Global Science Research Journals

ISSN: 2505-001X Vol. 4(9), pp. 254-260, September, 2016.

http://www.globalscienceresearchjournals.org/

Global Journal of Biological and Biomedical Research

Full Length Research Paper

An evaluation on antimicrobial activity of these nanoparticles against the human pathogenic fungus *A. fumigatus*

Naguib Zewail Mahfouz

Botany Department, Faculty of Science, Cairo University, Giza 12613, Egypt.

Accepted 10 August, 2016

In the past few decades, nanoparticles have emerged as a field in biomedical research. Four isolated *Aspergillus* species were tested for extracellular synthesis of silver nanoparticles using their cell free filtrate (CFF). Silver nanoparticles of the most potent producer, *Aspergillus terreus*, were further characterized. Transmission electron microscope (TEM) and atomic force microscope (AFM) revealed their spherical shape, homog eneity and size range between 20 and 140 nm. X-ray diffraction (XRD) showed the crystalline nature of the biogenic silver nanoparticles. Fourier transform infra-red (FTIR) spectroscopic analysis indicated that the coordination behaviors between amino groups of the secreted fungal proteins and other functional groups present in the CFF may be liable for the reduction of silver ions to form stabilized protein-capped silver nanoparticles. They were stable in aqueous solution for four months of storage at room temperature under dark conditions. The biogenic silver nanoparticles showed remarkable antifungal activity against the human pathogenic fungus *A. fumigatus*. The spore cell wall, plasma membrane and the inner constituents were damaged as shown by TEM. Furthermore, comet assay proved high breakage of DNA.

Key words: Silver nanoparticles, biosynthesis, fungi, antifungal, comet assay.

INTRODUCTION

Nanotechnology provides a good platform to modify and develop the important properties of metal in the form of nanoparticles having promising applications in diagnostics, biomarkers, cell labeling, contrast agents for biological imaging, antimicrobial agents, drug delivery systems and nanodrugs for treatment of various diseases (Marcato and Duran, 2008; Singh and Singh, 2011). The nanosize of material results in specific physicochemical characteristics different from those of the bulk materials or larger particles. This effect is mainly credited to high surface-area-to-volume ratio, which results in increased reactivity; hence, the nanoscale materials are more advantageous than their bulk materials. The metallic nano-particles such as copper, titanium, magnesium, zinc, gold and alginate have a strong bactericidal potential owing to their large surface-area-to-volume ratio (Gu et al., 2003; Ahmad et al., 2005). Among all, silver nanoparticles have proved to be the most effective antimicrobial agent against bacteria, viruses and other eukaryotic microorganisms (Gong et al., 2007).

In fact, the use of bulk silver as an antimicrobial dates back to ancient times when water and wine were stored in silver vessels to prevent spoilage. In the 17th century, silver nitrate was used to heal ulcers and chronic wounds. It was further used in the 19th century in the treatment of burns and prevention of ophthalmic diseases in newborns. In the beginning of the 20th century, Barns recognized that silver nitrate was caustic for the eyes of newborns and he invented agyrol, a protein-stabilized silver colloid. Around that same time, a similar colloidal

*Corresponding author: E-mail: dr.naguib770@gmail.com

silver solution was commercialized as collargol. These colloidal silver preparations were early nanosilver formulations (Silver et al., 2006; Nowack et al., 2011).

When silver nanoparticles are chemically produced, three main components are needed: a silver salt (usually $AgNO_3$), a reducing agent (e.g., sodium borohydride) and a stabilizeror capping agent (e.g., polyvinylpyrrolidone) to control the growth of the nanoparticles and prevent them from aggregating (Ledwith et al., 2007). In the case of the biological synthesis of silver nanoparticles, the reducing agent and the stabilizer are replaced by molecules produced by living organisms. These reducing and/or stabilizing compounds can be present in bacteria, fungi, yeasts, algae, or plants. The reduction can happen enzymatically or non-enzymatically and the stabilizer or capping agent is in many cases a protein (Gade et al., 2010; Narayanan and Sakthivel, 2010). One of the main advantages of biogenic silver compared to chemically produced nanosilver is its green synthesis avoiding organic sol-vents and toxic reagents. Moreover, the biological produc-tion of silver nanoparticles converts a waste stream into a product with an added value as biocide, catalyst or biosensor (Hennebel et al., 2009).

