Belgrade, 11th October, 2019

Dr. Olgica Nedić

Biochemistry & Biotechnology Sub Editor

Journal of the Serbian Chemical Society

Dear Dr. Nedić,

Please find enclosed the corrected version of manuscript ID 8394-46630-1.R1, entitled: Beta-glucan content and antioxidant activities of mushroom-derived food supplements “, by Milica Zrnić Ćirić, Nevena Dabetić, Vanja Todorović, Jelena Đuriš and Bojana Vidović for consideration for future publication Journal of the Serbian Chemical Society.

We have accepted the recommendations of reviewers and we believe that the revised manuscript has been improved. Additionally, the manuscript has been subjected to English correction and improvements, done by professional proofreading service. All corrected things have been marked in red in revised version.

A point-by-point reply follows:

**Response to Reviewer’s major concerns:**

**Reviewer A**

*Introduction: The paragraph starting at line 53 should be deleted.*

**Response:** Many thanks for your valuable comments. This has been amended in the revised version.

*You always compare data from each of the samples analyzed against the data obtained from shiitake. Please explain, as no explanation could be found elsewhere in the manuscript, the reason why you selected shiitake as a “reference”?*

**Response:** We strongly agree with the reviewer’s remark. The reason why we selected one portion of shiitake as a “reference” was based on its reported benefits by human intervention study (Dai et al. Journal of the American College of Nutrition 2015; 34 (6): 478-487). We have incorporated this explanation in the revised version of the Manuscript (page 3, lines 75-76). Therefore, new reference, which is highlighted in the revised Manuscript, is inserted relating to the additional text (ref. 20).

*Table I legend – maybe “commercially available mushroom-derived food supplements” and just omit “studied”. Separate the rows in the table, as it bit confusing to follow codes and the composition – e.g. insert new spacing*

**Response:** We appreciate the reviewer's suggestions and all proposed changes in the Table I were made in the revised version.

*Line 115 – what is meant by “properly diluted”? As stated in the paragraph that precedes this one, depending on the type of sample you made 50ml and 200ml dilutions in flasks? So, there are two questions. One, why did the authors make different dilutions? Is it based on the assumed content of beta-glucans coming from different sources? Second, back to line 115, what is “proper dilution” referred to, does it mean you used 10µl out of this 50/200ml, or you additionally diluted it? Please clarify this.*

*Line 116 – what is meant by “repeated volumes?*

**Response:**Based on the assumption of total polyphenol content in mushroom-derived food supplements as well as in shiitake, we have defined different dilutions for these two kinds of samples which were analyzed without additionally treatment. Namely, such extracts were 1g mushroom-derived food supplements (or the inner part of the capsules) filled up to 50 ml of water and 50g shiitake mushroom filled up to 200 ml of water*.* Additionally, lines 119-122 are replaced with next paragraph:

“Reaction mixtures (extracts (10 μL), ten times diluted Folin- Ciocalteu’s reagent (100 μL) and 1M Na2CO3 (80 μL) were incubated for 1 h in the dark conditions. The absorbance of the solutions was read at 630 nm on an MTP reader (BIOTEK, USA, ELx800 118 Absorbance Microplate Reader)”

*Line 117- I assume that gallic acid was used for the standard curve, not only as a standard. Just add this information.*

**Response:** We have clarified this in the revised version of the Manuscript (page 5, lines 122-123).

*Line 157 – Trolox was used just like a standard or also for a standard curve? Please add.*

**Response:** We have added this information in the revised version (page 6, lines 159-160).

*Line 166 – insert an equation*

**Response:** The equation was inserted in the revised version according to the reviewer's suggestion (page 6).

*Line 199 – as results for the alpha glucan content is not shown; just add this information in the brackets at the end of the sentence. One more thing to consider, don’t jump from one point to the other. In the current version, you start the first paragraph with B-glc., then switch to A-glc, and in the next paragraph you, again, discuss B-glc. Maybe it is better to first, in brief, discuss findings on the alpha glucan content and then all that is found regarding beta-glucans.*

**Response:** We completely agree with reviewer’s comment. We revised the Results and Discussion section according to reviewer's suggestion (Page 7 in revised version).

*Please re-write the conclusion as it is confusing. Pay attention to English grammar.*

**Response:** According to the Reviewer’s recommendation we have completely rewritten the Conclusion section (page 12, lines 301-308). All the grammatical errors have been checked and the text is carefully rewritten regarding spelling errors, too.

Finally, the authors would like to thank to Reviewer A for valuable remarks that have improved the manuscript and hope that each important reviewer’s point was taken into consideration.

