Author's response to reviews

Title: In vitro protection of biological macromolecules against oxidative stress and in vivo toxicity evaluation of Acacia nilotica (L.) and ethyl gallate in rats

Authors:

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Author's response to reviews: see over
To

Tom Rowles PhD,
Senior Executive Editor,
BMC Complementary and Alternative Medicine

Sub: Submission of the revised manuscript entitled “In vitro protection of biological macromolecules against oxidative stress and in vivo toxicity evaluation of Acacia nilotica (L.) and ethyl gallate in rats” – Reg.

Dear Sir,

We are glad to submit our revised manuscript (MS: 7432470912851020) with reference to your e-mail dated on 29.05.14.

In this regard, we are happy to inform that in light of the reviewer’s comments we have made the required changes with careful attention to each comments in the revised manuscript. These changes have been highlighted in red in the revised manuscript. We have also provided a point by point response to reviewer’s comments along with the cover letter.

We would be grateful if you consider the revised version of our manuscript and do the needful towards publication in your esteemed journal.

Thanking you

Yours sincerely,

Dr. C. Rajasekaran
Response to reviewer’s comments on the manuscript entitled: “In vitro protection of biological macromolecules against oxidative stress and in vivo toxicity evaluation of Acacia nilotica (L.) and ethyl gallate in rats” are:

Dear Reviewer: Mona Hetta
Thank you for your effort in correcting our manuscript by giving useful comments and suggestions needed for the revision of our manuscript. We have modified the manuscript accordingly, and the detailed corrections are listed below, point by point:

1. Discretionary Revisions (Recommended comments):
   • Addition of the name of the author (L.) each time after the plant name especially in figures and the table.
   Addition of the name of the author (L.) after the plant name has been incorporated wherever it is mentioned in the text and in figures and tables as well.

   • Addition of the range of normalcy in page 15.
   We have included the range of normalcy in page 15. The range of normalcy for total protein, albumin and glucose are reported to be 5.6-7.6 g/dL, 3.4-4.8 g/dL and 50-135 mg/dL respectively [37].

2. Minor Essential Revisions: (the corrections are highlighted in the manuscript)

- Page 5, line: 6: under "Plant material and extraction":
   It was mentioned "in our previous report [7]-------------please check this no. it is wrong.
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   As per the reviewer comment, the word gel in page 6, line 9: under “Protein damage assay”: has been corrected to Gel in the revised manuscript.

- Page 7, line 2: under "DNA interaction by FTIR and UV analysis": HCL----HCl (correction)
   In page 7, line 2: under “DNA interaction by FTIR and UV analysis”: HCL has been corrected to HCl in the revised manuscript.

- Page 8, line 1: under "Animals": mention the no. of rats used.
   Forty eight rats were used for the study and have been included in the experimental section under “Animals” in the revised manuscript (page 8, line 1).
- Page 10, line 7: under "Results and discussion": the same mention plant
In page 10, line 7: under “Results and discussion” the name of the plant *A. nilotica* (L.) has been mentioned in the revised manuscript.

**Please check the references no.**:

# There are mistakes in no. 4 – 7.
- References 4 & 5 have been replaced with correct reference number in the text as [4] and in reference section as well (Kalaivani and Mathew, 2010a).
- Reference 6 is changed to [5] in the text and as Hooda et al, 2009 in the reference section.
- Reference number 7 has been changed to [6] in the text and remains the same in the reference section as it matches with the text.
- Due to the changes made in reference numbers 4-7, the whole reference order has been carefully modified in the revised manuscript.

# Ref. 10 and 12 (exchange of letters; 2010a for ref. 10 and 2010b for ref. 12.
As rightly pointed out by the reviewer, we have changed the letters for references 10 and 12 as 2010a and 2010b in the text and also in the reference section. The new reference number for 10 and 12 are 4 and 11.

# Ref. 18 and 19 have to be interchanged "Lee et al for 19 and "Laemmli et al for 18; as in text.
The references Lee et al and Laemmli et al has been corrected and highlighted in the reference section. The revised reference number for Laemmli et al is 16 and Lee et al is 17.

