Author’s response to reviews

Title: Norartocarpetin from a folk medicine Artocarpus communis plays a melanogenesis inhibitor without cytotoxicity and skin irritation in mice

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Version: 4 Date: 4 July 2013

Author’s response to reviews: see over
Response letter

Referee 2

Reviewer's report

Title: Norartocarpin from a folk medicine Artocarpus communis plays a melanogenesis inhibitor without cytotoxicity and skin irritation in mice

Version: 2 Date: 15 May 2013

Reviewer: Antonella Canini

Reviewer's report:

Dear Editor,

our decision about the MS “Norartocarpin from a folk medicine Artocarpus communis plays a melanogenesis inhibitor without cytotoxicity and skin irritation in mice” by Horng-Huey Ko, Yi-Ting Tsai, Ming-Hong Yen, Chun-Ching Lin, Chan-Jung Liang, Tsung-Han Yang, Chiang-Wen Lee, Feng-Lin Yen is: ACCEPTED after Major Compulsory Revisions.

1) In the text, authors sometimes wrote in vivo and in vitro not in italics. Sometimes they wrote the genus name Artocarpus and not its abbreviation A. They should modify it.

Reply:
We thank the reviewer for pointing this out and we have rewritten in vitro and in vivo in italics throughout the manuscript. In addition, we have also rewritten the genus name Artocarpus with abbreviation A. such as A. heterophyllus, A. lakoocha, A. communis (Page 5, Line 3-4).

2) References should be checked (some mistakes are present for ex. in ref. 11 and 12).

Reply:
We are deeply thank that reviewer perused our manuscript. As suggestion, we have corrected reference 11, 12 and 21.

3) The most important contradiction of the work is that authors describe the Norartocarpin as whitening agent on B16F10 cells because of its action in the reduction of melanin amount, tyrosinase and MITF activity and levels. In reality, MITF, tyrosinase and melanin are melanoma cells differentiation markers and the decrease in their levels is considered a bad indicator of melanoma inhibition. A lot of work in literature (*) support this theory and demonstrate that an induction of differentiation in B16F10 cells is highly associated with the decrease of tumoral melanoma cells. Therefore, authors should justify and clarify the effect of Norartocarpin on B16F10 cells: in fact, if this molecule could be a whitening compound, on the other hand, it might also have a pro-tumoral effect on cells.
Reply:

We thank the reviewer’s comment and our opinion as follows, On a different note, Alesiani et al. demonstrated that high concentrations of 5,7-dimethoxycoumarin (100-500 µM) showed the in vitro anticancer activity in melanoma cells through cell cycle arrest, differentiation induction and the compound can also inhibit the ERK 1/2 phosphorylation led to the B16 cell differentiation and melanogenesis process [33]. Chen et al. revealed that α-MSH is a cancer stem cell-associated marker in melanoma through upregulating Wnt-1, β-catenin and MITF expression. Resveratrol at 15 µM could downregulate α-MSH stimulated cancer stem cell-associated molecules (Wnt-1, β-catenin and MITF expression) in melanoma B16 cells and finally decreased the cell proliferation, migration, and differentiation [34]. Moreover, Yajima et al. mentioned that MITF plays a “Two Faced” function role in melanoma development and progression. A low level of MITF expression promotes cell proliferation but a high level enhances cell differentiation through induction of cellular senescence and melanogenesis [35]. In the present data, norartocarpetin can downregulate the MITF expression and inhibit the melanogenesis and therefore it implicated that the anticancer activity of norartocarpetin is similar to resveratrol but the mechanism of norartocarpetin merits further investigation for cancer prevention application. This statement is added into the Discussion section (Page 21, Line 19 to Page 22, Line 16).

In fact, in the MTT assay, sometimes proliferation is enhanced with respect to the control.

Reply:

We thank the reviewer’s comment. The result of MTT assay indicated that the cell viability of norartocarpetin concentrations ranging from 5 to 40 µM did not show significance with control group. As suggestion, we also performed the Trypan blue test for checking the in vitro cytotoxicity of norartocarpetin. The result also did not present obvious cell proliferation in B16F10 cell.

4) MTT assay is a test able to determine a reduction in cell growth but it does not demonstrate the cytotoxic effect of a molecule on cells. Therefore, authors should perform a Trypan blue exclusion test to support their data.

