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Research article

Biosynthesis of gold nanoparticles by two bacterial and fungal strains, *Bacillus cereus* and *Fusarium oxysporum*, and assessment and comparison of their nanotoxicity *in vitro* by direct and indirect assays

Parastoo Pourali [a], Seyyed Hossein Badiee [b,*], Sahebali Manafi [b], Tahereh Noorani [b], Azadeh Rezaei [c], Behrooz Yahyaei [a]

[a] Department of Medical Sciences, Shahrood Branch, Islamic Azad University, Shahrood, Iran
[b] Department of Engineering, Shahrood Branch, Islamic Azad University, Shahrood, Iran
[c] Department of Biology, Neyshabur Branch, Islamic Azad University, Neyshabur, Iran

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ABSTRACT

Background: Although nanoparticles (NPs) have many advantages, it has been proved that they may be absorbed by and have toxic effects on the human body. Recent research has tried to evaluate and compare the nanotoxicity of gold nanoparticles (AuNPs) produced by two types of microorganisms *in vitro* by two different methods. AuNPs were produced by *Bacillus cereus* and *Fusarium oxysporum*, and their production was confirmed by visible spectral, transmission electron microscope, and X-ray diffraction (XRD) analyses. The human fibroblast cell line CIRC-HLF was treated with AuNPs, and the induced nanotoxicity was measured using direct microscopic and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.
Results: The results showed that the produced AuNPs had a maximum absorbance peak around 510–530 nanometer (nm), with spherical, hexagonal, and octagonal shapes and average sizes around 20–50 nm. The XRD results confirmed the presence of GNPs in the microbial culture supernatants. An MTT assay showed that GNPs had dose-dependent toxic effects, and microscopic analysis showed that GNPs induced cell abnormalities in doses lower than the determined half-maximal inhibitory concentrations (IC50s).
Conclusions: In conclusion, the biologically produced AuNPs had toxic effects in the cell culture, and direct techniques such as microscopic evaluation instead of indirect methods such as MTT assay were more useful for assessing the nanotoxicity of the biologically produced AuNPs. Thus, the use of only MTT assay for nanotoxicity evaluation of AuNPs is not desirable.

1. Introduction

Nanotechnology encompasses the production of substances that are produced at atomic scales, and nanoparticles (NPs) are products whose sizes are less than 100 nanometers (nm) [1]. Although NPs have advantageous applications in human life, there is a possibility that they have some toxic effects if they are absorbed into the body through the lungs, the skin, open wounds, and the intestinal tract [2]. It was proved that NPs are introduced to the environment and animal bodies through effluents, disposals, *etc.* [3,4]. Therefore, NPs may impose health risks, and it is important to evaluate their nanotoxicity *in vitro* and *in vivo*.

Depending on the technique used for the production of NPs, there are three types of NPs: physically, chemically, and biologically produced NPs [5]. Each route of production has its own advantages and disadvantages [6]. Among the types of NP production techniques, the biological method is widely accepted because the use of the living organisms in the production pathway is safer than other methods. Moreover, various bacterial and fungal strains have the ability to produce NPs. The types of reduction differ depending on the nature of the active components that are responsible for the bio-reduction process. That is, if microbial enzymes are responsible for the bio-reduction of the induced toxic ions, then the reaction is an enzymatic one, and if the active sites of the microbial products (*i.e.*, different types of polysaccharides or polypeptides) are responsible for the bio-reduction, then the reaction is non-enzymatic [7].

Among the different types of NPs that are produced by the biological systems, the gold ones are known as the most compatible and harmless ones with the human body [8,9]. However, so far there is no available

* Corresponding author.
E-mail address: drh_badiee@yahoo.com (S.H. Badiee).
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report regarding the nanotoxicity of biologically produced gold NPs (AuNPs) *in vitro* and *in vivo*. The present research has been conducted to evaluate the nanotoxicity of biologically produced AuNPs in cell culture. To achieve this, first, microbial strains with the AuNP production ability were purchased. Then AuNPs were produced, and after demonstrating the AuNP production through several techniques, NPs were used for *in vitro* studies.

2. Materials and methods

2.1. Biosynthesis of gold nanoparticles

To produce AuNPs, *Bacillus cereus* (PTCC) and *Fusarium oxysporum* (PTCC) were purchased from the Pasteur Institute of Iran. *B. cereus* and *F. oxysporum* were cultured in a flask containing 150 ml of sterile nutrient broth (NB) and Sabouraud's dextrose broth (SDB) (Merck, Germany), respectively. The bacterial culture was incubated in shaking conditions at 37°C, 150 rpm, for 24 h, and the fungal culture was incubated in shaking conditions at 30°C, 150 rpm, for 72 h. After incubation, the media containing the microbial biomass were centrifuged at 6000 rpm for 10 min, and 100 μl of 1 M HAuCl4 (Sigma Aldrich, USA) solution was added to 100 ml of the obtained microbial supernatant. Then both flasks were incubated in a shaker incubator at 37°C, 200 rpm, for 24 h. Negative control flasks containing 100 ml of sterile NB and SDB with 100 μl of 1 M HAuCl4 at a final concentration of 1 mmol were incubated under the same conditions [10].

