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Using Soil Fungus, *Fusarium Oxysporum* for Green Synthesis of Silver Nanoparticles and Evaluation of Their Antimicrobial Effects

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ABSTRACT

Synthesis of nanoparticles by using micro-organisms is a promising future achievement in the field of green nanotechnology. In this respect, the extracellular biosynthesis of silver nanoparticles (AgNPs) by using culture supernatants of soil fungus *Fusarium oxysporum* was studied. The bioreduction of AgNPs was detected by ultraviolet-visible spectroscopy, and the synthesized AgNPs were characterized by transmission electron microscopy and Zeta potential. In addition, evaluation of antimicrobial activity of the synthesized AgNPs was tested. The synthesized AgNPs appeared as polydispersed spherical particles, stabilized in the solution and ranging in size from 9 to 24 nm. Also, the results showed that the synthesized AgNPs had inhibition effects on various pathogenic microorganisms including bacteria and fungi. Synthesis of AgNPs by soil fungus *Fusarium oxysporum* may represent a safe, non-sophisticated and economic method for production of antimicrobial agents.

Keywords: Fusarium oxysporum - Silver nanoparticle -Transmission electron microscopy - Zeta potential- Antimicrobial activity

INTRODUCTION

Nanotechnology refers to the ability to design, characterize and produce structures, devices and systems by controlling size and shape at the nanometer scale (Mansoori, 2005).

Presently, there is a great demand for using eco-friendly synthesis protocols that do not produce toxic wastes in production of nanoparticles. This is briefly the nanobiotechnology. In this context, different types of microorganisms have been used in biosynthesis of nanoparticles. (Kowshik *et al*., 2002; Bhattacharya and Rajinder, 2005). Nanoparticles synthesized by microorganisms have the advantage of being better size controlled and better stabilized by peptides as phytochelatins that prevent aggregation (Kang *et al*., 2008). The synthesis of these short peptides occurs in response to heavy metal stress and have been concerned as a well-known mechanism to sequester metal ions in bacteria (Pages *et al*., 2008), fungi (Guimaraes-Suares *et al*., 2007) and plants (Cobbett, 2000)

In comparison with bacteria, fungi have been known to produce more amounts of bioactive substances, so fungi are considered to be more appropriate for large-scale production (Narayanan and Sakthivel, 2010). Additionally, the extracellular biosynthesis by fungi has the ability to make downstream processing much easier than by bacteria (Mohanpuria *et al*., 2008). A reliable example for fungal biosynthesis is the biosynthesis of silver nanoparticles by *Fusarium oxysporum* (Ahmad *et al*., 2003 Hassan *et al*., 2013; Selvi and Sivakumar, 2012), *Fusarium acuminatum* (Ingle *et al*., 2008) and *Penicillium fellutanum* (Kathiresan *et al*., 2009).

Silver nanoparticles (AgNPs) have many interesting applications, starting from electronics (Gratzel, 2001) and catalysis (Shiraishi and Toshima, 1999) to infection prevention (White and Budarin, 2011) and medical diagnosis (Groneberg and Giersinag, 2006). AgNPs are characterized by having excellent antimicrobial and anti-inflammatory effects, so, they were used to enhance wound healing (Elliott 2010)

Antimicrobial effects of silver may be improved by changing its size at nano scale (Dattu *et al*., 2014). Due to the mentioned change in physical and chemical properties, silver nanoparticles (AgNPs) have been considered as antimicrobial agents.

Several strains of *Fusarium oxysporum* can produce the extracellular silver nanoparticles with the help of enzyme nitrate dependent reductase and by a shuttle quinone extracellular process (Balaji *et al*., 2009).

The aim of the current work is to study the using of *Fusarium oxysporum* for green synthesis of silver nanoparticles and to evaluate their antimicrobial effects. This biosynthesis process seems to be safer and cheaper than other alternative processes.

MATERIALS AND METHODS

Microorganisms

Three fungal species; *Fusarium oxysporum*, *Aspergillus niger* and *A. oryzae* were cultured in potato dextrose agar at 28°C and maintained at 4°C.

Three bacterial species; *Staphylococcus aureus*, *Bacillus cereus* and *E. coli* were grown on nutrient agar at 37 °C and maintained at 4°C.

