Author's response to reviews

Title: Chemical profile and in vivo hypoglycemic effects of Syzygium jambos, Costus speciosus and Tapeinochilos ananassae plant extracts used as diabetes adjuvants in Puerto Rico

Authors:

Jannette Gavillán-Suárez (jannette.gavillan@upr.edu)
Alexandra Aguilar-Pérez (alexandra.aguilar@uccaribe.edu)
Natalie Rivera-Ortiz (natalie.rivera.ortiz@baxter.com)
Lorelein Morales-Santiago (lorelein.morales@upr.edu)
Karla Rodríguez-Tirado (karla.rodriguez5@upr.edu)
Wanda Figueroa-Cuilán (wanda.figueroa89@gmail.com)
Gerónimo Maldonado-Martínez (geronimo.maldonado@gmail.com)
Luis A. Cubano (lacoardgs@gmail.com)
Michelle M. Martinez-Montemayor (mmmtz92@gmail.com)

Version: 4
Date: 17 June 2015

Author's response to reviews:

We have provided these comments in the attached cover letter.

May 26, 2015

Tom Rowles, Ph.D.
Senior Executive Editor
BMC Complementary and Alternative Medicine
e-mail: editorial@biomedcentral.com
Web: http://www.biomedcentral.com/

Dear Dr. Rowles:

We deeply appreciate the questions and comments of the reviewers during the analysis of our manuscript MS 12221727774159583. We hope that our revisions and answers fulfill the reviewer’s assessment and make our manuscript suitable for publication. This work is unique and describes the validation of herbal remedies used as diabetes adjuvants by communities in the Caribbean.

Below, we have included the reviewer’s comments followed by our point-by-point response to their concerns and a description of the text changed in the manuscript to make the content more descriptive and accurate.

Title: Chemical profile and in vivo hypoglycemic effects of Syzygium jambos, Costus speciosus and Tapeinochilos ananassae plant extracts used as diabetes adjuvants in Puerto Rico.
1. Figures

a) Figure numbers don’t match with the results.
   • We have edited the content accordingly.

b) Figure 1 Schematic drawing of in vivo experimental procedures. Figure 1 is not clear and it appears to be too much pixilated therefore, it would be recommended to the Authors to redraw the Figure in PowerPoint and save it a Tiff file.
   • We have followed the recommendation re-drawing the figure. Please refer to Figure 1.

c) Figure 2.B Blood glucose level for Syzygium 2.2 after 90 min after 10% glucose injection is between 200 to 300 mg/dl whereas, it shots up to more than 400 mg/dl after 120 min. Similarly Costus 2.2 pre incubation glucose level is between 300 to 400 mg/dl and it shoots up to more than 450 mg/dl it would be great if the authors can elaborate more on this anomaly.
   • In the discussion, for the db/db mouse model, where all of the mice displayed pre-incubation blood glucose levels (BGL) greater than 200mg/dL, we mentioned that this is an indication of an established diabetic state and included the following references to account for the remark:


To address the results for S. jambos we included in the Results section the following comment: “Interestingly, mice treated with S. jambos 2.2 mg/kg showed decreased BGL at 90min (BGL 267 mg/dL) post injection. These results suggest that mice receiving 2.2 mg/dL of S. jambos display gradual blood glucose modulation (increased and decreased) peaks.” And in the Conclusion: “At 5wks, individual mouse BGL readings show that depending on the timepoint either none (90 min), or only one or two animals have BGL > 500mg/dL. This data substantiates our results that show that S. jambos at levels consumed by humans (2.2mg/kg) tends to modulate BGL better than the rest of the plants.”

To address the Costus blood glucose results we include in the Results section the following statement: “After the glucose injection, most of the animals treated with C. speciosus 2.2 mg/kg_BW have glucose levels greater than 500mg/dL and were not able to reduce their BGL to basal state, indicating that glucose tolerance in these mice is impaired (Figure 2B).”

d) Figure 2. D and H and Figure 3 D data points for Syzygium 220 are missing.
   • The 220mg/kg BW S. jambos treatment in db/db mice was not performed. This has been clarified in the Methods section as follows: “db/db mice were orally
gavaged daily during 10 wks with 100µL of control (sterile water) or 0.2, 2.2, 22 mg/kg_BW doses of T. ananassae, C. speciosus and S. jambos aqueous extracts or 100µL of 220 mg/kg_BW doses of T. ananassae, and C. speciosus aqueous extracts, for a total of 12 treatments.”

- In Figures 2 and 3 legends as follows: “Figure 2 IP-Glucose Tolerance Test (IP-GTT) in the C57BLKS/J (db/db) mouse model. A-D. IP-GTT performed at 5 wk post-treatment with (A) 0.2 mg/kg_BW, (B) 2.2 mg/kg_BW, (C) 22 mg/kg_BW of S. jambos, T. ananassae and C. speciosus aqueous extracts or water (control), (D) 220 mg/kg_BW of T. ananassae, C. speciosus aqueous extracts or water (control).” The same legend was used in Figure 3.

e) Figure 3 C and D- After treating mice with Costus 22 blood glucose level drops down to less than 200 mg/dl after 30 mins of treatment whereas we do not see such effects when mice were given Costus 220.

