Author’s response to reviews

Title: A screening for antimicrobial activities of Caribbean herbal remedies

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Version: 3 Date: 4 January 2013

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Version: 2 Date: 21 December 2012

Author’s response to reviews: see over
December 21st, 2012

Tom Rowles PhD
Executive Editor
BMC Complementary and Alternative Medicine

Dear doctor Rowles:

We deeply appreciate the questions and comments of the reviewers during the analysis of our manuscript MS 2097252080810840. We hope that our revisions and answers fulfill the reviewer’s assessment and make the manuscript suitable for publication. This is a unique work that describes the validation of herbal remedies used as antibiotics by communities in the Caribbean.

First, I would like to comment about the critical issue related to the publication online of a previous version of the study. A preliminary version of this study was published in the Website of the Institute of Interdisciplinary Research (IIR) as part of their Technical Reports Series, called Cuadernos (roughly translated as workbook or bulletin). [http://webs.oss.cayey.upr.edu/iii/content/cuadernos](http://webs.oss.cayey.upr.edu/iii/content/cuadernos).

The IIR web-site where the Cuadernos are published is linked to the general University of Puerto Rico at Cayey (UPR-C) – Web site and is primarily visited by UPR-C faculty and students. The Cuadernos series was created to report preliminary, informal and non-peer reviewed accounts of the on-going studies of our faculty in order to promote interdisciplinary dialog and to account for the funding received by the research projects. They are also a provisional source of information that can be removed from the web-site at any time at the author’s request.

The preliminary and temporary nature of the report and the fact that the IIR does not follow a peer-reviewed process or actively publicize or circulate these reports; can account for our oversight when we failed to cite ourselves in the paper we were submitting for publication in the peer-reviewed journal.

Furthermore, the manuscript submitted for publication to BMC Complementary and Alternative Medicine is a longer, much more comprehensive study that will also
incorporate the recommendations of the reviewers. The preliminary technical report will be removed from the webpage and a reference to the published version (if the paper is published) will be included instead.

I look forward to your decision about continuing processing the manuscript and to advance the review process. Thanks for the opportunity to reply to this issue.

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.

1. Preparation and extraction of plant material

a) The preparation of extracts should have been clearly explained. The main concern with the paper is the preparation of the extracts used for the determination of MICs. The determination of concentrations is not clearly explained. Lines 2/3: What was the final concentration used? Concentration to 15 mL is confusing.

Decoctions were prepared by boiling 30 g of plant material in 100 mL of distilled water. After concentration to 15 mL, to lyophilize 3 x 5 mL replicates, the decoctions were filtered through cheesecloth, and freeze-dried using a Freezone 4.5 lyophilizer. Plant juices (3 x 5 mL replicates) were lyophilized after the plant part was crushed in a mortar and filtered through cheesecloth. The solids obtained after freeze-drying were re-dissolved in autoclaved water to known concentrations for antibacterial determination. The concentrations of the lyophilized extracts in mg/mL were calculated as the average of the total solids obtained per mL of lyophilized decoction or plant juice. The plant part, preparation form, concentration of the re-dissolved extracts and the extraction yields are reported in Table 1.

The extract concentrations (mg/mL) and extraction yields (% w/w) obtained were added to Table 1. We clarified the procedure in the section “Extracts preparation and extraction of plant material” as described above and changed the title of the section to “Plant material and preparation of decoctions, juices and aqueous extracts”.

b) Why did the authors lyophilize the plants extracts instead of storing in the refrigerator?

By lyophilizing the extracts we wanted to minimize contamination and increase their viability. In addition we wanted to determine the final concentration of the extracts in order to prepare stock solutions and dilutions at known concentrations.
c) The authors dissolved all the plant extracts in autoclaved water. Were all the extracts completely soluble? It is usually common practice to use solvents that can readily dissolve the extracted materials e.g. DMSO.

We dissolved the extracts in water to validate the traditional remedies (teas) used by the population as suggested by Ríos and Recio (2005) in http://www.ff.ul.pt/FCT/EXPL/QEQ-MED/0406/2012/Ref15.pdf.

Given that we did not encounter any solubility problems with the lyophilized extracts, DMSO was not needed to dissolve the extracted material. Also, DMSO might interact with some of the potential antibacterial compounds originating diffusion problems in the aqueous media of the agar-based plates.

2. **Disc Preparation**

What was the final concentration of the plant materials in the filter paper discs?