Reply:

We thank the reviewer’s comment but we have different opinion with it. According to UNI/EN ISO 10993-5: 2009 (E), the MTT assay (colorimetric test) is performed to evaluate the potential release of cytotoxic substances by a medical device/cosmetic product. A cytotoxicity test on scalar concentrations of the investigated product is performed using in vitro cell cultures of human fibroblasts/ keratinocytes. The statement is from http://www.biobasiceurope.it/en/. As suggestion, we performed a Trypan blue and crystal violet test for checking the in vitro cytotoxicity of norartocarpetin. As shown in Figure A, the result of the trypan blue and crystal violet test indicated that a serial concentration of norartocarpetin did not find obvious cell death or cell proliferation when compared with normal control group (DMSO treatment). Therefore,
norartocarpin at 1 to 40 µM is a safe molecule in B16F10 cell.

Figure A. Trypan blue and crystal violet test of norartocarpin.

5) Western blot analysis was also performed by authors but images are all very ugly!! For example: Figure 5, TYR spots are cut and GAPDH image is highly modified by authors, in fact it appears compressed!!! Authors should repeat these experiments or use original and complete images of their immunoblotting!

Reply:
We thank the reviewer's comment. As suggestion, we repeated the western blot assay and we also added the new images in Figure 4 (D), Figure 4 (E), Figure 5 (B), Figure 6 (B) and Figure 6 (C). Please review it, thanks for your careful reviewing.
We thank the reviewer gratefully for his/her detailed examination on our manuscript and for his/her kind suggestions. We hope that these answers are satisfactory.
Referee 3

Reviewer's report

Title: Norartocarpetin from a folk medicine *Artocarpus communis* plays a melanogenesis inhibitor without cytotoxicity and skin irritation in mice

Version: 2 Date: 23 May 2013
Reviewer: Léocadie KAMAGAJU

Reviewer's report:

Please find below my comments after reviewing the manuscript: Norartocarpetin from a folk medicine *Artocarpus communis* plays a melanogenesis inhibitor without cytotoxicity and skin irritation in mice”

Major Compulsory Revisions

1. Rephrase the title to make it more clear, it looks like the inhibition of melanogenesis, cytotoxicity and skin irritation are all done in mice.

Reply:

We thank the reviewer’s comment. As suggestion, we rephrased the title as “Norartocarpetin from a folk medicine *Artocarpus communis* plays a melanogenesis inhibitor without cytotoxicity in B16F10 cell and skin irritation in mice”.

2. For all plants in your paper, give full identification (ex. *Artocarpus communis* Forst. or *Artocarpus communis* J.R.Forst. & G.Forst. Moraceae)

Reply:

We are deeply thank that reviewer perused our manuscript. The plant material in the present study is *Artocarpus communis* and its full identification is *Artocarpus communis* J.R.Forst. & G.Forst. (Page 7, Line 19)

3. Background: you say: “Natural products used in preventive medicine have also been developed as cosmeceutical ingredients in skin care products”. Generalization, I do not think that all plants used in preventive medicine have developed as cosmeceutical ingredients in skin, if it is in your country, please specify. If it is in others country, please give references.

Reply:

We apologize for not making this clear and we have rewritten the Background section as follow, Many natural products used in preventive medicine have also been developed as cosmeceutical ingredients in skin care products, such as *Scutellaria baicalensis* and *Gardenia jasminoides*.

“Norartocarpetin is one of the antioxidant and antityrosinase activity compound in *Artocarpus communis*; however, the cytotoxicity, skin irritation and antimelanogenesis mechanisms of norartocarpetin ………..”. Tyrosinase is the key enzyme of melanogenesis, its inhibition means the melanogenesis inhibition, if tyrosinase inhibition was studied, where the foundation of your work? what is the novelty of your work?
We thank the reviewer’s comment. We think our foundation in science and the novelty of the present study as follow; previous study only demonstrated that norartocarpetin inhibits mushroom tyrosinase activity but no publication study the molecular biological mechanism in cell model. Our present study **firstly demonstrated** that norartocarpetin inhibits melanogenesis by downregulating microphthalmia associated transcription and tyrosinase expression via the MAPK signal pathway. In addition, we firstly demonstrated that norartocarpetin at 5-40 µM did not present cytotoxicity in *in vitro* model. Moreover, we also firstly used *in vivo* model to determine the skin irritation of norartocarpetin and our result indicated norartocarpetin at 0.1% and 0.2 % did not present skin irritation. Therefore, if norartocarpetin used as cosmeceutical ingredient and added in cosmetic product, its concentration at 0.2% is safe for skin.

