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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Response to reviewer’s comments.

Reviewer 3:

1- Written English language should be revised by a native English language speaker/writer;
Response: The manuscript has been carefully revised by a native English language writer.

2-Line 183, change "105" to 10⁵;
Response: Corrected.

3-Probability symbol should be represented by a lower case "p" throughout the manuscript;
Response: Corrected.

4-Nitric oxide (NO) was a part of the discussion "not detected" (line 362-365) but there was no indication that it was included in the study or mentioned in the methodology part;
Response: Kindly note that we have performed the NO assay, but we have not included this in the methodology as NO was not detected at measurable amounts in U937 macrophages. The question of whether U937 possesses the complete machinery necessary for NO synthesis remains controversial. It has been reported that U937 macrophages contain most of the cofactors needed for NO synthesis, except BH4 which might be one of the major limitations for not producing the NO in these cells (Eur J Pharmacol 2010; 628: 247-254). We have added this statement in the discussion (line 362-365) because it is important to clarify and let the scientific community knows about the issue as NO is one of the imperative inflammatory mediators besides the cytokines we studied. If the reviewer feels wise that the assay should be stated in the methodology section despite no outcome, authors are ready to include it.
The discussion and interpretation of COX-2 assay is rather misleading (line 359-362). COX-2 is a pro-inflammatory mediator and attenuation of its activity is desirable in chronic inflammation rather than its up-regulation. I hope the authors could review this section and the concept on this assay.

Response: Agreed. COX-2 is a pro-inflammatory mediator and attenuation of its activity is desirable in chronic inflammation as well as autoimmune disorders. But its upregulation is anticipated in terms of boosting immune response or immunostimulation (ref.: International Journal of Biological Macromolecules 108 (2018): 1310-1321). Kindly note that, in addition to its involvement in inflammatory responses, COX-2 and its products, prostaglandin E2 (PGE2), have been reported to be important in inhibition of apoptosis (Ref.: Cell 83.3 (1995): 493-501; Molecular and Cellular Biology 20.22 (2000): 8571-8579; Cancer Research 58.2 (1998): 362-366). In fact, literature reports also suggest that the expression of COX-2 is anticipated as it plays protective role as a survival factor and protects the cells from uncontrolled apoptosis (inappropriate induction of apoptosis) (Ref.: Molecular and Cellular Biology 20.22 (2000): 8571-8579; The FASEB Journal 11.11 (1997): 887-895; Journal of Biological Chemistry 275.16 (2000): 12095-12101; Journal of Biological Chemistry 276.52 (2001): 48997-49002). Regarding the lines 359-362, the authors carefully reviewed the section as suggested and humbly disagreed with the reviewer about the misleading interpretation issue. It was neither misleading rather it is an established fact and supported by the published literature that pro-inflammatory cytokines play crucial role in inducing iNOS and COX-2 in LPS-activated macrophages (International immunopharmacology 17.3 (2013): 698-703; Mediators of inflammation 2014 (2014); International immunopharmacology 60 (2018): 141-151). And based on these established facts and our present findings, we suggested the upregulation of COX-2 is associated with the upregulation of the pro-inflammatory cytokines expression in the U937 macrophages. We have justified this hypothesis experimentally where the pro-inflammatory cytokines were found to be upregulated with T. crispa treatment as well as the products of COX-2 i.e., PGE2 was also found to be upregulated. Above and beyond, the outcomes were further validated and confirmed using specific inhibitors.