Author's response to reviews

Title: Inhibition of Raf-MEK-ERK and Hypoxia pathways by Phyllanthus Prevents Metastasis in Human Lung (A549) Cancer Cell Line

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Author's response to reviews: see over
The manuscript entitled “Inhibition of Raf-MEK-ERK and Hypoxia Pathways by Phyllanthus Prevents Metastasis in Human Lung (A549) Cancer Cell Line” details very interesting data to demonstrate the mechanisms account for anti-metastatic effect of Phyllanthus extracts in lung cancer cell line A549. Phyllanthus extracts down-regulated ERK and hypoxia pathways may contribute to the inhibition of metastasis in A549 cells. However, there are several concerns that need to be more thoroughly addressed.

**Major comments:**

1. Lack of significant data is the weakest part of this manuscript. Figures are very difficult to see, especially Figure 4. The images with higher resolution are required. The reviewer does not agree with the conclusion the authors made based on this poor quality of western blot analysis.

   **Response:** We thank you for your comments and we have increased the resolution of all the figures to 600dpi. Since it is a 2D-gel electrophoresis-based western blot, the proteins were observed as spots instead of bands and hence might not appear too clearly after scanning and conversion into a .tiff image file. Nevertheless, we have tried to increase the clarity of the western blot images in this revision and this is the clearest image that we can produce from the 2D-WB blots.

2. a) It seems like the authors are trying to distinguish the difference between aqueous and methanolic extracts from Phyllanthus. Since the authors’ previous study had shown that methanolic extracts seem to be more effective than aqueous extracts, an explanation is necessary to address why the authors kept using these two extracts in this present study. It makes more sense to only investigate the anti-metastatic effect of methanolic extracts, or even more specifically focus on certain pure components of methanolic extracts, such as tetragalloylglucopyronside which is not present in aqueous extracts (Lee, S.H. et al., 2011, PLoS ONE).

   **Response:** Although the previous toxicity study suggested that methanolic extracts were more effective than aqueous extracts, they are not feasible to be used for human therapy since methanol is detrimental to human health and we intend to develop this plant into a chemotherapeutic or chemopreventive agent. However, we were also interested to compare their activities with the aqueous extracts and hence, both of them were continuously studied in all the experiments.
We also agree that pure components are desirable chemotherapeutic compounds. However, they are often less specific and tend to cause toxicity to the normal healthy cells leading to harmful side effects, as demonstrated by the current chemotherapeutic drugs. Similar phenomena was also observed from our previous toxicity studies whereby the semi-purified Phyllanthus extracts were causing considerable toxicity to the normal cells. Contrarily, the mixture of components present in the Phyllanthus crude extracts allow specific killing of the cancer cells with minimal effects on their corresponding normal cells (Lee, S.H. et al., 2011, PLoS ONE). This is believed to be a result of the synergistic activities of the various bioactive components in the extract and hence, we would like to investigate the activity of the plant as a whole instead of as fractions or pure compounds.

b) Is it possible that the different results obtained from aqueous and methanolic extracts are due to the effect of DMSO? The authors should include DMSO treatment as a control in this study.

Response: DMSO was only used as a solvent for methanolic extracts and hence the results obtained from aqueous extracts were solely due to the activity of the plant itself. As for methanolic extracts, we did include DMSO as one of the controls in the toxicity study and within the range of concentrations tested on the cells, the percentage of DMSO present in the methanolic extracts were not exhibiting cytotoxicity and therefore we believe that the cell-killing activity displayed was because of the methanolic extracts itself.

3. The authors state that the extract from P. urinaria is the most effective one. However, the 2-D gel analysis was performed using P. watsonii extracts-treated samples. An explanation is needed. Additionally, the results from four different Phyllanthus species are not consistent and confusing. It is recommended to focus on only either P. urinaria or P. watsonii.

Response: P. urinaria extract is indeed showing the highest effectiveness among the four species, but it was closely followed by P. watsonii and their differences in activity is not significant. Unlike P. urinaria which is more easily found in other countries and was well reported to exert anticancer activities, P. watsonii is an endemic plant only found in Malaysia and my research group actually produced the first publication on its anticancer activities in the year 2010. Therefore, we would like to highlight this species by including its 2D protein expression profile as a representative image for other species which we also worked on. Besides that, a variation in the results obtained from all the four Phyllanthus species tested was anticipated since each of them has their own composition of polyphenol components and hence might be exhibiting their activities differently. Since we intended to
compare the activities of all these four species, all of them were included in the experiments instead of focusing only on either one of the species.

