Author's response to reviews

Title: Ssanghwa-tang, an oriental herbal cocktail, exerts anti-melanogenic activity by suppression of the p38 MAPK and PKA signaling pathways in B16F10 cells

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Title: Ssanghwa-tang, an oriental herbal cocktail, exerts anti-melanogenic activity by suppression of the p38 MAPK and PKA signaling pathways in B16F10 cells

We greatly appreciate the helpful comments of the reviewers that have led to changes to substantially improve the manuscript. In response to the comments of the reviewers, we added and edited some sentences in the revised manuscript to answer the comments. All of these changes are highlighted in Red color in the revised manuscript. In addition, the English in this document has been checked at least two professional editors, both native speakers of English.

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Responses to specific comments from referee 1

Comment #1
The retention time of each compound has been identified. Please provide the concentration of each compound in SHT. Which one is the most abundant compound in SHT?

Response
In our previous study (Ref. 15), content of constituents from SHT was already identified (Table 3). Therefore, we added the concentration of each compound in SHT in the ‘Result’ section (p14) and more discussed about the compounds (p16-17).

p14, Results
The content of each compound in SHT was identified as follows: paeoniflorin, 1.136 μM; liquiritin, 0.122 μM; nodakenin, 0.130 μM; benzoic acid, 0.415 μM; nodakenetin, 0.003 μM; decursinol, 0.010 μM; cinnamyl alcohol, 0.032 μM; cinnamaldehyde, 0.033 μM; decursin, 0.009 μM; decursinol angelate, 0.010 μM [15].

p16-17, Discussion
Ten marker components in SHT, including paeoniflorin, liquiritin, nodakenin, benzoic acid, nodakenetin, decursinol, cinnamyl alcohol, cinnamaldehyde, decursin, and decursinol angelate, were identified by HPLC analysis (Figure 4) and the most abundant was paeoniflorin (1.136 μM). The extract of the Paeonia lactiflora flower, with paeoniflorin as the primary ingredient, has a whitening effect [27]. In addition, some compounds…. 
Comment #2
P.16 line 12-14 (Of course, several herbs........a little cytotoxic). Please provide the reference.

Response
In response to the comment, we added some sentences with references (p16).

p16, Discussion
Several herbs in SHT, including *A. gigas*, *C. officinale*, *Z. officinale*, and *Z. jujube*, have been reported to modulate melanogenesis; however, the effective doses were much higher and potentially cytotoxic compared with the doses used in our experiments. The methanol extract of *C. officinale* exhibited tyrosinase inhibitory activity with an IC$_{50}$ of 9.6 mg/ml [24], whereas the ethanol extracts of *Z. officinale* and *Z. jujube* inhibited tyrosinase activity by approximately 40% at 330 µg/ml and by 20.3% at 4 mg/ml, respectively [25, 26]. Although the ethanol extract of *A. gigas* (AGE) at 5–30 µg/ml remarkably inhibited melanin synthesis in a dose-dependent manner, AGE at 20, 25, and 30 µg/ml reduced cell viability to 90, 80, and 60%, respectively, compared with untreated control cells [12]. In contrast, SHT is a comparatively safe formulation; at concentrations up to 2000 µg/ml, it did not cause cytotoxicity in murine melanoma cells or normal hepatocytes (Figure 1A, B).

Comment #3
Please label the concentration of each treatment in Table 1.

