Author’s response to reviews

Title: Effects of unaltered and bioconverted mulberry leaf extracts on cellular glucose uptake and antidiabetic action in animals

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Response to Reviewer’s Comments on Manuscript “BCAM-D-18-01249R1”

General Comments – We thank the reviewers for their positive consideration of our manuscript (BCAM-D-18-01249R1). We have addressed all issues raised by reviewers with adding some information, reformatting the texts, and changing some sentences to improve the presentation. All changes were highlighted and all page numbers noted below refer to the revised version. We hope that these efforts will make the points to clear to the reader and improve the overall presentation of the data.

Comments & Issues Answers & Modification

Reviewer 1

1) The method for preparation of bioconverted mulberry leaf extract (BMLE) with VIS is not clear. What is the VIS (full name)? What is its principle for bioconversion?
2) In the methods section, it mentioned that the ITT followed the same protocol as the OGTT, except for the subcutaneous administration of insulin (1 U/kg) 30 min after glucose injection. Why glucose is injected, but not oral such as OGTT?

3) This study used NA+STZ+HFD to induce obese type 2 diabetic mouse model. However, the results showed that was not a type 2 diabetic model, because the blood glucose level was higher to 400 mg/dL (Fig. 3B) and the plasma insulin level was lower than that in control mice (Fig. 5B). Therefore, the type 2 diabetic condition was exactly not to be induced successfully. It is more like type 1 diabetic state. The dosage of STZ (100 mg/kg) may be too high.

4) The results in in vitro experiments are still not convincing. The increased glucose uptake levels under insulin stimulation by tested compounds compared to insulin alone group in skeletal muscle cells or adipocytes are really limited (Figs. 1C, 2A, B). Similarly, the increased insulin secretion levels under glucose stimulation by tested compounds compared to glucose alone group in beta-cells are also limited (Fig. 2C).

5) In Table 1, the name of compounds should be listed, but not number 1 and 2.

6) In Fig. 3 legend, the descript “After checking for high blood pressure, mice were…” is not correct, which blood glucose is checked but not blood pressure.1) Thank you for your comments. We described the principle of bioconversion in the body of manuscript (lines 61-63) and how to perform bioconversion in lines 126-132. We added new information about VIS, Viscozyme L, in lines 71-74 with new reference (24) and lines 82-85.

2) Thank you for your comment. The ITT followed the same protocol as the OGTT, except for the subcutaneous administration of insulin (1 U/kg) 30 min after glucose administration (2 g/kg, PO). We changed this information in line 183 of page 7.

3) We previously reported that the co-administration of STZ and NA with or without HFD exerted type 2 diabetic condition which is different from type 1 diabetic condition induced by STZ administration (Choi et al., Arch Pharm Res 2011; Nan et al., J Drug Target 2010). To confirm whether a type 2 diabetes condition was established in animals,
OGTT was performed and the results were compared with type 1 diabetic mice induced by STZ administration (please see Additional file 1). Thus, STZ-NA-induced diabetic mice had different conditions from STZ-induced ones, indicating a type 2 diabetes which was similar to previous reports. Furthermore, our condition looks similar to that in several reports using STZ-NA-induced type 2 diabetic mice (Arya et al., J Ethnopharmacol 2015; Matsuyama-Yokono et al., Metabolism 2009; Tahara et al., Eur J Pharmacol 2011).

4) Yes, the rate for increasing glucose uptake into cells and releasing insulin out of cells is so limited in vitro condition. Each cell might have the limited capacities for uptaking glucose and releasing insulin stimulated by insulin or glucose. However, the results produced by repeated experiments showed statistically significant which might have good physiological relevance. Our previous reports (Han et al., J Nat Prod 2015; Han et al., PLoS One 2014) also showed these limitations, and the results still showed statistically significant. If the condition is in vivo, the increasing values might be much bigger, however, owing to each cell’s capacity, the values might be limited.

5) We changed numbers to the name of compounds, trans-caffeic acid and syringaldehyde in Table 1.

6) We changed hypertension to glucose in Fig. 4 legend. We added new experimental data in new Fig. 3.

Reviewer 2

1) Based on the dramatic reduction of body weight and increase in blood glucose concentrations following the STZ and NA administration as shown in Fig. 3A, these results indicate that the diabetes induced in this study is more likely to be a type 1 DM. The authors need to carefully interpret results obtained in this study.