**Reviewer B**

*Lines 53-56: It is a repetition of the following paragraph on the lines 57-60.*

**Response:** We agree with the reviewer and this paragraph has deleted in the revised version of the Manuscript.

*Line 178: β-glucan content was expressed as milligrams of β-glucan per 100 g of the weight samples. In the Table II and in the section results and discussion, β-glucan content was expressed as grams per 100 g of the weight samples.*

**Response:** Thanks to the reviewer for the detail evaluation and remarks.We have corrected made a mistake and additional clarified expression results of β-glucan content (page 7, lines 179-180).

*Line 216: The accepted daily dose is 1500-6000 mg of biomass or mushroom extracts. According to Wasser, reference 16, accepted daily dose is 3000-6000 mg.*

*Reference 16: "Numerous clinical trials have established that six capsules (three capsules two times per day or two capsules three times per day), of 500 1000 mg each (biomass or extracts), is the accepted dosage of MM preparations."*

**Response:** We agree with the reviewer’s notice and corrected mistake in the revised version (page 8, line 212).

*Line 224: Table II, total polyphenol contents should be expressed per gram of analyzed samples (mg GAE/g).*

**Response:** Corrected (Table II in revised version)

*Table II, for shiitake: the content of the β-glucan (2.87) was expressed as milligrams or grams per serving size (72.5 g)? On the line 179 are written mg of β-glucan per serving size for the shiitake mushrooms, in the Table II are grams.*

**Response:** We thank reviewer for the remark. The additional text to more clarifies the expression results of β-glucan per serving size for the shiitake mushrooms was marked in red in the revised version of the Manuscript (Table II in revised version, page 8).

*Lines 260-262: The values of antioxidant activity of water extracts of mushroom-derived supplements evaluated by CUPRAC assay were higher not only those obtained by DPPH and ABTS than by FRAP assays too.*

*On the Table III it could be seen that exceptions are S2, S3 and P2. Values for DPPH scavenging abilities are higher than for CUPRAC assay...for S2- almost 2 times, for S3 5 and for P2 11 times. Please correct or explain.*

**Response:** According to the reviewer’s suggestion this paragraph was completely reformulated in the revised version (page 10, lines 262-263) (suggested by another reviewer too). Therefore, new reference, which is highlighted in the revised Manuscript, is inserted relating to the additional text (ref. 39).

We would like to thank to Reviewer B for valuable comments and suggestions that improved our manuscript.

**Reviewer C**

*The main problem of the study is methodology; instead of treating whole products as samples, the products were extracted with water. So the obtained glucan fraction was only water-soluble glucan fraction; there are also water insoluble glucans that have the same immunomodulating properties, although water-soluble are (logically) regarded as more potent (Wasser, S. P. (2017). Medicinal Properties and Clinical Effects of Medicinal Mushrooms. Edible and Medicinal Mushrooms, 503-540. doi:10.1002/9781119149446.ch22). The fact that only water soluble glucans were taken into account should be stated somewhere in the introduction section. Products were dissolved at concentration of 100mg/mL and then centrifuged – even water soluble beta glucans are of different solubility and part of the glucan fraction might had been be lost that way. It would be correct to give a comment somewhere in the discussion/conclusion section that there are still no established protocols for preparation of such samples and that protocols themselves need to be improved/established.*

**Response:** We strongly agree with the reviewer's observation that there are still no established protocols for the preparation of water extracts for subsequent analysis of glucans content. Therefore, we used the original mushroom supplements (or the inner part of the capsules) for the determination of β-glucan content. To clarify this fact, we added the new sentence at the end of Sample and sample preparation sub-section (pages 5 in revised version).

*Keywords – I would suggest that words “glucan(s)” or “β-glucan(s)” be included*

**Response:** Including these words would not be in line with the JSCS guidelines that propose not using words appearing in the manuscript title as keywords.

*line 45 – “among β-glucans of different plant origins and species impact their bioactivities”*

*Mushrooms are not plants, I would suggest that you rephrase the statement (for example, that difference in beta glucan structure impacts its biological activity), or find a more suitable reference, for example: Wasser, S. P. (2017). Medicinal properties and clinical effects of medicinal mushrooms. Edible and Medicinal Mushrooms, 503-540. Ruthes et al., D-Glucans from edible mushrooms: A review on the extraction, purification and chemical characterization approaches.*

**Response:** We agree with the reviewer's criticism. We carefully revised the Introduction section according with reviewer’s suggestion (page 2). Therefore, new reference (ref. 7), which is highlighted in the revised Manuscript, is inserted relating to additional text.