4. What are exact concentrations of these extracts at IC50? It would help readers to easily appreciate this study if the authors can provide clear information in this present study. A table may be useful since each extract has its unique IC50. Meanwhile, in some cases, the IC50 is close to the high dose, for example, IC50 of aqueous extracts from P. niruri is ~466.7 µg/ml and methanolic extract is ~128.3 µg/ml in A549 cells (Lee, S.H. et al., 2011, PLoS ONE). The authors should choose different range of doses for each Phyllanthus species.

Response: We thank you for your comment as we have previously neglected the need to include the IC_{50} values for each extract in this manuscript. Admitting to our mistake, we have already included Table 1 with the IC_{50} values in this revision (Lines 140 and 800). We also agree that it would be more relevant to choose a different dose range for each species in the dose-dependent study. However, this will then not allow us to do a comparison study between the activities of different species due to the different dose range chosen and hence we decided to use a single high and low treatment concentration for each species in the experiments.

5. What buffers did the authors use to perform western blot analysis and zymography? Please specify lysis buffer (protein lysate), rehydration buffer (protein lysate), 4x sample buffer (western blot), 2x sample buffer (zymography), renaturing buffer (zymography) and developing buffer (zymography).

Response: We thank you again for your comment and hence we have already included the recipes for each of the buffers in the methods section.

6. Figure 1: How to determine the significance between non-treated and treated groups since the data include different extracts from four Phyllanthus species? Did the author combined all four extracts-treated samples as a “treated group”? Please provide a clearer description regarding statistic analysis. Additionally, it is recommended to perform real-time qPCR or RT-PCR to confirm the data from the reporter gene array.

Response: We would like to apologize for this misunderstand. The statistical analysis was performed individually for each of the Phyllanthus species by comparing the extract-treated samples with the non-treated sample and the differences were only deemed significant with p value less than 0.05. For ease of discussion however, the treatment of cancer cells with all four Phyllanthus species were clustered and mentioned as “treated group” instead of mentioning them one-by-one in each section throughout the manuscript.
We also do understand that results from this cancer pathway reporter gene array is not conclusive enough and therefore, we further confirmed these data with western blotting that detects specific protein which is representative of the pathways affected instead of RT-PCR.

7. The authors should explain the reason to use cisplatin and doxorubicin as positive controls in METHODS section.
Response: The sentence “Cisplatin and Doxorubicin (MERCK – 1mg/ml) as standard anticancer drugs for lung and breast carcinomas respectively were included as the controls” has been included in the Methods section (Lines 142-143) to explain the reason for using Cisplatin and Doxorubicin as positive controls.

8. Cancer Reporter Array is a good tool to detect changes in signaling pathways following chemical exposure. However, low reporter gene activity in E2F/DP1 in Cell cycle/pRb-E2F pathway found in Figure 1 is not enough to explain the reason why no changes in cell cycle were observed after Phyllanthus extract treatment (Page 16, first paragraph).
Response: We agree that the low reporter gene activity of E2F/DP1 in cell cycle/pRb-E2F pathway as reported by the cancer reporter array is not conclusive. Therefore, a standard flow cytometry-based cell cycle analysis was further carried out by staining the treated and non-treated cancer cells with Propidium Iodide dye. From the data obtained, there were no significant changes between the percentages of treated-cells gated at each cell cycle phases as compared to the non-treated cells after each incubation period. Based on this data, we reasoned that Phyllanthus do not exhibit cell cycle arrest on the cancer cells (Lines 323-326).

9. It is recommended to remove Figure 3.
Response: Figure 3 has been removed as suggested.

10. Figure 4: Lacking internal control in western blots. The bands are too faint to see and quantify the signals. No error bars were shown in Figure 4K.
Response: In the 2D gel electrophoresis-based western blotting experiment, the first part of the assay is the same as a standard 2D gel electrophoresis assay whereby the isomers and subunits of each protein will be fully separated based on their pI value and molecular weight. Therefore, it is not possible to include an internal control for this 2D-WB assay. Nevertheless, being a standard 2DGE assay, it is crucial to load the same amount of proteins into each IPG strip to ensure comparable analysis and hence, an internal control
will not be necessary. Since the proteins are appearing as spots in 2D-WB, they might not appear too clearly after scanning and conversion into a .tiff image file. However, we have tried to increase the clarity and resolution of the image in this revision and this is the clearest image that we can produce from the 2D-WB blots. We would also like to apologize for the mistake and hence have already included the error bars in Figure 3K [The previous Figure 4 is now named as Figure 3 after removal of the pathway diagram (previously Figure 3) as suggested].

