Author's response to reviews

Title: Potent alpha-glucosidase and alpha-amylase inhibitory activities of standardized 50% ethanolic extracts and sinensetin from Orthosiphon stamineus Benth as anti-diabetic mechanism

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Version: 3 Date: 3 September 2012

Author's response to reviews: see over
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Version: 2 Date: 24 August 2012
Reviewer’s report (reviewer 1)

Title: Potent alpha-glucosidase and alpha-amylase inhibitory activities of standardized 50% ethanolic extracts and sinensetin from Orthosiphon stamineus Benth as anti-diabetic mechanism

Version: 1 Date: 11 June 2012

Reviewer: Prasenjit Manna

Reviewer’s report:

Major Comments

The authors did not include the inhibitory effect of two other compounds, eupatorin and 3-hydroxy-5,6,7,4-tetramethoxflavone, which they identified as the active components of the extract in their earlier investigation using diabetic animal model. It would be helpful for the reader to compare the antidiabetic effect of sinensetin with other active components of the extract.

Thank you for the suggestions, basically this manuscript is one part of the whole study, we studied the antidiabetic activity of O. stamineus base on bioassay-guided fractionation and isolation principle. Base on the HPLC profile (unpublished) as below, after sub-fractionation we found that content of sinensetin is hight (arrow), so for the reason why we isolate sinensetin for this in vitro study. The result support the finding of sinensetin is one of the active ingredient for antidiabetic activity.
The authors should include the NMR as well as HRMS figures of isolated sinensetin. Similar isolation has been published before from our research team, it is not nice to publish similar data again even though different isolation method and bioactivity were study. For the proof please find the NMR profile as below:

Table 1: $^1$H-NMR spectral data for sinensetin, (400 MHz, CDCl$_3$)

<table>
<thead>
<tr>
<th>Proton</th>
<th>Sinensetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-3</td>
<td>6.61 (1H, s)</td>
</tr>
<tr>
<td>H-8</td>
<td>6.81 (1H, s)</td>
</tr>
<tr>
<td>H-2'</td>
<td>7.35 (1H, s)</td>
</tr>
<tr>
<td>H-5'</td>
<td>6.99 (1H, d, J=8.54 Hz)</td>
</tr>
<tr>
<td>H-6'</td>
<td>7.52 (1H, d, J=8.47 Hz)</td>
</tr>
<tr>
<td>3'-OMe</td>
<td>4.00 (3H, s)</td>
</tr>
<tr>
<td>4'-OMe</td>
<td>3.98 (3H, s)</td>
</tr>
<tr>
<td>5-OMe</td>
<td>3.89 (3H, s)</td>
</tr>
<tr>
<td>6-OMe</td>
<td>3.94 (3H, s)</td>
</tr>
<tr>
<td>7-OMe</td>
<td>4.01 (3H, s)</td>
</tr>
</tbody>
</table>

Table 4.12: $^{13}$C-NMR spectral data for compound NC 2 (100 MHz, CDCl$_3$).

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Sinensetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2</td>
<td>162.1</td>
</tr>
<tr>
<td>C3</td>
<td>108.4</td>
</tr>
<tr>
<td>C4</td>
<td>178.1</td>
</tr>
<tr>
<td>C5</td>
<td>152.9</td>
</tr>
<tr>
<td>C6</td>
<td>141.4</td>
</tr>
<tr>
<td>C7</td>
<td>157.8</td>
</tr>
<tr>
<td>C8</td>
<td>97.3</td>
</tr>
<tr>
<td>C9</td>
<td>154.8</td>
</tr>
<tr>
<td>C10</td>
<td>108.1</td>
</tr>
<tr>
<td>C1'</td>
<td>121.2</td>
</tr>
<tr>
<td>C2'</td>
<td>109.8</td>
</tr>
<tr>
<td>C3'</td>
<td>149.9</td>
</tr>
<tr>
<td>C4'</td>
<td>150.3</td>
</tr>
<tr>
<td>C5'</td>
<td>112.2</td>
</tr>
<tr>
<td>C6'</td>
<td>120.6</td>
</tr>
<tr>
<td>OCH$_3$1</td>
<td>63.2*</td>
</tr>
<tr>
<td>OCH$_3$2</td>
<td>62.5*</td>
</tr>
<tr>
<td>OCH$_3$3</td>
<td>57.3*</td>
</tr>
<tr>
<td>OCH$_3$4</td>
<td>57.2*</td>
</tr>
<tr>
<td>OCH$_3$5</td>
<td>57.1*</td>
</tr>
</tbody>
</table>

* These assignments may be interchanged
We do not analyze the HRMS of the isolated compound but we study it mass spec with LCMS/MS. For the proof, below are the profile:

The column used for chromatographic analysis is Phenomenex Syngeri, 4um MAX-RP 80A (30 mm x 2 mm). The LCMS/MS model is Varian 320MS.

