Author’s response to reviews

Title: Anti-viral activity of culinary and medicinal mushroom extracts against dengue virus serotype 2: an in-vitro study

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Author’s response to reviews:

April 20, 2019
Dr. Deepa Nath,
Editor,
Journal of BMC Complementary and Alternative Medicine

Dear Dr. Deepa Nath,

Thank you very much for reviewing our manuscript. We greatly appreciate the editor and reviewers for their comments and suggestions. We had carefully reviewed the comments and had revised the manuscript accordingly. We had enclosed our point-by-point response to editor’s and reviewer’s concerns as below.

The followings are our point-by-point responses to Wildriss Viranaicken (Reviewer 3) comments:

1/ Cytokines are markers of inflammation and do not demonstrate an antiviral effect. The anti-inflammatory effect can have a beneficial effect when organisms are infected. For example, dexamethasone is an anti-inflammatory, and yet it is not an anti-dengue. Please modify accordingly. Also these results are presented in the discussion, they should be presented in the result section under a table and experimental procedure should be adapted (ELISA and macrophage infection).
Response: We agreed with the reviewer, we had excluded few statements about the anti-inflammatory
of mushroom extract presented in the discussion section. We had submitted the findings on the anti-inflammatory effect of mushroom extract against inflammatory cytokine expressed in dengue infected monocytes for publication in Journal of tropical biomedicine under titled “Anti-Inflammatory effect of mushrooms in dengue-infected human monocytes”. We decided not to include anti-inflammatory effect of mushroom extracts in the experimental procedure and result for this manuscript.

2/ Figure 1 is incorrectly mounted. A graph with PFU would be more informative with statistics. In general, the presentation of the results in table (CC50 and IC50) is synthetic but seems to limit the ability of readers to clearly identify the effects described. Graphs presenting the data in dose response and with statistical analyses would be more informative and easier to interpret. Response: As recommended by reviewer, the figures were mounted in separate files, were submitted as a single composite file.

Here, we had tested cytotoxic and anti-dengue activities of five types of extract isolated from five different mushrooms. The data were extensive and summarized into CC50 and IC50 values which were estimated from the dose response study for cytotoxic assay and plaque reduction assay, respectively. The CC50 and IC50 values were calculated from probit sigmoid dose response curve generated from log concentration of mushroom extract versus probit probability value using Probit regression analysis using SPSS version 20 (SPSS Inc. an IBM company, United States). The values of IC50 or CC50 values corresponding to a probability of 0.50.

3/ In Figure 2, the experiment was done in n=2, so the shape of the graph is not adequate. No error bar and no significance on a repetition. Please change. n=3 will be fine. This experiment is very important and suggests by comparing with the Plaque reducing assay, an action of the extract tested in figure 1 on the egress of the DV2. please complete and see the point 4 below. Response: We agreed with the reviewer, unfortunately we only able to conduct real time RT-PCR using duplicate samples due to cost constraint.

4/ An MTT test alone seems to me insufficient to conclude on the cytotoxic effect moreover to make plaque reducing assay, the cells are incubated 7 days in the CMC with the extracts and the CC50 was tested over 48 hours. It is therefore necessary to make a cytotoxicity test with LDH assay, neutral red assay or ideally clonogenicity assay with violet crystal on a time course of more than 48 hours. Otherwise, it is necessary to review the flow chart of the plaque reducing assay experiment: Infect Vero cells for 1 hour, then treat the cells with the extracts for 48 hours, then determine the viral progeny in the culture supernatants by PFU and/or RT-qPCR. Do the RT-qPCR in parallel on the cells. Response: Here, we had selected MTT assay to test the cytotoxic effect of mushroom extract on vero cells. It is a conventional method that rapid and cost-effective. This method had been used by many researchers prior to test their compound for anti-viral effect. However, as suggested by reviewer we will include suggested assays in our future study.

As suggested by reviewer, we had treated the infected vero cells for 48 hours with mushroom extract and tested the anti-dengue effect using real-time RT-PCR. The methodology of this assay was illustrated at page 10, line 241-244. “Vero cells were grown at about 90% confluency and infected with DENV-2 and cultured in the presence of 2000µg/mL mushroom extract. After 48 h post-infection, the infected cells were collected by centrifugation at 1500 rpm for 10 minutes” We are unable to repeat plaque reduction assay due to time and budget constraint.

5/ Also the authors focus on glycans, proteins or polyphenols but aqueous extracts has many molecules of other nature capable of antiviral activity.
A "time of addition" experiment must be carried out to determine whether the action is on the entry, replication or egress. This experiment is crucial and important. These results will help to better value the chemical characterization in the discussion part.

