In vitro cytotoxic effect of *Aspergillus clavatus* generated silver nanoparticles on RAW 264.7 cells

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Abstract

Aspergillus clavatus (A. clavatus) was utilized for the synthesis of silver nanoparticles (AgNPs) isolated from the soil. . About one (1) mM silver nitrate (AgNO_3) was gradually added to the aqueous extract of A. clavatus, the colour of the extract becomes dark brown indicates the AgNPs formation. The UV -Vis spectrophotometry showed the lambda max ($-\lambda_{\text{max}}$) at 430 nm. Fourier-transform infrared spectroscopy (FTIR) results revealed the peaks at 3221.40 cm N-H symmetric amide, 1645.78 NH bend amine, 1557.02 N-H bend amide groups are associated with AgNPs. The powder form of AgNPs was recorded for X-ray diffraction (XRD) confirms the crystalline nature of AgNPs. Energy dispersive X-rays (EDX) showed the presence of silver, while zeta potential (ζ -potential) -65mV value confirms AgNPs are stable. Transmission electron microscopy (TEM) revealed AgNPs size ranges from 23.65 to 36.99 nm and is spherical in shape, showing no agglomeration. For cytotoxic effect AgNPs showed IC50 value at 125 $\mu\text{g}/\text{ml}$ against RAW cells through MTT assay.

Keywords

Aspergillus clavatus, X-ray diffraction, Transmission electron microscopy, Cytotoxic effect

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1. Introduction

Nanobiotechnology is the branch of science and technology that controls the materials at a smaller level, especially at nano-level from 1–100 nm called nanoparticles [1]. Nanoparticles can be prepared by using different methods like physical, chemical, and biological (natural). Chemical and Physical methods used for the preparation of nanoparticles are quite laborious and contain some hazardous chemical that is not environmentally friendly [2]. The biological approach used to produce nanoparticles involves using the biological organism as nano factories as a source of reduction (reducing agent), which is non-toxic and environment-friendly [3]. Fungus especially *Aspergillus* are a good source for the reduction of metal ions into metallic nanoparticles which contain a lot of essential enzymes, especially reductase enzymes [4,5]. Different metals are reduced into nanoparticles but using AgNPs has its unique properties with useful application in the biological area, especially biotechnology and biomedical field [6,7]. There are various metallic nanoparticles possess good biological activity, however silver metals are well known for their antibacterial, antifungal, antiviral and anticancer properties and were used as an antibacterial material for a long time [8–11]. These silver nanoparticles also help in the faster growth of hair [9].

Inflammation involves the swelling, redness, pain etc, at a particular area because of the response due to fracture, injury, infection, and toxic bites, etc. The inflammation occurs because of our bodies' immune system activates that cause the dilation of the local blood vessels and neutrophils and monocytes moving towards the affected area. Sometimes this inflammation remains for the longer times causes various harmful severe effects [12]. Many metal nanoparticles, especially AgNPs, contain a good anti-inflammatory property to reduce inflammation [13].

Presently many treatments are available to cure the malignant cells, especially chemotherapy, but however these drugs not only destroy the cancerous cells but also healthy cells. Hence this study was carried on the synthesis of nanomaterials specific to the cancerous cells to minimize the side effect with higher availability of the drug to the target cells. Therefore, this study reported the synthesis of AgNPs from *Aspergillus clavatus* through the extracellular method. Characterization of these AgNPs was performed by

FTIR, UV–Vis spectrophotometer, Zeta potential, XRD, TEM, and EDX techniques. These AgNPs were evaluated for cytotoxic effect against RAW 264.7 cells and compared with healthy cells through MTT assay were studied.

2. Material and methods

2.1. Sample collection

Soil sample was collected from UNIKL RCMP Green town Ipoh Malaysia. The soil sample was brought to a microbiology laboratory and dried for 2–3 days. The soil sample was serially diluted from 10^{-1} to 10^{-5} to decrease the fungal spore load.

2.2. Isolation and identification of fungal species

Soil sample 1 g was serially diluted and spread on PDA (potato dextrose agar) media and incubated for 3–4 days. *A. clavatus* was separated from the other species, and pure culture was grown on separate media. The *A. clavatus* was identified by the morphology of the culture and observed through the microscope and according to laboratory manuals.

2.3. Preparation of AgNPs

The isolated *A. clavatus* was used for the synthesis of AgNPs. The fungal biomass was produced by inoculating fungi in the 100 ml of PDA broth in a conical flask. The entire broth was put on the orbital shaker to let the growth of fungi. After three days the entire biomass was filtered through Whatman filter paper to remove the entire media components by washing the biomass with Milli-Q water. The entire biomass was again, resuspended in the 100 ml of Milli-Q water and put on an orbital shaker for 72 h so that all the necessary enzymes will be released from the biomass. After three days, again the whole biomass was filtered, and the filtrate was utilized for the biosynthesis of AgNPs by adding 1 mM (0.017 g) silver nitrate [14].

2.4. Characterization of silver nanoparticles (AgNPs)

The change in the fungal extract is the first indication for the synthesis of AgNPs. These synthesized AgNPs were characterized by UV–visible

Fig. 1. Synthesis of AgNPs from *A. clavatus*- colour change after the addition of AgNO_3 at different intervals of time.

spectrophotometer. The lambda max was taken from 300 to 600 nm wavelength. The FTIR (Nicolet) was used to check the various proteins and other functional groups associated with AgNPs by using infrared spectroscopy. Zeta potential was used to determine the stability of nanoparticles measuring the electrostatic charge of nanoparticles. For XRD (Rigaku Smart Lab) analysis, the powder form of AgNPs was subjected to

the X-ray diffraction pattern to check the crystalline nature of the AgNPs. The EDX was used to determine the elemental chemical composition of the AgNPs. TEM (Zeiss Libra ®120) analysis, used to reveal the nanoparticles dispersity, size and shape. For TEM analysis, 5 mL of AgNPs solution was sonicated for 3 min, and one drop of the AgNPs was put on the copper coated-grid and observed under TEM.

Fig. 2. UV–Visible spectrophotometric analysis of AgNPs synthesized from *A. clavatus*.

Fig. 3. FTIR analysis of silver nanoparticles showed various functional groups associated with AgNPs.

2.5. Cytotoxic effect through MTT assay

2.5.1. Cell culture collection

The RAW 264.7 cells and L929 cells were brought from the national center of cell studies (NCCS) Poona India. Dulbecco's Modified Eagle's medium (DMEM) was used to grow the cells containing antibiotic antimycotic 1 M solution in the presence of fetal bovine serum (FBS) 10% under carbon dioxide (5%) environment at 37 °C.

2.5.2. MTT assay of AgNPs

The RAW 264.7 cells and L929 cells were grown on the 96 well plate with the concentration of

1×10^4 cells/well. Each well was treated with AgNPs concentration ranges from 25, 50, 75, 100, 125 µg/ml media free from serum, and later incubated for 24 h. After the incubation medium was aspirated at the end of the treatment period, after this, 0.5 mg/mL MTT dye was prepared in 1 M phosphate buffer saline (PBS). It was added gently to all wells and again incubated for 4 h at 37 °C in presence of CO₂ environment. After incubation, the MTT dye was discarded from each well and later washed with 200 µL of PBS. The formazan crystals formed were dissolved with 100 µL of DMSO and mixed thoroughly. The colour development intensity was measured at a wavelength of 570 nm. The conversion of formazan crystals into purple-blue

Fig. 4. Zeta potential showed the higher value confirms the stability of AgNPs.

Fig. 5. XRD diffraction pattern of AgNPs showed the theta value at 40 kV, 30 mA.

colour and absorbance was measured using a 570 nm microplate reader.

3. Result and discussion

A. clavatus was used for the synthesis of AgNPs. About 1 mM (0.017 g) silver nitrate (AgNO_3) was put

into the 100 ml of fungal extract, the colour of the solution alters into dark brown indicates the formation of AgNPs, as shown in Fig. 1. The UV–visible spectrophotometry showed the lambda max ($-\lambda_{\text{max}}$) at 435 nm specific for the AgNPs confirms the formation of nanoparticles (see Fig. 2). The effect is due to the plasmon surface resonance and interband transition of

Fig. 6. EDX analysis of AgNPs confirms the presence of silver.

the AgNPs [15]. The colour production of the nanoparticles in which light acts as the primary source of the catalyst induces the transfer of electrons and causes the reduction of metal ions into metallic nanoparticles [15]. It has been reported from the previous research that fungal species produce the NADPH reductase involves the reduction of silver ions (AgNO_3) into AgNPs [16].

The FTIR analysis was used to determine the various proteins, functional groups associated with the AgNPs. FTIR analysis showed sharp peak at 3421.40 cm^{-1} N–H stretch primary amine, 1645.78 cm^{-1} NH bend amine, 1557.02 cm^{-1} N–H bend amide, 1384.68 cm^{-1} CH_3 C–H bend alkanes and 1067.29 cm^{-1} C–O stretch alcohol. These

functional groups and proteins are associated with nanoparticles not only helps in the stabilization but also prevent them from agglomeration [17] Fig. 3.

Zeta potential (ζ -potential) was used to check the stability of AgNPs based on the nanoparticle charge due to electrostatic repulsion. The result showed the high value -65 mV confirms the nanoparticles are stable (Fig. 4).

The XRD analysis of AgNPs showed the sharp diffraction pattern of 2-theta (deg) value at 27, 32, 46, 54, 57, and 67 shown in Fig. 5. XRD analysis confirms that AgNPs are in well-defined, precise dimensions and show purity and crystalline nature of the AgNPs. Energy dispersive X-rays were used to determine the various constituents present with AgNPs. The EDX

Fig. 7. TEM analysis of AgNPs showed particles are spherical and well distributed at different magnifications.

results clearly showed the sharp silver peaks confirm the presence and purity of the AgNPs, as shown in Fig. 6.

The TEM analysis of AgNPs shown was well dispersed, no sign of agglomeration and particles were spherical with size ranges from 23.65 to 36.99 nm Fig. 7. The smaller size of these nanoparticles playing a pivotal role in biological activity shows good promising results like antibacterial, antifungal, and cytotoxic function.

These biologically synthesized AgNPs were evaluated for its cytotoxic effect against RAW 264.7 cells through MTT assay. These RAW 264.7 cells are macrophage derived from the mice BALB/C. Five concentrations of AgNPs were used from 25, 50, 75, 100, and 125 $\mu\text{g/ml}$ on dose-dependent manner. These AgNPs showed IC 50 value at 125 $\mu\text{g/ml}$, as shown in Figs. 8 and 9.

However, these AgNPs were evaluated for its cytotoxic effect on healthy L929 cells using the same concentration as used on RAW cells and results were shown in Figs. 10 and 11. This cytotoxic effect is due to the formation of ROS generated by the AgNPs that damage proteins and nucleic acid, which has a strong

affinity towards the sulfur group. Hence interferes with functional aspects of proteins, and nucleic acids make them vulnerable and dysfunctional thereby causes the inhibition and apoptosis of cell [18,19]. One study suggested that smaller silver nanoparticles induces great production of ROS formation and larger the size of AgNPs induce less ROS production with less toxicity and showed that 15 nm produces more cytotoxicity than 55 nm particle size on macrophage cell [20]. Another study reported that smaller-sized silver nanoparticles 5 nm generates more cytotoxicity than 15 nm particle size on the cells [21]. Another study on different cell lines like J774.1, RAW 264.7, A549, HepG2, A498, and neurons by using the silver nanoparticles from 5 to 43 nm showed some unique results on macrophages for its good sensitivity towards these cells [22]. The silver nanoparticles enter the cell by scavenger pathway and then into cytoplasm thereby releasing the Ag^+ ions to induce the cytotoxicity. The cytotoxicity of the silver nanoparticles is due to the release of silver ions into the biological and environmental media, thereby enters the cell through diffusion causes mitochondria dysfunctional [23]. This study, clearly showed the effect of AgNPs on both

Fig. 8. Cytotoxicity effect of AgNPs on RAW cells by applying different concentrations where C-control, A-125 $\mu\text{g/ml}$, B-100 $\mu\text{g/ml}$, D-75 $\mu\text{g/ml}$, E-50 $\mu\text{g/ml}$, F-25 $\mu\text{g/ml}$.

Fig. 9. Graphical representation of AgNPs effect on RAW cells on the dose-dependent manner.

Fig. 10. Effect of AgNPs on healthy L929 cells by using different concentrations where C-control, A-125 µg/ml, B-100 µg/ml, D-75 µg/ml E-50 µg/ml, F-25 µg/ml.

macrophage RAW cells and healthy cells synthesized from an aqueous extract of *Aspergillus. clavatus*. AgNPs synthesized from, aqueous extract of *A. clavatus* showed the good effect on RAW cells, and AgNPs synthesized were well distributed, spherical and stable.

Also fungal extract contains necessary proteins that played an important role in the stabilization of nanoparticles. These nanoparticles could become good cytotoxic agent but need further cytotoxicity study on healthy cells before using as cytotoxic agent.

Fig. 11. Graphical representation of AgNPs effect on healthy cells on dose-dependent manner.

4. Conclusion

A. clavatus isolated from soil showed robust capability for the production of AgNPs. Microscopic examination reveals particles are spherical and are well dispersed with no agglomeration. These AgNPs showed good cytotoxic effect against macrophage RAW cells. Thus, these AgNPs could be used as a cytotoxic agent but needs further toxicity studies on healthy cells and animal models before using them as a cytotoxic agent.

Conflicts of interest

No conflict of interest among authors.

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