Author’s response to reviews

Title: Gamma-tocotrienol and hydroxy-chavicol synergistically inhibits growth and induces apoptosis of human glioma cells

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Author’s response to reviews: see over
Dear Sir,

SUBMISSION OF REVISED MANUSCRIPT

Following the review of our Research Article titled “Gamma-tocotrienol and hydroxy-chavicol synergistically inhibits growth and induces apoptosis of human glioma cells” by Amirah Abdul Rahman, A Rahman A Jamal, Roslan Harun, Norfilza Mohd Mokhtar and Wan Zurinah Wan Ngah, we have revised the manuscript by taking into account the changes requested by the reviewers.

Here are the replies and the corrections made based on the reviewers’ comments:

Reviewer 1 - HoKeun Kwon:

1. In this manuscript, author showed no cytotoxic effects of GTT+HC in normal embryonic liver cells (WRL68) compared with tumor cells. However, this cell line is also transformed cell line and also regarded as HELA (Cervix Adenocarcinoma) derivative. Hence, it should be pointed out this by using normal primary cells such as hepatocyte or splenocyte from human or at least mice to confirm selectivity of these compounds for not normal but cancer (or glioma) cells.  
   In addition to normal embryonic liver cells (WRL68), we have also shown that no cytotoxic effects of GTT+HC were observed using normal primary fibroblast (discussed in page 10 & 15) cells.

2. In introduction, authors mentioned pathways which have been shown to be affected by these two compounds. Some of them are specific but others are common in both GTT and HC. However, based on these synergistic results of two compounds, we can assume that each compound might touch different pathways which can make synergistic effects for cell growth inhibition and induction of apoptosis. This specificity of each compound will be very critical for the future usage of these compounds with other drugs or compounds at the clinic. Hence, I strongly recommend to check and show specific pathways affected by single GTT, HC and GTT+HC to answer how this synergisms can be possible and to get better understanding which pathways are the major targets of each or both compounds treatment.

The actions of GTT towards specific pathways were mentioned in:
   a. page 11 “..GTT induces growth arrest through several pathways such as through the suppression of β-catenin/Tcf signaling in human colon cancer HT29 cells [21] and has been reported to modulate a novel pathway through down-regulation of TGFβ2 in prostate cancer”.
   b. Page 12 “..revealed that GTT induced apoptosis by caspase-8 and caspase-3 but not caspase-9 activation in neoplastic mammary epithelial cells..”
   c. Page 14 “..GTT down-regulates cyclin D1 and cyclin E levels in several cancer cell lines..”

The actions of HC towards specific pathways were mentioned in:
   a. Page 14 “HC elicit cytotoxicity through mitochondrial failure related to mitochondrial membrane potential at an early stage and subsequently lipid peroxidation through oxidative stress at a later stage..” “This causes release of cytochrome c from mitochondria, cleavage of caspase 9, 3 and poly-adenosine diphosphate-ribose polymerase (PARP) leading to apoptosis..”
   b. Page 15 “HC also showed scavenging properties toward H₂O₂, superoxide and hydroxyl radicals and is possibly an antioxidant at low concentrations whereas at higher concentrations, HC induces glutathione (GSH) depletion, reactive oxygen species (ROS) production, cell cycle arrest and apoptosis of oral KB epithelial cells (cell death mediated by oxidative stress)..”
The possible mechanism of action of synergism between GTT+HC were discussed in page 13: “Synergism between the GTT and HC bioactives may be due to the fact that the bioactives acting in concert to reach different targets of the same signalling pathway, controlling apoptosis, thus accelerating cell death process”.

We cannot speculate which pathways will be affected by the combination of GTT+HC. However, studies on the effect of GTT+HC on whole gene expression of these cell lines are ongoing; as we have suggested in page 15 “Future studies on the elucidation of mechanisms induced by combined GTT+HC are warranted”.

Reviewer 2 - Shubhankar Suman:

1. Discretionary Revisions: It would be worthwhile to comment on the bioavailability of these compounds in brain. Is there any data available showing that these compounds can cross blood brain barrier? If yes, should be discussed in the discussion section.

   Tocotrienols and hydroxychavicol has been reported to cross the blood brain barrier (BBB) (page 15).

Reviewer 3 – Suzana Makpol:

Minor Essential Revisions

1. There have been many studies on the effects of tocotrienol on different types of cancer which should be highlighted to show the importance of using combined compounds for cancer treatment. The difference between this study and previous reported findings shall also be highlighted.

   Studies on the effects of tocotrienol on different types of cancer have been highlighted in the manuscript (page 4, 11). However, to our knowledge, the combination of bioactives used in this study (GTT+HC) has not been reported yet. The difference between this study and previous findings (on combined therapeutic treatments) were discussed on page 12, 13: “..Evidence have shown that combined low dose tocotrienol treatment with specific chemotherapeutic drugs such as statins [33] and celecoxib [34] showed significantly enhanced therapeutic response compared to that observed from individual treatments alone. It is possible that combination of GTT+HC is more effective than narrowly focused therapies as each bioactive are likely to impact several aspects of tumor progression. Our results further confirmed and extended the findings from previous studies where a synergistic anti-proliferative response were observed in grade II, III and IV human glioma cell lines after exposure to lower dose combination treatment with GTT and HC compared to individual treatment alone.”.

