Author's response to reviews

Title: Anti-diabetic potential and antioxidant activities of traditional medicinal plants

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Author's response to reviews: see over
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The Editor, BMC Complementary and Alternative Medicine

Re: Revised submission of manuscript "Anti-diabetic potential and antioxidant activities of traditional medicinal plants" (MS: 3368017925852625)

Dear Sir,

We have pleasure in submitting our revised manuscript in which we have made the following amendments as recommended by the nominated reviewers. The reviewer’s comments are followed point-by-point by our revisions and corrections. We thank the reviewers for their insightful and very helpful comments and hope that the changes made meet with your approval.

Kind regards,

Vandana Gulati
Environment and Biotechnology Centre
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Reviewer 1: Ashwell Ndhlala

Materials and methods

**Total phenolic content assay**
Please state the strength of Folin C reagent used i.e. 1N or 2N
The strength of Folin C reagent was 2N (Line 197, p. 8).

Please state the % of Na2CO3 used.
Percentage of Na2CO3 used was 5% (Line 198, p. 8).

**Antibacterial assay**

Why were positive controls not included?
For future assays, it is much accurate to use indicators such as iodonitrotetrazolium chloride (INT) to detect the bacterial or fungal growth/inhibitions.
The starting concentrations used in the study were too high for MIC antibacterial assays (250 mg/ml) Usually a concentration of 50 mg/ml is used as a starting concentration to give the first well a conc of 12.5 mg/ml.
It is pointless to report disc diffusion methods in a paper when MIC methods were also used.
Please state which agar was used i.e. Mueller-Hinton???? or which one.

We understand that the results we obtained with the anti-microbial assays were not promising, so we are happy to omit this part from our manuscript.

Please include positive controls for the Glucosidase inhibition assay and the Amylase inhibition assay.

Acarbose was used as a positive control in the quantitative $\alpha$-amylase (Line 151-152, p. 7) and $\alpha$-glucosidase inhibition assays (Line 165, p. 8). The disk diffusion $\alpha$-amylase inhibition assay was semi-quantitative so we did not consider that the use of a positive control was necessary.

Results section

**Glucosidase inhibition assay**
The authors reported that there was no dose dependant activity.... this is because they started the assay at very high concentrations. The authors were supposed to lower the concentration until a meaningful curve is observed. That way, then their results can be meaningful. As suggested we carried out the assay at lower concentration range (0.35 $\mu$g/ml to 100 $\mu$g/ml) and IC50 values for the assay is shown in Figure 2.

The authors should not compare plant extracts tested at 1 mg/ml e.g. Mucuna pruriens to ascorbic acid which was tested at 0.1 mg/ml and the authors went on to say the extracts were equally as potent as ascorbic acid. **We have corrected this by comparing IC50 values for ascorbic acid and plant extracts.**

**Antibacterial assays**
The authors should pre-define the levels of activity e.g. what is mild activity. The activity less than 70% was considered to be mild. However, as this is not strong activity, we are happy to omit this part of the manuscript.

Table 1: Please indicate which plants are from India and which ones are from Australia to enable the reader to make good comparisons. The plants have been segregated in the tables and figures.

Table 2: Please include the standard errors
As mentioned above, we have omitted the antimicrobial activity sections of the manuscript.

Table 3: But it is mentioned that percentage inhibition was calculated for the zones so why is it not presented here? Please use percentage inhibition instead of the symbols as it is more informative.

It was zone of inhibition, not percentage inhibition. Again, this section has been removed from the manuscript.

**Minor Essential Revisions**
The author can be trusted to make these. For example, missing labels on figures, the wrong use of a term, spelling mistakes.

We have made all necessary corrections.

Please insert a reference to the statement ‘ROS contribute to the development of diseases such as cancer, cardiovascular,….’
The reference has been added (Line 80, p. 4).

Please add species after ‘streptococcus’
Not needed as we have removed the antimicrobial sections.

Please insert a hyphen ‘-‘ between ‘Gram’ and ‘negative’ or ‘positive’ i.e. Gram-negative
Not needed as we have removed the antimicrobial sections.

For future assays, it is much accurate to use indicators such as iodonitrotetrazolium chloride (INT) to detect the bacterial or fungal growth/inhibitions.
Thank you for the valuable feedback.

Please be consistent with decimal places
This has been corrected.

The statement ‘…. Were unable to kill this species,….’ Can be written as ‘were unable to inhibit this species…’
Not needed as we have removed the antimicrobial sections.

Please italicize ‘in vitro’ or ‘in vivo’
This has been corrected.

Please define the phrase ‘fasting blood sugar’
This phrase has been removed from the manuscript.

Please remove E. coli, S. aureus etc from the ‘List of abbreviations’
Not needed as we have removed the antimicrobial sections.

