Author's response to reviews

Title: Modulation of apoptosis in human hepatocellular carcinoma (HepG2 cells) by a standardized herbal decoction of Nigella sativa seeds, Hemidesmus indicus roots and Smilax glabra rhizomes with anti-hepatocarcinogenic effects.

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Author's response to reviews: see over
To,
The Editor.
BMC Complementary and Alternative Medicine,

Sub: Submission of Revised Manuscript for publication

Dear Sir,

The manuscript entitled “Modulation of apoptosis in human hepatocellular carcinoma (HepG2 cells) by a standardized herbal decoction of *Nigella sativa* seeds, *Hemidesmus indicus* roots and *Smilax glabra* rhizomes with anti- hepatocarcinogenic effects.” has been revised according to reviewer comments and suggestions. Our responses for reviewers comments have been included point by point in a MS word file. On behalf of all the contributors I will act and guarantor and will correspond with the journal from this point onward. All authors have read and agreed to your editorial and peer review policies.

Thanking you,

Yours’ sincerely,

Signature
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Reviewer: Nizar Mhaidat

Minor Essential Revision: Author has reported in the discussion section that caspase-3 and -9 are the major caspases being activated by non-receptor mediated stimuli. I think it is largely a system-dependent fact. In melanoma for example, caspase-2 was activated and was the apical caspase following treatment with docetaxel. To exclude caspase-8 and caspase-2 as initiating caspases, I would recommend to study the kinetics of these caspases in addition to caspase-3 and -9 to show which caspase is activated first.

Reply:

I agreed with reviewer’s comments and suggestions. It is very useful if we can show which caspase is activated first by the decoction. However, now we are not in a position to carry out kinetics study of caspases because of funding constraints. We have already shown in this manuscript that the decoction has the ability to induce apoptosis in HepG2 cells via activation of caspase 3 and caspase 9.
Reviewer: AR Khuda-Bukhsh

1. HepG2 should normally be grown without the presence of CO2, but the authors have grown the cells in the 5% CO2.

2. In the sets of experiments, the author haven’t shown any cytotoxicity assay to check the viability of the cells in the presence of the drug, the assays like MTT assay, trypan blue assay or LDH activity assay should have been done to make the findings more authentic and scientifically sound.

3. The LD50 value of the drug hasn’t been determined or mentioned in the article, without which the actual drug activity couldn’t be understood properly.

4. The activity of the drug on the normal cells hasn’t also been shown in the sets of experiments, so the author should include that part in this article. At least, author should include the cytotoxic assay on the PBMC (peripheral blood mononuclear cells), as one arm of the control.

5. The authors have shown the gene and protein level expressions of Bcl-2 and BAX, but the ratio of BAX/Bcl-2 should be included in a tabular form. Then the actual difference of the ratio of them would be understandable to the readers. (The altered ratio of BAX/Bcl-2 is responsible for the change in the membrane potential of mitochondria which in turn releases the cytochrome c, responsible for the downstream activity of the caspases).

6. In figure 3 [A], the resolution and the magnification of the cells are not clear, so, if the author could change those 4 sets (Control, 1200 µg/ml, 1500 µg/ml, 1800 µg/ml) of figures, then the morphological changes may be more clear.

7. For RT-PCR and Western blot analyses, the authors have added the results of 12, 24 and 48 hrs, but for the other experiments like detection of morphological changes related to apoptosis and DNA fragmentation analysis, they have shown the concentration-dependent figures only, not the time-dependent ones, which are more important. The author should maintain a uniform time point for performing the experiments and for presentation of results.

8. The author should add the FACS analysis with Annexin-V (FITC conjugated) to find out the actual percentage of apoptosis (both late and early apoptosis) to make the result more understandable, complete and convincing.

9. In Discussion part, the authors have mentioned many cross-talks of cell signaling events. But If the authors want to cover-up that part, more signal proteins will have to be studied for accurate linking up of events of cell-signaling, otherwise the present study of only three signal proteins and commenting too much makes the study more speculative than scientifically acceptable and convincing.

10. In fluorescent microscopic study (0-1800 µg/ml) concentration range was chosen, where as in RT-PCR, western blotting, caspase assay (0-1200 µg/ml) a different concentration range was used. Make a proper dose selection to all experiments.
11. Chromatographic separation study could also have been included to spell out the active principles of each component or the actual ingredients present in the decoction could have been mentioned from existing literature to make the paper more informative.

