Author's response to reviews

Title: An experimental study on providing a scientific evidence for seven-time alcohol-steaming of Rhei Rhizoma when clinically used

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Author's response to reviews: see over
Responses to Reviewers

Dear Reviewers,

We appreciate your kind and wise comments, the opportunity to revise our work, and your review of this work. We have responded to the comments as follows and revised the manuscript after duly considering your recommendations.

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Regarding the reviewer #1’s reports

Question 1:
The goal of this study is unclear. It seems that it is to prove that Dongeuibogam is correct. If not, the sentences in line 86-89 “Dongeuibogam, the most famous book on Korean traditional medicine, is an encyclopedic guideline of medical knowledge and treatment techniques first compiled by Joon Heo in 1613. As stated in this book, RR should be alcohol-steamed seven times when used for elderly patients” as well as the phrase in line 213 “as recorded in Dongeuibogam” should be removed.

Response:
Thank you for the reviewer’s comments. In this study, we were to investigate the effects of seven-time alcohol-steaming on RR constituents and hepatotoxicity to provide a scientific evidence of the recommendations in traditional oriental medicine, which RR should be alcohol-steamed seven times to reduce the toxicities when used for elderly patients (Heo, Translated Dongeuibogam. 1999). Most previous studies on RR processing have focused on changes in the chemical constituents of processed RR and there was no study on effects according to the number of processing cycles. So we performed this study and got the results that the seven-time alcohol-steaming process almost completely reduces RR hepatotoxicity. These results provide a scientific evidence for conducting systemic investigations of the recommendations of traditional oriental medicine. We submitted this paper to BMC Complementary and Alternative
Medicine that considers articles related to interventions or resources in the complementary medicine field, including toxicological studies. Because processing of RR has been commonly used in Korea, China, Japan and other Asian countries, we removed the phrase “as recorded in Dongeuibogam” in the conclusions section.

-Page 8, line 232-234 in the conclusions section.

Using modern pharmacological, toxicological and chemical analyses, the present study confirms that RR should be alcohol-steamed seven times and provides a scientific evidence for conducting systematic investigations of the recommendations of ancient documents.

**Question 2:**
This manuscript did not state whether the efficacy of RR-7 is also decreased when its toxicity is decreased. It is necessary to make readers aware of its efficacy, especially when the sennoside A and B were decreased (It is not stated but I suppose that sennosides are bioactive constituents like emodin).

**Response:**
We agree that it is necessary to make readers aware of efficacy after processing. In traditional medicine, it is stated that RR should be alcohol-steamed seven times in SopungSunkiwon, a prescription used for neurodegenerative disorders in elderly patients (Heo, *Translated Dongeuibogam*. 1999; Choi et al., Neurosci Lett. 2011). To compare their efficacy after processing, we performed supplementary experiments including assays for anti-oxidative and neuroprotective activities. Because oxidative damage inflicted by reactive oxygen species is deeply associated with neurodegenerative diseases (Berlett and Stadtman, J Biol Chem. 1997), we firstly performed 2,2-azinobis-(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS) radical cation and 2,2-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity assays. The activities of RRs were expressed as half maximal inhibiting concentration (IC\(_{50}\)) which is defined as the concentration of RRs required to scavenge 50% of ABTS radical cation and DPPH free radicals, respectively. In both assays, although anti-oxidative activities decreased by processing, all processed-RRs still exhibited strong radical scavenging...
activities, even which of RR-P7 were higher than those of Scutellariae Radix (SBE) which is well-known to have a strong anti-oxidative and neuroprotective effects (Gao et al., Biochim Biophys Acta. 1999; Shang et al., Phytother Res. 2006). Then, we compared the neuroprotective effects of RRs on H₂O₂-induced toxicity in PC12 neuronal cells. Decreased cell viability as 69.24 ± 3.15% induced by 75 µM H₂O₂ was prevented by RRs pre-treatment (89.81 ± 1.85 – 93.67 ± 1.94%), showing a better effect compared with SBE (88.44 ± 1.55%). These results suggested that the efficacy after processing is still potent, exhibiting better than SBE, a positive control. All together, we have supplementary figure and revised our manuscript as followings;

<table>
<thead>
<tr>
<th></th>
<th>RR-U</th>
<th>RR-P1</th>
<th>RR-P4</th>
<th>RR-P7</th>
<th>SBE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS assay (IC₅₀ value)</td>
<td>7.74 µg/mL</td>
<td>9.23 µg/mL</td>
<td>16.88 µg/mL</td>
<td>21.83 µg/mL</td>
<td>32.44 µg/mL</td>
</tr>
<tr>
<td>DPPH assay (IC₅₀ value)</td>
<td>11.35 µg/mL</td>
<td>15.57 µg/mL</td>
<td>17.89 µg/mL</td>
<td>46.71 µg/mL</td>
<td>48.07 µg/mL</td>
</tr>
</tbody>
</table>

