

**EXTRACELLULAR BIOSYNTHESIS OF SILVER NANOPARTICLES USING THE MYCORRHIZAL MUSHROOM *TRICHOLOMA CRASSUM* (BERK.) SACC.: ITS ANTIMICROBIAL ACTIVITY AGAINST PATHOGENIC BACTERIA AND FUNGUS, INCLUDING MULTIDRUG RESISTANT PLANT AND HUMAN BACTERIA.**

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In this study we show for the first time the green biosynthesis of silver nanoparticles with the help of the mycorrhizal mushroom *Tricholoma crassum*(Berk.) Sacc. This is a simple and reproducible method of silver nanoparticle production using mycelial mats which results in monodispersed nanoparticles of high concentration. The particles were studied with spectrophotometry showing absorbance peak at 440nm. Scanning and transmission electron microscopy showed particles were mostly spherical with a few of hexagonal shape. With a TEM software, histogram was constructed showing the size range of the particles to be of 5 - 50 nm diameter, the average being 21.91nm. These nanoparticles had potent antimicrobial effect on human bacteria *E.coli* (DH5 $\alpha$ ) , plant pathogenic bacteria *Agrobacterium tumifaciens* (LBA4404) and the plant pathogenic fungus *Magnaporthe oryzae*. Multi-drug resistant (MDR) *E.coli* was generated by transformation with pUC19 and pZPY112 and selection with 100 $\mu$ g/ml Ampicillin and 35 $\mu$ g/ml Chloramphenicol. *A. tumifaciens* which is resistant to 25 $\mu$ g/ml Rifampicin was transformed with pCAMBIA2301 and selected with 50 $\mu$ g/ml Kanamycin. The nanoparticles had potent inhibitory effect on these MDR pathogenic bacteria. Thus this source of silver nanoparticles has the potential to be produced on a large scale and find application in the field of medicine and crop protection.

(Received June 6, 2011; accepted August 30, 2011)

*Keywords:* Silver nanoparticles, green synthesis, UV-Vis, TEM, antibacterial activity.

## **1. Introduction**

In the past decade there has been a tremendous amount of research interest in nanomaterials with respect to its production, properties and applications [1,2,3,4]. Nanoparticles are particulate dispersions or solid particles with a size in the range of 10-1000nm. The characteristic physical, chemical, electronic, electrical, mechanical, magnetic, thermal, dielectric, optical and biological properties of nanoparticles are distinct from the bulk material of the same element [3,5]. Optoelectronic, physicochemical and electronic properties of nanoparticles vary with differences in their size, shape and crystallinity. Therefore, the production of monodispersed nanoparticles with diverse size and shape has been a goal of numerous investigations in the field of nanotechnology [3].

Artificially made metal nanoparticles are typically produced on a small laboratory scale using methods such as chemical vapor deposition, irradiation or chemical reduction of metal salts. However most of these processes give rise to harmful byproducts [6]. Therefore stress is laid on benign biosynthesis process which results in environmentally friendly nanoparticles of biological origin. The use of microorganisms as nano-factories enables us to use simple large scale

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production of nanomaterials which does not give rise to toxic waste products<sup>1,6</sup>. Microbial assisted biosynthesis of nanoparticles is therefore a rapidly progressing area of nanobiotechnology [7].

Recently several studies have shown that nanoparticles can be produced from virus [8], bacteria [9] and fungi like *Trichoderma reesi* [10], *T. viride* [11], *Phytophthora infestans* [12], *Aspergillus niger* [7], *A. flavus* [13], *A. clavatus* [14] *Fusarium oxysporum* [15], *Verticillium* sp. [16,17], *Penicillium* sp. [18], *Pleurotus sajor-caju* [19] etc. Production of nanoparticles using filamentous fungi has some advantages over other organisms. Filamentous fungi are easy to handle, require simple raw materials and has high wall-binding capacity.

