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RESEARCH ARTICLE

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BIOSYNTHESIS OF SILVER NANOPARTICLES USING FUNGI AND BIOLOGICAL EVALUATION OF MYCOSYNTHESIZED SILVER NANOPARTICLES

ABSTRACT:

Eight fungal species were screened for mycosynthesis of silver nanaoparticles (AgNPs), by visual observation of fungal filtrate only six fungal species were found to reduce the silver salt into silver nanoparticles. The UV-visible spectra of the biosynthesized nanoparticles (AgNPs) by *Aspergillus ochraceus* (RCMB 036254) cell filtrate showed characteristic surface plasmon absorption at 420 nm. Transmission electron microscopy (TEM) micrograph showed the formation of spherical AgNPs ranging from 5.5 to 24.5 nm diameter. The Qualitative, as well as quantitative status of elemental silver was characterized by Energy Dispersive Analysis of X-ray (EDX). The optimum conditions for maximum production of AgNPs were obtained using 0.75 mM silver nitrate at 40°C and pH 6. The antimicrobial activity of the mycosynthesized nanoparticles under optimum conditions were investigated alone and in combination with commonly used antibiotics against Methicillin-resistant *Staphylococcus aureus* (MRSA). Out of thirteen commonly used antibiotics, the antibacterial efficiency of only five antibiotics has been increased as a result of combination with AgNPs. Biosynthesized silver nanoparticles showed dose-dependent antitumor activity with IC50 at 1.4, 2.1, and 1.2 µg/ml against human colon carcinoma, human breast cancer and human hepato-cellular carcinoma cells, respectively, while 39.6 µg/ml was required to induce 50% of normal Vero cell mortality.

KEY WORDS:

Biosynthesis, silver nanoparticles, mycosynthesized silver nanoparticles

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INTRODUCTION:

The prefix nano is derived from the Greek word *Nanos* refers to things of one billionth (10^{-9}m) in size (Narayanan and Sakthivel, 2010). A nanoparticle is defined as having one dimension 100 nm or less in size. Environmentally toxic or biologically hazardous reducing agents are typically involved in the chemical synthesis of nanoparticles (Ghorbani *et al.*, 2011).

There has been a search for greener production alternatives of metal nanoparticles (Abou El-Nour *et al.*, 2010) many researches have shown that microorganisms, plant extracts, and fungi can produce nanoparticles through biological pathways (Ahmed *et al.*, 2003; Abou El-Nour *et al.*, 2010; Popescu *et al.*, 2010).

Both unicellular and multicellular organisms are known to produce inorganic materials either intra- or extracellularly (Shankar *et al.*, 2004). The ability of microorganisms like bacteria and fungi to control the synthesis of metallic nanoparticles is employed in the search for new materials. Biosynthesis of nanoparticles of different elements is reported from both pathogenic and nonpathogenic fungi (Vigneshwaran *et al.,* 2007).

The fungal systems or myco-nanofactories have been exploited for the synthesis of metal nanoparticles of silver, gold, zirconium, silica, titanium, iron (magnetite) and platinum. A large number of

fungal strains are capable to synthesize silver nanoparticles (AgNPs) extracellulary, among which *Fusarium oxysporum* (Ahmad *et al*., 2003), *Aspergillus fumigatus* (Bhainsa and D'Souza, 2006), *Aspergillus niger* (Gade *et al.,* 2008), *Fusarium semitectum* (Basavaraja *et al*., 2008), *Penicillium brevicompactum* (Shaligram *et al*., 2009), *Cladosporium cladosporioides* (Balaji *et al*., 2009), and *Aspergillus clavatus* (Verma *et al.,* 2010) have been previously described. Fungi are more advantageous compared to other microorganisms in many ways. Fungal mycelial mesh can withstand flow pressure and agitation and other conditions in bioreactors or other chambers compared to plant materials and bacteria. These are fastidious to grow and easy to handle and easy for fabrication. The extracellular secretions of reductive proteins are more and can be easily handled in downstream processing. And also, since the nanoparticles precipitated outside the cell is devoid of unnecessary cellular components, it can be directly used in various applications (Narayanan and Sakthivel, 2010).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the major nosocomial pathogens responsible for a wide spectrum of infections, including skin and soft tissue infections, pneumonia, bacteraemia, surgical site infections (SSI), catheter related infections (de San *et al*., 2007). Intensive care unit characteristically has higher rates of infections and increased transmission rates, high antibiotic use and large numbers of vulnerable patients. The emergence of bacterial resistance to antibiotics and its dissemination, however, are major health problems, leading to treatment drawbacks for a large number of drugs (Schito, 2008). Consequently, there has been increasing interest in the use of inhibitors of antibiotic resistance for combination therapy (Gibbons, 2008).

It has been demonstrated that AgNPs have effective antimicrobial activity (Aymonier *et al*., 2002; Sondi and Salopek-Sondi, 2004; Baker *et al*., 2005; Melaiye *et al*., 2005; *Lok et al*., 2006). AgNPs have been applied to a wide range of health care products, such as burn dressings, scaffold, water purification systems and medical devices (Thomas *et al*., 2007).

In vitro cytotoxicity testing procedures reduce the use of laboratory animals and hence use of cultured tissues and cells have increased (Byrd *et al*., 2000). The discovery and identification of new antitumor drug with low side effects on immune system has become an essential goal in many studies of immuno-pharmacology, it is a challenge to find drugs for the effective treatment of various types of cancers (Xu *et al*., 2009).

In the present study, we attempted to evaluate antibacterial and antitumor activities of mycosynthesized AgNPs; evaluation of the antibacterial activity singly and in combination with commonly used antibiotics against Methicillin-resistant *Staphylococcus aureus* (MRSA); while antitumor activity was evaluated against different cancer cell lines *in vitro*.

