Author's response to reviews

Title: Kalanchoe tubiflora extract inhibits cell proliferation by affecting the mitotic apparatus.

Authors:

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Version: 2 Date: 29 June 2012

Author's response to reviews: see over
Dear Professor Rowles:

We enclose the revised manuscript “Kalanchoe tubiflora extract inhibits cell proliferation by affecting the mitotic apparatus” for your consideration. The work is original, has not been published in whole or in part elsewhere, and has not been submitted for publication elsewhere except as a brief abstract in the proceedings of a scientific meeting or symposium. All authors have contributed significantly, and all authors are in agreement with the content of the manuscript.

We are gratified and flattered by all three reviewers’ positive statements that this article is well written and is important to related field. Overall, we have revised our paper according to the suggestions made by all three reviewers: (1) change the manuscript title and removed the phrase “mitotic catastrophe” in the text according to reviewer’s concerns; (2) introduce more information about possible mechanisms that may involved in the action of KT-NB; (3) add more labels in figures 3 and 4; (4) How KT-NB toxicity may be used to target cancers specifically is described in last paragraph of the discuss section. A detailed “point-by-point” answer was enclosed after this letter.

Since we have complied completely with the suggestions made by the reviewers, we hope that the changes introduced in the manuscript will address the reviewer’s queries satisfactorily.

We look forward to hearing from you in due course,

Chih-Jui Chang
Reviewer's report
Title: Kalanchoe tubiflora extract inhibits cell proliferation by inducing multipolar spindles, leading to mitotic catastrophe
Version: 1 Date: 31 May 2012
Reviewer: Gopal Chakrabarti

Reviewer's report:
The manuscript “Kalanchoe tubiflora extract inhibits cell proliferation by inducing multipolar spindles, leading to mitotic catastrophe” by Hsieh et al. are described anti-cancer property of n-BuOH extract of Kalanchoe tubiflora by loss of cell viability and cell cycle arrest at G2/M phase and formation of multipolar spindle microtubules. Manuscript is well written and contribute in field of mechanism of anticancer property of natural plant derived compounds through disruption of microtubule network which is one of the important targets for anti-tumor therapy. Some comments below

Major Comments:
1) The IC50 for KT-NB of A549 is 1.5625 μg/ml for 72 h incubation. Then how come it is possible for A549 cells to remain almost in unaltered morphology (cells shown in the migration wounding healing assay) after 72 h incubation at as high dose as 50.0 μg/ml. As KT-NB has effect on microtubule network, it would interested to see effect of KT-NB on cell morphology of A549 cells and effect of interphase microtubule structure. The authors experiemntal evidence mechanism of cell death by KT-NB, e.g., Apoptosis and some apoptosis protein markers.

Response:
(I) In most of the experiments, dosage of drug treatment is based on the drug concentration in the culture medium. However, the cell number in different assays are different. IC50 for KT-NB of A549 was performed in 96-well plates. The wounding healing assay was performed in 6-well plates. According to our experience, comparison of experimental results can only be done when experiments are performed in the same culture conditions (same kind of culture plates, same number of cells seeded, same drug concentrations). Based on the immunofluorescent images, the morphology of interphase cells treated with KT-NB are normal compared to the control cells. Multipolar spindle cells are almost impossible to be observed under low power objects and bright field microscopy used in wound healing assay. This will explain the cell morphology looks quite normal in wound healing assay. To reveal this question, we used higher power objects to exams A549 cells after KT-NB treatment. The morphology of A549 cells were changed after a 72-hour incubation at 50.0
μg/ml of KT-NB. The images are showed below. Considering the length and the structure of this manuscript, we suggest not adding these data in this manuscript. We hope reviewer agree with us. This is only for reviewer's information.

(II) A-549 cells were incubated for 24 and 48 hours with 1.5625 μg/ml, 3.125 μg/ml, 6.25 μg/ml, 12.5 μg/ml and 25 μg/ml of KT-NB, apoptotic cells were evaluated by Annexin V binding and propidium iodide (PI) staining. The number of apoptotic cells was determined by flow cytometric analysis. No significantly early apoptotic cells were observed. The data is showed below. Actually, the fates of defective mitotic cells could be apoptosis, senescence, quiescence and even necrosis. Different cell lines might response different. It also depends on the genetic background of these cells. These will need to be carefully analyzed in the future.

