***Chemical Industry***

Editor-in-Chief

Prof. Dr Bojana Obradović

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Dear Editor,

with great gratitude to You and the reviewers I am pleased to provide You with answers to the reviewers’ comments of the manuscript titled “Synthesis and biological activity of silver nanoparticles stabilized by aqueous extract of cultivated strawberry leaves (*Fragaria x ananassa* Duch.)“.

All changes are highlighted in yellow in the manuscript.

Please, find the answers attached on the next pages.

Sincerely,

Marija Stevanović

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| --- | --- |
| Reviewer's comment: | Answer: |
| **Reviewer A:** | |
| TITLE | |
| Consider changing the title of the manuscript to less match already presented work. | The title is changed as:  Aqueous extract of strawberry (*Fragaria x ananassa* Duch.) leaves as stabilizing agent in synthesis of bio-active silver nanoparticles |
| ABSTRACT and INTRODUCTION: | |
| Consider improving the abstract by clarifying in the beginning that this work aimed to investigate the possibility to obtain AgNPs by aqueous extract of strawberry leaves during synthesis at room (RT) and boiling temperature (BT), as all further characterization was made referring to these conditions. | The first part of the abstract is rearranged according to the reviewer’s suggestion clarifying the aim of the presented work in the beginning as:  The aim of the presented work was to investigate the potential of aqueous extract of cultivated strawberry (*Fragaria x ananassa* Duch.) leaves for stabilization of silver nanoparticles (AgNPs-E) synthesized at room (RT) and boiling temperature (BT).  The adequate changes are made in the part in Serbian as well. |
| Consider removing the size of AgNPs (app. 20 nm) from the abstract as this information is just prediction according to literature data, not precisely defined value within this study (by TEM analysis e.g.). | The information about AgNPs size is removed from the abstract as:  “The synthesis and stability of AgNPs-E were monitored by UV-Vis spectroscopy confirming high stability of the AgNPs-E in the dark at room temperature”. |
| The Introduction section should be rewritten to describe current methods for AgNPs synthesis, followed by the disadvantages of these methods and the reasons for investigating aqueous extract of cultivated strawberry leaves for AgNPs synthesis. Consider focusing more on the biomedical application of the AgNPs, while removing less relevant parts (such as “One of the potential uses is based on proven catalytic properties of silver nanoparticles thus can be utilized for dye degradation since the release of organic dyes is one of the major concerns for the environment [2]” | The Introduction section is completely rewritten according to reviewer’s suggestion.  The part “One of the potential uses is based on proven catalytic properties of silver nanoparticles thus can be utilized for dye degradation since the release of organic dyes is one of the major concerns for the environment [2]” is removed. The reference [2] is replaced with Cakić et al. 2018 [4] and all references are rearranged. |
| MATERIALS AND METHODS: | |
| Please check that all materials are reported. | Potassium-bromide FT-IR grade, ethanol (HPLC grade), in house strains and sterile cellulose discs 9 mm diameter are added. |
| Correct manuscript by reporting all suppliers and manufacturers correctly. | Materials part is complemented with:  Silver nitrate (AgNO3) was obtained from RTB Bor Grupa, Serbia; ATCC strains obtained from American Type Culture Collection, Manassas, USA  Other suppliers: Sigma-Aldrich (Merck KGaA), Capricorn Scientific, Carl Roth GmbH + Co. KG, Torlak (Institute of Virology, Vaccines and Sera, Belgrade, Serbia) etc. are reported correctly. |
| In section 2.2, the complete extraction process is described; thus, potentially, “aqueous extract of strawberry leaves” could be omitted from materials. | The sentence “aqueous extract of strawberry leaves” is deleted from materials. |
| Lines 116/117: I suggest becoming clear that UV/Vis analysis was taken to confirm the presence of AgNPs. Written like this does not indicate the reasons for doing this analysis (it is mentioned later). | The sentence is rearranged as:  “In order to confirm the presence of AgNPs, the same aliquots of the samples were taken during the synthesis procedure, 20 times diluted and UV-Vis spectra measured”. |
| 2.4.1. UV-Vis spectroscopy: Complement the method with dilution ratio. | The sentence is complemented as:  “The UV-Vis spectra of 20 times diluted reaction mixture were measured on a spectrophotometer Varian Cary-100 Konc…” |