MATERIAL AND METHODS:

Fungal species:

Fungal organisms namely, *Aspergillus fumigatus* (RCMB 02568), *Aspergillus flavus* (RCMB 02426) *Candida albicans* (RCMB 05031), *Penicillium italicum* (RCMB 03924), *Syncephalastrum racemosum* (RCMB 05922), *Fusarium oxysporum* (RCMB 08213), *Alternaria solani* (RCMB 07324) and *Aspergillus ochraceus* (RCMB 036254); kindly provided from the Regional Centre for Mycology and Biotechnology, Al-Azhar university, Egypt were screened for production of silver nanoparticles AgNPs.

Biomass preparation:

Fungi were grown on malt extract broth at 28°C on a rotary shaker (120 rpm) for 96 h. The biomasses were harvested by filtration using Whatman filter paper No. 1, followed by washing with distilled water to remove any components of the medium. The biomass (25 g) wet weight was placed in individual flasks containing 100 ml water and incubated for 24 h. The biomass was filtered, and the cell filtrate was collected and used for biosynthesis of AgNPs (Devi and Joshi, 2012).

Biosynthesis of AgNPs:

For biosynthesis of AgNPs, 50 ml of cell filtrate was mixed with 10 ml $AgNO_3$ solution (1mM) and reaction mixture without $AgNO_3$ was used as control. The prepared solutions were incubated at 28°C for 24 h. All solutions were kept in dark to avoid any photochemical reactions during the experiment. The AgNPs were purified by centrifugation at 10,000 rpm for 10 min twice, and collected for further characterization (Devi and Joshi, 2012).

Characterization of AgNPs:

After 24 hours of synthesis, the sample of AgNPs was centrifuged at 14,000 rpm for 30 minutes at room temperature. Repeated rinses were performed to remove impurities. The residue of AgNPs was re-suspended in 1 ml sterile water. The production of AgNPs in aqueous solution was monitored at the Regional Centre for Mycology and Biotechnology (RCMB) using:

i. UV-visible Spectroscopy Analysis:

Change in colour of the cell free filtrate incubated with silver nitrate solution was visually observed over a period of time. Absorption measurements of the filtrate were

carried out after 24 h. using UV-visible spectrophotometer (Milton-Roy Spectronic 1201). UV-Visible analysis of several weeks old samples was also carried out to check the stability of synthesized AgNPs (Ingle *et al.*, 2008).

ii. Transmission Electron Microscopy (TEM):

For TEM analysis, a drop of the cell filtrate was placed on the carbon coated copper grids and dried by allowing water to evaporate at room temperature. Electron micrographs were obtained using GEOL GEM-1010 transmission electron microscope at 70 kV (Jain *et al.*, 2011).

iii. Energy Dispersive Analysis of X-ray (EDX):

The presence of elemental silver was confirmed through EDX. The EDX microanalysis was carried out by X-ray micro-analyzer (Oxford 6587 INCA) attached to JEOL JSM-5500 LV scanning electron microscope at 20 kV. The EDX spectrum recorded in the spot profile mode from one of the densely populated silver nanoparticles region on the surface of the film. The nano crystallites were analyzed using Quanta 200 FEG (Devi *et al.*, 2012).

Effect of reaction parameters on the production of AgNPs:

To obtain the optimized reaction parameters giving maximum synthesis of AgNPs, firstly, $AgNO_3$ ranging from 0.25 to 7 mM (final concentration) was added to the fungal filtrate and incubated for up to 120 hours. After this, $AgNO_3$ was added to the fungal filtrate and incubated at (5°C–50°C) and at pH (2-10) for temperature and pH optimization, respectively. Then UV-Vis spectra were carried out to study the production with varying reaction parameters.

The antimicrobial activity of AgNPs:

The antibacterial activity of synthesized AgNPs was investigated against Methicillin-resistant *Staphylococcus aureus* (MRSA). The overnight grown MRSA culture was plated on Muller-Hinton agar (MHA). Wells were cut on the plates using cork porer and 50 µl of AgNP solution was dispensed in each well. The mycelia-free water extract alone and $AgNO_3$ were used as control. The plates were incubated overnight at 37°C for 24 h. and observed for the presence of zones of inhibition. The minimum inhibitory concentration (MIC) of mycelia-free water extract alone, AgNPs and $AgNO_3$ were determined by broth microdilution method given by the Clinical Laboratory Standards Institute (CLSI, 2004). Two-fold serial dilutions of AgNPs were made using Muller-Hinton (MH) broth (Roy *et al.* 2010).

Antibiotic susceptibility:

Thirteen antibiotics, namely, Ampicillin (AM10), Penicillin (P10), Oxacillin (OX1), Amoxicillin (AX25), Amoxicillin/clavulanic acid (AMC30), Cephalothin (KF), Cefoxitin (FOX30), Ceftriaxone (CRO30), Vancomycin (VA30), Amikacin (AK30), Tobramycin (TOB10), Erythromycin(E15) and Ciprofloxacin (CIP5) were used to assay the antibacterial efficiency of commonly used antibiotics alone and their combined effect with extra-cellularly mycosynthesized AgNPs against MRSA using disc agar diffusion method (DAD) on Muller-Hinton agar, according to the guidelines recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2002). To determine the combined effects, each standard paper disc was further impregnated with sub-inhibitory concentration of AgNPs. A single colony of MRSA was grown overnight in Muller-Hinton broth medium on a rotary shaker (200 rpm) at 37°C. The inocula were prepared by diluting the overnight cultures with 0.9% NaCl to a 0.5 McFarland standard and were applied to the plates along with the standard antibiotics alone and that combined with sub-inhibitory concentration of AgNPs. After incubation at 37°C for 24 h., the zones of inhibition were measured. The assays were performed in triplicates.

The antitumour activity of AgNPs

The activity of AgNPs was assayed on three tumour cell lines and normal Vero cells.