2) Figure 4B is showing ~10-12% (2 fold) increase of cells in the mitotic phase due to treatment, where as figure 5C is showing almost ~ 60% (6 fold increase) cells are showing multipolar spindles for the same treatment. How is it possible?

Response:
In figure 5C, it means that ~60% of the “mitotic phase cells” showed multipolar spindles.
Minor Comments:
1) It is important to write how many gms of dry n-BuOH extract was obtained from what amount of raw plant tissue. What are major compounds in KT-NB?
   Response:
   (I) 53.79 grams of dry n-BuOH extract were obtained from 6638.76 grams of raw plant tissue in the experiment. This information was also added in the materials and methods.
   (II) In the present work, investigation on n-BuOH extract of Kalanchoe tubiflora led to the isolation of 18 compounds, including 5 new flavonoids, 4 known flavonoids, 4 benzenoids, and others (data not shown). We believe that it is more suitable to publish this part of work alone in chemistry journals.

2) In the “KT-BN decreases cell viability” section in “The IC50 of KT-NB in A549 cells IC50 was less than 1.5625μg/ml.” IC50 was written twice.
   Response: It was corrected.

3) Figure 4B and 4C; Y axis is to be marked properly. Only “%” is not sufficient to show the axis in the figures.
   Response: It was improved as advised.
Reviewer's report

Title: Kalanchoe tubiflora extract inhibits cell proliferation by inducing multipolar spindles, leading to mitotic catastrophe

Version: 1 Date: 29 May 2012
Reviewer: Ling-Huei Yih

Reviewer's report:

This manuscript reports interesting findings regarding the cytotoxic and anti-mitotic effects of the BuOH extract of Kalanchoe tubiflora (KT-NB) on cancer cell lines. The major concerns are:

1. In the title, the authors state that “Kalanchoe tubiflora extract inhibits cell proliferation by inducing multipolar spindles, leading to mitotic catastrophe”. However, there is no description regarding how mitotic catastrophe is measured and there is no results describing what mitotic catastrophe is and whether Kalanchoe tubiflora extract induces mitotic catastrophe.

Response:
Mitotic catastrophe is a form of cell death that results from abnormal mitosis and leads to formation of interphase cells with multiple micronuclei. Abnormal mitotic spindle and chromosome misalignment are part of the possibilities leading to mitotic catastrophe. We agree with reviewer’s concern. It is a risk to come to this conclusion. Therefore, we changed our title as” Kalanchoe tubiflora extract inhibits cell proliferation by affecting the mitotic apparatus”.

2. The authors conclude that KT-NB reduced cancer cell viability by exclusively inducing spindle multipolarity and chromosome mis-alignment. The cause-and-effect relationship of spindle multipolarity/chromosome mis-alignment and cell death is not characterized in this study.

Response:

In 2009, David Pellman group used live cell images to study the fate of cells with extra centrosomes. This work was published in Nature. According to their study, most of progeny of multipolar cells died or arrested regardless of whether the cells were mono- or poly-nucleated. However, they did not provide any information of how these cells died. Actually, the fates of defective mitotic cells could be apoptosis, senescence, quiescence and even necrosis. It depends on the genetic background of these cells. The link between defective mitotic cells and fates of these cells is still a big question for cell biologists.

3. The authors have carried out detailed studies showing the cytotoxic effect of KT-NB in a variety of cancer cell lines. Why the BuOH extract is being used for
the study is not justified.

**Response:**
n-BuOH is the abbreviation of n-butanol. We have displaced all this abbreviation with full name in the article. KT-NB means n-butanol extract of KT. It is our responsibility to avoid confusion. We apologized for this misleading.

