Author’s response to reviews

Title: Antioxidant activity of rosemary (Rosmarinus officinalis L.) essential oil and its hepatoprotective potential

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Author’s response to reviews: see over
Dear Reviewers,

Firstly, on behalf of the co-authors, I would like to thank you for helping us with your kind and precise comments and suggestions to improve our initial version of the manuscript.

We accepted all suggestions and they are included in this modified and improved version of our manuscript. We have highlighted these changes in the manuscript.

In this letter we tried to state point by point the changes we made to the manuscript in accordance with your comments.

Reviewer #1

Dear Dr. Moselhy,

Thank you for your precise comments. We accepted all your suggestions and they are highlighted in the manuscript. Several grammatical, spelling and other language-related errors have been corrected by the consultation with native English speaker. Statistical review has been accomplished by consultation with a statistician.

1. **Reviewer’s comment:** The rational of the study should be clear.

   **Our action:** We have accepted your suggestion and rearranged the last paragraph of ’Introduction’ section in order to clarify the aim and highlight the rationale and significance of our study.

2. **Reviewer’s comment:** The number of animals in each group not enough for statistical analysis

   **Our action:** We performed our *in vivo* experiments on animals in accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes (published on 20 October 2010) that includes an explicit reference to the 3Rs principle [Replacement, Reduction and Refinement]. The principle of reduction refers to the reduction of the number of animals used to a minimum, to obtaining information from fewer animals or more information from the same number of animals. Our study was approved by the Ethical Committee of the University of Novi Sad that also includes a statistician, and 6 animals was considered acceptable from ethical and statistical perspectives. We also found several studies with the same or similar methodology that included 6 animals in the group, such as:

3. Reviewer’s comment: GC/MS chart figure should be supplemented.

Our action: Please find supplemented gas chromatogram and mass spectrum of investigated rosemary essential oil (Suppl_1, Suppl_2).

4. Reviewer’s comment: The discussion is not clear. The authors should make interpretation linked to other recent studies.

Our action: We have largely rearranged the 'Discussion' section and added results of several recent studies that are highlighted in both text and Reference list in order to provide more detailed explanation of our obtained results.

Reviewer #2

Dear Dr. Singh,

Thank you for your precise comments. We accepted all your suggestions and they are highlighted in the manuscript. Several grammatical, spelling and other language-related errors have been corrected by the consultation with native English speaker. Statistical review has been accomplished by consultation with a statistician.

1. Reviewer’s comment: Authors have not investigated which component is responsible for the observed hepatoprotective effects? Is it eucalyptol alone or in combination with other major component or was it synergistic or antagonistic effect? These details should be provided and discussed.

Our action: We have largely rearranged the 'Discussion' section and added results of several recent studies that are highlighted in both text and Reference list in order to provide more detailed explanation of our obtained results. In the last paragraph of 'Discussion’, we have focused on established effects of eucalyptol as the major compound in investigated essential
oil, but have also emphasized the possible contribution of other components and the importance of further investigations on their mechanisms of action.

2. **Reviewer’s comment:** Include details about various enzymes and stress markers in abstract and their results.

**Our action:** The abstract has been changed and the details about enzymes and stress markers have been included.

3. **Reviewer’s comment:** Please provide details about the number of replicates, sample size for REO chemical analyses, oxidative stress studies and others.

**Our action:** We have added those details in 'Materials and Methods' section. All measurements were performed in triplicate for every sample.

4. **Reviewer’s comment:** Please add the names of the libraries used for identification.

**Our action:** National Institute of Standards and Technology (NIST) mass spectra library for GC-MS (IBM-AT, version 2, 1990) was used in our experiment.

5. **Reviewer’s comment:** Did authors use co-GC for identification?

**Our action:** No, retention indexes and MS were used for identification.

6. **Reviewer’s comment:** Did authors use REO as such for phenolic and DPPH assay or it was dissolved in some solvent or an emulsion was used? Please explain.

**Our action:** REO was used as such in DPPH and TPC assays. We replaced the term 'sample' in the text with 'essential oil' in order to avoid misunderstandings.

7. **Reviewer’s comment:** REO was applied as emulsion – please elaborate.

**Our action:** The essential oil was administered orally by gavage, previously suspended in saline in order to avoid using very small volumes. The variability in the volume of administered doses was managed by adjusting the concentration to ensure a constant volume (1 ml/kg). This has been added in the 'Animals and treatment' section.
8. **Reviewer’s comment:** Pl present data as mean with SE and not deviation.

**Our action:** We have changed standard deviation with standard error of the mean (SEM) for all results (presented in Table 2 and Figure 1). It was changed also in the 'Statistical analysis' section.

9. **Reviewer’s comment:** Pl replace hours with h.

**Our action:** 'Hours' is replaced with 'h', which is highlighted in the text.

10. **Reviewer’s comment:** Replace N-hexane with n-Hexane.

**Our action:** 'N-hexane' is replaced with 'n-Hexane' in 'Materials and Methods' section.

Reviewer #3

Dear Dr. Jeong,

Thank you for your precise comments. We accepted all your suggestions and they are highlighted in the manuscript. Several grammatical, spelling and other language-related errors have been corrected by the consultation with native English speaker.

1. **Reviewer’s comment:** The authors should provide explanations, and references if possible, on why REO 10mg/kg co-treatment resulted in a dramatic increase in bilirubin that is a maker of an impaired excretory function of the liver. In addition, among the three antioxidant enzymes why only GPx activity increased by CCl4 treatment? If this is due to upregulation of the enzyme in response to oxidative damage, why other antioxidant enzymes do not respond same way? Any references with similar results?

**Our action:** We have highlighted and tried to explain result regarding bilirubin in 'Discussion' section. We proposed camphor, a bicyclic monoterpene which is present in our REO in a relatively high amount (12.53%), as a potentially toxic compound that might contribute to hepatic excretory impairment after administration of REO in the dose of 10 mg/kg. REO in the dose of 5 mg/kg did not exert that effect, which is in agreement with previous findings that many monoterpenes do not exhibit dose-dependent effects and that it is necessary to find the most appropriate dose range that shows effectiveness.

Regarding antioxidant enzymes, similar results were obtained in the study of Hsiao et al. 2003, where it was demonstrated that CCl4 induced increase of GPx activity and reduction of CAT activity. It was explained that under oxidative stress conditions GPx can be upregulated
as the adaptive response of the hepatocytes to oxidative damage, and GSH is then largely consumed, which is in accordance with results of our study. On the other hand, CAT is easily inactivated by reactive oxygen species.

2. Reviewer’s comment: It is hard to understand that the REO contains phenolic compounds since the essential oil was collected with "Hexane" after hydrodistillation. Hexane is one of the most non-polar solvent and the polar phenolic compounds would not be extracted with hexane. Have the authors analyzed the phenolics profile? Also, give the proper rationale for measuring phenolics content in this study.

Our action: In earlier studies, the antioxidant activity of rosemary was shown to be attributed mainly to carnosic acid, carnosol and rosmarinic acid components. It was also demonstrated that the antioxidant activity of rosemary essential oil may be also related to two phenolic diterpenes - carnosic acid and carnosol which are considered as two effective free-radical scavengers (Estévez et al. 2007; LWT-Food Science and Technology, 40(1), 58-65). We haven't analyzed the phenolics profile in our study, but we measured total phenolic content in order to determine if this class of chemical compounds contributes to antioxidant capacity of REO. It is obvious that the solvent used may be important for the antioxidant activity, depending on the phenolic content, but it was shown, for instance, in the study of Liu et al. (Food Chemistry, 2007, 105(2), 548-554) that phenolic content was 5-fold lower in hexane extract in comparison to methanol extract of Xylaria sp. from Ginkgo biloba, but still present. The results of TPC assay in our study demonstrated that REO contains phenolics in a relatively small amount, which is in accordance with results of the study by Viuda-Martos et al. 2010, in which it was shown that phenolic content in REO was lower when compared to essential oils isolated from oregano, thyme and clove.

3. Reviewer’s comment: Is the radical scavenging activity of REO from its oil components such as volatile terpenes or from the phenolics?

Our action: We added information in 'Results' and 'Discussion' sections that phenolics are present in the studied REO in a small amount and that monoterpenes mostly contribute to free radical scavenging capacity of REO.

Our team hopes that we have managed to properly consider and act upon the comments and suggestions raised.

Yours sincerely,

Nebojša Pavlović, corresponding author