Author's response to reviews

Title: Antioxidant, alpha-glucosidase inhibitory activity and sub-chronic toxicity of Derris reticulata extract: its antidiabetic potential

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Author's response to reviews: see over
Dear Editor:

We are submitting our revised manuscript, entitled “Antioxidant, α-glucosidase inhibitory activity and sub-chronic toxicity of Derris reticulata extract: its antidiabetic potential”. We thank the reviewers for their constructive comments, and have revised the manuscript in response to the suggestions in red font, as detailed on the separate pages. Thank you for your consideration of our revised manuscript.

Sincerely yours,

Nuannoi Chudapongse, Ph.D.
Associate Professor
Responses to the Reviewers' comments: Reviewer; **Oyinlola O. Olaokun**

(1) In section 2.4.2 in the last line “ABTH” ought to be “ABTS”.
   We have corrected the misspelling as suggested.

(2) In section 2.5 in the second paragraph, authors did not cite source for method used for the assay.
   We have added the original reference that we followed their method to analyze cell viability, instead of our previous published paper.

(3) In section 3.4, the IC50 for acarbose (1.378.99 ± 0.13 mg/ml) is quite high, indicating that the positive control was not active. Studies have reported that acarbose may not be the appropriate positive control when using *S. cerevisiae* as source of α-glucosidase. Acarbose only weakly inhibit or is inactive against the enzyme. Thus the conclusion of extract’s inhibitory potential may not be valid. Authors may have to re-validate with α-glucosidase from other sources.

There is a typing error in the above comment which said that the IC50 for acarbose was 1.378.99 ± 0.13 mg/ml. So we are not sure whether the reviewer think that the IC50 of acarbose was 1,378.99 mg/ml which we accept that it is too high. In fact, the IC50 of acarbose found in our study is 1,378.99 µg/ml (or 1.379 mg/ml). In case the reviewer think that 1,378.99 µg/ml is still too high, we would like to explain as the followings.

Yeast α-glucosidase from *S. cerevisiae* has been widely used for studying inhibitory effect of several antidiabetic compounds including plant extracts (Choi et al., 2010, Mohamed Sham Shihabud et al., 2011; Elsnoussi et al, 2012; Kim et al., 2013; Shai et al., 2010). There is evidence showing that acarbose inhibits both intestinal and yeast α-glucosidase with the IC50 of 34.11 and 36.89 µg/ml, respectively (Mohamed Sham Shihabud et al., 2011). However, the IC50 of acarbose against yeast α-glucosidase greatly varied among laboratories from about 40 µg/ml (Mohamed Sham Shihabud et al., 2011) up to 17 mg/ml (Shai et al., 2010), probably depend on the different activities of enzyme, sources of acarbose, and conditions used. The IC50 of acarbose found in our study (about 1.38 mg/ml), was similar to (or lower than) the results previously reported by several different laboratories. For example, Elsnoussi et al. (2012) reported in this journal, BMC
Complementary and Alternative Medicine, that the IC50 of acarbose against yeast α-glucosidase enzyme was found at 1.93 ± 0.281 mg/ml whereas that of acarbose found by Choi and coworker (2010) was 5.88 mg/ml (9.11 mM). Kim et al. (2010) showed that 500 µg/ml of acarbose inhibited yeast α-glucosidase activity only 29.6 ± 1.1%. We would like to argue that yeast α-glucosidase from *S. cerevisiae* should be acceptable for studying the effect of antidiabetic agents on α-glucosidase activity.

**References**


(4) In the section of Figure legends, Figure 1 was not clearly described. Figure 1 has two sub-sections; 1A and 1B but this has not reflected in the description of the Figure. The authors should note where the description of Figure 1A starts and label appropriately and the same should be done for Figure 1B.

We have revised the legend of Figure 1 as suggested.
Responses to the Reviewers' comments; Reviewer: David Katerere

(1) Ln 65 – 66 – Re-phrase this sentence.
We have re-phrased the sentence in lines 65-66 as suggested.

(2) Ln 72 – Were standards used to validate the phytochemistry screening assays; if so, which ones?
Usually, in phytochemistry screening, standard agents are not required to validate each groups of phytochemicals. Each chemical reaction recommended is specific for functional group in a particular phytochemistry. So we did not use any standards in these methods.

