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Biological synthesis of gold nanoparticles using endophytic fungi

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ABSTRACT

The myriad uses of gold nanoparticles in the field of medicine have forwarded research to find the best possible way to synthesize them with better size manipulation. Biological synthesis of nanoparticles has attracted lot of attention in the recent past as this offers a convenient procedure of synthesizing them in a "green and facile manner". In the present study, the less explored endophytic fungi were employed in the synthesis of gold nanoparticles. Extracellular gold nanoparticle formation was observed by the change in colour of the solution to dark pink. This was followed by characterizing the nanoparticles using various instrumental analyses like UV-Vis spectroscopy, TEM and FTIR. The obtained gold nanoparticles were found to be spherical with slight aggregation and their size was found to be in the range of 15 - 35 nm. Their biocompatibility has been assessed using cytotoxicity assay and the results have shown that these biogenic gold nanoparticles do not induce significant cytotoxicity in normal and cancer cell lines. This study highlights the use of endophytic fungi as potential synthesizers of biocompatible gold nanoparticles in a benign fashion.

Keywords: Microorganisms; Endophytic fungi; Gold nanoparticles; UV-Vis spectrum; TEM; Cytotoxicity

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INTRODUCTION

The term “Gold nanoparticles (GNPs)” is frequently used in medical field especially cancer diagnosis and treatment. This increased interest is due to the unique properties displayed by them at nanoscale. The role played by them ranges from biomarkers to biodelivery vehicles in medicine, anti-aging components to biosensors. Such wide real time applications of GNPs make their synthesis, on a large scale under facile conditions with defined morphology, all the more valuable.

Many strategies have been developed for the synthesis of metal nanoparticles and nanomaterials. The existing chemical and physical methods raised concerns over environmental contamination as these procedures are known to generate large amounts of hazardous by-products [1]. In this context, there is a greater need to develop safe, reliable, clean and eco-friendly methods for the preparation of nanoparticles and other high structured nanomaterials. The answer lies in “Green chemistry” methods which include clean, nontoxic and environment-friendly process of nanoparticle synthesis with precise control over the shape and size. Biological synthesis using microorganisms like bacteria, fungi and plants seems to be an efficient route for benign synthesis.

It is well known that extracellular or intracellular inorganic materials were produced using various biological organisms like iron oxides and various metal nanoparticles. Eukaryotic organisms like fungi are successfully used

in the synthesis of nanoparticles with different chemical composition and size as they display the ability to secrete large amounts of enzymes. Additionally, the fungi are found to display as intracellular uptake of metals and also show high tolerance towards metals [2].

In this perspective, the present study focuses on the synthesis of gold nanoparticles using the less explored endophytic fungi. Three endophytic fungi isolated from medicinal plants [3] were used for the synthesis of gold nanoparticles in a facile manner and these were characterised using various instrumental analysis.

MATERIALS AND METHODS

Endophytic Fungi

The endophytic fungal cultures that were employed in the synthesis of gold nanoparticles were isolated from medicinal plants and described elsewhere [3]. These endophytic fungi successfully synthesised silver nanoparticles thereby displaying the property of bioreduction of metals [4, 5].

Culture conditions and synthesis of gold nanoparticles

The endophytic fungi were grown aerobically in liquid broth containing malt extract powder, glucose, yeast extract and peptone. The culture flasks were incubated at 27°C. The culture supernatant was obtained by separating the biomass after 7 days of growth by sieving using plastic sieve. 10 mL of the culture filtrate was challenged with 20 mL of $HAuCl_4$ and incubated under dark conditions at room temperature for 48 hr.

Characterization of gold nanoparticles

The formation of gold nanoparticles (AuNPs) was initially observed by the colour change from pale yellow to pink which was later confirmed using UV-Vis spectrum of the reacting solution using spectrophotometer, in a 1 cm path quartz cell at a resolution of 1 nm from 250 to 800 nm. This solution was centrifuged at 5000 rpm for 15 min. The dried samples were ground with KBr and made into pellets. The spectrum was recorded in the range of 4000-400 cm^{-1} using Perkin Elmer FTIR spectrophotometer operating at resolution of 4 cm^{-1}. Further characterization of the gold nanoparticles involved the use transmission electron microscope (TEM) to comprehend the morphology, size and the distribution of nanoparticles.

