Author's response to reviews

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

Authors:

Chia-Hung Lin (james07212001@yahoo.com.tw)
Ching-Yao Chang (cychang@asia.edu.tw)
Kuan-Rong Lee (krlee@mx.nthu.edu.tw)
Hui-Ju Lin (d2396@mail.cmu.edu.tw)
Ter-Hsin Chen (thc@dragon.nchu.edu.tw)
Lei Wan (leiwan@mail.cmu.edu.tw)

Version: 4 Date: 14 September 2015

Author's response to reviews: see over
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Version: 2 Date: 14 Sep 2015
Author’s response to reviews: see over
1. The first paragraph in Background is too superficial for readers in cancer field.
   We have reorganized and added some in-depth information on breast cancer as well as FOXO3a. The added words are marked in red words in the manuscript and are listed below:

   Paragraph 1, line 3-7
   Despite the new promising breakthrough in therapeutics, the annual breast cancer mortality rate continues to increase, and one million new cases are diagnosed every year. Numerous risk factors for breast cancer etiology have been identified, including genetic, gender, age, alcohol consumption, smoking and obesity.

   Paragraph 2, line 1-5
   Breast cancer develops as a consequence of cellular changes that increase the rate of cell division and metastasis, decrease the rate of apoptosis, or both. These changes often involve dysregulation of key signal transduction pathways within the cell that transmit extracellular signals to transcription factors, resulting in changes in gene expression.

   Paragraph 2, line 7-9
   Forkhead box O3 (FOXO3a), a downstream target of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, belongs to a family of transcription factors that are characterized by a distinct forkhead DNA-binding domain.

   Paragraph 2, line 22-24
   Several anti-cancer drugs have been shown to increase the expression of FOXO3a, which suggest it is a tangible therapeutic target for breast cancer therapy.
Surgical resection, radiation therapy, and chemotherapy are among the main treatment options for breast cancer patient. In addition, there is growing need to discovery new chemopreventive agents that are effective in preventing/treating breast cancer.

2. The second paragraph in Background introduced the signaling pathway of AKT/FOXO3a but at the end suddenly jumped to their hypothesis of flavone, apigenin and luteolin may increase the expression of FOXO3a. I just do not follow the logic reasoning here.
   Please refer to question 1.

3. The colony formation experiment lasted for 21 days. I was wondering how stable the compounds are?
   The colony formation assay is an assay to test every cell in the population for its unlimited growth ability. After treated with cytotoxic agent, only a fraction of cells retained the capacity to produce colonies. We did not have the data on the stability of the compounds in the media. However, we did add compounds at days 7 and 14 to maintain the compound concentrations.

4. In Fig 2F, both total PARP and cleaved PARP should be shown. Also, positive control (eg. puromycin treatment) should be added. A major flaw is that even MCF-7 bear WT p53 loci, Hs578 and MDA-231 cells have mutant p53. In other words, high level of p53 in these two cell lines does not mean p53 is activated. The authors should show that p53 is truely activated if they argue that the apoptosis is induced via p53.
   A positive control indicates that drug treatment with a known response, so that this positive response can be compared to the unknown response of the treatment. Previous studies indicated flavonoids could inhibit the growth of tumor cells by inducing apoptosis.
As shown in these references, flavonoids inhibited cell growth through inducing apoptosis by showing PARP cleavage. Accordingly, we do not think a positive control as well as total PARP should include in this manuscript.

In most cancers, p53 is either lost of function or mutated to allow cancer cells to expand and progress.

Recent reports raised the possibility to suppress tumor growth by restoring wild-type p53 to cancer cells

Our results indicated the flavone, apigenin and luteolin restoring the function of WT p53 in MCF-7 cells. In Hs578 and MDA-231 cells, p21 and p27 activation were probably through the p53 independent pathways.


3. T. L. Chiu, et al., Curcumin inhibits proliferation and migration by increasing the Bax to Bcl-2 ratio and decreasing NF-κBp65 expression in breast cancer
Another question is: Did these compounds show specific toxicity only in breast cancer cells? In other words, if the authors believe these compounds are potent drug candidates, they should show that these compounds are much less toxic in normal cells eg MCF-10A or HMEC.

Recently, natural plant extracts and compounds have received widespread attention because of their potential beneficial effects on human health. Natural compounds provide a wide array of potential drug candidates for cancer therapy with various roles and targets. Accumulating evidence indicates that flavones of natural origin induce apoptosis and inhibiting proliferation in multiple tumor cell lines including A549 lung cancer cells (1), hepatoma HepG2 cells (2), ovarian cancer cells (3), prostate cancer cells (4) and gastric cancer cells (5).

