Author’s response to reviews

Title: Anti-stress and neuronal cell differentiation induction effects of Rosmarinus officinalis L. essential oil

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Version: 1 Date: 05 Nov 2017

Author’s response to reviews:

November 6, 2017

Maurice D Awouafack, PhD
Editor
BMC Complementary and Alternative Medicine
BioMed Central, UK

Dear Dr. Awouafack:

We would like to submit the revised version of our manuscript entitled, “Anti-stress and neuronal cell differentiation induction effects of essential oil derived from Tunisian Rosmarinus officinalis L.” for publication in your BMC Complementary and Alternative Medicine as a research article.
As required, we provided a point-by-point response letter that provides the detailed response to each reviewer/editorial point raised, including where the changes made may be found. We hope that we have satisfactory replied to the said reviewers’ comments and we have also edited the manuscript for any grammatical and spelling errors. The revised parts of the manuscript are in red letters. We sent a request for changing the list of authors to add Dr. Kazunori Sasaki because we did not add his name to the previous list of authors despite his contributions to the study, and added his name in the authors list during the submission of this revised manuscript. Thank you for the consideration given to our submission and we hope that the revised manuscript is now acceptable for publication in your journal.

Sincerely,

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REPLY TO THE REVIEWERS’ COMMENTS/QUESTIONS

We are sincerely grateful to all our reviewers for your comments that have helped us improve our manuscript.

A. Ephrem Engidawork, PhD (Reviewer 1):
This is a very interesting study that included both in vivo and cell line studies, used to substantiate findings obtained from animal studies.

Here are some of my comments.

*How is the volume related to the amount of EO inhaled by the animal? The MTT assay revealed that the EO was toxic at 50 and 100 ug/ml. How can the authors prove that the amount inhaled by the animal was not toxic?*

**REPLY:**

We acknowledge that your concern for the toxicity of the essential oil was valid however, based on our observations, inhalation of the ROEO did not cause any adverse effects on the mice based on our observations. There was no significant differences between groups in terms of the animal weight and food consumption as shown in the following figures that are not included as one of the figures in the submission:

*Statistical analysis: t-test could produce a false positive result if the groups within the experiment are more than two. Please, use One way ANOVA followed by an appropriate post hoc test.**

**REPLY:**

As suggested, in addition to t-test, we performed ANOVA, followed by pairwise comparison test using the JMP Statistical Discovery software from SAS version 13.2.0 to determine the significance in the differences between the means of the treatment groups. We revised the manuscript to add the results of ANOVA for Table 2.

*Treatment was done for 14 days and TST was performed every other day. What was the rationale for doing the test every other day? Why not daily or at the end of treatment? What does the value presented in Figure 1 represent? I am asking this because various measurements had been taken. I think if time is taken as a factor, ANOVA with repeated measure should be done.*

**REPLY:**
The tail suspension test is known to induce the stress itself and it was done every other day to induce chronic stress. Depending on the study and intensity of the stress, some reports perform TST daily (Manhaes et al., 2014) others advise not to repeatedly test animals (> 3 times) receiving drug regimens, for the reason that it will become difficult to distinguish repeated drug effects from drug-behavior interactions (Castagne et al., 2010), this precaution was given because the drugs were orally administered. In this study, the animals inhaled the essential oil and the amount that entered the animals was relatively lower compared to orally administered drugs. Aromatherapy has been known to have no adverse effect on human health and in fact is effective in reducing anxiety levels (Fayazi et al., 2011).

We changed the figure to include only the data for the last TST done on the last day of the study (Figure 1A). The data for previous figure was the average measurements of all the TSTs done which is not an accurate reflection of the state of the animal at the end of the study (i.e. the initial times were not reflective of the chronic stress that the repeated TST was designed for).

References:


*Noradrenergic input from the Locus ceruleus to the hypothalamus controls the production of corticosteroids. Depression is characterized by decreased noradrenaline, which is believed to contribute to enhanced production of corticosterone. I presume this notion is the basis for determination of both the steroid and the transmitter. However, amelioration of corticosterone was not accompanied by increase in noradrenaline. How do the authors explain these seemingly conflicting findings? Moreover, how does the dopamine story fits into the stress induced depression-like activity?