In the field of medicine also, with the rise in multidrug resistant bacterial strains, nanoparticles have become the alternate choice for the future of disease control [20]. Nanoparticles exhibit increased chemical activity due to their large surface to volume ratios and crystallographic surface structure [20]. The mechanism of antimicrobial property of nanoparticle lies with the fact that the extremely small size means a large surface area relative to the volume, which effectively covers the microorganisms and reduce oxygen supply for respiration. Silver nanoparticles are therefore a safer alternative to antibiotics [21]. In fact silver nanoparticles have been shown to enhance the activity of several commonly used antibiotic such as kanamycin, erythromycin, chloramphenicol and especially of ampicillin [11]. Hence, silver nanoparticles is a promising candidate for diverse medical applications ranging from silver based dressings, silver coated medicinal devices, such as nanogels, nanolotions, etc.[22, 23].

In the present study we show for the first time the green biosynthesis of silver nanoparticles from the mycorrhizal fungus *Tricholoma crassum* (Berk.) Sacc. The silver nanoparticles were of the size range 5-50 nm and were mostly spherical in shape, a few appearing hexagonal. These silver nanoparticles have potent antimicrobial activity against bacteria, fungi as well as multidrug resistant pathogenic bacteria.

## **2. Experimental details**

### **2.1 Preparation of cell free filtrate of *Tricholoma crassum* (Berk.) Sacc. mycelial mats**

The mycelium of *Tricholoma crassum* was cultured *in vitro* and was used for production of silver nanoparticles. Tissue from basidiocarp was first cultured on potato dextrose agar (PDA). For liquid culture, the mycelium from solid substrate was inoculated in 50ml potato dextrose broth in 250 ml flasks. The biomass was harvested after 72 h of growth in 28°C by straining through a sieve. The biomass was washed with sterilized distilled water to remove medium component. 1 g of biomass (fresh weight) was added to 10 ml of deionized water in a 250 ml Erlenmeyer flask and agitated in the same condition for 72 h at 28°C.

The resulting cell filtrate was collected by passing it through Whatman filter paper no. 1. This filtrate was used for nanoparticles synthesis.

### **2.2 Biosynthesis of silver nanoparticles by filtrate of *Tricholoma crassum***

For the synthesis of silver nanoparticles, 50 ml of 1mM AgNO<sub>3</sub> solution was mixed with 50 ml of cell filtrate in a 250 ml Erlenmeyer flask and agitated at 28°C in dark. Control (without the silver ion, only biomass) was also run along with the experimental flask (method taken from Jha and Prasad, 2010) [24].

### **2.3 UV–visible spectroscopic analysis of silver nanoparticles**

The reduction of silver ions was confirmed by qualitative testing of supernatant by UV–visible spectrophotometer. 1 ml of sample supernatant was withdrawn after 24 hr, 48 hr, 72 hr. and absorbance was measured by using UV–visible spectrophotometer between 350-600 nm.

#### **2.4 Scanning Electron Microscopic analysis of silver nanoparticles**

The filtrate with silver nanoparticles was taken on glass slide dried under vacuum. The sample was subjected to scanning electron microscopy (Spectrophotometer FPI, Model: Quanta200).

#### **2.5 Transmission Electron Microscopic analysis of silver nanoparticles**

For TEM measurement for particle shape, size and its distribution, a 50  $\mu$ l drop of solution containing biosynthesized silver nanoparticles was placed on the carbon-coated copper grids and subjected to vacuum desiccation before loading onto a specimen holder. TEM micrographs were taken by analyzing the prepared grids on TECHNAI G TEM instrument having a low voltage (100 kV) construction.

#### **2.6 Analysis of silver nanoparticles using OLYMPUS MEASURE IT software:**

The TEM measurements of particle sizes and construction of a histogram was done by OLYMPUS software MEASURE IT tool. The concentration of nanoparticles was calculated according to Marquis et al., 2009 [25], and was found to be 28mg/L.

#### **2.7 Transformation of human and plant pathogenic bacteria to develop multi-drug resistance, using plasmids carrying antibiotic resistance markers:**

The plant pathogenic *Agrobacterium tumefaciens* strain LBA4404 and human bacteria *E. coli* strain DH5 $\alpha$  was used for these experiments. Both of the bacteria were made competent by using 100mM CaCl<sub>2</sub>. *A. tumefaciens* which is resistant to Rifampicin (concentration 25 $\mu$ g/ml) was transformed with pCAMBIA 2301 and selected on plates containing 50 $\mu$ g/ml Kanamycin. *E. coli* DH5 $\alpha$  was transformed with pUC 19 and pZPPY112 and selected with 100 $\mu$ g/ml Ampicillin and 35 $\mu$ g/ml Chloramphenicol.