3. H. Luo, et al., Inhibition of Cell Growth and VEGF Expression in Ovarian Cancer Cells by Flavonoids, Nutrition and Cancer, 60(6), 800-809, 2008.

Flavone, apigenin and luteolin showed low cytotoxicities against MCF-10A cells compared with MCF-7, Hs578t and MDA-MB-231 cells. The IC$_{50}$ results are shown in Table 1.
6. I was also wondering if the authors know whether these compounds suppress Akt activity via direct binding or indirectly eg via PTEN or PI3K. Also, the authors should show more stringent experiment that these compounds specifically target Akt/FOXO3a.

Activated PI3K and Akt are considered the focal point of a survival pathway known to protect cells from apoptosis by several stimuli. Our results shows that treatment of MCF-7 cells with flavone, apigenin and luteolin resulted in downregulation of PI3K pathway, due to a decrease in PI3Kp85 subunit expression. Furthermore, we found that in the MCF-7 had a decreasing level of total PI3K and phosphorylated
PI3K treated with flavone, apigenin and luteolin, whereas its downstream effector phosphorylated Akt was inhibited.

**Level of interest:** An article of limited interest

**Quality of written English:** Acceptable

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

**Declaration of competing interests:** I declare that I have no competing interests.
Reviewer's report

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

Version: 3 Date: 10 July 2015

Reviewer: Zhanwei wang

Reviewer’s report:

Major Compulsory Revisions This manuscript has been improved a lot with revision according to the reviewers’ comments. I think there are still some issues to be addressed.

1. In this study, three of breast cancer cell lines, Hs578T, MDMB-231, and MCF-7 were used. Could the authors explain the reason?

There are significant differences between these cells. Hs578T and MDA-MB-231 are both basal-like breast cancer cell lines, which are both ER, and HER2 negative. MCF-7 is luminal type breast cancer cell and ER and HER2 positive. Since flavonoids are considered as phytoestrogens, we tempt to understand whether the cytotoxic activities are through ER and the possibility of through inhibiting HER2 activity. According to our results, ER and HER2 were not played roles in the cytotoxic effect of flavone, apigenin and luteolin. There are no significant differences in the IC$_{50}$ between different cell lines. We are seeking the possibility of common pathways of these compounds against breast cancers.

2. In the Fig 5B and 5D, loading controls are labeled as #-actin and PCNA, respectively, but the bands are actually the same.