Antibacterial Activity of Gold Nanoparticles

The potential of gold nanoparticles as antimicrobial agents were checked using the agar well diffusion assay method [6]. The test organisms used were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Salmonella typhi* ATCC 6539 and *Klebsiella pneumoniae* NCIM 2883. The respective test organisms were prepared by spreading 500 µL of revived culture on the nutrient agar plate. 6 wells were cut with the help of a sterilized stainless steel cork borer into which different concentrations of AuNP solution (10, 20, 30, 40, 50 and 100 µL) was loaded and incubated at 37° C. The plates were examined for presence of zones of inhibition indicative of the antibacterial activity.

Cytotoxicity of AuNPs using MTT assay

The cell lines were procured from the Kings Institute of Preventive and Medicine, Guindy, Chennai and the cell lines were subcultured and maintained in CO_2 incubator at 37°C. 20% serum containing RPMI growth media was used for growing the cells. The cells were continuously monitored under inverted microscope for their confluence and to confirm the absence of the bacterial and fungal contaminants.

Cell lines were subcultured and $1x10^4$ cells were transferred to the 96 wells and incubated at 37° C in CO_2 incubator for 2 days to form confluence. The spent media was removed and 150 µl of fresh media was added. 150 µl of AuNPs (1mg/ml) was added and serially diluted to get 1 fold dilution in each wells till 6^{th} dilution to get varying concentration from 75 µg to 1.14 µg. The plates were incubated at 37°C for 4 hrs in CO_2 incubator. After incubation the drug was removed and 20 µl of MTT solution (5mg/ml) and 180 µl of media were added to the wells. The plates were incubated at 37°C for 3.5 hrs in CO_2 incubator. Without disturbing the cells the media was removed carefully. The insoluble formazon crystal was dissolved by adding DMSO and the absorbance was read at 570 nm with reference filter at 630 nm. The percentage cytotoxicity was calculated and used for finding the IC_{50}. The untreated cells were considered as control.

$$\%\,Cytotoxicity = \frac{(Abs_{Control} - Abs_{Test})}{Abs_{Control}} \times 100$$

RESULTS AND DISCUSSION

Nanosized inorganic particles, of either simple or composite nature, display unique physical and chemical properties and represent an increasingly important material in the development of novel nanodevices which can be used in numerous physical, biological, biomedical, and pharmaceutical applications [7]. The problem with most of the existing chemical and physical methods of nanomaterial production is that they are extremely expensive and also involve the use of toxic, hazardous chemicals, which may pose potential environmental and biological risks. Further, it is an unavoidable fact that the metal nanoparticles synthesized have to be handled by humans and must be available at cheaper rates for their effective utilization. Thus, there is a need for an environmentally and economically feasible way to synthesize these nanoparticles. This paved a way to use biomimetic approaches for the benign synthesis of these nanoparticles [8].

Biosynthesis of nanoparticles is a kind of bottom-up approach, whereby the main reaction occurring involves reduction/oxidation of substrates, giving rise to colloidal structures. Microbial enzymes or plant phytochemicals with antioxidant or reducing properties are usually responsible for reduction of metal compounds into their respective nanoparticles. Fungi pose certain additional traits compared to their bacterial counterparts. For example, studies on the scaling up of fungal cultures by optimization have shown that fungal mycelia can endure the culture condition deviations which perhaps the plant and bacterial based extracts cannot. Furthermore, fungi with their fastidious nature of growth enable them to release certain enzymes and proteins of vital importance which inturn facilitate easier bioreduction of corresponding metal salts to form reduced metallic ions as zero-valent nanoparticles [9]. Based on these earlier revelations, the present study aims to harness the ability of less explored endophytic fungi as nanoparticles synthesizers.

Synthesis of gold nanoparticles

The three endophytic fungal isolates named GX2, GX3 and ARA that were successful in reducing $AgNO_3$ [4, 5] earlier were used to synthesize the gold nanoparticles using $HAuCl_4$ as the starting material (Fig. 1). The extracellular synthesis of gold nanoparticles was successfully achieved by using the fungal culture filtrate as this mode of synthesis would facilitate easy extraction. The formation of AuNPs was initially observed by the change in colour from pale yellow to pink indicating the formation of AuNPs. Their role as potential *in vivo* diagnostic and therapeutic agents, as X-ray contrast agents, drug delivery vehicles and radiation enhancers [10] makes their synthesis mechanism all the more important.