- The results displayed in these figures correspond to effects seen at 10wks post-treatment in complement with insulin. We have edited the Conclusion section to address a possible explanation for these results as follows: “In some cases, we see that lower doses in complement with insulin have better glucose modulation than the same plant at higher doses (C. speciosus 22 vs. C. speciosus 220). It is possible that the plants display a synergic or additive effect with insulin that is lost by increasing its concentrations. However, without performing proper combinatorial index analysis [54-56], we cannot be certain that such effect indeed occurs.” We have included three references that address our comment through combination therapy and synergistic effects.


2. Plant extracts and remedies

a) Plant extracts were dissolved in different solvents such as methanol, ethanol and DMSO. Can authors explain why they had to use three different solvents for solubilisation when the extraction had been done in methanol?

- Extractions of dry leaves were performed with Methanol and of fresh leaves were performed with ddH2O. DMSO and other solvent systems were used for the quantitative analysis of phytochemicals in methanolic or aqueous extracts. To clarify the use of different solvents we have edited the Preparation of decoctions, methanolic and aqueous extracts subsection in the Methods section as follows:

“For in vitro and quantitative analysis, fresh leaves (50 g) were weighted, freeze-dried using a Freezone 4.5 lyophilizer and extracted overnight with Methanol (MeOH) (350 ml) using a Soxhlet apparatus. The resulting extracts
were concentrated to dryness by rotatory evaporation (Yamato RE-200) at room temperature to yield from 1 – 11 % w/w of extract (1-5 g extract/ 45 – 200 g fresh leaves). Stock solutions of dry plant extracts were re-dissolved in various solvents (depending on the phytochemical to be tested). Alternatively, for the preparation of decoctions used in vitro, in vivo and quantitative analysis, fresh leaves (30 g) were boiled in 100 mL of distilled, deionized water, concentrated to 15 mL, to lyophilize 3 x 5 mL replicates, filtered through cheesecloth and freeze-dried. The resulting solids were re-dissolved in ddH2O and used to prepare aqueous extracts of known concentrations. All extracts were filtered using a 0.4µm Nanopure® filter syringe before assayed [21].

- We eliminated ethanol, which was not used to re-dissolved plant extracts.

b) Abbreviation used for the plant extract need to be mentioned clearly in figure legends or in the materials and method section.

- We have made these changes; please see Figures 2 – 4.

c) In the results section, authors have mentioned nine different plant remedies were used among the study population the last time that one of the family members was treated for diabetes; What do they mean by treatment of diabetes, was there any specific time frame of treatment? If yes, what was the time frame?

- As described in the Methods section, (Ethnopharmacological survey) during the structured interviews, participants were asked to provide information, if appropriate, about the botanical remedies used by the family as the first treatment for the ailments included in the survey. Besides a detailed description of the treatment preparation and application, including dosage and contraindications or side effects, participants were asked to describe the health problem surveyed in order to assess the perceptions of these ailments by the study population.

In the Results section we have included the descriptions for diabetes offered by the surveyed participants as follows:

“Nine different plant remedies were used among the study population the last time that one of the family members was treated for diabetes. The more frequent descriptions of diabetes as a health problem were uncontrolled sugar levels (high or low) and dizziness. Other descriptions included dry mouth, excessive thirst, sleepiness, restlessness, not feeling the legs, blindness or poor vision and frequent urination.”

- The time frame given by the participants was described as follows in the first paragraph of the results section: “Dosages were variable, with most families reporting the use of one cup of tea (decoction) daily over several days or weeks.” The time frame has been edited to describe specifically the use of the plants studied in this report as follows: “Dosages were variable, with most families reporting the use of one cup of tea (decoction) daily or during one week.”

d) Page 7 lines 11 and 21: There are no literature references for “Preparation of decoctions, methanolic and aqueous extracts and dosage calculation”.

• The following reference has been included in the Preparation of decoctions, methanolic and aqueous extracts paragraph in the Methods section. The calculation of the percent yield of total solids obtained from decoctions needed for the dosage calculation is also included in this reference.


• The reference Alvarado Guzman et al., 2009 has been included after the first sentence in the Dosage calculation paragraph of the Methods section to account for the source of the following statement: “consensus of dosage and administration reported during the TRAMIL interviews from Puerto Ricans who self-medicate with the medicinal plants.”

e) Page 30 line 13: Authors should describe the meaning of the (*) asterisk shown on graphs) on the legends.

• We have included the meaning of the asterisk in Figure 5.

3. Study plan and treatments

a) It is not clear from the study plan what the 12 treatment groups were being administered. It should be given in more detail what each group was administered?

• We have clarified the description of the Treatment Administration Study 1 subsection in the Methods section as follows: “db/db mice were orally gavaged daily for 10 wks with 100µL of control (sterile water) or 0.2, 2.2, 22 mg/kg_BW) doses of T. ananassae, C. speciosus and S. jambos aqueous extracts or 100µL of 220 mg/kg_BW doses of T. ananassae, and C. speciosus aqueous extracts, for a total of 12 treatments.”. In the Results section we added the following sentence: “Blood glucose levels (BGL) were monitored in mice treated with plant extracts or control (total of 12 treatments) at 5 and 10 wks post-treatment”.

b) Number of mice per cage as suggested in study varied between 4 to 5. Was there any specific reason for doing so and why a constant number of four animals per cage not maintained?

