Author’s response to reviews

Title: Attenuation of inflammatory-mediated neurotoxicity by Saururus chinensis extract in LPS-induced BV-2 microglia cells via regulation of NF-kappa B signaling and anti-oxidant properties

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Author’s response to reviews: see over
Response to reviewer’s comments

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Reviewer #1:

**Major Compulsory Revisions**

The study entitled as “Attenuation of inflammatory-mediated neurotoxicity by *Saururus chinensis* extract in LPS-induced BV-2 microglia cells via regulation of NF-κB signaling and anti-oxidant properties by Kim and others showed that *Saururus chinensis* (SC) can attenuate inflammation by LPS in murine cell line.

1. I believe this is an important finding in the field of alternative medicine as the findings provide scientific basis of the SC treatment. However, conclusion made out of the finding is misleading. The authors argue the plant extract may have “synergistic effect” (in Abstract and Discussion of the manuscript) yet, I do not find any reason to believe this. If there is any evidence to support this, authors should make it more clearly

**Response:** We appreciate for your kind suggestion. Since the extract contained various potential compounds analyzed by HPLC we thought that these compounds might act in a synergistic way. Since we do not show any experimental evidence we agree your suggestion and omitted the sentence arguing that the extract may have synergistic effect.

2. Likewise, the last sentence of the manuscript (“…SC can be developed as a therapeutic target in treating…”) is misleading as well; observations made by the study provide scientific explanation of the traditional claims, yet do not suggest any new therapeutic target in treating neurodegenerative disease.

**Response:** As suggested we have modified the statement in both abstract and in the manuscript.

**Minor Essential Revisions**

**Methods** (Isolation of total RNA and RT-PCR)

1. Listing of full primer sequences is recommended.
Response: As suggested a separate table listing the primers used in the study was included as table 1.

Statistical analysis
2. “at least three separate experiments conducted in triplicate”.
To gain better credibility of the readers, “n value” of each experiment and the definition of “separate experiments” (i.e. number of blots vs number of culture dishes, wells etc)” is recommended to describe in figure legends or in texts.

Response: As suggested “n” values were included in statistical analysis section and figure legends.

Results
3. Significance should be labeled more efficient way; Significances between experimental groups need to be indicated.

Explanation: As suggested the significance values between groups were indicated in the revised manuscript.

4. Explanation on Figure 2 cannot be found in the text.

Explanation:-
As mentioned we have rectified the errors in indicating the Figure numbers in the revised manuscript.

5….nitrite production in BV-2 microglial cells
The amount of NO produced should be dependent on the number of cells in the culture dish. Example image shown in Figure 4A seem to have more number of cells in LPS group. It is recommendable to normalize the production of nitrite by the number of cells or total protein contents etc.

Explanation:- As indicated we have normalized the production of nitrite by the number of cells and the same was included in the revised manuscript

6. SC regulated NF-kB activation in LPS-induced…
(Figure 5B, 5C) -> The relevant data is not found. Mislabeled?

Explanation:- As indicated, we have rectified the errors in mentioning the Fig numbers in the revised manuscript
7. In Figure 6
(C) For completion of the data set, 10 uM QCT without LPS is recommended to show.
(D) 10 uM QCT induced COX-2 without inflammatory insult. This needs to be discussed.

**Explanations:** As suggested we have now included the 10 uM QCT without LPS data in the revised figure 6C. For Fig 6D, since the control cells also showed slight expression in COX-2 as like the 10 uM QCT alone treated group, we performed the experiment again with freshly cultured BV-2 cell lines. The new data did not show any changes in the expression of COX-2 in control groups and in 10 uM QCT alone treated group. The new data for Fig. 6D was included in the revised manuscript.

**Reviewer #2:**

**Major Compulsory Revisions**

The authors used LPS-treated BV-2 microglial cells as an in vitro model for microglia-mediated neuroinflammation. The authors analyzed the effects of the Saururus chinensis extract (SC) and one of its components quercetin. They reported microglial inhibiting effects of these compounds: the attenuation of NO and ROS generation; cytokines (TNFalpha, IL-6) production; NF-kappaB activation (concomitantly with the inhibition of IkappaB degradation and p65 nuclear translocation).

The authors suggest Saururus chinensis as a promising plant when looking for therapeutic compounds preventing microglial-mediated neuroinflammation, which is currently suspected to play a crucial early role in various neurodegenerative diseases. The manuscript is well written and the experiments are well done, but some points require authors’ attention.

1. Authors should explain the rationale of selecting a specific range of concentrations for SC (µg/mL). Did they select these specific concentrations out of a broader panel based on NO assay or any other basis? Did authors perform cell viability assay for quercetin also?

**Response:** We have selected SC concentration dose based on NO and cell viability assay. We have also performed NO/MTT assay for quercetin but we did not input the data as several authors earlier reported the potential benefits of quercetin in neuroinflammation in cell lines and animal models.

2. The statistical comparison between groups was done by using one way ANOVA and Dunnett’s test. Generally, Dunnett’s test is used to compare each of many treatments with a single control. Please, correct the statistical analysis.
**Explanation:-**
In our study we compared different groups for significance data; as suggested we have crosschecked and realized it should be Tukey’s test and accordingly we have corrected where ever necessary.

