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RESEARCH ARTICLE

BIOMIMETICS OF SILVER NANOPARTICLES FROM GANODERMA LUCIDUM (CURTIS) P.KARST AND ITS ANTICANCER POTENTIAL ON BREAST CANCER CELLS

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ABSTRACT

Ganoderma lucidum is a fungus belonging to family Ganodermataceae of polypore mushrooms which grows on woody root region of a tree. These mushrooms are extensively used as a traditional Asian medicine and are well known for their potential in bioremediation of metals. The present study was carried out with the objective of using the above mushroom for developing silver nanoparticles(Ag-NPs) and characterization of the same using ultraviolet-visible spectroscopy (UV-Vis), Fourier transform infrared (FT-IR) spectroscopy ,X-ray diffraction (XRD), energy dispersive X-ray (EDX), and scanning electron microscopy (SEM), followed by High resolution-Transmission Electron Microscopy(HR-TEM).Surface Plasmon resonance showed the formation of silver nanoparticles in UV-Visible spectra at 438 nm. The Fourier transform infrared spectroscopy (FTIR) analysis was carried out to identify and study the functional groups responsible for the bio-reduction of silver ions. The XRD study showed that the particles as crystalline in nature with a Face Centered Cubic (FCC) structure. The synthesized Ag-NPs were poly dispersed spherical particles as confirmed by EDAX-SEM and stabilized in the solution with the spherical shapes further confirmed by HRTEM analysis to be in the range of 12-20 nm. The present findings of Ag-NPs of *Ganoderma lucidum* promisingly proved have strong anti-cancer activity on MCF-7 cell line; in future these findings may contribute to the improvement of a suitable anticancer drug.

INTRODUCTION

Breast cancer is a diverse disease which is categorized by the propagation and unusual variation of malignant adolescent cells that often carry abnormalities that liberalize hundreds or even thousands of genes (Ghodake and Lee, 2011). It has perceived an enormous proliferation in most recent times, predominantly in emerging nations like India and it accounts for 30% prevalence frequency in the new female cancers (Siegel *et al*., 2012). Age, family history, reproductive aberrations, exogenous hormone contraceptives or hormone replacement therapies and environmental localities are some of the risk dynamics (American Cancer Society, 2007). Numerous clinical trials have been conducted to treat breast cancer but the trials have revealed minimum eminent effects .To overcome these complications, investigators have introduced the usage of nanoparticles therapy in breast cancer treatment (Yezhelyev *et al*., 2006) *Ganoderma lucidum is* commonly known as Lingzhi or Reishi which belongs to the family Ganodermatace, recognized across the world as an oriental fungus with medicinal properties for over 2000 years and its prevailing effects have been recognized in ancient scripts (Wasser *et al*., 2005).

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In addition to these, mushrooms have been found to contain an extensive variety of bioactive molecules such as terpenoids, steroids, phenols, nucleotides and their derivatives, glycoproteins, and polysaccharides. *G. lucidum* has been used for hundreds of years as a health promotion and treatment strategy; there are now many published studies that are based on animal and cell culture models and on *in vitro* valuation of the health effects of *G. lucidum*, and there are also some reports of human trials in the field. Silver nanoparticles (Ag-NPs) can perform unique communications with biomolecules both on the outside and inside the body cells, which may bring help in cancer identification and treatment (Liu *et al*., 2012). It has shown to be efficacious for antimicrobial, antifungal, antioxidant, and anti-inflammatory effects and nanoparticles are believed to play a major role in cancer diagnosis and treatment (Seigneuric *et al*., 2010). The shape of the NPs plays a significant role in tuning the properties and is vital to manipulate systematically (Gupta, 1998) in the past few decades, numerous chemical and physical approaches for synthesis of silver nano particles have been described. The drawbacks of chemical methods of nanoparticle synthesis include exclusive instruments and the discharge of toxic chemicals.Hence, biological methodologies using micro-organisms, mushrooms and plants extracts for metal nanoparticles synthesis are appreciable replacements to chemical methods (Sastry *et al*., 2004).

There is very little literature supporting the use of mushrooms in nanoparticles synthesis. The present work is based on the biosynthesis of silver nanoparticles from the aqueous extract of *G.lucidum* and is the first study to evaluate its anticancer potential. The current study defines the *G.lucidum* Mushroom-mediated *in vitro* one-step transformation of Ag-NPs, characterization, and its cytotoxic effects on the breast cancer cell line MCF-7.

