Author's response to reviews

Title: Evaluation of direct antiviral activity of Deva-5 herb formulation and extracts of five Asian plants against Influenza A Virus H3N8

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Author's response to reviews: see over
Dear Victor Kuete,

We are grateful to all the reviewers for their critical comments that help us to improve the manuscript. We corrected, where it was appropriate, the manuscript according to comments of the reviewers. Please find our detailed reply below. Also, please find the proof reading certificate attached.

Reviewer 1

**R1:** “Authors of this manuscript intended to evaluating antiviral activities of extracts of five Asian plants and their formula Deva-5. However, they didn’t focused the work on antiviral activity evaluation, but involved many irrelevant researches such as viral subtype identification. In addition, some methods for assaying cytotoxicities and antiviral activities of extracts are not classic methods. Therefore the results make little meaning. A large number of extracts from plants have antiviral activities in vitro, but few of them has activities in vivo.”

**Authors' reply:** The virus subtype identification is an integral part of the research that provides the basement for the critical analysis of the results. We had chosen the methods for evaluation of cytotoxicity and direct antiviral activity of the herbal preparations because they are widely used by scientists who work in similar field of research. Using the same methods we can compare our results with results of other groups (e.g. Badmaev V et al., 2000 or Hour MJ et al., 2013). Three extracts significantly reduced the infectivity of virus in 2 independent experiments, and in one case this effect was dose dependent. The evaluation of protective ability of the extracts in vivo was out of scope of this study.

Reviewer 2

**Major Compulsory Revisions:**

**R2:** “The results in Fig. 2 show that H. erectum extract was toxic to MDCK cells at 2% (20% viability), and T. chebula extract was toxic to MDCK cells at 0.25% (40% viability) or higher concentrations. In Fig. 3, the extract concentration used to detect the antiviral activity of the materials was 1% according to the figure legend (though seem to be 1% for H. erectum and 0.5% for T. chebula according to the information in the Methods). In Fig. 4, a 2-fold serial dilution was started at 1% or 0.5% in the experiment according to the information in the Methods (though such information is not included in the figure legend). The results in Fig. 4 show the reduction in the titers of the virus treated with H. erectum at the concentrations of 1% and 0.5%, which are only 2-4 times lower than the cytotoxic dose of 2%. Fig. 4 also shows the reduction in the titers of the virus treated with T. chebula at the concentrations of 0.5% and 0.25%, which are cytotoxic doses. Since the cellular growth reflects substantially to the proliferation of the virus infected to the cells, the virus titer reduction obtained at the toxic doses or the doses slightly lower than the cytotoxic dose could not be regarded as “consistent direct antiviral activity”.

**Authors' reply:** This revision is not completely relevant to the design and methods of our research. For virus neutralization test (fig.3), the virus samples were titrated ten-fold after incubation with tested extracts. Thus, the highest concentration of extracts in the inoculums applied to cells was not 1% (as stated by Reviewer 2) but 0,1% and lower. For PRNT assay (fig 4), the concentration of the extracts was indeed just below the cytotoxic dose. However, the inocula were applied to cells for short period of time and were discarded after one hour of contact with cells. Toxic effect was significant after several days of co-incubation of cells with extracts (see “Methods” section).

Thus, either the concentrations of extracts were lower than toxic or time of contact with cells was too short to interfere with establishment of virus infectivity. This design provides significant determination of direct antiviral action.

The consistency of direct antiviral activity is supported by the fact that some extracts inhibit virus in two independent experiments with different concentrations of virus and of extracts applied to cells.

**R2:** “It may be interesting to test the extract materials after concentrating the biologically active components and removing the toxic substances, as the authors described in the paper.
However, based on the data presented in the manuscript, it is difficult to accept their conclusion: “the extracts of H. erectum and T. chebula are promising potential sources of new antiviral drugs”.

Authors' reply: This set of experiments might be a subject of further research.

R2: “2. Abstract/Methods/Line 4-5: “…in the presence and absence of these five plants and the drug extracts…”. The “five plants and the drug extracts” sounds different from the materials described in the paper.”

Authors' reply: The sentence is re-stated.

R2: “3. In the Fig. 4, the concentration of the extract used to test the antiviral activity is not presented.