REPLY:
Regarding the corticosterone and noradrenaline, ROEO at 50 μL significantly increased the noradrenaline level (Table 2). ROEO at 100 μL had slightly higher NAD than control however the difference was not significant. Re: dopamine, ROEO at both volumes (50 and 100 μL) significantly increased the dopamine levels of the mice (Fig. 2). Dopaminergic signaling has a significant role in mice’s appraisal of threats from social environments. Dopaminergic neurons and its projections to the nucleus accumbens (NAc) make up the mesolimbic dopamine pathway. The NAc are linked cognitive processes the alterations of which may cause social withdrawal that is common to human affective disorders including depression and post-traumatic stress disorders (Berton et al., 2006)

Reference:

*Page 18, Line 291-291: "ACh is produced from ingested choline." I do not think that's an appropriate statement. In fact, much of the choline used for ACh synthesis comes from the recycling of choline from metabolized ACh. Another source is the breakdown of the phospholipid, phosphatidylcholine. One of the strategies to increase ACh neurotransmission is the administration of choline in the diet. However, this has not been effective, probably because the administration of choline does not increase the availability of choline in the CNS.

REPLY:
As pointed out, choline may also “come from the recycling of choline from metabolized ACh.”. We revised that paragraph to improve the manuscript by editing that part of the manuscript to “Since ACh is produced from ingested choline (Ch) and from recycled choline in the synapse …” (Page 17, Line 313).

*AChE activity is similar in both undifferentiated (Figure 2) and differentiated (Figure 4) PC12 cells. It is known that differentiation of PC12 cells with NGF or other agent is associated with an increase in the cholinergic system.

REPLY:
We observed that ROEO induces cell differentiation (Fig. 2) that is why the undifferentiated (+ROEO) and the differentiated (NGF+ROEO) have more or less the same AChE activity. Addition of NGF further increased the AChE activity (by ~20%). We revised the manuscript to add this hypothesis in the discussion: “It can also be noted that in undifferentiated PC12 Ach was increased by ROEO, even without NGF to induce differentiation, providing further evidence of its differentiation induction effect that was also shown in Fig. 3B.” (Page 22, line 400-402).

*Is the value given for Gap-43 mRNA expression a normalized value?

REPLY:

Yes, the expression of Gap43 was normalized to the expression of Gapdh (page 12, Methods).

* What was the basis for inferring that ROEO regulates the sympathetic nervous system?

REPLY:

The sympathetic nervous system is responsible for the fight or flight response so that in the presence of stress, it stimulates the adrenal glands to secrete adrenaline and noradrenaline.

We inferred that ROEO can regulate the sympathetic nervous system based on its ability to alleviate stress as shown by the reduced time in the TST. Moreover, ROEO affects acetylcholinesterase which has an important role in both the sympathetic and parasympathetic nervous system as neurotransmitters. In addition, the increase in Gap43 mRNA expression following treatment with ROEO signifies that ROEO also affects the hypothalamo-pituitary-adrenocortical (HPA) axis since Gap43 modulate the HPA axis. Gap43 plays an important role in embryonic growth and axonal regeneration of the sympathetic nervous system…

B. Shih-Shun Chen (Reviewer 2):

Comments for BCAM-D-17-01149 The manuscript has provided a mechanism of Rosmarinus officinalis EO (ROEO)-induced rat renal medulla-derived PC12 cell differentiation. The authors also show that ROEO inhalation did decrease the levels of immobility time and serum corticosterone as well as increase the level of brain dopamine in ICR mice.
There are few concerns/comments that needs to be addressed:

1. It is not clear how control the volume and rate of ROEO inhalation in ICR mice.

REPLY:

To control the volume and rate of ROEO inhalation, the mice in treatment groups were made to inhale the same amount of essential oils and for the same duration.

2. They mainly based on the results of acetylcholine, choline, and Gap43 gene expression to examine the ROEO-induced neuronal differentiation in PC12 cells. The authors need to examine neuronal markers to bolster their claims.

REPLY: We acknowledge that checking for the other differentiation markers would further bolster our claim. We used acetylcholinesterase (AChE) activity as marker for differentiation because it has long been established as a marker for neuronal differentiation. In a study using pluripotent stem cells, AChE activity was not present in the undifferentiated stem cells, no AChE mRNA was also not detected. Commitment to a neuronal differentiation pathway results in increased levels of AChE mRNA, a tetrameric form of the enzyme was produced, and AChE was secreted into the culture medium (Coleman and Taylor, 1996).

