Author’s response to reviews

Title: Malaysian Endophytic Fungal Extracts-Induced Anti-inflammation in Lipopolysaccharide-Activated BV-2 Microglia is Associated with Attenuation of NO Production and, IL-6 and TNF-alpha Expression

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Author’s response to reviews: see over
We wish to thank the Reviewers for the comments. We accept all recommendations/ suggestions raised by the Reviewers, and have revised the manuscript accordingly. Page (Pg) numbers refer to the pages in the revised manuscript, unless stated otherwise. The amendments made are highlighted in yellow.

Comments:

In general, the authors have discussed the anti-inflammatory effects of five fungal endophytic extracts in microglia. The topic is rather interesting; however a major rectification is required to improve the quality of the work before it can be recommended for publication in the journal.

<table>
<thead>
<tr>
<th>No</th>
<th>Section</th>
<th>Comments/Recommendations</th>
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<tbody>
<tr>
<td>1.</td>
<td>General</td>
<td>The English language used in the manuscript writing requires proof-reading by a proficient English speaking scientist. A number of grammatical and syntax errors were detected throughout the text.</td>
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<td>The manuscript has been proof read. Grammatical and syntax errors were addressed.</td>
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<td>2.</td>
<td>Methods</td>
<td>Griess assay for Nitric Oxide Production</td>
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<td></td>
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<td>i. In the background and discussion, the authors have repeatedly mentioned that the extracts inhibited pro-inflammation in LPS stimulated microglia. However, in the study, microglia were first treated with the extracts and then only stimulated with LPS. The study design seems contradict to the order of action that claimed by the authors. Please explain and rectify.</td>
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<td>ii. The authors applied pre-treatment of the five extracts to microglia. The cells were first treated with the extracts for 24 h and then the media was replaced with a fresh one. Subsequently, LPS was added to the cells in order to initiate stimulation. Why such steps were taken? As mentioned before, the employed methods contradict with that of claimed. Please justify the rationale of the pre-treatment.</td>
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<td>Griess assay for Nitric Oxide Production</td>
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<td>i. The study was designed with the intention to assess as to whether pre-treatment was able to exert protective effect against neuroinflammation. As such, we have clarified in text that the NO lowering effect was attributed to pre-treatment.</td>
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<td>ii. This study intended to measure NO production by pre-treated BV2 under LPS stimulation. The replacement of media with fresh one was necessary to facilitate pre-treatment effect. The extracts should not be present when challenged with LPS.</td>
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iii. What was the cell confluency when the treatment was carried out? Please state.

iv. How long was the treatment? As mentioned in the methodology, 18, 24 and 48 h incubation were meant for LPS stimulation in microglia.

**Cell viability assay**

i. Commonly, cell viability assay is carried out before a drug/compound/chemical can be used to test its biologic effects in vitro or in vivo. This is to make sure that the compound concentration used in treatment causes the least or does not cause detrimental effects to cells. Please rearrange the order of experiments, otherwise, justify why cytotoxicity of the five extracts was not determined before the extract treatment.

ii. Please elaborate the steps in order to allow experiment reproducibility by others.

**Immunophenotyping**

i. The steps were only briefly described. Please also include how fixing, blocking and washing were carried out for the experiment.

ii. How many cells were selected for the analysis?

iii. Did the authors test on the effect on the extracts on CD40 expression in non-stimulated cells? If yes, please include the result; otherwise explain why this step was not considered.

iv. The duration of pre-treatment was 24 h. This was then followed by exposure to LPS for 18, 24 and 48 h, respectively. Griess assay was conducted further to LPS challenge.

**Cell viability assay**

i. The cytotoxicity of the five extracts was performed simultaneously with Griess assay using the same 96-well plate but different wells. The present study intended to assess the effect of very high concentration (1 mg/mL). Given, its cytotoxic effect, the highest subtoxic concentration of the extract (0.1 mg/mL), which exhibited good NO inhibitory activity without affecting cell viability, was chosen for subsequent anti-inflammatory study instead.

ii. The steps have been elaborated in the methods as suggested by the reviewer (Pg 6 Lines 19-23).