2. ‘Hydroxy-chavicol (4-allyl-catechol, 1-allyl-3,4-dihydroxybenzene, HC has been shown to induce oxidative stress, glutathione (GSH) depletion and cell cycle deregulation.’ Can the authors please provide the reference for this statement. How this finding supports the anti tumor properties of HC?

   “..HC has been shown to induce cell apoptosis by the induction of oxidative stress, glutathione (GSH) depletion and cell cycle deregulation [10].”. The reference for this statement has been provided (page 4).

3. The reason for choosing gamma-tocotrienol and HC as combined treatment for glioma cancer is not well explained.

   GTT and HC were chosen as combined treatment for glioma based on the effectiveness of individual compounds in reducing the glioma cell viability tested in this study. The screening of several compounds including GTT and HC individually was done on 1321n1, Sw1783 and Ln18 cell
lines prior to choosing the compounds for combination. However, the data of screened compounds were not shown.

4. Although this study is interesting, the argument in the discussion is not convincing. It is possible that the two compounds act differently towards the different stages of glioma. Moreover, the two compounds may possess different biological properties. The possible mechanism for the synergistic effects shall be elaborated to justify your findings and not just discussing the effects of single compound.

The possibility of these two compounds act differently towards different stages of glioma were discussed on page 12: “...specific characteristics such as different mutations involved in different grades of glioma cell lines influenced the choice of route of signaling pathway mediated by GTT and HC in order to inhibit the proliferation of glioma cells [31]. This is further supported by evidence which have shown that tocotrienols mediated its apoptotic effect through activation of different intracellular signaling mechanisms in different types of cancer cells [8]. This may provide an explanation for the different doses of each bioactives required to inhibit proliferation of the cancer cells in culture [31].”.

The possible mechanism of action of synergism between GTT+HC were discussed in page 13: “Synergism between the GTT and HC bioactives may be due to the fact that the bioactives acting in concert to reach different targets of the same signalling pathway, controlling apoptosis, thus accelerating cell death process”.

We cannot speculate which pathways will be affected by the combination of GTT+HC. However, studies on the effect of GTT+HC on whole gene expression of these cell lines are ongoing; as we have suggested in page 15 “Future studies on the elucidation of mechanisms induced by combined GTT+HC are warranted”.

5. ‘Combination therapy optimizes the effectiveness of each treatment by their complimentary action that will eventually result in synergistic therapeutic response’ – please explain the complimentary action of GTT and HC that eventually result in synergistic response to show the connections between these two compounds.

The complimentary mechanism(s) of combined GTT and HC is yet unknown. However, studies on the effect of GTT+HC on whole gene expression of these cell lines are ongoing; as we have suggested in page 15 “Future studies on the elucidation of mechanisms induced by combined GTT+HC are warranted”.

Other minor corrections:

6. How the combination dose (the ratio) of GTT and HC is determined? Probably shall be mentioned in the methodology.

Mentioned in page 6: “As for combined compounds (GTT+HC), ½ or ¼ of IC50 of GTT were initially titrated to a range of concentrations (1, 10, 50, 100 μg/ml) of HC”.

7. The concentration of single GTT or HC and combined treatment that results to 90-95% inhibition shall be explained in results section.

Changes have been made in Page 8: “..cytotoxicity induced by GTT and HC was dose dependent with 90-95% inhibition achieved at maximum concentration of 200 μg/ml after 24h of treatment..”.

8. The morphological changes indicating apoptosis with single GTT or HC treatment shall be included in results section as a comparison to combined treatment (Figure 2).

The morphological changes with single GTT or HC treatment were included in the results section (Figure 2) compared to combined treatment. Similar apoptotic changes were observed in the single treatment but with less percentage of apoptotic cells compared to combined treatment.
9. What is CPT mentioned in Figure 4?
Camptothecin (CPT) (a well known apoptosis inducer) was used as a positive control for apoptosis induction (mentioned in page 7, under the Materials and methods section).

10. Change compound A and B in Table 2 to GTT and HC.
The text (compound A or B) in Table 2 has been changed: “IC50 of compound A or B” to “IC50 of individual compound”.

11. The label for figures (capital and small letters are used in the same figure) such as Figure 2A(a), 2B(b) or 5C(b) etc. is not appropriate. Perhaps can be changed to Figure 2A(i) etc.
The labelling has been changed.

12. When writing your results please indicate specifically which figures are you referring to such as Figure 2A(a) or Figure 5A(b) and not just referring to Figure 2 or Figure 5 only.
Changes have been made.

13. Please check the format for writing correct citation in the text as it is not standardised throughout the manuscript.
We have utilized Endnote, using the style specific for BMC complementary and alternative medicine for the citation/reference format.

Thank you for considering our manuscript for publication. We hope that the editorial board will consider our revised manuscript for possible publication and look forward to a positive response from you.

Sincerely yours,
Amirah Abdul Rahman
(On behalf of the authors)