Reviewer’s report

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

Version: 2 Date: 09 Jun 2020

Reviewer: Trkulja V

Reviewer's report:

I have reviewed the revised version (Rev 02) of the paper BCAM-D-20-00429R2.

The manuscript has been rather extensively revised and improved. Still, there are further drawbacks / comments.

Minor comments.

1. The language has been greatly improved. still, there are typos or grammatical errors. A few examples:

a) Abstract. Objectives: "To find an………. molecules - "an" refers to ONE, while "molecules" is plural.

b) Abstract. Methods. "locomotors assays" -?. rather - animal assay for screening the locomotor effects (or something similar).

c) Abstract. Results. 1st sentence - you have past and present tense in the same sentence.

d) Background. Line 55 - " it usually grows up to 600 m in height, and….up to 2000 m". I am not familiar with the plant - does it really grow to be 2000 m high? or the point is that it could be typically found at the altitude up to 600 m or, in some parts of the world, up to 2000 m?

e) Methods. Line 152 - animal cannot be "sedative" - it could be "sedated". Also - the test employed should be named. "placed in a special box…” - is it a validated known assay? It should be named.

f) Results. Line 206. Table 1 shows hot plate assay results, not the "sedative effect" (this is Table 2).

.etc..

Major comments.
1. Objective (in Abstract). The current definition of objective ("to find and effective, safe and…") simply does not fit the reported material. The material reports on 2 phyto preparations evaluated in a limited set of animal paradigms. The objective could be: "To evaluate analgesic, anti-inflammatory and sedative potential of D.lotus extracts in animal paradigms" or something like that.

2. Although the authors replied that "% yield of compound 1 is reported" (DDG extract) - it is not.

3. The anti-inflammatory experiment is still not clear (methods and Fig 1, Fig 2). The authors (again) mostly refer to their "previous published work" regarding how data were processed and reported. This is really not relevant - if the procedure here is not clear - it could have been published 1000 times: a reader needs to be able to get (and understand) what and how was done, in order to be able to assess whether data are indeed indicative or meaningless. It is unclear: a) whether there was a group that received saline as a "negative control treatment" + "saline" as a "sham carrageenan/histamine". This would represent paw edema due just to volume injection; b) how was the effect of "carrageenan/histamine assessed"? - the actual effect of inflammation inducer is a difference between "saline + saline" vs. "saline + inflammation inducer". Their difference is the "extent of induced inflammation". The volume of the paw of an animal injected with "saline + inflammation inducer" is NOT the measure of the effect of the inflammation inducer: it is a sum of (i) injected volume + (ii) induced inflammation. 3) The effect - that is intended to be presented as a "percent inhibition of the effect of inflammation inducer" - of any anti-inflammatory agent is seen as the following: (("saline + inflammation inducer" - "saline + saline") - ("treatment + inflammation inducer" - "saline + saline") / ("saline + inflammation inducer" - "saline + saline")) x 100%. The authors show "formula for % inhibition" as (paw edema of saline + inflammation inducers) - (paw edema of treatment + inflammation inducer)/(edema of saline + inflammation inducer) - which actually does NOT provide the measure of an inhibitory effect (expressed as percentage). It says for how much (in %) is the paw edema with treatment smaller than the paw edema with saline - but DOES NOT show "percent inhibition", since it does not include "inflammatory inducer effect" as a reference value. Finally, the experiment was designed so that each treatment group included different animals (it is not that each animal was tested under different treatments). Hence, "percent inhibition" can only be generated as an OVERALL ESTIMATE for the group! It cannot be determined for an individual animal! (you need to subtract things...values...so, which animal is "paired to which)? With independent groups (as here) - "relative" or "percent inhibition" - can only be estimated for the entire group and the calculations that I explained are based on means (SDs). How did authors generate "individual percent inhibition"?

I am afraid that data in Figure 1 and 2 - should simply be disregarded as they do not show or quantify - what they claim. To wrap-up: also - data in Figure 1 and 2 can meaningfully be analyzed ONLY in a two-way anova (factor 1 treatment, factor 2 time; + treatment-time interaction). Everything else is erroneous.
4. For all results - authors keep "summary data" in the form of "mean (SEM)". The reply that this is how they "usually do it" - is meaningless. This is erroneous and should not be done and is not acceptable.

5. Hot plate assay. This assay (methods and Table 1) is also unclear, in a way similar to that elaborated for "paw edema" assay. a) Methods state that results are "percent effect as previously published". However (i) Table 1 provides latency times in seconds. No sign of "percent effect". What was analyzed? percentages, seconds? (ii) In relation to "previously published" - the comment is the same as for "paw edema". This means absolutely nothing. (iii) In respect to "percent effect" - the comment is the same as for "paw edema"; (iv) The Methods state that "time on the hot plate was limited to 25 seconds". And then times are summarized (erroneously!) as mean (SEM). This is not appropriate, nor is ANOVA appropriate - with this "upper limit" of time on the hot plate - data are "un-naturally" trimmed - and clearly cannot meet the need for ANOVA (that residuals are normally distributed). Next - if there is the "upper limit" - than the only appropriate way to quantify the results of the hot plate test is to use analgesia index (AI), which is calculated as (for each animal) (latency under treatment - baseline latency)/(cut-off time of 25 seconds - baseline latency). Finally - (although likely not appropriate), one-way ANOVA to compare different treatments to "saline" was done at each time point (there were 4 time points). This is erroneous - two-way ANOVA (assuming that data have adequate properties) is in place: treatment is factor 1, time is factor 2 + treatment*time interaction. Within each ANOVA, authors stated that a post-hoc test was done for multiple comparisons (per time point) - however, across different time-points there was no adjustment for multiple testing. Also, exact p-values were not reported. The fact is that Table 1 shows data for which at least 32 statistical null-hypothesis tests were done, and type 1 error control was inappropriate. To conclude - the methodology, analysis and presentation of this assay is highly questionable.

6. Sedative effect assay. methods and Table 2 - this assay is not sufficiently described. It certainly has a name. Also, it certainly has a time-constraint on its duration. It is unclear to which comparisons (to saline? to..diazepam to..what?) to P-values pertain. P-values should be given as exact numbers.

7. Discussion- lines 242-248 is speculative, with no grounds in the data and should be removed.

8. Considering the methodological and data analysis issues - it is highly uncertain that any of the suggested effects were "true effects" or, at least, their extent is questionable.

**Are the methods appropriate and well described?**
If not, please specify what is required in your comments to the authors.

Unable to assess
Does the work include the necessary controls?
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Unable to assess

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