Author's response to reviews

Title: Anti-diabetic Indian Ayurvedic medicinal plants as potent inhibitors of pancreatic alpha-amylase activity

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Author's response to reviews: see over
To,
The Editor-in Chief
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Dear Sir,

Thank you, for considering our manuscript entitled ‘Anti-diabetic Indian Ayurvedic medicinal plants as potent inhibitors of pancreatic alpha-amylase activity’ manuscript Ref no.- MS: 1909338916428534 via e-mail October 4, 2010.

We are submitting a revised version of the manuscript taking into account all the comments made by the reviewers. A colleague from the English department has helped us to copyedit the paper and all changes are highlighted in the revised manuscript.

Replies to reviewers comments are attached separately below. The covering letter along with the answers to the reviewers comments and the, revised manuscript have been uploaded.

Thanking you,

Yours sincerely,

Dr. Ameeta Ravi Kumar
**Reviewer 1: Isao Kouno**

**Query 1:** Within the analysis of 126 extracts from 17 plants, only three extracts were quantified with respect to PPA inhibition. But, these three plants have been chemically well investigated, so far. The authors must make consider which compound is active.

Answer 1: The phytochemical analysis followed by the GC-MS analysis of the three plant extracts quantified with respect to PPA inhibition has been done in the study and probable active chemical components identified (Tables 3 and 4). However, unless a bioactivity guided isolation and characterization of the lead inhibitor molecule is performed, it is difficult to predict as to which compound is active. These studies are currently underway.

**Query 2:** P14: line 16, 2-heptanpl, 5-ethyl ???

Answer 2: The correction has been made and is highlighted in the revised manuscript page 14, line 24.

**Query 3:** P13: bottom, double period.

Answer 3: The correction has been made and is highlighted in the revised manuscript page 14, line 8.

**Query 4:** Table 1, Cinnamomum verum Rdeuces ^^^ Reduces

Answer 4: The correction has been duly made and is highlighted in the revised manuscript.

**Reviewer 2: Mauro Carai**

**Query 1:** The entire name, in brackets, of each abbreviation should be provided.

Answer 1: The corrections have been incorporated and are highlighted in the revised manuscript.

**Query 2:** The rationale of the choice of porcine pancreatic amylase for use in testing extract activity should be indicated.

Answer 2: Control of Human pancreatic $\alpha$-amylase (HPA) activity with inhibitors would lead to control of post prandial hyperglycemia and in turn lead to Type 2 diabetes management. According to reference No. 16 (revised version) wherein the structure of HPA is elucidated and compared with amylases from different sources, close structural homology occurs between Porcine pancreatic $\alpha$-amylase (PPA) and HPA. Moreover PPA is available at a low cost as compared to HPA. Hence for initial screening of 126 plant extracts PPA was used as the target.

Appropriate changes are made in the revised manuscript, page 6, line 19.

**Query 3:** In the Introduction and in the Results and Discussion paragraphs, a more appropriate literature on type 2 diabetes should be reported (to replace references no. 3, 5, 18, and 19), and a brief review of the pharmacological properties of $\alpha$-amylase inhibitors is needed.

Answer 3: Appropriate changes have been made in the references. Reference No. 3, 5, 18, 19 are changed in the revised manuscript. Pharmacological properties of $\alpha$-amylase inhibitors are mentioned on page 5, line 23 to page 6, line 3. An additional reference No.15 is cited.

**Query 4:** In different paragraphs, appropriate spaces must be introduced when referring to concentrations or quantities (0.02-M, 100-$\mu$l, etc.)
Answer 4: The corrections have been made and are highlighted in the revised manuscript.

Query 5: Authors should clarify which kind of controls was included (page 8, line 16).
Answer 5: The extract control is elaborated and clarified in the text as “To eliminate the absorbance produced by plant extract, appropriate extract controls with the extract in the reaction mixture except for the enzyme were also included.” Page 8, line 23-25 and page 9 line 19 (revised version) and the correction made is highlighted.

Query 6: Was the starch soluble in the buffer? Or it was suspended in the medium (page 8, line 23).
Answer 6: Soluble starch which is soluble in buffer is used for the assay. It is mentioned in the Methods: subtitle Materials. page 7, line 3 and is highlighted in the revised manuscript.