Cell lines:

Human colon carcinoma (HCT-116) cells, Human breast cancer (MCF-7) cells and Human hepatocellular carcinoma (HepG2) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium components supplemented with 10% inactivated foetal calf serum and 50 µg/ml gentamycin. The cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and were sub-cultured two to three times a week (Mosmann, 1983).

Evaluation of the antitumor activity:

The antitumor activity was evaluated on MCF-7, HCT-116 and HepG2 cells. The cells were grown as monolayers in growth RPMI-1640 medium supplemented with 10% inactivated foetal calf serum and 50 µg/ml gentamycin. The monolayers of 10,000 cells adhered at the bottom of the wells in a 96-well microtiter plate incubated for 24 h. at 37ºC in a humidified incubator with 5% CO_2. The monolayers were then washed with sterile phosphate buffered saline (0.01 M pH 7.2) and simultaneously the cells were treated with 100 µl from different dilutions of the test sample in fresh maintenance medium and incubated at 37ºC. A control of untreated cells was made in the absence of the test sample. Six wells were used for each concentration of the test sample. Every 24 h., the observation under the inverted microscope was made. The number of the surviving cells was determined by staining the cells with crystal violet

(Muthumary, 2007) followed by cell lysing using 33% glacial acetic acid and read the absorbance at 490 nm using ELISA reader (Sun Rise, TECAN, Inc, USA) after well mixing. The absorbance values from untreated cells were considered as 100 % proliferation. The number of viable cells was determined using ELISA reader as previously mentioned and the percentage of viability was calculated as [1-(ODt /ODc)] x 100% where ODt is the mean optical density of wells treated with the test sample and ODc is the mean optical density of untreated cells. The 50% inhibitory concentration (IC_{50}); the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots.

RESULTS AND DISCUSSION:

Biosynthesis of silver nanoparticles (AgNPs):

Out of the eight fungal species screened, only six fungal species; namely *Aspergillus fumigatus* (RCMB 02568), *Candida albicans* (RCMB 05031), *Penicillium italicum* (RCMB 03924), *Syncephalastrum racemosum* (RCMB 05922), *Fusarium oxysporum* (RCMB 08213) and *Aspergillus ochraceus* (RCMB 036254) were found to reduce silver salt into silver nanoparticles by visual observation of the fungal filtrates. These six fungal filtrates exhibited a gradual change to brown colour, clearly indicating the formation of AgNPs. The colour of the culture filtrate with silver nitrate solution changed to intense brown after 24 h. of incubation, whereas, the control (without silver nitrate salt) did not exhibit any colour change. *Aspergillus ochraceus* (RCMB 036254) exhibited the most intense brown colour compared to the other five fungal species (Fig. 1). The colour changes observed can be attributed to the surface plasmon resonance of deposited AgNPs (Mulvaney, 1996).

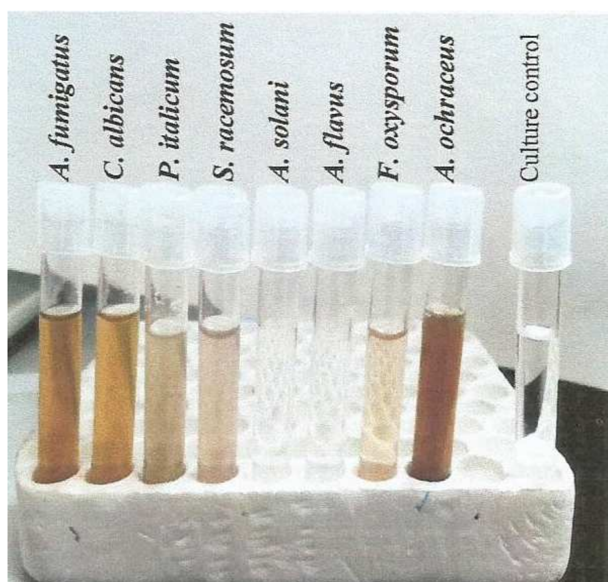

Fig. 1 Colour observed in fungal extract of different fungal species after exposure to silver nitrate solution.

Characterization of Ag NPs:

- UV-visible spectroscopy analysis:

The UV-visible spectra of *Aspergillus ochraceus* (RCMB 036254) filtrate treated with the silver nitrate solutions showed characteristic surface plasmon absorption at 280 and 420 nm (Fig. 2). The absorption at 280 nm indicates the presence of tryptophan and tyrosine residues present in the protein, this observation indicates the release of proteins into filtrate that suggests possible mechanisms for the reduction of silver ions present in the solution as demonstrated by Bhainsa and D'Souza (2006). Fungal cell filtrate treated with silver nitrate solution is known to show peak around 420 nm with high absorbance as demonstrated by Ingle *et al*. (2008), which supports our finding of the peaks observed for absorbance at 420 nm, indicating the bio-synthesis of nanoparticles by *A. ochraceus* (RCMB 036254).

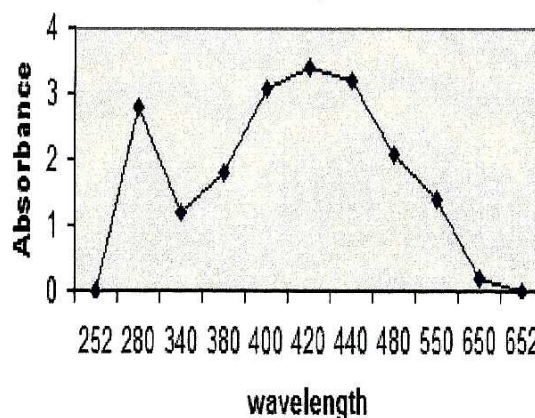

Fig. 2. UV-visible absorption spectra obtained for silver nanoparticles synthesized by *Aspergillus ochraceus* (RCMB 036254)

- Microscopic characterization by TEM:

The data obtained from transmission electron-micrograph showed distinct shape and size of nanoparticles. The particles were spherical in shape with mean of 13.88 ± 4.11 nm (Fig. 3 & Table1). AgNPs uniformly distributed with some agglomeration which revealed pattern similar to the biosynthesized AgNPs by Kathiresan *et al*. (2009) and Jain *et al*. (2011).