4. The paper also describes about the isolation of the H3N8 virus from teal, and the genetic analysis of the isolate. However, those data are not related to the topic of this paper. In fact, nothing is mentioned about those data in Abstract and Discussion.”

Authors' reply: The virus subtype identification is an integral part of the research that provides the basement for the critical analysis of the results. However, detailed discussion of genetic properties of A/Teal/Tunka/7/2010 (H3N8) does not fit the scope of this research and may distract the reader’s attention from antiviral activity of the studied herbal preparations.

R2: “5. The manuscript includes words such as “very low”, “extremely low”, “quite toxic”, which do not sound scientific.

6. The information for the commercial products is not provided sufficiently.

7. Figure legends do not include enough information, especially for Figure 2 and Figure 4.

8. The paper includes careless mistakes such as: using both “double distilled water” and “ddH2O”; using both lg10 PFU and Lg PFU; “Chiazospermum erectum” in Table 1 seems to be H. erectum.”

Authors' reply: Indicated errors are corrected according to the recommendations of the reviewer.

Reviewer 3

Major Compulsory Revisions

R3: 1. I have a major problem with the logarithmic representation of viral titer (lg10 PFU/ml). This is not the accepted means of reporting titer. Instead, the titer should be reported as 10^n PFU/ml. Also, a clearer explanation is needed for the following statement of Results in the Abstract “The extracts of …. reduced the titre of A/Teal/Tunka/7/2010 (H3N8) by 0.7-0.9 lg10 PFU/ml (p#0.05), which indicates a 4–6 fold decrease in viral infectivity.”

Authors' reply: Logarithmic expression of virus titer is often used to represent the viral infectivity and neutralization index is usually estimated from logarithmic expression as well (e.g., see “Virology: A Practical Approach.” by B. W. J. Mahy (Editor). Oxford University Press, USA (1985), 280p.). However, to make the reading convenient for non-virologists, we amended the expression of virus titer in the text to 10^n PFU/ml.

Minor Essential Revisions

R3: “2. I disagree with the statement that M. cochinchinensis is well tolerated by MDCK cells at concentrations 1% or less. This statement is not supported by data reported in Figure 2.”

Authors' reply: The text is revised according to fig. 2 and reviewer’s recommendations.
R3: “3. There are minor problems with usage throughout the paper that need to be corrected. For example, the medicinal properties of Deva-5 are characterized in the Background as being used to treat “infectious heat” (not an accepted expression of a disorder). In the discussion authors state that it is used to “cure fever”. Do the authors mean that Deva-5 is used to treat infections diseases that cause fever? This section is critical in establishing a rationale for testing Deva-5 in terms of traditional medicinal uses. Neither usage (infectious heat and cure fever) is standard and both are ambiguous.”

Authors' reply: The usage of medical terms is revised throughout the manuscript.

R3: “4. There are also numerous instances of incorrect verb tense. Additionally, the last statement in the Conclusions should be re-stated. Following is another example of a usage error: "should be studied deeply".

Authors' reply: The proofreading of the manuscript is performed. The last statement is re-stated.

Discretionary Revisions
R3: 5. Authors state in discussion that their preliminary study showed bacterial inhibition. They did not lay groundwork to deviate focus from antiviral properties to bacterial inhibition (S. aureus). A better transition statement is needed. Authors need to show the relevance for including this in the Discussion. The final statement in the paragraph does make a case for studying Deva-5 and its components as antimicrobial agents, but because the reader does not know what is meant by “infectious heat” or “cure fever” (see comment #3), the rationale for including this is not apparent.

Authors' reply: Relevance of the antibacterial and antiviral effects of Deva-5 was explained in the Discussion. The usage of traditional medical terms is revised throughout the manuscript.

Reviewer 4
Major Compulsory Revisions:
R4: “A major concern is the considerable effort and space devoted in this work to the molecular and phylogenetic analysis of the virus strain because the data obtained are not discussed in relation to the rest of the work and no rationale is given for performing this extensive analysis. This part of the manuscript should be more fully integrated and its relevance to the rest of the work should be clearly explained.”

Authors' reply: The virus strain used in our study was previously characterized in very restricted set of experiments. Therefore, we sequenced the HA and NA genes to exclude possible misidentification of virus subtype or misplacement of virus stock. The genetic identification of virus subtype was performed by phylogenetic analysis in comparison with reference strains of each subtype.

