

Dear editor,

We deeply appreciate all the comments made by the editors and reviewers. We revised the manuscript very carefully according to the comments as following:

1. The certificate of the funding agency for the grant has been provided.
2. We provided the original editable figures as PowerPoint format.
3. The highlights of this article have been included at the end of the main text in the revised manuscript.
4. We have used superscript numbers for illustration, and for statistical significance, superscript letters were used.

We thank once again for the attention and comments from the editors and reviewers to improve our manuscript.

We are looking forward to your kind reply.

Sincerely yours,

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Answering reviewers

Name of Journal: *World Journal of Gastroenterology*

Manuscript NO: 58505

Title: Nimbolide inhibits tumor growth by restoring hepatic tight junction protein expression and reduced inflammation in an experimental hepatocarcinogenesis

Author: Amit Kumar Ram, Balasubramaniyan Vairappan, Bheemanathi Hanuman Srinivas

We would like to thank the reviewers for their fruitful comments to improve our manuscript further. The point by point replies to all the comments raised by the reviewers are given below.

Response to Reviewer #1:

S. No.	Comments	Response
	I'm glad to read this manuscript as a basic study. By macroscopic examinations of hepatic nodules, measuring liver histology and HCC tumor markers, analyzing	We deeply appreciate and thank the reviewer for supporting our work.

	<p>expression of TJ proteins, cell proliferation and cell cycle markers, inflammatory markers and oxidative stress markers, and insilico analysis to confirm binding and modulatory effect of nimbolide on ZO-1, NF-κB, and TNF-α, they showed for the first time that nimbolide exhibited anticancer effect by improving TJ proteins, ameliorating inflammation and oxidative stress, and suppressing cell cycle progression in HCC mice. Over all, your study contains a lot of information. Readers with an interest in nimbolide will find this paper beneficial and informative. I really appreciate your great work and it's my great honor to read this paper.</p>	
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Response to Reviewer #2:

S. No.	Comments	Response
1	<p>The submitted manuscript about the releasing of zinc ions that inhibit calcium channel signaling contains several interesting points. I would suggest the following:</p> <p>The title should be concise.</p> <p>According to recent studies, that would favor future citations to the paper. What is really timely and new in the paper? Nimbolide</p>	<p>In the current study, Nimbolide supplementation to HCC mice significantly reduced tumor growth (Fig 2, a-c) .</p>

	inhibits tumor growth?	
2	<p>Abstract results would eventually be improved if more quantitative information is referred for rapid comparison with similar studies. Avoid imprecise terms: significantly reduce? But how much: 2 fold; 20%? From 50 to 45%? It should be a mirror of the paper and not a type of discussion or/and introduction or track of the research. Even quantitative information for nimbolide concentrations should refer as well as the percentage of tumor reduction for rapid comparison with similar studies.</p>	<p>We thank the reviewer for this suggestion. The abstract results have been revised. We have avoided imprecise terms. Quantitative information for nimbolide concentration as well as the percentage of tumor reduction has been included in the abstract results section in the revised manuscript.</p>
3	<p>The results are not properly described. The authors should first describe in a quantitative manner the data before jump to conclusions. Imprecise terms such</p>	<p>As suggested, the results have been revised. We have avoided imprecise terms and have provided a detailed description of the results in our revised manuscript. (Page 13 - 18)</p>

	as significantly reduced should be avoided and replace by the description of the results.	
4	The figures should be clearly globally improved, as possible, once WJG deserves high quality figures and with rigor would avoid lacking of interest for the data. Scale bars should be inserted in all the figures panels.	As suggested, we have provided high-quality figures in PowerPoint format. Scale bars have been inserted in all the figures panels in our revised manuscript.
5	Discussion should be more assertive and concise and eventually be divided in sections with titles highlighting the major results.	As per the suggestion of the reviewer, the discussion section has been revised. Titles highlighting major results have been included in our discussion section of the revised manuscript.

