Author's response to reviews

Title: Alpha-Glucosidase Inhibitory Activity and Antioxidant Activity of Flavonoids Compound and Triterpenoid Compound from Agrimonia Pilosa Ledeb.

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Author's response to reviews: see over
Nov 10, 2013
Dear Editor,
We are highly appreciated for these helpful reviews from you and the reviewers about our manuscript entitled “α-Glucosidase Inhibitory Activity and Antioxidant Activity of Flavonoids Compound and Triterpenoid Compound from Agrimonia Pilosa Ledeb.” (MS: 4023323951046452). We have revised carefully for whole manuscript according to reviewers’ comments. The major revised portions and response to reviewers’ comments were listed below. We hope that the revised manuscript is acceptable for publication.
Thank you very much for your continued attention.
Best wishes,

Liancaï Zhu

Response to reviewers’ comments:

Reviewers' comments:
Reviewer #1: Major Compulsory Revisions
1. Additional experiments should be done in compounds analysis.
Answer: The compounds in TC and FC have been analyzed by using HPLC. In FC, 10 flavonoids were identified and quantified (See Table 1 and Figure 1) and 6 triterpenoids in TC were identified and quantified (See Table 2 and Figure 2).

2. There are no correlation statics, so the conclusion is uncorrect.
Answer: When we study the bioactivities of one compound, we only need to test the activities of the compound at different concentrations and the correlative analysis isn’t essential.

3. The total flavonoids content in FC is 316.53±6.37 mg/g by using the aluminum chloride colorimetric method with quercetin as a standard. And the total triterpenoid content in TC is 415.97 ± 5.15 mg/g with Ursolic as a standard. What other components are? So, the author could not conclude that flavonoids and triterpenoid responde for that bioactivities.
Answer: In this paper, we put forward that the bioactivities is attributed to the FC and TC. FC consists of some flavonoids and other ingredients, and TC consists of some triterpenoids and other ingredients. So we think that FC or TC as a whole, not just flavonoids or triterpenoids, are responsible for the good clinical effects of APL on T2MD through targeting oxidative stress and postprandial hyperglycaemia.

Reviewer #2:
1. Many minor errors are revised in PDF, please check and revise.
Answer: Some wrong English grammars have been modified, and some incorrect expressions have been corrected in the relevant places.

2. Time collected plant sample and identifier should be given.
Answer: The dried entire plants of APL were purchased from Western Medicine City (Chongqing, China) in 2011 and verified by Changhua Wang (Chongqing Academy of Chinese Materia Medica, China). We have added these contents in the section “Plant materials and preparation of the extract”.

3. Reference “Antioxidant Activities of Aqueous Extract from Agrimonia pilosa Ledeb and Its Fractions” was reported by author in Chemistry and Biodiversity, 2009. which is the difference between both works?
Answer: The differences between both works are as follows:
(1) Their subjects are different. In the reference article, the aqueous extract (AE) from Agrimonia pilosa Ledeb were partitioned successively by petroleum ether, AcOEt, and BuOH to the corresponding soluble fractions. The subjects were AE and its fractions. In this paper, we separated and enriched flavonoids compound (FC) and triterpenoid compound (TC) from Agrimonia pilosa Ledeb by using liquid chromatogram as subjects.
(2) Their contents are different. In the reference article, we only studied the antioxidant activities of AE and its fractions. In this paper, we played emphasis on the \( \alpha \)-glucosidase inhibitory activity of FC and TC from Agrimonia pilosa Ledeb, which was reported for the first time. Our study clarified that the FC and the TC could be responsible for the good clinical effects of APL on T2MD through targeting oxidative stress and postprandial hyperglycaemia.
4. The method for FC and TC collected is difficult to understand. How to determine that the method can collect all or partly FC and TC?

**Answer:** (1) We have drawn a diagram to clarify the collected procedure of FC and TC, and please see Figure 1. (2) In the entire collection process, thin layer chromatography control with 10% sulfate in ethanol as color-developing agent was adopted in order to collect all flavonoids compounds and triterpenoid compounds.

5. Phytochemistry for this plant is abundance in reference, especially triterpenoids and flavonoids, compounds in TC and FC should be analyzed using HPLC or HPLC-MS. It is essential for publication when compounds are determined in FC and TC, activities for antioxidant and α-glucosidase inhibitory activities should be discussed to elucidate the clinical effects of Agrimonia pilosa.

**Answer:** The monomeric compounds in TC and FC have been analyzed by using HPLC. In FC, 10 flavonoids were identified and quantified (See Table 1 and Figure 2) and 6 triterpenoids were identified and quantified in TC (See Table 2 and Figure 3). And the activities of these quantified flavonoids or triterpenoids have been discussed.

**Reviewer #3**

In my opinión this manuscript needs a major compulsory revisión.

The sections "Methods" and "Results and Discussion" should be rewritten, because there are a lot of inaccuracies, particularly in relation to the fractionation process, isolation and thin layer chromatographic identification of fractions FC and TC (?). The manuscript does not explain how to prepare the stock solutions for use in various tests. Likewise, it is not explained what it is and what implications mixtures FC: TC 4:1, 1:1 and 1:4. In Figures, the results are presented as data outside of the scale level, or simply in a distorted scale.

