Author's response to reviews

Title: Protective effect of AVS073, a poly herbal formula, against UVA-induced melanogenesis through a redox mechanism involving glutathione-related antioxidant defense

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Author's response to reviews: see over
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Dear Editor,

I wish to submit my revised manuscript to be considered for publication as a Research Article in *BMC Complementary & Alternative Medicine*.

‘Protective effect of AVS073, a poly herbal formula, against UVA-induced melanogenesis through a redox mechanism involving glutathione-related antioxidant defense’

This work assessed antimelanogenic effects and redox mechanisms of a traditional Thai herbal formula, Ayurved Siriraj Brand Wattana (AVS073), and gallic acid, a phenolic compound present in the formula extracts, in association with modulation of glutathione (GSH)-related antioxidant defenses including GSH, glutathione S-transferase (GST) and γ-glutamate cysteine ligase catalytic (γ-GCLC) and modifier (γ-GCLM) subunit using G361 human melanoma cells irradiated with UVA. We demonstrated for the first time that AVS073 extracts exerted inhibitory effects on UVA-mediated melanogenesis in G361 cells through improving the redox state by upregulation of GSH and GST at the cellular and molecular level. Pharmacological activity of the polyherbal formula would be attributed to combined action of different phenolic compounds present in the formula.

We have followed suggestions of the editor in repeating our experiment on chromatogram fingerprint analysis of AVS073 extracts using UHPLC.

Please also find enclosed my responses to the reviewers’ comments below.

**Reviewer #1:**

The effect of AVS073 formula against UVA-induced melanogenesis can’t be due to the GA content in the formula. Authors found a 0.0342% of GA in AVS073, that mean that the maximum GA present in the tyrosynase inhibition and melanin content assay is of 0.02 µg/ml of GA at 60 µg/ml of AVS073. Data shows in Figure 4 reflect that at least 5 µg/ml are needed to have a similar effect to AVS073. For a 0.02 µg/ml of GA nearly to none effect should be show.

The same occurs with ROS formation and the rest of assays. This is a very complex formula with a lot of compounds most of them are unknown. One of known tyrosinase inhibitors present in the formula is Kojic acid. Kojic acid have IC50 of 3-5 µg/ml for tyroxynase. Other
compounds such as flavonoids and simple phenols have also tyrosinase inhibition activity and probably some of them can be present in the AVS073. In my opinion is not a good idea to use a complex and uncharacterized formula if we want know the compound responsible of activity and their mechanism of action.

Answers to the reviewer#1:

We appreciate the valuable comments and have repeated our experiment on chromatogram fingerprint analysis of AVS073 extracts using UHPLC. We observed the same and reproducible results for GA content at 0.03% in the formula. We agree with the reviewer that the effect of AVS073 formula against UVA-induced melanogenesis should not be due to only GA content in the formula but may also be attributed to other phytochemicals present in the formula.

This work aims to investigate antimelanogenic effects of AVS073 formula (page 5, underlined text) in order to give pharmacological and biological evidence for the traditional use of the herbal formula and therefore further studies are needed to identify active ingredients responsible to the depigmenting activity of the formula. We agree that the formula extract is a complex mixture of multiple plant extracts, which also contain various types of phytochemicals with different polarities, separation of herbal extracts remains a challenge for identifying bioactive compounds and improvement in extraction efficiency and in analytical techniques with high sensitivity and resolution is needed. The fingerprint chromatogram analysis of AVS073 extracts using both HPTLC and UHPLC was performed in this study to ensure consistent quality of preparations of the herbal extracts studied and showed the presence of several phenolic compounds including GA in the AVS073 formula (page 17). Generally, if an active component cannot be identified, chromatographic fingerprint, which can represent characteristic constituents of herbal extracts, should be obtained to ensure quality of the herbal preparation. One or two markers of phytochemicals in herbal extracts could be employed for assessment of quality and authenticity of herbal preparations, although such phytochemicals may be primary active compounds of the herbal extracts studied. It is thus important to further identify active components and/or ingredients of the whole formula and its constituent herbs responsible for the pharmacological activities of the AVS073 formula (page 17).