Response to Reviewer #3:

S. No.	Comments	Response
	This is an interesting manuscript about the effect of nimbolide on TJ	

1	<p>protein expression, cell cycle progression, and inflammation in diethylnitrosamine (DEN) and N-nitrosomorpholine (NMOR) induced experimental HCC in mice. The manuscript is well written, experiments are well design and the findings are according with the objectives. However, authors should made some changes in order to improve the quality of the manuscript:</p> <p>In the manuscript, all data are expressed as mean \pm SEM (Standard Error of Mean) and statistical analysis are made using one-way ANOVA. This strategy is right when normality of data can be assumed or the number of experimental subjects by group is large. Most of data analyzed in the manuscript usually do not follow a</p>	<p>We thank the reviewer for the valuable comment. We analyzed the normality of the data and based on normality distribution we performed either the parametric test (one-way ANOVA followed by Tukey's multiple comparison post-hoc test) or non-parametric test (Kruskal-Wallis followed by Dunn's multiple comparison post-hoc test) for the statistical analysis in our revised manuscript.</p>
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	<p>normal distribution and the number of experimental subjects by group is very small. In this conditions, the use of parametrics test such as ANOVA may be problematic. Authors should evaluate the normality of data (residuals for ANOVA) before each analysis and in the case of non-normality use a non-parametric approach to analyze the data.</p>	
2	<p>the Turkey's multiple comparison post-hoc test is in fact the Tukey's multiple comparison post-hoc test</p>	<p>We have fixed the typo error in our revised manuscript as suggested.</p>
3	<p>the discusión section is very long and most of information in this section appeared in the introduction or results section. Most of the information in the paragraphs from the start of discusión on page 18 (from "Our study demonstrated for the first time that nimbolide...") to the paragraph on page 21 "In the</p>	<p>We appreciate the reviewer for the comment. We have rewritten and shortened the discussion section as suggested. (Page 19 to 20)</p>

	<p>present study, we found that nimbolide treatment to HCC mice significantly upregulated hepatic protein expression of ZO-1 and occludin. Since HCC is a type of epithelial cell carcinoma, modulation of TJ proteins by nimbolide may induce hepatocyte (epithelial cells) polarity thereby reversing the phenotypic transformation and metastatic characteristics of cancerous cells and might inhibit HCC progression[7]" has been previously described in the results and introduction sections. This paragraph must be rewritten and shortened without repeat information and data described in other sections of the manuscript.</p>	
4	<p>Authors must describe and discuss the following limitations of the</p>	<p>We deeply appreciate for this comment. We have included the</p>

	<p>manuscript: - With data obtained in this work it is not possible to establish the mechanism or mechanisms (from all the mechanisms that have been studied) involved in the anticancer effect of nimbolide. The study shows different mechanisms that occur at the same time than the anticancer effect but a direct relationship between them has not been shown. - The efficacy of doxorubicin is very limited in the treatment of human HCC so a limited effect of nimbolide could be also expected.</p>	<p>limitations in the revised manuscript as suggested. (Page 23 line 417- 425)</p>
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Response to Reviewer #4:

S. No.	Comments	Response
1	Abstract – “Our previous study in hepatocellular carcinoma (HCC) patients identified that decreased hepatic zonula occludens (ZO)-1 expression was positively correlated with inflammation and promotes disease severity, albeit a causal relationship remains unclear.” – this sentence does not seem to be linked with Abstract Aims and therefore should be deleted.	We thank the reviewer for this comment. As suggested, we have removed the lines in the revised manuscript.

2	Abstract, Conclusion - “exhibits anticancer effect by improving TJ proteins” - this is data overinterpretation. The observations were correlative. You did not provide any link between the observed effects on TJ proteins and the anticancer activity of nimbolide.	We thank the reviewer for this valuable point. We have modified abstract conclusion as suggested.
3	Introduction - “Hepatocellular carcinoma (HCC) is one of the most lethal malignancy” - language check by a native English speaker is necessary. Despite the manuscript was claimed to be already checked, there remain grammar and phrasing issues.	We appreciate the reviewer for the suggestion. The revised manuscript has been proof-read and corrected the typo error.
4	Introduction - “ <i>Azadirachta indica</i> ” - use italics for Latin names	As suggested, we have now used italics for Latin names “ <i>Azadirachta indica</i> ” in the revised manuscript. (Page 7 line 45)
5	Introduction - use upper index for	We have now used the upper index