11. a) Page 16, Last paragraph: If the authors did not use antibody against FUSE-binding protein, how do they know the band shown on the blot (#5) is FUSE-binding protein rather than just a result of non-specific binding? Did the author mix all antibodies together to perform western blot analysis?
Response: All the antibodies were indeed mixed together to perform western blot analysis in this study and they were first identified based on their molecular weight. However, to determine accurately the identity of each protein spot, they were then carefully excised out from their positions in the acrylamide gel and sent for identification using mass spectrometry analysis. Upon analyzing the data obtained, we realized the presence of an additional protein spot which is the FUSE-binding protein although an antibody against this particular protein was not included. After a thorough literature search, we found out that this protein has similarity with the c-Myc protein due to its regulatory role and hence we postulated that c-Myc antibody used in this study could be detecting this FUSE-binding protein as well.

b) What about JNK1/2 expression?
Response: Although an antibody against JNK1/2 protein was included in this western blotting experiment, its protein spot was not detected. This could be due to its low expression in A549 cells which makes it difficult to be detected and therefore, we speculated that this JNK pathway has very little involvement in anticancer activity of Phyllanthus.

12. a) Figure 5: Zymography results indicated the reduction in MMP2 and MMP9 following Phyllanthus extract treatment. However, the bands are too faint, and it is hard to tell the changes in either pro and active forms of MMP2 or MMP9.
Response: In zymography assay, the acrylamide gel embedded with a suitable substrate (gelatin or casein) is used to detect the MMP enzymes whereby the clear bands are the areas in the gel in which the substrate has been digested by the enzymes. A clearer band
therefore signifies a higher amount of MMP enzyme in the sample and therefore resulted in a higher degree of substrate digestion. Meanwhile, a faint band is due to a lower digestion rate of the substrate in the area as a result of a reduced expression of MMP enzymes after Phyllanthus treatment. Hence, the bands shown in the zymogram represents only the active forms of MMP2 and MMP9 since the pro/inactive forms of MMP will not have the capability to digest substrate. Another explanation for this faint bands is because A549 predominantly expresses MMP7 to induce invasion and metastasis with lower expression of MMP2 and MMP9 although all these three MMPs can degrade the type-4 collagen present abundantly in the basement membrane.

b) In addition, aqueous extracts seem to induce MMP7 activity in A439 cells, especially at low dose. Can the authors explain it?

Response: We would again like to apologize for the misunderstand in the figure as the control/non-treated bands in the Figures 4C and 4D (previously Figure 5C and 5D) should have almost the same intensity and thickness since they are originated from the same sample. We therefore have already changed to a clearer zymogram for Figure 4C in this revision. As shown in the figure, the control band has almost the same intensity and thickness as the low dose-treated band. This is mostly due to the low dosage of the Phyllanthus extracts which had very little effect on the MMP7 expression. However, as the extracts dosage increases, the expression of the MMP7 was reduced as shown in the Figures 4C and 4D.

13. Did the authors detect major changes in MMP2, MMP9 or MMP7 in proteomic results?

Response: The changes in MMP2, MMP9 and MMP7 expressions were not detected in this proteomic results since cell lysate was used as the sample type for this experiment. Meanwhile, MMP enzymes are expressed and mainly secreted extracellularly (into the culture supernatant) to carry out their activities. Therefore, modulation of MMP expressions were not observed in the proteomic analysis.

14. What did the numbers in table 1 and 2 mean? Please provide the details in the analysis.

Response: The sentence “The negative (-) symbol in both Tables 2 and 3 signified suppression of the proteins and a value of 1.00 indicates the complete absence of this protein in the Phyllanthus-treated sample as compared to the untreated-control sample and the degree of suppression reduces as the value increases” has been included in the results section (Lines 404-407) to explain the meaning of the numbers in Tables 2 and 3.
(previously Tables 1 and 2). A similar explanation has also been included as a footnote for these tables (Lines 810-812 and 824-826) to further clarify the meaning of the numbers.

15. Bcl-2 is an anti-apoptotic protein and primarily regulates intrinsic apoptotic pathway; however caspase-8 is also able to interact with Bcl-2 (Poulaki, V. et al., 2001, Cancer Res). If authors attempt to determine whether Phyllanthus extracts induces extrinsic or intrinsic pathway, the changes in either the expression of cytochrome c in mitochondria or the activities and expression of caspase-3, -8, and -9 are needed. 

Response: We thank you for your suggestion and we do agree that either the cytochrome c or the caspases-8 and 9 expression studies are needed to determine whether Phyllanthus induces extrinsic or intrinsic apoptotic pathway. In this study however, we are not attempting to determine how Phyllanthus induces apoptosis (via extrinsic or intrinsic pathways) in the treated-cancer cells as our intent is to show that Phyllanthus does reduce the bcl-2 expression in order to cause cancer cell death. One of the main mechanisms for bcl-2 protein is by inducing a release of cytochrome c to stimulate intrinsic apoptosis. However, as shown by Poulaki et al (2001, 61(12), Cancer Research), bcl-2 also activates caspase 8 to trigger Fas-mediated extrinsic apoptotic pathway and therefore, further supports our findings whereby reduction of bcl-2 expression by Phyllanthus helps to promote cancer cell death. Meanwhile, caspase-3 is one of the executioner caspases in the caspases cascade and its activation indicates that the cell is committed towards an irreversible apoptotic cell death. This experiment has already been done in my previous study to show that Phyllanthus induces apoptosis in cancer cells (Lee et al., 2011, PLoS ONE).