MATERIALS AND METHODS

Sample collection and extraction

$AgNO_3$ (Silver nitrate) was purchased from HI-MEDIA, Mumbai, India. All aqueous solutions were prepared using de-ionized water. In the present study, *Ganoderma lucidum* was collected from Western Ghats and foot hills of Maruthamalai. The collected sample was authenticated by the Mycology Division of IFGTB (Indian Forest Genetics and Tree Breeding Institute) Coimbatore, Tamilnadu, South India and the voucher specimen (RT-25406/9-1-2015) was retained in our laboratory for future reference. Samples were brought to laboratory in polythene bags and cleaned thoroughly with fresh water to remove adhering debris and associated biota. The mushrooms were cleaned using brush for the removal of the epiphytes with distilled water. After cleaning, the fungi were dried in shade at room temperature for three days to one week. The dried fungal material was homogenized to fine powder and further subjected to extraction.

Preparation of aqueous extraction

The whole *Ganoderma lucidum* was initially rinsed thrice in distilled water and dried on paper towelling, and samples (25 g) was cut into fine pieces and boiled with 100 ml of sterile distilled water for 5 minutes. The crude extract is passed through Whatman No.1 filter paper and the filtrate was stored at 4 °C for further use.

Synthesis of silver nanoparticles (Ag-NPs)

In the synthesis of silver nanoparticles, 10ml of the aqueous extract of *G.lucidum* was added to 90ml of 1mM aqueous $AgNO_3$ solution in 250 ml conical flask and kept at room temperature for 48hrs at 1200 rpm. Suitable controls were maintained throughout the conduct of experiments.

Characterization of Silver nanoparticles

UV-Vis spectral analysis

The colour change in reaction mixture (metal ion solution + fungal extract) was recorded through visual observation. The bio reduction of Ag^+ ions in aqueous solution was monitored by periodic sampling of aliquots (0.5 ml) and subsequently measuring the UV-Vis spectra (200 to 800nm) of the solution. UV-Vis spectra of these aliquots were monitored as a function of time reaction on UV-Vis spectrophotometer (UV-100Cyberlab USA).

Fourier Transform infra-red (FT-IR) spectroscopy analysis

To remove any free biomass residue or compound that is not the capping ligand of the nanoparticles, the residual solution of 100 ml after reaction was centrifuged at 5000 rpm for 10 min. The supernatant was again centrifuged at 10000 rpm for 60 min and the pellet was obtained. This was followed by re dispersion of the pellet of Ag-NPs into 1 ml of deionised water. Thereafter, the purified suspension was freeze dried to obtain dried powder. Finally, the dried nanoparticles were analysed by FTIR Nicolet Avatar 660 (Nicolet, USA).

XRD (X-ray diffraction analysis)

The Ag-NPs solution thus obtained was purified by repeated centrifugation at 5000 rpm for 20 minutes followed by redispersion of the pellet of Ag-NPs in 10 ml of deionised water. After freeze drying of the purified Ag-NPs, the structure and composition was analyzed by XRD. The dried mixture of Ag-NPs was collected for the determination of the formation of Ag-NPs by an X'Pert Pro x-ray diffract meter (PAN analytical BV, The Netherlands) operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation in a θ- 2 θ configuration. The crystalline domain size was calculated from the width of the XRD peaks, assuming that they are free from non-uniform strains, using the Scherer's formula:

$$D= 0.94 \lambda / \beta \cos \theta$$

1) where D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the full width at half maximum (FWHM), and θ is the diffraction angle. To eliminate additional instrumental broadening, the FWHM was corrected using the FWHM from a large grained Si sample:

$$\beta \text{ corrected} = (FWHM2sample- FWHM2si)1/2$$

2) This modified formula is valid only when the crystalline size is smaller than 100 nm.

EDX-SEM (Energy Dispersive X-ray Analysis) (Scanning Electron Microscopy)

Electron microscopy is another commonly used method of characterization. Scanning electron microscopy and transmission electron microscopy are used for morphological characterization at the nanometer to micrometer scale. The bio functionalized *G.lucidum* Ag-NPs were characterized using high resolution Scanning Electron Microscope (JSM-5600LV; JEOL, Tokyo, Japan). The samples were prepared by a simple drop coating of suspended silver solution on to an electric clean glass and allowing the solvent (water) to evaporate and the samples were left to dry at room temperature. EDAX analysis was carried out to confirm the presence of elemental Silver bio functionalized *G. lucidum* Ag-NPs using the drop coated bio functionalized Ag-NPs of *G.lucidium* on to carbon film using EDAX (S-3400N; Hitachi, Tokyo, Japan).

