March 12, 2021

Dear Dr. Patel,

Attached please find our revised manuscript entitled "Thymoquinone Anticancer Activity is Enhanced when Combined with Royal Jelly in Human Breast" (**Manuscript NO: 62945**)**.** We have provided a point by point response to the comments of the reviewers and uploaded the manuscript with tracked changes as a supplementary file. In addition, we have revised the title page, and the introduction section and updated the reference list in addition to providing the missing PMID and DOI numbers. As per the suggestions of the Editorial Office, the article highlights section was added, and figures and figure legends were relocated to the end of the manuscript. The original figure documents were uploaded as a Powerpont file along with the approved grant applications.

We believe that our paper is now in a form that is acceptable and we look forward to your positive response.

Sincerely,

Hala Gali-Muhtasib

Answers to Reviewer Comments

Abstract Section

Comment 1A: Abstract: Methods: all abbreviations should be explained (MTT, IC50).

Answer 1A:

We thank the reviewer for this comment. We have added the full terms of the used abbreviations by including the following statement in the METHODS section of the Abstract: " Using MTT (3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay we determined the half-maximal inhibitory concentration (IC50) of TQ".

Comment 1B: Results: The need for investigating the effect of RJ on FHS 74 Int small intestinal cells should be explained before; in the background or aim. The use of this cell line appears here for the first time.

Answer 1B:

We have commented on the need for investigating the effect of RJ on FHS 74 Int small intestinal cells by rephrasing the AIMS section of the Abstract as follows: "Investigate RJ's cytotoxicity in FHs74 Int cells and anticancer effects of TQ, RJ and their combinations in MDA-MB-231 cell line".

Introduction Section:

Comment 2: Introduction: Page 5: The first 2 sentences on this page are a repetition. I suggest to reconsider the 2 sentences starting with TQ.

Answer 2:
We thank the reviewer for this comment. We have addressed this comment by rephrasing the first paragraph of the INTRODUCTION section, page 4.

Materials and Methods Section

Comment 3A: Materials: You should explain the need for use of FHs 74 cell line in the Introduction. Furthermore, the reader could better follow if you mentioned that the toxicity experiments on these cell lines were already performed in a previous study with TQ (also in the Introduction).

Answer 3A: We thank the reviewer for this comment. Accordingly, we have clarified the need for use of FHs 74 cell line in our study in addition to mentioning the

findings of a previous study regarding TQ cytotoxicity in this cell line by including the below paragraph on page 6:

"In a previous study, we have shown that TQ exerted a dose-dependent antitumor effect against a panel of human colon cancer cell lines with minimal cytotoxicity against FHs 74 Int non-tumorigenic human intestinal cells. Here, to assess the cytotoxic effects of RJ, FHs 74 Int intestinal cell line was used as a model of non-tumorigenic epithelial cells".

Comment 3B: Drug preparation and treatment: You wrote: 16,4 mg/ml of TQ crystals in 1 mL methanol. It should rather be: 16,4 mg of TQ crystals in 1 ml methanol to get a concentration of 16,4 mg/ml. Why does the stock solution have this concentration?

Answer 3B: As suggested by the reviewer, the typing mistake (mg/ml) in the first statement in the "*Drug preparation and treatment*" section was corrected by deleting the (/ml). The designated stock concentration is explained by including the following statement to replace the first sentence of the same paragraph page 8: "Directly before use, fresh stock of the purified synthetic compound TQ of 0.1mol/L concentration was prepared by dissolving 16.4 mg of TQ crystals in 1 mL methanol".

Comment 3C: For the assays: Why did you use the concentrations described, why max 15 µM TQ? What happened in a combination of RJ with 20 or more µM TQ? This remains unclear throughout the whole study. You should shortly explain the reason why you did not present the effects of TQ doses > 15µM.

Answer 3C:

We added the following paragraph to the DISCUSSION section page 15 to explain the reasons:: "To assess for any possible anticancer synergy (or additive effects), concentrations that are not highly cytotoxic to cells (i.e., less than 50% cell death) should be used".

Comment 3D: Immunfluorescence assay, page 10: Please declare the abbreviations (DAPI).

Answer 3D: Kindly, refer to the "*Materials*" in the "MATERIALS AND METHODS" section on page 7 where "DAPI" was declared as "(4',6-diamidino-2-phenylindole)" upon first mention in the manuscript.

Results Section

Comment 4: Results: page 11: RJ exerted mild inhibitory effects al low doses of RJ (...) - please define low doses. Consider splitting the sentence in 2 sentences as it is quite long and difficult to understand.

Answer 4: **In the RESULTS section on page 12, the fourth sentence in the paragraph entitled "Cytotoxicity of TQ and RJ on human breast cancer cells" was revised and the low doses of RJ were defined. The rephrased sentence is as follows: "Royal jelly (RJ) exerted mild inhibitory effects at low doses of RJ (below 5 µg/ml) on FHs 74 Int non-tumorigenic human intestinal cells and MDA-MB-231 human breast cancer cells".**

Comment 5: page 14, Figure 2: (...)values corresponding to the % cell death of FIVE different combinations - You use the arabic sign for 5 :).

Answer 5: **The Arabic sign for 5 is replaced with the word "five" in the legend of Figure 2 under the Figures Legends section page 30.**