4. In the “Background” of abstract, the authors state that Kalanchoe tubiflora (KT) is commonly used as a medicinal wound healing agent and that the underlying mechanisms regulating the healing property of KT, including tumor suppression, are unexplored. In the “Methods” of abstract, the authors state the use of scratch-migration assay to address the effect of KT on wound-healing. What is the correlation between KT as a medicinal wound healing agent and tumor suppression and induction of spindle multipolarity? Does scratch migration assay reflect the property of KT as a “medicinal wound healing agent”?

**Response:**
Schmidt C. et.al. (reference 44) investigated twelve plants used in traditional South Brazilian medicine as wounding healing agents. They performed different assays including antibacterial assay, anti-inflammatory assay, elastase assay and Scratch assay. Some plants do have wounding healing activity. However, some plants claimed for wounding healing do not have obviously activity, and KT is one of them. It is unfair to disclaim these plants in traditional usage as wound healing agents since the extraction or isolation conditions are not the same. Also, traditional wound healing treatments actually bases on the whole effects of anti-inflammatory activity, anti-bacterial activity, proliferation activity and apoptotic activity of the medical plants. All we can say is that in our extraction condition of KT, KT-NB inhibits wounding healing (Scratch assay).

5. In the “Results” of abstract, the authors state that “KT-NB inhibits cell proliferation and reduces cell viability by two mechanisms that are exclusively involved with cell division. First by disrupting centrosome integrity and inducing multipolarity; second by disrupting chromosome alignment during metaphase”. Please define “centrosome integrity” and how to measure centrosome integrity. Do the authors mean centrosome numerical integrity? Secondly, this paragraph contains incomplete sentences.

**Response:**
We agree with reviewer’s concern. Localization and patterns of Aurora A and gamma-tubulin were quite normal in KT-NB treated cells. Without further testing a number of essential centrosome components, we can not come to this conclusion.
was misleading by using “centrosome integrity” to describe multipolar spindle (extra centrosomes). Therefore, we removed the phrase“disrupting centrosome integrity” in the results and conclusion sections.

6. In the “Discussion” (page 9, line 2 of the 3rd paragraph) and the “figure legend” (Figure 7B), the authors state that “a few cells managed to form bipolar spindles by clustering centrosomes (Fig. 7B)”. Please describe the method to characterize whether the spindle pole and the ester-like structure are being clustered.

Response:
Centrosome clustering is a phenomena that occurs in polyploid cancer cells (i.e. that have failed cytokinesis and entered a new division cycle). The spatial arrangement of centrosomes was characterised as clustered depending on their proximity. “Centrosome clustering” can be analyzed properly by live cell imaging. In this study, we just tried to describe a phenotype that cells with more than two centrosomes could assemble almost bipolar spindles. To avoid confusing, we omitted the “Centrosome clustering” statement.

7. The results in this study showed that the localization of Aurora A and phospho-histone H3 (serine 10) were not affected in KT-NB-arrested mitotic cells. The authors state in the discussion that KT-NB induces multipolar spindles and mis-aligned chromosomes not by inhibiting Aurora A and B. Please consider adding information regarding other possible mechanisms that may involve in the action mode of KT-NB.

Response: Thanks for the suggestion. We have added three possible mechanisms in the discussion (Plk1, CENP-E, Mps1).

8. The scratch-migration data (Fig. 3) are difficult to follow. It would help to add appropriate quantification data of the wound closure. The effects of KT on cell cycle progression are also difficult to follow by the histograms shown in Figure 4A. It would be clearer to add the quantification data of each cell cycle stage over KT-NB treating time.

Response: Figure 3 and Figure 4A were improved as advised.