These are typos. The corrected figures are shown below.
3. In all figures, the authors showed the significant difference with p<0.05 instead of the exact number. I am wonder whether there are some p values with much less than 0.05, such as 0.001. If it dose, I think it is better to present them in the figure legend and figure. We have modified the p values accordingly. The p values are divided into three different groups which are $P < 0.05$ (*), $P < 0.01$ (#), $P < 0.001$ (□) compared with control.
Figure 1. Effects of flavone, apigenin and luteolin on cell viability of MCF-7, Hs578T and MDA-MB-231 breast cancer cells. (A) MCF-7, (B) Hs578T, and (C) MDA-MB-231 cells were cultured in 96-well plates and treated with varying concentration of flavone, apigenin and luteolin (12.5 - 100 µM) for 72 h as indicated. Cell viability was assessed with a MTS/PMS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium, inner salt)/phenazine methosulfate) assay. (D) Effects of treatment with IC_{50} concentrations (Table 1) of flavone, apigenin, and luteolin on for 21 d on colony formation in MCF-7 cells. (E) Quantification of colony numbers from colony-forming assays of untreated MCF-7 cells (control) and cells treated with flavone, apigenin, and luteolin. Results are the mean ± standard deviation of three independent experiments. *: P < 0.05; #: P < 0.01; #: P < 0.001.
Figure 2. Flavone, apigenin, and luteolin induced cell cycle arrest and apoptosis in human breast cancer cells. (A) MCF-7, (B) Hs578T, and (C) MDA-MB-231 cells were treated with the IC$_{50}$ concentrations (Table 1) of flavone, apigenin, and luteolin for 24 h prior to cell cycle analysis by propidium iodide staining. The percentage of cells in each phase of the cell cycle (sub G1, G0/G1, G2/M and S) is indicated. (D) The effects of flavone, apigenin, and luteolin on cyclin B and cyclin D1 protein expression. Western blot analyses were performed on cell lysates form MCF-7 cells treated with the IC$_{50}$ concentrations (Table 1) of flavone, apigenin, and luteolin for 24 h. (E) Flavone, apigenin, and luteolin induced apoptosis in MCF-7, Hs578T and MDA-MB-231 breast cancer cells as detected by Hoechst 33342 staining. The breast cancer cells were treated with the IC$_{50}$ concentrations (Table 1) of flavone, apigenin, and luteolin. (F) Quantification of Hoechst 33342 staining of untreated MCF-7, Hs578T and MDA-MB-231 cells (control) and cells treated with flavone, apigenin, and luteolin. (G) Western blot analyses for cleaved-PARP, tumor p53, and cytochrome c in MCF-7 cells treated with the IC$_{50}$ concentrations (Table 1) of the flavone, apigenin and luteolin from 24 h. Results are the mean ± standard deviation of three independent experiments. $P < 0.05$ is considered as statistically significant. Symbols: *: $P < 0.05$; #: $P < 0.01$; #: $P < 0.001$. 
Flavone, apigenin and luteolin inhibited cell motility. (A) Representative images showing wound healing assays for cells treated with flavone (88 µM), apigenin (30 µM) or luteolin (43 µM) and an untreated control for 24 h. (B) Average number of cells that had migrated after 24 h. (C) Effects of the flavone, apigenin, and luteolin on MCF-7 cells migration. MCF-7 cells were treated with the IC₅₀ concentrations (Table 1) of flavone, apigenin, and luteolin, and the real-time migration of the cells was measured using an xCELLigence system. The value of the open area at 0 h is 100%. Results are the mean ± standard deviation of three independent experiments. *: P < 0.05; #: P < 0.01; □: P < 0.001.
Figure 4. Flavone, apigenin, and luteolin activate FOXO3a, which is associated with a change in the signal transduction pathway. (A) Real-time PCR for FOXO3a. HS578T, MDA-MB-231 and MCF-7 cells were treated with the IC$_{50}$ concentrations (Table 1) of flavone, apigenin, and luteolin for 0 h (control) and 24 h. (B) Western blot analyses of total FOXO3a in cytoplasmic and nuclear extracts isolated from MCF-7 cells treated with the IC$_{50}$ concentrations (Table 1) of flavone, apigenin, and luteolin for various time from 0 to 48 h. Loading controls: cytoplasmic, β-actin; nuclear, proliferating cell nuclear antigen (PCNA). (C) Densitometric quantification of the FOXO3a expression from the western blot analyses. (D) MCF-7 cells were treated with IC$_{50}$ concentrations (Table 1) of flavone, apigenin, and luteolin for 0-48 h. Western blot analyses of the total protein kinase B (Akt) and Ser-473-phosphorylated Akt (p-Akt) in cytoplasmic and nuclear extracts isolated from MCF-7 cells treated with the IC$_{50}$ concentrations (Table 1) of flavone, apigenin, and luteolin for 0-48 h. Loading control: β-actin. (E) Densitometric quantification of the Akt and Ser-473-phosphorylated Akt expression from the western blot analyses. Results are the mean ± standard deviation of three independent experiments. $P < 0.05$ is considered as statistically significant. Symbols: *: $P < 0.05$; #: $P < 0.01$; □: $P <$
Figure 5. Flavone, apigenin and luteolin regulate the expression of the cyclin-dependent kinase inhibitors p21 and p27 through the Akt-FOXO3a signaling axis. (A) Real-time PCR for p21 and p27 expression in untreated MCF-7 cells (control) and cell treated with the IC50 concentrations (Table 1) of flavone, apigenin, and luteolin for 0 and 24 h. (B) Western blot analyses of total p21 in cytoplasmic and nuclear extracts isolated from MCF-7 cells treated with the IC50 concentrations (Table 1) of flavone, apigenin, and luteolin from 0-48 h. (C) Densitometric quantification of the p21 expression from the western blot analyses. (D) Western blot analyses of total p27 in cytoplasmic and nuclear extracts isolated from MCF-7 cells treated with the IC50 concentrations (Table 1) of flavone, apigenin and luteolin for 0-48 h. (E) Densitometric quantification of p27 expression from western blot analyses. Loading control (B and C): cytoplasmic, β-actin: nuclear proliferating cell nuclear antigen (PCNA). Results are the mean ± standard deviation of three independent experiments.
\( P < 0.05 \) is considered as statistically significant. Symbols: *: \( P < 0.05 \); #: \( P < 0.01 \); \( \square \): \( P < 0.001 \).

4. **In the line 159, “Softeare” should be Software.**
   
   We have corrected the typo.

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

**Quality of written English:** Acceptable

**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
Reviewer's report
Title: Flavones inhibit breast cancer proliferation through the Akt/FOXO3a signaling pathway

Version: 3 Date: 12 July 2015

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?
We did three times MTS assay.

