Author’s response to reviews

Title: In vivo analgesic, anti-inflammatory, and sedative activity and a molecular docking study of dinaphthodiospyrol G isolated from Diospyros lotus

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Version: 2 Date: 13 May 2020

Author’s response to reviews:

Dear Professor Yogendra Nayak
Associate Editors
BMC Complementary Medicine and Therapies

We gratefully thank you for your e-mail dated 05.05.2020 and for the opportunity to revise our manuscript entitled "In vivo analgesic, anti-inflammatory, and sedative activity and a molecular docking study of dinaphthodiospyrol G isolated from Diospyros lotus by” Moreover, we also thank the reviewers for having spent their time reviewing our manuscript and providing helpful comments to improve our review paper. All the suggested changes are addressed and have been highlighted as yellow in revised manuscript. The given inputs are copied and answered following. Editor Comments:

We operate a transparent peer review process for this journal where reviewer reports are published with the article but the reviewers are not named (unless they opt in to include their name).
Reviewer reports:
Reviewer 1: The manuscript entitled "In vivo analgesic, anti-inflammatory, and sedative activity and a molecular docking study of dinaphthodiospyrol G isolated from Diospyros lotus" is a very interesting study. The asian countries have large consumption rate of Diospyros lotus. Therefore, the study is highly valuable. Also inflammatory, analgesic pharmacotherapy identification is important as it affects all the people globally and better outcome in drug identification in this area is always better. I congratulate the author and his team for successful completion of such valuable research work. The English is correctly used throughout the manuscript and the data presentation is properly done.

Few minor corrections suggested to the author:
Line 23: Since this is the lead natural compound for the study, the name Diospyros lotus can be highlighted in bold and italics in abstract
Reply: Bold and italics
Line 25: Experiment model is more appropriate than using the work experimental animal.
Reply: corrected
Line 27: Briefly write about the name of the animal model.
Reply: corrected
Line 33: Incomplete sentence: sedative activity
Reply: corrected
Line 41: The writing style of Diospyros lotus should me maintained throughout the manuscript.
Reply: corrected
Line 52: This statement is not required in this manuscript as it is not adding any additional information in the study. Since the compound is obtained from the extraction of the fruits of the plant, so description about the fruit is sufficient.
Reply: Agree and corrected
Line 79: Highlight due to its anti-oxidant property, its reported to have anti-inflammatory activity.
Reply: the needful corrections has been done

79 have been reported. Similarly, naphthoquinones class has been reported with antihistaminic effect
Reply: Done
Line 81: Incomplete sentence: pharmacological activity reported.
Reply: Corrected
Line 135: Briefly write about the method also
Reply: corrected
Line 144: Methodology of the preparation should be mentioned in details.
Reply: Methodology for the preparation has been added in the revised manuscript.
Line 149: Time period of MD study should be mentioned. All the computational methods used should be described.
Reply: Time period of MD study has been mentioned. All the computational methods used in the study has been described in the revised manuscript.
Line 150: write the licensing agency name for the in silico tool used in the study
Reviewer 2: The authors report on a series of animal experiments intended to evaluate anti-inflammatory, analgesic and sedative effect of a chloroform extract of Diospyros lotus, as well as of one specific (extracted) compounds - DDG. There is a reasonable background that supports such an effort - considering the traditional use of various preparations of the plant for similar conditions in humans.

I have several comments.

1. English is not my native language, but thorough language editing is needed by English-proficient person acquainted with the biomedical terms (in addition to quite some typos across the manuscript).

Reply: Manuscript has been edited and corrected for possible mistakes.

2. Materials and methods. Extraction and isolation. The authors refer to several previous publications considering the process of extraction (chloroform), which is OK. But, at the end - no particular specification of the extract is available. Since DDG (seemingly) is the main component, it would be at least needed to state the estimated % content of DDG in the extract, and at least (qualitatively state) - what would other constituents include. DDG is referred to as a "single extracted" compound (that is - there were two "test" items - the extract, and "purified"? DDG?) -
but it should be stated what is meant by "DDG". Extract "enriched in DDG"?..Extract with...e.g. 97% w/w DDG? "Pure DDG"?. WHen things are being tested, then the item(s) tested should be well described.