2.2. Characterization of gold nanoparticles

2.2.1. Visible spectral analysis

The bio-production of AuNPs was confirmed using a Nanodrop spectrophotometer. The solutions showed maximum absorbance at around 500–550 nm due to the surface plasmon resonance (SPR) of the produced AuNPs. The wavelength ranges were from 400 to 700 nm against the blanks (*i.e.*, sterile NB and SDB) [11].

2.3. Transmission electron microscopy

The exact sizes and shapes of the produced AuNPs were obtained using a Zeiss Leo 910 transmission electron microscope. Briefly, 10 μl of each sample was placed on a carbon-coated grid, and the excess sample was removed using a piece of blotting paper. After drying the sample under an infrared lamp, the electron micrographs were obtained at 80 kV accelerating voltage, and photographs were obtained using a Gatan SC1000 camera [12].

2.4. X-ray diffraction analysis

X-ray diffraction (XRD) was used to confirm the presence of elemental gold in the bacterial and fungal culture supernatants. Briefly, each AuNP sample was freeze-dried, and the obtained powder was heated at 300°C for 1 h. This pre-treatment was used for obtaining sharper Bragg peaks. Then each powder was analyzed using a Philips automatic X-ray diffractometer equipped with Philips PW 1830 X-ray generator, and the diffracted intensities were obtained from 30° to 80° at 2°θ [10].

2.5. Purification of gold nanoparticles

The AuNPs were produced with some microbial secreted proteins, excess of HAuCl4, and other impurities that could affect the cytotoxicity results. Therefore, each AuNP solution was washed three times by centrifugation (14,700 rpm, 30 min) using ddH2O. Finally, each obtained pellets was suspended in phosphate-buffered saline (PBS) and used for further studies [12].

2.6. Sterilization of gold nanoparticles

It is important to prevent any contamination of AuNPs before the cell culture studies. Therefore, the AuNPs suspended in PBS were subjected to tyndallization, which was considered the safest technique for the sterilization of NPs. Briefly, the NP solutions were heated indirectly on the surface of a boiling water bath for 30 min over 3 d. During each interval, the samples were placed in an incubator at 37°C. Finally, the samples were freeze-dried [12].

2.7. In vitro cytotoxicity assay

Equal quantities of each AuNP sample (10 mg) were suspended in 1-ml PBS and used for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The non-cancerous human fibroblast cell line CIRC-HLF was purchased from the Pasteur Institute of Iran and used for the cytotoxicity test. First, the cells were cultured in a T75 flask containing Dulbecco's Modified Eagle's medium (DMEM, Sigma–Aldrich, USA) plus 10% fetal bovine serum (FBS, Sigma–Aldrich, USA) and 1% penicillin + streptomycin (Sigma–Aldrich, USA) at 37°C for 24 h with 5% CO2. In this study, this medium was referred to as the complete medium. After achieving 80% confluent monolayer cells, the cells were detached using trypsin–EDTA. After cell counting, 2×104 cells were seeded in each well of a 96-well microtiter plate containing 200 μl of the complete medium. The microtiter plate was incubated in the abovementioned conditions. After incubation, the obtained confluent monolayer cells were washed using PBS, and each well was filled with 200 μl of 2× concentration of the complete medium. In the first well, 200 μl of the AuNP solution was added, and after mixing, 200 μl of the solution was transferred to the next well. This titration was continued until the 11th well. Finally, 200 μl of the solution in the 11th well was discarded. The 12th well was the positive control and was loaded with cells and 200 μl of the complete medium. The plate was incubated at 37°C with 5% CO2. After 24 h of incubation, the medium was discharged, and micrographs of the cells were taken under a phase contrast microscope with 10× magnification. Subsequently, to evaluate the toxic effects of the AuNPs on the cells, the cells were subjected to 10 μl of MTT (Sigma–Aldrich, USA) dye solution at a concentration of 5 mg/ml in PBS. The cells were then incubated at 37°C for 4 h with 5% CO2. Following this, the dye was discharged and 100 μl of dimethyl sulfoxide (DMSO, Sigma–Aldrich, USA) was added to each well. The plate was placed in a shaker incubator, and after 20 min, the absorbance of each well was measured at 570 nm using the Nanodrop ELISA reader [7].

The half-maximal inhibitory concentrations (IC50s) of the AuNPs used were calculated using the formula described below:

$$IC50 = (\text{OD of 1th well} - \text{OD of 11th well} / \text{OD of 11th well}) \times 100$$

The first well was the well in which maximal growth inhibition was seen (*i.e.*, presence of the highest AuNP content), and the 11th well showed minimal growth inhibition (*i.e.*, presence of the lowest AuNP content) [13].

3. Results

3.1. Biosynthesis of gold nanoparticles

Bio-production of AuNPs was achieved by incubating the microbial culture supernatants with HAuCl4 at a final concentration of 1 mmol. A change in the color of the microbial supernatants from yellow to dark purple after incubation, in contrast to the negative controls, indicated the formation of AuNPs in the microbial culture supernatants.