HRTEM (High resolution Transmission Electron Microscopy) analysis

The structural characterization of the Silver nanoparticles was carried out by High Resolution Transmission Electron Microscopy (HR-TEM; JEM-1200EX; JEOL, Tokyo, Japan).). The sample was prepared by air-drying drops of diluted

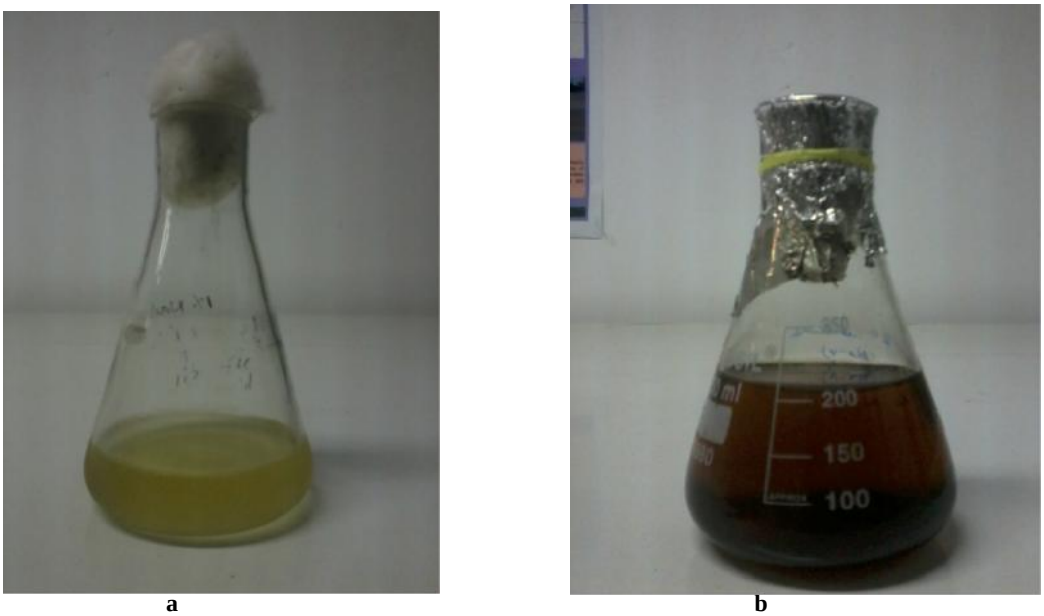
solution of the preparations on carbon films supported by copper grids.

In-vitro Cytotoxicity studies of bio functionalized *G.lucidium* Ag-NPs

Cell culture

Human MCF-7cells was purchased from NCCS, Pune, India. Cells were routinely grown at 37°C in a humidified atmosphere of 5% CO 2 and 95% air, in Dulbecco's Modified Eagle Medium (DMEM) Glutamax supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) and 1 mM Anti– -Inc A antibody (Invitrogen). MTT assay was performed to determine the cytotoxic properties of bio-functionalized Ag-NPs against MCF-7 cell lines.

The cell lines were seeded in 96-well tissue culture plates and the appropriate concentrations of Ag-NPs stock solutions were added to the cultures to obtain respective concentration of Ag-NPs and incubated for 48 hrs at 37°C. The non-treated cells were used as control. The incubated cultured cell was then subjected to MTT (3- (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. MTT assay is based on the measurement of the mitochondrial activity of viable cells by the reduction of the tetrazolium salt MTT (3-(4,5-dimethyathiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to form a blue water-insoluble product, formazan. MTT (5 mg/mL, 20 µL) was added to respective set of cells and the plates were incubated for an additional 4 h. After 4 h of incubation, the medium was removed and DMSO (200 µl, Sigma–Aldrich, USA) was added to dissolve the formazan crystals resulting from the reduction of the tetrazolium salt only by metabolically active cells.



a **b**

Figure 1. Silver nanoparticles synthesis of *G.lucidum* Figure 1.a (intial) and Figure1 .b (final) colour change

