Reviewer’s report

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

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Reviewer: Pei-Li Yao

Reviewer’s report:

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.

2. 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 tetragalloylglucopyranoside which is not present in aqueous extracts (Lee, S.H. et al., 2011, PLoS ONE). 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.

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.

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.

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).

6. Figure 1: How to determine the significance between non-treated and treated groups since the data include different extracts from four Phyllathus 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.

7. The authors should explain the reason to use cisplatin and doxorubicin as positive controls in METHODS section.

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).

9. It is recommended to remove Figure 3.

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.

11. 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? What about JNK1/2 expression?

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

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

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

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.

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.

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.

18. It is recommended to re-organize DISCUSSION section, regarding proteomic data.

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”.

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.

3. Page 4, Line 7: Please spell out “ENO, CEA, SCC, CA-125 or TPA”.

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.

5. Page 9, First paragraph: First sentence sounds odd.

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.

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.

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

Level of interest: An article of limited interest

Quality of written English: Needs some language corrections before being published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declared that I have no competing interests.