Fig. 1 Gold nanoparticles synthesized by endophytic fungi observed by colour change from pale yellow to pink. A) GX2-AuNPs B) GX3-AuNPs C) ARA- AuNPs

This change in colour was due to the collective coherent oscillation of conduction electrons at the surface of the gold nanoparticles when these particles interact with the oscillating electric field of the incident light, a phenomenon called surface plasmon resonance (SPR). This change in colour indicates the reduction of $HAuCl_4^-$ to nanogold which is the first step in the formation of AuNPs [11, 12]. The synthesis of gold nanoparticles using biological source is understood to be a type of bottom-up approach where in the key reaction is reduction/oxidation of substrates, thereby forming the colloidal structures. It was also reported that microbial enzymes with antioxidant or reducing properties are usually responsible for the reduction of metal compounds into their respective nanoparticles [12].

Characterization of gold nanoparticles

The preliminary information on the gold nanoparticle synthesis was initially gathered by observing the colour change in the reaction medium from pale yellow to pink. Further information about the presence of the AuNPs was determined by various instrumental analyses that delve into the size and shape of the nanoparticles.

UV-Vis spectrophotometry

The most commonly used method for confirming the formation of nanoparticles is UV-Vis spectroscopy. UV-Visible absorption measurements in the range 350-600 nm can provide an insight into size, distribution, surface properties and optical properties of the nanosized Au particles. The surface plasmon bands for the gold nanoparticles usually ranges between 510 and 560 nm in aqueous solution depending upon the function of their morphology, since plasmon bands are very sensitive to the length and sharpness of the tips of nanomaterials.

In the present study, the UV-visible spectra of the AuNPs synthesized by endophytic fungal isolates GX2, GX3 and ARA displayed clear peaks at 536 nm, 536 nm and 531 nm respectively (Fig. 2). The results obtained in the present study very well adhere to the standard peak SPR wavelengths. All the peaks obtained were in the range of 525 – 535 nm which indicates the spherical nature of the AuNPs. As substantiated by Huang and Yang [13], the spherical nanoparticles have strong absorption at about 520 nm with almost no absorption after 600 nm; however, the triangular shape has absorption at 540 nm which extends well in near infrared region (NIR). The wavelength of peak absorption depends upon several factors such as particle size, dielectric constant of surrounding media and the inter-particle distance.

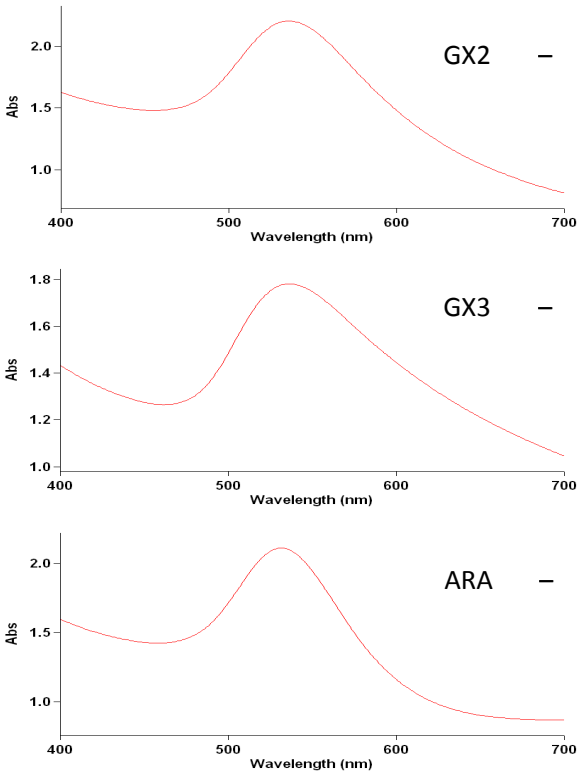

Fig. 2 UV-Visible spectra of the AuNPs synthesized by endophytic fungal isolates GX2-AuNPs, GX3-AuNPs, ARA- AuNPs with the absorption peaks

