**In vitro antimicrobial and cytotoxicity efficacy of gold nanoparticles synthesized from Alternaria brassicae (KF934409)**

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Received: October 4, 2016; Accepted: October 24, 2016; Published: November 4, 2016

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**Abstract**

Nanotechnology is emerging as one of the most revolutionizing area in research field. Biological methods of reduction of metal ions are often preferred because they are non-toxic, safe, clean, biocompatible and environmentally acceptable. Alternaria brassicae was isolated from rhizospheric region and used for the extracellular synthesis of gold nanoparticles (AuNPs). Further, the study was performed to estimate the antibacterial, antifungal and cytotoxic efficacy of AuNPs. The immunomodulatory efficacy of AuNPs was investigated on various pathogenic microorganisms and human macrophages cell lines (THP1-α). Stable AuNPs were produced when an aqueous solution of chloroauric acid (HAuCl₄) was reduced by fungal biomass. Production of nanoparticles was confirmed by the color change from yellow to pinkish violet after approximately 72 hr of reaction. The produced nanoparticles were then characterized by UV-vis spectroscopy, DLS and Transmission electron microscopic analyses. The TEM images of sample revealed that the AuNPs were spherical and hexagonal in shape. Biosynthesized AuNPs were ranged in size from ~15-72 nm. These AuNPs were also assessed for their antimicrobial and cytotoxic efficacy. The results indicated towards efficient antimicrobial activity of AuNPs against Enterobacter aerogenes, Bacillus cereus and Trichoderma sp. at higher concentrations. Cytotoxicity analysis of AuNPs on THP1 α cell lines revealed dose dependent behavior. In conclusion, this study proposed the inimitable character of AuNPs prepared from A. brassicae. AuNPs synthesized by this fungus can be either used as cost effective antimicrobial, insecticidal agents or nontoxic immunomodulatory delivery vehicle.

**Keywords:** Alternaria brassicae; Antimicrobial; Cytotoxicity; Gold Nanoparticle

**Introduction**

Nanotechnological study mainly focuses on the advancement of man-made as well as natural systems for the synthesis of materials at nanoscale level [1]. In recent years, metal nanoparticles have been the subject of interest due to their unique chemical, physical and optical properties [2]. These nanomaterials have a widespread range of implications not only from catalysis to agriculture but also from electronics to biomedicine which is due to their small dimension and large surface area. It was reported that the easiest method for the synthesis of nanoparticles is the reduction of their respective salts [4] (S-1). Nanoparticles can be synthesized from physical, chemical and biological methods. Physical and chemical methods like sonochemical processing, cavitation processing, UV irradiation and high energy ball-milling proved as an expensive, toxic and involve the use of harmful chemicals. Hence, in order to produce the nanoparticles by safe, secure, biocompatible eco-friendly methods, many biological systems have been used to produce the nanoparticles [5]. Some well known examples include the use of bacteria, fungi and plants for their production. Accumulation of metal ions by microbes has been regarded as low-cost, environment-friendly and easily achievable phenomenon. However, fungi, in particular, are a favored choice for the synthesis of different nanoparticles due to their high tolerance towards metals, high wall-binding capacity, can be easily scaled up, ability to secrete large amount of enzymes [6] and due to their capability of accumulating metals by physicochemical and biological mechanisms. The fungal supernatants were used for synthesis of different types of nanoparticles [7, 8]. Scientist used Fusarium oxysporum for both intracellular and extracellular production of gold nanoparticles [3, 9]. The rapid reduction of metal ions resulting in the formation of stable gold nanoparticles of variable sizes and shapes was reported [26]. It was reported that cell-free filtrate containing NADPH-dependent enzyme of Epicoccum nigrum, when react with the aqueous chloroauric acid solution, it has the ability to synthesize gold nanoparticles.
at ambient temperature [25]. However, AuNPs production by *Alternaria brassicae* was not reported yet. The present investigation is an effort to synthesize gold nanoparticles from *A. brassicae* (GenBank: KF934409), a phosphate solubilizing fungus, isolated from rhizospheric region of leguminous plant from Central Institute of Medicinal and Aromatic Plants (CIMAP) lawn (29ºN 79.38, 243.84 MSL). The potential antimicrobial and cytotoxic activity of these myco-nanoparticles synthesized has been evaluated.