Fig. 3. TEM micrograph of the silver nanoparticles synthesized by *A. ochraceus*. Scale bar = 100 nm

Table 1. Statistical measurements AgNP5 ranging from 5.5 – 24.4 nm

Statistical function	Distance (nm)
Count	53
Mean	13.88
Minimum	5.5
Maximum	24.43
Standard deviation	4.11

- Energy Dispersive Analysis of X-ray (EDX):

EDX gives qualitative, as well as quantitative status of elements that may be involved in the formation of AgNPs. Figure 4 shows the EDX spectrum recorded in the spot-profile mode. The optical absorption peak is observed at 3KeV, which is typical for the absorption of metallic AgNPs by Magudapathy *et al.* (2001). Strong signals from the silver atoms are observed, while weaker signals from Na, Si, S, P, Cl, and Ca atoms are also recorded. From the EDX spectrums, it is clear that AgNPs reduced by *Aspergillus ochraceus* (RCMB 036254) have the weight percentage of silver as 52.91% (Table 2).

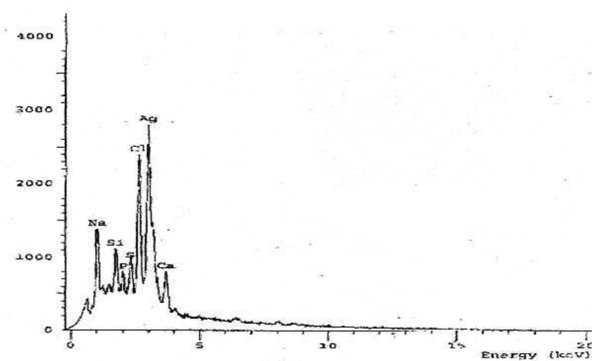

Fig. 4. EDX spectra of Ag NPs. Silver X-ray emission peaks are labeled. Strong signals from the atoms in the nanoparticles are observed in spectrum and confirms the reduction of silver ions to AgNPs

Table 2. The element composition of the AgNPs of EDX spectra

Element	Element%
Na	14.22
Si	4.93
P	2.52
S	4.08
Cl	14.89
Ca	6.44
Ag	52.91
Total	100

Effect of reaction parameters on the production of AgNPs:

Optimization studies revealed the significant effects of concentration of reaction parameters on the rate of bio reduction of silver ions to AgNPs. At a fixed temperature of 28°C, variation in reaction kinetics was observed for the synthesis of nanoparticles by varying the $AgNO_3$ concentration and reaction time (Fig. 5). Beyond 72 h. of incubation, no further increase in intensity was recorded indicating complete reduction of silver ions by the fungal cell filtrate. Maximum synthesis of nanoparticles occurred at 0.75 mM $AgNO_3$ in the reaction mixture, followed by 0.5 mM $AgNO_3$. Highest concentration (7 mM) $AgNO_3$ showed the least bio-reduction of silver ions to nanoparticles. This can be explained on the basis of enzyme-substrate kinetics; i.e. the active site in the key biomolecule responsible for reduction is already saturated with the silver ions, and no site is available for excess ions to get reduced, hence there is no further increase in synthesis of AgNPs despite the addition of more salt (Singh *et al.*, 2013). As compared with the UV-Vis spectrum obtained for different concentration of $AgNO_3$ after 72 h., 420 nm was the optimum wavelength for all concentrations (Fig. 6). At the optimized $AgNO_3$ concentration of 0.7 mM, rate of synthesis was found to increase with an increase in reaction temperature up to 40°C, which showed maximum synthesis (Fig. 7) after which a decline in the synthesis was observed and that may be due to deviations from the optimized parameters resulted in an increase in size and poly dispersity of AgNPs as demonstrated by Singh *et al.* (2013). pH 6 was found to provide optimal conditions for the maximal synthesis of nanoparticles (Fig. 8). Despite a large number of reports on the synthesis of Ag NPs, only few reports are available on the optimization. One such study was reported by Gurunathan *et al.* (2009) for *E. coli*-mediated Ag NPs synthesis, where 5 mM $AgNO_3$, 60°C temperature, and pH 10 were reported to provide optimal conditions for the maximal synthesis of small sized nanoparticles. In the present study, the enhanced rate of synthesis of AgNPs at optimized conditions might be the direct result of the effect of substrate (silver ions), pH and temperature on a key biomolecule responsible for the reduction present in the aqueous filtrate of *A. ochraceus.*

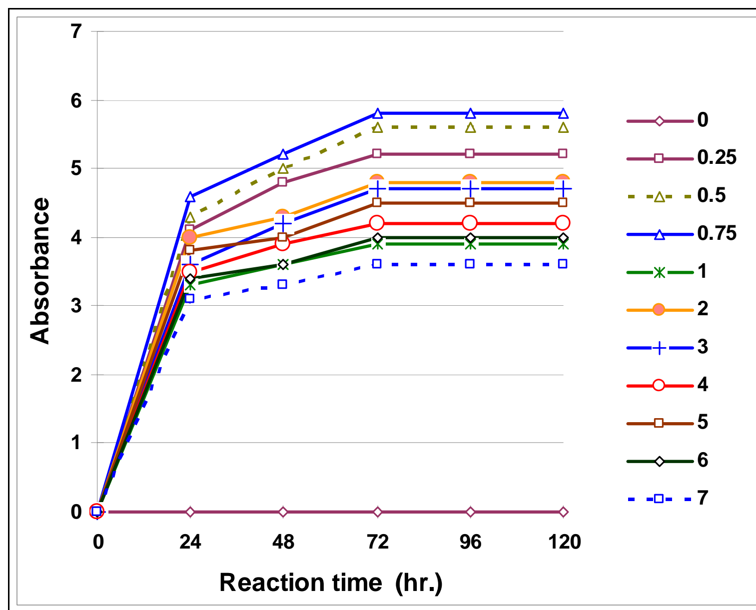

Fig. 5. Optimization of $AgNO_3$ concentration for AgNPs synthesis.