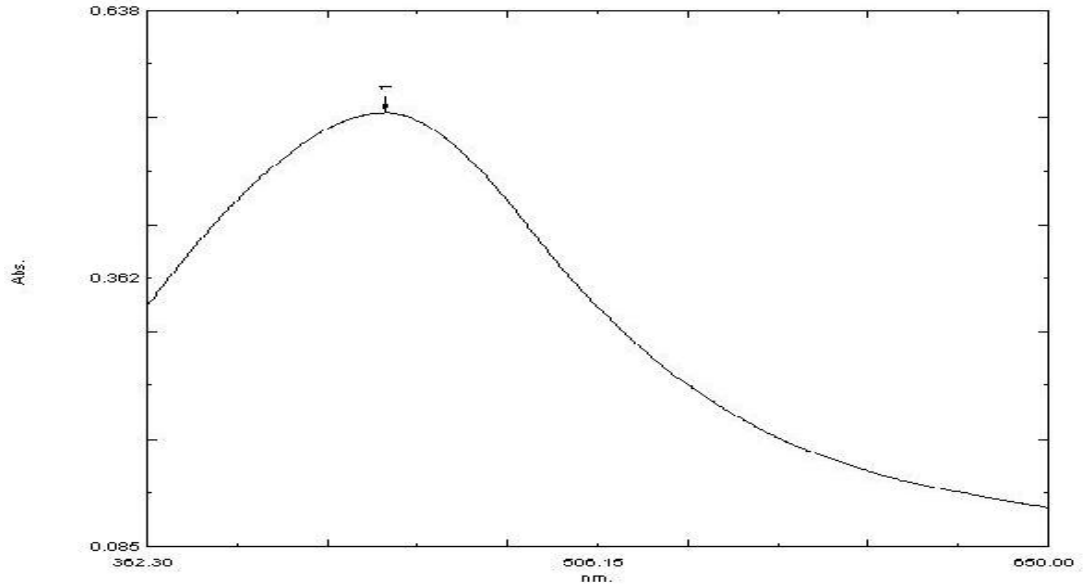


Figure 2. UV-Vis absorption spectra of Ag-NPs synthesized by *Ganoderma lucidum*

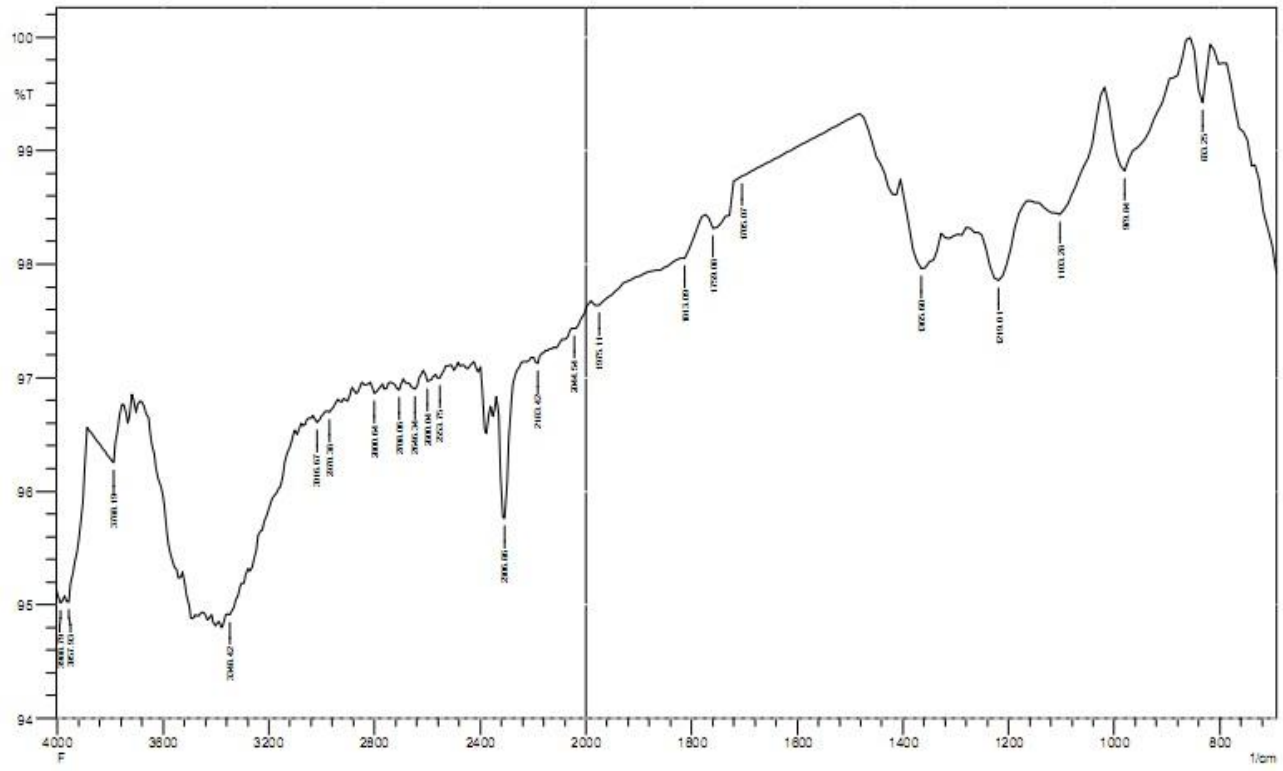


Figure 3. FTIR Spectrum of Ag-NPs synthesized from *Ganoderma lucidum*

MTT is reduced in metabolically active cells to yield an insoluble purple formazon product. The cells were harvested during the exponential phase and counted by a hemocytometer after staining with trypan blue solution. The cell suspensions were dispensed (100µl) in triplicate into 96-well culture plates at optimized concentrations of 1×10^5 cells/well after a 24- hr recovery period. Assay plates were read using a spectrophotometer at 592 nm. The spectrophotometrically absorbance of the samples was measured using a micro plate (ELISA) reader. The cytotoxicity data was standardized by determining the absorbance and calculating the correspondent Ag-NPs concentration. The data generated were used to plot a dose-response curve of the concentration of extract which was required to kill 50% of cell population (IC 50) was determined from formula as described below

$$\text{Cell viability (\%)} = \frac{\text{Mean optical density}}{\text{Control optical density}} \times 100$$

Since the absorbance was directly correlated with the number of viable cells, the percent viability was calculated from the absorbance. The IC_{50}, the concentration of the drug at which 50% cell growth is inhibited, was calculated by the curve fitting of the cell viability data using Prism 5.2. After bio-functionalized *G.lucidum* Ag-NPS treatment, the plates were observed under an inverted microscope to detect morphological changes and photographed.

RESULTS AND DISCUSSION