When compared with bacteria, fungi have been known to secrete much higher amounts of bioactive substances, which made fungi more suitable for large scale production (Narayanan and Sakthivel, 2010). In addition, the extracellular biosynthesis using fungi could also make downstream processing much easier than bacteria (Mohanpuria et al., 2008).

The present work was aimed at green production of silver nanoparticles by some species belonging to genus *Aspergillus*, due to its prevalence in the natural environment, ease of cultivation on laboratory media and economic importance. The aim was also extended to characterize the biosynthesized silver nanoparticles by the most potent producer *Aspergillus terreus*. In addition, the antimicrobial activity of these nanoparticles against the human pathogenic fungus *A. fumigatus* was studied.

MATERIALS AND METHODS

Isolation of fungal strains

Fungal species used in this study were isolated from an agricultural soil in Giza, Egypt. Soil samples were used as inoculums for soil dilution plate method. Fungal colonies were purified and identified by morphological and microscopic examinations (such as color, texture of mycelia, spore formation pattern, etc.) as described by Raper and Fennel (1965). Fungal species were grown on Potato Dextrose Agar (PDA) slants at 28°C for 4 days. Spores of each strain were harvested and stored at 4°C in sterilized spore suspension buffer containing 0.9% (w/v) NaCl and 1% (v/v) Tween-80.

Synthesis and detection of silver nanoparticles

The stock spore suspension of each fungal species (*Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus niger* or *Aspergillus terreus*) was inoculated in 100 ml of malt extract glucose yeast extract peptone (MGYP) medium (0.3% malt extract, 1.0% glucose, 0.3% yeast extract, 0.5% peptone; pH 6.8) in 250 ml Erlenmeyer flasks. The culture was incubated for 24 h at 28°C in a shaking incubator (150 rpm). This starter culture was used as an inoculum (10 %) for the same medium at the same culture conditions except for three days of incubation. The fungal mycelium was separated from culture me-dium by centrifugation (5000 rpm, 15 min and 4°C) and washed thrice with sterilized deionized water. The biomass was then auto-lysed to release the intracellular enzymes and other components into the aqueous solution. This was carried out by suspending 10 g fresh biomass in 100 ml sterilized deionized water and incubated at 28°C for 72 h in a shaking incubator (150 rpm). The contents of the flask were filtered through Whatman filter paper no. 1 and the cell free filtrate (CFF) was obtained. For the biosynthesis of silver nanoparticles, 50 ml of CFF were mixed with 10 ml of 10 mM $AgNO_3$ and incubated at 28°C in a shaking incubator at 150 rpm in the dark.

The reduction of silver ions to silver nanoparticles (by *A. flavus, A. nidulans, Aspergillus niger* or *A. terreus*) was noticed by UV-visible spectrophotometer (Perkin-Elmer, Hitachi 200) at 420 nm. *A. terreus* had highest absorbance rate. Silver nanoparticles produced by this species were chosen for further experiments.

Characterization of silver nanoparticles

FTIR spectrum and X-ray diffraction of freeze dried silver nanoparticles powder were studied using a Fourier Transform Infrared spectrometer (FTIR 6100) and an X-ray diffractometer (Type PANalyticalX'Pert PRO diffractometer) with *CuKα* radiation (λ = 0.15408 nm), operated at 30 mA and 45 kV. Morphology of the synthesized silver nanoparticles was observed by a Jeol JEM-1400 transmission electron microscope (TEM). Further morphological analyses of the particles were conducted by atomic force microscopy (AFM) imaging using a Shimadzu Wet-SPM (Scanning Probe Microscope) in a non-contact mode.

Antifungal activity

The ability of silver nanoparticles to inhibit spore germination of *A. fumigatus* was tested. The minimum inhibitory concentration (MIC) test was carried out. The concentrations of spore suspensions were determined in a hemocytometer and adjusted to 2×10^6 spores/ml. Fifty microlitres of spore suspension were transferred to each well of a microtiter plate containing 100 µl liquid Czapek-Dox medium with different concentrations of silver nanoparticles. Deionized water and amphotericin B replaced silver nanoparticles and were used as negative and positive controls, respectively. The plate was incubated at 30°C for 16 h. All tests were conducted in replicates. Spores were considered to be germinated when the germ tube extended to at least twice the length of the spore itself (Griffin, 1994). Germinated spores were counted using a hemocytometer. About 100 spores per replicate were observed to detect spore germination. MIC was determined as the lowest concentration of silver nanoparticles that inhibits spore germination.