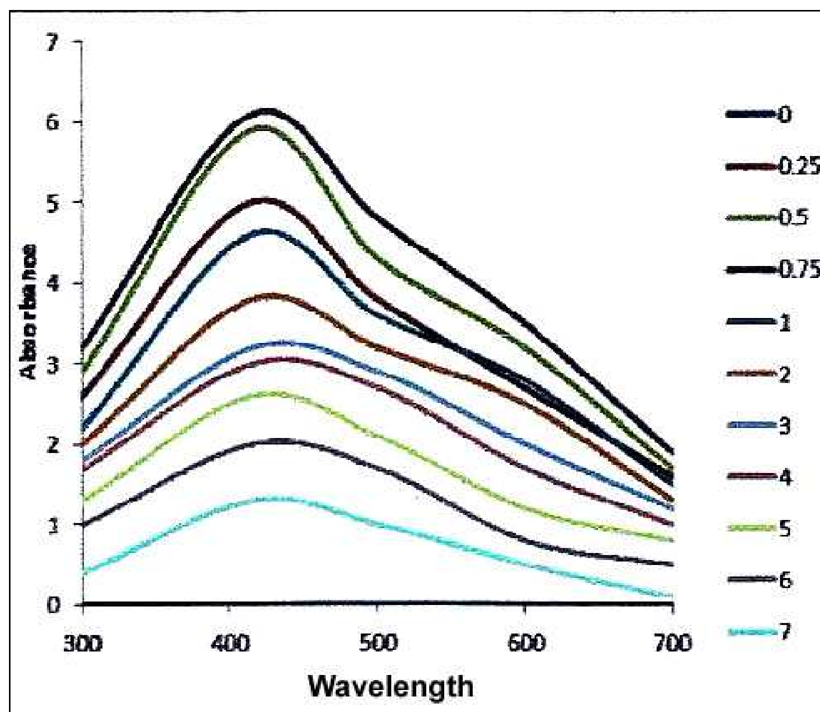

Fig. 6. UV-Vis spectra of AgNPs synthesis obtained with different concentrations of $AgNO_3$

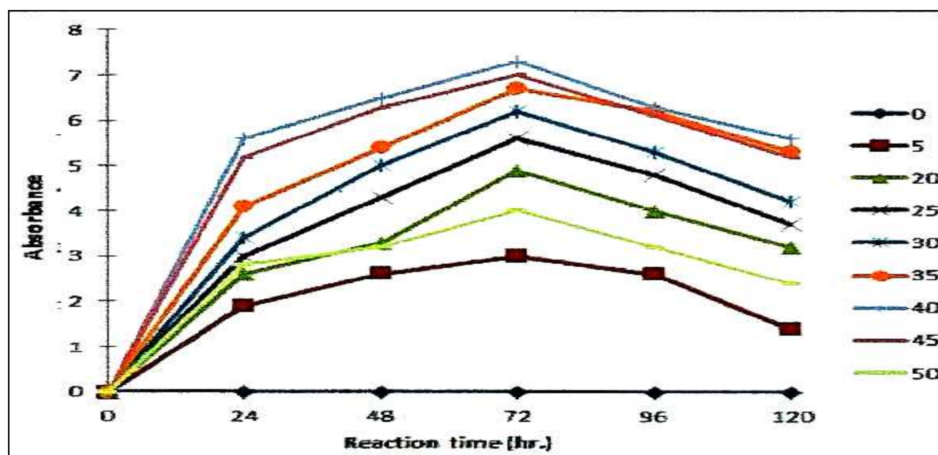

Fig. 7. Optimization of reaction temperature for AgNPs synthesis

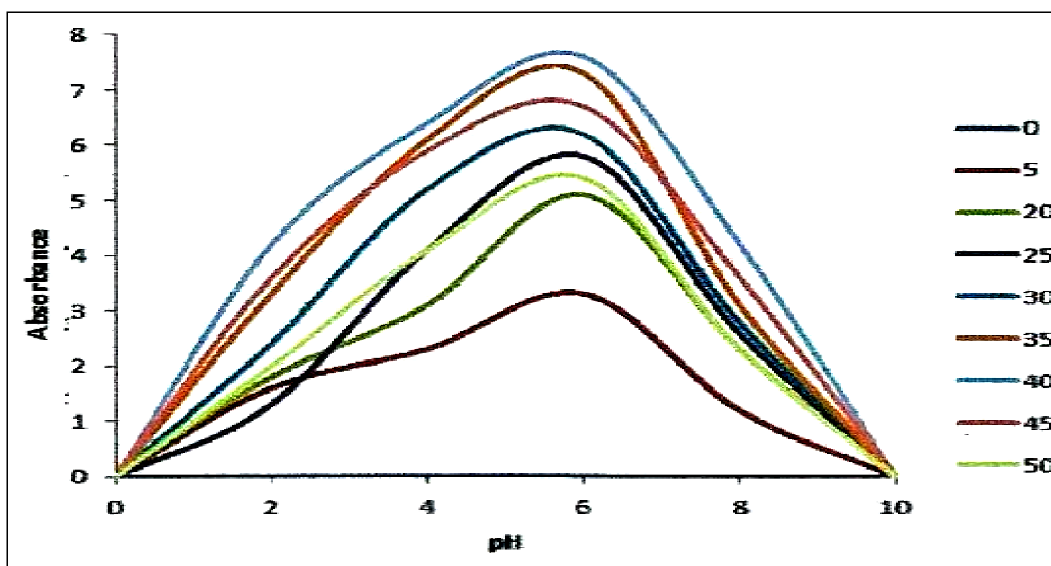

Fig. 8. Optimization of reaction pH for AgNPs synthesis

Antibacterial activity of AgNPs:

Silver and its compounds are known for their antimicrobial properties and for the treatment of burns and chronic wounds (Shakibaie *et al.,* 1998). High surface area to volume ratio cause high bactericidal activity of AgNPs compared with bulk silver metal (Cho *et al.,* 2005; Panyala *et al.,* 2008).