In this study, we attempted to use the extract of *G. lucidum* for synthesis of Ag-NPs as shown in Figure 1. It was observed that the *Ganoderma* extract had a pale-yellow colour before reaction with the silver ions, which transformed to a brownish colour at endpoint of the reaction.

The appearance of a yellowish-brown colour in solution containing the extract was a apparent clue for the formation of Ag-NPs in the reaction mixture, and was due to the excitation of surface plasmon vibrations in the NPs. These transformations confirms the presence of nanoparticles of Ag-NPs in *Ganoderma lucidium*, which is comparable with reports which was previously reported in Ag-NPs synthesis of *Ganoderma neo-japanicum,* a similar type of medicinal mushroom (Ahmad *et al*., 2003).

Characterization of Ag-NPs

UV-Vis spectroscopy analysis of Ag-NPs

The Ag-NPs were further categorized by UV- visible spectroscopy. UV-visible spectroscopy is a significant and precious technique for the characterization of NPs. The UV-visible absorption spectra of the Ag-NPs were measured in the range of 300–600 nm. A tough and broad surface plasmon peak located at 438 nm was observed for the Ag-NPs prepared using mycelia extract of *G.lucidum* (Figure 2). The strong surface plasmon reverberation centered at 438 nm clearly indicated the formation of Ag-NPs, which were tremendously stable with no evidence of flocculation of the particles even after 3 months. The band around 438 nm suggests that the particles were well dispersed lacking aggregation. A previous study was reported in which is analogous with our current research study which suggest us presences of Ag-NPs (Wani *et al*., 2013).

FTIR Fourier Transform infra-red (FT-IR) spectroscopy analysis of Ag-NPs

FTIR measurements were carried out to discover the possible biomolecules responsible for the stabilization of the newly synthesized Ag-NPs.

