Author's response to reviews

Title: Induction of p53-independent cell cycle arrest and apoptosis in caspase-3 deficient MCF-7 cells by Dillenia suffruticosa root extract via multiple signaling pathways

Authors:

Jhi Biau Foo (foojhibiau@gmail.com)
Latifah Saiful Yazan (latifahsy@upm.edu.my)
Yin Sim Tor (yinsim87@hotmail.com)
Nurdin Armania (armania_nurdeen@yahoo.com)
Norsharina Ismail (norsharinaibs@gmail.com)
Mustapha Umar Imam (mustyimam@gmail.com)
Swee Keong Yeap (skyeap2005@gmail.com)
Yoke Kqueen Cheah (ykcheah@upm.edu.my)
Rasedee Abdullah (rasedee@gmail.com)
Maznah Ismail (myhome.e@gmail.com)

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Author's response to reviews: see over
Dear Editor:

Following your letter regarding the manuscript MS:2003450862114442 entitled *Induction of p53-independent cell cycle arrest and apoptosis in caspase-3 deficient MCF-7 cells by Dillenia suffruticosa root extract via multiple signaling pathways*, we are sending the rebuttal letter explaining the changes performed on the manuscript. The changes incorporate the suggestions of the reviewers. We found the comments very helpful and constructive. We have addressed all the changes recommended by the reviewers and we are confident that the new version of the manuscript is easier to understand and has a more fluent scientific discourse. The revision is addressed as follows.

**Reviewer Dr. Parveen-Bansel:**

1. Methods 17 line change calorimetric to colorimetric
   
   The word calorimetric has been corrected to colorimetric (Page 2, Line 17).

2. Introduction 11 line synergetic to synergistic
   
   The word synergetic has been corrected to synergistic (Page 4, Line 11).

3. There seems to be a problem in communication when authors write that the fine powder of plant was obtained from a company. Then how the identification of plant is possible. It needs to be explained how the plant was identified and authenticated when the powder was ready made.

   Dear reviewer, thank you for your comment. I am pleased to clarify how the plant authentication was carried out. For your information, we received the fine root’s powder from the herbal company to carry out the present study. Prior to that, we have requested the herbal company to provide us the parts of the plant such as flowers and leaves for the purpose of plant authentication. Therefore, the plant authentication is actually based on the plant, not the fine root’s powder. With that, the write up in the manuscript has been corrected as follows (page 6, 21-24).

   “The plant’s authentication was performed using the parts of the plant (flower, leave, stem and root) at the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia, Malaysia (voucher specimen number SK1937/11)”.

4. I could not find the effect of Dichloromethane as such on viability of cell lines. Because during extraction despite pressurised removal of DCM there are still chances that DCM can have its own impact on viability of cells. If data for the same is available, it should be
Dear reviewer, thank you for your comment. The residue of the solvent in the extract is always our concern. Therefore, precautions in the steps have been carried out to make sure the solvent is removed from the extract. The reason for us to use DCM in our extraction is because DCM is a very volatile solvent that can be easily removed from the extract, unlike ethyl acetate or methanol which are difficult to be evaporated. After the pressurised removal of DCM with the rotary evaporator, the extract was not immediately tested for the anticancer activity. In fact, it was transferred to a glass vial and placed in a fume hood for the DCM residue in the extract to be evaporated. Just after the weight was constant for 3 days to indicate that the solvent has been completely removed from the extract, the study was then carried out.

For your information, we did not perform any experiment to evaluate the effect of DCM towards MCF-7 as it is a very volatile solvent and it is not miscible with the RPMI culture media (water). In fact, DCM has been widely used to decaffeinate coffee and tea as well as to prepare extracts of hops and other flavourings in the food industry\textsuperscript{1}. In addition, the use of DCM in the preparation of extract has been widely used in the pharmaceutical industry. Those data have been published in many reputed journals\textsuperscript{2,3,4}. With the precautions in the steps taken during the extract preparation, we believe that DCM was not toxic to the cells in the present study and the anticancer activity was solely due to the extract.