**Supplementary Figure 1.** (A) Radical scavenging activities of unprocessed or processed Rhei Rhizoma. (B) Neuroprotective effects of unprocessed or processed Rhei Rhizoma against H₂O₂ in PC12 cells. Cells were treated with RR-U, RR-P1, RR-P4, RR-P7 and SBE (1 µg/mL) for 1 h and incubated with H₂O₂ (75 µM) for a further 23 h. Cell viabilities are expressed as a percentage of the controls (cells treated with vehicle for 24 h). Values are indicated as the mean ± SEM. *** p < 0.001; mean values
were significantly different from the control group. ### $p < 0.001$; mean values were significantly different from the $\text{H}_2\text{O}_2$ only treated group.

-Page 7, line 213-227 in the results and discussion section.

Furthermore, to investigate the efficacy of RRs after processing, we performed supplementary experiments to compare the anti-oxidative and neuroprotective activities of RRs. RRs exhibited strong radical scavenging activities, even anti-oxidative activities of RR-P7 were higher than those of the water extract of Scutellariae Radix (SBE), a positive control, which is well-known anti-oxidant and neuroprotectant [17, 18] (Supplementary Figure 1A). Also, we compared the protective effects of RRs against $\text{H}_2\text{O}_2$-induced neurotoxicity in PC12 cells. Decreased cell viability induced by $\text{H}_2\text{O}_2$ was prevented by RRs pre-treatment, showing a better effect than SBE (Supplementary Figure 1B). These results suggest that the efficacy of RRs after processing may be still potent.

In this study, alcohol-steaming of RRs reduced their hepatotoxicity, which was normalized in vitro and in vivo after RR-P7 treatment due to the decreased sennoside A and B levels and maintained emodin levels. Sennosides have been reported to be related to hepatotoxicity [19]. Emodin, an anthraquinone derivative of RR, has antioxidant, anti-inflammatory and hepatoprotective effects [20-23]. Thus, the changes in chemical constituents suggest that the seven-time alcohol-steaming process reduced the hepatotoxicity of RR-U.

**Question 3:**
Why was rice wine used? It is suggested in Dongeuibogam? How about 14% ethanol in place of 14% rice wine?

**Response:**
Thank you for the comments. In the traditional medicine, many herbal medicines including RR have been processed using rice wine (Seo et al., *Medicinal Herbology*. 2012). In the case of RR, RR should be steamed with rice wine such as *huangjiu* (Chinese fermented wine) or *baijiu* (Chinese vodka) (Wang et al., J Sep Sci. 2014). Compared to unprocessed RR, wine processed RR is used to treat senile constipation.
and eliminate blood stasis (Wang et al., J Sep Sci. 2014). Also, in Dongeuibogam, RR should be wine steamed seven times before being used in elderly patients to treat neurodegenerative disorders. Therefore, we used rice wine for steaming of RR to follow the traditional processing method. Other recent studies also used rice wine for processing of RR (Doui et al., J Trad Med. 2009; Gao et al., J Ethnopharmacol. 2013).

**Question 4:**
What does it mean by “The yields were 24.80%, 28.70%, 26.70% and 27.61%, respectively”? I don’t believe that 100g RR can produce 24.8 g frozen-dried powder?

Response:
The yields of herbal extracts are very variable, ranging from 5 to 30% (Park and Oh, Exp Toxicol Pathol. 2014; Wang et al., PLoS One. 2011). The extract of RR has the high yield, for example, other report stated that the yield of RR is 29.3% (Wang et al., PLoS One. 2011). The weights of samples and the matching weights of freeze-dried powders in our study were shown in the following table.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Powder (g)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR-U</td>
<td>62.00</td>
<td>24.80</td>
</tr>
<tr>
<td>RR-P1</td>
<td>57.40</td>
<td>28.70</td>
</tr>
<tr>
<td>RR-P4</td>
<td>53.40</td>
<td>26.70</td>
</tr>
<tr>
<td>RR-P7</td>
<td>55.22</td>
<td>27.61</td>
</tr>
</tbody>
</table>

**Question 5:**
The authors gave the cell concentration (2.0 × 105/mL), but the reader still don’t know how many cells in each well.

Response:
Thank you for the kind comments. According to the reviewer’s suggestion, we have revised the manuscript as following:

-Page 5, line 136-137 in the methods section.