It is well known that Ag ions and Ag-based compounds have biological activities (Furno *et al.,* 2004). In the current study silver nanoparticles exhibited moderate antibacterial activity against MRSA better than that of $AgNO_3$ with zone of inhibition of 16.7 mm and 13.2 for AgNPs and $AgNO_3$, respectively, while the fungal extract did not show any activity against MRSA (Table 3). The reason for this is the structural composition of Gram-positive bacteria (Gram-positive bacteria possess a thick layer of peptidoglycan (20–80 nm), making it difficult for AgNPs to penetrate). Owing to their small size, AgNPs impair the sulphur and phosphorus containing essential macromolecules such as proteins and DNA (Wei *et al.,* 2009). Thus, action of AgNPs appears to be a consequence of adherence to and penetration inside the cell of the target cells.

Table 3. Zone of inhibition (mm) and Minimum inhibitory concentration (MIC) of $AgNO_3$, AgNPs and aqueous extract against MRSA

Test organism	$AgNO_3$	AgNPs	Aqueous extract	Reference drugs Ciprofloxacin
	Zone of inhibition (mm)			
MRSA	13.2	16.7	0	8
	MIC (µg/ml)			
	31.25	15.63	0	62.5

The effect of AgNPs on the antibacterial activity of thirteen antibiotics was investigated against MRSA using disk diffusion method. The antibacterial resistance of MRSA against various antibiotics decreases with nanoscaled AgNPs. The diameter of inhibition zones (mm) around the different antibiotic discs with and without AgNPs against MRSA are shown in (Table 4 & Figs 9 and 10). It should be pointed out that the AgNPs content of 7.81 µg/ disc was chosen to guarantee that the effect produced was due to the combination and not to the effect of the AgNPs itself. The antibiotic efficacy of some of tested antibiotics has been significantly improved in the presence of nano size silver against MRSA. The highest antibacterial activities increases in area were observed for Ceftriaxone (13 mm) followed by Amoxicillin/ clavulanic acid (10 mm) followed by Amikacin and Ciprofloxacin (6 mm in each). The lowest increase in inhibition zone area (3 mm) was reported for Tobramycin, Conversely, Ampicillin, Penicillin, Oxacillin, Amoxicillin, Cephalothin, Cefoxitin, Vancomycin and Erythromycin AgNPs showed no effect on the antibacterial activity of these antibiotics against MRSA. So the effect observed in this condition is due to the antibiotic-AgNPs combination. It is believed that microorganisms carry a negative charge while metal oxides carry a positive charge. This creates an “electrostatic” attraction between the microbe and treated surface. Once the contact is made, the microbe is oxidized and dead instantly. Generally, it is believed that nanomaterials release ions, which react with the thiol group (-SH) of the proteins present on the bacterial surface. Such proteins protrude through the bacterial cell membrane, allowing the transport of nutrients through the cell wall. Nanomaterials inactivate the proteins, decreasing the membrane permeability and eventually causing the cellular death (Zhang and Chen, 2009).

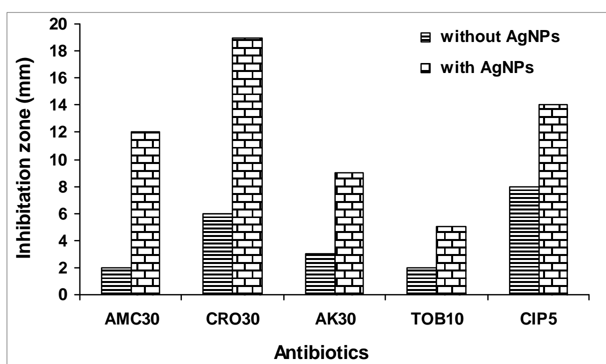

Fig. 9. Zone of inhibition (mm) of different antibiotics against MRSA in the presence and absence of AgNPs

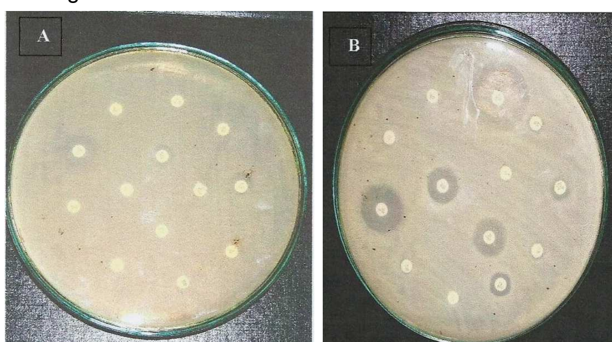

Fig. 10. Zone of inhibition (mm) of different antibiotics against the growth of MRSA on Muller-Hinton agar medium. (A): in the absence of AgNPs; (B): in the presence of AgNPs

Table 4. Zone of inhibition (mm) of different antibiotics against MRSA in the presence and absence of AgNPs

Antibiotics	Symbol	Inhibition Zone of Antibiotic (mm)	Inhibition Zone of Antibiotic with AgNPs (mm)	Increased Zone size (mm)
B-lactams				
Ampicillin	AM10	NA	NA	0
Penicillin	P10	NA	NA	0
Oxacillin	OX1	NA	NA	0
Amoxicillin	AX25	NA	NA	0
Amoxicillin/ clavulanic acid	AMC30	2	12	10
Cephalosporins				
Cephalothin	KF	NA	NA	0
Cefoxitin	FOX30	NA	NA	0
Ceftriaxone	CRO30	6	19	13
Glycopeptides				
Vancomycin	VA30	NA	NA	0
Aminoglycosides				
Amikacin	AK30	3	9	6
Tobramycin	TOB10	2	5	3
Macrolides				
Erythromycin	E15	NA	NA	0
Flouroquinolones				
Ciprofloxacin	CIP5	8	14	6