The effect of the tested silver nanoparticles on the ultrastructure of *A. fumigates* spores was studied. The control fungal spores at zero time and after 36 h and those treated with silver nanoparticles for 36 h were isolated, fixed and TEM was performed using a Jeol JEM-1400 transmission electron microscope.

Alkaline single-cell gel electrophoresis (SCGE) or alkaline comet assay was performed to elucidate the action of silver nanoparticles on DNA of fungal spores. The method followed was adopted from Miloshev et al. (2002) and Nemavarkar et al. (2004) with some modifications. Agarose (0.5%) was evenly spread on clean dry frosted slides, and the slides were air-dried. *A. fumigatus* spores or those treated with silver nanoparticles were washed once in sterilized

deionized water and resuspended in a suspending buffer (1 M sorbitol, 25 mM KH_2PO_4, pH 6.5). Approximately, 5 x 10^6 spores were mixed with 0.7% low melting agarose containing 2 mg/ml diluted Macerozyme R-10 (3 ml/100 ml buffer), and the cells were spread evenly on an agarose-coated slide. They were covered with cover slips and incubated for 30 min at 30°C to digest the spore cell wall and to obtain the spheroplast. All further procedures were carried out in a cold room at 10°C to minimize the activity of endogenous enzymes. After removing the cover slips, the slides were incubated in 30 mM EDTA (pH 12.4) to unwind the DNA. The gel-slides were then subjected to electrophoresis in the same buffer at 0.7 V/cm, 30 mA. After electrophoresis, the gels were neutralized by submerging the slides in 10 mM Tris HCl (pH 7.4) for 2-3 min followed by consecutive incubation in 76 and 96% ethanol. The slides were then air-dried and stained in ethidium bromide (2 mg/ml).

A fluorescence microscope (Carl Zeiss) at 400x, with an exciting filter of 515-560 nm and a barrier filter of 590 nm, was used to obtain images and quantify DNA strand breaks in the tested samples. The data are given as tail length, percentage of DNA in tail and tail moment. Tail moment, a commonly accepted unit of DNA damage, is the product of fraction of the DNA in the tail of comet and tail length. Standard errors were calculated on the basis of 30 comets/treatment.

RESULTS AND DISCUSSION

Isolation of fungal strains

In this study, five species belonging to the genus *Aspergillus* were isolated and purified. They were identified as *A. flavus, A. nidulans, A. niger, A. terreus* and *A. fumigatus*. The former four species were utilized as cell factories for the production of silver nanoparticles. *A. fumigatus* was used as a test organism to study the antifungal properties of biogenic silver nanoparticles.

Synthesis and characterization of silver nanoparticles

Using the supernatant formed from the autolysed biomass allowed the formation of nanoparticles in the absence of any mycelial materials. This resulted in a clearer solution and complications due to nanoparticle/cell interactions were avoided. The extracellular synthesis using CFF is thus more advantageous in isolation of silver nanoparticles from the solution and less number of steps is involved in both synthesis and purification of silver nanoparticles (Kalpana and Lee, 2013).

In this work, color of CFF changed to brown after mixing with $AgNO_3$ for each of the four tested fungal species. The produced brown color indicates the formation of silver nanoparticles. UV-visible spectroscopy at 420 nm (Jain et al., 2011; Alani et al., 2012) was performed to confirm the synthesis of silver nanoparticles in the reaction mixture. As shown in Figure 1, the absorbance increased with incubation time. Development of the brown color was a result of excitation of surface plasmon vibration in the metal nanoparticles and is typical

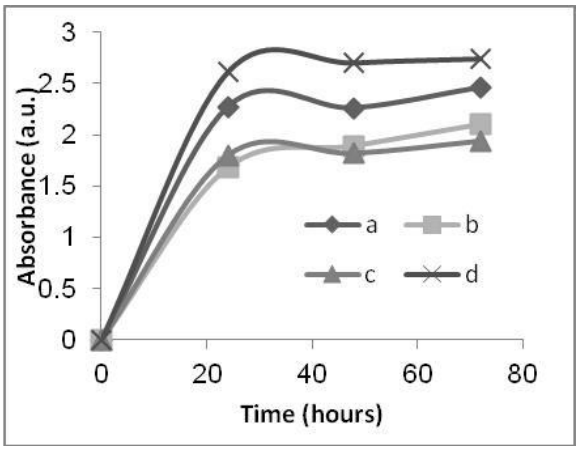

Figure 1. Increase in absorbance at 420 nm for silver nitrate solutions mixed with filtrates from autolysed (a) *A. flavus*, (b) *A. nidulans*, (c) *A. niger* and (d) *A. terreus*.