In my opinion, this article should be re-written and must be resubmitted for review. It is not publishable without further major review.

**Answer:**

(1) The sections "Methods" and "Results and Discussion" has been checked and revised. In this paper, the fractionation was NOT used. In addition, the isolation process has been rewritten and a diagram to clarify the collection procedure of FC and TC has been drawn in Figure 1.

(2) The preparations of test samples including solvent and concentration have been
clarified in the section "Methods".

(3) The sentence “mixtures FC: TC 4:1, 1:1 and 1:4” has been revised as “three complexes with mass ratio of FC and TC as 4:1, 1:1 and 1:4”.

(4) All the figures have been re-drawn in order to avoid data outside of the scale level.

Reviewer #4:

(1) The chemistry part of the paper has either not been described in full or kept untold. What is the authenticity of calling two fractions a flavonoid and triterpenoid fractions unless those were further purified? What is the purity of the fractions?

Answer: (a) The chemistry researches about FC and TC have been added by using HPLC. In FC, 10 flavonoids were identified and quantified (See Table 1 and Figure 2) and 6 triterpenoids were identified and quantified in TC (See Table 2 and Figure 3).

(b) FC and TC had been purified during the process of collection (see Figure 1). (c) The percentage of flavonoids in FC is 31.6%, indicating that the flavonoids are the dominant component. And the percentage of triterpenoids in TC is 41.6%, indicating that the triterpenoids is the dominant component.

(2) Over two decades, “α–Glucosidase inhibitory activity of many crude preparations from various medicinal plants have been reported with IC50 values. Where is the position of the crude from the plant Agrimonia pilosa ladeb? Does the preliminary data could justify further processing of the crude for the identification of any new bioactive molecule?

Answer: (a) FC and TC from Agrimonia pilosa ladeb showed exciting inhibitory effects on α-Glucosidase with IC50 values of 8.72µg/mL and 3.67µg/mL respectively compared with the crudes from other plants such as the 50% ethanolic extract from Orthosiphon stamineus Benth with IC50 values of 4.63mg/mL (Mohamed EAH, et al. BMC Complement Altern Med., 2012,12:176), Nelumbo nucifera leaf flavonoids with IC50 values of 1.86mg/mL (Liu S, et al., J Ethnopharmacol., 2013, 149(1): 263-269.), the extract from Moringa oleifera leaf with IC50 value of 0.78 mg/mL (Adisakwattana S, et al. Eur Rev Med Pharmcol Sci, 2011, 15(7):803-808) and so on. (b) Yes, our data can.

(3) EC50 and IC50 Value TC and FC fractions were not compared with those of drugs already used T2 diabetes therapy. The statement of “excellent” or “good” has no
value unless values were compared with those of drugs.

**Answer:** (a) In radical scavenging assays, the positive control substances were used for appraising the radical scavenging activities of TC and FC. (b) In inhibition assay for α-glucosidase activity, the pNPG method was adopted. The pNPG method has been so far riper that the positive control drug could not be indispensable in researches such as the literatures “In vitro inhibitory effects of plant-based foods and their combinations on intestinal alpha-glucosidase and pancreatic alpha-amylase” (Adisakwattana S et al. BMC Complement Altern Med. 2012, 31(12):110), “Changes in phytochemical compositions, antioxidant and α-glucosidase inhibitory activities during the processing of tartary buckwheat tea” (Qin PY et al. Food Res Inter, 2013, 50(2):562-567).

(4) The whole work was done with yeast α-Glucosidase as target enzyme. Use of Mammalian intestinal enzyme, available commercially, as target enzyme is more justified.

**Answer:** As mammalian intestinal enzyme, yeast α-Glucosidase is also used commonly for evaluating the bioactivities of α-Glucosidase inhibitors in many researches such as “Screening α-glucosidase inhibitor from natural products by capillary electrophoresis with immobilised enzyme onto polymer monolith modified by gold nanoparticles” (Zhang AZ. et al. Food Chem, 2013, 141(3):1854-1859), “Comparison of Inhibitory Activities and Mechanisms of Five Mulberry Plant Bioactive Components against alpha-Glucosidase”(He H et al. J Agric Food Chem. 2013, 61(34):8110-8119) and so on.

(5) Authors have isolated a few grams of TC and FC from 2kg of plant and determined EC50 and IC50 values of those fractions. Unfortunately authors have not indicated how many inhibitory units were originally present in whole plant.

**Answer:** The inhibitory units presented in whole plant isn’t essential because that we will take TC and FC as the subjects to develop functional food or drugs for T2DM and to isolate the active components.

(6) No data presented on “in vivo” activity of FC or TC fraction on rat/mouse Model. Is there any data available on the toxicity level of the plant extract?

**Answer:** The object of this study is to clarify the mechanism and the substance base
of *Agrimonia pilosa* Ledeb treating type 2 diabetes mellitus *in vitro*. And our study showed that the FC and the TC could be responsible for the good clinical effects of APL on T2MD through targeting oxidative stress and postprandial hyperglycaemia. Further investigations will be carried out to appraise the effect and the safety *in vivo*. 