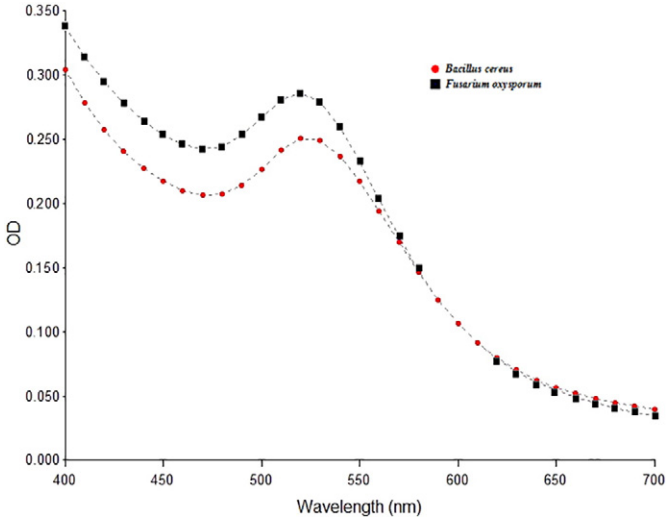

Fig. 1. Visible spectra that were obtained from the diluted culture supernatants of *Bacillus cereus* and *Fusarium oxysporum* after AuNP production.

3.2. Characterization of gold nanoparticles

3.2.1. Visible spectral analysis

Another method for confirming the production of AuNPs in the microbial culture supernatants was the use of a spectrophotometer. Sterile NB and SDB media were used as the blanks, and optical densities (ODs) of the solutions were obtained from 400 to 700 nm. The results showed that the color of both supernatants changed. The supernatant showed maximum absorbance at around 510–530 nm because of the SPR of the produced AuNPs, which was caused by the collective oscillation of free electrons. Fig. 1 shows the visible spectra obtained from the 1:3 diluted microbial culture supernatants after AuNP production.

3.3. Transmission electron microscopy

Transmission electron microscopy (TEM) micrographs that were obtained from both the microbial culture supernatants showed that the produced AuNPs were spherical, hexagonal, and octagonal with irregular contours and sizes of around 20–50 nm (Fig. 2).

3.4. X-ray diffraction analysis

To prove the presence of elemental gold in the microbial culture supernatants, XRD analysis was performed. The XRD results showed the presence of the Bragg peaks of elemental gold at 2°θ values in the microbial supernatant powders. Other peaks seen in the spectra belong to the impurities present in the microbial culture supernatants as the microbial culture supernatant powders were directly used after AuNP production. Fig. 3 shows the XRD result obtained from the *B. cereus* culture supernatant.

3.5. In vitro cytotoxicity assay

The toxicity of pure and sterile AuNPs with sizes of around 20–50 nm that were produced by two different types of microorganisms was measured by microscopic and MTT assays. To achieve this aim, a human fibroblast cell line was used, and the cells were incubated with decreasing doses of AuNPs (decreased by a half). After incubation, morphological changes in the cells were analyzed by phase-contrast microscopy. Fig. 4 and Fig. 5 show the obtained micrograph results. As shown, the morphology of the cells after incubation with different doses of AuNPs had changed, and irregular cells were observed in the wells containing higher doses of AuNPs in each row of the plate. These unusual cells were seen until the 8th well in the case of *B. cereus* and the 10th well in the case of *F. oxysporum*. Some of the unusual cells are shown in Fig. 4 and Fig. 5.

A cytotoxicity assay showed that after incubating the cells with decreasing doses of AuNPs for 24 h, the NPs showed dose-dependent toxic effects, and IC50 of the NPs produced by the culture supernatant of *F. oxysporum* was in the 3rd well (which contained 2.5 mg/ml of AuNPs) and that for *B. cereus* was in the 2nd well (which contained 5 mg/ml of AuNPs) of the microtiter plate (Fig. 6). This indicated that the toxicity of the AuNPs that were obtained from both culture supernatants of the microorganisms were similar to each other.