#### **2.8 Antimicrobial assays**

The antibacterial assays were done on *E. coli* (DH5 $\alpha$ ) and *A. tumefaciens* strain (LBA4404) by disc diffusion method. Luria Bertani (LB) broth/agar medium was used to cultivate bacteria. Fresh overnight cultures of inoculum (100 $\mu$ l) of each culture was spread on to LB agar plates. Quantitation of silver nanoparticles showed the concentration to be 28mg/L. Sterile paper discs of 5mm diameter with increasing amount of silver nanoparticles in each disc such as 0.28 $\mu$ g/10  $\mu$ l, 0.56 $\mu$ g/20  $\mu$ l, 1.4  $\mu$ g/50  $\mu$ l, 1.96 $\mu$ g/70  $\mu$ l, 2.8 $\mu$ g/100 $\mu$ l (total volume made up to 100 $\mu$ l with water) was used for the assay .

### **3. Results and discussion**

From published data it is known that nanoparticles exhibit brown color in aqueous solution due to excitation of surface plasmon vibrations in silver nanoparticles [26]. Fig. 1.A shows the cell -filtrate of *T. crassum* (control 1). The second control was 1mM AgNO<sub>3</sub> solution without cell filtrate (Fig. 1 B). When the cell filtrate was added to the 1 mM AgNO<sub>3</sub> solution and incubated at 28°C for 24hrs. the color changed to brown due to reduction of silver ions, indicating production of silver nanoparticles (Fig 1, C).

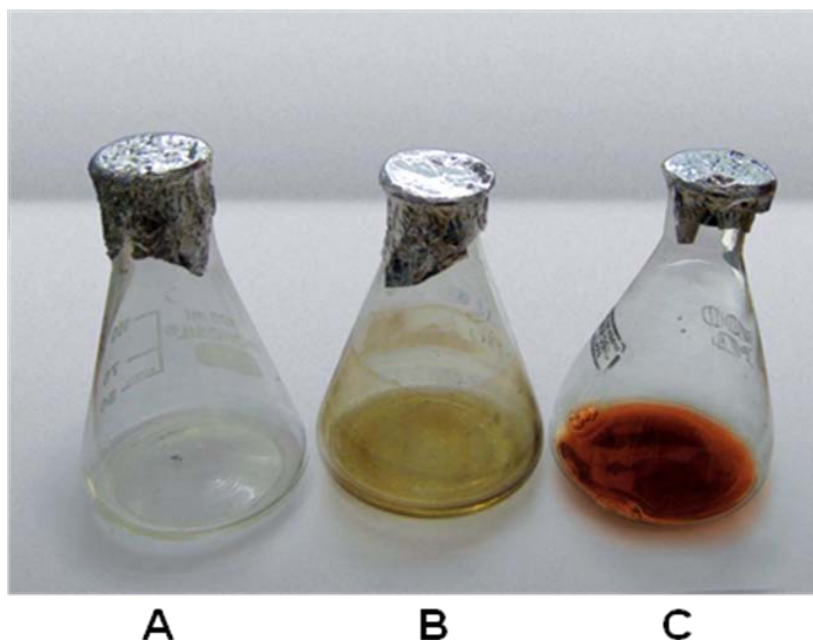


Fig. 1. Digital photograph of A. Mycelia-free cell filtrate of *Tricholoma crassum* (control 1). B. 1mM AgNO<sub>3</sub> without cell filtrate (control 2) C. 1mM AgNO<sub>3</sub> with cell filtrate after 24 hours incubation at 28°C.

It is accepted that UV-Vis spectroscopy can be used to determine size and shape-controlled nanoparticles in aqueous suspensions [27, 28]. Fig. 2 shows the UV-Vis spectral analysis of silver nanoparticles produced after 24, 48 and 72 hours of incubation. The absorption spectra shows absorbance peak at 440nm (Fig. 2). The narrow peak indicates that the particles are monodispersed.