**Material and methods**

**Isolation, screening and characterization of A. brassicae (KF934409)**

The fungus was isolated from soil of vegetative region near Central Institute of Medicinal and Aromatic Plants (CIMAP) lawn (29ºN 79.38, 243.84 MSL). Lucknow, India and screened by its phosphate solubilizing efficiency on Pikovskaya’s medium by plate assay [15]. The fungal colonies were subculture on fresh potato dextrose agar (PDA) media and the plates were further incubated in inverted position for 72 hr at 28 ± 4º C for halo formation around the fungal colony. Further the biochemical characterization of the fungus was tested on the basis of colony morphology, conidial microscopic analysis, and phosphate solubilization index (SI), starch and cellulose hydrolysis test according to standard protocols [16]. However, the molecular characterization of fungus was done by using 18S rRNA sequencing. Genomic DNA of selected fungus was isolated and characterized by using 18S rRNA gene segments determined in the present study. Evolutionary analysis was performed. The nucleotide sequences were further aligned by means of ClustalW2 program and its molecular and phylogenetic evolutionary analysis was performed. The nucleotide sequences of 18S rRNA genes segments determined in the present study have been submitted in GenBank database under accession number KF934409.

**Biomass preparation and extracellular biosynthesis of gold nanoparticles**

MGYP (Maltose, Glucose, Yeast and Peptone) broth comprising of malt extract (0.5 %), glucose (1 %), yeast extract (0.3 %) and peptone (0.5 %) was used for the growth of fungus aerobically. The culture was incubated at 28º C and harvested after 120 hr of growth by sieving through a plastic sieve followed by washing with sterile double-distilled water. Initially, 20 g of biomass (wet weight) was transferred to 100 ml deionized water for 48 hr at 28º C in an Erlenmeyer flask and agitated at 120 rpm for the release of secretory proteins. 1 mM of gold tetra hydrate was added to the Erlenmeyer flasks and the reaction was allowed to proceed under dark conditions for synthesis of AuNPs. Time-dependent formation of AuNPs was observed by using ultraviolet-visible spectrophotometer (Beckman DU-20 spectrophotometer). The scanning range was 400-800 nm for AuNPs at a scan speed of 420 nm/min. The data was performed using “UWWinlab” software.

**Characterization of gold Nanoparticles**

**Differential light scattering (DLS):** AuNPs suspensions were prepared in distilled water (dH₂O) by using a bath-sonicator (ULTRasonik 57 X, 50/60 Hz, California, USA) for measurements of nanoparticles size. Viscosity measurements were performed on dH₂O with the help of a Viscometer SV-10 (A&D Instruments Ltd., UK) at 25ºC and the recorded values were used in all DLS size estimations. The viscosity of dH₂O at 25º C was 0.887 centipoise. DLS size measurements were performed with the aid of a Malvern Zeta Sizer Nano ZS (Malvern Instruments, Worcestershire, UK) working at version 5.03 of the systems Dispersion Technology Software (DTS Nano). The samples for DLS were equilibrated at 25º C for 3 min before each measurement. The refractive index (RI) of AuNP, dH₂O was 1.430.

**Transmission electron microscopy (TEM):** For TEM analysis, the synthesized AuNPs were prepared by placing a drop of synthesized nanoparticles over gold coated negative grid followed by evaporation of the solvent [18]. TEM analysis was performed on Perkin-Elmer model, which was operated at an accelerating voltage of 1000 kV (JEOL, JEM-1000).

**Antibacterial efficacy:** *Pseudomonas sp, Enterococcus faecalis, Klebsiella pneumonia, Escherichia coli, and Strepptococcus pyogenes* were procured from National Chemical Laboratory (NCL), Pune following by their sub culturing on nutrient broth media at 37ºC. The turbidity (OD600) was sustained at 0.8 corresponding to ~1 × 10⁸ CFU/ml. Agar well diffusion assay was used to analyze the antibacterial efficiency of synthesized AuNPs [19] where 1 ml of each bacterial culture was placed on Muller Hinton agar medium (bactef extract 2 gm/l; casein acid hydrolysate 17.5 g/l; starch 1.5 g/l; agar 17.0 g/l). 5 mm diameter wells were prepared and filled with a range of concentrations of AuNPs (15 μM, 75 μM, 150 μM, 250 μM). Antibiotic tetracycline at a concentration of 25 μg/ml was used as control (Ab). Further potential of AuNPs at a concentration of 200 μM was analyzed along with 25 μg/ml antibiotic tetracycline (Ab + AuNPs). Plates were incubated overnight at 37º C for overnight. All experiment was carried out in triplicate and the average zone of inhibition, excluding the diameter of well, was recorded for each bacterial species in centimeters.

**Antifungal efficacy:** The antifungal activity of AuNPs was tested against *Aspergillus fumigatus* and *Penicillium marneffei* by agar well diffusion method [20]. Aliquot of 50 μl spores suspension (~1 x 10⁸ spores/ml) of each isolate was streaked in radial patterns on the surface of PDA media plates (per liter: sliced washed unpeeled potatoes 200 g, dextrose 20 g, agar 20 g). Further, 5 mm of agar wells in diameter were prepared and filled with a range of concentrations (100 μM, 150 μM and 200 μM) of AuNPs. Further potential of AuNPs at a concentration of 200 μM was analyzed along with 25 μg/ml antibiotic Amphotericin B (Ab)
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+ AuNPs). The cultured plates were incubated at 28˚C ± 4˚C for 7 days. The average inhibition zone, excluding well, for each case was measured in centimeters.

**Maintenance of cell lines:** THP1 α (Human macrophage cell line) was obtained from the National Center of Cell Sciences, Pune, India and further preserved at Animal Tissue Culture facility of Central Drug Research Institute (CDRI). Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % Foetal calf serum (FCS) and 1 % antibiotic-antimycotic solution was used for cell culture maintenance at 37˚C and 5 %CO₂ using standard cell culture methods.

**Cytotoxicity assay:** In order to analyze the cell viability of human macrophages cell lines (THP1 α) was used. It was determined by MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] conversion assay [21]. 1 x 10⁶ cells/ml were plated in 96-well culture plates and incubated with rising concentrations of nanoparticles (10 μM, 25 μM, 50 μM, 100 μM, 150 μM and 200 μM) for 24 hr at 37° C in CO₂ incubator. The MTT dye was further added to each well and plate was incubated at 37° C for 4 hr. The absorbance of insoluble formazan salts was evaluated at 550 nm using spectrophotometer “Powerwave XS “BIOTEK, USA” [22]. Data analyzed were used to plot a dose-dependent reaction curve and the metal nanoparticles concentration required to kill 50 % of cell population (IC₅₀) was determined.

**Cell viability (%) = Mean OD × 100 /Control OD**

**Statistical analysis:** All the experiments were performed in triplicates and results were expressed as mean ± SD. One-way analysis of variance (ANOVA) with a Dunnett’s test was performed for the multiple comparisons for normally distributed samples with homogenous variance. Statistically significant differences were set at p < 0.05.

**Results and discussion**

**Synthesis of fungal AuNPs**

Alternaria brassicae, secretes numerous enzymes like nitrate reductases, lignin peroxidase, laccase and phosphatase whose potential have been checked in various fields of industrial, pharmaceuticals and commercial purposes [23]. Fungi have been reported a better nanoparticles producers because of their toxicity reception, bioaccumulation, simple downstream processing, comparatively economic in comparison with others, and easy biomass handling [24]. Considering this fact AuNPs were synthesized from fungus A. brassicae where the supernatant of fungus was used as reducing and stabilizing agent for 1 mM of gold tetrahydrate salt. The appearance of a pink color in solution gave the clear indication of the formation of AuNPs (Figure 1). Reduction of gold ions was reflected in the color of the solution, which varied from pale-yellow to pink. One mM HAuCl₄ used as control, when subjected to similar conditions did not demonstrate any color change.

**Characterization by spectrophotometric, DLS and Transmission electron microscopic analyses (TEM)**

No indication of absorption in the range of 400-800 nm for the fungal extract was shown by UV-vis spectrophotometer while the fungal extract when exposed to HAuCl₄ showed a distinct absorption at around 540 nm (Figure 2a). Such type of peak is in accordance with the earlier report on metal nanoparticles which ranged between 10-100 nm [10]. DLS spectra showed an intensity of 79.82 nm for AuNPs (Figure 2b). This technique enables the particle size determination by measuring the changes in the light intensity scattered from a suspension or solution. TEM micrographs of AuNPs depicted two different forms viz, spherical and hexagonal formed. This was further confirmed by the representative images recorded from the drop-coated film of the gold nanoparticles uniformly dispersed on grid. The size of the AuNPs ranged from 72 nm (hexagonal), 28 nm and 15 nm (spherical), respectively (Figure 3). The size variation is due to oxidation of metal salts into their relevant nanoparticles in the presence of reductase enzymes.

**Antibacterial activity**

The bactericidal activity of AuNPs were studied using the pathogenic strains of bacteria, namely E. faecalis, E. coli, K. pneumonia, Pseudomonas sp., S. pyogenes using agar well diffusion method. The results recorded in centimeters for

**Figure 1:** Nanoparticles formation on the basis of colour change due to surface Plasmon

**Figure 2:** (a) UV-VIS spectrophotometry of AuNPs (b) DLS spectrum of AuNPs
AuNPs are shown in Figure 4. The comparative histogram demonstrated that the best antibacterial efficacy of AuNPs was against *Pseudomonas sp.* and *E. coli*, followed by *K. pneumonia*. Marked increase in antibacterial activity was demonstrated with increasing concentration of AuNPs. In addition, the efficacy of AuNPs was found to be enhanced in combination with the antibiotic tetracycline rather than alone.

Nanogold is an effective and a fast-acting microbicide against various pathogenic bacteria and fungi that have been utilized in various fields like in the industrial, medical field [11]. In the present study, somewhat similar results were also obtained where gold nanoparticles exerted antibacterial activities along with the standard antibiotic (tetracycline) against *Pseudomonas sp.* and *E. coli*. It is reported that AuNPs reacting with sulfur containing proteins in the cell interior as well as compounds containing phosphorous such as DNA will affect the cell division and respiratory chain in bacteria, ultimately causing the cell death [12].

**Antifungal activity of AuNPs**

The colloidal AuNPs inhibited the growth of the fungus (*A. fumigates* and *P. marneffei*) which was seeded in the Muller Hinton agar plate forming a zone of inhibition around the central cavity. The zone of inhibition with diameter of 1.3 cm was recorded in case of *A. fumigates* and 1.5 cm in *P. marneffei* (Figure 5). The antifungal activity is due to the disruption of membrane bound enzymes and lipids or formation of insoluble compounds by inactivation of sulfhydryl groups in the fungal cell wall which causes cell lysis [13].

**In vitro cytotoxicity assay**

The effect of gold nanoparticles on the cell viability of THP1 α (human macrophage cell lines) was assessed by MTT assay. Various concentrations of gold nanoparticles for cells treatment (10 μg/ml, 25 μg/ ml, 50 μg/ml,100 μg/ml and 150 μg/ml) for 24 hr. There is no significant difference on cell viability at concentration of 10 μg/ml. The cell viability was reduced in a dose-dependent manner, in the cell lines and the significant cytotoxicity was observed from 25 μg/ml and above concentrations (Figure 6), for cell line which might be due to over-accumulation of metal nanoparticle inside the cell.

Metal nanoparticles can bring toxicity at various degrees. However, it is suggested that higher concentrations of AuNPs are toxic and can cause various health problems. AuNPs, however, possess moderate toxic activity against cell line. This might be due to small particle size of AuNPs with enormous specific surface area, which assisted further expression and ion dissolution thus, potentially leading to increased toxicity. Since these NPs are exhibit oxidative potential, reactive in nature and have ability to bind with proteins and DNA, resulting in generating the disturbance in the working of biomolecules [14].

The present study supported the notion as higher doses of gold nanoparticles exhibited the cytotoxicity on cell line used. These results direct towards the potential use of biologically synthesized gold nanoparticles as an anti-microbicide, occupying

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**Figure 3:** Transmission electron microscopic analysis of AuNPs

**Figure 4:** Comparative analysis of antibacterial activity AuNPs for different pathogenic bacteria. Results are presented in relative units compared with controls (Ab). Data represent the mean ± standard deviation of the mean of three individual experiments. *p* < 0.05.

**Figure 5:** Comparative analysis of antifungal activity AuNPs for different pathogenic fungi. Results are presented in relative units compared with controls (Ab). Data represent the mean ± standard deviation of the mean of three individual experiments. *p* < 0.05.

**Figure 6:** Dose-dependent effect of AuNPs over cell viability using MTT assay on THP1 α cell line. Results are presented in relative units compared with controls. Data represent the mean ± standard deviation of the mean of three individual experiments. *p* < 0.05.
In conclusion, a simple, stable and eco-friendly method of biosynthesizing AuNPs was successfully developed using Alternaria brassicae fungus, identified it on the basis of morphological, biochemical and molecular techniques. This fungus produces extracellular, protein capped, water dispersible and highly stable gold nanoparticles, when reacted with aqueous solution of HAuCl₄. Gold nanoparticles produced by A. brassicae were in the size range of 15 nm to 72 nm and found to be spherical or hexagonal in shape and had irregular morphologies which were confirmed by SEM analysis. Furthermore, these functionalized AuNPs were more effective as antibacterial agents both against gram positive and gram negative bacteria. The AuNPs generated here also proved as a promising antimicrobial agent against both Gram-positive/Gram-negative bacteria and pathogenic fungi also. All AuNPs samples showed, good free radical scavenging activity. Finally, the AuNPs showed significant cytotoxicity against THP1α (human macrophage cell lines). On the contrary, AuNPs, which were found to be nontoxic in cytotoxic assays, can be used as a vehicle for drug delivery. However, higher doses of gold nanoparticles exhibited the cytotoxicity on THP1α cell lines. The fungal strain used in this study is likely to provide broad-spectrum benefits such as its efficacy in the solubilization of insoluble phosphate into soluble form, used to generation of insoluble phosphate into soluble form which enhances the activity of antibiotics against selected human bacterial pathogens. Int J Pharm Sci Res. 2012;3(5):1415–1422.

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