Aliquots (20 µL) of the re-dissolved extracts at their final concentration and at 25% and 50% dilutions were applied to separate discs for the antimicrobial activity assays. Table 1 was edited to include the concentration of the extracts that were applied to the discs. The “Disc Preparation” section was edited to include the reference to Table 1 and the preparation of separate discs with known dilutions of the extracts.

3. **Antimicrobial activity determination using disc diffusion assay**

What was the final bacteria concentration after adjusting the density to 0.5 McFarland units? This should be stated clearly.

The concentration of 0.5 McFarland units is $1.5 \times 10^8$ CFU/mL. We included this concentration in the corresponding section of the methodology.

4. **Minimum inhibitory concentration (MIC) determination**

a) Is there any reference to the definition of MIC provided here? I think there are better definitions of MIC in the literature. E.g. MIC is the lowest concentration of a specific antimicrobial agent that prevents the growth of an organism in vitro.

We agree and changed the definition in the manuscript to the one publish by the British Society for Antimicrobial Chemotherapy, which states that MIC is the lowest concentration of antimicrobial that will inhibit the visible growth of a micro-organism after overnight incubation.
b) What is the growth medium described in line 7 and what quantity was added?

The growth medium is Luria Broth. We used 100 µL per 96-wells plate.

The following sentence was added to line 7 in the section: “Wells in a sterile 96-well plate were inoculated with 100 µL Luria Broth (LB) medium”.

c) How were the issues with the diffusion of the extracts in the medium addressed?

The issues of low solubility and poor diffusion of the extracts were first addressed by dissolving the extracts in autoclaved water prior to the preparation of the discs. Also, the analysis of the MICs support the data from the disc diffusion assay, suggesting that interference by particulate matter is less than expected with the filter paper disc reservoir. Many reports rely in the determination of the MIC to validate that their disc diffusion experiments showing inhibition were accurate.

Other methods suitable for the disc diffusion assay with aqueous extracts, such as the hole-punch method, can carry problems of leakage that we wanted to eliminate in our analysis (Costa P, Vlietinck AJ, Vanden Berghe D, Maesa L: Anti-infective potential of natural products: How to develop a stronger in vitro ‘proof-of-concept’. J. Ethnopharmacology 2006, 106: 290–302).

d) What do the authors mean by bacterial suspension containing 5X10^4 CFU? Is this CFU per mL? I do not also see the ATCC numbers which should be mentioned after the bacteria named here. The authors mentioned this only once (when they described the isolates tested) but omitted it in all other sections of the paper.

We corrected the bacterial suspension concentration to 5X10^4 CFU/mL. Since the same isolates were used throughout the study we did not mentioned the ATCC numbers after the bacteria name throughout the manuscript. Nevertheless, the revised manuscript complies with the reviewer’s remark.

e) In the last four lines, authors should clearly define what the MIC was and how they arrived at it? They mainly stated that the absorbance of the extracts were subtracted from the absorbance of the experimental wells but did not say how they arrived at the MIC from these figures? Is this difference in absorbance the MIC?

The MIC was the concentration in the wells that gave an absorbance lower to the control well, that contained only bacteria and medium, and that also gave a p-value of
0.001 or less after statistical analysis.

We edited the final sentences in this section to clearly specify how the MIC was obtained, as follows: “The absorbance at 625 nm was measured for each plate using a microplate reader and background-subtracted with the background of the control (bacteria and medium). Then, the absorbance of the extracts was compared to the absorbance of the positive and negative control (Streptomycin 10 mg/mL) wells. The MIC was the concentration in the wells that showed an absorbance lower to the control well and that also gave a p-value of 0.001 or less after statistical analysis.

5. **Minimum bactericidal concentration**

In this section (line 5), the authors mentioned that there were positive and negative controls but did not specifically mention what these controls were?

We included the specific reference to the controls in the MBC section as follows: “Plates containing positive (bacteria with medium) and negative (bacteria with Streptomycin, 10mg/mL) controls for each bacterial species were also prepared”.

6. **Results and discussion**

a) From Fig 1A-D, it is not clear to me how the authors arrived at the various extract concentrations discussed. For example, how was the 139 µg/ml in line 9 arrived at?

The concentrations of the lyophilized extracts in mg/mL were calculated as the average of the total solids obtained per mL of lyophilized decoction or plant juice. The concentrations in the 20 µL applied to the filter paper discs were calculated based on the re-dissolved extracts at their final concentration and at 25% and 50% dilutions of the re-dissolved extracts. Given that the final concentration of the extracts was different for each plant the data presented for the dilutions varied from plant to plant.