Figure 3 represents the FTIR spectrum of *G lucidum* spectra of Ag-NPs obtained from *G.lucidum* that showed a strong transmission band at 3412 cm^{-1} consequent to the intermolecular H bonds. Another band observed at 2316 cm^{-1} was assigned to the stretching vibration of the SH Stretching Vibrations. Besides, a small shift from 1641cm^{-1} is assigned to the C=O stretch and the 1085 cm^{-1} peak in the Ag-NPs extract is assigned to the C–O stretch. The bio reduction of $AgNO_3$ by *G. lucidum* resulted in shifting of C=O peak from 1641 cm−1 which designated the reduction of Agionsto Ag^0 and shift in the O-H peak from 3240cm−1 to 3587cm−1 is accredited to participation of hydroxyl group of *G. lucidium* in capping of silver nanoparticles.

The shift in the spectra of silver nanoparticles indicates that hydroxyl (AOH) group of *G. lucidum* might have interacted with silver surface making silver nanoparticles highly stable. Prior literature reveals that the hydroxyl group (OH) has a strong ability to interact with Ag ions .Hence, the affinity of *G .lucidum* containing hydroxyl group (OH) to Ag+ and NO_3^- is studied by comparing the FTIR spectra of *G. lucidum* stabilized Ag-NPs. (Mukherjee *et al*., 2001)

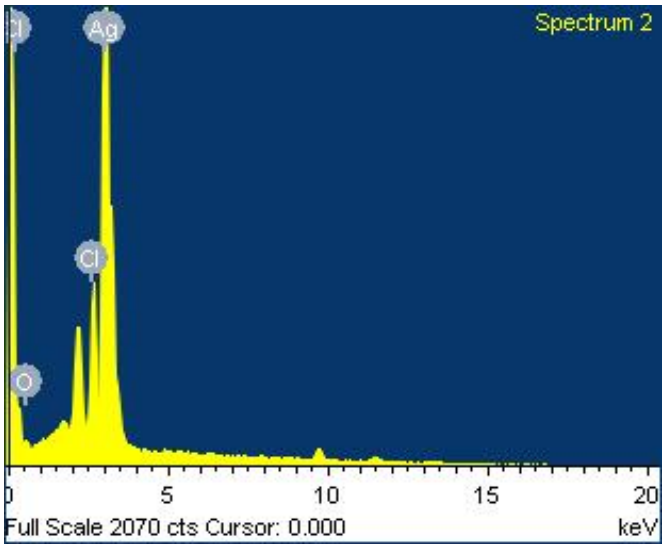


Figure 4. EDX spectrum of Ag-NPs synthesized from *Ganoderma lucidum* with two dominant peaks for Cl, and Ag respectively

XRD analysis of Ag-NPs

Further, we examined the verification of the crystalline nature of Ag-NPs using XRD. Figure 3 shows the XRD blueprint obtained for Ag-NPs synthesized using the mycelia extract of *G.lucidum*. Concerning the crystalline nature of the Ag-NPs, two intense XRD peaks were observed, subsequent to the (111) and (200) planes at 2θ angles of 38.28° and 44.38° correspondingly (Figure 3). XRD spectra of the nano- particles derived from *G.lucidum* extract displayed the configuration of metallic silver. The width of the (111) peak was engaged to calculate the average crystallite size using the Scherrer equation. It was found that the calculated average size is,6 nm, which matches the particle size obtained from a TEM image of Ag-NPs using *Ganoderma* extract. In addition to the Bragg peaks representative of face-centered cubic Ag-NPs, additional as-yet-unassigned peaks (marked with stars) were also observed, suggesting that the crystallization of the bioorganic phase occurred on the surface of the Ag-NPs.

Our results represent a noteworthy consent with earlier findings reporting synthesis of Ag-NPs using geranium. Nevertheless, Vikneswaran *et al*. (Vigneshwaran *et al*., 2007) recommended an intense diffraction peak at 2θ angles of 57.3°, due to the chloride ions involved during the preparation of the cell filtrate, and also possibly due to residue from an extract of the biomass. In addition, three new peaks were produced due to the communication of silver nitrate with the fungal cell-wall matrix.