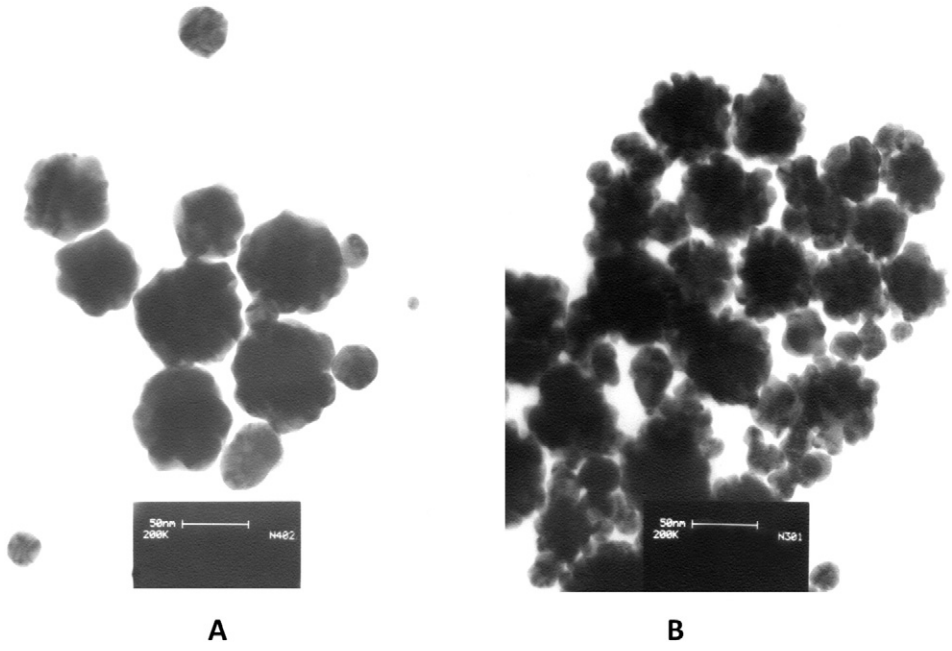

Fig. 2. TEM micrographs of AuNPs that were produced by (A) *Fusarium oxysporum* and (B) *Bacillus cereus* (Scale bars = 50 nm).

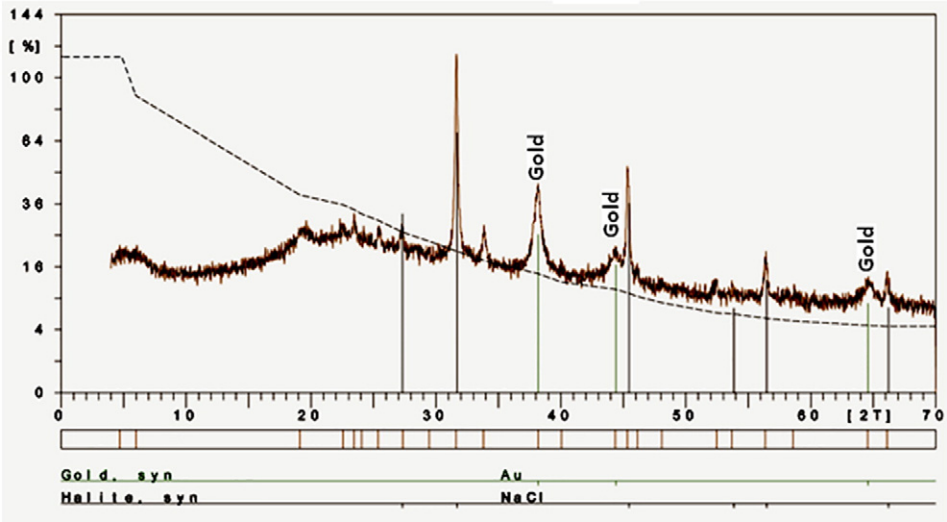

Fig. 3. XRD result after color changing of the culture supernatant of *Bacillus cereus*. The peaks that belong to the elemental gold are indicated in the figure.

4. Discussion

Nanotoxicity is the toxic effects of different types of NPs in biological systems [14]. Each day we are exposed to different types of NPs such as silver NPs that are used in refrigerators, washing machines, and clothes. Some other types of NPs such as titanium dioxide (TiO2) are used in sunscreen lotions [15]. In the field of nanotechnology, it is reported that NPs, because of their nanoscale size and tiny physical dimensions, may have toxic effects [16]. One of the important areas in the nanotoxicity field is discovering safer and well-characterized