Reference:
Reviewer Dr. Mohammad Rahmati Yamchi:

Major Compulsory Revisions:

1. In page 7, lines 6 & 22: using 0.3% of DMSO is so high and it must be less than 0.1%. I don't know why author used this concentration because it has cytotoxic effects.

   Dear reviewer, we have carried out a simple cell proliferation assay (MTT assay) to evaluate the inhibitory effects of DCM-DS towards MCF-7 cells with DMSO at both 0.1 and 0.3% (unpublished data). For your information, we obtained similar IC$_{50}$ of DCM-DS at both percentages of DMSO. In addition, the growth rate of MCF-7 cells at 0.1 and 0.3% DMSO was not significantly different from the untreated-MCF-7 cells (the control without DMSO and DCM-DS). Similar inhibitory effect by DCM-DS towards MCF-7 and MCF-10A at both percentages of DMSO was also noticed. Furthermore, the control cells treated with 0.3% of DMSO (morphological study) grew healthily. Therefore, we strongly believe that inhibitory effects on both MCF-10A and MCF7 cells were not due to DMSO. Wang et al. (2010)$^1$ studied the chemopreventive properties of a plant extract on MCF-7 cell line by introducing a final concentration of 0.5% of DMSO in the control (untreated cells). The cells grew exponentially without any sign of toxicity. In view of the potential negative effect of DMSO, we thank you for the suggestion to keep DMSO’s concentration below 0.1% and will consider it for our future works. We thank you and appreciate your precious time and efforts to provide such insightful guidance.

2. Page 21, lines 1 & 2: The author couldn't write about induction of oxidative stress by DCM-DS in MCF-7 cells because the result showed that the role of ROS was unclear.

   The sentence has been removed from the conclusion section.

3. The therapeutic dosage normally is very less than IC$_{50}$. The most IC$_{50}$ was 25 µg and I don't know why did the author use 50 µg dose to study?

   Dear reviewer, thank you for the comment. The use of 50 µg/mL concentration in the present study was to show the effect of the high dose of DCM-DS towards MCF-7 cells. We thank you for your information about the therapeutic dosage is normally less than IC$_{50}$. We will take your suggestion for our future study.
Minor Essential Revisions:

1. The author could use "total extract" instead "extract" at the whole body of text.
   
   We seek advice from a few chemists about the use of word “extract” or “total extract”. The comments we obtained that the word “extract” is more appropriate and therefore remains in the whole body of text.

2. Page 20, 7 &9: "nf-kb" must be corrected.
   
   The word “NF-kB” been corrected.

Reference:

Reviewer Dr. Yasmin Anum Mohd Yusof

Major Compulsory Revisions

1. The research question as to why this extract was used in the present study is not well defined according to the title of this study.

   Dear reviewer, the research question to use this extract in the present study has been mentioned in the introduction. The extract was used because our previous study has shown that DCM-DS possessed strong cytotoxicity towards MCF-7 breast cancer cells. Please kindly refer to page 6, lines 7-16.

2. The authors would like to propose a mechanism using multiple pathways, however the pathways were not defined in the Introduction and as to why were these pathways chosen? The justification to study the pathways has been included in the introduction. Please kindly refer to page 4 lines 16-23 and page 5 lines 1-22).

3. Since this is an extract (D. suffruticosa) which is unique to local interest, the study should be compared to a well-known breast cancer chemotherapy drugs such as tamoxifen.

   Dear reviewer, the reference drug tamoxifen has been included in the MTT assay for comparison and validation of the cytotoxicity of DCM-DS. Please kindly refer to Figure 1c. Nevertheless, the use of reference drug as the positive control was not included in subsequent experiments. In our point of view, the experiments and techniques performed in this study were established and well-recognized procedures in investigating agent with similar mode of action\textsuperscript{1}\textsuperscript{3}. Therefore, we strongly believe that the obtained findings in this study were well supported despite the lack of inclusion of reference drug. We thank you for your constructive comment and we will consider the use of reference drugs for future experiments.

4. The methods used in this study is appropriate and well described, however the authors did not state the name and place of the manufacturing company.