Response: As suggested by reviewer, we had included time addition assay experiment. The methodology of this assay was illustrated at page 9 & 10, line 227-230. The effect of addition time of mushroom HAE and ASE on the replication of DENV2 was determined as described previously [9]. Overlay containing 2000µg/ml of extracts were then added to the Vero cell monolayer either 2h before viral adsorption or 0, 1, 2 or 4h after viral adsorption (80-100PFU). Percentage of inhibitory activity of fractions was determined. The result for time of addition assay was presented at page 15, line 356-362.

The antiviral mechanisms of mushroom extract were further assessed by time of addition assay. We selected S.commune HAE and ASE to study this effect. From the result we found that the extracts showed highest percentage of inhibitory activity against DENV-2 infection when added immediately after the virus adsorption (Figure 2). Percentage of plaque inhibition decreased when extracts were added after viral adsorption at 2h, 3h or 5h of time interval. Pre-treating the Vero cells with extract for 2h prior to dengue infection did not significantly inhibit dengue plaque formation.

6/ The part on the correlation between composition and anti-DV2 activity and must be straightforward because it reaches a dead end. It is possible to test the major compound beta-D glycan in your tests or alternatively support your chemical composition analysis with recent study by Song et al, 2018 "Assessment of activity and mechanism of action of β-Dglucan against dengue virus" in Tropical Journal of Pharmaceutical Research.

Response: We are agreeing with the suggestion of reviewer, we had isolated the polysaccharide from the selected mushrooms and tested the anti-dengue effect. We had drafted a manuscript on the findings of this experiment will be submitted to BMC complementary and alternative medicine as soon as possible.

7/ Line 562 to 570, just because the level of NS5 and E decreases does not mean that the extract can have an effect on replication or entry. Please change and see point 5.

Response: As suggested by reviewer, we had included attachment assay and penetration assay experiments, to prove the inhibitory effect of mushroom extract which was during entry. The methodology of both assays was illustrated at page 9, line 211-220. For attachment assay, Vero cells were infected with 500 PFU of DENV-2, in the absence or in the presence of mushroom extracts. After the viral adsorption for 60 minutes at 4 °C, both extract and unabsorbed virus were removed and overlaid with carboxymethylcellulose. Meanwhile, in penetration assay, Vero cells were adsorbed with 500 PFU of DENV-2 for 1 h at 4°C. Unbound viruses were removed and the plates were shifted to 37°C to allow penetration. Cells were treated with mushroom extracts and incubated for 1 h at 37 °C. Extracts were discarded, cell were treated with 0.1ml of citrate-buffer (40 mM citric acid, 10 mM potassium chloride, 135 mM sodium chloride, pH 3) for 1 min to inactivate adsorbed but not internalized virus. Cells were washed and overlaid with carboxymethylcellulose [12].

8/ The manuscript must be reviewed for multiple type error.
Response: As suggested by reviewer, manuscript had been reviewed for multiple type error.

The followings are our point-by-point responses to Ying-Ray Lee (Reviewer 4) comments:
1. What are the mechanisms of the extracts on anti-DENV2.
Response: The anti-DENV2 mechanisms of mushroom extracts had been illustrated in the discussion section at page 20, line 474-481. Here, we had proved that the anti-viral potential of mushroom extracts by inhibiting of viral attachment and penetration of DENV. Extracts showed more prominent inhibitory effect during penetration assay tested using plaque reduction assay and real time RT-PCR. We assumed that the inhibitory activity may be initiated from attachment and gradually increased during penetration stage. The findings presented here in agreement with those published by other authors, who stated that the mechanism underlying the anti-viral activity of mushroom extract, may be related to the inhibition of viral adsorption and penetration [6,9,39].

2. The extracts can block which step of the life cycle of DENV2 (attachment, entry, uncoating, replication, assembling, release)?
Response: Mushroom extracts block the attachment and entry of DENV2.

3. The human cell lines are recommended to verify the anti-DENV2 activity of these extracts.
Response: Here we report, the preliminary study to evaluate anti-DENV2 effect of mushroom extract. We had screened the effect using plaque reduction assay using Vero cells, an African green monkey kidney cell. Vero cells had been certified by the WHO to produce live virus dengue vaccine candidates and use in the plaque reduction neutralization test. However, the anti-viral effects of mushroom in human cell line will be verified in our future study.