All cultures were kindly provided by Microbiology Dept. Faculty of Agric., Mansoura University, Egypt.

Media used for AgNPs synthesis

Three different media were prepared for AgNPs synthesis: potato dextrose broth (Medium 1) containing 200 g potato and 20 g dextrose, Sabouraud broth (Medium 2) containing 40 g dextrose and 10 g peptone and Malt yeast broth (Medium 3), containing 20 g malt extract and 5 g yeast extract peptone.

Preparation of fungal supernatants

F. oxysporum was cultivated on potato dextrose agar slants at 28°C. To prepare fungal supernatant for biosynthesis of silver nanoparticles, 250 mL flasks each containing 100 mL of tested medium were inoculated with 5ml of fungal spore suspension (10^6 spores /ml). All flasks were incubated at 28 °C at 120 rpm for 96 h. The biomass was collected by filtration through filter paper (Whatman filter paper No.1) and then was washed

with distilled water to get rid of any components of the medium. The washed mycelia were resuspended into 100 mL sterilized distilled water and were incubated at 28°C for 72 h at 120 rpm. Again, biomass was collected by filtration through Whatman filter paper No.1. The supernatants were used for biosynthesis of silver nanoparticles.

Biosynthesis of AgNP.

To synthesize AgNPs, 150mL of aqueous solution of $AgNO_3$ (0.1 mM) was mixed with 100mL of the supernatants of *F. oxysporum* and stirred for 1h at 60 °C. The reaction was carried out in dark conditions. The bioreduction of the Ag^+ occurred rapidly as indicated by a reddish brown color after 24h indicating the synthesis of AgNPs. The mixture of AgNPs obtained was purified by repeated centrifugation at 12000 rpm for 15 min and the pellet was collected and dried (Poopathi *et al*., 2014).

Synthesized AgNPs characterization.

Analysis by UV–Vis spectral

AgNPs surface plasmon resonance was characterized using UV-2550 spectrophotometer (UV-Visible spectrophotometer Shimadzu, Japan) by operating at the resolution of 1 nm with a range from 190 nm to 700 nm (Arokiyaraj *et al*., 2015).

Analysis by TEM

Characterization of the structure of AgNPs was done by transmission electron microscopy (TEM) (JEOL- JEM-2100, Japan). The sample was put on the carbon coated copper grid, making a thin film of sample on the grid and extra sample was excluded using the cone of a blotting paper and kept in grid box sequentially (Durán *et al*., 2005).

Zeta potential

Malvern Zeta sizer Nano ZS90 (Malvern Instruments Ltd., UK) was used to measure silver nanoparticles zeta potential. Zeta potential cell were washed with ethanol and deionized water followed by AgNPs sample. Finally, the average distribution of nanoparticles based on intensity, volume, and number weighting was studied comparatively (Amit *et al*., 2014).

Antibacterial effects of synthesized AgNPs.

The antibacterial effects of silver nanoparticles were tested by agar well diffusion method (Cruickshank, 1968). One ml of cell suspension (10^6 CFU/ml) was introduced into Muller-Hinton Agar medium then four wells were made after the medium hardened. Different volumes 10, 20, 50 and 100 µl of AgNPs solution (which contain 5ppm of AgNPs for each volume) were poured into the wells and kept for incubation at 37°C for 24 hours. All experiments were performed in duplicate. The antibacterial effects were evaluated by measuring the diameter of the clear zone around every well. In addition, 100 µl of a pure silver nitrate solution and standard antibiotic (Norfloxacin and Ofloxacin for *E. coli*, Erythromycin and Oflaxacin for each of Staphylococcus *aureus* and *B. cereus*) with concentration 5ppm for each were also done parallel.

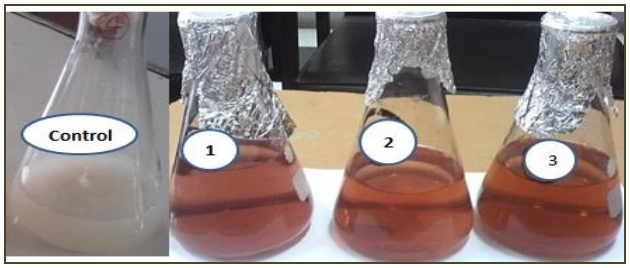
Antifungal effects of synthesized AgNPs.