Graph Pad Prism allows you to remove the bold text on Figures 1 and 2 to make neat graphs
This has been corrected.
Reviewer 2: Alejandro Tapia

1- Antioxidant activity determined by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) Radical Inhibition. The authors should look at some paper, which reported antioxidant activity of plants. A single in vitro method (DPPH assay) is not sufficient to inform the antioxidant activity.

To confirm the anti-oxidant activity, we carried out the ferric reducing power assay as well and have included the results of same (Line 202 to 213, p. 9 & 10).

Some antioxidant compounds should be included in the graph. On the other hand, the Figure 2 legend is unclear. That means the following paragraph:

were screened for enzyme inhibitory activity
We have included the standard ascorbic acid and Butylated hydroxytoluene (BHT) in the graph (Fig. 3 & 4).

Fig. 2 legend need to be checked……………………………….
This has been checked and corrected.

Title: DPPH Assay
Figure Legend: Fig. 2. Percentage Inhibition of DPPH by plant extracts. For all extracts, concentrations of 20 – 1000 µg/ml were screened for enzyme inhibitory activity and the highest activity is presented in the results shown. (n=3; data expressed as mean ± SD)
Checked and corrected.

2- Minimum inhibitory and bactericidal concentration of Indian Ayurvedic plant Extracts. The most MIC values shown in the table 4 are high, they are in the order of 3.9-15.6 times or more compared to the values considered promising for an extract (< 1000µg/ml). The MBC are very high, with values between 62000µg/ml and 125000. These results should be deleted from main text of the paper. Authors should include positive controls for bacteria in Table 4.
Not needed as we have removed the antimicrobial sections.

A paper must be based on a thorough and extensive study, using proper controls. Some data presented are lack of novelty. The study must represent a novel approach to the study of the activity, i.e. not more or less repeating what has already been published with similar results, but e.g. only using another extract of the same plant, or, in case of antimicrobial activity, some other microorganisms. The Aboriginal Australian plants had never been used for management of hyperglycemia and we found promising results for enzyme inhibition and anti-oxidant studies. Thus, we feel that these are novel observations because this is the first study of Australian aboriginal plants in this context. The Indian Ayurvedic plants are known for their traditional use in controlling diabetes but, with the exception of two plants, no previous data exist to our knowledge for the enzyme inhibition assays we have performed.

It has been reported that seeds of Eugenia jambolana have glucose-lowering
potential, -glucosidase and -amylase inhibition and antioxidant activity [37, 38]. The antibacterial activity has also been reported with an MIC of 250 µg/ml against E. coli and P. aeruginosa, 125 µg/ml against S. aureus and 62.5 µg/ml against B. subtilis for methanol extracts [30]. The MIC values reported in the current study were higher against these bacteria which might reflect differences in the chemical composition. The aqueous solution of Boerhaavia diffusa leaf extract has been reported to decrease blood glucose and increase insulin activity in normal and diabetic rats and possessed good antioxidant activity [40]. The phenolics from Pterocarpus marsupium (marsupin and pterostilbene) lowered the blood glucose levels of hyperglycemic rats [41] and in vivo -glucosidase inhibitory activity was found with water extracts of latex [42].

This section has been rewritten (p. 12).

3. Inhibition of -amylase by Australian aboriginal and Indian Ayurvedic plant extracts. The enzyme inhibition data should be expressed in terms of their IC50. The authors should ask, if an extract that inhibits an enzyme at high concentrations is an inhibition or a false positive. Values are between 43000 and 326000 µg/ml. The disk diffusion amylase inhibition assay was semi-quantitative so we did not calculate the IC50 in this assay. However, a colorimetric-based assay was carried out and the IC50 values were calculated for the same (Fig. 1).

4. There are sections that are not necessary to explain Pearson’s correlation coefficient between total flavonoids and total phenolic was 0.796 hence we can postulate there is direct relationship between phenolic and flavonoids content. Thank you for the valuable feedback.

5. Statistical analysis
All samples were analysed in triplicate. Data are presented as mean ± standard deviation (SD). Data were analysed by GraphPad Prism Software version 5.0 (GraphPad Software Inc., San Diego, USA) there are no references in the text on the statistical analysis of data. We have now included the relevant reference (Line 221, p. 10).

The authors should rewrite the work, eliminating the very high values of the various tests. This has been corrected.

control compounds should be included in all trials.
For amylase and glucosidase assays, we included acarbose as a positive control whereas buffer was used as a negative control (Line 151 & 165, p. 7 & 8). Ascorbic acid and BHT were used for DPPH and FRAP antioxidant assays (Line 199 & 211, p. 9 & 10).
Reviewer 3: Savarimuthu Ignacimuthu

Major comments

1. **Abstract is missing.**
   The abstract was submitted but perhaps was missing in this reviewer’s version.