(3) In a previous paper by the same authors (J. Physiol. Biochem 2014) an oral toxicity study was performed, how does this differ from what is reported in this paper?
In our previous report, the toxicity test was acute toxicity which is aimed to find LD50 or median lethal dose, whereas in the present study, sub-chronic toxicity was tested. Sub-chronic toxicity test is aimed to determine the toxic effect of longer exposure of agents.

(4) Ln 281 – Saponins and terpenoids are generally not associated with anti-oxidant activity
We have removed saponins and terpenoids from the sentence as suggested (page 9 line 288).

We have added discussion about phytochemistry of D. reticulate Benth including that of other plants in Derris genus on page 9 lines 278-286.
This is a controversial point “May cause fewer clinical events of hypoglycaemia”
This cannot be an advantage because the whole treatment of diabetes is to induce normoglycemia by secreting insulin. It appears that D. reticulate has a different mode of action i.e. glucosidase inhibition which would be useful in management of Type 2 Diabetes.

Now, there are 9 groups of oral drugs for the treatment of diabetes. They are sulfonylureas, glitinides, biguanides, α-glucosidase inhibitors, thiazolidinediones, GLP-1 receptor agonists, DDP-4 inhibitors, amylin analog and bile acid-binding sequestrant (Nolte Kennedy, 2011). The main objective of antidiabetic drugs is glycemic control to euglycemia, definitely not hypoglycemia. Only the first two groups mentioned are insulin secretagogues of which mechanism are an increase of insulin secretion from beta cell of pancreas. With this mechanism, the serious side effect of these groups of antidiabetic drugs is hypoglycemia, especially in elder patients. The other groups of antidiabetic drugs are less likely to cause hypoglycemia. So any drugs which produce less hypoglycemic event is claimed to be advantage over insulin secretagogues such as sulfonylureas which cause the highest incidence of hypoglycemia. Note that an α-glucosidase inhibitor itself rarely causes hypoglycemia.

References

(7) 347 Cytoprotective effect of D. reticulata. This is another controversial point. Alloxan induces Type I diabetes (which has a genetic basis and not due to chemical insult / oxidative stress), so is it scientifically valid to use a cytoprotective drug in the management of Type I diabetes? This should be discussed and supported by relevant literature

Type I diabetes usually is severe and insulin dependent type. Without receiving exogenous insulin, the patients or animals cannot survive even taking oral hypoglycemic drugs. There is a wide variability in the dose of alloxan required to produce diabetic state. Different doses of alloxan produce varying intensities of hyperglycemia depend on the severity of cell damage. It has been suggested in animal model that rats with fasting
blood glucose level between 150 and 250 mg/dl are considered as mild diabetic (resemble type II diabetes) and severe diabetic rats (resemble type I diabetes) have fasting blood glucose level more than 250 mg/dl (Gupta et al., 2009). In this study, the dose of alloxan used and the duration of exposure did not cause damage of all cultured cells. In addition, the results from our previous study in vivo showed that the extract prevented further degeneration of islet cells in alloxan-induced diabetic rats. There were several studies using alloxan-induced diabetic rats to study protective effect of plant extracts (Ju et al, 2008; Ramkumar et al., 2009; Shabeer et al., 2009; Venkatesh et al., 2010). We explain this issue in more detail in our response to the editor’s comment.

References


Responses to the Editor's comments:

1) The question raised by the reviewer on the clinical relevance of the data in a Type-II diabetic patient, where the damage is already present needs to be discussed (aloxan type chemical damage is not clinically relevant in the usual pathophysiology of the disease). One would assume that an anti-oxidant would be too late for therapeutic success.

Type-II diabetes is characterized by hyperglycemia, resulting from either a decrease of insulin release from pancreas or insulin resistant. It usually takes long period of time to develop as its occurrence mainly in people over 40. It has been suggested that reduced insulin production and release by the pancreas is probably due to the death of the organ's beta cells (Maecher et al.,1999; Robertson et al., 2004). One important mechanism of cell apoptosis is excessive generation of reactive oxygen species produced in our own body which may occur continuously through life time. This suggests that an anti-oxidant from plants would be useful to promote β-cell regeneration and protection (Zhang et al., 2010; Gandhi et al., 2011). Note that aloxan is widely used as diabetic inducing agent in animal model because it produces reactive oxygen species to damage beta cells of pancreas.