The data presented in Table 1 as concentration in µg/ml at different dilutions of the re-dissolved extracts was incorrect. The correct units for the concentration of the solutions applied to the filter paper discs are µg/µL. We corrected this inaccuracy in the table, changed the heading of the column to “Concentration of extracts/disc (µg/µL)” and added the following note: “Extracts concentration/disc: disc 1 - µg/µL in the re-dissolved lyophilized extract at the final concentration, discs 2 and 3 - µg/µL in 50% and 25% dilutions of the re-dissolved extract, respectively”.

We also corrected the data in the table and in the manuscript that was expressed as µg/20µL calculating the concentration in µg/µL.

b) Page 9. What does this statement mean: ‘….they failed to meet the p-value used in
our analyses?

The statement means that the values did not meet the p-value of 0.001 or less used in the analysis. The statement was clarified in the manuscript as described above.

c) On page 11, lines 7-10, the authors should compare experimental and control values using the same data units. A situation where % is used for extracts and then μg/mL is used for the control antibiotic Streptomycin and both compared together is not acceptable. Authors should express concentrations in the same units e.g. μg/mL for both experimental and control values. This was pretty consistent through this section.

The percentage is used to compare the changes in inhibition between the control Streptomycin and the extracts. In this case the inhibition observed with the antibiotic was taken as 100%. The manuscript was edited to change the concentration of Streptomycin to the percentage of inhibition of this control (100%) followed by the concentration at which the percentage inhibition was observed (5 mg/mL).

d) I also observed that many other plant extracts were tested but barely mentioned in the body text (e.g. Capraria biflora). Why were these data not captured in any of the Tables in the paper?

The plant species listed in Table 1 were identified during TRAMIL surveys in the Caribbean. We tested all the plant extracts listed but focused our analysis on those extracts that showed antimicrobial activity in the disc diffusion test. Likewise, we performed the MIC and MBC studies with the plant extracts that showed antimicrobial activity in the disk diffusion assay.

Plant extracts that did not showed antibacterial activities are mentioned in the manuscript (page 13 and forward) to compare the reports by other authors with the results obtained in our study. For example, for *Capraria biflora* we presented the following discussion: “Serrulatane quinonoid biflorin, which is isolated from the root tissue of *Capraria biflora*, has been reported to exhibit antimicrobial activities against Gram-positive bacteria [38]. This activity is consistent with the traditional use to treat conjunctivitis, which may be associated with infections by *S. aureus*, *S. epidermidis* and *Propionibacterium spp*, among others. Thus far, the activity of aqueous extracts from the leaves has not been reported. The concentrations tested in this study range from 0.56 to 2.25 mg/mL.

We changed the title of Table 2 to clarify that the plants listed were the only that showed antimicrobial activity in the disc diffusion test.
e) The list of abbreviated items mentioned in page 16 should be deleted and should be explained when first mentioned in the text.

We deleted that list and explained the abbreviations when first mentioned in the manuscript.

7. Tables and Captions

a) The dilutions arrived at in Table 1 is not very clear. For instance, why would 100% of one plant extract be 1000 µg/mL and 100% of another is 573 µg/mL? Is this based on the extract yield? The extract yield was not indicated on this Table and this is usually necessary.

The concentrations are based on the final concentration of the re-dissolved lyophilized extracts not in the extracts yield. The extracts yield (% w/w) was reported in a new column added to Table 1.

We reviewed the “Dilution concentration (%))” section in Table 1 as described in 6a above. In Table 1 of the previous manuscript we described the dilutions tested in the 20µL applied to the paper discs in the disc diffusion assays as: 100% - the final concentration of the re-dissolved lyophilized extracts, and as 25% and 50% the corresponding dilutions of the re-dissolved extracts. Given that the final concentration of the extracts was different for each plant the data presented for the dilutions varied from plant to plant.

In the revised manuscript the reference to the dilutions in terms of percentage was removed from Table 1. The column heading “Dilution concentration (%)” was changed to “Concentration of extracts/disc (µg /µL)” and the following note was added to the table: “Extracts concentration/disc: disc 1 - µg/µL in the re-dissolved lyophilized extract at the final concentration, discs 2 and 3 - µg/µL in 50% and 25% dilutions of the re-dissolved extract, respectively”.