Thus, this section is integral part of research that provides the basement for the critical analysis and discussion of the results. However, detailed description of phylogenetic relationships of A/Teal/Tunka/7/2010 (H3N8) or amino acid structure of HA and NA genes does not fit the scope of this research and may distract the reader’s attention from antiviral properties of the studied herbal extracts.

Minor Essential Revisions:
R4: “It is suggested that the authors include statistical evaluation of results in Figure 2 and Figure 4 (either in Figure legends or incorporated into the Figures). It would be helpful to establish the lowest concentrations at which toxic (or enhancing) effects reach statistical
significance (Figure 2). Regarding Figure 4, it would be helpful to identify the highest dilutions of extracts that correspond to statistically significant reduction of infectivity.”

Authors' reply: Figures 2 and 4 are revised according to recommendations of reviewer. The text is revised according to figures 2 and 4.

Minor suggested corrections:

R4: “Abstract - Conclusions, last sentence: The results suggest that the former two plants contain substances with high antiviral activity and could be promising…”

Methods – Plant material and preparation of extracts, line5: To minimize the effect of preparation procedures on bioactive compounds, …

- Virus maintenance and plaque titration assay, line 10: …the inocula were…

- Nucleotide sequencing and phylogenetic analysis, line 7/8:…for HA and NA gene fragments, respectively.

- Evaluation of toxicity of herb extracts for MDCK cells, line 5: …viability of cells was evaluated daily…

- Plaque reduction neutralization test, line 5: …antibodies to the homologous H3 subtype of influenza A virus were used as controls as described above.

Line 10: …and then inocula were discarded…

Results – Virus neutralization by plant extracts, last line: …the extract and infectivity of the virus…

Discussion, line 6: This effect can be explained…”

Authors' reply: Minor errors are corrected according to the recommendations of reviewer.

Reviewer 5

Minor Essential Revisions:

R5: “1-1- Title: The authors described Deva-5 as compound. The word compound must be replaced by “herb formulation”, herb prescription” or even omit the word compound from the title.”

2- Also, the term “Deva-5 compound” mentioned along all the different sections of the manuscript where the authors must be manipulating as correction no (1).

Authors' reply: word “compound” is replaced by “herb formulation” throughout the manuscript.

R5: “3-Abstract:
The virus isolate abbreviated as A/H3/Teal/Tunka/7/2010 in the abstract where it mentioned along all manuscript as A/Teal/Tunka/7/2010. The authors must be written the correct abbreviation.

4- Statistical analysis:
The authors must represent the standard deviation as “PFU±SD” instead of “PFU ±st.dev” when mentioned along all parts of the manuscript.

5- The authors must cite Figure 1, Figure 2 and Figure 3 in the manuscript.”

Authors' reply: errors corrected according to the reviewer' recommendations.

R5: “6-The figures must be footnoted by the significance of the results relative to the control and editing "*" to indicate the P value <0.05.

7-Results:
The authors mentioned Only two preparations, H. erectum and T. chebula, exhibited significant negative correlations between the concentration of the extract and the infectiveness of virus (Fig. 4). The mentioned note would be confirmed after editing the significance notes in the figures.”
Authors' reply: Figures 2 and 4 are revised according to recommendations of reviewer. The text is revised according to figures 2 and 4.

8- The authors must revise all the manuscript to obey the standards of the binomial names of the plants where it appears sometimes in normal and non italic format.

Authors' reply: the binomial names are revised.

Maha: Please replace the highlight with the following:
“The water extracts of the herbal parts of G. decumbens, H. erectum, and P. bistorta, seeds of T. chebula and M. cochinchinensis and Deva-5”
Maha: Please replace the highlight with the following: “according the previously mentioned report [23].”
Maha: Please replace the the highlight word with the word "respectively' 
Maha: “AF091309 (H1), AY633196 (H2), L11129 (H2)” Please check both symbols carefully. Both are designated with H2
Maha: Please unify as above by replacing "Lg PFU/ml" with "lg10 PFU/ml"
Maha: Please clarify your evaluation to mention this statement: Deva-5 did not shown reproducible antiviral activity.
Maha: Please replace the word "compounds" with species or components
Maha: Please unify the shape of standard deviation in this figure also.

Authors' reply: errors corrected according to the reviewer’ recommendations.