*lines 53-60 – “In addition to polysaccharides, the presence of a wide range of bioactive compounds, including phenolics, tocopherols, carotenoids, glycosides, ergothioneine and ascorbic acid, all together contribute to higher antioxidant potential of mushrooms than most vegetables and fruits have it. Besides polysaccharides, mushrooms contain a wide range of bioactive compounds, including phenolics, tocopherols, carotenoids, glycosides, ergothioneine and ascorbic acid, which all together contribute to a higher antioxidant potential of mushrooms than most vegetables and fruits have”. These two paragraphs represent the same statement, just differently said*

**Response:** We agree with the reviewerand we have delated the sentence “Besides polysaccharides, mushrooms contain a wide range of bioactive compounds, including phenolics, tocopherols, carotenoids, glycosides, ergothioneine and ascorbic acid, which all together contribute to a higher antioxidant potential of mushrooms than most vegetables and fruits have” in the revised version (suggested by other reviewers too).

*lines 62-64 - These products contain refined, or partially refined extracts, or dried biomass from fruiting body or mycelium of mushrooms,…*

*“dried fruiting bodies (mushrooms) or mycelium” – saying mycelium of mushrooms is not correct as mushroom is a fruiting body of a fungi*

**Response:** We thank the reviewer for the remark. It has been corrected in the revised text (page 2, lines 59-60).

*line 138 - DPPH radical solution. Please define the concentration*

**Response:** The concentration of DPPH solution has been added in the revised version (page 6, line 140).

*lines 199-201 - Since that mushrooms naturally contain a low amount of starch polysaccharides, this reflects the absence of starch-based excipients in food supplements.*

*Change starch polysaccharides to starch-like polysaccharides or α-glucans*

*Please clarify, what excipients? Did you mean “the content of α-glucans”? Mushrooms may contain considerable amount of α-glucans (glycogen) (Kalač, A review of chemical composition and nutritional value of wild-growing and cultivated mushrooms, 2013)*

**Responses:** According to the reviewer’s suggestion, the revised Manuscript is complemented with this information. Additionally, this sentence was rewritten, and changes are marked in red in the revised version of the Manuscript (page 9, lines 223-226).

*lines 202 & 203 – Thus, obtained results for α-glucan content also indicated that analysed supplements did not contain mycelium-derived active ingredients. No sufficient evidence for this claim*

**Response:** We have accepted this suggestion and have removed this sentence from the manuscript.

*line 227 – “pyrogallol, myricetin, caffeic acid, quercetin, and catechin, among others”. Phenolic acids commonly found in plants are present in very low amounts in mushrooms, it’s better not to specify phenolic compounds. Statement about them being a good source of phenolics is enough (see Barros et al. Phenolic acids determination by HPLC–DAD–ESI/MS in sixteen different Portuguese wild mushrooms species). Also, it was shown that fungi do not synthesize flavonoids (Gil-Ramirez, Mushrooms do not contain flavonoids)*

**Response:** We accepted all suggestions and sentence was rewritten. Additionally, we have replaced reference in the revised manuscript with one of the proposed (Barros et al, 2009) (page 9).

*TABLE III. Antioxidant activity in maximum daily dose of food supplements and one serving of shiitake mushrooms (μmol TE)*

*How was antioxidant activity calculated? Did you use aliquots equivalent to daily dose?*

**Response:** Based on the obtained results, antioxidant activity was mathematically recalculated by one unit of products (gram or milliliter) and then multiplied by the maximum daily amount available on the product package. We have added this explanation in the revised manuscript too (pages 9-10, lines 256-258).

*lines 260-263 – “The values of antioxidant activity of water extracts of mushroom-derived supplements evaluated by CUPRAC assay were higher not only those obtained by DPPH and ABTS than by FRAP assays too. This observation can be explained by higher stability of copper (II)-262 neocuproine reagent than other chromogenic radical reagents (e.g., ABTS, DPPH”. There is no point of comparing values of antiradical and metal reducing assays; you can simply state if the order of sample activity is the same.*

**Response:** We appreciate the reviewer's suggestion. We rewritten the Discussion section (Page 10 in revised version)

*lines 263-265 – “Also, advantages of the CUPRAC over FRAP assay are based on complete redox reaction, for most common flavonoids, and its capability to measure thiol antioxidants”. Confusing and unnecessary*

**Response:** Accepted.We have delated this sentence from the text.

*lines 304-306- “As one of the scarce that offers insight in the chemical composition of mushroom-derived food supplements, this study provides data on β-glucan and phenolic contents as well as antioxidant activity as parameters of quality related to its potential biological effects”. The sentence needs to be rephrased*

**Response:** The whole the Conclusion section was rephrased (page 12, lines 301-308).

We would like to thank to Reviewer C for the detail evaluation and your constructive comments and suggestions.