All experiments were performed 24 h after cells were seeded on 96-well plates at a
density of $2.0 \times 10^4$ cells/well.

**Question 6:**

Why the treatment was only for 4 h?

**Response:**

In the preliminary test, we investigated the effect of RR-U on hepatotoxicity in HepG2 cells using MTT assay in a time-dependent manner. HepG2 cells were treated with RR-U at 0.1–100 µg/mL in serum-free media for 4 h, 8 h and 24 h. Treatment with RR-U at 100 µg/mL significantly reduced cell viability comparing with the control group at all-time condition. However, treatment with RR-U at 100 µg/mL for 8 h and 24 h showed severe toxicity in HepG2 cells, while treatment with RR-U at 100 µg/mL for 4 h showed adequate toxicity to compare the effects of RRs. Therefore, we chose 4 h as a proper for treatment time.

![Graph](image)

**Question 7:**

HepG2 is human hepatoma cell, it is not suitable for drug toxicity. In contrast, do RRs have anticancer effect?

**Response:**

We think the reviewer’s point is reasonable, but HepG2 cell line is reported to retain many specialized liver functions and drug metabolizing enzyme activities comparable with human hepatocytes and to have suitability as an *in vitro* model for studying liver toxicity. Actually, recent studies on hepatotoxicity of drug using HepG2 cells have been published (Song et al., Free Radic Biol Med. 2015; Tag. BMC Complement Altern Med. 2015; Haegler et al., Toxicology. 2015; Gayathri et al., Food Chem Toxicol. 2015).
**Question 8:**
Rats were treated single or twice per day?

Response:
Thank you for the kind comments. Rats were treated with 3 g/kg of RRs dissolved in saline once per day for consecutive 21 days. We have revised the manuscript as following:

- Page 5, line 149-150 in the methods section.
  
  Vehicle or 3 g/kg/day of each sample dissolved in saline was administered orally once a day for 21 days.

**Question 9:**
Why 21 days for the rats while the cells were only 4 h?

Response:
As stated in response to question 6, we investigated the effect of RR-U on hepatotoxicity in HepG2 cells using MTT assay in a time-dependent manner in the preliminary test. Treatment with RR-U at 100 µg/mL for 4 h showed adequate toxicity to compare the effects of RRs. Therefore, we chose 4 h as a proper for treatment time. In the case of rats, we referred to other report demonstrated that repetitive administration of RR for 3 weeks at 3 g/kg per day had exhibited a clear toxicity to normal rats (Wang et al., J Ethnopharmacol. 2009). Therefore, we treated 3 g/kg/day of RRs for 21 days and compared the effects of RRs on hepatotoxicity in rats.

**Question 10:**
How did the authors identify the cholestasis in HE stained liver sections? The arrows should be superimposed.

Response:
Cholestasis is defined as a disturbance of bile secretion that can result from a functional defect in bile formation at the level of hepatocytes or from impaired bile secretion and
flow at the bile duct level (El-Sisi et al., PPAR Res. 2013). Under a microscope, we found the yellowish brown areas of intracellular bile pigment (El-Sisi et al., PPAR Res. 2013). According to the reviewer’s suggestion, we have superimposed the white dashed circles on the representative photomicrograph and revised the manuscript as followings;

-Page 7, line 209-212 in the results and discussion section.

Stained liver tissues showed swelling and yellowish brown bile pigments indicating cholestasis in the rats treated with RR-U, while this phenomenon improved in the groups treated with processed RRs as the number of processes increased (Figure 3).

**Figure 3.** Liver morphologies of unprocessed or processed Rhei Rhizoma. Typical histopathological features of the rats treated with RR-U, RR-P1, RR-P4, and RR-P7. The yellowish brown bile pigments (white dashed circles). Scale bar = 50 µm.

**Question 11:**
Quite frankly, from table 2, I didn’t see much difference between RR-P1 and RR-P7. RR-U seems to have influence on cholestasis as indicated by gamma-GT and bilirubin. RR-P7 did exhibit an increase in gamma-GT although insignificantly statistical analyses.

**Response:**
We think the reviewer’s point is reasonable. In this study, the levels of T-BIL, ALT and AST, highly sensitive and specific preclinical and clinical biomarkers of hepatotoxicity, clearly increased in the RR-U, while those in the RR-P7 decreased significantly. T-BIL is a marker of hepatobiliary injury, especially cholestasis and biliary effects (Ozer et al., Toxicology. 2008). Damaged hepatocytes release their contents including ALT and AST.
into the extracellular space and the released enzymes ultimately enter into circulation resulting increase in their levels. γ-GT is another marker of hepatobiliary injury, especially cholestasis and biliary effects (Ozer et al., Toxicology. 2008). Even though the reason for the result that RR-P7 exhibited a slight increase in γ-GT compared to those of RR-P1 and RR-P4 was unclear, the increased level of RR-P7 was statistically insignificant and still lower than that of RR-U. Also, it is considered that γ-GT enzymatic measurement is a less reliable assay in rats rather than other species (Ozer et al., Toxicology. 2008). Therefore, we have made a conclusion after overall consideration of the results. We have revised the manuscript as following:

-Page 7, line 203-205 in the results and discussion section.