• We had n=50 db/db mice to do an initial pilot study to test a range of doses smaller or up to 100 times higher than the dose used by humans. Since we had a total of 12 treatments, we decided to randomly assign 4 mice per treatment and add one more mouse in Costus 2.2 and Tapeinochilus 2.2, for a total of 50 mice.

c) Authors have presented the phytochemical profiles and hypoglycemic effects of Tapeinochilus ananassae, Costus speciosus and Syzygium jambos performed on two models of type 2 diabetes. Why they did not performed the tests on classical models of type 1 diabetes, for example streptozotocin?

• Since Puerto Rico has highest rate of Type 2 diabetes within all the states and territories of the United States and Puerto Ricans commonly use plants as diabetes adjuvants we decided to perform our pilot studies on two models of
Type 2 diabetes.

We have edited the Abstract and the Background sections to reflect this rationale adding the following phrase: “... highest rate of Type 2 diabetes within all the states and territories…”

d) Authors should describe the basal glucose limit considered to include db/db or ob/ob mice as diabetic animals.

• We have clarified that 200mg/dL is the basal limits in diabetic mouse models by including the following sentence in the Discussion section and references listed in 1c above: “Importantly, studies describe that the experimental basal BGL limit for diabetic animals is 200mg/dL [8, 46].”

d) On page 4 line 5 authors said 'Puerto Rico has the highest diabetes rate within the United States', what kind of diabetes they are specifically considering?

• Puerto Rico has the highest rate of Type 2 diabetes as described in 3c above.

e) Page 19 lines 7 and 8: Authors said “Our results show that the plant extracts do not exert adverse effects in db/db mice”. It is not clear if they are referring to the present work because they did not performed toxicological and hematological test that are crucial to guarantee the safety of the plants extract.

• According to TRAMIL, toxicity studies should be done using three or more spaced doses, evaluated throughout time (i.e. 14, 28, 30, 90 days), followed by histopathological results and visual organ change via necropsy.

In our study we used various spaced doses (more than three: less than used by humans, the concentration used by human and 10 or 100 times this concentration), evaluated the treatments for 60 days (blood parameters at 0, 30 and 60 days), performed visual organs observations, which looked undamaged, but did not complete post-mortem histopathological analysis.

To account for the fact that adverse effects cannot be confirmed, we edited the Discussion section as follows: “Our results show that the plant extracts do not affect the weight, food or water intake of db/db mice”.

Minor Revision:

All minor corrections were taken into consideration and appropriate revisions were incorporated in the manuscript as described below.

1. Figure legends are not same as they have been discussed in the results section e.g. Figure 3A, need to be corrected.

• We have edited Fig 3A accordingly.

2. Various grammatical mistakes are there in the manuscript.

• We have asked a native English-speaking scientist to read and edit grammatical mistakes in the manuscript.

3. Significance of doing TLC plants needs to be explained?
• TLC data provides the chemical profile for the plant extracts characterization and identification of relevant marker compounds used for standardization as required by the National Center for Complementary and Integrative Health (NCCIH) in the policy to establish product integrity of different types of products used in both mechanistic and clinical research. The products include complex botanical products such as plant extracts.

Other information such as: a) identification of the source plant for the product using the scientific taxonomic nomenclature; b) description of the parts of the plant from which the product is derived and c) identification of the extraction solvents used in the preparation of the product is also required by NCCIH product integrity guidelines, is also included in our manuscript.

• The significance of TLC analysis of plant extracts has been explained in the Results section. Major groups of phytochemicals were found in plant extracts subsection, as follows:

“To establish product integrity during the scientific investigation of complex botanical products, the National Institutes of Health (NIH), National Center for Complementary and Integrative Health (NCCIH) has published guidelines that required the characterization (chemical profile or fingerprint) and identification of relevant marker compounds used for standardization. Qualitative chemical profile by TLC analysis of methanolic and aqueous extracts of the leaves of S. jambos, T. ananassae and C. speciosus showed the presence of flavonoids, alkaloids, phenolic compounds, saponins, terpenoids, tannins and cardiac glycosides (Table 1).

• The following reference has been included in the Reference section:

NCCIH Policy: Natural Product Integrity

4. In vivo should always be in italics.
• This has been corrected throughout the manuscript.

5. Authors can also look at the effect of selected plant extract on glucose uptake using cultured C2C12 myotubes and 3T3-L1 adipocytes. They could also carry out chromatographic fraction of Syzygium and isolating pure compound showing the blood glucose lowering potential.
• We will consider these studies for future directions.

We are looking forward to your final decision. If you have another inquiry we are in the best disposition to answer them.

Cordially,

Michelle M. Martínez-Montemayor, Ph.D.
Associate Professor – Department of Biochemistry