 354-358 do not relate to the article and should be omitted.

Response: We thank the reviewer for the suggestion. We have omitted them.

On the other hand, comparison between freshwater and marine organisms would be appropriate.

Response: We thank the reviewer for the suggestion. We have improved the discussion based on your suggestion. Please check line 386-391 and 462-468.

Ad Conclusion
This chapter is much better that the others. Finally, some aim of the study is presented. However, the sentence in L 444-446 should be shortened or re-written providing some more suitable information than “various concentrations of phlorotannin and phloroglucinol“.

Response: We thank the reviewer for the compliment and suggestion. We have improved conclusion part. Please check line 489-492.

Ad Materials and Methods:
L 503-504: Although reducing the sample volume is important in studies with compounds such as phlorotannins, in my opinion, using 20 individuals per only 2 mL total volume is really not enough.

Response: We thank the reviewer for the suggestion. We will consider your suggestion for our next experiment. In this study we used the method from Kim and Choi (2017). We have added the reference in line 575.

L 520: “*The germination bioassays were performed from July to December 2021*.“ This sentence should be rather omitted as the information is not necessary. Moreover, no other time information on other biotests is included in the article.

Response: We thank the reviewer for the correction. We have deleted them.

Ad Germination bioassays: Was there special reason for the toxicant concentration preparation procedure? Why did the authors dry the soaked paper prior to addition of other media components? Could the phlorotannins adsorb on the paper? The procedure of root measurement is not described. Was it made by scanning and image analysis or manually measured under sterile conditions?

Response: We thank the reviewer for the suggestion. We have added some information based on your suggestion. Please check line 591-592 and 601-602.

Minor revisions:
L 6, L 11: *Ulva lactuca* mentioned in the abstract gave a wrong idea about the test organism used in the study since *Ulva lactuca* is a scientific name for marine green macroalga, not for the terrestrial seed plant lettuce (*Lactuca sativa*, as correctly used in further text).

Response: We thank the reviewer for the correction. It should be *Lactuca sativa*. We have revised it. Please check line 28 and 32

L 21-23: Scientific names of brown macroalgae should be written in italic font.

Response: We thank the reviewer for the correction. We have revised the scientific names of brown macroalgae.

L 43: „even though this is one of the most popular species used in aquatic toxicity tests“ – this part of the sentence is quite unnecessary.

Response: We thank the reviewer for the correction. We have deleted the sentence.

L 81 (and further): LD50 – I suppose, that the authors meant rather LC50 since the lethal concentration (50%) was calculated on the concentration in water instead of concentration

per *Artemia* body mass (or other organisms, as further). LD50 has been used in toxicology field usually after oral or skin administration of the toxic substance.

Response: We thank the reviewer for the correction. It should be LC50. We have revised them.

L 397: "*In this study, both CD and DCW increased with increasing exposure time in all groups*". There is no wonder, that both endpoints (Cd and DCW) had similar trend as both of them are calculated based on the same measured data (as written before). This is also an example of repeating results which should be omitted in the discussion part. However, describing the results in a similar way (less details on many values including two digits) in the Results chapter would be more appropriate.

Response: We thank the reviewer for the suggestion. We have revised the discussion part.

L 522, L 526, L 529: Petri dish should be written with uppercase in Petri (name)

Response: We thank the reviewer for the correction. We have corrected them.

C. RESPONSE TO THE COMMENTS OF REVIEWER 3

REVIEWER 3

The research work entitled "Evaluation of toxicity of crude phlorotannins and phloroglucinol using different model organisms" presents on the demonstrate a comprehensive study of phlorotannin from *Ecklonia cava* with four toxicity test models. This work has made forward-looking progress in the development of a comprehensive and credible approach to toxicological assessment. However, there are several concepts and arguments proposed by the author in the article is poor in presentation. I still believe that this work can make a great contribution for researchers to perform toxicological testing of marine bioactive compounds. I suggested this work may be **"major revision"** for publication in "*Toxins*". Specific comments and general comments are given below:

Specific comments

Abstract: Authors are advised to rewrite most of the content

1. I suggest that the authors mention 1) the potential applications of phlorotannins, 2) denoted by LD 50 for larvicidal and inhibitory effects, and 3) the positive implications of this study for researchers in related field.

   Response: We thank the reviewer for the correction. We have improved the introduction based on the suggestions 1) Please check line 24-26, 2) Please check line 35-40, 3) Please check line 40-42.

Introduction: Authors are advised to add new information and rewrite most of the content

2. I suggest that the authors explain and emphasize the reasons why phlorotannin deserves study in the introduction. For example, multiple biological activities with pharmacological application value, such as antioxidant, anti-inflammatory, anti-diabetic, and anti-cancer properties…etc.

   Response: We thank the reviewer for the insightful suggestion. We have added some explanation based on your suggestion. Please check line 52-54.

3. It is recommended to explain in the introduction that phlorotannins from different seaweed sources contain the same monomer (phloroglucinol units), but there may be some differences in bioactivity and toxicity due to their structural composition, additional modifications, and molecular weight.

   Response: We thank the reviewer for the insightful suggestion. We have added some explanation based on your suggestion. Please check line 75-79.

4. Although the rationale for toxicity testing with these four model organisms (i.e., *Artemia salina*, *Daphnia magna*, *Ulva lactuca*, and *Chlorella vulgaris*) has been mentioned in the discussion, I suggest that this should also be briefly described in the introduction.

   Response: We thank the reviewer for the insightful suggestion. We have added some explanation based on your suggestion. Please check line 84-99.

Result: Overall well written

5. Line 65-69, to increase readability, I suggest moving the math formulas to the **Materials and Methods section**.

   Response: We thank the reviewer for the insightful suggestion. We have moved the math formulas based on your suggestion. Please check line 559-562.

6. Line 256, the related figures and sentences for Figure 5c do not exist in this article and are recommended to be removed.

   Response: We thank the reviewer for the correction. We have corrected. Please check line 317.

Discussion: Authors are advised to add new information and rewrite some of the content

7. Line 174-180, the information in these sentences have not been effectively organized. The authors are advised to rewrite the effect of crude phlorotannin and phloroglucinol on the germination rate of *Lactuca sativa*

   Response: We thank the reviewer for the correction. We have corrected. Please check line 399-400.

8. Line 285-311, 1) Authors are advised to additionally state the research value of phlorotannin from *Ecklonia cava*, not just because it has not been studied.
   Response: We thank the reviewer for the suggestion. We have added some explanation based on your suggestion. Please check line *477-480*

   2) I suggest that the authors explain here the possible reasons for the lower larvicidal activity of the crude phlorotannin of *cava* relative to the phlorotannin of other seaweeds.

   Response: We thank the reviewer for the suggestion. We have added some explanation based on your suggestion. Please check line *469-476.*

General comments

1. Line 125, to the control. (font error)
2. Figure 3a and 6a, the title of the y-axis, Cell density à Cell Density (to uppercase)
3. Table 1, 2, and 3, Bold fonts are inconsistent.

   Response: We thank the reviewer for the correction. We have corrected.