Query 7: When a concentration is indicated in the text, its weight and volume should also be provided (page 9, line 5).
Answer 6: The corrections have been made and are highlighted in page 9 line 22 in the revised manuscript as 6.5 – 32.8 µgml⁻¹.

Query 8: The Results and Discussion paragraph should be shortened omitting (page 11, from line 7 to line 13) information already provided in previous paragraphs; some of the details may be more appropriately placed in the Methods section (page 11, from line 18 to line 24).
Answer 8: Results and Discussion paragraph has been shortened. Lines 7-13 on page 11 are deleted. The section from page 11 lines 18-24 is shifted to the Methods section Page 9, line 1-7 in the revised manuscript.

Query 9: Several typing mistakes (..) should be removed from page 13, line 25.
Answer 9: The correction have been made and are highlighted in the revised manuscript (Page 14, line 8).

Query 10: The References should be accurately checked (i.e. ref. 7 “potntials” a “e” has been omitted)
Answer 10: The correction has been made and is highlighted in the revised manuscript as ref 9.

Query 11: The Results section should also be rewritten, particularly as very important statistical data are missing, such as, for example, the results of ANOVAs (only the results of post hoc tests are reported in the tables).
Answer 11: The results of ANOVA have been incorporated in the manuscript and highlighted in the revised manuscript.

Query 12: In Table 1, the V. No should be omitted, these data may be reported in the Additional files section.
Answer 12: The V.No has been omitted from Table 1 and an Additional file named Plants names and their Voucher No. has been made.

Query 13: Table 2 adds no further understanding of the activity of extracts, particularly as the reader is not able to evaluate which concentration range performed an effective inhibition. A reference compound should however be used in evaluating the true potential of these extracts.
Answer 13: The concentration range of the plant extracts used for the starch iodine assay is added in the title of Table 2. The results of starch-iodine assay performed with positive reference standard Acarbose is also included in the Table 2 as well as in Page 8, line-25 to Page 9, line-1 in Methods. The changes are highlighted appropriately in the revised manuscript.

Query 14: The Authors should revise the style of the manuscript; as an example, shorter sentences might help the reader to grasp the meaning better.
Answer 14: The manuscript has been checked by a faculty member of the English Department.

Reviewer 3: Emmanouil Apostolidis
General Comment
Prof. Emmanouil Apostolidis suggests that - “the main target for management of type 2 diabetes is inhibition of α-glucosidase and NOT α-amylase”. However, a number of relevant references given below as well as in the manuscript show that pancreatic α-amylase is also a potential target for diabetes type 2 therapy. Postprandial hyperglycaemia (PPHG) can be partially controlled by delaying digestion and absorption of carbohydrates through the inhibition of the carbohydrate hydrolyzing enzymes, α-amylase and/or α-glucosidase, in the digestive tract.

In humans, upon reaching the small intestine, starch is first acted on by pancreatic α-amylase and extensively hydrolyzed into smaller oligosaccharides. The resultant mixture of oligosaccharides consists of maltose, maltotriose, α-(1-6) and α-(1 - 4) oligoglucans which are then acted on by a number of α-glucosidases and further degraded to glucose which enters the blood-stream (see diagram).

![Starch Metabolism Diagram](image)

Starch (from diet) 
\[\xrightarrow{\text{Salivary } \alpha-\text{amylase}}\] Partially digested starch
\[\xrightarrow{\text{Pancreatic } \alpha-\text{amylase}}\]
Lumen
\[\xrightarrow{\text{Brush border } \alpha-\text{glucosidase}}\]
Maltose, Maltotriose, Oligodextrans
\[\xrightarrow{\text{Membrane in the Gut}}\]
Glucose

One approach to control PPHG is by inhibition of α-glucosidase activity using acarbose the \(K_i\) for which is 5 μM. Such an inhibition leads to accumulation of maltose and its oligosaccharides as shown in the diagram. This causes side effects such as abdominal pain, flatulence, diarrhoea and soft faeces in colon (Toller, M; Muller L and Pul W).
The other approach is based on the inhibitory action of pancreatic α-amylase which have also been targets for suppression of PPHG. It has been shown that the activity of pancreatic α-amylase in the small intestine correlates to post-prandial glucose levels, the modulation of which through therapeutic use of inhibitors would be of considerable medical relevance in type 2 diabetes management (Tarling, 2008). Inhibitors of α-amylase are expected to be better suppressors of PPHG, since the inhibitor would not result in abnormal accumulation of maltose leading to lower side effects (Takahashi, 1998).

