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PeerJ

Dear Pedro Silva, Academic Editor:

Thank you for your e-mail of May 11, with regard to our manuscript (Article ID: 36484) entitled “Effects of shokyo (*Zingiberis Rhizoma*) and kankyo (*Zingiberis Processum Rhizoma*) on prostaglandin E₂ production in lipopolysaccharide-treated mouse macrophage RAW264.7 cells” by Toshiaki Ara *et al.* Moreover, we would express our thanks to you again and reviewers for reviewing this manuscript and helpful comments. I am sending therewith our revised manuscript with changes indicated by using red color and figures, with sheet detailing our response to the points raised and the changes we have made.

I hope you will find the data interesting and would consider whether this manuscript is acceptable for publication in PeerJ.

Thank you for your kind consideration.

Sincerely yours,

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Comments to Editor

We would express our thanks to the editor for helpful comments.

1. As the reviewer2 pointed out, the concentrations of 6-shogaol in Table 1 and original Figure 4 were wrong. Therefore, we corrected the unit of concentration properly (μM to nM) in Table 1 and Figure 5 (in revised version).
2. We evaluated the inhibitory effect of shokyo and kankyo on COX-2 using the *in vivo* method by Wilborn *et al.* (1995) in which arachidonic acid is added. In the first submission, we denoted the activity as the amount of PGE_2 production, but this representation was difficult to understand. Therefore, we converted the amount of PGE_2 to percent (%), and indicated as COX-2 activities.
3. As the reviewer2 pointed out, we did not provide the direct evidences about the inhibitory effects of shokyo, kankyo, and 6-shogaol on cPLA_2 activity. In fact, we tried to evaluate cPLA_2 activity in several conditions using the commercial product (cPLA_2 Assay kit, Cayman Chemical). However, unfortunately we could not detect cPLA_2 activity in RAW264.7 cells, and therefore, we could not evaluate the inhibitory effects of these components on cPLA_2 activity. For these reasons, we concluded that shokyo and kankyo may inhibit cPLA_2 activity although the methods we used in this study are indirect.

Answers to comments of Reviewer 1

We would express our thanks to the reviewer for reviewing this manuscript and helpful comments.

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- 1) As the reviewer pointed out, we used the indirect experimental method to evaluate COX-2 activity, and therefore the method is inconsistent with Figure 3. We described the reason that we used this indirect experimental method in answer 5). However, to clarify “the inhibitory effect on COX-2 activity”, we converted the amount of PGE₂ to percent (%), and indicated as COX-2 activities (**Figures 2B and 2C**).
- 2) As the reviewer pointed out, the expression that “the effect of kankyo were stronger than those of kankyo” is wrong. Therefore, we deleted the concerning sentence in Abstract and Results sections.
- 3) As the reviewer pointed out, the concentrations of 6-shogaol in Table 1 and Figure 4 were wrong. Therefore, we corrected the unit of concentration properly (μM to nM).
- 4) We corrected the vague sentences of results that the review pointed out (**Page 5, lines 187–188, Page 6, lines 259–261**).
- 5) As the reviewer pointed out, we did not provide the direct evidences that shokyo, kankyo, and 6-shogaol inhibit cPLA₂ and COX-2 activity. The reasons that we did/could not evaluate cPLA₂/COX-2 activities are described below.
 - In fact, we tried to evaluate cPLA₂ activity using the commercial product (cPLA₂ Assay kit, Cayman Chemical) in several conditions. However, we could not detect cPLA₂ activity in RAW264.7 cells, and therefore, we could not evaluate the inhibitory effects of these components on cPLA₂ activity. For these reasons, although the methods we used in this study are indirect, we concluded that shokyo and kankyo may inhibit cPLA₂ activity. We added the sentences concerning this result that we could not detect cPLA₂ activity in Materials and Methods (**Page 3, lines 103–115**), Results (**Page 5, lines 209–210**), and Discussion (**Page 7, lines 281–283**).
 - In addition, we have used the commercial product (COX Inhibitor Screening Assay Kit, Cayman Chemical) to evaluate the effect of several kampo medicines on COX-2 activity. However, we could not obtain the adequate inhibitory effects — perhaps due to the short reaction time in this method. Therefore, to evaluate inhibitory effect of shokyo and kankyo on COX-2, we used the *in vivo* method by Wilborn *et al.* (1995) in which arachidonic acid is added. In fact, the phrase “Determination of COX activity” is present in Materials and Methods section of this literature.

- 6) As the reviewer pointed out, we included positive controls (dexamethasone for COX-2 and annexin1, BAY 11-7082 for p65, and PD98059 for ERK) in Western blotting analysis. Moreover, we quantified the relative gray values of bands and indicated the values below each band (**Figures 3–6**). We added the sentence of this methods in Materials and Methods (**Page 2, lines 77–79, Page 4, lines 141–142**) and Figure legend (**Figures 3–6**).
- 7) As the reviewer pointed out, we added the data concerning p65 translocation to nucleus by immunohistochemistry to confirm the effect of shokyo, kankyo, and 6-shogaol on NF- κ B activity. We observed the results that shokyo, kankyo, and 6-shogaol did not affect LPS-induced p65 translocation to nucleus. We add these results to Abstract (**Page 1, line 23**), Materials and Methods (**Page 4, lines 153–165**), Results (**Page 6, lines 226–230 and 264-266**), and Discussion (**Page 7, lines 276–281, Page 8, lines 327–328**).

Answers to comments of Reviewer 3

We would express our thanks to the reviewer for reviewing this manuscript and helpful comments.

- A) The reviewer requested the addition of the paragraph about the effects of aqueous-extracts of ginger. Therefore, we added their paragraph and literatures such as Alsherbiny *et al.* (2019), and clarified the purpose of this study in Introduction (**Page 2, lines 57–64**), and Discussion (**Page 6, line 269**).
- B) The reviewer requested the discussion that PGE₂ production was reduced in macrophages and inflammatory sites but increased in gastric ulcer region. Therefore, we added their paragraph (**Page 8, line 366 – Page 9, line 373**).
- C) As the comments of the reviewer, We added 3D-HPLC profiles of shokyo and kankyo (**Supplemental Figure 1**) and related sentence (**Page 2, lines 69–70, Page 7, lines 298–299**), and changed thereafter figure numbers.
- D) As the comments of the reviewer, we added the paragraph about the limitation of this study and the ideas for future research (**Page 9, lines 374–378**).