Reply: The needful correction has been done, the % yield has been included now.

3. Materials and methods. Animals. There is some confusion in this section. The last sentence of this paragraph states that n=8 animals were assigned across each of the exp. group (in several experiments), but then - under the next sub-heading on the anti-inflammatory screening it is stated that n=6 per group were used. Etc.

Reply: This is our typo mistake. The correct one is n=6. Thank you for corrections.

So - it should be clear (by stating it in the methods, or under each table/figure reporting on a particular experiment) what was the exact number of animals per (each) group.

In one place, the authors state that animals were treated with "six compounds" - but actually, there were 2 tested compounds (extract and DDG), but each applied at 3 different dose-levels. This should be corrected.

Reply: corrected

4. Materials and methods. Anti-inflammatory screening. Authors refer to their previous publication to more detailed description of the procedure. In this respect, I would like to point-out:

a) the fact that something, some procedure or a protocol was already published does not automatically mean that it is valid; b) I agree that it is not really needed to always repeat all the details about a particular procedure. But, it could be stated "In brief,..." and key points of the procedure need to be outlined. It is not really practical for anyone reading this or any other paper, to search and try to acquire the published paper in order to be able to figure-out whether the methodology was appropriate or not. In this section, DDG is addressed as "compound 1"...you should use the same term for the tested item..always. So, it is either "compound 1" or DDG.

Loratadine dose is missing. The formula for "%inhibition" is not completely clear: what is "A" - the mean value for the "negative" control group? (saline?) or? And the value for each animal from the "tested group" is then "converted" into a "percent inhibition" using this mean as 100%? Or?. This should be explained. Also - when reporting results (e.g., Figure 2) - the Figure legend should contain numerical values from the plethysmometry measurements: "mean baseline volume" (intact animals prior to carrageenan/histamin); saline-treated animals at 1 hours: xyz (represents 100%), at 2 hours:...etc.). So that it is clear what "100%" actually means. at certain time-points. Figure 1 and Figure 2- WHAT was the DOSE of the "extract"? and what was the DOSE of DDG to which the data in these figure refer?

Reply. Methods were explained briefly. DDG was corrected throughout the MS to compound 1, dose of lortidine is mentioned, the control means negative control ie. Saline treatment, the plethysmometer volume is not mentioned here, we just calculated the percent inhibition of edema from that volume.

5. Materials and methods. Hot plate. Again, the authors refer to "previously published method". But, many points remain unclear (unless a reader is willing to search for and acquire "previous publication").

Reply. Corrected

The unknown points: 1. each treatment group was tested repeatedly over 120 minutes, or at each time point a new group with the same treatment was used?

Reply. Each group was tested repeatedly after 30, 60,90 and 120 min of post administration.
2. Table 1 shows results. Without any units. What are the numbers? Seconds of latency time? index of analgesia?.

Reply. Corrected

The method should be at least to some level explained. For mice included into hot-plate testing, it is accustomed to a) have "conditioning" a day before; b) that all animals (groups) are tested at baseline (before treatment), in triplicate; c) that they are re-tested after received treatment. d) that index of analgesia is calculated for each animal based on "baseline" and tested value. Baseline "runs" are needed to ascertain that all animals show comparable reactivity. Triplicates are needed to "smooth" the response, because there are intra- and inter- individual variabilities in latency times that can really be huge. The procedure needs to be explained. The meaning of "percent effect" needs to be explained.

Reply. Actually we have mentioned that we followed our published procedure. However we explained in brief.

6. Materials and methods. Sedative activity. Sedative effect is typically assessed by observing spontaneous locomotor activity. E.g., in the open-field test. The employed test certainly has a name (i.e., the employed paradigm). It should be hence termed like that. The result of the number of "crossed lines" - but this is given within the certain time-frame. Method should be explained.

Reply. Corrected and highlighted.