**Immunophenotyping**

i. The steps have been elaborated in the methods as suggested by the reviewer (Pg 7: Lines 9-22).

ii. 10,000 cells were selected for analysis (10K events acquired) (Pg 7 Lines 21-22).

iii. The test was conducted on the effect on the extracts on CD40 expression in non-stimulated cells (Pg 11: Lines 15-19).
iv. In the third sentence, the authors mentioned “extracts exhibited >70% cell viability”. I suppose it is meant to be “extracts allowed >70% cell viability”.

**Cytokine assay**

It is understood that detection of pro-inflammatory cytokines was performed using cytometric bead assay. However, determination of the cytokine expression level was not described. Please improve.

**Statistical analysis**

i. The statement of “Differences between the extracts…” is quite confusing. I suppose the authors meant to say “Differences in the anti-inflammatory effects of the extracts after treatment…” Please improve.

ii. “Significance was p<0.05…” I suppose it to be “The differences are significant when p<0.05…”.

3. **Results**

**Endophytic extracts inhibited NO production in LPS stimulated BV2 microglial cells**

The authors should consider:
(a) L-NAME was added simultaneously with LPS to the cells,  
(b) cells were first treated with the extracts, then only stimulated with LPS, when  
(a) discussing the inhibitory effects of the extracts on the microglia,  
(b) comparing the inhibitory effects of the extracts with that of LNAME in microglia.  
Please improve.

**Endophytic extracts inhibited NO production in LPS stimulated BV2 microglial cells**

The statements were improved (throughout text) as suggested by the reviewer
Endophytic extracts altered CD40 expression on unstimulated BV2 microglial cells but not on their stimulated counterparts

(a) The authors described that CD40 expression level was altered by the extract treatment in unstimulated cells. The changes were not discussed; instead the alterations were only mentioned in the discussion. Please improve.

Endophytic extracts suppressed IL-6 and TNF-α in stimulated BV2 microglial cells

(a) Pg. 11, lines 5-7. The authors described the expression level of IL-6 in unstimulated BV2 cells was below the detection limit. How the quantification was done? The result has not been shown in the table. Please state “data not shown” if the authors do not intend to include the result in the table.

4. Discussion

(a) The finding was only vaguely discussed. Rather, data were repeatedly mentioned throughout the section. Please discuss each finding in breadth in order to highlight the importance of the findings.

(b) Please include the previous comment when comparing the inhibitory effect of the extracts with that of L-NAME.

Endophytic extracts suppressed IL-6 and TNF-α in stimulated BV2 microglial cells

Quantitation was done as described in methods. The expression of the cytokines, however, was very low. As such, it was stated “Data not shown” (Pg 12: Line 11).

The discussion has been improved as advised by the reviewer (Pg 13-17).

We have highlighted the pre-treatment nature of the extracts when compared to L-NAME “Interestingly, pre-treatment with extracts at 0.1 mg/ml was generally more effective than L-NAME, an iNOS inhibitor.
(c) Alteration of CD40 expression level by the extracts on unstimulated cells was not discussed in the section. Please discuss accordingly.

(d) Pg. 14, lines 11-21; Pg. 15, lines 1-10. The authors justified the use of the endophytic extracts as anti-inflammatory agents. Please shift the justification to the early part of discussion.

Overall, the authors have produced several interesting findings. However, the findings were weakly and inappropriately discussed. Please improve accordingly before an acceptance by the journal could be recommended.

in lowering NO production in activated microglia. The mechanism underlying the NO lowering effects by endophytic extracts remains poorly understood. There is, however, a large number of studies have considered that the antagonistic effects of natural compounds on NO production are due to suppression of NFκB”. (Pg 14:Lines 19-22; Pg 15: Lines 1-2)

The relationship between the pharmacological effects of the present tested endophytic extracts with CD40 expression on BV2, be it at resting or activated state, remains unclear and requires further investigations. This is because the extracts, which were found to affect baseline expression of CD40, exhibited no effect against elevated expression of the said antigen on activated cells. The overall effect of the tested endophytic extracts against CD40 expression on unstimulated BV2, in particular, was bizarre and failed to exhibit an apparent trend. (Pg 15: Lines 8-14)

We have shifted the justification as suggested by the reviewer (Pg 13: Lines 2-23).