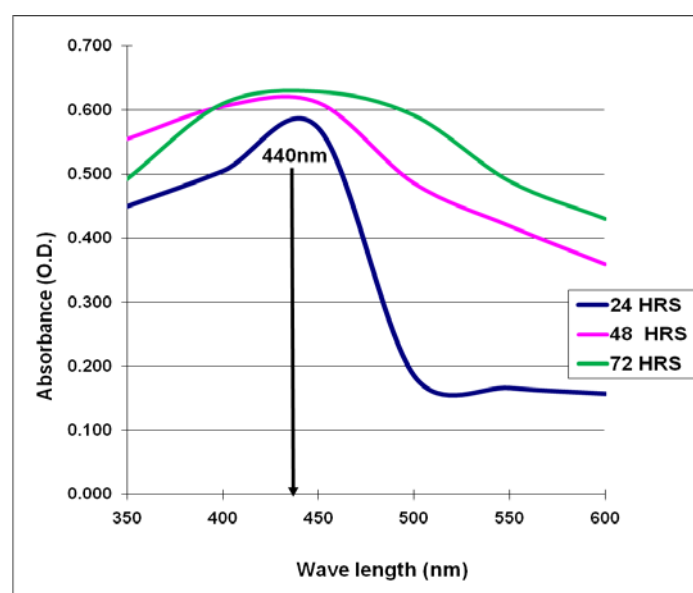


Fig 2. UV-Vis spectra recorded as a function of time of reaction at 24 hrs, 48 hrs, 72 hrs of incubation of an aqueous solution of 1mM AgNO<sub>3</sub> with the *Tricholoma crassum* fungal cell- filtrate. Showing absorption peak at 440 nm.

The morphology of the nanoparticles, including shape, size and concentration of the particles, was further studied with scanning electron microscopy and transmission electron microscopy. The scanning electron micrograph shows presence of silver nanoparticles magnified 30,000 times (Fig 3). The shape appears to be mostly spherical.

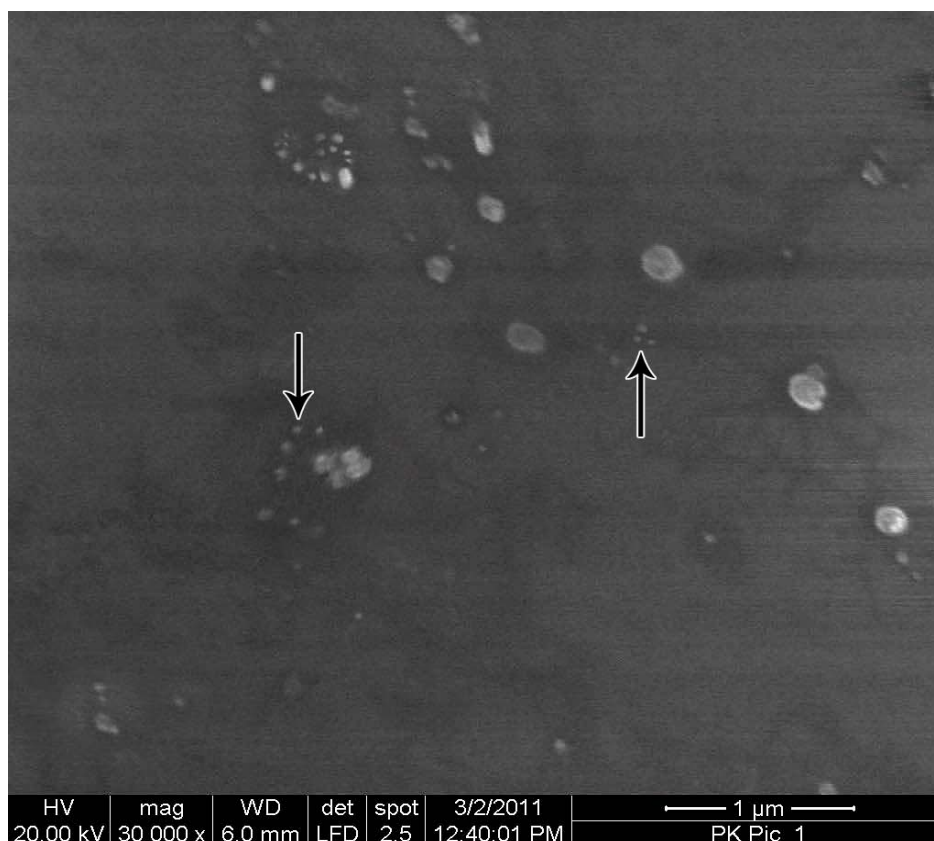


Fig 3. SEM micrograph of silver nanoparticles at 30,000 magnification.