12. Did you check the extrinsic pathway of the caspase activities as well?. If yes, provide evidence.

13. The references should again be checked for accuracy of citation and style.

Minor points:

1. In page 3, “Apoptosis can occur….or a mitochondrial (intrinsic a) pathway. The “intrinsic a” should be corrected.
2. Write the full form of abbreviations at the first mention, e.g. MTT assay, TAE buffer, etc
3. Primer sequences can be presented in a table.
4. SHIMATZU, Japan) should be SHIMADZU
5. Glyceraldehydes-3-phosphate …should be …glyceraldehydes-3-phosphate
6. Fluorescent microscopy…should be”’fluorescence microscopy..
7. Fig. 1 is showing only the graphical representation of m-RNA level expression, the PCR bands should better be incorporated in the figure as well.
8. Fig. 2a. Photograph of histochemical staining not very clear, better photograph should be more convincing.
9. Fig. 3a, the photograph of morphological features of the cells presented some impression that the control cells were not properly maintained. As they had their cell outlines not in the proper shape.
10. In Fig. 3c, no fragmented DNA is visible in the photograph.
11. In Fig. 3d, it is difficult to conclude whether the cells are undergoing apoptosis or necrosis.
12. The statistical part be consulted with a statistician whether both forms of the post-hoc tests are necessary.

Reply

1. We follow the ATCC guidelines for HepG2 cell cultures which clearly indicate that the cell should be grown in 5% CO2 environment.

2. Previously we have shown that this decoction has a cytotoxic effect on HepG2 cells by using MTT and SRB assays. We published our results in Pharmacognosy Research 2010. Please check reference 3 of revised manuscript. However in this manuscript we have included effects of the decoction on HepG2 (MTT assay) and peripheral blood mononuclear cells (trypan blue assay).
3. We selected these doses for the experiments according to our previous results which have been reported in the Reference no. 3 of the revised manuscript. EC 50 value of the decoction was also published in the Reference no. 3.

4. Anti-proliferative effects of the decoction in peripheral blood lephocyte was carried out and results are expressed in results section of the manuscript.

5. BAX/Bcl-2 ratio of mRNA expression has been calculated and indicated in table 2.

6. High resolution figures for Fig.3 are provided and magnification included in the revised manuscript.

7. Our observation on regulation of selected genes by the decoction shows most significant effect at 24 h post-incubation. Therefore we selected the same post incubation period to support our data on gene regulation by (a) cell morphology, and (b) DNA fragmentation. However we used 24 and 48 h post incubations for DNA fragmentation analysis.

8. We accept that the FACS analysis is good suggestion. Unfortunately, due to financial and other constraints we do not have the facility to carry out this analysis at present. However a sentence to reflect this suggestion has been included in discussion section of the revised manuscript (page 17,2nd paragraph, line2).
9. We have mentioned many cross-links to cell signaling events in discussion with the purpose of describing the importance of the role of Bax, Bcl-2, caspase 3 and caspase 9 in cancer treatment.

10. The doses at which genes are regulated are not reflective of morphological changes and DNA fragmentation in cells, as gene regulation occurs as an early apoptotic event. Therefore, we selected low decoction dose to document gene expression while selecting higher dose for cell morphological changes and DNA fragmentation.

11. Active components in the decoction have not been characterized before. We are currently in the process of isolating and characterizing the active components in the decoction. Since the aim of this manuscript was to show the ability of the decoction to induce apoptosis, this aspect was not discussed. The decoction was standardized based on WHO guidelines (refer ref. 3).

12. Caspases 3 activity occurs in both extrinsic and intrinsic pathways as it is the effectors of these pathways. In this study, regulation of caspase 3 is the only evidence for extrinsic partway.

13. References have been re-checked.

Minor points:
1. Corrected in the revised manuscript
2. Full names have been given for MTT and TAE in the manuscript.
3. Primer sequences presented in a table in the revised manuscript
4. Corrected in the revised manuscript
5. Corrected in the revised manuscript
6. Corrected in the revised manuscript
7. Figures of the RT PCR has been provided as a supplementary data

8. High resolution figures are provided in revised manuscript.

9. High resolution figures are provided in revised manuscript.

10. High resolution figures are provided in revised manuscript.

11. High resolution figures are provided in revised manuscript.

12. Statistical part was discussed with a statistician and she advised to put both tests for these results.

Reviewer: Akiko Kojima-Yuasa

Results
1. The concentration of the extract exposed to cells was very high. Authors should examine the effect of this extract on normal cells.
2. Authors should measure the cell viability and the ratio of apoptotic cells after 24 hrs and 48 hrs incubation. Did this extract inhibit cell proliferation than induction of apoptosis?
3. Authors should measure intracellular ROS levels, and discuss about intracellular redox.
4. Authors should describe the reason why the concentration of the extract was different in each experiment.