9. Figures 1 and 7E contain unexplained abbreviations.

Response: Abbreviations in figure 1 were replaced with full names. Explanations of abbreviations in figure 7E were added in the figure legend.
Reviewer's report
Title: Kalanchoe tubiflora extract inhibits cell proliferation by inducing multipolar spindles, leading to mitotic catastrophe
Version: 1 Date: 12 June 2012
Reviewer: sharon wald krauss
Reviewer's report:
Major Compulsory Revisions
1. Since the major message of this paper is that KT-NB is a promising anti-cancer agent candidate, acting by perturbing accurate mitosis, the authors should present analysis of KT-NB effects in non-cancerous cell lines (if possible, analogous to tumor lines used) to assess if they are relatively more refractory. If they are, this would greatly strengthen this manuscript and the potential for therapeutically attacking cancer cells using KT-NB (and these results should be added to the Abstract). If they are not, then the authors should speculate how KT-NB toxicity may be used to target cancers specifically.
Response:
(I) How KT-NB toxicity may be used to target cancers specifically is described below, and this description is also included in last paragraph of the discuss section. Targeting of mitotic cells is one of the bases of therapies for patients with multiple types of solid tumors. Some antimitotic agents, taxanes or vinca alkaloids, affect both dividing and nondividing cells. An essential characteristic of the ideal new generation of antimitotic agents is that they target proteins required in dividing cells but not in nondividing cells. As cancer cells have fast dividing rate compared to normal cells, these drugs target cancer cells preferentially. Perturbing mitotic apparatus by KT-NB may be used to selectively target cancer cells.
(II) We had analyzed the effects of KT-NB on cell proliferation by treating A-549 lung carcinoma cell line and WI-38 normal female embryonic lung cell line with five different concentrations of KT-NB. After 72 hours of treatment with 10 µg/ml of KT-NB, the growth of the lung carcinoma cell line A-549 was fully inhibited, when WI-38 cells were treated with 50 µg/ml of KT-NB, about 20% of cells still survived. These results showed that KT-NB has low toxicity to normal cells, but it was only a preliminary data in our laboratory. This part of work need to be further carefully analyzed, and this will be combined with mouse model experiments for another manuscript in the future. Therefore, we suggest not adding these data in this manuscript. We hope reviewer agree with us. This is only for reviewer’s information.
2. The authors concluded that “KT-NB inhibits cell proliferation and reduces cell viability”. How has reduced viability been conclusively demonstrated? For instance, decreased MTT assays could result from decreased metabolism and/or cell death. Before making this claim of reduced viability throughout the text, they should consider whether KT-NB treatment results in non-proliferating but viable inactive cells versus cell killing. If there is ambiguity, this should be acknowledged (and the phrase “and cell death” should be omitted from the Conclusion). This distinction is important although, in either case, KT-NB may be a valuable anti-cancer agent.

Response:
It is true that MTT assay can not distinguish decreased metabolism or cell death. It is also ambiguity whether the reduced cell growth rate resulted from cell death or non-proliferating in fig 2A. We agreed with reviewer’s concern. Therefore, we have pointed out this concern in the results (“KT-NB decreases cell viability” section) and removed the phrase “cell death” from the conclusion. Live cell technique will be an ideal approach to answer this question in the future. A good approach was done by David Pellman group. They used live cell images to study the fate of cells with extra centrosomes. This work was published in Nature in 2009. According to their study, most of progeny of multipolar cells died or arrested regardless of whether the cells were mono- or poly-nucleated.

3. Discussion, paragraph 3 “Rather than acting on microtubule dynamics, we found that KT-NB disrupts centrosome integrity and induces multipolar spindles”. How has disruption of centrosome integrity been shown? To draw this conclusion, a number of essential centrosome components would have to be tested. In fact, the authors may have some preliminary evidence regarding this point if they revisit their Aurora A- and gamma-tubulin- stained slides and
examine centrosome immunofluorescent patterns at interphase cells. Do they mean that multipolar spindles are a hallmark of altered centrosome integrity? Multipolar spindles may result from many disfunctions such as multinucleate cells, cytokinesis failure, centrosome splitting, centrosome amplification etc. In the Conclusion, the phrase “disrupting centrosome integrity” should also be omitted.

Response:
Localization and patterns of Aurora A and gamma-tubulin were quite normal in KT-NB treated cells. We agree with reviewer’s suggest. It was misleading by using “centrosome integrity” to describe multipolar spindle (extra centrosomes). In this article, we have omitted the phrase” disrupting centrosome integrity”.