Gallic acid was one of phenolic compounds identified in AVS073 formula by comparing its Rt and absorption spectrum with those of GA marker. Thus, GA was used as the positive control for investigating antimelanogenic effect of AVS073 formula and not be
solely accountable for the biological activity of the formula. We also agree with the reviewer’s comment that other compounds such as flavonoids and simple phenols also having tyrosinase inhibition activity can probably be present in the AVS073 and therefore the tyrosinase inhibition activity found in this study may be due to combined effect of multiple flavonoid compounds.

We have revised our manuscript according to the reviewer’s comments as follows:

-Page 2-3 (underlined text) in abstract section;

Methods: The standardization of AVS073 extracts was carried out by TLC and UHPLC to obtain fingerprinting profiles of the formula, which identified several phenolic compounds including gallic acid (GA) in the formula.

Conclusions: AVS073 formula exerted antimelanogenic effects possibly through improving the redox state by upregulation of GSH and GST. Moreover, pharmacological activity of the polyherbal formula would be attributed to combined action of different phenolic compounds present in the formula.

-Page 17 (underlined text) in discussion section;

In addition, the fingerprint profile of the AVS073 extracts was carried out using TLC and UHPLC analysis to ensure consistent quality of preparation of the herbal extracts studied. While the chromatogram showed the presence of several phenolic compounds including GA in the AVS073 formula, which was then used as the positive control to screen antimelanogenic effects of the herbal formula, GA content in the AVS073 extracts were observed to be 0.0342 %w/w, which was very low, and its concentration at 5 µg/ml was required to achieve a significant inhibition of UVA-induced tyrosinase activity (Fig. 4B) and melanin content (Fig. 4B) in G361 cells. Therefore, GA could not be solely a phenolic compound contributed to biological activity of the AVS073 extracts and antimelanogenic effect of the formula may be attributed to combined effect of multiple phytochemicals present in the formula. It is thus important to further identify active components and/or ingredients of the whole formula and its constituent herbs responsible for the pharmacological activities of the AVS073 formula.

Reviewer #2:
A well-described study demonstrating the anti-melanogenic potential of a commercial herbal extract. A few minor comments follow:
1. The quantitation of the GA, at 0.03 % of the extract, seems very low. Other phenolics appeared to be present at higher levels from the TLC chromatograms. Is the GA content critical for anti-melanogenesis compared with the other compounds identified? Also on page
7, para 1 last line, ‘...AVS073 powder (100 mg/ml)....’, is this unit meaningful, when the GA concentration is given in %w/w, that must apply to the whole dried extract?

**Answers to the reviewer#2:**

We appreciate the valuable comments of the reviewer and have repeated our experiment on chromatogram fingerprint analysis of AVS073 extracts using UHPLC. We observed reproducible results for GA content at 0.03% in the formula and agree with the reviewer that the quantitation of the GA at 0.03% in the extract seems very low. We then have revised our manuscript as follows.

-Page 2-3 (underlined text) in abstract section;

Methods: The standardization of AVS073 extracts was carried out by TLC and UHPLC to obtain fingerprinting profiles of the formula, which identified several phenolic compounds including gallic acid (GA) in the formula.

Conclusions: AVS073 formula exerted antimelanogenic effects possibly through improving the redox state by upregulation of GSH and GST. Moreover, pharmacological activity of the polyherbal formula would be attributed to combined action of different phenolic compounds present in the formula.

-Page 17 (underlined text) in discussion section;

In addition, the fingerprint profile of the AVS073 extracts was carried out using TLC and UHPLC analysis to ensure consistent quality of preparations of the herbal extracts studied. While the chromatogram showed the presence of several phenolic compounds including GA in the AVS073 formula, which was then used as the positive control to screen antimelanogenic effects of the herbal formula, GA content in the AVS073 extracts were observed to be 0.0342 %w/w, which was very low, and its concentration at 5 µg/ml was required to achieve a significant inhibition of UVA-induced tyrosinase activity (Fig. 4B) and melanin content (Fig. 4B) in G361 cells. Therefore, GA could not be solely a phenolic compound contributed to biological activity of the AVS073 extracts and antimelanogenic effect of the formula may be attributed to combined effect of multiple phytochemicals present in the formula. It is thus important to further identify active components and/or ingredients of the whole formula and its constituent herbs responsible for the pharmacological activities of the AVS073 formula.

-Page 7, underlined text in method section;
Additionally, quantitative analysis of GA present in AVS073 extracts was carried out using the UHPLC method and GA content in the whole dried AVS073 extract was found to be 0.0342 %w/w.