# Ref. 20; not italic
The reference number 20 (old ref no) has been corrected to 18 (revised ref no) without italics in the revised manuscript.

# Ref. 34 Chin is capital.
In reference 34 (old ref no) the name *chin* has been replaced to *Chin* [35] (revised ref no).

# Ref. 36 the name of the journal is italic.
The name of the journal 36 (old ref no) has been italicized *J Toxicol Environ Health* and replaced with 37 (revised ref no) in the revised manuscript.

- Table 1: DMRT: to be clarified in the footnote without abbreviations.
- Duncan’s multiple range test (DMRT) has been clarified in the footnote for Table 1 and also in the Figure legends.

- Resolution of fig. 3 & 4 to be improved.
- Resolution of figures 3 and 4 has been increased to 600 dpi for good clarity.
3. Major Compulsory Revisions:
- The authors didn't mention any reported work on toxicity: the following is an example:
  - We have mentioned the previously reported work on toxicity in page 14 under “Acute toxicity study in rats” in the text [31 and 34] (old ref no) which is revised to [30 and 35] (new ref no).
- Apart from this based on the reviewer comment the other previously reported work on toxicity and lethal dose LD_{50} of Acacia nilotica (L.) has been included in the revised version of the manuscript under results and discussion section and cited in page 14.
  - This includes:
- The last paragraph in page 12, starting with "Second – order, needs more clarification, it is not compatible with figures 5 & 6.
  - Based on the reviewer comment, we have replaced the term second-order in the revised paper and replaced the term with UV spectral analysis for better understanding in page 13.
  - Figures 5 and 6 have been explained carefully in the results and discussion section. The change in wavelength has been described appropriately in relation with interaction of DNA.
  - UV-Vis spectral analysis of A. nilotica (L.) leaf extract and ethyl gallate to varying concentrations of CT-DNA are shown in Figure 5 and 6. The maximum absorbance for A. nilotica (L.) leaf extract was reduced with a shift from 278 nm to 260 nm indicating hypochromism with hypsochromic or blue shift effect (Figure 5) [27]. Similarly, the maximum absorbance for ethyl gallate becomes lesser with a shift from 271 nm to 268 nm indicating hypochromism with blue shift effect (Figure 6). This shift in wavelength indicates that the interaction of A. nilotica (L.) leaf extract and ethyl gallate to DNA is by intercalation.

A question to the authors:
Why you choose only ethyl gallate and not other antioxidants phenolic compounds present in the plant?
  - Ethyl gallate is a good antioxidant and an emerging anticancer agent with challenging cancer cell specific cytotoxicity (Kalaivani et al, 2011; Kim et al, 2012).
  - The mechanism of cancer growth inhibition was reported to be apoptosis at low concentrations indicating a direction for future anticancer research. It is also reported to be an approved food additive (Kim et al, 2012).
- *Acacia nilotica* (L.) is rich in phenolics and therefore reported previously against different free radical scavenging activities. Out of the many compounds present in *A. nilotica* (L.), ethyl gallate was found to be the major bioactive phenolic present and hence we established the ethyl gallate equivalence for crude extract. Therefore, the differences in response between the two could be attributed to constituents other than ethyl gallate.

- As there are no reports available on its interaction with DNA and hydroxyl radical scavenging activity against DNA damage, we have made an effort to describe its protection offered against DNA or protein damage and its mode of interaction with DNA.

- As ethyl gallate proves to be a natural multipurpose bioactive, the anticancer activity and neuroprotection studies of ethyl gallate in animal models are also under progress.
Dear Reviewer: Omer KOZ

We are happy to receive your comments and thank you for the suggestions in order to improve our manuscript. The following suggestions have been revised and are given as point to point clarification.

