Author's response to reviews

Title: Anti-inflammatory effects of sargachromenol-rich ethanolic extract of Myagropsis myagroides on lipopolysaccharide-stimulated BV-2 cells

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Response to reviewer’s comment  
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We appreciate the reviewers for their deep and insightful comments. We have revised the current research paper about “Anti-inflammatory effects of sargachromenol-rich ethanolic extract of Myagropsis myagroides on lipopolysaccharide-stimulated BV-2 cells” based on their useful suggestions. We added discussion on the inhibition of ERK and JNK in the revised manuscript. We hope that these modifications have improved the manuscript to a level of their satisfaction. Our answers to their suggestions/comments are as follows.

**Reviewer #1:** This paper reports on the anti-inflammatory activity of the ethanol extract of Myagropsis myagroides to LPS-stimulated BV-2 cell line. The authors describe several such activities of M. myagroides ethanol extract to BV-2 cells.

1. The authors should describe the merit or background of BV-2 cell line to investigate such anti-inflammatory activity of natural products in Introduction section. Are BV-2 cells common to use to study the anti-inflammatory activity? If so, please introduce BV-2 cell characteristics in Introduction section.

**Answer:** Thank you for reviewer's comment. To follow reviewer's comment, we have added the sentence “BV-2 cells, derived from primary mouse microglia cells, are considered as a reasonable model for in vitro pharmacological studies, since their response to LPS showed a similar pattern to primary microglia in vivo based on transcriptome and proteome analysis [26]. With respective to neurodegeneration studies, activated BV-2 cells by LPS secret proinflammatory cytokines, which have been shown to promote neuronal injury at high level [27].” in Introduction section.

2. In Table 1, What does "N/A" mean?  
**Answer:** Thank you for reviewer's comment. We deleted “N/A” in Table 1.

3. In Table 1, Are these data from all seaweed ethanol extracts? If so, please describe this in Table 1 because these look like all phenols.  
**Answer:** Thank you for reviewer's comment. We changed the Table title to “Phenolic contents, NO and ROS suppressive activities, and yields of ethanolic extracts from the selected brown seaweeds”

**Reviewer #2:** In this study, the authors evaluated the inhibitory effect of sargachromenol-rich ethanolic extract of Myagropsis on BV-2 cells, and also investigated the possible molecular mechanisms underlying its anti-inflammatory action. The results showed the sargachromenol-rich MME inhibits the production of NO, PGE2, and pro-inflammatory cytokines as well as iNOS and COX-2 at transcriptional and translational levels. Moreover, the inhibitory effect of MME was associated with inactivation of the NF-κB pathway via blocking the phosphorylation of ERKs and JNKs. The authors had used similar experimental design to evaluate effects of different fraction extract of MME on RAW 264.7 cells, murine macrophages and mouse ear edema. And also obtained positive and rational results. However, this manuscript was adequately defined, described, and the discussion and conclusions were supported by the data with well balanced.
1. In P18 line14, the authors mentioned “However, anti-inflammatory activity of MME in this study would not due to fucoxanthin, fatty acid or phlorotannins, since anti-inflammatory activities of those compounds were much lower than MME.” How to prove this speculation?

**Answer:** We think that the reviewer’s comments are very insightful. We changed the sentence to “However, anti-inflammatory activity of MME in this study would not due to fucoxanthin, fatty acid or phlorotannins, since the peaks of those compounds were not detected in the chromatogram (Figure 6) [20,21].”

As based on the chromatogram (Figure 6), fucoxanthin would be eluted around 42 min (Reference 20) and phlorotannins would be around 36-85 min (Reference 15) at the same chromatographic condition. However, we did not find any appreciable peaks after 36 min in HPLC chromatogram. Also fatty acids would be eluted later than sargachromenol, since fatty acids are strong lipophilic compounds based on our previous experiments. Specifically, fucoxanthin should be degraded during the sample preparation (sun-dry) and extraction process (around 70°C) since it is highly susceptible to degradation by heat and light (Reference 31). In addition, we isolated fucoxanthin and 8 kinds of phlorotannins from brown algae and determined their anti-inflammatory activities using BV-2 and RAW 264.7 cells. As shown in the previous papers (Reference 9, 14, 15, 20), fucoxanthin and phlorotannins showed lower anti-inflammatory activities compared to MME based on inhibition of NO production. n-3 PUFA and hexadecanoic acid have been shown immunomodulatory or anti-inflammatory activity and their activities were shown at higher concentration compared with MME (Wall et al., 2010, Nutr Rev 68: 280-289; Aparna et al., 2012, Chem. Biol. Drug Des 80: 434-439). Thus, we speculated that MME has other strong anti-inflammatory compounds and we identified sargachromenol as a strong anti-inflammatory component in MME.

**Reviewer #3:** This study investigated the anti-neuroinflammatory activities of Myagropsis myagroides ethanolic extracts (MME) in lipopolysaccharide (LPS)-stimulated BV-2 cells. The experiment is well designed. The detection methods and conclusion are reasonable. However, there are some problems should be point out.

1. Methods. The collected Myagropsis myagroides, Undaria pinnatifida, Saccharina japonica, Sargassum horneri, and S. fulvellum should be checked by professional certification.

**Answer:** Thank you for reviewer’s comment. We have added the sentence :” Taxonomic identification of the collected seaweeds was confirmed by an agal taxonomist (C.G. Choi), at the Department of Ecological Engineering, Pukyong National University, Korea.” in Materials and Methods section.

2. Figure 1C (viability assay). There are two groups marked by LPS(+) and MME (50 ug/ml), I think it’s a slip of the pen, please check it.

**Answer:** Thank you for reviewer’s comment. To follow reviewer’s comment, we have corrected the Figure 1C.

3. Figure 4A. Scale should be added into the picture.

**Answer:** Thank you for reviewer’s comment. To follow reviewer’s comment, we have added the scale bar into the picture.
4. Figure 5A. Statistical assay should be performed for p-p38 and p-Akt.

**Answer:** Thank you for reviewer’s comment. We have marked with * having statistically significant groups. However, the level of Akt phosphorylation was not changed by MME MME treatment. Also that of p38 MAPK phosphorylation was slightly changed by MME compared with those of ERK and JNK phosphorylation, which may be disregarded on the NF-kB activation.

5. Whether MME directly regulated NF-kB activity or indirectly via ERK and JNK signal? The results are ambiguous. The authors should discuss more about it.

**Answer:** Thank you for reviewer’s deep and impressive comments. In this experiment, MME inhibited NF-kB translocation and activation and IκB phosphorylation as shown Figure 4. Those result indicated that MME directly regulated NF-kB activity. Additionally, we found that MME inhibited the phosphorylation of ERK and JNK, which means MME indirectly regulated NF-kB activity. Thus, we have revised several parts in Discussion and Conclusion section with yellow paint.

6. If sargachromenol inhibits inflammation via targeting NF-kB pathway or MAPK pathway, this compound should be able to transfer into the cells? Please show the evidence.

**Answer:**
In the present study, we focused on the biochemical/pharmacological actions of MME. This study initially reports its pharmacological characteristics in vitro. Our preliminary results indicated that sargachromenol in MME has unique physicochemical characteristics which are hydrophilic and hydrophobic properties. This property may suggest reasonable absorption into the cell as suggested NO suppressive activity of sargachromenol in LPS-treated BV-2 cells in Table 1. In this point, this is the first report to show the potential anti-inflammatory activity from *M. myagroides*. In addition, we found that sargachromenol reduced inflammatory responses to LPS stimulation in BV-2 and RAW 264.7 cells and mouse. These results will be published in future.