7. Statistics. 1. All raw data are summarized as means (SEM). This is not appropriate. SEM is NOT a measure of data scatter - it is a measure of precision. Hence, if mean is the measure of central tendency that fits the data - it should be given with SD. 2. One-way ANOVA is appropriate (if residuals were not skewed) for the "sedation test" - since the only factor was "treatment". For the hot-plate and inflammation paradigms - one-way anova is appropriate ONLY if at each time point there was a different animal group by treatment. E.g., positive control, negative control, 3 doses of an extract, 3 doses of DDG. That is 8 independent groups a each time-point. Then, at each time point one uses one-way ANOVA with 1 factor - "treatment" with 8 levels. But, if the same animals (per treatment) were repeatedly evaluated at different time-points, then TWO-WAY anova is needed: because there are two factors (1) treatment and (2) time. And the model needs to include the "treatment*time interaction", and differences between treatments at different times are generated from the interaction term.

Reply. we compared step by step therefore used ONE way.

EXACT P-values with clearly depicted comparisons to which they pertain - need to be presented for each experiment.

8. Results. Anti-inflammation (Figure 1 and Figure 2). The two figures were already commented-on. But: i. what are points? individual animals? what are horizontal lines? If you want to show data scatter - show all individual data, or mean(SD). Figure legend should declare numerical values which served as "100%". Textual part: it is not really obvious form the figure when and what "had an effect". Data should be reorganized and more clearly shown. There is no reason not to use a Table (like for other data).

9. Results. Pain. Table 1. As already mentioned - what are the data? Seconds? percentages...of what? How determined?

Reply. These are seconds of latency time find from hot plat.

To which comparisons do p-values refer?

Reply. The statistical level of significant find through graphpad, find by comparison of tested group results with negative control.
Use SD to illustrate data scatter. I have done quite some hot plate testing. I have never seen such consistent means...eg., for saline group - always around "9.0" (something) at different times of testing. This is an unclear experiment. Should be better explained (methodology etc.). And - two-way ANOVA should be used (see above).

10. Results. Sedation. Table 2. Again..completely uncelar...values for treatments are compared..to what? where is the "saline group"?...use SD for data scatter.

   Reply. Thank for these points but we normally using SEM.

11. DESIGN of EXPERIMENTS. The "sedative" effect (e.g., diazepam) in the used paradigm is evidenced by reduced spontaneous locomotion. Hot-plate test actually quantifies a COMPLEX response to a thermal stimulus (i.e, a complex behavior, not only "reaction to pain") - if a compound reduces spontaneous locomotion..could it influence the behavioral response in hot-plate?...A diazepam-treated group in the hot-plate test would help to "separate" the true analgesic and behavioral effects.

   Reply. Thank you for these valuable suggestions. However we can think so in our laboratories future study. Due to lockdown issue in Pakistan with respect to covid-19, our Labes are closed till further announcement of Gov. of Pakistan.

12. Results - computer simulations.- I am not qualified to assess this.

13. Discussion - there is a lot of speculation there. The experiments WERE NOT designed in a way that would allow you to claim ANYTHING about the potential mechanisms of the DDG effect. You DO NOT HAVE "material" to speculate that DDG might be affecting "central opioid system". The hot plate experiment would need to include e.g., opioid antagonists and you would need to show that e.g. naloxone could abolish the anti-nociceptive effect of DDG - in order to imply the possible "opioid mechanism". The same for sedation and GABA. You did not use, e.g., flumazenil to "remove" the sedating effect. So - there is no grounds for any speculation about the mechanisms. YOu can only state- that data are suggestive, and deserve further investigation of the involved mechanisms.

   Reply. Due to lack of research fund these mechanistic experiments were left out, also was not the aim of current project.

14. Discussion -in line with this and the limitations of the experiments and the fact that there was only one paradigm used for pain and sedation - the entire Discussion should be less "enthusiastic" about what was actually shown.

   Reply. Due to lack of research fund these mechanistic experiments were left out, we will do that in future study. Also was not the aim of current project.