For example, cells are treated with 0, 12.5, 25, 50 and 100 μM flavone and their respective growth inhibition percentages are 0, 28, 60, 72 and 82 %. The 50% of inhibition should fall between the doses 12.5 and 25 μM. By using Microsoft EXCEL program, we use the following formula to calculate IC50. FORECAST(50,log25(Concentration):log12.5(Concentration), 60(Inhibition%):28(Inhibition%).
The IC50 value is 20 μM in this experiment.

We did three times 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.

The original flow cytometry graphs are listed below.
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?

We have at least three different technical replicates for each experiment. Different technical replicates reveal the same trend of cell cycle distributions.

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. The results were analyzed by Chi-Square statistical comparisons to see the overall distribution profiles of the samples. The results are listed in the following table.

<table>
<thead>
<tr>
<th>Breast cancer cell lines</th>
<th>Type of Flavones</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Flavone</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>Apigenin</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>Luteolin</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Hs578T</td>
<td>Flavone</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>Apigenin</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>Luteolin</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Flavone</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>Apigenin</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>Luteolin</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

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?

Cyclin D1 is accumulated during G1 phase and is degraded by ubiquitin-mediated proteolysis pathway as the cell enter S phase.


Since MCF-7 cells were arrested in S phase, it is reasonable that we observed the down-regulation of cyclin D1. For cyclin E, which highly expressed during G1-S transition, degrades throughout S phase and at the lowest level at the end of S phase.


As shown in the figure below, we observed significantly down-regulation of cyclin E when MCF-7 cells treated with luteolin, apigenin and flavone. Cyclin A is associated with CDK1 at the end of S phase into G2 phase and then it is replaced by cyclin B.


Therefore, without functional cyclin B, cells may accumulate in S or G2 phase. Flavone, apigenin and luteolin caused a dose-dependent cancer cell growth inhibition, and this antiproliferative effect appears to be due to its ability to induce G2-M and S-phase arrest. To further characterize the effects of flavone, apigenin and luteolin, we examined by Western blot analysis the levels of Cyclin B, Cyclin D1 and Cyclin A after treating MCF-7 cells with flavone, apigenin and luteolin for 24 and 48h. We found that cyclin B expression levels were decreased after 48 h of treatment. Cyclin D1 expression decreased within 24 h of treatment. In addition, we did not find significant differences in the expression level of cyclin A, but significantly down-regulated cyclin E2 (Figure below).
The effects of flavonoids on cell cycle control proteins have been studied previously. A human promyelocytic cell, HL60, was treated with resveratrol and found S phase arrest with the decrease expression level of cyclin A, B1 and cyclin D.


In U937 lymphoma cells, resveratrol increased the levels of cyclin E, cyclin A, and cyclin D3 whereas decreased the level of cdk2, and did not affect the levels of cyclin B1, although the cells were arrested in S phase too.


Therefore, the effects of flavonoids on the expression of cell cycle control proteins appear to vary considerably between cell systems.

There are significant differences between these cells. Hs578T and MDA-MB-231 are both basal-like breast cancer cell lines, which are both ER, and HER2 negative. MCF-7 is luminal type breast cancer cell and ER and HER2 positive. Since flavonoids are considered as phytoestrogens, we tempted to understand whether the cytotoxic activities are through ER and the possibility of through inhibiting HER2 activity. According to our results, ER and HER2 were not played roles in the cytotoxic effect of flavone, apigenin and luteolin. There are no significant differences in the IC\textsubscript{50} between different cell lines. We are seeking the possibility of common pathways of these compounds against breast cancers. There are about 20% of breast cancers are HER2-positive. Younger women are more likely to be HER2-positive than older
women. HER2-positive breast cancer tends to be more aggressive and to spread more quickly than other cancers. Therefore, we mainly focused on MCF-7 cells.

**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.

There are several references using Hoechst dye to detect apoptosis.

4. J. Peng, et al., Calcium phosphate-uracil, a synthesized pyrimidine derivative agent, has anti-proliferative, pro-apoptotic and anti-invasion effects on multiple tumor cell lines, Molecular Medicine Reports, 10(5), 2271-2278, 2014.

The quantitation of apoptosis has been shown in following figure (Figure 2F).

![Figure 2F](image-url)  

**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.

We did three times western blot.

The original blot for cleaved PARP was showed in the following figure.

The original blot for p53 was showed in the following figure.

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?

