Author's response to reviews

Title: Preliminary antimycobacterial study on selected Turkish plants (Lamiaceae) against Mycobacterium tuberculosis and search for some phenolic and flavonoids from the plants

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Author's response to reviews: see over
Jean de Dieu TAMOKOU

1) The manuscript is overall poorly written.

Authors answer: we checked all the manuscript

2) There are some typographical and grammatical errors throughout the manuscript.

Authors answer: We checked it again

3) The title is not appropriate.

Authors answer:

The title “Phenolic and flavonoid profile, antimycobacterial properties of six selected Turkish plants (Lamiaceae) used for tea, spice and folk remedies” is changed as below.

“Preliminary antimycobacterial study on selected Turkish plants (Lamiaceae) against Mycobacterium tuberculosis and search for some phenolic and flavonoids from the plants”.

4) The abbreviations: anti-TB leads, LC-MS, MICs, etc. were used in the manuscript even earlier than their description in the methods; therefore these abbreviations should be described in full form at their first use in the manuscript.

Authors answer: The abbreviations were described in full form at their first use in the manuscript.

5) Please rewrite the background of the abstract and also summarize it.

Authors answer: Background was checked again and also shortened.

6) Also, rewrite the following sentence to summarize it: “Six Turkish medicinal plants of the family Lamiaceae (Stachys t molea Boiss., Stachys thirkei C. Koch, Ballota acetabulosa (L.) Benth., Thymus siptorpii Benth., Satureja aintabensis P.H. Davis, Micromeria juli ana (L.) Benth. ex Reich.) were collected in 2009 – 2010 and subjected to a sequential extraction with different solvents according to their increasing polarity: petroleum ether, ethyl acetate, and methanol”.

Authors answer: This sentence is rewrited as below.

“Six Turkish medicinal plants of the family Lamiaceae (Stachys tmolea Boiss., Stachys thirkei C. Koch, Ballota acetabulosa (L.) Benth., Thymus siptorpii Benth., Satureja aintabensis P.H. Davis, Micromeria juli ana (L.) Benth. ex Reich.) collected in 2009 – 2010.

Dryed and crushed plant samples were subjected to a sequential extraction with petroleum ether, ethyl acetate, and methanol as to their increasing polarity.”

7) Line 16, What does " were found to develop significant activity against… " mean?

“The three fractions of S. aintabensis exhibited significant activity.”
Authors answer: This Word “significant” is depend on MIC and MBC concentration values. This is not depended on any statistical value.

8) Line 21, What does " … pronounced antimycobacterial activity… " mean?

Authors answer: “pronounced antimycobacterial activity” used as clear / distinctive/ prominent /marked significant or evident activity means. This Word changed as “prominent” in the manuscript

9) The following statement " …not only inhibits but kills all the…." is repetition.

Authors answer: It is changed and signed as red in the manuscript.

10) Please revise the keywords

Authors answer: In this section, some keywords are omitted and some keywords is added and signed as blue in the manuscript.

11) The Background has been poorly written. There is need for a major review.

Authors answer: see below (14)

12) There is much literature on biological activities of the studied plants that should help the authors in writing the background for their work.

Authors answer: see below (14)

13) Paragraph 2 sounds more appropriate to start the introduction. “Tuberculosis (TB) is an infectious bacterial disease caused by………. have provided an alternative source for the development of antimicrobial drugs [17].”

Authors answer: see below (14)

14) The 1st statement in the background should be moved and made the next statement of this section. “Lamiaceae members have been……………..such as antifungal [11], antibacterial [12], anticholinesterase and antioxidant [13], and anti-HIV-1 [14].”

Authors answer: For 11, 12, 13, and 14;

The background is changed. Paragraph 1 and 2 In the beginning, we started to explain about the facts how serious tuberculosis is all over the World, what is the incidence of sick and infected people in the World, why standard treatment is difficult. After giving some information about tuberculosis, we build up other paragraphs about Lamiaceae and usage of our species such as how these plants traditionally used by people for diseases.