Even though the reason for the result that RR-P7 group exhibited a slight increase in γ-GT compared to those of RR-P1 and RR-P4 groups was unclear, the increased level of RR-P7 group was statistically insignificant and still lower than that of RR-U group (Table 2).

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Regarding the reviewer #2’s comments
The manuscript by Yeomoon Sim et al. performs the seven-time alcohol steaming process to attenuate RR-caused hepatotoxicity. The manuscript raises several interesting issues while there are some defects in the present study.

Question 1:
First of all, the toxicity of RR should be mainly caused by its purgative effect rather hepatotoxicity. Therefore, I suggest the authors might also evaluate the dysfunction of kidney.

Response:
We think the reviewer’s point is reasonable. In the previous other report, treatment of RR at 3 g/kg for 3 weeks showed protective effect against chronic renal failure in rats (Wang et al., J Ethnopharmacol. 2009). Therefore, we focused on comparing the effects of RRs on hepatotoxicity.
Question 2:
If the alcohol steaming process would interfere the pharmaceutical effects?

Response:
We agreed with reviewer’s comment and performed supplementary experiments to investigate pharmaceutical effects after processing. In traditional medicine, it is stated that RR should be alcohol-steamed seven times in SopungSunkiwon, a prescription used for neurodegenerative disorders in elderly patients (Heo, *Translated Dongeuibogam*. 1999; Choi et al., *Neurosci Lett.* 2011). To compare their efficacy after processing, we performed assays for anti-oxidative and neuroprotective activities. Because oxidative damage inflicted by reactive oxygen species is deeply associated with neurodegenerative diseases (Berlett and Stadtman, *J Biol Chem.* 1997), we firstly performed 2,2-azinobis-(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS) radical cation and 2,2-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity assays. The activities of RRs were expressed as half maximal inhibiting concentration (IC\(_{50}\)) which is defined as the concentration of RRs required to scavenge 50% of ABTS radical cation and DPPH free radicals, respectively. In both assays, although anti-oxidant activities decreased by processing, all processed-RRs still exhibited strong radical scavenging activities, even which of RR-P7 were higher than those of Scutellariae Radix (SBE) which is well-known to have a strong anti-oxidative and neuroprotective effects (Gao et al., *Biochim Biophys Acta*. 1999; Shang et al., *Phytother Res.* 2006). Then, we compared the neuroprotective effects of RRs on H\(_2\)O\(_2\)-induced toxicity in PC12 neuronal cells. Decreased cell viability as 69.24 ± 3.15% induced by 75 µM H\(_2\)O\(_2\) was prevented by RRs pre-treatment (89.81 ± 1.85 – 93.67 ± 1.94%), showing a better effect compared with SBE (88.44 ± 1.55%). These results suggested that the efficacy after processing is still potent, exhibiting better than SBE, a positive control. All together, we have supplementary figure and revised our manuscript as followings;
Supplementary Figure 1. (A) Radical scavenging activities of unprocessed or processed Rhei Rhizoma. (B) Neuroprotective effects of unprocessed or processed Rhei Rhizoma against H$_2$O$_2$ in PC12 cells. Cells were treated with RR-U, RR-P1, RR-P4, RR-P7 and SBE (1 µg/mL) for 1 h and incubated with H$_2$O$_2$ (75 µM) for a further 23 h. Cell viabilities are expressed as a percentage of the controls (cells treated with vehicle for 24 h). Values are indicated as the mean ± SEM. *** $p < 0.001$; mean values were significantly different from the control group. ### $p < 0.001$; mean values were significantly different from the H$_2$O$_2$ only treated group.

Furthermore, to investigate the efficacy of RRs after processing, we performed supplementary experiments to compare the anti-oxidative and neuroprotective activities of RRs. RRs exhibited strong radical scavenging activities, even anti-oxidative activities of RR-P7 were higher than those of the water extract of Scutellariae Radix (SBE), a positive control, which is well-known anti-oxidant and neuroprotectant [17, 18] (Supplementary Figure 1A). Also, we compared the
protective effects of RRs against H$_2$O$_2$-induced neurotoxicity in PC12 cells. Decreased cell viability induced by H$_2$O$_2$ was prevented by RRs pre-treatment, showing a better effect than SBE (Supplementary Figure 1B). These results suggest that the efficacy of RRs after processing may be still potent.

