**Author’s response to reviews**

**Title:** Standardized ethanol extract of Tinospora crispa upregulates pro-inflammatory mediators release in LPS-primed U937 human macrophages through stimulation of MAPK, NF-κB and PI3K-Akt signaling networks

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Reviewer 5

I have reviewed the manuscript BCAM-D-20-00353_R2. It is a revised version of a previously reviewed manuscript (not by me). It provides quite a comprehensive evaluation of the topic - mechanistic analysis of the effects of T. crispa standardized extract in a permanent cell line of human macrophages (U937). It is overall a comprehensive and informative work with adequately characterized extract, adequately designed stepwise approach to the problem, adequately designed experiments. Authors well recognize the limitations and suggest a direction for future work.

My comments are only minor.

1. It appears that one of the previous comments was on the language. It apparently is much improved - still, further "polishing" is needed (maybe at the editorial level), because there are syntax and sporadic spelling errors as well as typos. For example (just page 1): I would not call immunopharmacology tools "imunodrugs" - these are drugs with the effect on the immune system/reactions; 2) in the same sentence, listed are glucocorticoids and two words later (again) "steroidal" (- where - glucocort = steroidal), 3) "there use ...is with limited success" - should be revised; 4)"...agents with additional safety and efficacy"...- is not really the way to say that drugs with improved efficacy/safety.. would be welcome. 5) All latin names (T. crispa, for example) should be always italicized; 6) P-index was suggested to be "lower case" - where still on some
places it is "P". 7) On several points, exponents should be in superscript; or on p.4, "there are little mechanistic.." -

should be something like " a few…" etc.; 8) or on p.11 "concentrations used in this experiment were extending from 75 or bellow" - there were many experiments. The concentrations extended from 75 to../ OR - concentrations $\leq$75 were used; 9) the difference in pcr yields is expressed as "fold expression" (so...528 fold; not folds); So, the entire paper should be thoroughly re-checked.

Authors response: We have addressed and revised accordingly. All changes are marked in red font.

2. In footnote to Fig.5, 7 authors direct the reader to go and see footnote to Table 3 - in order to "read" the symbols flagging results of statistical tests. This is legitimate but not really practical. Each Fig. together with its legend should be a stand-alone unit.

Authors response: We have addressed and corrected accordingly. All changes are marked in red font.

3. I am aware that statistics here is not the "crucial part". And that is a rather common thing to present data on 3 values as "mean (SEM)" and use parametric stats. However, strictly speaking, this is not really an adequate approach. For example, figs. showing percent viability or concentrations are presented as bars - in fact..there are only 3 values per group (treatment regimen)...and they could (should - for full information) - be shown as individual values (e.g., dots). So - one gets the proper idea. The test - ANOVA (one-way anova) - the non-parametric version is clearly more suitable than the parametric one. Also, to my understanding, all comparisons were "vs. control" - so, in the Methods, authors should state the method of adjustment for multiple comparisons. The main findings (about the effect of 75 TCE) will not be changed - but some other p-values will be different. These are, in a way, "discovery" experiments...and type I error should be adequately controlled.

Hence - I am sure that that main findings will stay unchanged, but the info will be communicated in a proper way.
Authors response: Yes, statistics here is not the crucial part in this experiment and according to most published literatures, this is a rather common thing to present data on 3 values as mean +/- SEM. We have carried out the statistical analysis following the published methods of relevant articles. Specifically, a one-way variance analysis and Tukey’s multiple-comparison test were employed for the statistical analysis and this analysis is supported by the most published literatures. However, we would carefully consider reviewer’s valuable suggestions during our next experiments with in-depth statistical analysis.

4. mRNA expression is typically done having a technical triplicate. Then by the -delta,delta method "signal" from the experimental (corrected for the housekeeping) is relatively quantified vs. the control; the calculation retrieves fold change (or fold difference) as an point with a bar indicating SD of the fold difference. Footnote to figure 3, for example, suggests that the bars are SEMs..which is a bit "non-standard". And it remains unclear (as for any other mRNA level analysis) - did you have, for each treatment condition, a set of 3 plates with cultured cells - and then each processed for mRNA with technical triplicates?...Or?

Authors response: The experiment was conducted with 3 independent replicates (n = 3) for each condition. Thus, each condition will have 3 sets of data. The SEM were calculated between the 3 sets of data, which is also common way of statistical presentation according to the most published literatures.

5. Finally - the point or "problem" of western blots. WB is actually a semiquantitative method...and this seems to be commonly disregarded in the sense that researchers tend to derive a lot of "quantitative-statistical" inference from the results. But, this is a minor point. The main one - is the quality (e.g., the problem of overexposure) of the blots and image analysis. To my understanding, the authors used ImageLab (by BioRad) that "processes" the "native" blots and provides in a way "processed" images. It seems that in the image analysis, the authors did not use the option for overexposure control. This review is accompanied with an attachment that contains re-analysis of submitted blots in Figures 7 (suggesting overexposure of beta-actin; and not indicating really an effect on MyD88 and TLR4 - as read from the submitted blots: treatment conditions 2 - just lps, and 3 - lps + 75 TCE appear closely similar) - and Figure 4 - COX2..where conditions 2 (just lps) and 3 (lps +75 TCE) - actually seem to be very close).

My suggestion - not only "representative" - but all blots should be supplemented (suppl. material) - i.e., their "native forms" - for all those who are interested in a possibly more detailed insight into the matter.
Otherwise - one could only provide bar graphs, ie., image analysis results, since the blots (in the form that they are) - per se are not really informative.

Authors response: Yes, we have presented the band and bands intensities analyzing with the Image Lab™ software of BioRad. Yes, we agree that it is impossible and hard to assume the immunoblotting band intensities in apparent view except analyzing. Therefore, beside the blots, we have presented the data in bar graphs for the clear understanding of readers. Regarding availability of the native blots, we have already informed the editorial manager the unavailability of the supplementary data due to the Covid-19 situation.