Silver ions have been used in many kinds of formulations, and recently it was shown that hybrids of AgNPs with amphiphilic hyperbranched macromolecules exhibit effective antimicrobial surface coating. The most important application of silver and AgNPs is in the medical industry, such as topical ointments to prevent infection in burns and open wounds. Newly devised AgNPs-coated wound dressings have been a major breakthrough in the management of wounds or infections. To prevent or reduce infections, a new generation of dressings incorporating antimicrobial agents like silver has been developed. Impregnation of wound dressings impregnated with colloidal silver resulted in a strong decrease of pathogen-specific alterations in infected epithelium. The delivery of silver and AgNPs to infected keratinocytes in a moist healing environment is efficient, fast, and active as compared to wound dressing without silver. Similar results with *E. coli* were obtained with AgNPs (Mudasir *et al.*, 2013).

Antibacterial activity of AgNPs and its combined effects with antibiotics was assessed by disk diffusion method. Each disk was impregnated with 20 µl of AgNPs, to check the combined effect the disk was impregnated with 10 µl of the antibiotic and 10 µl of AgNPs. AgNPs showed enhancing antibacterial property when used in combination with an antibiotic (Mudasir *et. al.*, 2013). In the present study also, combined effect of AgNPs with antibiotics is assessed and the use of AgNPs is found to be useful in most results.

Antitumor activity:

The anti-tumor effect of AgNPs and Ag+ was reported (Ahamed *et al.,* 2008; Rahman *et al.,* 2009). In this study, *in vitro* antitumor activity of the AgNPs was evaluated against HCT -116, MCF-7 and HepG2 cell lines at different concentrations. The cytotoxicity analysis of the AgNPs showed a direct dose-response relationship; cytotoxicity increased at higher concentrations (Tables 5& 6). The result revealed that $AgNO_3$ and AgNPs have potent antitumor activity with IC50 values of 12.4 and 1.2 µg/ml, respectively against HepG2 cells; 14.9 and 1.4 µg/ml, respectively against HCT -116 cells; 3.0 and 2.1 µg/ml, respectively against MCF-7 cells (Fig. 11). The enhanced cytotoxicity of AgNPs may be due to their size which facilitates their subsequent penetration in tumor cells. The cytotoxic effects of AgNPs, probably due to the fact that AgNPs are likely to interact with thiol rich enzymes (Morones *et al.,* 2005), other researchers suggest that AgNPs may interfere with the proper functioning of cellular proteins and induce subsequent changes in cellular chemistry (Rogers *et al.,* 2008); Therefore, it is possible that once penetrated into cells, AgNPs may attack functional

proteins of cells which results in partial unfolding and aggregation of proteins as it is the case in the bovine haemoglobin. Toxicity of silver nanoparticles is concentration-size-shape dependent; In the green process for synthesis of nanoparticles, these factors are affected by chemical compositions of fungal extracts, accordingly this will lead to variability in the biological activities of such extracts (Elechiguerra *et al.*, 2005; Morones *et al.*, 2005; Okafor, 2013).

Table 5. In vitro cytotoxicity effect of AgNO3, Ag nanoparticles and aqueous extract on HCT - 116 and MCF-7 cell lines

Sample concentration (µg/ ml)	Viability% of HCT-116			Viability % of MCF-7		
	$AgNO_3$	Ag NPs	Aqueous extract	$AgNO_3$	Ag NPs	Aqueous extract
100	11.52	1.34	87.52	9.74	3.22	85.18
50	15.86	4.36	92.14	12.46	6.75	91.36
25	34.64	8.91	98.67	19.18	11.94	96.23
12.5	53.67	13.74	100.00	26.29	18.31	98.92
6.25	68.90	28.11	100.00	37.92	37.43	100.00
3.125	74.23	37.25	100.00	48.16	45.94	100.00
1.56	83.62	47.38	100.00	67.98	52.11	100.00
0.78	91.28	59.82	100.00	75.42	63.78	100.00
0.39	96.20	73.34	100.00	81.37	79.34	100.00
0	100.00	100.00	100.00	100.00	100.00	100.00
IC 50	14.9	1.4	-	3.0	2.1	-

Table 6. In vitro cytotoxicity effect of $AgNO_3$, and Ag nanoparticles aqueous extract on HepG2 cell line

Sample concentration µg/ml	Viability % of HepG2		
	$AgNO_3$	AgNPs	Aqueous extract
100	11.43	2.33	68.94
50	13.94	4.92	83.16
25	24.85	8.74	92.68
12.5	49.74	10.22	98.32
6.25	71.38	26.98	100.00
3.125	80.24	38.46	100.00
1.56	87.34	46.74	100.00
0.78	94.15	53.16	100.00
0.39	98.22	64.47	100.00
0	100.00	100.00	100.00
IC 50	**12.4**	**1.2**	**> 100**

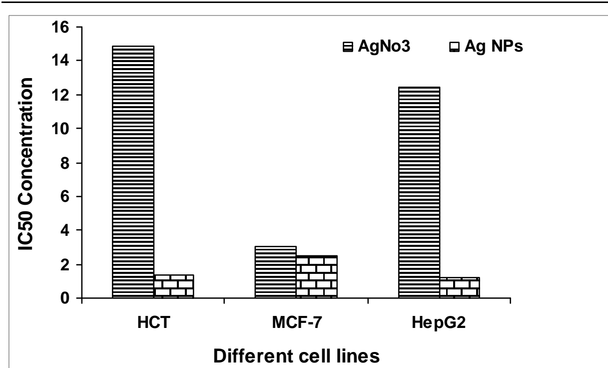

Fig. 11. IC50 con centration of AgNPs against different cell lines

The effect of cytotoxicity was compared with a normal Vero cell line on which the same concentrations were used. The results indicated that the sensitivity of human cancer cell line for AgNPs is higher than that of Vero cell line (Table 6) for the same cytotoxic agents (AgNPs). It was found that 39.6 µg/ ml are enough to induce 50% of cell mortality (Table 7 & Fig. 12). These results are potentially promising because they suggest that, by using non-cytotoxic amounts of silver salt with a convenient, eco-friendly and cheap method using *Aspergillus ochraceus* aqueous extract; AgNPs can be synthesized with good anticancer activities.