The transmission electron micrograph distinctly shows the presence of a large population of silver nanoparticles formed after incubation of *T.crassum* mycelia-free cell filtrate with 1mM AgNO<sub>3</sub> for 72 hours (Fig. 4 and Fig. 5A).

Vast majority of the particles are of symmetrical spherical or quasi- spherical shape while a few appears to be hexagonal. The image also confirms that the particles are single crystalline in nature. The particle size distribution histogram of nanoparticles was obtained from TEM analysis using the OLYMPUS software MEASURE IT tool (Fig 5A). The histogram shows that the particle size ranges from 5nm to 50nm in diameter with an average size of 21.91 nm (Fig.5B). This result also indicates that the particles are monodispersed without aggregation.

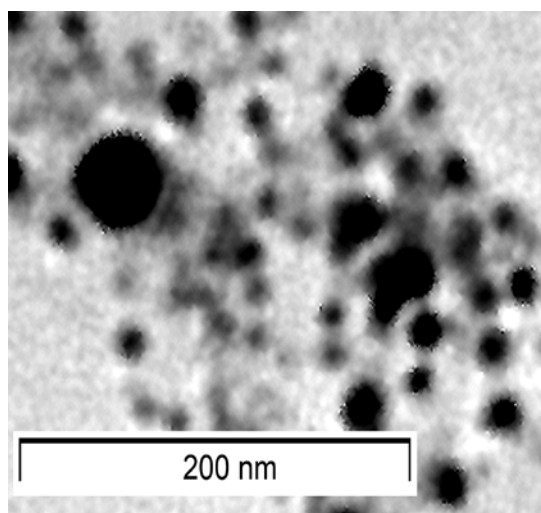


Fig 4. TEM micrograph at 100,000 times magnification recorded from a drop-coated film of an aqueous solution incubated with cell free extract of *Tricholoma crassum* and reacted with 1 mM AgNO<sub>3</sub> for 72h at 28°C. Scale bar corresponds to 200 nm.

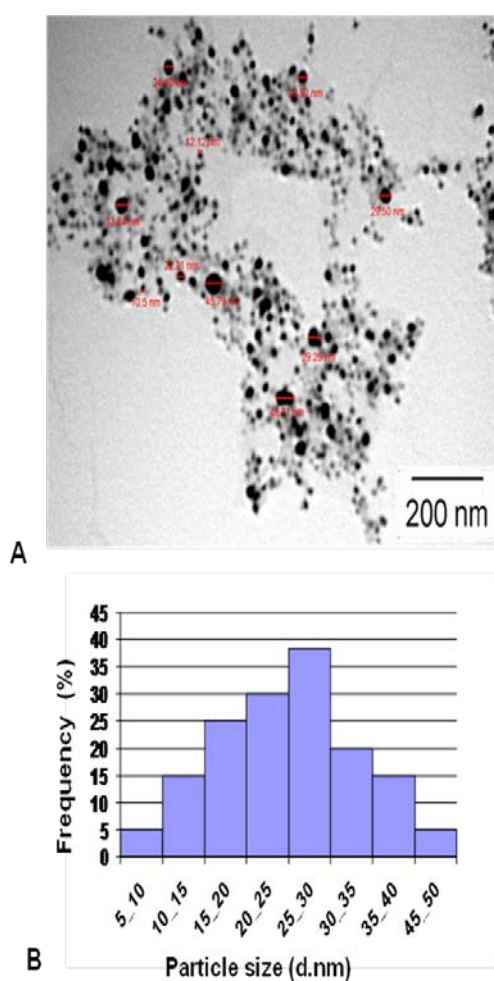


Fig. 5 Analysis of TEM micrograph using Olympus Measure It software. A. Enlarged view of TEM micrograph showing measurements. B. Particle size distribution histogram of silver nanoparticles from transmission electron microscope (TEM) analysis.