References


2) I would like to see more discussion of the in vitro effects in relation to the tox study. The product appeared to have no toxic effect, yet it physiologically meant to alter glucose
digestion. How can the animals remain healthy and have normal parameters if its prime mechanism is meant to be an alteration in oral glucose absorption i.e after 42 day one would expect some changes

The inhibition of α-glucosidase activity by an inhibitor results in a delay of carbohydrate digestion. It does not completely stop such digestion. The delay of carbohydrate digestion prevents rapid increase in blood glucose level after meal. The question why animals remain healthy after oral administration of the extract would be best explain by using the case of acarbose.

Acarbose is a competitive inhibitor of α-glucosidase which is clinically used as chronic treatment of type II diabetic patients. The most common side effects caused by acarbose is gastrointestinal upset. The most serious side effect of acarbose is that it may cause hepatic enzyme elevation. This toxicity was found after several large clinical trials have been done. However, these studies reported no occurrence of clinically apparent liver injury. So acarbose is recommended to use with caution in patients with hepatic disease as it is relatively safe for uncomplicated patients. Please note that the alteration of hepatic enzyme by acarbose occurs with unknown mechanism, not by the alteration of carbohydrate digestion (http://livertox.nih.gov/Acarbosee.htm).

Some of parameters in the present study were higher than control group, however, all of them were within the normal ranges as we already indicated in the Tables 2 and 3.

3) Please justify why the tox study did not include insulin and an oral glucose tolerance test as a marker

We did measure fasting blood glucose level and found that all animals that received the extract even at the highest dose had normal level of blood glucose. So the measurement of blood insulin was not considered. In addition, based on the guidelines of the OECD (2008), only overnight fasting glucose level, BUT NOT the level of insulin and oral glucose tolerance test, is recommended for any toxicity tests, including acute, sub-acute, sub-chronic and chronic toxicity tests.

4) Please justify why an incomplete sub-acute toxicity was undertaken: Histopathology- these studies require an evaluation of all organs, and not just liver and kidney; clinical
pathology parameters monitored: Urea, creatine, albumin are some of the pivotal markers. I fail to see how this study can justify a statement that the product is safe. At best, all it can show is that no changes occurred in the parameters monitored.

The measurement of parameters in toxicity study varies in different laboratories depend on the facilities, instruments and funding budget. We do agree that complete report on all parameters are the best. However, due to our budget limitation, we chose to measure what are most important and acceptable. Urea (blood urea nitrogen; BUN) and creatinine are useful indicators of renal health. The best is to examine both parameters, however, some study measured only BUN (Andreia et al., 2014), whereas some study analyzed only creatinine (Mukinda and Eagles, 2010). We chose to measure the level of creatinine to study the effect on kidney as shown in Table 4 due to the instruments we have. In the case of albumin, even though it is recommended by the guidelines of the OECD (2008), alteration of albumin level by drugs or medicinal plants does not occur frequently so several reported studies on toxicity did not measure albumin level, for example the toxicity studies reported by Andreia et al. (2014), Mukinda and Eagles (2010) and Rhiouani et al. (2008). We provided safety data only for the parameters tested. Only one study may not be able to provide all of the safety data.

**References**

Andreia RH, et al. *Biochemical and hematological effects of acute and sub-acute administration to ethyl acetate fraction from the stem bark Scutia buxifolia Reissek in mice*. *J Ethnopharmacol* 2014, **153**:908-916.

Mukinda JT, Eagles PFK *Acute and sub-chronic oral toxicity profiles of the aqueous extract of Polygala fruticosa in female mice and rats*. *J Ethnopharmacol* 2010, **128**:236-240.


5) The manuscript should also make it more clear, that a 42 toxicity study, is not an indication of safety. For a diseases like diabetes, where treatment is prolonged, two-year chronic toxicity studies in rats may be required to give an indication of chronic exposure effects of the product.
We do agree that a two-year chronic toxicity study is better indication for the safety of antidiabetic drugs than 42-day treatment. However, subchronic toxicity studies are also important for the approval of pharmaceutical products for human use (Echobichon, 1992). More importantly, two-year chronic toxicity study is very costly and time consuming. Now, only few researchers have reported two-year chronic toxicity, except for pharmaceutical company as it is required for new drug application. Usually it is recommended to treat animal only 28 day for sub-chronic study, however, in the present study we extended our treatment to 42 days which has been done previously in the study of plant extract with antidiabetic activity (Tchamadeu et al., 2011).

For the reviewer’s concern, we have emphasized that we provided safety data only from sub-chronic toxicity test in this study in the Conclusion on page11 line 356.

References