| Lines 136/137: I suggest specifying concentrations of AgNPs for which the DPPH test was performed. | The concentrations are specified as:  Ethanolic solution of DPPH radicals (1.0 mL, 3×10-4 mol/L) was added to 2.5 mL of AgNPs-E solutions prepared in different concentrations (0.1; 0.085; 0.075; 0.05; 0.01; 0.005 and 0.001 mg/mL). |
| In section 2.8: In my opinion, it would be easier to follow if different dilutions of AgNPs colloid solution would be named differently; “extract” could cause confusion with reducing and stabilizing extract from strawberry leaves. | The “extract” is replaced with AgNPs-E in all sections to avoid confusion. |
| Line 177: I suggest presenting final concentrations, not final dilutions. According to this, Fig. 6 should be corrected. | The final concentration is presented instead dilutions as:  The AgNps-E were examined in the following concentration 0.05, 0.025, 0.015, 0.01, 0.005 and 0.002 mg/mL.  The sentence “The amount of formed formazan is in direct correlation with the percentage of viable cells” is added in section 2.7.3. as well as sentence “Cytotoxicity of higher examined concentrations (0.025 and 0.05 mg/mL) on both cell lines was noticed, although more pronounced on MDCK compared to HeLa cells” in section 3.5.  Fig. 6 is corrected. Consequently, the concentrations are replaced throughout the text in section 3.5. |
| Consider reorganizing sections; section 2.7 could be named “*In vitro* cytotoxicity” and then all sections from 2.7 to 2.10 could be organized under 2.7. | The section 2.7. is named “*In vitro* cytotoxicity testing” and all sections are organized under 2.7. section. |
| Lines 197/198: Consider presenting the formula separately from the main text, such as Eq. 1. | The formula is presented separately as Eq. 1. |
| RESULTS AND DISCUSSION: | |
| Absorbance maximum in UV/vis spectra is proportional to the concentration of AgNPs. Fig. 1 and Fig. 3 are similar and indicating a higher concentration of AgNPs after synthesis at BT. In Fig. 2 absorbance maximum is significantly higher compared to Fig.1a and Fig. 3a. Please check. | The spectra in Fig. 2 were mistakenly described as synthesis at room temperature.  The mistake is corrected in the Fig. 2 caption and throughout the manuscript as boiling temperature. |
| Fig. 3: It would be interesting if the stability of AgNPs synthesized at RT and BT was observed for the same period of time. Consider presenting time in days, not in hours. | The Fig. 3 is corrected and the presenting time is given in days according to data available. |
| Line 236 and Figure 1B: It is not clear why the authors concluded that the synthesis of AgNPs at BT is completed after 1,5 h. Please check that the lines and legend in Figure 1B are correct. | The completion time is corrected in 2 h, since spectrum no.5 indicate the synthesis after 2h. The lines in legend of Fig.1b were labeled correctly. |
| Please check that all procedure explanations are moved from results to methods. | The sentence “The dialysis process was carried out for about 48 h with occasional replacement of redistilled water” is moved from results to methods. |
| Please check that procedure explanations are not duplicated in results. | Line 215-216: The sentence “Termination of dialysis process has been determined according to nitrate band (200 – 350 nm) disappearance” is removed.  Line 238: The sentence is rearranged as:  “The effect of dialysis was monitored by changing of UV-Vis spectra configuration (Figure 2)”.  Line 253: The sentence “The spectra were measured by pressing method in KBr pastille” is removed.  Line 285: The sentence “Based on the absorbance of control, sample and blank, the DPPH radical scavenging activity (%) of synthesized AgNPs-E, was calculated” is removed. |
| - Fig. 5. It would be more statistically relevant if the standard deviation will be added. “EC50 values are shown in the figure”? | The error bars are inserted in the Fig. 5  The sentence “EC50 values are shown in the figure” is removed from Fig. 5 caption and the adequate change is made in the text. |
| It would be more consistent to use term “antibacterial” instead of “antimicrobial.” Generally, please make sure that multi-word terms are denoted in the beginning and consistently used throughout the manuscript. | The “antibacterial” term is used throughout the whole text.  The same action is taken for term “antioxidant” instead “antioxidative” activity. |
| “Inhibition of growth has not been observed only of *B. cereus* bacteria” – according to Table 1, inhibition of growth has not been observed either for 4 types of Gram (-) bacteria. It would be beneficial to explain the resistance of Gram (-) bacteria to used AgNPs. | Although the literature data showed higher activity against Gram (-) bacteria, susceptibility of certain bacterial strains depends on the characteristics of particular AgNPs regarding their size and charge. |
