Author's response to reviews

Title: Anti-angiogenic effects of total flavonoids from Scutellaria barbata D. Don

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Version: 4 Date: 22 March 2013

Author's response to reviews: see over
The revision letter

Dear Editor:
Thank you very much for your letter and advice. We have revised the manuscript, and would like to re-submit it for your consideration. We have addressed the comments raised by the reviewers, and the amendments are highlighted in red in the revised manuscript. Point by point responses to the reviewers’ comments are listed below this letter. We hope that the revised version of the manuscript is now acceptable for publication in your journal.

I look forward to hearing from you soon.
With best wishes,

Yours sincerely,

Zhi-Jun Dai
2013. 3. 22
Editorial comments:

- Please could you confirm whether your institutional ethics committee granted permission for the animal experiments performed in your study. Please include a statement to this effect in your Methods section. We would also ask you to ensure that the name of your ethics committee is included in this statement.

  **Answer:** We confirm our institutional ethics committee granted permission for the animal experiments performed in this study. We have added the statement in the Methods section.

In revised manuscript: The animals were housed and handled in strict accordance with the guidelines of the institutional and national Committees of Animal Use and Protection. The protocol was approved by the Committee on the Ethics of Animal Experiments of Xi’an Jiaotong University College of Medicine (Certificate No. 22-9601018). All efforts were made to minimize animals’ suffering and to keep the numbers of animals used to a minimum.

- Please remove the figure legends from your uploaded figures, and instead include these in your manuscript. Further information on our formatting guidelines for figures can be found through the following link:

  http://www.biomedcentral.com/bmccomplementalternmed/authors/instructions/researcharticle#preparing-figures

  **Answer:** We have made correction according to your comments.

- We recommend that you ask a native English speaking colleague to help you copyedit the paper.

  **Answer:** As to language problems, I have worked jointly with other two colleagues to look through the full text and made revisions. Furthermore, we have invited a native English speaker for professional help to revise my texts.
Reviewer's report 1
Title: Anti-angiogenic effects of total flavonoids from Scutellaria barbata D. Don
Version: 3 Date: 9 February 2013
Reviewer: Nattanan Panjaworayan
Reviewer's report:
Major Compulsory Revision
1. As MTT assay has been reported to give false positive results, how the authors performed experiment to exclude false positive results.
   Answer: In our study, the MTT assay was performed in triplicate. Furthermore, three independent experiments were done over multiple days. The results represented as the average of three experiments.

2. Did cells were treated with any solvent at 0 µg/mL in every experiments?
   Answer: Yes, we had used 0 µg/mL as the blank control group in every experiments.

2. How many independent experiments were performed for Figure 1 and Figure 6?
   Answer: Dear Professor, there were three independent experiments were performed for Figure 1 and Figure 6.
   In revised manuscript: This assay was performed in triplicate. The results represented as the average value of absorbance from three independent experiments done over multiple days.

3. The authors should indicate the meaning of “*” and “**” in the Figure 1.
   Answer: Sorry for my careless. I have added the meaning of “*” and “**” in the Figure 1.
   In revised manuscript: *p<0.05, **p<0.01 versus the control group.

4. According to MTT results, high concentration of TF-SB could significantly caused cell death. Could it possible that the results of cell migration and tube formation are due to cell cytotoxicity?
   Answer: Thanks for your problem. Our results suggested that TF-SB inhibited both the proliferation and the migration of endothelial cells. We consider that the cytotoxicity and anti migration effects exist simultaneously.
   It could not denied anti- migration effect because of the cytotoxicity of TF-SB.
Because the drug is only added to the middle of the slide in the *in vitro* scratch assay, it just affects migration of HUVECs.

5. The authors need to pay more attention on wording and should keep consistency in article i.e. h or hour, ml or mL. Make sure that there is a space between number and a unit. Typo error should also be corrected i.e. side effect.

**Answer:** As to language problems, I have worked jointly with other two colleagues to look through the full text and made revisions. Furthermore, we have invited a native English speaker for professional help to revise my texts.
Reviewer's report 2
Title: Anti-angiogenic effects of total flavonoids from Scutellaria barbata D. Don
Version: 3 Date: 14 February 2013
Reviewer: Edmund Narre
Reviewer's report:
REVIEWER’S REPORT-Edmund Narre
Major Compulsory Revisions:
1. A positive control should be used in the methods to compare the activity of the plant extract to a known/standard anti-angiogenic agent.
   Answer: We are very sorry for our negligence of the positive control. However, we demonstrated the anti-angiogenic effects of TF-SB in this study.

2. There should be HPLC analysis of the extract.
   Answer: Thanks for your advice. We have added HPLC analysis of TF-SB in the revised manuscript.

In revised manuscript:

*Figure 1. HPLC analysis of TF-SB.* There was a main peak in HPLC, which was identified as Scutellarin (A). There were also some other flavonoids in TF-SB which were identified as apigenin(B), baicalein(C), luteolin (D). (Wavelength=280 nm)

3. The voucher specimen number/reference should be cited after identification.
   Answer: As you suggested that, we have added the voucher specimen number/reference in “Preparation of TF-SB from Scutellaria barbata D. Don”
In revised manuscript: Dried plant materials of S. barbata were purchased from Yi Shan Tang Chinese Herbal medicine store (Xi’an, China) and authenticated according to the descriptions found in the Chinese Pharmacopoeia. The original herb was identified as Scutellaria barbata D. Don (SB) by Prof. Run-Xia Liu at Medical School of Xi’an Jiaotong University (Xi’an, China). The voucher samples, ZLK-ZY-05 (S. barbata) was deposited at the department of oncology, the Second Affiliated Hospital of Xi’an Jiaotong University.

