

March 23, 2023

To whom it may concern,

We thank the reviewers for their valuable comments on our manuscript entitled "**Thymoquinone enhances the antioxidant and anticancer activity of Lebanese propolis**" (Manuscript NO.: 82834, unsolicited manuscript, basic study). We have provided a point-by-point response to the comments of the reviewers, and uploaded the revised paper with changes in red. Thus, we hope our paper is now in a form that is acceptable for publication.

We look forward to receiving your positive response.

Sincerely,

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Answers to Reviewers Comments

Reviewer #1

Comment1: Initially the authors used distilled water for extraction of bioactive compounds from raw propolis, followed by adding methanol to the residue for extraction to obtain the methanol extract. Did the authors collect the water extract? If not, the contents of both phenolic acids and flavonoids can be decreased substantially.

Answer 1: We did not collect the water extract as it is known that the water extract of propolis will contain much less components and thus we did not feel that it is worthwhile to test. Indeed, methanol is more effective than water to extract phenolic acids and flavonoids as per literature below and this is why we opted to test the methanolic extract.

According to Arceusz et al. (2015), “ natural phenolic acids show a solubility preference basically to alcohols rather than to the more polar water” [1]. Park et al. (1998) demonstrated that **water was not effective in extracting phenols from propolis** [2]. Miguel et al. (2014), extracted propolis with water as we did and analyzed the phenolic and flavonoid content of the water extract. Then they extracted the residue with methanol. They demonstrated that that phenols and flavonoids from propolis were better extracted with methanol than with water. They also showed that **phenol content were about 3-4 times higher in methanolic extracts than in the aqueous extracts** [3].

1. Arceusz A, Wesolowski M, Ulewicz-Magulska B. Flavonoids and Phenolic Acids in Methanolic Extracts, Infusions and Tinctures from Commercial Samples of Lemon Balm. *Nat Prod Commun.* 2015; 10(6): 977-981 [PMID: 26197530]
2. Park YK, Ikegaki M, Abreu JAS, Alcici NMF. Study of the preparation of propolis extracts and its applications. *Ciência e Tecnologia de Alimentos* 1998; 18: 313-318 [DOI:10.1590/ S0101-20611998000300011]
3. Miguel MG, Nunes S, Dandlen S A, Cavaco A M, Antunes M D. Phenols, flavonoids and antioxidant activity of aqueous and methanolic extracts of propolis (*apis mellifera* L.) from algarve, south portugal. *Ciência e Tecnologia De Alimentos* 2014; 34(1): 16-23 [DOI: 10.1590/S0101-20612014000100002]

Comment 2: The authors only studied the inhibition effect of the methanol extract on breast cancer cells and colorectal cancer cells, which should be questionable. It would be better for the authors to study the effect of methanol on these cancer cells and normal cells as well as demonstrate the inhibition effect is mainly from bioactive

compounds in the extract. However, no experiment dealing with normal cells and methanol is performed.

Answer 2: We did not test the effect of methanol alone on normal and cancer cells because the concentration of methanol in our extract which was tested on cancer cells did not exceed 0.25%. Only the stock solution of propolis extract was prepared in methanol. However, after serial dilution in media as a solvent the percentage of methanol in the tested solution was less than 0.25%. This concentration is not toxic to cells as documented by our previous findings.

Comment 3: In Figures 1 and 2, the superscript letters should be provided on the top of each bar for significance in comparison.

Answer 3: We placed the superscript letters on the top of each bar in figures 1 and 2.

Reviewer #2

Comment 1: It is advised to check all manuscript body (especially references section) as shape carefully and correct it kindly.

Answer 1: We thank the reviewer for this comment. All the manuscript body and references are now written as per the journal guidelines.