	“-/-“	for “-/-“ in the revised manuscript. (Page 7 line 40)
6	Introduction – “Furthermore, nimbolide has emerged as one of the most promising chemotherapeutic agents in oral squamous cell carcinoma[24],” – it is the overinterpretation of the results provided by ref. #24	We have modified the sentence in the introduction section of our revised manuscript. (Page 7 line 49 to page 8 line 51)
7	Introduction – The whole manuscript is focused on a phytochemical. This reminds me that the concept of multitherapies should be mentioned somewhere in the Introduction as the curcumin therapy will typically be used as a part of such multitherapies. Check and cite Seminars in Cancer Biology 35 (2015) S276–S304 for more details.	We thank the reviewer for the kind suggestion. We have added the concept of multi-therapies in the introduction section of our revised manuscript. (Page 6 line 11-13). We have also cited the reference Seminars in Cancer Biology 35 (2015) S276–S304. Ref. #4 as suggested.
8	Materials – “Sigma-Aldrich, USA.” – provide city and state for	As suggested, we have included all the details in the materials section of

	every manufacturer mentioned.	our revised manuscript.
9	Materials - “Nimbolide of 97% purity” - what were the impurities? Why did you avoid the nimbolide distributed by Sigma, which is of >98% purity, despite you used other chemicals from Sigma?	The information about impurities in the datasheet was not provided by the manufacturer. The manufacturer estimated the purity of Nimbolide to be 97% by HPLC. Since the purity was determined by HPLC the impurities present might be organic (intermediate or by-products), inorganic (inorganic salts or filter aids), or solvents used for purification. The cost of nimbolide distributed by Sigma-Aldrich was 5 times higher than the manufacturer we procured the compound from. Moreover, we have used the nimbolide from the same manufacturer which has been cited by the previous literature [1]
10	Animals - “Swiss mice” - provide more detailed definition of the strain used.	As suggested, mouse strain given in the revised manuscript. (Page 8 line 65)
11	Animals - “pathogen-free	After obtaining mice from the central

	environment” – be more specific. Free of all pathogens?	animal facility, these mice were maintained in a specific pathogen-free environment. The animal room was sterilized by fumigating and all the cages were disinfected before housing the mice. (Page 8 line 66)
12	Animals – “After tumor formation at 28th week” – how did you know that the tumors formed exactly at the 28th week?	We thank the reviewer for this comment. The hepatocellular carcinoma (HCC) mice model has been previously established in our lab by conducting a pilot study where the intermittent monthly sacrifice of the animals was done. We found tumors were macroscopically visible at 28 th -week on the liver surface and histological analysis showed nodules exhibiting features of HCC. Based on these findings, we started the nimbolide treatment from 28 th week.
13	Results – “slight increased body weight at the end of the 32nd week which was not significant compared to HCC alone” – if it was	Again we thank the reviewer for this comment. As suggested we have modified the test outcomes in the result section of the revised

	not significant, then it was not an increase. Perhaps a trend. Report the test outcomes.	manuscript. (Page 13 line187-188) “a trend in increased body weight from 28 th to 32 nd week ($P = 0.6781$)”
14	For any statistical test outcomes, report the type of the test, test statistics, n, and the p value.	We have reported the type of test, test statistics, n, and the p-value for all the statistical test outcomes in our revised manuscript as suggested.
15	Results – “showed much lesser hepatic nodules” – is it possible to provide any quantification?	We counted the number of nodules for each mouse in all the experimental groups. Since we randomized the mice in all the study groups, we assumed that HCC was initiated uniformly by DEN and NMOR across all the groups before nimbolide treatment. From a clinical point of view once the tumor is established it might not disappear completely because of chemotherapy alone, however, there might be a regression of established tumor or stoppage of further growth in tumor size which was observed in our study. In certain cases complete tumor

		<p>regression might happen when a combination of neoajuvant therapy, primary treatment (surgery), and adjuvant therapy is followed [2,3]. Therefore, we could not find the statistically significant difference ($P = 0.6182$) for the average no. of nodules per animal in each group. Quantification of no. of nodules in the experimental groups is depicted in figure 1. It could have been better if we could monitor the progression of HCC through non-invasive methods. Unfortunately, due to lack of facilities we were unable to perform the same.</p>
16	<p>The authors failed to identify recent studies on the study topic that were published in top-tier journals. They cite some good studies that were published in the past. However, the recent citations consist mostly of papers that were published in marginal journals.</p>	<p>As suggested, we have cited some recent references on the study topic from 2020 in our revised manuscript. Ref #1, 3, 5, 32, 33, 34.</p>

	References from 2020 are completely absent.	
17	Fig. 1A – nimbolide is incorrectly visualized in one of the treatment types.	We have corrected Fig. 1A in our revised manuscript as suggested.
18	Fig. 1B – the figure should contain clear indication that nearly whole graph focuses on animals, which were not provided nimbolide (including the “nimbolide-treated” groups). Nimbolide was added only during the last several weeks.	We have now added a specific indication to refer for the weeks with nimbolide treatment in Fig. 1B of the revised manuscript as suggested.
19	Fig. 2 – How exactly did you measure tumor burden (tumor volume)?	<p>We measured tumor volume and tumor burden using the following formula:</p> <ol style="list-style-type: none"> Length (L) and width (W) for each superficial tumor nodules per animal was measured by using a digital vernier caliper. No. of nodules = N Tumor volume (V) = $L \times (W)^2 / 2$. Tumor volume of each nodule per