It is well known that spherical nanoparticles of Au should exhibit single-surface plasmon bands whereas anisotropic particles should exhibit two or three bands, corresponding to the quadrupole and higher multipole plasmon excitations [14, 15]. Similarly, Skirtach et al [16] reported that the gold NPs synthesized using *P. aeruginosa* showed a peak at 560 nm. The reduction of gold ions occurs comparatively slowly, but the gold nanoparticles are found to be very stable in the colloidal suspension. Since extracellular gold nanoparticle synthesis is more

advantageous in terms of extracting the nanoparticles, this would be a much preferred methodology. Verma et al [11] have initiated the use of endophytes in biologically synthesizing gold nanoparticles. Another study reported the synthesis of gold nanostructures using the extract of *Trichoderma koningii* [17]. Similarly, bacteria were also used for the extracellular biosynthesis of gold nanoparticles using *Klebsiella pneumoniae* as the source [18]. Even marine bacteria are explored for their ability to produce gold nanoparticles. Stable, monodisperse AuNP formation with around 10 nm dimension occur upon exposure of $HAuCl_4$ solution to whole cells of a novel strain of *Marinobacter pelagius* [19]. This very much justifies the use of microorganisms in the synthesis of nanoparticles as the procedure involved is simple and benign.

TEM analysis

The morphology and size of the nanoparticles play an important role in their functional properties. Hence the biologically synthesized AuNPs were subjected to TEM analysis (Fig. 3). The TEM images revealed the AuNPs to be spherical in shape with slight aggregation. The size was observed to be in the range of 15 – 30 nm. Similar results were obtained when C albicans where the size of the gold nanoparticles was found to be in the range of 15 – 30 nm [12]. Further, studies conducted by Verma et al [11] also substantiate these results wherein an endophytic fungus *Aspergillus clavatus* was used for the synthesis of gold nanoparticles.

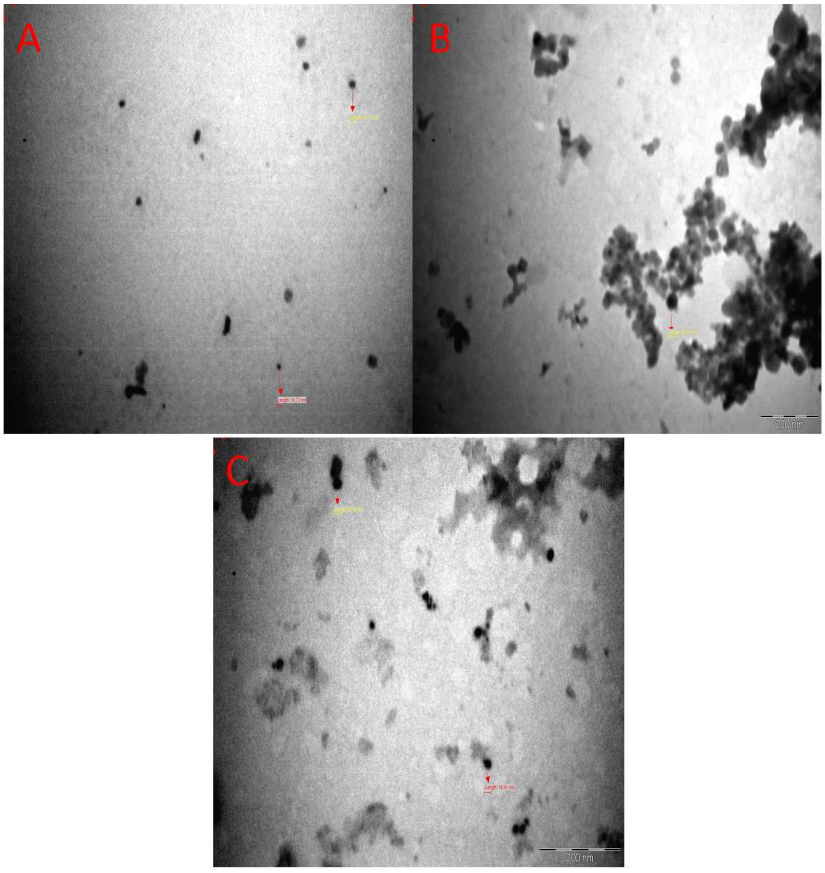

Fig. 3 TEM micrographs of AuNPs synthesized by endophytic fungal isolates A) GX2-AuNPs B) GX3-AuNPs C) ARA- AuNPs