To test the antimicrobial properties for the nanoparticles, we used cultured *E.coli* (strain DH5 $\alpha$ ) and the plant pathogenic *Agrobacterium tumifaciens* (strain LBA4404) and plant pathogenic fungus *Magnaporthe oryzae* as well as multidrug resistant *E. coli* and *A. Tumifaciens*. The normal *E.coli* and *A. tumifaciens*, which were initially susceptible to antibiotics, were cultured on LBA without antibiotics. After transformation of the *E.coli* with pUC 19 and pZPPY112 the colonies were selected on LBA with 100 $\mu$ g/ml Ampicillin and 35 $\mu$ g/ml Chloramphenicol. Similarly *A. tumifaciens* transformed with pCAMBIA 2301 was selected with 25 $\mu$ g/ml Rifampicin and 50 $\mu$ g/ml Kanamycin. The antimicrobial activity was assayed on LBA plates using the paper-disc method. Different amounts of silver nanoparticles like 0.28 $\mu$ g /10  $\mu$ l, 0.56 $\mu$ g/20  $\mu$ l, 1.4  $\mu$ g/50  $\mu$ l, 1.96 $\mu$ g/70  $\mu$ l, 2.8 $\mu$ g/100 $\mu$ l (total volume made up to 100 $\mu$ l with water) were placed on the discs and the inhibition zones of the different pathogenic microbes were measured. Fig 6 shows that all the three microbes that were tested were inhibited at low concentrations of nanoparticles. Fig. 6A, B and C shows that the inhibition zone gradually increased with increasing amounts of nanoparticles for the *E.coli*, *A. tumifaciens* and *M. Oryzae*. Fig. 6.D, E and F show the graph of the inhibition zones of three microbes as a function of increasing amounts of silver nanoparticles. When assayed against multidrug resistant *E.coli* and *A. tumifaciens*, the result showed that the bacteria which could withstand 100 $\mu$ g/ml Ampicillin and 35 $\mu$ g/ml Chloramphenicol or 25 $\mu$ g/ml Rifampicin and 50 $\mu$ g/ml Kanamycin were inhibited with low concentrations silver nanoparticles (Fig. 7 A, B). Fig 7 C and D shows the graph of the same. Fig. 7 E shows the trend of inhibition for the two MDR microbes. On the whole, in these assays *A. tumifaciens* and *M. oryzae* showed greater sensitivity to the silver nanoparticles than *E.coli*.