Comment 2: Please give a little information about antioxidants agents in the introduction section to improve your paper with to cite of some papers below:

1. Z. Selamoglu, I. Ozdemir, O. Ciftci, M. F. Gulhan, and A. Savci, "Antioxidant effect of ethanolic extract of propolis in liver of L-NAME treated rats," *Advances in Clinical and Experimental Medicine*, vol. 24, no. 2, pp. 227–232, 2015.
2. G. Badr, E. A. Sayed, H. Waly, K. A. H. Hassan, M. H. Mahmoud, and Z. Selamoglu, "The therapeutic mechanisms of propolis against CCl₄ -mediated liver injury by mediating apoptosis of activated hepatic Stellate cells and improving the hepatic architecture through PI3K/AKT/mTOR, TGF- β /Smad2, Bcl2/BAX/P53 and iNOS signaling pathways," *Cellular Physiology and Biochemistry*, vol. 53, no. 2, pp. 301–322, 2019.
3. Ozdemir Betul, Gulhan Mehmet Fuat, Selamoglu Zeliha, Sahna Engin. The investigation of antioxidant and anti-inflammatory potentials of apitherapeutic agents on heart tissues in nitric oxide synthase inhibited rats via N ω -nitro-L-arginine methyl ester. *Clinical and Experimental Hypertension*. 43(1): 69-76, 2021.
4. Ekhteiari Salmas Ramin, Durdagi Serdar, Gulhan Mehmet Fuat, Duruyurek Merve, Abdullah Huda I, Selamoglu Zeliha. The effects of pollen, propolis, and caffeic acid phenethyl ester on tyrosine hydroxylase activity and total RNA levels in

hypertensive rats caused by nitric oxide synthase inhibition: experimental, docking and molecular dynamic studies. 36(3): 609-620, 2018.

5. Selamoglu Zeliha, Ozdemir Ilknur, Ciftci Osman, Cakır Oguz, Gulhan Mehmet Fuat, Oguz Murat Pasaoglu. Role of Propolis on Biochemical Parameters in Kidney and Heart Tissues against L NAME Induced Oxidative Injury in Rats. Clinical and Experimental Hypertension. 7(36): 492-496, 2014.

6. RE Salmas, MF Gulhan, S Durdagi, E Sahna, HI Abdullah, Z Selamoglu. Effects of propolis, caffeic acid phenethyl ester, and pollen on renal injury in hypertensive rat: an experimental and theoretical approach. Cell biochemistry and function 35 (6), 304-314.

7. Gülhan Mehmet Fuat, Özdemir Betül, Selamoğlu Zeliha, Şahna Engin. The effects of apitherapeutic agents on oxidative stress in serum metabolic parameters of hypertensive rats created by nitric oxide synthase inhibited. Sains Malaysiana 50(6): 1745-1754, 2021.

8. Göğebakan Ayşe, Selamoğlu Zeliha, Özdemir İlknur, Şahna Engin. Role of Propolis on Tyrosine Hydroxylase Activity and Blood Pressure in Nitric Oxide Synthase Inhibited Hypertensive Rats. Clinical and Experimental Hypertension. 34(6): 424-428, 2012.

9. Selamoglu Zeliha. The Natural Products and Healthy Life. Journal of Traditional Medicine Clinical Naturopathy. 7(2): 1-2, 2018.

10. Selamoglu Zeliha. Polyphenolic Compounds in Human Health with Pharmacological Properties. Journal of Traditional Medicine Clinical Naturopathy. 6(4): 137, 2017.

11. Selamoglu Zeliha. Biotechnological Approaches on Anticancer Activity of Flavonoids. Modern Approaches in Drug Designing. 1(2): 510, 2017.

12. Doğan H, Akyol E, Akgül H, Selamoğlu TZ. 2014. Biologic Activity of Honeybee Products Obtained From Different Phytogeographical Regions of Turkey. Turkish Journal of Agriculture - Food science and Technology 2(6): 273-276.

13. Amin K, Ozgen S, Selamoglu Z. Stevia rebaudiana: A potential boon for human health. SM J Med Plants Stud. 2017;1:1005.

Answer 2: We added to the introduction of the paper page 5 lines 26-28 the following sentence and cited the relevant references (1, 2, 3, 6 from above):

“, and carbon tetrachloride^[22]. In addition, propolis was demonstrated to reduce the blood pressure and suppress oxidative stress in heart, liver, and renal tissues in animal models of hypertension^[23-25].”