   Dear reviewer, the name and place of the manufacturing company have been thoroughly stated in the Materials and Methods section. Please kindly go through the section especially the 2.2 chemicals.

5. The discussions and conclusion however is not well balanced. The authors needs to justify figure 6 (proposed mechanism) further by extracting from own's experiment and
other related experiment from authors previous study as well as other similar studies by other authors. There seemed to be no flow of thoughts in the discussion to lead to the conclusion and the proposed mechanism.

Dear reviewer, thank you for the comments. We have rewritten the introduction and discussion parts about the cell signalling pathways induced by the extract in the MCF-7 cells. Now there is a flow of thoughts in the discussion lead to the conclusion and the proposed mechanism. Please kindly read through it. We look forward to receiving your feedback soon.

6. The discussion on MCF-7 cells being caspase 3 deficient may be inappropriate here since the authors did not evaluate caspase 3 in their study

Dear reviewer, in our point of view, the discussion on MCF-7 cells being caspase-3 deficient is appropriate as we did not observe any typical characteristics of apoptosis such as membrane blebbing, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies in the morphological study. Nevertheless, Annexin-V analysis has confirmed that DCM-DS induced apoptosis in MCF-7 cells. This phenomenon was due to the caspase-3 deficiency in MCF-7 cells originated from ATCC, USA. Therefore, it is important to explain the phenomenon to the potential readers of our manuscript.

7. The authors should declare in the Methods section that this extract is still in the process of patenting or has it been patented?

Dear reviewer, the extract used in the present study has been patented. The declaration has been stated in the Methods section (Page 7, line 20).

8. Limitations of the work are not clearly stated in the study.

Dear reviewers, the limitations of the work have been stated in the Discussion section. Please kindly refer to page 22, line 1-13.

9. The title of the study "Induction of cell cycle arrest and apoptosis in MCF-7 cells by Dilleniasuffruticosa root extract via ROS formation and multiple signalling pathways" does not reflect fully to the abstract and the introduction, in which the authors were emphasizing
multiple pathways in the mechanism of anti-tumour of this extract. The authors did not mention which pathways they are suggesting in the Introduction section and why were these pathways chosen?

The justification to study the pathways has been included in the introduction. Please kindly refer to page 4 line 16-23 and page 5 line 1-22). The title of our manuscript has been improved to reflect the content of the abstract and introduction.

References:


Reviewer Dr. Jong Bin Kim

Major revision
Authors observed anti-cancer effect of *Dilleniasuffruticosa* root extract on breast cancer cells. Authors reported same results for figure 1, 2, 3, and 4 on their previous reports (Journal of Ethnopharmacology 146 (2013) 525–535, Molecules 2013, 18,13320-13339). On the manuscript, authors showed reactive oxygen species level and related molecules after *Dilleniasuffruticosa* root extract treatment unlike their previous reports. This is interesting and novel effect of *Dilleniasuffruticosa* root extract on breast cancer. The manuscript should be revised to publish by focusing on the cell death and related molecules by reactive oxygen species stress.

Dear reviewer, thank you for rising up the issue. We are pleased to explain the inclusion of Figure 1-4 in our present manuscript.