Major Compulsory Revisions:
Firstly the title of the manuscript should be changed. because the authors did not isolate ethyl gallate from a. nilotica leaves. they used ethyl gallate from sigma aldrich and leaves ext. directly for study. the leaves ext. contains many compounds not only ethyl gallate.
- The title has been revised according to the suggestion given by the reviewer, “In vitro protection of biological macromolecules against oxidative stress and in vivo toxicity evaluation of Acacia nilotica (L.) and ethyl gallate in rats”.
- As mentioned by the reviewer, pure ethyl gallate was purchased from Sigma and used for the present study. This study provides a comparative analysis of pure ethyl gallate and Acacia nilotica (L.) leaf extract. As ethyl gallate was found to be the major bioactive phenolic present in Acacia nilotica (L.), we established the ethyl gallate equivalence for crude extract. Therefore, the differences in response between the two could be attributed to constituents other than ethyl gallate.

Minor Essential Revisions:

Abstract (Results):
in previous study (Free radical scavenging, cytotoxic, and hemolytic activities of an active antioxidant compound ethyl gallate from leaves of Acacia nilotica (L.) Wild. Ex. Delile subsp. Indica (Benth.) brenan) authors give that the yield of ethyl gallate was found to be 0.26174 µg/g leaves of A. nilotica. the results in this study for et gallate equivalent of leaves ext. seems quite high (20mg et. gallate/1g ext.).
- In our previous study, the yield of ethyl gallate was 0.26174 µg/g leaves of A. nilotica (L.). In the present study, we have mentioned about ethyl gallate/g of ethanol extract obtained from the leaves of A. nilotica (L.).

Results and Discussion (FTIR and wave scan analysis):
Figure 4A and 4B is the same spektra. (there is no evidence for binding. All peaks are exactly same)
- Figure 4A and 4B are not the same and few variations observed have been carefully highlighted in page 12 of the revised manuscript. The details are given below:
- As shown in Figure 4A and B, upon addition of CT-DNA to ethyl gallate, we observed a variation in the intensity of 1469 cm\(^{-1}\) indicating the positive peak vibration of cytosine binding to ethyl gallate [27]. In addition, a moderate shift and the intensity vibration of 1533 cm\(^{-1}\) to 1535 cm\(^{-1}\) indicates the structural change that occurred in CT-DNA. Moreover, the appearance (553 cm\(^{-1}\)) or disappearance (750 cm\(^{-1}\), 732 cm\(^{-1}\) and 655 cm\(^{-1}\)) of peaks demonstrates a plausible interaction of ethyl gallate to CT-DNA [18].
- In order to confirm the interaction of ethyl gallate to CT-DNA, the UV-Vis spectral analysis was also carried out.
Also for UV analysis part, the uv spektra (Fig 5 and 6) did not show the peaks at 692 nm, 530 nm etc.

- We would like to thank the reviewer for his effort in pointing out the necessary corrections.
- UV-Vis spectral analysis of *A. nilotica* (L.) leaf extract and ethyl gallate to varying concentrations of CT-DNA has been interpreted carefully in the revised manuscript.
- The maximum absorbance for *A. nilotica* (L.) leaf extract and ethyl gallate was 278 nm and 271 nm respectively. The previously mentioned wavelengths (692 nm or 530 nm) did not show the maximum absorbance and hence removed from the text in page 13 under FTIR and wave scan analysis.
- The maximum absorbance for *A. nilotica* (L.) leaf extract was reduced with a shift from 278 nm to 260 nm indicating hypochromism with hypsochromic or blue shift effect (Figure 5) [27]. Similarly, the maximum absorbance for ethyl gallate becomes lesser with a shift from 271 nm to 268 nm indicating hypochromism with blue shift effect (Figure 6). This shift in wavelength indicates that the interaction of *A. nilotica* (L.) leaf extract and ethyl gallate to DNA is by intercalation.
- These points have been included in page 13 of the revised manuscript.
General comments

In general, the research constitutes a useful contribution to the field and well prepared.

1. Is the question posed by the authors well defined? **yes**
2. Are the methods appropriate and well described? **yes**
3. Are the data sound? **yes**
4. Does the manuscript adhere to the relevant standards for reporting and data deposition? **yes**
5. Are the discussion and conclusions well balanced and adequately supported by the data? **yes**
6. Are limitations of the work clearly stated? **yes**
7. Do the authors clearly acknowledge any work upon which they are building, both published and unpublished? **yes**
8. Do the title and abstract accurately convey what has been found? **yes**
9. Is the writing acceptable? **Yes**

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