Author's response to reviews

Title: Evaluation of antibacterial and cytotoxic activity of Artemisia nilagirica and Murraya koenigii leaf extracts against mycobacteria and macrophages

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Author's response to reviews:

To

Prof. Tom Rowles
Executive Editor
BMC Complementary and Alternative Medicine

Dear Prof. Tom Rowles,

As suggested by you, herewith we are re-submitting a manuscript (Ref. MS: 1708304829111889) enti-tled “Evaluation of antibacterial and cytotoxic activity of Artemisia nilagirica and Murraya koenigii leaf extracts against mycobacteria and macrophages”, to consider for publication in BMC Complementary and Alternative Medicine journal. In this manuscript, we have addressed all the comments given by the editor and the reviewers. The changes made in the text are highlighted in RED. The responses to Editorial and Reviewers comments are given below.

Tuberculosis, caused by Mycobacterium tuberculosis (Mtб), kills more than two million people worldwide each year. An increase in the prevalence of drug-resistant strains of Mtб has renewed focus on the development of new drugs that can treat both drug-sensitive and resistant Mtб infections. Moreover, Mtб persists for extended period of time by modulating host immune responses and drug regimes by entering dormant phase within macrophages. Therefore, anti-TB agents that can penetrate the macrophage and kill the intracellular persisters without harming the host cell is essential. A vast number of medicinal plants have been recognized as valuable resources of natural antimicrobial
compounds. In this study we screened several edible and medicinal plants for their anti-mycobacterial activity. Among them, Artemisia nilagirica and Murraya koenigii were found to be more effective against Mycobacterium smegmatis. Ethanol extracts were found to be more effective against Mycobacterium smegmatis as compared to petroleum ether and water leaf extracts. Moreover, M. koenigii extract showed maximum activity against M. bovis- BCG in combination with first line anti-tuberculosis drug rifampicin. M. koenigii leaf extract also exerted more cytotoxic, DNA damage and apoptosis in mouse macrophage RAW 264.7 cell line. Treatment of mouse macrophages with A. nilagirica extract also increased intracellular killing of M. smegmatis by inducing the production of reactive oxygen species and nitric oxide.

We think that the manuscript may be of interest for the audience of Journal of BMC Complementary and Alternative Medicine. The manuscript has been approved by all authors and is not being submit-ted concurrently elsewhere.

Yours sincerely,

Avinash Sonawane

Responses to Reviewer’s Comments

Responses to Editorial comments:

The changes made in the text are highlighted in RED. Also, the line numbers are given throughout the text.

Q 1: Please provide details in your manuscript on who formally identified the plant material used in your study. Please also confirm whether a voucher specimen of the plant has been deposited in a publicly available herbarium, and include this information in your manuscript. Please also include a deposition number, if available.

Answer: All the plants were identified by a taxonomist Dr. Udhab Behera from Regional Plant Resource Centre, Odisha and these plants have been deposited in the herbarium at Regional Plant Resource Centre, Keonjhar, Odisha, India. (Page No. 06 , line 129-131).

Q2: We would suggest that you include an Acknowledgements section in your manuscript.

Answer: The acknowledgement section has been included in the revised manuscript. (Page No. 18, line 404)
Responses to reviewers comments:

Reviewer # 1 Lyndy McGaw

Minor essential revisions:

Q1: The spelling of the microorganisms should be carefully checked and corrected where necessary, for example “Pseudomonas” and “pneumonia”.

Answer: We have checked and corrected all the spelling of the microorganisms throughout the manuscripts.(Page No. 05, line 109).

Q2: In the abstract Methods, include the test organisms against which antibacterial activity was evaluated and the cell line for cytotoxicity testing.

Answer: We appreciate the reviewer comment here and we have mentioned the microorganisms (M.smegmatis and M.bovis BCG) and mouse macrophage RAW264.7 used in our study.(Page No. 02, line 37-39).

Q3: In the abstract results, the mention of ten plants screened does not follow from the Background and Methods. How were these ten plants selected? Also the macrophages and intracellular activity assay is not mentioned in the methods. The best results in terms of MIC values should be given in this section.

Answer: According to the reviewer comment we have removed the “ten plant” statement and only named the two plants with major activity. Name of ten plants is mentioned in the introduction section. These plants were selected on the basis of their usage in the tribal areas for its edible and medicinal property.(Page No.03, line 50-54).

In the corrected manuscript we have incorporated the macrophages and intracellular activity in the methods.(Page No. 10).

The MIC values are given in the main result part of the manuscript.

Q4: In the abstract Conclusions, change “Ethanol extract” to “Ethanol extracts”. The combination with rifampicin is mentioned for the first time here and this should be addressed.

Answer: We appreciate the reviewer’s comment here and we have changed the text to “Ethanol extracts” in the corrected form. The combinatorial experiment with rifampicin is mentioned in the abstract methods and results as first line anti TB drugs and rifampicin respectively.(Page No.03, line 59 ; Page No. 02 ,line 38 ; Page No.03, line 53).
Q5: One of the anti-TB drug is referred to as either “rifampin” or “rifampicin” and this need to be consistent.