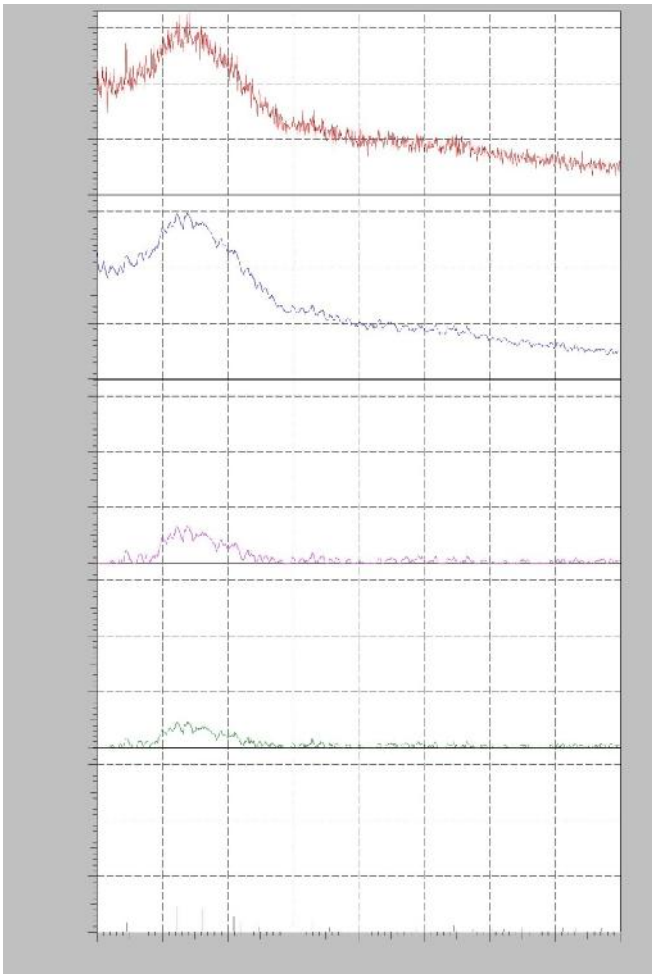


Figure 5. XRD patterns of capped of Ag-NPs synthesized from *Ganoderma lucidum*

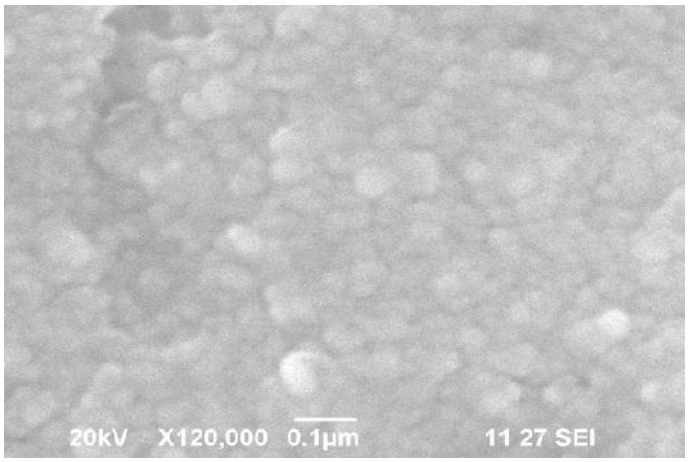


Figure 6. SEM images of Ag-NPs synthesized from *Ganoderma lucidum*

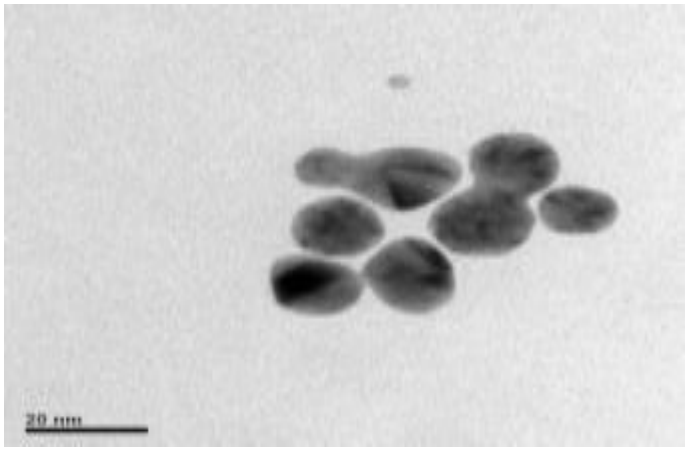


Figure 7. HRTEM images of Ag-NPs *Ganoderma lucidum*

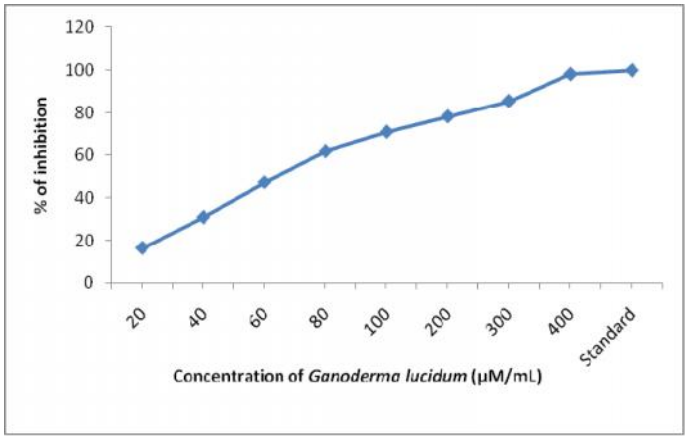


Figure 8. MTT Assay- anti cancer activity of Ganoderma lucidum against MCF-7 breast cancer cells

EDX-SEM analysis of Ag-NPs

In this study, EDX was used to confirm the occurrence of silver in the suspension of nanoparticles purified by ultracentrifugation. The EDX result exhibited a small peak of silver that confirmed its presence in the suspension. In EDX analysis, strong Ag peaks were observed in *G. lucidum*. Peaks for Cu and Cl were also recorded in EDX (Figure 6).The results obtained from EDAX gives a clear suggestion regarding the other elements involved in the synthesizing nanoparticles.

The existence of (Cu) Copper peak may be due Copper grids used in our analysis and Cl derivatives along with Ag suggests the participation of the biomolecule in the synthesis of Ag-NPs and they might have served as stabilizing molecules since similar peak were observed in Ag-NPs of *Ganoderma neo-japanicum* (Vigneshwaran *et al*., 2007). Scanning electron microscopy (SEM) investigation of silver nanoparticles was done using Hitachi S-4500 Scanning Electron Microscopy. The exterior morphology (ie. shape and size) of the silver nanoparticles is shown in Figure 7. The identical spherical shape Ag-NPs were obtained with the sized ranging from ~5-30 nm. Correspondingly, the spherical shaped (silver nanoparticles) Ag-NPs with a diameter ranging from 30-40 nm have been synthesized using *Boswellia ovalifoliolata* (Sangiliyandi gurunathan *et al*., 2013) and with identical species analogous result were observed in the previous reports of Ag-NPs of *Ganoderma lucidum* (Savithramma *et al*., 2011)

High Resolution Transmission Electron Microscopy analysis of Ag-NPs (HRTEM)