FTIR spectra of gold nanoparticles

The nature of possible biomolecules in the synthesis and stabilisation of nanoparticles is identified by FTIR spectra. The singular advantages of FTIR over other techniques are that spectra can be obtained for proteins in a wide range of environments, requiring less time and sample, and direct correlations between the IR amide I band frequencies and the secondary structure components can be found [20]. The FTIR spectra of the biogenic gold nanoparticles synthesised by endophytic fungi are given in Fig. 4

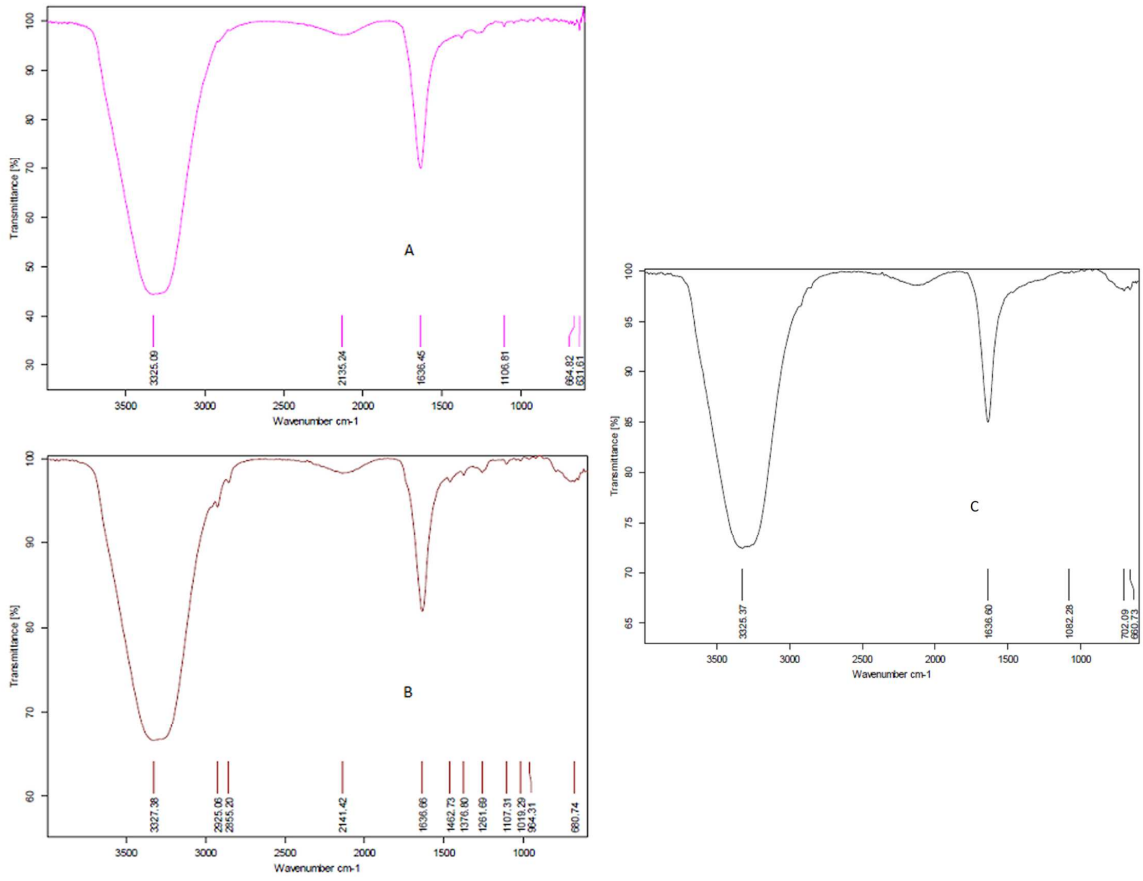

Fig. 4 FTIR spectra of fungal AuNPs A) GX2-AuNPs B) GX3 –AuNPs C) ARA-AuNPs

All the spectra documented two very prominent peaks at 3325 cm^{-1} and 1635 cm^{-1} with small peaks at around 600 – 700 cm^{-1}. The peaks at 3325 cm^{-1} corresponds to amide absorption band which is characteristic of the N-H stretching vibrations. The peaks at 1636 cm^{-1} correspond to C=O stretching vibrations. Gold nanoparticles can bind to proteins through free amine groups or carboxylate groups in the protein. The presence of the intense peak at C=O stretching mode indicates the presence of carboxylic groups in the material bound to gold nanoparticles [21]. These spectra confer to the standards of FTIR spectra of the proteins and peptides thus validating the role of extracellular proteins in synthesis of gold nanoparticles [22]. Though the exact mechanism of formation of nanoparticles is still not clear, it was proposed as a nitrate reductase-mediated synthesis where in the enzyme, nitrate reductase would bring about bioreduction of metal ions to nanoscale [23].