Discussion
5. Authors should discuss about the active compounds in this extract, if possible. If this extract was also related with inhibition of cell proliferation, authors should discuss the mechanisms.

Figures
Authors should describe the result of statistical analysis.

Miner correct:
Authors should rewrite reference number in text.

Reply
1. The doses at which genes are regulated are not reflective of morphological changes and DNA fragmentation in cells, as gene regulation occurs as an early apoptotic event. Therefore, we selected low decoction dose to document gene expression while selecting
higher dose for cell morphological changes and DNA fragmentation. In this study we used 0-1800 µg/ml dose range. Higher doses were used to show DNA fragmentation and apoptotic changes, however in gene expression analysis, genes were regulated from 150 µg/ml doses. An anti-proliferative effect of the decoction in peripheral blood lephocyte was carried out and results are expressed in results section of the manuscript.

2. Flowcytometric analysis is the reliable method to evaluate ratio of apoptotic cells and cell viability. Unfortunately, due to financial and other constraints we do not have the facility to carry out this analysis at present. However a sentence to reflect this suggestion has been included in the revised manuscript.

3. Anti-oxidant potential of the decoction has been extensively studied using in-vitro and in-vivo models and the data were published previously (refer Ref. 5).

4. For gene expression analysis we used non-toxic doses of the decoction and for DNA fragmentation and apoptotic detection we used high doses of the decoction. After 1200 µg/ml dose fragmented DNA and apoptotic cells were clearly observed. At low doses it was demonstrated that the decoction can exert a significant influence in gene regulation. For DNA fragmentation analysis and apoptotic detection by fluorescent microscopy we used dose 0-1800µg/ml.

5. Isolation of active compounds of the decoction is in progress and we are not in a position to compile the data for this manuscript. Previously we have shown that thymoquinone which is isolated from Nigella sataiva oil is not present in the decoction (Please check reference no 4). Active compounds isolated from water extracts of this plant is not reported in the literature.
Reviewer: Rajan Somasundaram

Major Compulsory Revisions:

1. Most important it is of interest if the decoction also affects apoptosis in “normal” liver cell lines. Please perform some additional experiments using e.g. hepatic stellate cells or other cell types.
2. The apoptosis inducing effects of the decoction should be tested in at least one other HCC cell line.
3. In the whole manuscript it is not stated how the decoction was standardized. A section where the standardization is described (which internal standards, which concentrations, which methods) should be added to the manuscript.
4. Derived from the new standardized-section, some information or discussion about the active ingredients of the decoction should be added.

Minor essential revisions:
1. The discussion would be easier to follow if own results in the text are recommitted to the respective figures.
2. Please discuss the differences in the concentrations of the decoction between earlier studies and the present study (0-50 mg/ml vs. 0-1200 µg/ml).
3. Please add the gel pics of the PCR in the supplemental data.
4. Maybe a table or a bar chart instead of the present Fig. 1 would be better?
5. In the title, the three components of the decoction should be named.
6. Please support higher resolution images of the figures.

Reply

1. Anti proliferative effects of the decoction on peripheral blood mononuclear cells have been included in the revised manuscript.

2. HepG2 cell line is a well recognized hepatocellular carcinoma cell line and has been used by many other researchers. All previous anti-cancer studies carried out by us have also been with HepG2 cells. Hence they were selected for the present study. In this manuscript, our main objective was to evaluate the apoptotic effects of the decoction in HepG2 cells. We hope to study effects on other cancer cell lines in the near future.

3. We standardized the decoction according to the WHO guidelines and published the results in Pharmacognosy Research 2010 (reference no 3). We have added a sentence in
the Background section (page 3, paragraph 1, line 6) of the revised manuscript about standardization of the decoction.

4. Isolation of active compounds of the decoction is in progress and we are not in a position to compile the data for this manuscript. Previously we have shown that thymoquinone which is isolated from *Nigella sataiva* oil is not present in the decoction (Please check reference no 3). Isolated active compounds from water extracts of this plant is not reported in the literature.

Minor essential revisions:
1. This manuscript has been written according to the BMC guidelines.
2. In this experiment the standardized decoction was completely dissolved in 1 % DMSO in water while in previous studies it was dissolved only in water. When freeze dried decoction is dissolved in pure water it dose not dissolve completely; therefore dose range may be varied in the two experiments.
3. Gel pictures of RT PCR have been submitted as supplemental data
4. This graph is giving a clear idea of time and dose dependent regulation of genes than bar chart. Bax/Bcl-2 ratio was included in a table (table 2). we have included bar graph of rt pcr in suplimentary data.
5. We included the names of the three plants in the title.
6. Resolutions of the figures have been increased in the revised manuscript.