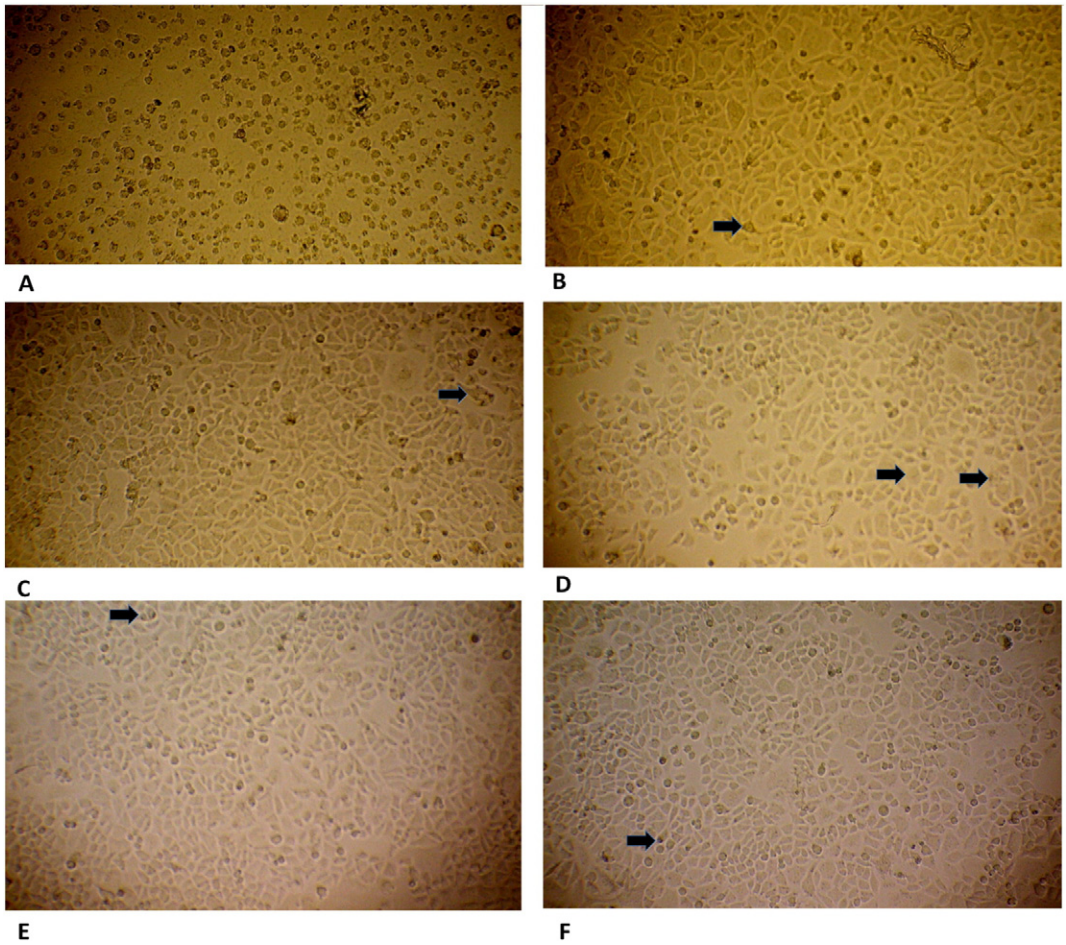

Fig. 4. Micrographs of the cells treated with decreasing doses of AuNPs that were produced by the culture supernatant of *Bacillus cereus*. A to L represent the first to 12th wells in one row of a 96-well microtiter plate. Arrows indicate the unusual cells.

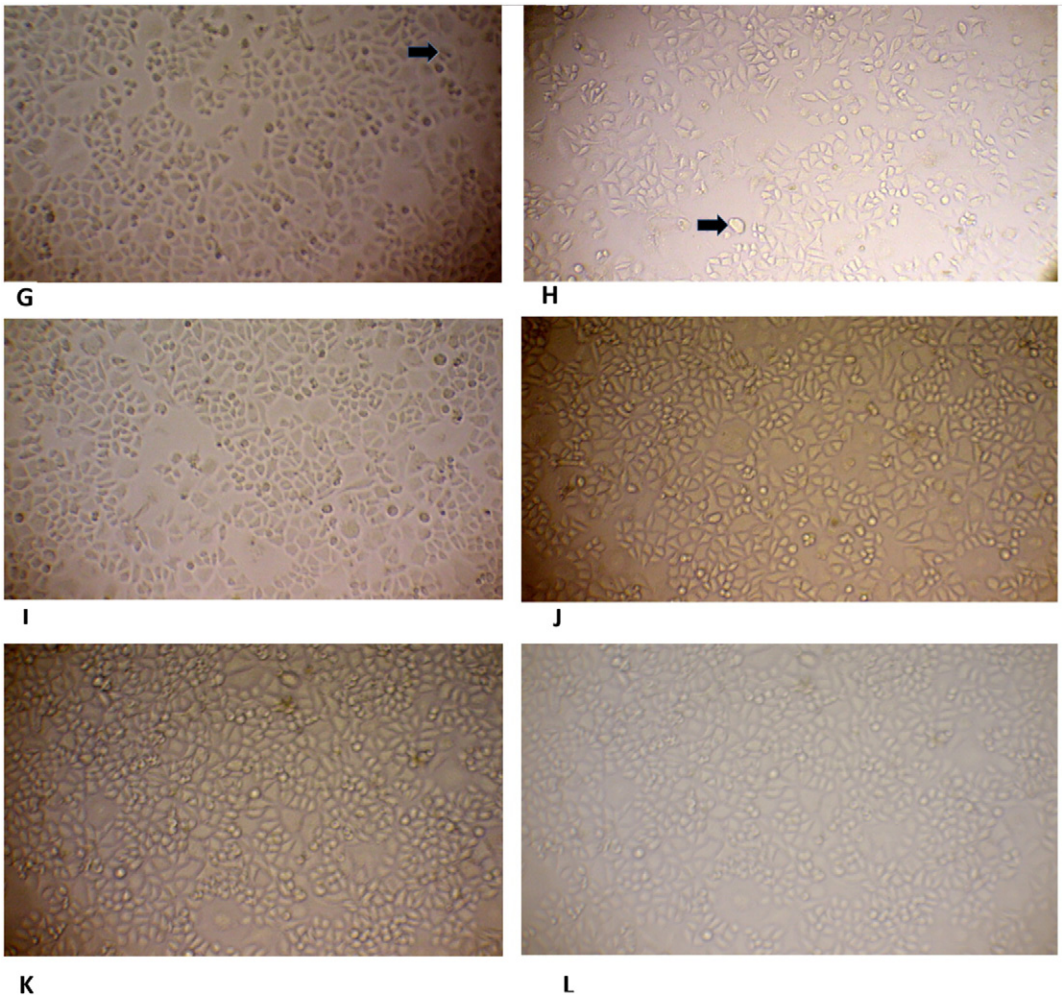

Fig. 4 (*continued*).

physicochemical NPs [14]. Therefore, recently, a new method of NP production called green synthesis has drawn attention and is being researched. In this field, different types of NPs are produced by using different plant extracts and microorganisms. Given the use of these natural agents, the possibility of *in vivo* toxic effects of the produced NPs is very low [9].