2) Many mistakes and contradictions appear in the experimental methods. For instance, Ŷ texts in the section of “Induction of obese type 2 diabetic mice” (lines 142-151) differ from that seen in the legend of Fig. 3 (lines 577-580). STA and NA are given at the 1st week (legend of Fig. 3) or the 9th week (line 144).
Lines 147-150 are hard to realize how animals are grouped. Rewritten of this section is needed. In addition, MLE and BMLE are given for a time period of 6 weeks, but not for 7 weeks (line 151).

BMLE and MLE are given by oral route (line 151) or injection (line 580).

According the standard protocol, it should be the “confluence” 3T3-L1 preadipocytes are used for initiation of the differentiation of adipocytes (line 204).

3T3-L1 cells are grown in DMEM with 10% FBS (line 208) or 1% FBS (line 216).

“Mean ± standard error of the mean” appear twice in lines 229-230.

Diabetes animal model is not simply induced by STZ and NA, HFD-induced obesity is also employed in this study. This needs to be addressed in the abstract.

Add 3T3-L1 adipocytes in line 80.

Move “unaltered mulberry extract” appeared in line 84 to line 81.

Fig. 6A and B, significant symbols need to be added in case of 600 mg/kg BMLE is significantly higher than that noted in the MLE-treated mice.

Briefly describe the characteristics of VIS in the Materials and Methods.

The treating time for trans-caffeic acid and syringaldehyde as well as rosiglitazone and GLM needs to be given in the section of “glucose uptake measurement” and/or the legend of Figs. 1 and 2.  
1) We previously reported that the co-administration of STZ and NA with or without HFD exerted type 2 diabetic condition which is different from type 1 diabetic condition induced by STZ administration (Choi et al., Arch Pharm Res 2011; Nan et al., J Drug Target 2010). To confirm whether a type 2 diabetes condition
was established in animals, OGTT was performed and the results were compared with type 1 diabetic mice induced by STZ administration (please see Additional file 1). Thus, STZ-NA-induced diabetic mice had different conditions from STZ-induced ones, indicating a type 2 diabetes which was similar to previous reports. Furthermore, our condition looks similar to that in several reports using STZ-NA-induced type 2 diabetic mice (Arya et al., J Ethnopharmacol 2015; Matsuyama-Yokono et al., Metabolism 2009; Tahara et al., Eur J Pharmacol 2011).

2) We changed or modified all information as follows:

- Ÿ sentence in Fig. 4 (we added new experimental data in new Fig. 3) legend in lines 614-616 in page 21.
- Ÿ We added new “Experimental design” part in Methods section in lines 159-170 to make clear. All reagents or vehicles were daily oral-administered to animals in all groups for 7 weeks and this information also added in lines 170-171 of page 7.
- Ÿ Both MLE and BMLE were orally administered. We changed the sentence in line 617 of page 21.
- Ÿ Yes, we added this information in line 226 of page 9.
- Ÿ 3T3-L1 preadipocytes were grown in 10% calf serum and then changed to 10% FBS to induce differentiation.
- Ÿ We changed this in line 266 of page 10.

3) Yes, we added these information in lines 33 and 35 in Abstract section.

4) We added 3T3-L1 adipocytes in line 86 in Introduction section.

5) Thank you for your comment. We rewrote the sentence in lines 85-89 in Introduction section.

6) Thank you for your comment. We added symbols in Fig. 7.
7) We added new information about VIS, Viscozyme L, in lines 71-74 with new reference (24) and lines 82-85.

8) We added these information in legends of Figs. 1 and 2.

Reviewer 3

1) It is suggested that the authors should provide more relevant experimental data or references to illustrate the antidiabetic activity of major active compounds from bioconverted mulberry leaf extracts. If more data and more discussion, the manuscript should be better.

To provide the mechanism of antidiabetic action of two active compounds, trans-caffeic acid and syringaldehyde, we performed new experiment using Western blot. The results shown in Fig. 3 presented that trans-caffeic acid increased insulin-stimulated Akt phosphorylation, which could activate the translocation of GLUT-4 from cytosol to membrane. We added the results in Fig. 3.