Author’s response to reviews

Title: Neuritogenic effect of standardized extract of Centella asiatica ECa233 on human neuroblastoma cells

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Version: 2 Date: 20 June 2013

Author’s response to reviews: see over
Dear Editor;

Enclosed, please find a revised manuscript entitled “Neuritogenic effect of standardized extract of Centella asiatica ECa233 on human neuroblastoma cells”. We are grateful for all valuable comments and advices that editor and reviewers have given and carefully amended the manuscript accordingly. All corrected contents were labeled in red.

I am pleased to further revise the manuscript if the need has arisen.

Sincerely yours,

Pithi Chanvorachote and Mayuree H. Tantisira

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We are grateful to the reviewers for their valuable comments to improve our manuscript. As suggested, we have conducted additional experiments and corrected some minor mistakes as followed.

Reviewers’ comments:

Reviewer #1:

Reviewer's report:

In the article 'Neurotrophic activity of standardized extract of Centella asiatica ECa233 on human neuroblastoma cells' Tantisira et al. investigates the potential neuritogenic effect of a standardized extract of the plant Centella asiatica using the human neuroblastoma cell line IMR-32. They show that the extract does not affect cell viability but does promote neurite outgrowth. They also provide data indicating that the neuritogenic effect is mediated via MEK/ERK and PI3K/Akt. The article has a well-defined aim and the chosen methods are appropriate. For the most part, methods and results are adequately described. The discussion is well-balanced and conclusions are in general backed up by the presented data. However there are some questions that need to be clarified (see below).
Major compulsory revisions:
1. It is clearly seen from figure 5 that the applied inhibitors statistically significantly decreases the basal level of neurite outgrowth indicating that the inhibitors in the applied concentrations inhibits neurite outgrowth in non-specific manner. This makes the interpretation of the decreases in ECa233-induced neurite outgrowth in the presence of the inhibitors difficult. The decreased neurite outgrowth may reflect a need for PI3K and MEK activity, respectively, but may also just be caused by non-specific inhibitory effects of the inhibitor. Authors must therefore repeat this experiment using the inhibitors in lower concentrations which they can show not affects the basal level of neurite outgrowth. Authors do not have to test the lower concentrations for all concentrations of ECa233. It will be enough to use just one concentration of ECa233.

Response:
*We have conducted the experiment using the lower concentrations of the inhibitors, which exhibited no significant effect on the neurite outgrowth (Figure 5 of the revised manuscript) and it was found that the pre-treatment of the inhibitors could completely abolish the neurite outgrowth stimulating effect of Eca 233.*

Minor essential revisions
2. The term neurotrophic is generally used to describe a molecule or compound that affects the survival and growth of a neuron, thus I find it misleading that the title of the paper refers to the neurotrophic effect of ECa233, when it is only the neuritogenic effect of this compound that is demonstrated. I suggest the authors change neurotrophic to neuritogenic in the title. Likewise authors should in the text refer to the neuritogenic effect of ECa233 when referring to the data obtained in the present paper. If authors wish to conclude that ECa233 has a neurotrophic or neuroprotective effect in vitro they should in my opinion provide data demonstrating that ECa233 promotes the survival of neurons in vitro.

Response: *We have amended the revised manuscript, accordingly.*

3. The authors use the expression IP3K/Akt. This must be a mistake. It is not the IP3K (Inositol-trisphosphate 3-kinase) but the PI3K (Phosphoinositide-3-kinase) that plays a role in activation of Akt. In accordance, the authors used an inhibitor of PI3K (the LY294002). Authors should therefore correct IP3K/Akt to PI3K/Akt.

Response: *We have amended the revised manuscript, accordingly.*
4. The number of independent experiments is stated for the Western blot analyses in the figure legend. For figure 1 the information on number of independent experiments is found in the methods section. The authors should also provide this information for figure 3 and 5, and it would be preferable if the information was presented in a more uniform way.

Response: The additional information for figure 3 and 5 was added in the revised manuscript.

5. It is not possible to see from the description of the neurite outgrowth assay how many cells were actually analyzed for each experimental condition. The text says “cells selected randomly from 3-a4 fields of each well”. If fields here are equal to fields of view, the authors are likely to have analyzed to few cells. Authors should state approximately how many cells were analyzed for each condition in each experiment.

Response: The data of number of cells counted per condition were added in revised manuscript.

6. In the main text authors should remember to include units (µm) for the neurite outgrowth data.

Response: We have amended the units for neurite outgrowth in the revised manuscript.

7. In the last sentence of the section “preparation of tested substances” the concentration of inhibitors are said to be 10 µM/ml. This is confusing – does it mean 10 µmol/ml or 10 µM? If authors mean µmol/ml they should write it like this, as M means mol/l.

Response: Unit of the concentration of inhibitors is µM. We have amended the units for the concentration in the revised manuscript, accordingly.

In addition, we would like to thank the reviewer once again for your additional material in PDF file.

Reviewer #2:
In this manuscript, Wanakhachornkrai and colleagues provided evidence to demonstrate that the standardized extract of Centella asiatica ECa233 have the neurotrophic activity compareble to BDNF. They used the human neuroblastoma IMR-32 as the cell model. Morphologically, they demonstrated that Eca233 promotes neurite outgrowth similar to the effect of BDNF. In terms of mechanisms, they demonstrated that ECa233 promotes neurite
outgrowth through ERK1/2 and Akt signaling pathways. The manuscript is correctly written, however there are some points that need to be addressed to improve the quality of the manuscript.

1. What is the time point of the cell lysates in Figure 4? It was not mentioned when were the cells harvested after the treatment of Eca233? Since the neurite outgrowth assay is performed at 24 and 48 h, the increase of pERK and pAkt levels should be characterized in a time-dependent manner.

Response: The time for cell lysates collection was 6 h after ECa 233 treatment. We do agree with the reviewer that the increase in the phosphorylated proteins should be increased in a time-dependent manner; however, our results indicated that after treatment with the compound for 3 h the phosphorylated protein showed the increased trend; however, only slightly (not significant). The first time for clear detection of such increase was at 6 h after treatment and again later on at 12 and 24 h, there was no significantly change found compared to those of 6 h. These phenomenon may happen because the increase of phosphorylated protein was due to the reaction of the kinase enzymes but not involving with the protein production as the total form of both ERK as well as Akt were steady in all observation.

We are pleased to discuss more with the reviewer regarding this observation or if the reviewer wishes us to included this time-dependent study in the revised manuscript.
Cells were treated with ECa 233 (100 µg/ml) for 0, 6, 12, 24 h and the named proteins were evaluated by western blotting. The densitometry was used to quantify the level of each protein related to the non-treated control.

2. In Figure 5, 10 µM of LY and PD compounds already inhibit neurite outgrowth. It is possible that Eca233 promotes neurite outgrowth independent of ERK1/2 and Akt pathways. The authors should use a smaller concentration of these two compounds which themselves cannot inhibit neurite outgrowth but can decrease the enhancement of Eca233 to claim that it functions through these two pathways.

Response: We conducted the experiment using lower concentrations of these inhibitors which showed no significant effect on the neurite outgrowth. We found that the inhibitors still could decrease the effect of ECa 233 on neurite outgrowth of the cells.
3. N and number of cells counted per condition and individual experiment in Figure 3 and 5B should be described in the figure legends. This is important to assess the appropriateness of data analysis and related statistical measurements.

Response: We have amended Figure legends of Figure3 and 5B in the revised manuscript, accordingly.