As mentioned previously, NPs are produced by enzymatic and non-enzymatic processes. Moreover, depending on whether the corresponding NPs are present within or outside the microbial cells, the NP production type can be classified as intracellular or extracellular [17]. The latter is used widely because the extraction of the produced NPs is easier than with the intracellular method. It means that there is a need for additional techniques for the extraction of the produced NPs from the microbial cells [17]. Therefore, in the present study, the extracellular method of NPs production was used.

Among bio-compatible elements, gold is extraordinarily bio-compatible and known as the safest element. The nanotoxicity of biologically produced AuNPs is not fully understood. Moreover, because AuNPs are used for targeting and imaging in drug delivery, it is important to evaluate their toxicity both *in vitro* and *in vivo* [8]. Recent studies have attempted to understand whether AuNPs that were produced by two types of microorganisms have toxic effects on cell culture.

The first step of this study was to choose microorganisms that were previously reported to have the ability to produce AuNPs. The color of the microbial supernatants after incubation with HAuCl4 changed from yellow to dark purple because of the SPR of the produced AuNPs. It was previously reported that the color ranges are from red to purple and blue or from green to brown depending on the solution's refractive index and shape of the nanoparticles [18,19].

Burda et al. [19] reported that by increasing the size of the produced AuNPs, the plasmon band shifts to red. They reported that if the nanoparticles have spherical shapes, they will have one broad absorption band with a deep red color, but if the nanoparticles are rod shaped, their SPR will split into two bands, whereas for triangular nanoparticles, this will split into three bands with a blue color. In our study, the produced AuNPs were generally spherical in their shape, and their sizes were less than 50 nm.

The presence of AuNPs was confirmed by visible spectrophotometry, TEM, and XRD. The spectrophotometry results showed that AuNPs because of their SPR had a maximum absorbance at around 510–530 nm. The TEM images revealed that AuNPs were present in the microbial culture supernatant, and the sizes and shapes of AuNPs produced by both cultures were similar to each other. Using various magnifications of TEM images, it revealed that the AuNPs were uniform in their sizes. The obtained images showed that the sizes of AuNPs were around 20–50 nm, which fits with the obtained

maximum absorbance peak from the spectrophotometry results according to the study by Link S, El-Sayed MA [20].

The XRD results indicated the presence of AuNPs in the freeze-dried powder of the culture supernatants. The resulting three intense peaks at 38.3°, 44.3°, and 64.8° observed in the XRD spectrum matched the Bragg's reflection of AuNPs (Fig. 3).

The next part of the experiment was the MTT assay. To use the produced AuNPs in the cell culture, it was important to wash and sterilize them. Therefore, the produced NPs were washed using a simple method (using ddH2O and a centrifugation process) and were sterilized using a harmless method (*i.e.*, the tyndallization technique). As mentioned previously, ethanol can be used for cleaning the surface of the NPs, but the most reliable method is the washing method because ethanol as a denaturing agent may affect the nature of the capping proteins that are present on the surfaces of the biologically produced NPs [12]. It means that the surfaces of the biologically produced AuNPs are coated with some microbial secreted proteins, which are called "capping agents"; capping agents are responsible for the stability of the NP structure. The presence of these proteins enables NPs even in the close contact to not clump together. Therefore, this type of NP production can be easily scaled up [21].

To sterilize the washed NPs, the tyndallization process was used because it was reported that the function and structure of biologically produced NPs is altered when the heat treatment is used [12]. Tyndallization is known as a safe method. Using this technique, the NPs will be retained intact [6].

For the *in vitro* toxicity assay, non-cancerous fibroblast cells (CIRC-HLF) were used. This cell line was chosen because fibroblast cells travel throughout the human body and are present in all types of tissues and organs. Therefore, the results obtained from the nanotoxicity assay of AuNPs can be generalized to many organs [22].