4. The study procedure to detection of the expression of DENV-E and NS5 by Q-PCR, the data suggested that extracts might reduce the viral transcription or replication in the cells, but not the explain "inhibited the viral attachment" in the page 25 lines 571 to 572.
Response: As suggested by reviewer, we had included the findings for attachment assay and penetration assay. The anti-DENV2 mechanisms of mushroom extracts had been illustrated in the discussion section at page 20, line 474-481. Here, we had proved that the anti-viral potential of mushroom extracts by inhibiting of viral attachment and penetration of DENV. Extracts showed more prominent inhibitory effect during penetration assay tested using plaque reduction assay and real time RT-PCR. We assumed that the inhibitory activity may be initiated from attachment and gradually increased during penetration stage. The findings presented here in agreement with those published by other authors, who stated that the mechanism underlying the anti-viral activity of mushroom extract, may be related to the inhibition of viral adsorption and penetration [6,9,39].

5. The data of cytokines modulation of these extracts in human monocytes are recommended to include in this manuscript.
Response: The anti-inflammatory effect of mushroom extract against inflammatory cytokine expressed in dengue infected monocytes had been submitted for publication in Journal of tropical biomedicine under titled “Anti-Inflammatory effect of mushrooms in dengue-infected human monocytes”. We decided not to include anti-inflammatory effect of mushroom extracts in the experimental procedure and result for this manuscript.

6. If authors explain that glucan and protein complex is the reason for the anti-DENV activity, this is difficult to be used as an agent for anti-DENV therapeutic in the further.
Response: We disagree with the comment from reviewer, a protein bound glycan isolated from mushroom of Polystictus Versicolor, PSK or Krestin have been used as a drug for treating acute nonlymphocytic leukemia, colorectal cancers, gastric cancers, lung cancer, primary liver cancer,
hepatitis, and hyperlipidemia. Further research on the structural characterisation of glucan and protein complexes and testing these compounds in suitable murine models will lead to the discovery of new anti-dengue therapeutic agents from the culinary and medicinal mushroom.

7. The discussion section is too long, and needs to summarize. – Response: We agree with the reviewer, we had summarized the discussion section.

The followings are our point-by-point responses to Rafidah Hanim Shueb (Reviewer 5) comments:

1) The effect of the mushroom extracts on inflammatory cytokines in infected monocytes was elaborated in the discussion. However, nothing was mentioned in the Methods and Results Sections. If additional experiments were done and the data was meant for this manuscript, the authors should add further information in Methods and Results sections. However, if the data was meant for another publication/manuscript, the authors should mention that a separate study/experiment had been done to evaluate the inflammatory cytokines and results should be described in brief. The current writing style in the discussion part gives an impression that additional experiment was for this particular publication.
Response: We agreed with the reviewer, we had excluded few statements about the anti-inflammatory effect of mushroom extract presented in the discussion section. We had submitted the findings on the anti-inflammatory effect of mushrooms in dengue-infected human monocytes for publication in Journal of tropical biomedicine under titled “Anti-Inflammatory effect of mushrooms in dengue-infected human monocytes”.

2) The authors speculate that the reduced expression of ENV gene indicates that the mushroom extracts inhibit virus attachment. However, in my opinion, using plaque reduction reduction assay as mentioned in the Methods, one could only speculate that the mushroom extracts exert their antiviral activity during the early phase of dengue virus replication. It is not known at this stage whether the inhibition of viral replication at early phase involve viral attachment, entry or others. In addition, doesn't reduced viral replication in general would have resulted in the reduced ENV expression?
Response: As suggested by reviewer, we had included attachment assay and penetration assay experiments, to prove the inhibitory effect of mushroom extract which was during entry. The methodology of both assays was illustrated at page 9, line 211-220.
For attachment assay, Vero cells were infected with 500 PFU of DENV-2, in the absence or in the presence of mushroom extracts. After the viral adsorption for 60 minutes at 4 °C, both extract and unabsorbed virus were removed and overlaid with carboxymethylcellulose. Meanwhile, in penetration assay, Vero cells were adsorbed with 500 PFU of DENV-2 for 1 h at 4°C. Unbound viruses were removed and the plates were shifted to 37°C to allow penetration. Cells were treated with mushroom extracts and incubated for 1 h at 37 °C. Extracts were discarded, cell were treated with 0.1ml of citrate-buffer (40 mM citric acid, 10 mM potassium chloride, 135 mM sodium chloride, pH 3) for 1 min to inactivate adsorbed but not internalized virus. Cells were washed and overlaid with carboxymethylcellulose [12].

3) The authors still need to revise the manuscript for minor linguistic errors
Response: As suggested by reviewer, the manuscript had been revised for linguistic errors.

We hope that you find our responses satisfactory and that the manuscript is now acceptable for publication.

Sincerely,
Ms. E. Kavithambigai,
Corresponding author