15) “This study investigated six plants of the Lamiaceae family (Stachys tmolea Boiss.,Stachys thirkei C. Koch, Ballota acetabulosa (L.) Benth., Thymus sipthropii Benth., Satureja aintabensis P.H. Davis, Micromeria juliana (L.) Benth. ex Reich.)
with traditional claims for several diseases.” This statement is not a scientific presentation. Please specify what you want to do.

**Authors answer:** This sentence is corrected as
“In this study, six plants of the Lamiaceae (*Stachys tmolea* Boiss., *Stachys thirkei* C. Koch, *Ballota acetabulosa* (L.) Benth., *Thymus sipthorpii* Benth., *Satureja aintabensis* P.H. Davis, *Micromeria juliana* (L.) Benth. ex Reich.) were investigated with traditional claims for several diseases.”

16) The authors need to justify the choice of microorganisms according to their traditional use.

**Authors answer:** This justification is present in the discussion part. please refer to the discussion and get the following sentence: “Our rationale for using H37Rv (ATCC 27294) and H37Ra (ATCC 25177) was that they have drug susceptibility profiles that are reasonably representative of the majority of drug-susceptible clinical isolates; In addition, they are used around the world as a standard, and could provide an opportunity to identify new compounds effective against strains of *M. tuberculosis*.

17) Any reference? Antimycobacterial assay Determination of Mycobactericidal Effect

**Authors answer:** We re-organized the subtitles about antimycobacterial assay in order to get well understanding and added two references for MBC.


18) Can the authors summarize these sections with references?

**Authors answer:** Two references are added to these section.


19) Results have not been scientifically reported. The tables need to be explained in the text, for the different strains and how these compare with other Lamiaceae species as presented in available literature should be highlighted. The authors need to scientifically present and discuss this section.

**Authors answer:** Results section is rewritten and signed as blue in the manuscript.
Conclusion section:
20) The authors should revise this section.

Authors answer: Conclusion section is developed as below. 
Tuberculosis is a very serious disease in the world. The toll was 1.4 million people in 2011. A total of 1.4 million people died from TB in 2011. One third of the world’s population, especially from in low- and middle-income countries, is infected with *Mycobacterium tuberculosis*. In addition, approximately one in four deaths among people with HIV is due to TB. MDR-TB, 630 000 people in the world, is caused inappropriate use of TB-drugs, does not respond to standard treatments due to costly and ineffective to treat [46].

In the present investigation, we also demonstrated for the first time the efficacy of PEF and EAF of two plant species, *S. aintabensis* and *T. sibthorpii*. In addition, PEF extract of *Ballota acetabulosa* was also efficient against *M. tuberculosis* strains including isolates taken from patients. These extracts displayed very high activity not only by inhibiting but also by killing all of the *Mycobacteria* strains. The phenolic and flavonoids might represent promising sources of drug for anti-TB. The LC-MS spectra of all the extracts revealed their main phenolic composition, which was closely associated with the observed antimycobacterial activity [41]. However, advanced researches will be necessary to confirm this hypothesis. Consequently, compounds in the extracts and potential activity may guide the future isolation and antimycobacterial evaluation of the active principles. Further phytochemical and pharmacological studies of these plants are clearly worthwhile. Flavonoids might be “a modal” for the drug design. Identification of the antimycobacterial mechanism of action might be the key to the development of these compounds.

Reviewer: Mariam Degani

1. The solubility of the 100mg/mL stocks in DMSO has not been mentioned. Also, the cell counts of the bacteria used for the various strains have not been mentioned.

Authors answer: Stock solutions of each extract are given under the “Stock and working solutions” subtitle. Suspensions for inoculum was adjusted to a 0.5 McFarland standard by a visual comparison to a 0.5 McFarland Turbidity standard and diluted 1 mL of the adjusted suspension in 4 mL of sterile saline (1:5 dilution). Inocula prepared from a Day 1 to Day 5 positive MGIT 7 mL tube range were between $0.8 \times 10^5$ to $3.2 \times 10^5$ CFU/mL. All details of inoculum preparation are added to “Preparation of inoculums for anti-tuberculosis assay” section.