		<p>animal = V1, V2, V3</p> <p>e. Mean tumor volume per animal (MTV) = Average of tumor volume of each nodule (V1+V2+V3)/3</p> <p>f. Tumor burden per animal = MTV x N</p>
20	Fig. 3 - How did you measure AST?	We measured AST by a kinetic method using commercially available kit from the Beckman Coulter (autoanalyzer).
21	Fig. 4 - provide uncropped blots as supplementary files	We have provided uncropped blots of fig. 4 as supplementary file of the revised manuscript. (Supplementary Figure S5)
22	Fig. 5A - the difference between the third and fourth column is much larger than what is presented in the evaluation. Did you subtract the background? Disclose all the blots (uncropped) used for the evaluation. The same issue is with	We have revised our analysis after subtracting the background and provided uncropped blots for Fig. 5 as a supplementary file of the revised manuscript (Supplementary Figure S6)

	Fig. 5C.	
23	Fig. 6B – provide image with more equal loads.	We have provided an image with more equal loads for Fig. 6B in our revised manuscript.
24	Figs 7/8 – where are the proofs that these are the real binding partners? Why is, e.g., TNF-alpha tested in silico but no co-IP or pull down experiments are disclosed?	We thank the reviewer for this comment. We have not provided the evidence for the real binding partners as mentioned in figure 7/8. We have discussed these issues as limitations of the current study in the discussion section of our revised manuscript. (Page 23 line 417 - 425). However, as suggested by the reviewer we will be performing in vitro study or pull-down experiments in the next phase of our study.

Reference:

1. **Sophia J**, Kowshik J, Dwivedi A, Bhutia SK, Manavathi B, Mishra R, et al. Nimbolide, a neem limonoid inhibits cytoprotective autophagy to activate apoptosis via modulation of the PI3K/Akt/GSK-3 β signalling pathway in

oral cancer. Cell Death Dis. 2018 23;9(11):1087. [PMID: 30352996 DOI: 10.1038/s41419-018-1126-4]

2. **Langer R**, Ott K, Feith M, Lordick F, Siewert J-R, Becker K. Prognostic significance of histopathological tumor regression after neoadjuvant chemotherapy in esophageal adenocarcinomas. Modern Pathology. 2009 Dec;22(12):1555–63. [PMID: 19801967 DOI:10.1038/modpathol.2009.123]
3. **Akateh C**, Black SM, Conteh L, Miller ED, Noonan A, Elliott E, et al. Neoadjuvant and adjuvant treatment strategies for hepatocellular carcinoma. World Journal of Gastroenterology. 2019 Jul 28;25(28):3704–21. [PMID: 31391767 DOI: 10.3748/wjg.v25.i28.3704]

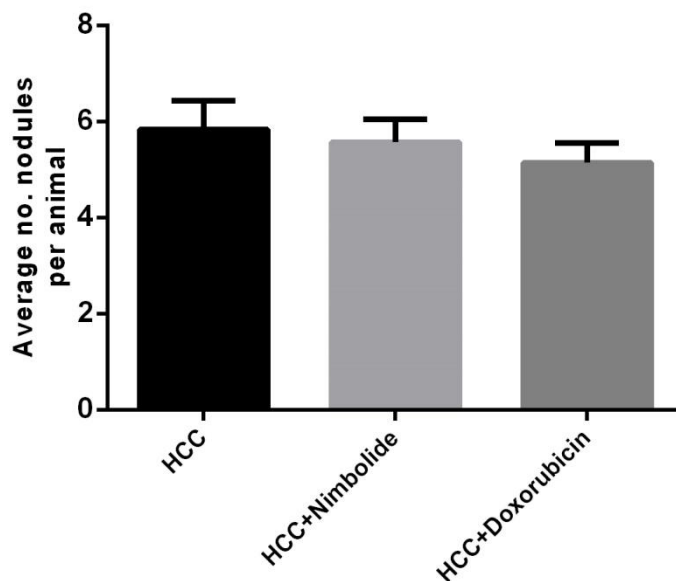


Figure 1: The comparison between the groups was analyzed by one way ANOVA followed by Tukey's multiple comparison test (n = 6-7). $P = 0.6182$