4. For different inhibition tests, what is the positive control (a product known as tyrosinase inhibitor, for example)?

Reply:
We thank the reviewer’s comment and our opinions are as follows, kojic acid is commonly used as positive control to compare the melanin content in cell model. Sato et al have demonstrated that 200 µM of kojic acid was obviously decreased the melanin content (36%) in B16 cell (Figure B) [Reference 1]. According that, our lab also used the concentration of kojic acid as positive control group but our result did not find the similar melanin content inhibition. Therefore, we survey several references about it activity. Ahn et al reported that 1.6 mM (1600 µM) of kojic acid had the 50% inhibition of melanin content (Table 1) [Reference 2]. Criton et al revealed that the melanin content inhibition of 100, 300 and 1000 µM were 4.5%, 17.7% and 22.2%, respectively (Table 2) [Reference 3]. Finally, we can conclude that the melanin content inhibition of kojic acid had positive and/or negative result. The difference among these studies may be come from different concentration of kojic acid in different experimental environment or cell model.

![Figure B. Effect of ASA from melanogenesis in B16 melanoma. (Reference 1: Sato et al., Down-regulation of tyrosinase expression by acetylsalicylic acid in murine B16 melanoma. Biological and Pharmaceutical Bulletin, vol. 31, no. 1, pp. 33-37, 2008.)](image-url)
Table 1. Depigmenting activities of kojic acid derivatives.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Inhibitory activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Depigmentation (IC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>Cytoxicity (IC&lt;sub&gt;50&lt;/sub&gt;)</td>
</tr>
<tr>
<td>Kojic acid</td>
<td>1.60 mM</td>
<td>3.50 mM</td>
</tr>
<tr>
<td>Trolox</td>
<td>&gt;300 μM</td>
<td>300 μM</td>
</tr>
<tr>
<td>3a</td>
<td>17.70 μM</td>
<td>56.47 μM</td>
</tr>
<tr>
<td>3b</td>
<td>&gt;200 μM</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>3c</td>
<td>&gt;200 μM</td>
<td>&gt;200 μM</td>
</tr>
<tr>
<td>3d</td>
<td>&gt;200 μM</td>
<td>&gt;200 μM</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values were determined from logarithmic concentration-inhibition curves and are the means of three experiments.

(Reference 2: Ahn et al., Inhibitory activity of novel kojic acid derivative containing trolox moiety on melanogenesis. *Bioorganic and Medicinal Chemistry Letters*, vol. 21, no. 24, pp. 7466-7469, 2011.)

Table 2. Effect of compound 1 on Melanin synthesis in normal human melanocyte.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (μM)</th>
<th>Melanin content NHM (% inhibition)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 1</td>
<td>1</td>
<td>ni</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.7±0.04</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>14±2.1</td>
</tr>
<tr>
<td>Kojic acid</td>
<td>100</td>
<td>4.5±0.7</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>17.7±1.1</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>22.2±1.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are represented as % inhibition over control cells, means±S.E. of three independent tests; ni: no inhibition.


5. P7: why do you describe the methods only and not the material?

Reply:

We thank the reviewer’s comment. According to the manuscript format of BMC Complementary and Alternative Medicine, we only wrote the Methods section.

6. Describe the cell lines used

Reply:

We thank the reviewer’s comment. As suggestion, we have rewritten the description of cell lines as follow, “B16F10 melanoma cells and human fibroblast cells (Hs68 cell line) were purchased from...”
BCRC (Bioresource Collection and Research Center, Hsinchu, Taiwan), which originally purchased them from ATCC (USA).” Please check it on Page 9, Line 7.

7. Be sure to check the figures legend; ex. Fig 3A and B is not “The cellular melanin (A) and tyrosinase activity (B) of norartocarpentin in B16F10 cells but inhibition of cellular melanin (A) and tyrosinase activity (B) of norartocarpentin in B16F10 cells.

Reply:
We are deeply thank that reviewer perused our manuscript and we are carefully checked the figure legend again.

We thank the reviewer gratefully for his/her detailed examination on our manuscript and for his/her kind suggestions. We hope that these answers are satisfactory.