Also, in the methods section (“Disc preparation”) the preparation of the dilutions was described as follows: “. . . paper discs that were incubated with 20 µL aliquots of the re-dissolved extracts from lyophilized decoctions or juices at their final concentration and at 25% and 50% dilutions”.

b) In Table 2, why did the authors test some extracts against 4 bacteria isolates and others on 3 or 2 isolates?

Table 2 presents the plant extracts that showed MIC values with p-value of 0.001 or less for a given bacteria isolate. It also presents the corresponding MBC analysis. For
the MIC determination we tested all plants extracts that showed inhibition in the disc diffusion assay against the four bacteria isolates: *S. saprophyticus* (ATCC 15305), *S. aureus* (ATCC 6341), *P. aeruginosa* (ATCC 7700) and *P. vulgaris* (ATCC 6896). For example, *P. calomelanos* extract only inhibited *P. aeruginosa*, but was screened against the four bacteria isolates.

We clarified this in Table 2, adding the following note: “a) All the extracts that showed inhibition in the disc diffusion assay were analyzed against the bacteria isolates *S. saprophyticus* (ATCC 15305), *S. aureus* (ATCC 6341), *P. aeruginosa* (ATCC 7700) and *P. vulgaris* (ATCC 6896).

In addition in the “Methods” section (MIC determination sub-section) we added the following text to explain this point: “Extracts that showed inhibition in the disc diffusion assay were further analyzed according to the procedure of Velmonte et al. with some modifications [16]. The bacterial species tested were *S. saprophyticus* (ATCC 15305), *S. aureus* (ATCC 6341), *P. aeruginosa* (ATCC 7700) and *P. vulgaris* (ATCC 6896)”.

c) The authors generally ignored to mention the reference numbers of the isolates tested in many parts of the manuscript. It normal to write the ATCC numbers of isolates each time they are mentioned. I have highlighted before but I am mentioning it again here because of the consistency of this omission. I also did not find any test data for *H. influenzae*, *C. albicans* and *E. coli* even though the authors mentioned that these were tested (see page 16).

We have included the ATCC numbers, across the manuscript except when citing others studies.

We also included at the beginning of the “Results and Discussion” section (page 8), the following statement to make clear that test data from the plant extracts that did not showed inhibition on the disc diffusion assay is not presented: “The plant extracts listed in Table 1 were studied for their antimicrobial activities against *Staphylococcus saprophyticus* (ATCC 15305), *Staphylococcus aureus* (ATCC 6341), *Pseudomonas aeruginosa* (ATCC 7700), *Proteus vulgaris* (ATCC 6896), *Escherichia coli* (ATCC 4157), *Haemophilus influenza* (ATCC 8142), and the fungus *Candida albicans* (ATCC 752). Of the 13 plant extracts tested, data corresponding to those that showed microbial growth inhibition in the disc diffusion assays will be discussed. These extracts include *G. barbadense* fruit juice and the decoctions of *P. calomelanos*, *T. ananassae*, and *S. jambos* (Figure 1). The bacterial species inhibited by these extracts were *S. saprophyticus* (ATCC 15305), *S. aureus* (ATCC 6341), *P. aeruginosa* (ATCC 7700) and *P. vulgaris* (ATCC 6896)”. 

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d) The “Results and Discussion” section should have been written in a more comprehensive manner as it is rather too elaborate in relation to the research data. Table 3 may not be necessary.

Table 3 has been deleted. We agree with the reviewers’ comment about the extent of the section. Some of the citations are used to stress the point that the activities reported for some of the plant species are not evidence towards the validation of the traditional uses reported during the interviews since the antimicrobial activities were obtained for essential oils, hydroalcoholic or methanolic extracts, among others. Nevertheless, we have edited the discussion on the antimicrobial activities of *P. amboinicus*, *A. trilobata*, *C. moschata*, and *H. rosa-sinensis* in the revised final manuscript.

All minor corrections were taken into consideration and appropriate revisions were incorporated in the manuscript with one exception: the suggestion to add “Caribbean” to the text in Page 9, Line 7: “of the recommended herb for infectious diseases in the Caribbean”. Instead we change the text and use the author’s description: “of the commonly used herb for infectious diseases, *Echinacea purpurea*”.

We look forward to your concluding comments and evaluation of the revised manuscript.

Cordially,

[Jannette Gavillan-Suárez, PhD]

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