cal of silver nanoparticles (Gurunathan et al., 2009). Most of the silver nanoparticles (Figure 1) were synthesized by 24 h and there was almost no increase in absorbance after 48 h for the four tested *Aspergillus* species. This loss of activity may be related to a loss of nitrate reductase activity or cofactor availability.

The role of NADH-dependent nitrate reductase from micro-organisms in the biosynthesis of silver nanoparticles was previously discussed (Kumar et al., 2007; Bai et al., 2011). In this study, *A. terreus* was the most potent producer of silver nanoparticles, accordingly, its silver nanoparticles were chosen for further experiments. Stability of silver nanoparticles, produced by *A. terreus*, was detected after four months using UV-visible spectro-scopy at 420 nm. It was found that the nanoparticle solution was highly stable at room temperature under dark conditions, without flocculation. Biological mole-cules, mostly proteins, stabi-lize biogenic silver nanopar-ticles which allow functiona-lization of the particles with other biomolecules (Hennebel et al., 2009). Functiona-lization can increase the antimicro-bial activity by impro-ving the interactions with microor-ganisms (Botes and Cloete, 2010). Chemically produced nanoparticles on the other hand, are known to aggregate to larger clusters (Mafune et al., 2000). This decreases their high specific area and therefore also their catalytic and antimicrobial activity.

FTIR analysis of the freeze-dried sample was carried out to identify the possible interactions between silver and bioactive molecules, which may be responsible for synthesis and stabilization (capping material) of silver nanoparticles. The amide linkages between amino acid residues in proteins give rise to well-known signatures in the infrared region of the electromagnetic spectrum (Jain et al., 2011). The FTIR spectrum (Figure 2) revealed a peak at 3423 cm^{-1} (no. 1) which could be attributed to strong stretching vibrations of hydroxyl functional group

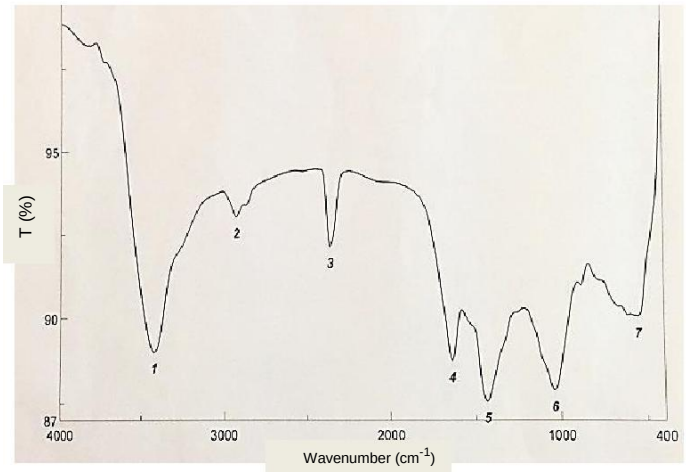

Figure 2. FTIR spectrum of silver nanoparticles synthesized by *A. terreus*.

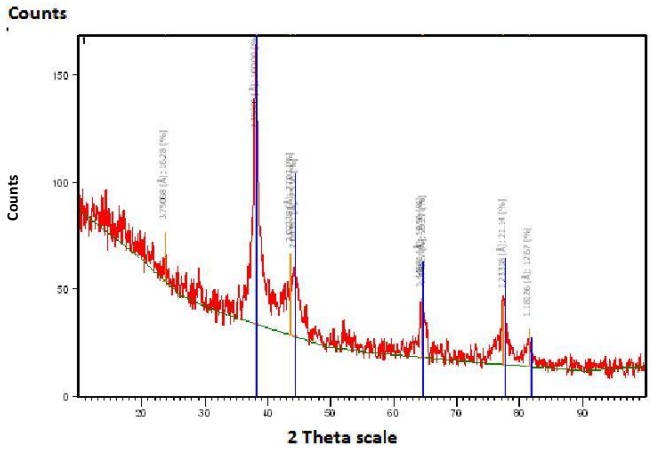

Figure 3. X-ray diffraction (stick pattern) of silver nanoparticles synthesized by *A. terreus*.