Answer: We have used rifampicin throughout the manuscript.

Q6: In the second paragraph of the introduction, there are many other references concerned with the recognition of medicinal plants as source of antimicrobial compounds, particularly antimycobacterial compounds.

Answer: In the second paragraph of the introduction we’ve given the references where Ríos JL et al, Kokoska L et al, Khan R et al, describing antimicrobial activity of plant extracts and we have added B. A. Adeniyi et al in references for antimycobacterial activity of Cola plant extract. (Page No. 19, line 421 and line 428).

Q7: In the second paragraph of the introduction, reference need to be added for several statements which remain unreferenced.

Answer: We appreciate the reviewer’s comment here. We have made the necessary changes and reference Adeniyi et al has been incorporated in the second paragraph of the introduction. (Page No. 04, line 91).

Q8: The name of the family “Rutaceae” should not be in italics.

Answer: The word “Rutaceae” has been corrected throughout the manuscript. (Page No.05, line 106).

Q9: In the third paragraph of the introduction, include the MIC values of the compounds and extracts of Rutaceae plant species. What about antibacterial activity of members of Asteraceae family or more closely related species of the one under investigation?

Answer: We appreciate reviewer’s comment here. We have mentioned the references for the antimicrobial activity of Rutaceae and Asteraceae plant species in the third paragraph. (Page No. 05, line104).

Q10: “M. Bovis BCG” should not be typed with hyphen.

Answer: As per the reviewers valuable suggestion we have removed the “hyphen” mark in “M. Bovis BCG” in the manuscript. (Page No.06, line 118-119 ; Page No.07, line 156 ; Page No.12, line 258 and 264 ; Page No.16, line 356 ; Page No.18, line 395).

Q11: In the methods, a voucher specimen for each species should be identified names and deposited in a recognised herbarium.
Answer: In the revised manuscript, we have included the information on identification of plants by a recognized taxonomist and also given the information on deposition of voucher specimen in the recognized herbarium. As mentioned in the text all the plants were identified by Dr. Udhab Behera from Regional Plant Resource Centre, Odisha and these plants have been deposited in the herbarium at Regional Plant Resource Centre, Keonjhar, Odisha, India. (Page No. 06, line 129-130).

Q12: In the methods cite the centrifuge speed in "x g" not in "rpm" and mention the make of centrifuge used.

Answer: As per the reviewer's instruction we have cited the centrifuge speed in "g" in the method section and also mentioned the centrifuge model and source. (Page No. 06, line 135).

Q13: In the cytotoxicity assay method description, how many cells were seeded per well?

Answer: In the cytotoxicity assay method we have seeded 10,000 cells per well in a 96 well cell-culture plate. (Page No. 08, line 174).

Q14: In the apoptosis assay method, was the cell density mentioned per well? Don't start a sentence with a number.

Answer: As per the reviewer's comment we have modified the sentence. Cell density $2 \times 10^5$ RAW264.7 cells per well were seeded in a six well cell culture plate. (Page No. 08, line 186).

Q15: In the comet assay method, what density of cells was treated? How were the concentrations of the plant extract selected? After treatment with trypsin, how many cells/ml were there in the cell suspension?

Answer: We appreciate the reviewer's comment here. In the comet assay $5 \times 10^5$ RAW264.7 macrophages per well were treated in a 6 well culture plate. Concentration of the plant extracts were selected on the basis of in vitro data. These concentrations were found to be effective against M. smegmatis under in vitro killing assay. So for comet assay we took 100µg/ml concentration for M. Koenigii and 300µg/ml concentration for A. Nilagirica ethanol extract. After treatment with trypsin, 5000 cells/µl were there in the cell suspension. (Page No. 09, line 197-201).

Q16: In the NO method description, change “calorimetrically” to “colorimetrically”.

Answer: As per the reviewer's comment, we have rectified “calorimetrically” to “colorimetrically” in the NO method description. (Page No. 11, line 240).
Q17: In the result again, mention is made of several plant extracts but results are not reported.

Answer: In the revised text we have added the names of plant tested in our study in the result section and we have included the results of the two plant ethanol extracts (M. Koenigii and A. Nilagirica) which are showing positive result for antimycobacterial activity. Other plants did not show any significant antibacterial activity. For this reason we have not included data of other plants.

Q18: Reference 6 is incorrectly written- the first names should be given as initials with the surname “Cowan” first.

Answer: As per the reviewer’s suggestion, we have corrected the indicated reference .(Page no. 19 , line 431).

Q19: There are many reviews of the use of medicinal plants as antimycobacterial agents and some of these should be included.

Answer: We have added some references on antimicrobial activity of plant extracts and reference 6 describing about antimycobacterial activity of plant extract.

Major Compulsory Revision

Q1: There are inconsistencies in the logical progression of the manuscript, for example in the abstract as mentioned in the minor revisions, experiments are not mentioned in the methods before being discussed in the Results or Conclusions. In the last paragraph of the introduction mention is made of screening of several plant leaf extracts. What were these and how were they selected? The results should not be explained in the Introduction. This section should focus on the motivation for the study and what was done. The motivation for the apoptosis and genotoxicity should be strengthened.