Reference:


3. The effect of the ROEO on the AChE activity need to be shown in Figure 4.

REPLY:

As suggested, we added the results for “ROEO alone” in Figure 4 and revised the Methods (Page 13, Line 231-237) and Results (Page 18, Line 324-330) parts of the manuscript.

4. The authors need to explain the rational of using PC12 cells and male (not for female) ICR mice as the study models?

REPLY:

Neuroendocrine cell line PC12 cells are derived from a rat pheochromocytoma. In culture medium containing horse serum, PC12 cells undergo mitosis; when nerve growth factor (NGF)
or epidermal growth factor (EGF) is included in the medium, the cells cease multiplication and extend neurites. PC12 cells have a requirement for NGF, similar to that of normal sympathetic neurons (Vaudry et al., 2002, vol. 296, pp. 1648-1649). Regarding the in vivo test, female mice are not usually used for experiments because of the possible influence that estrogen (female hormone) might have on animal’s response to the treatment. It has been reported that estrogen level influence mood disorders such as depression (Wharton et al., 2012).

Reference:

C. Andrea Bugarcic (Reviewer 3):
This is an interesting study that looked at the effect of essential oils in vivo and in vitro.

The conclusions from the in vitro study show elevation of the stress response, while in vitro experiments speak to the neuronal plasticity. These two conclusions may be a completely separate conclusions or may be connected through the nervous-endocrine system interaction, but this needs to be stated fully in the paper.

Currently, the conclusions are putting the two conclusions together and no testable model has been suggested on the interrelationship of these two findings.

REPLY:
Thank you for pointing this out. We revised the manuscript to give a conclusion to put the summaries for the in vivo and for the in vitro together (Page 23, Line 429-431).

Experimentally:
1. TST assay was done without appropriate controls (both negative and positive - can another compound or situation act as a positive control for the assay itself?, also no control was used where immobility was increased)

REPLY:
In the TST, lavender oil was used as a positive control while almond oil was used as a negative control. This is described in the Methods part of the manuscript, page 8, Line no. 143.
2. why are corticosterone levels and immobilization times (Fig 1 a and b) at 50ul and 100ul showing inverse relationship?

REPLY:

We changed the figure to include only the data for the TST done on the last day of the study (Figure 1A) because for the previous figure, the data was based on the average measurements of all the TSTs done which is not an accurate reflection of the state of the animal at the end of the study. The initial times were not reflective of the chronic stress that the repeated TST was designed for.

3. Figure 2a shows increase in PC12 numbers in the presence of 5 and 10ug/ml ROEO while 2b shows increased AcHE activity similar the level of cell number increase. NGF is known to act on division cycle - is this also the case with ROEO? What are cell numbers at the point of assay? Simple counting of the cells in the Fig 3b may suggest the increase in numbers is also contributing the level of AcHE activity assay - this needs to be addressed for publication.

REPLY:

MTT assay is the technique to measure the activity of phthalate dehydrogenase in cells. So, the increase of cell viability does not directly reflect the increase cell numbers. We thought that the increase of cell viability by ROEO indicating the activation of mitochondoria in PC12 cells. This means that ROEO increases the AChE activity by increasing the mitochondrial activity of PC12 cells.

4. Figure 3b - the outgrowths are difficult to see. It would be more convincing if the outgrowths were quantified and better contrasting picture presented.

REPLY:

As suggested, we adjusted the photographs contrast and changed the arrows’ color to black so they will be easy to see. Thank you for pointing it out.

5. formatting issue - Figure 4 - x-axis does not need to have 2 label types for the same message

REPLY:
As pointed out, indeed we labeled the x-axis twice. We changed the figure to delete the redundant label.

The paper adds to the body of knowledge and has a potential to unlock the level of different system involvement and molecular pathways influenced by the EOs in a range of mental and neurodegenerative disorders (e.g. authors note the different NT secretion in presence of different EOs).

The experimental data needs to be addressed as above and a presentation of a testable model would be beneficial to see.

REPLY:

We made the necessary changes as requested and if there are anything we can do to improve the manuscript, kindly let us know. Regarding a testable model, if you are referring to a mouse model for stress, one of our collaborators in the UK are developing a mouse model of stress and depression and we hope we can use it in our future studies.