The HR-TEM images authenticated the construction of Ag-NPs (Figure 7) and it was observed that the recently synthesized nanoparticles were poly dispersed with spherical, triangular and hexagonal rod shapes. The size of the particles ranged from 17 to 85.2 nm. The higher magnification of TEM images exposed the shape and size of Ag-NPs. These particles were predominantly spherical in shape and the shape of metallic nanoparticles significantly changed their optical and electronic properties. On careful surveillance of TEM images, all the particles lacked physical contact and were separated from each other by an identical allocation of inter particle distance. These result confirms the presences of Ag-NPs in *Ganoderma lucidum* the results were similar when compared with the preceding reviews of nanoparticles (Mohan Kannan *et al*., 2014).

MTT Assay for Ag-NPs

Different concentration s and dilutions of silver nanoparticles of *Ganoderma lucidum* ranging from 20,40,60,80,100,200,300,400 (µM/ml) were used to study the viability of MCF-7 cells and the toxicity was measured. Positive control included DMSO treated cells which did not show the lethal effect in all the tested concentrations. Silver nanoparticles *of Ganoderma lucidum* showed promising activity on MCF-7 cell lines with the IC_{50} value being 64.714 µM/ml(Figure 8).This study suggested that the Cytotoxicity of biological synthesized silver nanoparticles increased with increasing concentrations of nanoparticles. The observations confirms and promisingly related to Krishnaraj *et al.* (2014) in MDA-MB-231 breast cancer cells when treated with the extracts of Silver nanoparticles of *Acalypha indica* (Zhou *et al*., 2007).

Conclusion

In conclusion, we effectively synthesised and characterised Ag-NPs of *Ganoderma lucidum* which exhibited strong anti-cancer potential on MCF-7 cell lines. Hence Ag-NPs of medicinal mushrooms like *G.lucidum* hold tremendous promise as anti-cancer agents in the near future.

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