References reporting pancreatic α-amylase as a potential target for type 2 diabetes.


Major Essential Revisions:

Query1: The authors should distinguish in the results section when they are using the DNS and when the iodine assay. As the manuscript is now it appears that only the iodine assay was used.
Answer 1: Reviewer 2, Mauro Carai, in his general comments says – “Preliminary screening was conducted by means of a starch-iodine colour assay. The activity of the most promising extracts on porcine pancreatic amylase was quantified using the chromogenic DNSA method”. Hence it seems to be clear in the manuscript. However, further clarification has been incorporated in the revised manuscript (page 12 line 3-10).

Query2: The authors should specify why they are using 2 assays for alpha-amylase. Why use a separate assay for screening?
Answer 2: There are mainly two types of assays that are used to determine the activity of α-amylase. One is based on measuring the amount of reducing sugars by the dinitrosalicylic acid (DNSA) assay while the other is based on the decreased staining value of blue starch–iodine complexes. Both methods generate equivalent values for the number of enzyme units present as stated in the reference No. 17. So preliminary high throughput screening was conducted on a microtitre plate by means of a starch-iodine color assay with a positive inhibitor acarbose included as a standard. This has been mentioned in the Methods section. (page 8, line 14– Page 9, line 7). The activity of the most promising extracts exhibiting porcine pancreatic amylase inhibition was quantified using the chromogenic DNSA method which is more robust in terms of evaluating the kind of inhibition.

Query 3: A major revision should be performed in the down-toning the manuscript and mentioning that excessive alpha-amylase inhibition have side effects.
Answer 3: Appropriate changes are highlighted on page 5, line 2-10. An additional reference No.7 is been added and accordingly all other references numbers are changed which are highlighted. Also see general comments.

Query 4: The authors should make clear in the manuscript that the desired enzyme to be inhibited for type 2 diabetes management is alpha-glucosidase which results to alpha-amylase inhibition.
Answer 4: Please see the general comments above.Appropriate changes are made in the manuscript which are highlighted page 4, lines 14-18. An additional reference is added Ref. No 6 (revised version) and accordingly all other Ref. No. are changed.
Minor Essential Revisions:

Query 1: Page 3, line 9: The authors should state what this study endorses. However, they should be very careful on the word selection, since they should not state that they endorse these plants for type 2 diabetes management. Maybe they can endorse these plants for further studies to determine the potential for type 2 diabetes management.
Answer 1: Appropriate changes made in the revised manuscript (page 3, line 10).

Query 2: Page 5, line 4: The authors mention that the currently used drugs for type 2 diabetes treatment have side effects. They should also mention that they mainly have side effects due to excessive alpha-amylase inhibition.
Answer 2: Appropriate changes are highlighted on Page 5, line 2-10. An additional reference is been added and accordingly all other references numbers are changed which are highlighted.

Query 3: Page 5, Lines 18-20: The reference used to cite this statement (13) does not mention anything about discovery of new alpha-amylase inhibitors. This statement should be removed or the citation should change.
Answer 3: Appropriate reference [6] has been cited for the statement on page 5, lines 21-23. The changes made are highlighted in the revised manuscript.

Query 4: Page 8, Line 18: The title should be changed to DNS assay (since the first assay was titled starch-iodine assay). The current title is not correct since both assays described are alpha-amylase inhibition assay and there is no other way to differentiate them.
Answer 4: The appropriate change is made and highlighted in the revised manuscript (page 9, line 9). The title has also been changed to DNSA assay.

Query 5: Page 11, Line 4: Appropriate references should be provided for the claimed several studies that were performed on these plants.
Answer 5: The references for the statement claimed are cited in the Table 1 under the section Hypoglycemic and medicinal properties of the plants.

Query 6: Page 15, Lines 16-18: This research does not show that blood glucose levels are reduced. The phrase should be changed accordingly. The Results and Discussion section is confusing as presented. This reviewer suggests to divide in two sections (Separate results and discussion).
Answer 6: An appropriate change is made and highlighted in the manuscript page15, line 22-page 16, Line 1 (changed version)

Discretionary Revisions:

Query 1: Page 11, Lines 18-24: Move this to the Materials and Methods section.
Answer 1: Lines 18-24 Have been moved to Materials and Methods section in the revised manuscript (page 9, line 1-7)