(Priyadarshini et al., 2013). The peak no. 5 at 1432 cm^{-1} may be related to COO- symmetrical stretch from carboxyl groups of the amino acid residues (Gajbhiye et al., 2009). Hydroxyl groups of Tyr residues and carboxyl groups of Asp and Glu residues are the most active functional groups for Ag^{+} reduction and silver nanoparticles anisotropic growth (Xie et al., 2007). The bands no. 6 and 4 at 1039 and 1635 cm^{-1}, respectively, may correspond to -N-H and carbonyl (C-O-) stretching vibrations in amide linkages (amide I and amide II) of proteins (Suresh et al., 2011). The peak no. 2 at 2931 cm^{-1} could be due to C-H stretch of methylene groups of proteins (Ghaseminezhad et al., 2012).

The X-ray diffraction study confirmed the crystalline nature of synthesized silver nanoparticles. Figure 3 shows four distinct peaks at 38.29, 44.51, 64.77 and 77.81° which were assigned to the (111), (200), (220) and (311) lattice planes of face-centered-cubic (fcc) silver, respectively (Gopinath et al., 2013). The obtained diffraction spectrum suggested the presence of silver nanoparticles which is in accordance with the UV-visible spectrum analysis.

TEM analysis of silver nanoparticles (Figure 4) revealed their homogeneity, well dispersion and spherical morphology indicating their stabilization. On the other hand, atomic force microscope imaging (Figure 5) showed silver nanoparticles to be uneven due to the presence of some of the aggregates along with individual nanoparticles. It proved the morphological homogeneity with the grain size falling in the submicron range. Spherical shape of the synthesized silver nanoparticles was confirmed and their size lied mostly in the range of 20-140 nm. A variety of different-sized silver nanoparticles has been reported depending on the biological system used. Also, diverse shapes have been noticed with spherical particles being predominant. Alani et al. (2012) found that silver nanoparticles from *A. fumigatus*

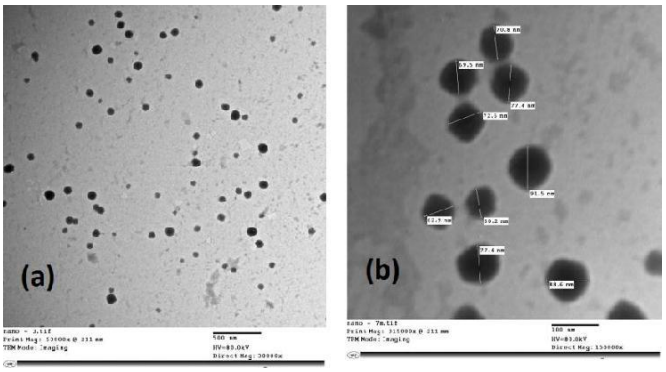

Figure 4. TEM photographs of silver nanoparticles synthesized by *A. terreus*. (a) Low magnification image (scale bar: 500 nm) and (b) high magnification image (scale bar: 100 nm).

had a diameter ranging from 15 to 45 nm meanwhile those from *Streptomyces* sp. showed a narrower size distribution of 15-25 nm. The silver nanoparticles synthesized by *Puccinia graminis* (Kirthi et al., 2012) were shown to have a size between 30 and 120 nm while those from the white rot fungus *Pycnoporus sanguineus* had an average diameter of 52.8-103.3 nm (Chan and Don, 2013).

Antifungal activity

A. fumigatus is a fungal saprophyte that is ubiquitous throughout the world. It is also an opportunistic pathogen of immunocompromised hosts causing invasive aspergillosis, a usually fatal infection. Germination of inhaled conidia (spores) is an early and crucial event in the infection process of *A. fumigates* (Rohde et al., 2002). The antifungal test was performed against this pathogen with different concentrations of silver nanoparticles (from