**Minor Essential Revisions**

Discussion could be improved further focusing mainly on the present study.

**Explanation:** As suggested we have modified the discussion part.

**Minor comments**
- In background section, the second paragraph there is redundancy. Please, modify accordingly.
- There are some typographical mistakes in the manuscript. Please proof read and correct: e.g., copunds should be compounds, antioxidant or anti-oxidant, proinflammatory or pro-inflammatory
- In the discussion section, suddenly aminoguanidine appears. How is it linked with SC or the present study?

**Explanation:**
We have crosschecked manuscript for redundancy as well typographical mistakes. We have corrected at all places wherever it was required.

In the discussion section we would like to express to the readers that several agents possessing inhibitory action on inflammatory cytokines and mediators are also having free radical scavenging activities thereby acting as anti-oxidant agents. This discussion is to support our present study. Since our present study also falls in to that category we referred to that compound (aminoguanidine). However since it was not necessary to mention the compound we omitted and modified that section.

**Reviewer #3:**

**Major Compulsory Revisions**

I suggest only the Minor Essential Revisions to this manuscript which are attached here as a separate word file
Minor Essential Revisions

1. Results, SC suppressed LPS-induced iNOS and COX-2 expression in BV-2 microglia cells, Line 1: Modify the sentence, “As shown in Fig. 3A and 3B, treatment with LPS” as “As shown in Fig. 2A and 2B, treatment with LPS”

Explanation:- As indicated we have corrected in the revised manuscript.

2. Results, SC suppressed LPS-induced iNOS and COX-2 expression in BV-2 microglia cells, Line 6: Modify the sentence, “As shown in Fig. 3C and 3D, LPS treatment significantly” as “As shown in Fig. 2C and 2D, LPS treatment significantly”

Explanation:- As indicated we have corrected in the revised manuscript.

3. Results, SC extract inhibited LPS-induced TNF-α and IL-6 production in BV-2 microglial cells, Line 8: Modify the sentence “concentration-dependent manner (Fig.4A and 4B)” as “concentration-dependent manner (Fig.3A and 3B)”

Explanation:- As indicated we have corrected in the revised manuscript.

4. Results, SC regulated NF-kB activation in LPS-induced BV-2 microglial cells, Line 6: Modify the sentence, “translocation of the NF-kB p65 subunit (Fig. 5A)” as “translocation of the NF-kB p65 subunit (Fig. 4A)”

Explanation:- As indicated we have corrected in the revised manuscript.

5. Results, SC regulated NF-kB activation in LPS-induced BV-2 microglial cells, Line 11: Modify the sentence, “in a concentration-dependent manner (Fig. 5B, 5C)” as “in a concentration-dependent manner (Fig. 4B and 4C)”

Explanation:- As indicated we have corrected in the revised manuscript.

6. Results: Place the paragraph on “Free radical scavenging activities of SC” prior to the paragraph on “HPLC fingerprint -----------BV-2 microglia”

Explanation:- As indicated we have modified in the revised manuscript.
7. Results, Free radical scavenging activities of SC, Line 5: Modify the sentence, “and the results are shown in Fig. 2A” as “and the results are shown in Fig. 5A”

**Explanation:** As indicated we have corrected in the revised manuscript.

8. Results, Free radical scavenging activities of SC, Line 10: Modify the sentence, “observed with a dose increases of SC (Fig. 2B)” as “observed with a dose increases of SC (Fig. 5B)”

**Explanation:** As indicated we have corrected in the revised manuscript.

9. Results, HPLC fingerprint ----------BV-2 microglia, Line 2: Modify the sentence, “Earlier works indicated that Sc extract” as “Earlier works indicated that SC extract”

**Explanation:** As indicated we have corrected in the revised manuscript.

10. Results, HPLC fingerprint ----------BV-2 microglia, Line 6: Modify the sentence, “we identified that SC extract extract used” as “we identified that SC extract used”

**Explanation:** As indicated we have corrected in the revised manuscript.

11. References: Volume and page numbers are missing for the references 3, 50 and 52.

**Explanation:** As indicated we have included in the revised manuscript.

12. Figures 1A/2A/2C/3A, LPS (100 ng/mL): Modify, “- + - - + +” as “- + - + + +”

**Explanation:** As indicated we have corrected in the revised manuscript (Figures 1A/2A/2C/3A).

13. Figure 3: Changes associated with the treatments should also be presented at protein level (Western blot analysis) for the molecules TNF-α and IL-6. This data only will confirm that the changes occurred at mRNA level are translated.
Explanation:- We agree with your suggestion, however in this study we showed only the changes occurred in mRNA expressional levels.

We appreciate for the time and interest shown in reviewing our article. The comments by reviewers helped us to improve the manuscript for clear understanding and better presentation for the readers. I hope we answered all the comments raised by the reviewer’s appropriately point by point and the manuscript in its present form will be acceptable for publication in your esteemed journal “BMC Complementary and Alternative Medicine”.

Thanking you,

Dr. Dong Kug Choi
Corresponding author