Answer: We highly appreciate the reviewer’s comment here. We have made necessary changes and in the abstract methods the experiments are mentioned. Plant names have been added in the last paragraph of the introduction (Page No. 05, line 112-114). In the revised manuscript we have added that the selection of plants are based on their use as edible or medicinal property by the local tribal population of eastern India (Page No. 05, line 115). The motivation for the apoptosis and genotoxicity is to check for the cytotoxic effect of plant extracts on macrophages, which are primary resident cells for mycobacteria. Our interest was to identify the plant extract which will exhibit antibacterial activity without showing any toxic effect on host cells.

Q2: Where the “In vitro killing assay” is described, to what cfu/ml did the optical
density of 0.1 at 600 nm correlate for each species? What was the reference for the assay method used here? Not enough detail is given regarding the combinations tested and the respective concentrations used. Why was a minimum inhibitory concentration (MIC) method using serial broth dilution not used to obtain MIC values for each plant extract against each test organism? This could be included to allow easier comparisons of data with those published.

Answer: As per the growth pattern of mycobacterium 0.1 optical density at 600 nm corresponds to \(1 \times 10^7\) cfu/ml. We have added the reference number 13 (Sonawane et al., 2011, 13:1601-161), which describes about in vitro killing assay. As shown in Figure 1 MIC was determined by serial broth dilution method. (Page No. 07, line 152).

Q3: Plant extracts, particularly those with antioxidant activity, are well known to interact with MTT so the cells need to be washed before adding MTT. Alternatively a cell-free control needs to be added to preclude interference with the assay. See reference: Shoemaker M, Cohen I, Campbell M: Reduction of MTT by aqueous herbal extracts in the absence of cells. Journal of Ethnopharmacology. 2004, 93:381-384.

Answer: In our cytotoxicity experiment, before addition of MTT the cells were washed two times with 1X PBS.

Q4: Reference are missing for all the methods and these need to be included. How many replicates were included in each experiment?

Answer: As per the reviewer’s instruction, we have added references for all the methods (Page No. 20, Reference No. 12-20). As mentioned in the Figure legends all experiments were performed in triplicates. (Page No. 24).

Q5: There are concerns with the intracellular assay method. What is the reference for this method? As far as I am aware M. smegmatis lacks the capability to infect macrophages as it is a non-pathogenic species and evidence need to be provided that it is indeed able to infect the cells. Is it not possible for gentamicin to penetrate the macrophage cell membrane to kill intracellular bacteria? More details need to be given on this method in general.

Answer: In the revised manuscript we have incorporated the relevant references, which describes intracellular killing assay. Several reports such as sonawane et al Cell Microbiol. 2011, 13(10); Mishra et al Cell Microbiol. 2010. 12 (8) suggest M. smegmatis can infect macrophages and it is widely accepted model for mycobacterial infection studies. Our previous studies reported that the gentamicin concentration (20\(\mu\)g/ml) used in this study does not kill intracellular bacteria. The detailed process is mentioned in the method section.

Q6: For the synergistic activity study, a recognised method such as the
checkerboard or isobologram method should be used to identify synergistic, additive or negative interactions.

Answer: As per the reviewer’s suggestion, an isobologram study has been added in the result section indicating synergistic activity of M. Koenigii ethanol extract and anti-TB drug rifampicin. (Page No. 08, line 164-170).

Q7. With the MTT assay, results should be reported as IC50 values to make it easier to compare results with those published.

Answer: We highly appreciate the reviewer’s comment here. We have mentioned in the MTT assay result section about the 50% reduction in cell viability for both the plant extracts which is indicating the IC50 value. (Page No. 13, line 286).

Q8. In the discussion, references are missing for many statements and these should be included.

Answer: As per the reviewer’s suggestion we have added references in the discussion part.

Reviewer # 2 Naseer Ali Shah

Q1: Mention names of plants screened for the activities mentioned in the manuscript other than A. Nilagirica and M. Koenigii.

Answer: We have added the names of plants screened for the antibacterial activities. In addition to A. Nilagirica and M. Koenigii, other plants such as Eupatorium triplinerve, Nyctanthes arbor-tristis, Azadirachta indica, Barringtonia acutangula, Achyranthes aspera, Moringa oleifera, Artemisia nilagrica, Murraya koenigii, Lantana camara and Mentha spicata were used in this study. (Page No. 05, line 112-114).

Q2: Extract was dissolved in 1% DMSO (wt/vol). Add reference for this description or explain in revision.

Answer: As per the reviewer’s instruction we have added reference for the above issue (reference 12) where P. S. Luize et al has used 1% DMSO for extract preparation.

Q3: For statistical analysis use One WAY ANOVA followed by posthoc Dunnet test.

Answer: We have used Mann Whitney test for statistical analysis in our studies.
Q4: Add more references to introduction and discussion portion. Reference range should be above 30.

Answer: We have added required references to introduction and discussion portion. There are 32 references cited in the manuscript.