First of all, MCF-7 cell line used in the present study was recently purchased from the American Type Culture Collection (ATCC), USA, which is a caspase-3 deficient cell line. As reported by others, the cells originated from ATCC do not display typical characteristics of apoptosis such as membrane blebbing, chromatin condensation, nuclear fragmentation and formation of apoptotic upon treatment with anticancer drug that induces apoptosis\(^1\text{3}\). We were happy that the same observation was also noted in the MCF-7 cell line treated with our extract in the present manuscript. In contrast, the MCF-7 cells used in paper published in Molecules\(^4\) displayed typical characteristics of apoptosis such as membrane blebbing, nuclear fragmentation and formation of apoptotic bodies upon the treatment with extract’s fraction. We suspect that the cells were not caspase-3 deficiency. We then carried out PCR on the caspase-3 gene for the both cell lines to confirm our hypothesis. Our data have shown that the cell line published in Molecules is not a caspase-3 mutant, but the cell line used in the present manuscript is a caspase-3 deficient cell line! This is the reason why the effects of the extract towards the “original” and “not original” MCF-7 cell lines were totally different. Therefore, we think that it is crucial to repeat the experiment for MTT, morphological study, cell cycle and Annexin-V analyses and discuss on the caspase-3 deficient MCF-7 cell line in our present manuscript. We hope that the publication of our manuscript would give more information on the both cell lines. This will create awareness that the original MCF-7 breast cancer cell line that can be obtained from ATCC does not express the caspase-3 protein. Hopefully, this misconception can be avoided in the future study.
Second, the time-point strategy (24, 48 and 72 hours) was employed in the present manuscript for MTT, morphological study, cell cycle and Annexin-V analyses, which provides us more information about the effect of the extract towards the MCF-7 cell line (the previous manuscript just focused on 72 hours). In addition, the current manuscript contains the effect of the extract towards normal breast MCF-10A cell, which is valuable information used to compare the toxic effect of the extract towards cancer and normal cells. For the morphological study, the present manuscript employed the strategy of “capture the same spot” from 0 to 72 hours upon the treatment of the extract. This is important to observe the growth rate at the same spot as well as to avoid observation bias (capture the desired spot that contains the positive result). Moreover, the previous paper evaluated the fractions of DCM-DS towards MCF-7 cell line whereby the current manuscript employed the total crude extract.

As a conclusion, our current manuscript presented the caspase-3 deficient cell line and more informative data. We hope that our detailed explanation could convince you for the inclusion of Figure 1-4 in our current manuscript. We thank you for your comment.

Minor Revisions

1. For figure 3, 4, 5, 6, and 7, independent experiments should be performed more than three times to confirm statistical difference.

   Dear reviewer, the results of our experiments were from three independent experiments. For each independent experiment, at least 3 replicates have been performed to ensure that the results were reproducible. Therefore we believe that three independent experiments are enough to confirm the statistical difference for our work.

2. Figure 5 was not significant statically. Cell death by *Dillenia suffruticosa* root extract treatment did not block by anti-oxidant agents on breast cancer cells. These results showed that cell death by *Dillenia suffruticosa* root extract did not result from reactive oxygen species stress.

   Dear reviewer, we agree on your point that the cell death by the extract treatment was not blocked by the anti-oxidant agents at 12.5 and 25 µg/mL of DCM-DS, suggesting that ROS did not involve in the cell death at these two concentrations. Nevertheless, at high concentration of the extract (50 µg/mL), the co-treatment with α-tocopherol significantly blocked the cell death. Therefore, it shows that
ROS played a significant role at 50 µg/mL of the extract to induce cell death in MCF-7 cell line. With that, the discussion part about the role of ROS in the signalling pathway has been rewritten.

3. Figure 6 and 7 showed related signalling molecules after Dilleniasuffruticosaroot extract treatment. To observe signalling pathway after drug treatment, it should not be measured by gene level, but protein level.

Dear reviewer, our present manuscript is reporting observations, that appear to be important, based on the effects of DCM-DS on expression of growth, survival, apoptosis and stress genes in MCF-7 breast cancer cells. Even though the scope of our current research does not include protein expression analysis, we believe that the data on gene expression alone have given us good insights on the signalling pathway involved in the process. We agree on your view that the signalling pathway should be measured at protein level as it is more convincing.

We thank you for the constructive comment. Nevertheless, in order to perform the experiments, more funding are needed as the materials for Western blot analysis especially the antibodies are expensive, of which are currently not available. We hope that the publication of our current manuscript in BMC Complementary and Alternative Medicine would assist us to get more funding for the protein analysis as well as the isolation and identification of bioactive compounds responsible for the effects in the present manuscript.

4. Figure 6 and 7 should be combined.

Figures 6 and 7 have been combined.

5. Figure 8 is not correct.

Dear reviewer, the figure has been corrected. We look forward to receiving your feedback soon.

References: