Author’s response to reviews

Title: The Effects of thylakoid-rich spinach extract and aqueous extract of caraway (Carum carvi L.) in letrozole-induced polycystic ovarian syndrome rats

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Author’s response to reviews:

Dear Editor,

Thank you very much for your letter informing us of your decision about the manuscript (BCAM-D-19-01767) entitled: "The Effects of thylakoid-rich spinach extract and aqueous extract of caraway (Carum carvi L.) in letrozole-induced polycystic ovarian syndrome rats". We tried our best to revise the manuscript according to the reviewer’s comments and also the journal style. The point by point responses to the comments are provided in below. The changes in the manuscript are highlighted in yellow.

Yours Sincerely

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Reviewers’ comments:

Reviewer 1

Dear Jue Zhou,

We kindly appreciate your comments. Thanks for your attention and comments. Below you can see point by point answers:

1. The entire manuscript requires extensive language revision.
   Authors: We corrected all the manuscript's language errors. The changes are highlighted in the manuscript.

2. The background, lines 58 and 59 should indicate the pharmacological agents and the side effects stated
   Authors: It has been corrected and highlighted in the background section, lines 60-64, page 3.

3. The justification for the use of the natural products indicated for this study is poor
   Authors: Suggested modifications were done. (background section, lines 69-74 and 79-85, pages 3-4). Medicinal properties of both Thylakoid and Caraway as natural and herbal products were described based on scientific basic studies.

4. The products voucher numbers should be specified
   Authors: products voucher numbers were added in the manuscript (Methods section, lines 92-94 and line 116, pages 4-5). Herbarium number of 2617 and 2603 for spinach and caraway respectively.

5. How was it ensured that the thylakoid concentration was high enough as suggested?
   Authors: It should be noted that the dose of thylakoid treatment was determined according to the previous literature(1). Furthermore, we were ensured about the amount of thylakoid concentration based on our laboratory procedures conducted regarding the determination of quantity of chlorophyll for our thylakoid-rich spinach extract via spectrophotometry method(2). We admitted that this part was not clearly explained which has been modified and highlighted in the manuscript (Method section, lines 102-113, page 5).

6. Indicate month and year of collection of the products.
   Authors: It has been done (Methods section, line 91 and line 115, pages 4-5).

7. Authors indicate that they dissolved letrozole in water, this is highly unlikely as letrozole is sparingly soluble in aqueous solutions. How did the authors achieve this?
   Authors: Thanks for the editor's attention. Actually, it is true that letrozole is meagerly soluble in water, but in the present study, after mixing letrozole with normal saline, the obtained emulsion was poured in to the shaker before each session of the gavage, then the drug was rapidly gavage to the rats with a specific syringe (3). Moreover, it is noteworthy to mention that the induction of the disease was confirmed by determining the stage of cyclicity of rats via microscopic analysis
of the predominant cell type in vaginal smears. (Methods section, line 141, page 6: The word “emulsion” was added).

8. Wherever a method is referred to, authors should briefly indicate the procedure rather than simply refer readers to the article cited.
Authors: It has been corrected. The procedure described completely and in detail as performed. (Method section, lines 102-113, page 5)

9. The start and end times of letrozole administration as well as treatment is unclear.
Authors: All the time frames have been clarified (Methods section, line 142-143 and line 149, page 7).

10. Authors should justify the use of letrozole model of PCOS induction rather than the other PCOS models in their study.
Authors: It has been done. According to the earlier studies (4), the histology of ovaries taken from letrozole-treated animals bears remarkable similarities to human PCOS features than to other PCOS models (Methods section, lines 143-145, page 7).

11. It is imperative that authors use their own studies to prove chlorophyll content proportional to thylakoid.
Authors: We admitted this part was not clearly explained which has been modified in the manuscript (Method section, lines 102-113, page 5). We were conducted laboratory procedures regarding the determination of quantity of chlorophyll for our thylakoid-rich spinach extract via spectrophotometry method according to Ostbring et al.(2). Evaluation of the chlorophyll content is a method to discover the amount of thylakoid membrane. Thirty ml thylakoid extract was added to 2 ml ice-cold acetone (80 vol%). The samples were then incubated dark and centrifuged at 25 °C for 4 min at 12100xg. The chlorophyll a, b and total content was determined at the absorbance of 646.6 nm and 663.6 nm by a spectrophotometer (Varian Inc., Santa Clara, CA, USA):

\[\begin{align*}
\text{Chl a} & : 12.25 A_{663.6} - 2.550 A_{646.6} \\
\text{Chl b} & : 20.31 A_{646.6} - 4.910 A_{663.6} \\
\text{Chl (a + b)} & : 17.76 A_{646.6} + 7.340 A_{663.6}
\end{align*}\]

The total chlorophyll content in the non-treated thylakoid slurry was 0.767 ± 0.02 mg ml⁻¹.

12. Discussion is poor and does not sufficiently address the outcomes of the study.
Authors: We improved the discussion based on your statement. The changes in different parts were highlighted in pages 14 -17.

13. The Figures are poorly arranged and also unclear. The figures should be presented with better resolutions and labels.
Authors: All the figures have been corrected and also we improved the resolution of the study figures.

14. Figures should not be written within the discussion.
Authors: Figures were omitted from the discussion part.
Reviewer 2:

Dear Abdel Halim Harrath,

We kindly appreciate your comments. Thanks for your attention and comments. Below you can see point by point answers:

1: The authors performed a histopathological study for the right ovary collected from the different studied groups. The images of this study (Figs. 1, 2 and 6) are of poor quality. I strongly recommend the authors to change these images with other photos with high quality, especially that the journal is insisting on the fact to upload images with high resolution during submission (300dpi at least). Otherwise, I regret to inform the authors that the manuscript is likely to be of lower interest to the readership of BMC Compl Altern M.
Authors: We improved the resolution of all the figures in the manuscript as you wanted.

2: The authors mainly focused on ELISA analysis of some biochemical parameters and stereological measurements to show the corrective effect of caraway and thylakoid extracts on PCOS. If the authors' hypothesis is correct that caraway and thylakoid extracts corrected PCOS status, some reproductive parameters should expectedly be ameliorated. The authors found that the number of follicles increased among the caraway and thylakoid treated groups, but I guess this is too incomplete to generate such hypothesis! They should study some molecular markers of fertility such as the expression of some steroidogenic-related genes/proteins (Cyp19, Cyp17, GDF...), proliferative markers (cyclin D, PCNA, …) and so on.
Authors: You are completely right. Our findings in this study are based on biochemical parameters and stereological measurements indicative of PCOS. The increase in the number of follicles we observed in treated groups were just based on stereology and not molecular base. Unfortunately, due to the limited funding, we could not measure the precious molecular parameters including the expression of steroidogenic-related genes/proteins and proliferative markers. We admitted that it's one of the important part of our study limitations which we added it in our manuscript (Discussion section, lines 389-392, page 17).

3: I'm not clear on why the authors used the right ovary for their analysis? Why not the left or both ovaries right and left? Probably, most of readers also do not understand the stuff.
Authors: Since the data in the stereological studies are based on quantitative measurements, so the same conditions for each animal, such as weight, age... and so on, can greatly affect the results of the study. Therefore, throughout an internal team decision we choose the right ovary to ensure the same condition for collecting our stereological data. Actually, it should be noted that there is no difference between the right and the left ovary, and it was just our team decision to choose the right ovary. (Methods section, lines 188, page 9).

4: Page 2, Line 43:"Abstract section: Spell out the full name of "HDL-C".
Authors: Done ("Abstract section, line 44, page 2).

5: Background section: It seems that a relatively large body of knowledge already exists on the biological effects of caraway and even thylakoid extracts and, based on that, I advise the authors to develop the Background section substantially.
Authors: Background section has been modified as you suggested. We referred to more detailed biological effects of caraway and thylakoid extracts. Changes were highlighted in the manuscript. (background section, lines 69-74 and 79-85, pages 3-4)

6: Page 3, Line 52, Background section:"...affecting 2.2% to 26% of women during their reproductive years worldwide". Please revise the percentages given carefully!!
Authors: It has been corrected as you mentioned (based on NIH criteria). (Background section, lines 54-55, page 3).

References:
1. Köhnke R, Lindqvist A, Göransson N, Emek SC, Albertsson PÅ, Rehfeld JF, et al. Thylakoids suppress appetite by increasing cholecystokinin resulting in lower food intake and body weight in high-fat fed mice. Phytotherapy research. 2009;23(12):1778-83.
2. Östbring K, Rayner M, Sjöholm I, Otterström J, Albertsson P-Å, Emek SC, et al. The effect of heat treatment of thylakoids on their ability to inhibit in vitro lipase/co-lipase activity. Food & function. 2014;5(9):2157-65.
3. Noorafshan A, Ahmadi M, Mesbah S-F, Karbalay-Doust S. Stereological study of the effects of letrozole and estradiol valerate treatment on the ovary of rats. Clinical and experimental reproductive medicine. 2013;40(3):115-21.
4. Kafali H, Iriadam M, Ozardali I, Demir N: Letrozole-induced polycystic ovaries in the rat: a new model for cystic ovarian disease. Archives of medical research 2004, 35(2):103-108.