Method:
Mobile phase: Solvent A- 0.1 % acetic acid in deionized water
                Solvent B- Acetonitrile
Flow rate: 0.2 mL/min
Isocratic run program: 65 % solvent A and 35 % solvent B
Total run time: 5 minutes

MS/MS parameter:
Ion source: ESI
ESI needle voltage: 5250 V
Drying gas temperature: 300 C
pressure: 19 psi
Nebulizer gas pressure: 50 psi
Parent ion: 373.8
Product ion: Ion Collision Energy (CE) Capillary voltage

<table>
<thead>
<tr>
<th>Parent Ion</th>
<th>Product Ion</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>373.8</td>
<td>312.1</td>
<td>-22.5 V</td>
</tr>
<tr>
<td>339.9</td>
<td>-24.5 V</td>
<td></td>
</tr>
<tr>
<td>343.1</td>
<td>-26.0 V</td>
<td></td>
</tr>
<tr>
<td>357.7</td>
<td>-22.0 V</td>
<td></td>
</tr>
</tbody>
</table>

Standard (sinensetin purchased from Sigma)                      Isolated compound (sinensetin)

Both show the same profile.
In the present study, there is no statistical analysis of the experimental data. The authors should include the statistical analyses of their data to investigate at which dose sinensetin shows significant inhibitory effects.

Thanks for the suggestion, we have used one way anova followed by turkey comparison test to compare the IC50 of the different groups.

In their previous work, the authors established the antidiabetic effect of O.stamineus and they also reported the presence of the active ingredients, mentioned above. In this context it would be good to include any kind of cell culture studies or in vivo studies to investigate the antidiabetic effects of those isolated compounds.

We are working to synthesis sinensetin in large quantity for in vivo study, we are also planning to use in vitro cell culture method to study the activity of this compound.

Oxidative stress plays an important role in the pathogenesis of diabetic complications and flavonoids are well known antioxidant. The authors may wish to include the free radical scavenging effect of those compounds both in cell free system as well as in cellular system.

We did DPPH scavenging test for the identified flavonoids including sinensetin, eupatorin, tetramethoxyflavone but the antioxidative active is very low, so we did not further the study.

Level of interest: An article of importance in its field

Quality of written English: Acceptable
Author's response to reviews

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Version: 2 Date: 24 August 2012
Reviewer’s report (Reviewer 2)

Title: Potent alpha-glucosidase and alpha-amylase inhibitory activities of standardized 50% ethanolic extracts and sinensetin from Orthosiphon stamineus Benth as anti-diabetic mechanism

Version: 1 Date: 15 June 2012

Reviewer: Murad Faris

Reviewer’s report:

This paper described an interesting biological study (glucosidase and amylase inhibitory activity) of an ethanolic extract of Orthosiphon stamineus and one isolated compound, sinensetin with the aim to explain the mechanism of action of the antidiabetic activity.

This paper is acceptable for publication in BMC Alternative and complementary medicine after the following points have been taken into consideration.

1. What is the rationale that ethanolic extract, but not aqueous extract was examined in the current study?

   From our HPLC study, we found that the standard marker are highly found in 50% ethanol extract compare to other extract, and 50% ethanol extract was the more active among the other extracts in preliminary screen so we choose 50% for the study.

2. What about the effects of other fractions?

   Other extract are less potent compare to 50% ethanol extract, and the extract was then fractionated into chloroform, ethyl acetate, methanol and water. Among these fractions, ethyl acetate was the most active and contain high sinensetin and subjected for isolation.

3. Does ethanolic extract of Orthosiphon stamineus reveal more potent anti-hyperglycemic effects than its aqueous extract in in vivo experiments?

   Yes, in our experiment, we are not sure compare to Mariam et al., 1996 result.

4. What is the rationale that ethanolic extract, but not aqueous extract was examined in the current study?

   From our HPLC study, we found that the standard marker are highly found in 50% ethanol extract compare to other extract, and 50% ethanol extract was the more active among the other extracts in preliminary screen so we choose 50% for the study.
5. What about the effects of other fractions?

Other extracts are less potent compared to 50% ethanol extract, and the extract was then fractionated into chloroform, ethyl acetate, methanol, and water. Among these fractions, ethyl acetate was the most active and contained high sinensetin and subjected for isolation.

6. Does ethanolic extract of Orthosiphon stamineus reveal more potent anti-hyperglycemic effects than its aqueous extract in in vivo experiments?

Yes, in our experiment, we are not sure compared to Mariam et al., 1996 result.

In summary, it is an interesting finding that ethanolic extracts and sinensetin from Orthosiphon stamineus Benth exerts #-glucosidase and #-amylase inhibitory activities in vitro.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: Yes, and I have assessed the statistics in my report.

Declaration of competing interests:

I declare that I have no competing interests