Reviewer's report

Title: Flavones inhibit breast cancer proliferation through the Akt/FOXO3a signaling pathway

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Reviewer: Wangzhi Li

Reviewer's report:

The manuscript set to evaluate the effects of flavones on breast cancer proliferation and migration as well as the underlying signal pathways. The results would be of interest to the community. However, the qualities of many of the data need improvement and many of the interpretations the authors made on the current data are premature. Further clarification and verification are needed before making the conclusions. Languages are also still require improvement. Specific comments are below:

I. Concerns with data results and interpretation:

Figure 1:
1A-C: how many repeats of the MTS experiments? How is IC50 calculated?
1D: how many repeats of the colony formation assay?

Figure 2:
2A-C: Effects of Flavone seems different from Apigenin and Luteolin, also the effects of Flavone, Apigenin and Luteolin seem varied in different cell lines. It's not clear if the variations are due to technical variations or something real. Only consistence is an increase of apoptosis subpopulation. However, the authors should show original flow cytometry graphs in Supplements to verify the sub-G1 apoptosis peaks.

Also, how many technical replicates used for each sample during each flow cytometry cell cycle measurement? How big is the variability among the technical replicates?

Also, instead of pair-wised T-test of each peak/phase subpopulation vs control, the authors could also perform Chi-Square or other suitable statistical comparisons to see if the overall distribution profiles of the samples are significantly different.

2D: Western blot results seem not consistent with cell cycle results in 2A. If cell cycle results in 2A are correct, then MCF-7 cells treated by Apigenin and Luteolin seem to have normal G1 phase, but have more cells in S phase and less cells in G2-M phase. Thus, it would suggest normal Cyclin D/CDK4&6 and Cyclin E/CDK2 activity (required for G1-S progression), and deficient Cyclin A/CDK2 activity (required for S-G2 progression). However, the results in 2D showed huge
deficiency of Cyclin D1 at 24 hrs, why? Also, the authors didn’t show results for Cyclin A and E, as well as any CDKs. Why? Similarly, the results for Flavone treated cells are also not consistent with cell cycle results in 2A.

Also, cells are only treated by the compounds for 24 hrs, but the results showed cells continued to deteriorate from 24 hr to 48 hr in Cyclin D1 and Cyclin B expression. The authors need explain or discuss why the cells continue to deteriorate, instead of somewhat recover, after removing the compounds?

In addition, why the authors didn’t repeat the experiment on Hs578T and MDA-MB-231 cells? How many repeats were done for the Western blot experiments?

2E: Although the authors did cell cycle flow cytometry which can confirm the apoptosis, TUNEL assay would be preferred over Hoechst 33342 staining. Also, quantification should be shown for the apoptosis assays.

2F: How many repeats the Western blot was done? Also, given these blots are for cleaved PARP and p53, the authors also need to show to original blots with molecular weight markers labeled to show the identity of the bands, better with positive controls.

Figure 3:
3A-B: how is it quantified? Flavone picture in A doesn’t seem too much difference from control, but shown 60% reduction in B. Also, Figure 2A and 2E showed MCF-7 cells treated with the compounds for 24 hrs already show significant apoptosis, however, Figure 3A seem to show equal, if not better, cell proliferation for MCF-7 cells treated with compounds for 24 hrs compared to the control. Why?

3C: results seem inconsistent with 3A. Flavone seems to show the least effect In 3A, but the highest effect in 3C. The authors need explain/discuss the discrepancy. Also, the cell index value at each time points were averaged from three different wells or just simply three measurements of the same well?

Figure 4:
4B and 4D: The results seem inconsistent with the authors' interpretation. In 4B, Foxo3a protein levels boosted as early as 6 hours after compound treatment. However, Akt changes were not evident until mostly until 24 hours. If the Foxo3a changes are directed by Akt, Akt changes should precede Foxo3a changes, but it’s opposite, as Akt changes are much behind Foxo3a changes.

Also, Foxo3a levels increased in both cytosol and nucleus, so there is not sufficient evidence to indicate increased nuclear translocation of cytosol Foxo3a, as the authors interpreted. The authors need use other methods, e.g. label Foxo3a and do a real-time imaging experiment, to check that.

For Akt, 4D showed that total Akt protein levels appear to decrease around 24 hrs after treatment. Ser-473p-Akt levels also seem to decrease around 24 hrs
after treatment (except in Apigenin cells). Thus, it’s not clear if the decrease of Ser-473p-Akt is due to decrease of total Akt or also due to decreased phosphorylation ratio. The authors’ interpretations are premature and need do more experiment to clarify. At least, the authors should calculate the ratio of Ser-473p-Akt: Akt from the two blots.

PCNA may not be a good loading control for nucleus fraction in this case, given the cell cycle of these cells are affected by the treatment. Also, the authors also should provide results in Supplements to verify the quality/purity of cytosol and nucleus fractions with both positive and negative markers for cytosol and nucleus. Also, how many times these Western blot experiments were repeated?

Figure 5: Magnitudes of mRNA expression changes seem not consistent with protein level changes. mRNA expression of p27 and p21 in Luteolin treated cells are barely increased (5A), but their protein expression were greatly increased (5B, 5D).

Similarly, PCNA may not be a good loading control for nucleus fraction in this case, given the cell cycle of these cells are affected by the treatment. Also, the authors also should provide results in Supplements to verify the quality/purity of cytosol and nucleus fractions with both positive and negative markers for cytosol and nucleus. Also, how many times these Western blot experiments were repeated?

II. Concerns with writing:

Line 48-52: p53 is upstream of p21, to my knowledge, it’s not shown that p21 and/or p27 could increase p53

Line 86-90: please list references

Line 90-92: why suddenly hypothesize “that flavone, apigenin, and luteolin may increase the expression of FOXO3a in breast cancer cells.” Until Line 92, the Introduction hasn’t indicated any connection between the two, then authors suddenly stated this baseless hypothesis, need provide more reasoning and/or evidence

Line 101-103: need references

Line 105: should be “these” compounds

Many more to name..

**Level of interest:** An article whose findings are important to those with closely related research interests

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

**Statistical review:** Yes, but I do not feel adequately qualified to assess the
statistics.

Declaration of competing interests:
I declare that I have no competing interests