In this study, alcohol-steaming of RRs reduced their hepatotoxicity, which was normalized in vitro and in vivo after RR-P7 treatment due to the decreased sennoside A and B levels and maintained emodin levels. Sennosides have been reported to be related to hepatotoxicity [19]. Emodin, an anthraquinone derivative of RR, has antioxidant, anti-inflammatory and hepatoprotective effects [20-23]. Thus, the changes in chemical constituents suggest that the seven-time alcohol-steaming process reduced the hepatotoxicity of RR-U.

**Question 3:**
The MTT assays should be determined at 24 or 48 hour to evaluate the cell viability under RR treatment.

**Response:**
In the preliminary test, we investigated the effect of RR-U on hepatotoxicity in HepG2 cells using MTT assay in a time-dependent manner. HepG2 cells were treated with RR-U at 0.1–100 µg/mL in serum-free media for 4 h, 8 h and 24 h. Treatment with RR-U at 100 µg/mL significantly reduced cell viability comparing with the control group at all-time condition. However, treatment with RR-U at 100 µg/mL for 8 h and 24 h showed severe toxicity in HepG2 cells, while treatment with RR-U at 100 µg/mL for 4 h showed adequate toxicity to compare the effects of RRs. Therefore, we chose 4 h as a proper for treatment time.

![Cell viability graphs](image)

**Question 4:**
The detailed information for animal experiments should be provided.

Response:
Thank you for the kind comments. According to the reviewer’s suggestion, we have revised the manuscript as following:

-Page 5-6, line 142-165 in the methods section.
Thirty male Sprague-Dawley rats (5 weeks, 120–140 g) were obtained from Orient Bio (Sungnam, Korea). This study was carried out in accordance with the Principles of Laboratory Animal Care and Use Guidelines of Kyung Hee University, and was approved by the Ethics Committee of Kyung Hee University (Seoul, Korea). Animals were housed at an ambient temperature of 23 ± 1°C and at a relative humidity of 60 ± 10% under a 12 h light-dark cycle, and they were allowed free access to water and food. Animals were randomized into five groups: (1) control group; (2) RR-U treated group; (3) RR-P1 treated group; (4) RR-P4 treated group; (5) RR-P7 treated group. Vehicle or 3 g/kg/day of each sample dissolved in saline was administered orally once a day for 21 days. The treatment dose (3 g/kg) in rats was equivalent to the maximal clinical dose of RR in the Chinese pharmacopoeia (0.5 g/kg) [14, 15].

On the last day of treatment, blood samples were collected into non heparinized tubes, and centrifuged at 3000 rpm for 10 min. The serum separated was analyzed to evaluate the liver enzymes. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (T-BIL), and gamma-glutamyltransferase (γ-GT) were entrusted to Chemon (Yongin, Korea) for analyses.

Necropsies were performed on all animals. After sacrifice, pieces of all excised tissues were individually placed in neutral buffered formalin for histologic examinations. Tissue specimens were processed into paraffin-embedded 5-µm sections and stained with hematoxylin and eosin (H&E). Sections were fixed in 100% acetone at –20°C for 15 min. Sections were stained with hematoxylin for 1 min and destained by dipping in acid ethanol. Then, sections were stained with eosin for 30 s, dehydrated, and mounted. The images were photographed at 400× magnification using an optical light microscope (Olympus Microscope System BX51; Olympus, Tokyo, Japan).
Question 5:
The long-term drug toxicity (more than 90 days) should be also evaluated.

Response:
We think the reviewer’s point is reasonable. In the traditional medicine, RR should be stopped immediately, after the disease cured, because RR may damage the body by its extremely bitter and cold character (Seo et al., *Medicinal Herbology*. 2012). In our study, RRs were administered to rats orally once a day for 21 days, which equals to 560.7 human days (Sengupta, Int J Prev Med. 2013) and may be enough time to cure the disease. Furthermore, several studies have been already investigated the long-term toxicity of RR. Yan et al. reported that the total anthraquinones extracted from RR could induce swell and denaturation of renal tubule epithelial cells in Sprague–Dawley rats with 13 weeks of administration at dosage of 4.5 g/kg of body weight per day counted with the quantity of the extract (Yan et al., J Ethnopharmacol. 2006). Another 6-month study showed that the administration with 10 g/kg of RR induces renal lesion in rats (Wang et al., J Toxicol. 2007).