As the results show, using the MTT assay, the biologically produced AuNPs had a dose-dependent toxic effect on the cells (Fig. 6); however, this indirect cytotoxicity assay was not a reliable method. This is because, as shown in Fig. 4 and Fig. 5, although in the MTT assay the used AuNPs in the cell culture showed low toxic effects, a high load of unusual cells are seen in the wells with containing nontoxic doses of AuNPs (*i.e.*, lower doses of AuNPs than the determined IC50 wells). From the obtained results, we suggest that in future, for all types of NPs that will be used for the MTT assay, it is better to evaluate the cell shape and structure too. Therefore, only reporting the viability of the cells is not sufficient, and assessment of the effects of the NPs on the cell structure and its appendages are necessary. In previous studies, the nanotoxicity of AuNPs produced by the chemical method was investigated *in vitro*. The results showed that AuNPs enter cells through receptor-mediated endocytosis. Other studies indicated that AuNPs had low cellular toxicity in cultured cells [23,24]. However, it was reported that nontoxic doses of AuNPs had lethal effects *in vivo*. This may be because of the use of

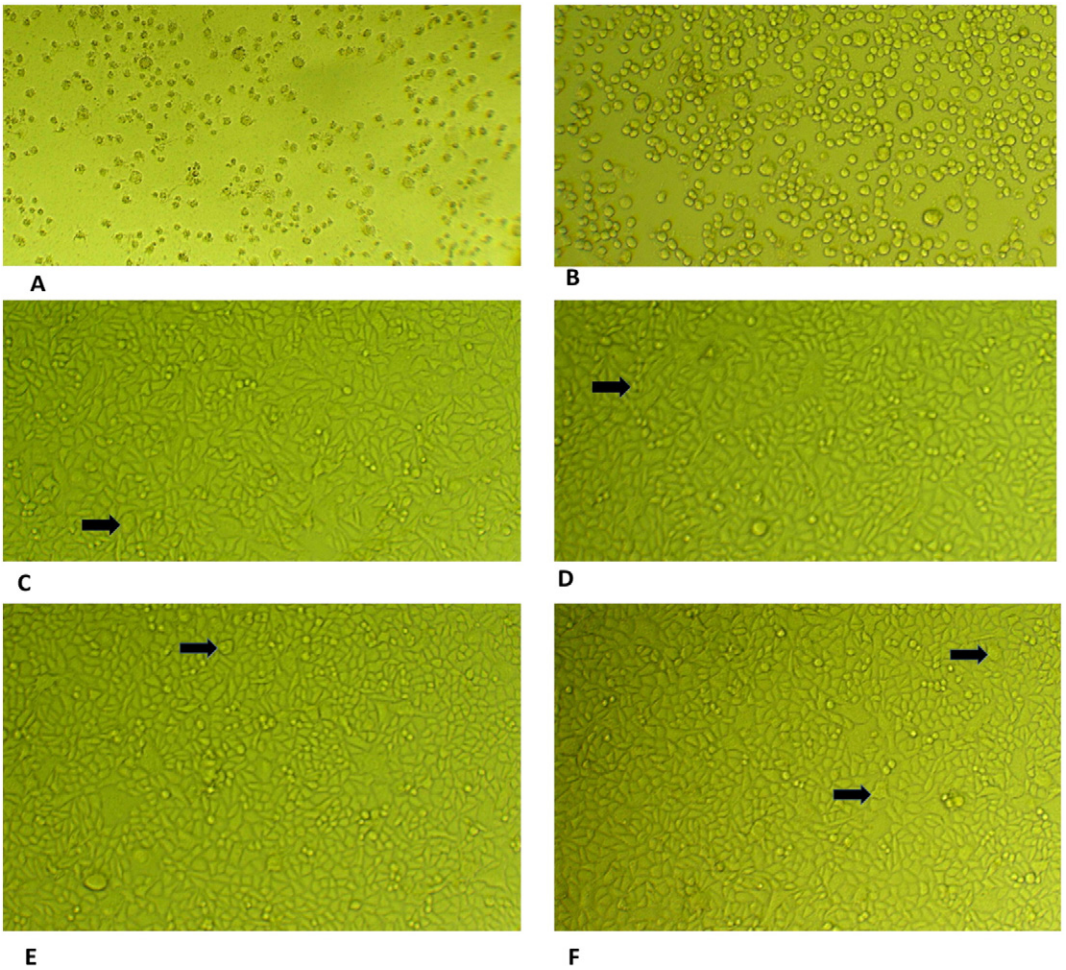

Fig. 5. Micrographs of the cells treated with AuNPs that were produced by the culture supernatant of *Fusarium oxysporum*. A to L represent the first to 12th wells in one row of a 96-well microtiter plate. Arrows indicate the unusual cells.