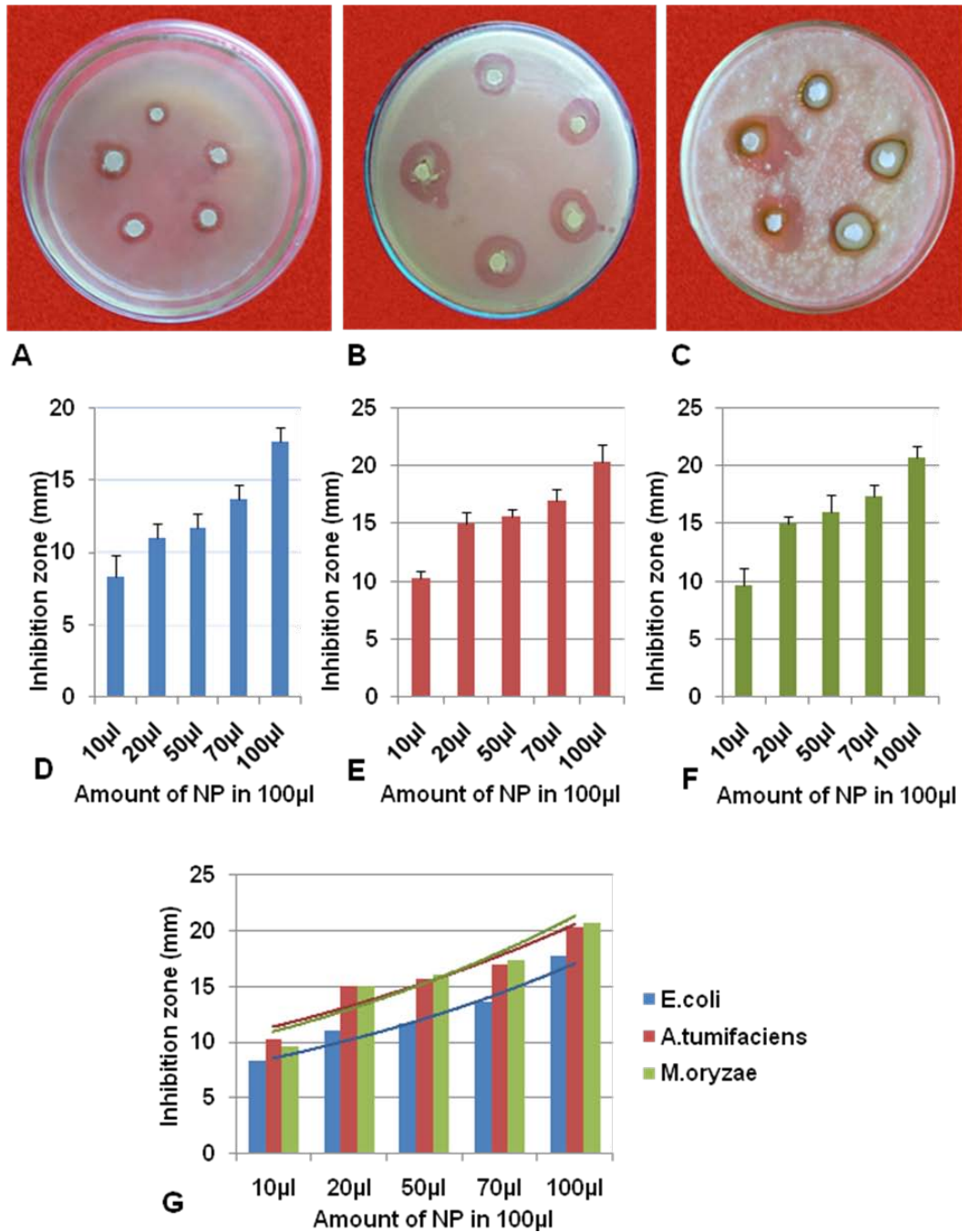


Fig 6. Antimicrobial effect of *Tricholoma crassum* silver nanoparticles on plant and human pathogenic microbes by disc-diffusion method. A. Plate showing increasing inhibition zone of MDR *E. coli* with increasing amounts of *T. crassum* silver nanoparticles ; clock-wise from top: 10µl, 20µl, 50µl, 70µl, 100µl with volume made up to 100 µl with water wherever needed. B. *Agrobacterium tumifaciens* C. *Magnaporthe oryzae* D. graph showing antimicrobial assay on *E. coli* E. Graph of antimicrobial assay on *A. tumifaciens* F. Graph of antimicrobial assay on *M. oryzae* G. Comparative trend of increasing inhibition zone of all three microbes.