Antibacterial activity of gold nanoparticles

Gold nanoparticles synthesized by the endophytic fungi were evaluated for antibacterial activity against the above mentioned test organisms. Though certain reports have stated good antibacterial activity against certain pathogens [22], in the current study the biologically synthesized gold nanoparticles didn't show significant antibacterial activity as the zones of clearance were negligible.

Cytotoxicity of AuNPs

A preliminary study on the cytotoxicity of AuNPs was carried out using the MTT assay with 4 hrs of incubation time (Fig. 5).

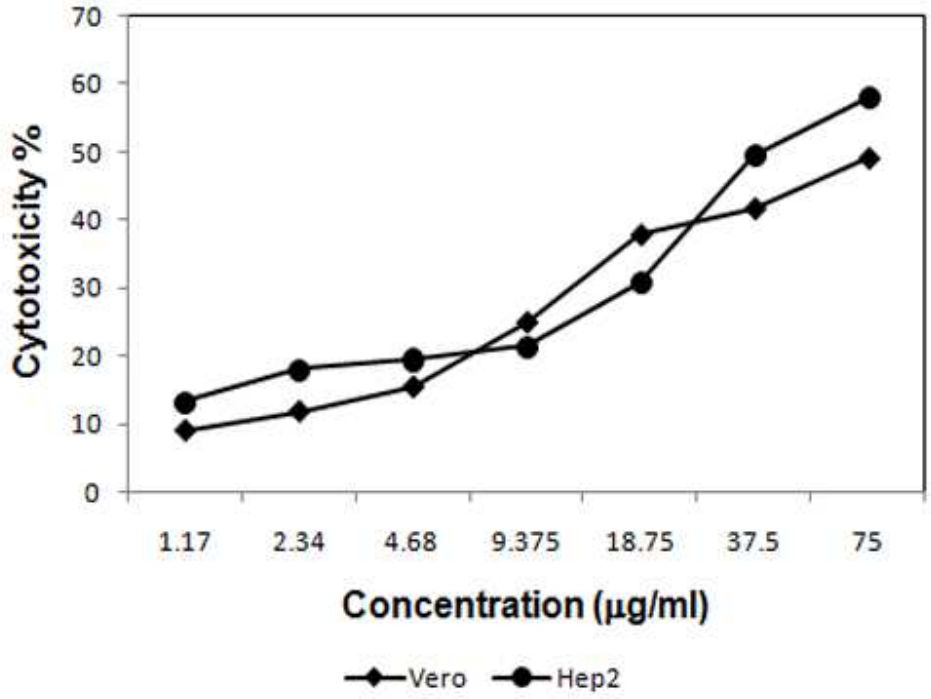

Fig. 5 Cytotoxicity of AuNPs against Vero and Hep2 cell lines

The test results indicate the toxicity to be very insignificant though the % cytotoxicity showed a gradual increase in both the cell lines (Vero and Hep2) with increase in the concentration of AuNPs

The IC_{50} value for Vero and Hep2 cells was found to be 12 µg/ml and 23 µg/ml respectively. Maximum cytotoxicity was observed to be 49 and 58 % for Vero and Hep2 at the concentration 75 µg/ml. These results reiterate the biocompatibility of the these gold nanoparticles and hence can be used for research in the medical field.

CONCLUSION

This study advocates the use of microorganism especially the endophytic fungi for the synthesis of gold nanoparticles as a valuable alternative for the existing physical and chemical methods. The worth of the synthesis method depends on the solvent medium used, reducing agent involved and a non-toxic stabilizing agent. In this context, the current approach attracts attention as biological synthesis is carried out in an aqueous solution doesn't require any additional reducing agent. The particles are stabilized by the proteins released by the fungi in the medium thus making this a viable option for the synthesis of nanomaterials. The AuNPs synthesised were in the size range of 15-30 nm with spherical shape and partial aggregation and didn't display significant antibacterial activity or cytotoxicity thus making them potential candidates in various medical applications.

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