Response
As the reviewer suggested, we added calculated concentration of each treatment included in 500 µg/ml of SHT in Table 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (µg/ml)</th>
<th>Viability (%)</th>
<th>Tyrosinase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No stimulation</td>
</tr>
<tr>
<td>control</td>
<td>0</td>
<td>100.0 ± 2.3</td>
<td>100.0 ± 4.6</td>
</tr>
<tr>
<td><em>P. luctifera</em></td>
<td>140</td>
<td>104.5 ± 1.8</td>
<td>130.8 ± 7.6</td>
</tr>
<tr>
<td><em>A. gigas</em></td>
<td>56</td>
<td>101.2 ± 0.6</td>
<td>125.6 ± 2.5</td>
</tr>
<tr>
<td><em>A. membranaceus</em></td>
<td>56</td>
<td>108.2 ± 1.3</td>
<td>152.9 ± 8.1</td>
</tr>
<tr>
<td><em>C. officinale</em></td>
<td>56</td>
<td>100.0 ± 1.1</td>
<td>96.5 ± 3.2</td>
</tr>
<tr>
<td><em>R. schisandrae</em></td>
<td>56</td>
<td>103.2 ± 0.8</td>
<td>194.7 ± 4.9</td>
</tr>
<tr>
<td><em>G. glabra</em></td>
<td>41.9</td>
<td>102.3 ± 0.3</td>
<td>122.5 ± 1.1</td>
</tr>
<tr>
<td><em>C. cassia</em></td>
<td>22.3</td>
<td>106.8 ± 0.6</td>
<td>140.4 ± 6.9</td>
</tr>
<tr>
<td><em>Z. officinale</em></td>
<td>41.9</td>
<td>104.3 ± 1.4</td>
<td>86.3 ± 9.2</td>
</tr>
<tr>
<td><em>Z. jujube</em></td>
<td>29.9</td>
<td>100.5 ± 0.2</td>
<td>78.9 ± 3.6</td>
</tr>
<tr>
<td>SHT</td>
<td>500</td>
<td>104.4 ± 2.4</td>
<td>58.9 ± 5.5</td>
</tr>
</tbody>
</table>

The data are representative of two independent experiments carried out in triplicate, and expressed as mean ± SD. *p<0.05 vs control, **p<0.05 vs α-MSH-treated cells
Responses to specific comments from referee 2

Comment #1
The “Treatment” items should be presented in italic type format. And, the capital letters should be paid attention.

Response
In response to the comment, we corrected word style of “Treatment” items into italic type. And some capital letters changed into lower case letters. In addition, the English in this document has been checked at least two professional editors, both native speakers of English. http://www.textcheck.com/certificate/GBxZcP

Comment #2
Table 2 and 3 are not necessary, and authors should write it into the Materials & Methods.

Response
As the reviewer recommended, analytical chromatographic condition was written in ‘Materials and methods’ section (p9). Table 2 and 3 were deleted.

The main components profile of SHT was analyzed at the 254 nm UV wavelength using the Elite Lachrom HPLC system (Hitachi High-Technologies Co., Tokyo, Japan) consisting of pump (L-2130), auto-sampler (L-2200), column oven (L-2350), and diode array UV/VIS detector (L-2455). System control and data analyses were executed by EZchrom Elite software (version 3.3.1a) system. The chromatographic separation was conducted with RS-tech C18 column (Optimapak C18, 4.6 × 250 mm, 5 µm, Daejeon, Korea) at 40°C and the injection volume was 10 µl. The mobile phase was a gradient elution of 1% acetic acid and acetonitrile at a flow rate of 1 ml/min, commencing with 5% acetonitrile for 5 min, linear gradient to 100% acetonitrile was applied over 70 min, and then maintained at 100% for 10 min.

Comment #3
In Figure 3A, it was quite strange that the tyrosinase protein didn’t show without SHT treatments.

Response
In response to the comment, we changed band image of tyrosinase (Figure 3A) and relative band intensities of all western blot data were quantified using ImageJ software.
Comment #4

There are about 20-30 published papers about melanogenic inhibitors within each year and reveal that some of these extracts or compounds may be potential. However, I know most of them may induce human skin side effects, and in these researches, the cytotoxicity is a very important issue. There are many platform studies for the melanin production inhibition assays of tyrosinase, including B16, human primary skin cell lines, zebrafish, mouse and human skin assays, such as Pigment Cell Research 20, 120-127, 2007; Bioorganic & Medicinal Chemistry, 18, 5241-5247, 2010; Experimental Dermatology, 20 (3), 242-248, 2011. As melanogenic regulatory compounds are being developed, potential safety shall be taken into consideration, such as the sensitivity, allergy, or toxicity tests in dermatological or cosmetics applications.

Response

We thank reviewer for this invaluable comment. We agree that adoption of human skin cell and in vivo screening system is more physiologically relevant method to elucidate the inhibition mechanisms of melanogenic inhibitors, and potential safety should be confirmed to be developed as melanogenic regulatory compound. We will keep in mind.

Our group investigated the acute toxicity after oral administration of SHT at the doses of 0 (control), 2560, 3200, 4000, and 5000 mg/Kg in ICR mice. Compared with the control group, we could not find any toxic alteration in all treated groups for survival rate, general toxicity, change of body weight, and autopsy, suggesting SHT is very safe. *Korean Journal of Oriental Medicine (2007), 13(1), 161-164*
Again, we greatly appreciate all of your great comments.

Sincerely yours,

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