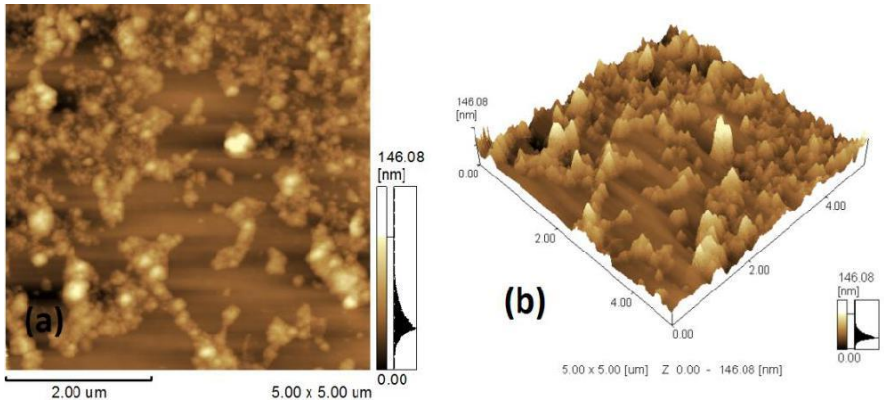

Figure 5. Atomic force microscope images of silver nanoparticles synthesized by *A. terreus* depicting the topography. (a) Top view and (b) 3-D view.

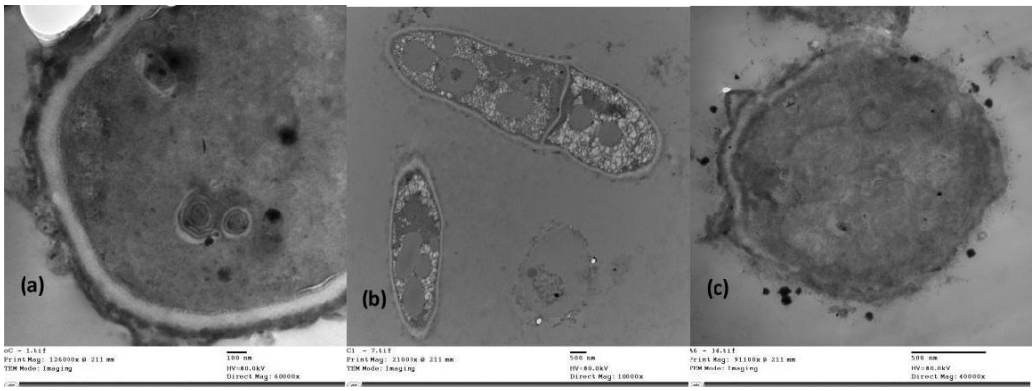

Figure 6. TEM micrographs of cross sections of *A. fumigatus* spores. (a) resting spores, (b) control spores after 36 hours of incubation in Dox's medium and (c) spores after 36 hours of incubation in Dox's medium containing silver nanoparticles (15 µg/ml).

1.88 to 94 µg/ml). The results revealed that the MIC of silver nanoparticles against germination of spores of *A. fumigatus* may be estimated to be between 7.52 and 11.28 µg/ml. The antifungal drug amphotericin B was used as positive control and it showed an MIC between 70 and 75 µg/ml. The significant antifungal activity shown by the biogenic silver nanoparticles suggests their potential against infections caused by *A. fumigatus*. Several studies proved the antimicrobial activity of silver nanoparticles (Kim et al., 2007; Jain et al., 2010; Lamsal et al., 2011; Kalpana and Lee, 2013; Chan and Don, 2013). Kora and Arunachalam (2012) compared the antibacterial effect of silver nanoparticles against some bacteria with erythromycin, a commonly used antibiotic. The antibacterial activity of silver nanoparticles was higher in most cases. Meanwhile, Li et al. (2012) studied the antimicrobial activity of silver nanoparticles against some bacteria, yeasts and fungi. They used silver nitrate as a control. In all tested microorganisms, silver nanoparticles showed higher inhibitory effect than silver nitrate.

It was thought advisable to study the effects of the biogenic silver nanoparticles on spores of *A. fumigatus* *via* TEM analysis. The main abnormalities noted *via* TEM study (Figure 6) was the alterations in the morphology and complete collapse of the spores after 36 h of exposure to the biogenic silver nanoparticles. The typical echinulate surface and inner constituents of the resting spore is shown in Figure 6a. Germinating spores (Figure 6b), after 36 h of incubation in Dox's medium, seemed to shed the outer surface layer before the cells start to swell, germinate and multiply. On the other hand, the spores incubated in Dox's medium containing silver nanopar-ticles for 36 h (Figure 6c) looked different. Silver nanopar-ticles appear to accumulate, adhere on and penetrate the outer surface of spore. Parts of the cell wall, plasma membrane and the inner constituents of the spores were obviously damaged which may be due to the toxic effect of the silver nanoparticles.