We used the Image J plugin to quantified wound healing assay. We once again quantified data. We obtain similar results.
Apoptosis was analyzed by Hoechst 33342 staining. The MCF-7, Hs578T, and MDA-MB-231 cells were seeded in 6-well plates (2.1 × 10^4 cells/cm^2) and treated with IC_{50} concentrations of flavone, apigenin and luteolin for 24 h. For cell cycle analysis, cells were seeded in a 10 cm dish (9 × 10^3 cells/cm^2) and allowed to adhere overnight. Cells were then treated with IC_{50} concentrations of flavone, apigenin and luteolin for 24 h. Cell migration was determined using the culture inserts. Cells were seeded plated into each well of the culture-inserts (15.9 × 10^4 cells/cm^2). It is because of the cell density that causes the differential responses to the flavonoids.

The cell indexes are from three independent wells. The cell numbers seeded in CIM-plate 16 are 7.5 × 10^4 cells/well, which indicate for 2.3 × 10^5 cells/cm^2. Although we use the same concentration of flavone, apigenin and luteolin as in the wound-healing assay, the different in cell density may cause altered results. The CIM-plate using an electrode sensor array to detect the cells migrate through the microporous membrane, which contains thousands electrode underside of the membrane. When cells attached to the electrode, it will cause changes in impedance that is directly correlated with the number of migrated/attached cells. Flavone, apigenin and luteolin may have different effect on the attachment ability to the electrode, which cause different responses compared with wound-healing assay.

**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.

Our results indicate that the protein expression levels of Akt were down-regulated with the treatment of flavone, apigenin and luteolin and also inhibit the phosphorylation of Akt at Ser473. Activated (phosphorylated) Akt promote the phosphorylation of FOXO3a, which in turn degraded. Our results showed that flavone, apigenin and luteolin treatment substantially suppressed Akt activation at 6h, which is consistent with the results. The effect of flavone, apigenin and luteolin were take effect gradually, which cause the significantly accumulation of FOXO3a at 6h. Moreover, there are ERK and IKKβ signaling pathway regulated FOXO3a expression.

We will conduct a more detailed study on the signaling pathway in the near future.

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.

In our results section, we did not mention that increased FOXO3a in the nucleus was due to enhance nuclear translocation. It is only mentioned in the Discussion section, which indicate that according to previous studies the increase in the FOXO3a in the nucleus may due to increase nuclear translocation.

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 (expect 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.

The absolute ratio of p-Akt to Akt does not always reflect the actual activation of Akt. For example, if the level of total Akt and p-Akt down-regulated by a factor of 2, the ratio of p-Akt to Akt would suggest that the actual level of Akt activation would remain the same, but the level of p-Akt was in reality reduced.

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?

We did three times western blot experiments. We used GAPDH (cytoplasmic protein marker) and Lamin A/C (nuclear protein marker) antibodies to verify the purity of cytosol and nucleus fractions.
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). There is a possibility of feedback mechanism in this case and also the half-life of protein seems to be more. When RNA levels increase it is corresponding to an increase in protein levels however the feedback loop inhibits further transcription. This subsequently leads to reduction in RNA level while the protein is maintaining itself at higher amount. Proteins perform a vast array of functions within living organisms, including catalyzing metabolic reactions, DNA replication, responding to stimuli, and transporting molecules from one location to another. The expression level of mRNA and protein levels may not always the same. We did found significant correlation in the mRNA and protein expression levels of p21 and p27.

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?

We did three times western blot experiments. Please refer to question Figure 4.

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

We have corrected the errors.

In Abstract, line 21-24.
This subsequently elevated the expression of FOXO3a target genes, including the Cyclin-dependent kinase inhibitors p21\(^{Cip1}\) (p21) and p27\(^{kip1}\) (p27), which increased the levels of activated poly(ADP) polymerase (PARP) and cytochrome c.

**Line 86-90: please list references**

In Background, Paragraph 2, line 19 and 21.

We have listed 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

We have corrected the errors.

In Background, Paragraph 2, line 21-24.

Moreover, FOXO3a is an important tumor suppressor that is underexpressed in many breast cancers. Several anti-cancer drugs have been shown to increase the expression of FOXO3a, which suggest it is a tangible therapeutic target for breast cancer therapy.

**Line 101-103: need references**

In Background, Paragraph 3, line 14.

We have listed references.


**Line 105: should be “these” compounds**
We have corrected the errors.
In Background, Paragraph 3, line 15-17.
Although different mechanisms and signaling pathways have been proposed as targets of flavone, apigenin, and luteolin, these compounds were studied individually and occasionally by using different model cancer cells.

Many more to name.