Response:
Localization and patterns of Aurora A and gamma-tubulin were quite normal in KT-NB treated cells. We agree with reviewer’s suggest. It was misleading by using “centrosome integrity” to describe multipolar spindle (extra centrosomes). In this article, we have omitted the phrase” disrupting centrosome integrity”.

4. Discussion, paragraph 5: Modify the statement that the image in Fig 7B shows centrosome clustering. This cannot be concluded without further analysis, eg of centriole numbers. It would be sufficient to say that centrosome clustering can produce bipolar structures.

Response:
Centrosome clustering is a phenomena that occurs in polyploidy cancer cells (i.e. that have failed cytokinesis and entered a new division cycle). The spatial arrangement of centrosomes was characterised as clustered depending on their proximity. “Centrosome clustering” can be analyzed properly by live cell imaging. In this study, we just tried to describe a phenotype that cells with more than two centrosomes could sometimes assemble almost bipolar spindles. We agreed with reviewers’ concerns. To avoid confusing, we omitted the “centrosome clustering” statement.

Minor Essential Revisions
1. Please indicate the units for KT-NB. Micrograms protein?

Response:
It is an extract from plant. Basically, there are almost no proteins in this fraction. However, it is still a mixture of different chemical compounds.

Response:
It is an extract from plant. Basically, there are almost no proteins in this fraction. However, it is still a mixture of different chemical compounds.

2. Methods, Reagents, source/composition of MTT solution

Response:
The information was described in the materials and methods (cell viability assay).

Response:
The information was described in the materials and methods (cell viability assay).

3. Fig 3, describe what lines indicate and how their placement was determined

Response:
Lines in wound healing assay indicated the border of the wounds. Imagines were
processed using Photoshop software. The contrast of the images was adjusted to that the border could be identified without ambiguity. The border lines were determined by eyes.

4. Labels are needed for FACS traces in 4A  
**Response:** revised as advised

5. It would be helpful to place a box or shaded area in Fig 4A to indicate the sub-G1 population quantitated in Fig 4C.  
**Response:** revised as advised

6. For Fig 5, state cells used and KT-NB concentration  
**Response:** Cell line and concentration were added in both text and figure legend.

7. Are data in Fig 7D,E redundant? If so, omit D. If not, please clarify how they differ.  
**Response:** Fig 7D only analyzed metaphase cells. It described the phenotype of misalignment chromosomes in metaphase. In Fig 7E, we analyzed all phases of mitotic cells. This analysis emphasized the high percentage of defective mitotic cells (bipolar spindles with misalignment chromosomes; multipolar spindles) after KT-NB treatment. In “mitotic language”, misalignment chromosome could only be defined in metaphase, in which metaphase plate could be seen. Otherwise, there is always an argument to distinguish prometaphase and chromosome misalignment. We have clarified clearer in the Results section.

8. Results, KT-NB induces multipolar spindles, paragraph 1: “analyzed by DAPI and microtubule staining”.  
**Response:** revised as advised

9. Discussion, paragraph 5: should use comma after taxol, not semicolon  
**Response:** corrected as advised

**Discretionary Revisions**

**Overall response:**

Thanks for the writing advice. We have revised the writing.

Schmidt C. et.al. (reference 44) investigated twelve plants used in traditional South Brazilian medicine as wounding healing agents. They performed different assays including antibacterial assay, anti-inflammatory assay, elastase assay and
Scratch assay. Some plants do have wounding healing activity. However, some plants claimed for wounding healing do not have obviously activity, and KT is one of them. It is unfair to disclaim these plants in traditional usage since the extraction or isolation conditions are not the same. Reviewer’s idea is right. Traditional wound healing treatments actually bases on the whole effects of anti-inflammatory activity, anti-bacterial activity, proliferation activity and apoptotic activity of the medical plants. All we can say is that in our extraction condition of KT, KT-NB inhibits wounding healing (Scratch assay).

Live cell technique is definitely the ultimate approach in the future to answer all of the questions including fates of the multipolar spindles cells, dynamics of microtubules of cells in interphase and mitosis, centrosome integrity and centrosome clustering.