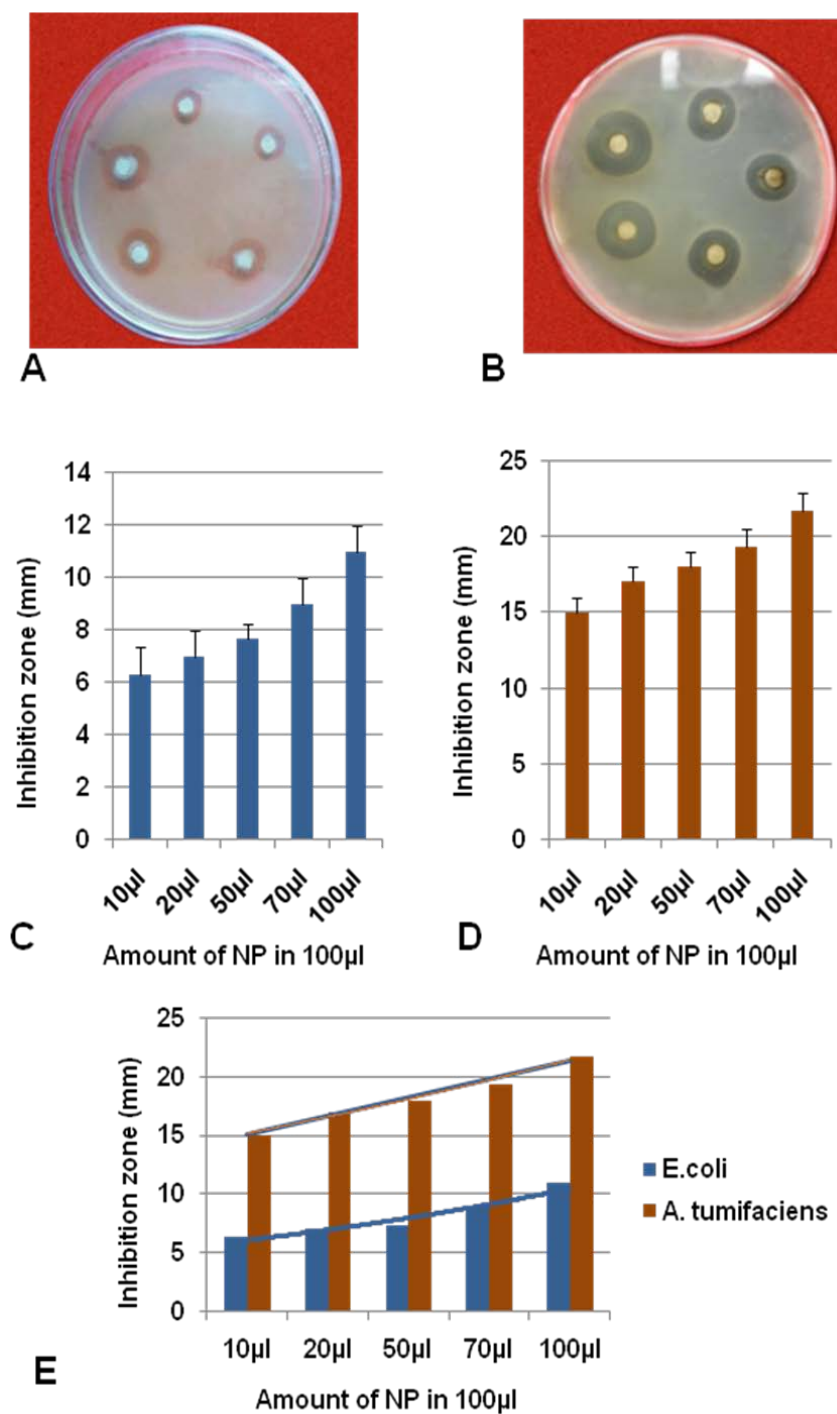


Fig 7. Antimicrobial effect of silver nanoparticles produced by *Tricholoma crassum* against multi-drug resistant (MDR) human and plant pathogenic microbes A. Plate showing increasing inhibition zone of MDR *E. coli* with increasing amounts of nanoparticles; clock-wise from top: 10µl, 20µl, 50µl, 70µl, 100µl with volume made up to 100 µl with water wherever needed. B. Same plate for MDR *Agrobacterium tumifaciens*. C. Graph showing antimicrobial assay on MDR *E. coli* D. Graph of antimicrobial assay on MDR *A. tumifaciens* E. Comparative trend of increasing inhibition zone for the two MDR microbes.

#### 4. Conclusion

The present study demonstrated the green or bioproduction of silver nanoparticles by mycelia-free cell-filtrate of *Tricholoma crassum* (Berk) Sacc. using 1mM AgNO<sub>3</sub>. This method results in the formation of monodispersed spherical nanoparticles of the size range 5-50 nm. The nanoparticles have been shown to have a potent antimicrobial effect against human pathogen *E.coli* and the plant pathogens like *A.tumifaciens* and *M. oryzae*. It is also very effective against MDR *E. coli* and *A. tumifaciens*. Thus these nanoparticles can be used both by the medicine industry as well as for control of plant diseases in the field.

This study also presents a simple and reproducible silver nanoparticle production using a filamentous fungus. This holds the potential for the large scale production of silver nanoparticles using big bioreactors resulting in minimal harmful by-products. This method would be both cost-effective and eco- friendly.

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