The alkaline SCGE technique was applied to *A. fumigatus*

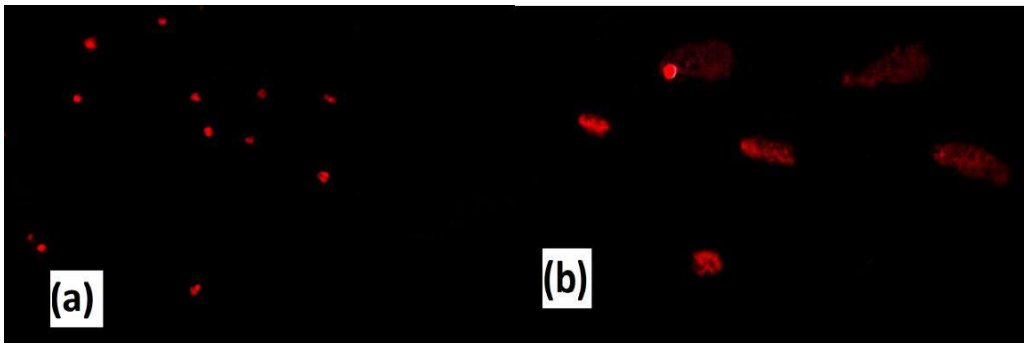

Figure 7. Comets from *A. fumigatus* spore suspension. (a) Control spores and (b) spores treated with silver nanoparticles (15 µg/ml).

Table 1. DNA damage parameters of comet assay of *A. fumigatus* spore cells exposed to silver nanoparticles.

Group	Tail length (px)	DNA in Tail (%)	Tail moment
Control	3.00 ± 0.54	21.54± 2.78	0.61± 0.09
Treated	13.87 ± 1.92	35.59 ± 3.14	5.39 ± 1.12

Data presented are mean ± standard error. 1 px = 0.24 µm.

spores to follow the silver nanoparticles-induced DNA breaks. A clear-cut nearly spherical image was seen in control spore cells illustrating undamaged DNA (Figure 7a). DNA was still contained in the cells which were protected by cell walls. On the other hand, a characteristic picture of comets was obtained for spores treated with silver nanoparticles (Figure 7b), which denotes DNA damage. The results in Table 1 reveal the increase of tail length, percentage of DNA in tail and tail moment of spores subjected to silver nanoparticles. There was a nearly 9-fold increase in tail moment due to presence of silver nanoparticles.

Some reports explained that silver nanoparticles attack bacterial cell by anchoring and penetrating cell wall. As a consequence, a structural change in cell membrane takes place which leads to an increase in cell permeability. Hence, uncontrolled transport through cytoplasmic membrane followed by cell death is the fate (Morones et al., 2005; Sondi and Salopek-Sondi, 2007). Kim et al. (2007) attributed the antibacterial mechanism of silver nanoparticles to the formation of free radical-induced membrane damage. Klueh et al. (2000) proposed that silver nanoparticles inhibit bacterial growth by binding to thiol groups (-SH) in enzymes, which deactivates the enzymes. They also hypothesized that the bactericidal activity of silver nanoparticles is due to silver ions which enter the cell and intercalate between the purine and pyrimidine bases of DNA. These base pairs showed disturbing effect on the hydrogen bonding between the two antiparallel strands, leading to denatu-ration of DNA. Accordingly, cell division and DNA replication are prevented which ultimately leads to cell death.

In conclusion, four *Aspergillus* species were isolated from soil and used in the production of silver nanoparticles. Using a simple autolysis method on the biomass, the released intracellular materials were used in the cell-free biosynthesis of silver nanoparticles from silver nitrate solution. *A. terreus* produced highest concentrations of silver nanoparticles. These biogenic silver nanoparticles were highly stable for a long storage period. They showed strong antifungal activity against the human pathogenic fungus *A. fumigatus*. Thus, it could be recommended that extracellular synthesis of silver nanoparticles from *A. terreus* could be used in developing novel antifungal agents which may find potential applications in the drug industry.

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