4. The percentage yield after extraction should be stated and the storage conditions of the extract mentioned including its shelf usage.
Answer: Thanks very much for your suggestions. The recovery of TF-SB was 69%, TF-SB were stored at 4 °C. We have added this section in the revised manuscript.

In revised manuscript: After treatment with AB-8 resin, the flavonoids purity increased with a recovery of 69%. The total flavonoids of S. barbata (TF-SB) were stored at 4 °C for use.

5. Were any phyto-chemical tests done to confirm the presence of flavonoids in the extract after extraction? Or otherwise cite the reference text for the extraction method used.
Answer: We have identified the component of TF-SB with HPLC.

In revised manuscript: The component of TF-SB was identified with high performance liquid chromatography (HPLC). As shown in Figure 1, there was a main peak in HPLC, which was identified as Scutellarin (A). There were also some other flavonoids which were identified as apigenin(B), baicalein(C), luteolin (D). The contents of these four flavonoids were 67.2% (A), 8.7% (B), 4.6% (C) and 4.3% (D).

6. Were statistical analysis done to compare the incubation time (48hr with 72hr) for the different doses of the extract. This should be clearly indicated in the figures before any conclusion on time-dependency.
Answer: Sorry for my careless. There were significantly difference between 48
h and 72 h.
In revised manuscript:
There were significantly difference between 48 h and 72 h in 20, 40, and 80 µg/mL groups.

7. The figures showed only statistical comparisons between individual extract concentrations and the control. There must be statistical analysis between the various concentrations (eg. 40 µg/mL to 80 µg/mL etc) before any conclusion on dose-dependency.

Answer: Thanks for your problem. We have added statistical analysis between the various concentrations.

In revised manuscript:

Fig 2

![Fig 2 graph](image1)

Fig 4

![Fig 4 graph](image2)
Fig 5

Inhibition of blood vessel growth (%)

Concentration (µg/ml)

0 40 80 120

Fig 7

A

VEGF (pg/mL)

Concentration (µg/ml)

0 40 80 120

B

VEGF (pg/mL)

Concentration (µg/ml)

0 40 80 120
Minor Essential Revisions
1. Insert the address of the software manufacturer used in the statistical analysis
Answer: As you suggested that, we have added the software manufacturer of the statistical analysis in the revised manuscript.
In revised manuscript: Statistical analysis of the data were performed with Student’s $t$-test, one-way analysis of variance (ANOVA) test and linear regression analysis using the Statistical Package for Social Sciences version 13.0 (SPSS Inc, Chicago, IL). $p$ value $< 0.05$ was considered statistically significant.

2. Insert List of abbreviations in the appropriate section
Answer: Thanks for your suggestions. We have added the list of abbreviations in the revised manuscript.
In revised manuscript:

**Abbreviations**

HCC, hepatocellular carcinoma; VEGF, vascular endothelial growth factor; HUVECs, Human umbilical vein endothelial cells; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; CAM, Chick embryo chorioallantoic membrane; TF-SB, total flavones of Scutellaria barbatae; HPLC, high performance liquid chromatography.

3. Insert the reference text in
   • Background Line 6 after “.....side effects”
   • Background Line 6 after” .....drug resistance”
Answer: As you suggested that, we have added the reference in the revised manuscript.
In revised manuscript:

3. Chang CY, Huang ZN, Yu HH, Chang LH, Li SL, Chen YP, Lee KY, Chuu JJ: The adjuvant effects of Antrodia Camphorata extracts combined with

4. In Methods- In-vitro scratch assay: Rephrase the 1st and 2nd sentences.
   **Answer:** Thank you a lot for your suggestions. We have rephrased these sentences.
   In previous manuscript: To assess the activity of TF-SB on migration of HUVECs by the scratch assay [24]. 2×10^5 HUVECs were seeded on a 12-well plate in complete medium overnight to obtain a full confluent monolayer.
   In revised manuscript: We used the *in vitro* scratch assay to assess the activity of TF-SB on migration of HUVECs [24]. HUVECs were incubated at 2 × 10^5 cells /well in 12-well plates with complete medium overnight to obtain a full confluent monolayer.

5. In general the Results Section should be re-written so as not to repeat the Methods in the Results Section.
   **Answer:** Thanks for your advice. We have made some amendments according to your suggestions.

6. The results should not include statements which could have being in the discussion eg. The 1st paragraph of the results sub-section “The relationship between leukocyte infiltration count and formation of blood vessels”
   **Answer:** We have made some amendments according to your suggestions.

7. Abbreviations should for the first time be stated in full text.
   **Answer:** Sorry for my mistake. The abbreviations have been stated for the first time.

8. The citation of references in Results is not appropriate.
   **Answer:** I have checked the citation of references, and revised this mistake it in the revised manuscript.

9. In figure 1 the meanings of the asterisks (* and **) should be clearly stated as done for the other figures.
   **Answer:** Sorry for my careless. I have added the meaning of “*” and “**” in the
Figure 1.
In revised manuscript: *$p<0.05$, **$p<0.01$ versus the control group.

10. The values stated in the Results Section were Mean ± SEM but in the Methods Section (under statistical analysis) they were supposed to be Mean ± SD.
Answer: Sorry for my careless. It should be Mean ± SD. We have revised this mistake it in the revised manuscript.

11. Rephrase the section “Chick chorioallantoic membrane (CAM) assay” to clearly indicate the age of the chick embryos before experimentation and the duration of the various incubation.
Answer: We have re-written this part according to the Reviewer’s suggestion.

Discretionary Revisions
Change the word “fluid” in Preparation of TF-SB from Scutellaria barbata D. Don to “infusion”.
Answer: I have revised this mistake in the manuscript.