Table 7. Cytotoxicity of Ag nanoparticles on Vero cell line

Viability % of Vero cell line	Ag NPs concentration (µg/ ml)
18.46	100
39.81	50
64.29	25
89.12	12.5
96.48	6.25
99.22	3.125
100	1.56
100	0.78
100	0.39
100	0
39.6	IC 50

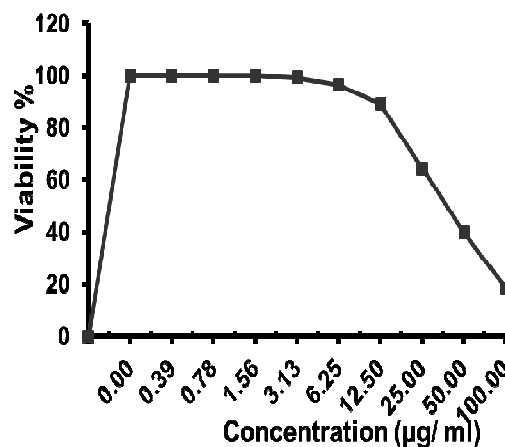

Fig. 12. Cytotoxicity of Ag nanoparticles on Vero cell line

CONCLUSION:

Nanoparticles can be produced by physical-chemical methods but it requires involvement of hazardous chemicals and many sophisticated techniques which are not easy. On the other hand, the biosynthesis of nanoparticles by microorganisms is quick, consumes less time, it provides satisfactory biosynthesis of nanoparticles and the whole process is very cheap and effective without the involvement of hazardous chemicals. In the present study, *Aspergillus ochraceus* (RCMB 036254) was exploited to biosynthesize silver nanoparticles by reducing silver nitrate. These

AgNPs were found to be more active against MRSA when used in combination with antibiotics. It is known that the nanoparticles are so small in size that they can pass through the cell membrane easily. This property is exploited to treat diseases like cancer which is a fatal disease. Chemotherapies which are used to treat cancer have many side effects so there is a need to find some other alternative which could treat it without causing side effects. The mycosynthesized AgNPs showed remarkable anticancer activity against human colon carcinoma, human breast cancer, and human hepato-cellular carcinoma cells. With the application point of view, a suitable pharmaceutical formulation using these nanoparticles, as well as studies on different biological activities in different fields should be strengthened in future studies.

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التكوين الحيوى لدقائق الفضة النانونية باستخدام الفطريات والتقييم البيولوجى لتلك الدقائق النانونية

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تم اختبار ثمانية أنواع فطرية من حيث قدرتها على إنتاج الدقائق النانو الفضية النانوية وقد لوحظ بالرؤية العينية تلون الراشح الفطرى لستة فطريات باللون البنى مما يدل على إنتاجها للدقائق الفضية النانوية وكان من أكثر الفطريات إنتاجاً هو فطر أسبرجيلس أوكريشياس والذى أعطى لوناً بنياً داكناً للراشح الفطرى مقارنةً بباقى الأنواع الفطرية. و تم توصيف الدقائق الفضية النانوية المنتجة بواسطة فطر أسبرجيلس أوكريشياس باستخدام جهاز المطياف الضوئى حيث أظهر امتصاصاً عند طول موجى 420 نانومتر. وباستخدام جهاز الميكروسكوب الإلكترونى النافذ أمكن تصوير الدقائق الفضية وقياسها وقد تراوح قطرها ما بين 5.5 ، 24.5 نانومتر.وتم التحليل الكمى والكيفى للعنصر الفضى باستخدام جهاز المحلل بأشعة إكس الملحق بجهاز الميكروسكوب الإلكترونى الماسح. وأيضاً تم تعيين الظروف المثلى لإنتاج تلك الدقائق الفضية وهى تركيز 0.75 ملى مول لمحلول نترات الفضة عند درجة 40°م وأس هيدروجينى 6. ومن ثم تم تقدير الفاعلية ضد الميكروبية لتلك الدقائق تحت هذه الظروف باستخدامها منفردة و بخلطها مع المضادات الحيوية الشائعة ضد بكتيريا ستافيلوكوكاس أورياس المقاومة للمثيسيلين (ميرسا) وقد أظهرت التجارب أنه من بين ثلاثة عشر مضاداً حيوياً وجد خمسة مضادات حيوية فقط أعطت زيادة فى النشاط ضد الميكروبى عند خلطها بالدقائق الفضية النانوية اتصفت أيضاً تلك الدقائق الفضية بقدرتها المثبطة للخلايا السرطانية وتعتمد تلك القدرة على تركيز الدقائق الفضية وكان التركيز المثبط لنصف عدد الخلايا هو 1.4, 2.1, 1.2ميكروجم/ ملى ضد كل من خلايا سرطان القولون وسرطان الثدى وسرطان الكبد على التوالى , فى حين أن التركيز المثبط لنسبة 50% من الخلايا الطبيعية هو 39.6 ميكروجم/ ملى. وقد أوضحت تلك الدراسة إمكانية إنتاج دقائق فضية نانوية من فطر أسبرجيلس أوكريشياس والتى يمكن استخدامها للقضاء على الخلايا السرطانية بتركيزات لا تضر بالخلايا الجسدية الطبيعية وأيضاً استخدامها ضد الميكروبات المقاومة للمضادات الحيوية.

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