| Please make attention to decimal units in Table 1. | Decimal units in Table 1 are corrected. |
| It is interesting that AgNPs (RT) induced a higher inhibition zone, although according to UV/vis spectroscopy, it could be expected to have a higher concentration of AgNPs (BT) in the same dilution as for AgNPs (RT). What could be a potential explanation? | Having in mind that the same dependence was noticed for antioxidant activity, it strengthens the assumption that phenolic compounds from extract exhibit simultaneous antibacterial activity with Ag nanoparticles (Bouyahya et al. British Biotechnology Journal 14(3): 1-10, 2016). The lower concentration of synthesized AgNPs at RT suggests a higher quantity of thermal not destroyed phenolic compounds with free functional groups able to exhibit antibacterial activity. |
| While comparing the present study results with previous studies' results, use more quantitative data. | Quantitative data are given in section 3.3. and highlighted in yellow. |
| Concentrations used in antibacterial tests are significantly higher than those used in cytotoxicity studies, which rase a concern if “safe” concentrations of AgNPs would be efficient against bacteria. | Having in mind that AgNPs are mainly designed as a basis for topical (cosmetic) preparations, and that the particles are larger than 20 nm, they probably cannot penetrate through the skin (Filon Larese et al. Toxicology 255 (2009) 33–37). Based on this, the tested concentration of AgNPs can be assumed as safe. In any case, more detailed safety tests must be performed and exact shape of AgNPs must be determined before using in any preparations (Kyung Tak et al. 2015. DOI: 10.1038/srep16908). |
| **Reviewer B:** | |
| 1. English language needs improvement. I would suggest additional editing by native speaker. | Language editing is performed by an expert. |
| 1. Page 3, line 62 “both gram‐positive and gram‐negative” should be changed to “both Gram‐positive and Gram‐negative”, as stated in Table 1, page 16. | The change in “both Gram‐positive and Gram‐negative…” sentence is made in text. |
| 1. The Authors make a valid statement that the increase of the band intensity in SPR is proportional to the amount of nanoparticles formed, while the band position is directly related to the particles size. Broadening of the SPR peak width is considered an agreeable detector of the nanometal size and its polydispersity, where the range of 320–580 nm is characteristic λmax for AgNPs biofabriction (Govindaraju et al. J Nanosci Nanotechnol 9, 5497–5501 (2009)), where frequency and band width of SPR is not only depending on both size and shape of the metal nanoparticles but also on the dielectric constant characterized the metal itself as well as adjacent medium. However, without TEM results, Authors should elaborate more on their assumption that synthesized particles are about 20 nm in diameter (Section 3.1). | The assumption of particles’ size is additionally elaborated, mostly based on already published results (cited as ref.4).  The inserted text “Based on the literature data that increasing diameter of nanoparticles from 10 to 100 nm leads to a shift of the absorption maxima from 400 nm to 500 nm [19], it’s possible to assume that synthesized particles are about 20 nm in diameter. The prediction of particles' size is additionally supported by already published results where AgNPs were synthesized and stabilized with aqueous extract of *Fumaria officinalis* L. by the same procedure, while the particles' size has been confirmed as 21±1 nm (synthesized at RT) and 18±1 nm (synthesized at BT) by XRD and SEM method [4].” is highlighted in yellow. |
| 1. For the Section 3. 4. Antimicrobial activity, even though the results of inhibition zone widths for the tested microorganisms are given in Table 1 it would be nice to see the photographs of actual plates in the Supplementary material. Also, the presentation of the results and the significant figures given in Table 1 needs to be corrected e.g. “14.5±0.551 for radial diameter of inhibition zone (mm) ± SD” - thus presented result does not make sense. | The photographs are submitted as Supplementary material with a caption:  Figure S1. The photographs of Petri plates illustrating antimicrobial activity of AgNPs-E in different concentrations against some bacterial strains: *B. luteus* (1), *S. aureus* (2), *B. cereus* (3), *B. subtilis* (4)  Radial diameter zone is corrected to Average radial diameter zone and all values are written with one decimal place. |