2. In the abstract the authors mention the Mycobacterial species whereas the term strains would be more appropriate. Also the origins of Strains 1 & 2 have not been mentioned in the material data. Some mention about the source and nature of these strains (MDR/XDR) is appropriate. In the list of abbreviations.
Authors answer: Thank you very much for this correction. Mycobacterial species is changed to Mycobacterial “strains”. The source and nature of strain 1 & 2 were added in the manuscript.

3. BACTEC has been mentioned without any reference in the paper

Authors answer: Related references for antimycobacterial assays were added.

4. The 37 in H37Rv is not uniformly depicted as subscript in the paper.

Authors answer: “$H_{37}Rv$” is corrected as “H37Rv” in the manuscript.

5. The term chemical flood appears in the Materials and Methods which needs to be explained.

Authors answer: This sentence is corrected as “Solvents were evaporated under reduced pressure using a rotavapor (Heidolph).”

6. Minor grammatical errors need to be rectified in the paper.
Authors answer: We got professional help for checking grammar of the manuscript again.

Discretionary Revisions:
1. If the individual standards, especially rosmarinic acid and chlorogenic acid are screened for the activity, it would confirm the proposition of the active/inactive component.

Authors answer: Thank you very much for your proposals and critics. They help us to make some corrections and additions.

2. The final conclusion states the importance of flavonoids as the models for drug design. However some the extracts which are active have components which are phenolic but not flavonoids. Further clarification is required for this statement.

Authors answer: Yes, you are right. Now, we have mentioned not only phenolic but also flavonoids. We changed “the phenolic” to “the phenolic and flavonoids”.

Cassandra Quave

1. Your methods don’t describe the use of a carrier control (drug excipient control) Did you also include this?

Authors answer: We used plant materials not drug. We solved it by DMSO. Due to higher plants are the source of potential substances of new drugs, we try to possess varied plant material according to their traditional use. We prepared plant extracts and assayed them according to standard methods. Broth microdilution and agar dilution methodologies are considered quantitative because they can measure the minimum inhibitory concentration (MIC). The MIC is defined as the lowest
concentration of an antibiotic that inhibits visible growth of a microorganism. Both quantitative methods are considered the reference methods for susceptibility testing because of their high levels of reproducibility.

2. You discuss rosmarinic acid as the major phenolic in most of these plants – this doesn’t necessarily mean that it is the active ingredient. You should include a control of rosmarinic acid (this doesn’t have to be isolated from the plants, but may be purchased) in your MIC testing. You can then compare the anti-TB efficacy of the rosmarinic acid alone to the complex extracts. Likewise – the other major phenolics listed (caffeic acid, chorogenic acid, etc – should be tested).

Authors answer: To detect phenolic and flavonoids in the extracts, we use analytical standards for chromatography (mostly LC-grade or HPLC grade). Plant extracts include a mixture of substances. At the preliminary stage, we can only determine the phenolic and flavonoids by LC-MS. We have also started to studying the biological activities of these analytical substances for our next research.

3. Statistical analysis is missing? Are these data significant? p-values?

Authors answer: In these assays, it doesn’t need statistical analysis.

Minor Essential Revisions:
• In the methods section, you refer to “gradient grade” solvents – do you mean to say LC-grade solvents? This needs to be corrected or clarified.

Authors answer: Yes, we called “gradient grade” but “LC-grade” is better. We changed the term as “LC-grade”.

4. In addition to testing your extracts against 2 highly sensitive ATCC strains, it would be much more interesting (and would add more value to the paper) if you also performed some tests on clinically-relevant isolates of TB (in particular, Isoniazid-resistant strains)

Authors answer: Testing against drug-resistant and MDR strains of *M. tuberculosis* is not critical in primary screening, because these strains are not “superbugs”, which are resistant to multiple antibiotics by virtue of a single mechanism, such as the effusion pumps found in other bacteria, but are instead the result of specific step-wise mutations to individual drugs.

5. Table 3 would be better illustrated as a bar chart

Authors answer: we were unable to built bar chart from the data. It seems very difficult and could not give something clear.