**Extraction**

1. Volume of plant samples taken and volume of solvent used for extraction should be mentioned
   5 gms of plant sample was soaked in 50 ml of ethanol.

2. How the Indian ayurvedic plant samples were used for extraction? (Shade dried or fresh plant sample).
   The Indian ayurvedic plant powders were gift from Promed Research Centre, Gurgaon, Haryana, India.

3. Percentage yield of each extracts and percent of ethanol used for extraction should be mentioned.
   Percentage of ethanol used was 100% which was same for all the extracts and percent yield was different for each sample.
   EJ – 56.4%, CO – 69.6%, MP – 59.8%, PM – 45.2%, BD - 57.2%

4. Concentrated at which temperature?
   The plant samples were concentrated *in vacuo* in rotary evaporator at 55 °C (Line number 118, p. 5).

**Glucosidase inhibition assay**

1. Carbohydrate source (sucrose, starch or glucose) should be added along with enzyme and test samples.
   Glucosidase inhibition assay - glucose is released when the enzyme is mixed with substrate i.e. absorbance of p-nitrophenol is measured which is released after enzyme is reacted with substrate p-NPG [Hogan S, Zhang L, Li J, Sun S, Canning C, Zhou K: Antioxidant rich grape pomace extract suppresses postprandial hyperglycemia in diabetic mice by specifically inhibiting alpha-glucosidase. *Nutrition and Metabolism* 2010, 7(1):71].


2. Glucose concentration should be determined at initial and final period of the experiment.
The initial and final absorbance's were measured (Line 163, p. 7).

3. Inhibition rates of extracts against blank control should be expressed in standard equations. The same formula was used to determine inhibitory percentage for the plant extracts tested in our manuscript.

The standard equation includes the blank control to calculate the percent inhibition of samples.

\[(\%) = \frac{A0 - A1}{A0} * 100\] where A0 is absorbance of blank control and A1 is absorbance of sample (Line 167 – 170, p. 8).

4. Inhibitory effect of #glucosidase should be presented as IC50 (µg/ml). This has been calculated now (Fig. 2).

5. To better understand the effect of extract, the experiment should be conducted in different concentrations of enzyme and plant extracts. We did use different concentrations of enzymes and plant extracts for optimization of the assay and finalized the concentration of enzyme to be used after optimization.

**Total phenolic content assay**

1. Concentration of sodium carbonate should be mentioned.  
   5% sodium carbonate was used in TPC assay (Line 176, p. 8).

2. Samples should be in five replicates.
   The papers we have referenced performed all assays in triplicate and this is standard practice in the literature [e.g. Sunil C, Ignacimuthu S: In vitro and in vivo antioxidant activity of Symplocos cochinchinensis S. Moore leaves containing phenolic compounds. *Food and Chemical Toxicology* 2011 49(7):1604-9].

**Total flavonoids determination**

1. Volume of sample or reagents taken should be expressed in uniform units (µl or ml).  
   It has been corrected in the manuscript (Line 185 & 188, p. 8 & 9).

2. Standard curve was prepared using 5-1000mg/l of quercetin. Aliquots of Concentration of standard seems to be incorrect.
   There appears to have been a typographical error; the standard curve was prepared using 25 to 1000mg/l of quercetin (Line 190, p. 9).

**DPPH radical inhibition**

1. The antiradical activity should be expressed as IC50 (µg/ml).  
   This has been calculated now (Fig. 3).
2. This study explains only about the reducing effect of stable free radical DPPH. It would be wise to refute the hypothesis by carrying out hydroxyl scavenging, nitric oxide reduced inhibition and lipid peroxidation assays together. To confirm the anti-oxidant activity, we carried out the ferric reducing power assay as well and have included the results of same (p. 9 & 10).

2. Where are the aliquots of standard??????
The concentration of BHT and ascorbic acid was in range of 3.125 - 250 µg/ml (Line 211, p. 10).

4. Percentage of inhibition should be expressed by the following equation:
DPPH scavenging effect (%) = A0 – A1/ A0

Where A0 is the absorbance of the control, and A1 is the absorbance of the sample.

The same formula was used to determine inhibitory percentage for the plant extracts tested in our manuscript (Line 199 – 201, p. 9).

5. All test samples should be analysed in triplicate.
Results shown are mean ± SEM of triplicates experiment (Line 215, p. 10).

Tables
Table 2, concentration of plant extracts against #-amylase inhibition (mg/ml) seems to be high; usually µg/ml is expressed.
The amylase inhibition assay was semi-quantitative as it was by the disc diffusion technique and since it was preliminary screening but we do understand that the concentrations used were high. However, we have included the more reliable and quantitative colorimetric amylase assay (Fig. 1).

Figures
Figure 2, No standard error bar has been shown.
Figure 2 has been replaced with IC$_{50}$ for the plant extract tested (Fig. 3 & 4).