2. In Fig 1A, it is not clear what are the 15 components? How does Fig 1B differ from these 15 analyses, and what are ‘Wattana recipe 1-3?’ The densitometric traces are not very informative unless the peaks can be labelled. How does this analysis compare with the scans in Fig 2? And what samples were used in the UHPLC analysis

-Figure 1. TLC fingerprints of AVS073 and its 15 components visualized at 254 and 366 nm under UV and visible light (after spraying with fast blue salt: FBS), respectively. (A) 15 components of AVS073 were P. nigrum, B. rotunda, C. rotundus, T. crispa, T. chebula, C. orientalis, D. scandens, A. cocculus, D. roxburghii, C. siamense, F. assa-foetida, A. maemelos, C. univitatum, S. lappa, C and buchanani; lane1-15: component no. 1-15; lane16: mixed phenolic markers (MP1): gallic acid, caffeic acid and p-coumaric acid; lane17: mixed phenolic markers (MP2): kojic acid, vanillic acid and ferulic acid (from bottom to top). (B) Wattana recipe; lane1-3; 3 replicates of AVS073; lane 4: MP1; lane 5: MP2. (C) Densitometric fingerprint of AVS073.

-Page 5 (underlined text in introduction section) and page 17 (underlined text in discussion section);

We agree with the reviewer that densitometric traces are not very informative and cannot be compared exactly with the scans in Fig. 2 obtained from UHPLC analysis. Since this work aims to investigate antimelanogenic effects of AVS073 formula (page 5, underlined text) in order to give pharmacological and biological evidence for the traditional use of the herbal formula.

TLC is a simple, rapid and inexpensive tool which can be used to separate phytochemical components of a mixture to ensure consistent quality of herbal preparation. In fact, the formula extracts are composed of multiple plant extracts, which also contain various types of phytochemicals with different polarities, separation of herbal extracts remains a challenge for identifying bioactive compounds and improvement in extraction efficiency and in analytical techniques with high sensitivity and resolution is needed. The fingerprint chromatogram analysis of AVS073 extracts using both HPTLC and UHPLC was performed in this study for the quality control of preparations of the herbal extracts studied and showed the presence of several phenolic compounds including GA in the AVS073 formula (page 17).
Generally, if an active component cannot be identified, chromatographic fingerprint, which can represent characteristic constituents of herbal extracts, should be obtained to ensure quality of the herbal preparation. One or two markers of phytochemicals in herbal extracts could be employed for assessment of quality and authenticity of herbal preparations, although such phytochemicals may be primary active compounds of the herbal extracts studied. It is thus important to further identify active components and/or ingredients of the whole formula and its constituent herbs responsible for the pharmacological activities of the AVS073 formula (page 17).

-Page 6 in method section;

The samples used in the UHPLC analysis were “the lyophilized powder (5 mg) dissolved in 1 ml of methanol (50%, v/v) was used” for thin layer chromatography (TLC) or ultra-high performance liquid chromatography (UHPLC) with photodiode array (PDA) detection.

3. In Results, page 14 para 1, the UVA dose for Fig 3A and B is shown on the graph as 8 J/cm² (not 16 J/cm²). In para 2, the ‘concentration-dependent inhibition’ for GA is not apparent in Fig 5B.

-Page 14 in result section, paragraph 1:

The antimelanogenic effects of the herbal extracts and GA were assessed by measuring tyrosinase activity and melanin production in G361 cells irradiated with a UVA dose of 8 and 16 J/cm², respectively.

-Page 14 in result section, paragraph 2:

We re-checked our data analysis concerning determination of ROS formation and observed that “pretreatment of cells with AVS073 (15-60 µg/ml) (Fig. 5A) and GA (2.5-10 µg/ml) (Fig. 5B) resulted in a dose-dependent inhibition of UVA-induced oxidant generation.”

4. Results page 15, para 2, line 4: ‘....a rise in tyrosinase mRNA expression (1.2 +/- 2.1-fold....) does not appear to be correct from the figure.

We re-checked our data and made a revision as follows;

In agreement with the data observed in the study of tyrosinase activity, a rise in tyrosinase mRNA expression (2.2 ± 0.2-fold change; p < 0.01) was observed in response to UVA irradiation (8 J/cm²), although AVS073 (Fig. 7A) and GA (Fig. 7B) significantly diminished tyrosinase mRNA levels.