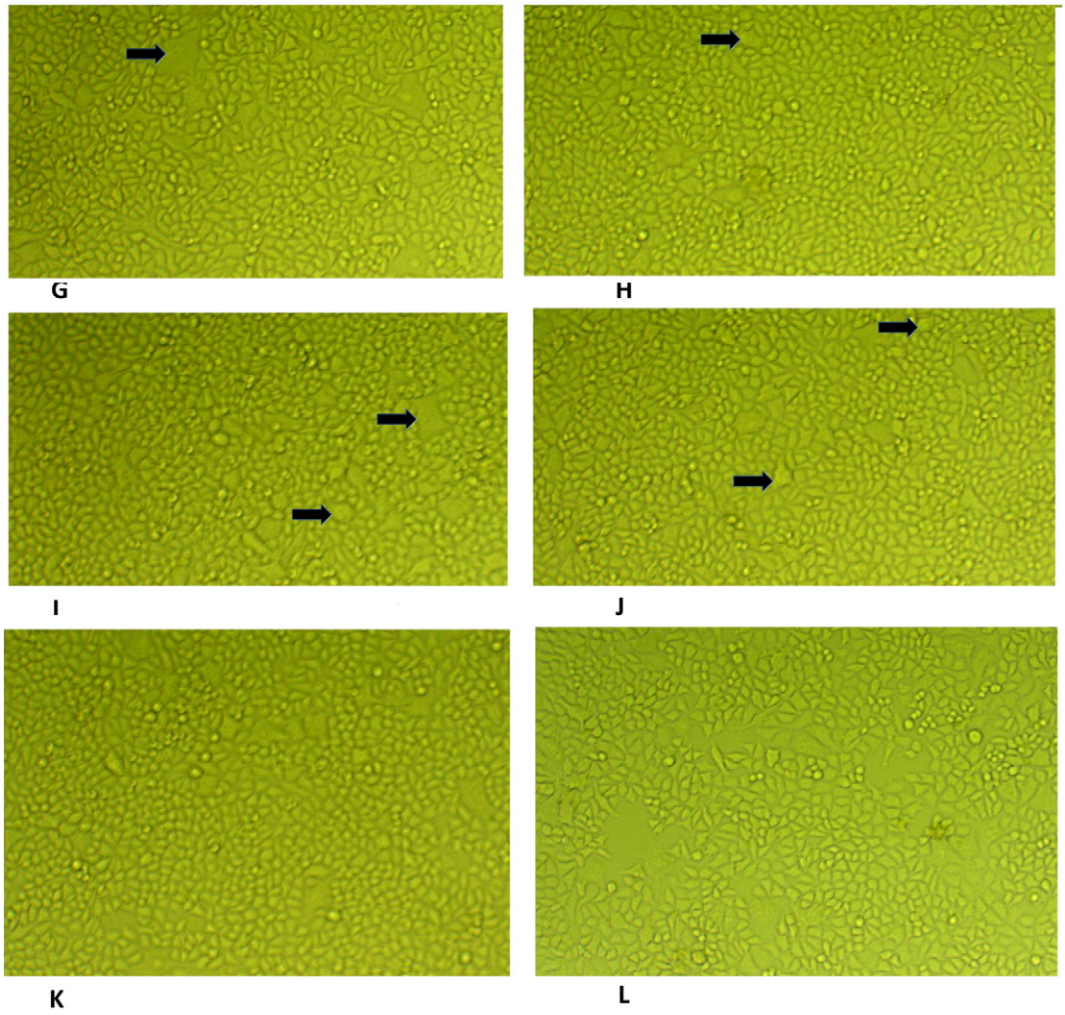

Fig. 5 (*continued*).

nontoxic doses of AuNPs, as determined by the MTT assay. In that research, if the AuNPs were administered at lower doses that do not induce any abnormalities in the cell structure, the obtained results may be altered.

In the future, it is recommended to use the AuNPs at doses that do not induce any abnormalities in the cell structure, and to assess their nanotoxicity *in vivo* compared to using the nontoxic doses obtained by the MTT assay result. More supporting evidence is needed to generalize this result for all types of biologically produced nanoparticles.

5. Conclusions

The current research evaluated the nanotoxicity of two biologically produced types of AuNPs that are known to be compatible in cell culture. The results showed that although the AuNPs were produced

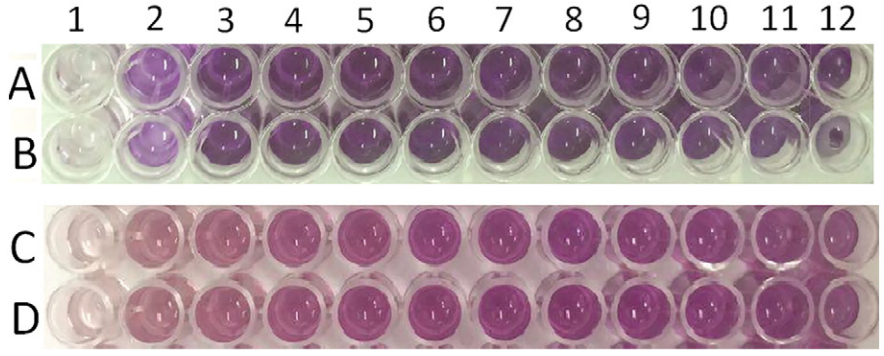

Fig. 6. MTT assay results of the cells treated with AuNPs produced by the culture supernatant of *Fusarium oxysporum* (A and B) and *Bacillus cereus* (C and D). The first well contains the maximum, while the 11th well contains the minimum dose of AuNPs. Lines A and C are the test and B and D are their repeats, respectively.

by the green method of synthesis (*i.e.*, microbial production), both the AuNPs used had toxic effects *in vitro*. The results showed that in contrast to the traditional method of IC50 measurement (*i.e.*, MTT assay), using another precise technique such as microscopic evaluation of the treated cells may be helpful for understanding the nanotoxicity of AuNPs. More supporting evidence is needed to generalize this result, and the nanotoxicity of the other biologically produced nanoparticles should be evaluated in future studies.

Transparency document

The Transparency document associated with this article can be found, in online version.

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