16. Since in this study, p53 is barely detected and the authors did not observe any changes in p53 after treatment, it does not make sense to discuss the role of p53 in A549 cells.

Response: We thank you for your comment and we have already removed the entire paragraph on the discussion of p53 role in A549 cells in the Discussion section.

17. Page 28, Conclusions: The authors mentioned in vivo study using very high dose of Phyllanthus extracts. Is it based on their unpublished data? It will be interesting to see if they can show some mechanistic data in vivo.

Response: The preliminary data on the in vivo study is indeed unpublished yet and the high dose stated (50g/kg) is a dosage that will cause 100% acute toxicity in the Balb/c mice. In addition, we also observed that Phyllanthus has a maximal non-toxic dose of 10g/kg.
Since this study is still at a preliminary stage, we were unable to disclose any further these mechanistic data in vivo.

18. It is recommended to re-organize DISCUSSION section, regarding proteomic data.
   
   **Response:** Thank you for your suggestion and we have edited some of the discussions to make it clearer.

**Minor comments:**

1. It is recommended that the authors pay attention to correct spelling mistakes and grammar throughout the paper. Non-standard, conversational language used throughout the text should be limited. Consistency in terminology is also necessary, i.e. “Cignal Finder Cancer” or “cignal finder cancer”.
   
   **Response:** We apologize for these mistakes and we have already requested for help from native-English speakers to correct and edit the English language of this manuscript. We have also made sure the terminology used are consistent throughout the manuscript.

2. The authors should sharpen BACKGROUND section in order to stay focused. Several sentences are not necessary, such as “Worldwide, cancer remain …… women” in page 3, first sentence.
   
   **Response:** The sentence “Worldwide, cancer remains a major public health problem with lung cancer ranked as the main cause of mortality due to cancer in both men and women” has been removed as suggested.

3. Page 4, Line 7: Please spell out “ENO, CEA, SCC, CA-125 or TPA”.
   
   **Response:** “ENO, CEA, SCC, CA-125 or TPA” have been spelled out in the Background section (Lines 75-76).

4. Page 7, Second paragraph: Since only one cell line was used in this study, the first sentence sounds very odd. A549 cells are a model cell line to study cancer biology; thus, it is not necessary to describe the development of A549 cells.
   
   **Response:** The first sentence was edited to “Human lung carcinoma (A549) cell line was purchased from American Type Culture Collection (ATCC, USA) and was grown in RPMI-1640 (Roswell Park Memorial Institute)” (Lines 146-147) and the description for development of A549 cells has been removed as suggested.
5. Page 9, First paragraph: First sentence sounds odd.

Response: The first sentence in that paragraph has been edited to become “DNA content analysis is performed by Propidium iodide (PI) staining which binds to the distinct amount of DNA content in the cells at different phases of the cell cycle” (Lines 176-177).

6. Page 11-12, Immunoassay: It is recommended to simply state “following the manufacturer’s instructions” or provide details in blocking buffer, substrates F1 and F2, etc.

Response: The brief description for the immunoassay methodology has been removed as recommended and is replaced by the sentence “In order to measure the total iNOS in whole cell, a cell-based ELISA, Human Total iNOS Immunoassay (R&D Systems, USA) was performed according to the manufacturer’s instructions” (Lines 243-245).

7. Page 15, Line 8-10: The statement “…..suggesting their major role to ensure their growth and survival” is too general and does not mean anything. It is recommended to delete it.

Response: The sentence “Among the 10 pathways, NFκB, Myc/Max, Hypoxia, and MAPK (ERK and JNK) pathways were highly expressed in untreated A549 as compared to the other pathways, therefore suggesting their major role to ensure their growth and survival” has been deleted as recommended.

8. Page 15, Last paragraph: It is recommended to delete the first sentence (Other pathways…….. in both the treated and untreated cells)

Response: The sentence “Other pathways that could possibly have a role are Wnt, Notch, p53/DNA damage, TGFβ as well as cell cycle/pRb-E2F as they were lowly expressed in both the treated and untreated cells” has also been deleted as recommended.
Editorial comments
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Response: We thank you for your comment as well as the discounted offer for English-editing service by Edanz. We will try our best to improve the English language of this manuscript as recommended. Hence, we have requested for help from our native-English speaking colleagues to read and edit the language of this manuscript and hope that you will find it satisfactory.