Antifungal effects of the synthesized AgNPs against three fungal species *F. oxysporum*, *Aspergillus niger* and *A. oryzae* were determined by agar well diffusion method (Magaldi *et al*., 2004). A positive control drug (Nystatin) and a pure silver nitrate solution were also done parallel at concentration 5 ppm (100 µl) for each. The inhibition zone which appears as a clear area around the well was observed and measured.

RESULTS AND DISCUSSION

Visual inspection

In current study, biosynthesis of silver nanoparticles by the supernatants of *F. oxysporum* (from three selected media) was tested. After mixing the fungus supernatant with an aqueous $AgNO_3$ solution, a change in color to reddish brown was observed within a few hours, but no color change was appeared in the culture supernatants without $AgNO_3$ (Picture 1). The presence of a reddish brown color in the silver nitrate treated flask was a good indicator of the synthesis of silver nanoparticles in the reaction mixture because of the reduction of metal ions and formation of surface Plasmon resonance, while there was no color change observed in the control flasks.



Picture 1. Chang in color to brown indicated to the synthesis of AgNPs. Supernatants (1, 2 and 3) treated with silver nitrate. Control as a supernatant silver nitrate free. 1. Supernatant of Potato dextrose broth medium, 2. Supernatant of Sabouroud broth medium and 3 Supernatants of malt yeast broth medium.

It is well known that in different culture media conditions and compositions, microbial cell responds in different ways and secretes diverse metabolites and diverse proteins. Also, it is known that the biological synthesis of AgNPs is enzymes catalyzed reaction (Kumar *et al*., 2007). In case of maximum formation of AgNPs, fungi should secrete specific enzymes or metabolites that are responsible for of silver ions reduction, considering the high growth rate and low cost requirement (Kumar *et al*., 2007). In addition, there is a slight difference in color between the three tested media. The supernatants obtained from the fungus growth on sabouraud broth (2) and malt yeast broth (3) media were more effective than potato dextrose broth medium (1). These two media (2 and 3) may promote the extracellular nitrate reductase secretion and hence enhance the synthesis of AgNPs (Sonal *et al*., 2013).

This experiment provides a new evidence that the synthesis of silver nanoparticle by *Fusarium Oxysporum* is purely extracellular (Praseetha and Panda, 2011). In this work, a well stabilized AgNPs solution were prepared using 100 mL of the soil fungus supernatants

of *F. oxysporum*, as a reducing agents for silver ions to produced AgNPs. It is well known that the AgNPs show brownish color in aqueous solution due to excitation of surface plasmon vibration in silver nanoparticles (Mulvaney, 1996). Consequently, reduction of silver ions to nanoparticles through experiments could be followed by color change from pale yellow to dark brown which was checked periodically by UV–vis spectroscopy.

UV–vis spectroscopy study

The addition of $AgNO_3$ to soil fungus supernatants of *F. oxysporum* resulted in a bioreduction reaction and a change in color to reddish brown and this was an indicator for formation of AgNPs. UV–vis. spectroscopy is a primary step for analyzing the synthesis of silver nanoparticles. The silver surface plasmon resonance was observed at 450 nm (Fig.1). Sabouraud broth (2) and malt yeast broth (3) media showed maximum surface plasmon intensity and broad peak at 450 nm. Potato dextrose broth medium gave the lowest surface plasmon intensity.

Earlier report (Gengan *et al*., 2013) stated that maximum absorbance occurred at 448 nm due to presence of silver particle. The characteristic of silver surface plasmon resonance bands were detected around 400–450 nm (Njagi *et al*., 2011). Even though the actual mechanism is not properly understood, the reduction process of silver ions to silver nanoparticles using fungi may be due to the presence of the extracellular enzymes and metabolites responsible for AgNPs formation. The nitrate reductase and hydrogenase were actually essential for ferric iron reduction (Ottow and Von, 1969). In addition these extracellular enzymes, several naphthoquinones (Duran *et al*., 2002) and anthraquinones (Baker and Tatum, 1998) with excellent redox properties were observed in *Fusarium oxysporum* that could act as electron shuttle in reduction reactions of metals (Kumar and McLendon, 1997).

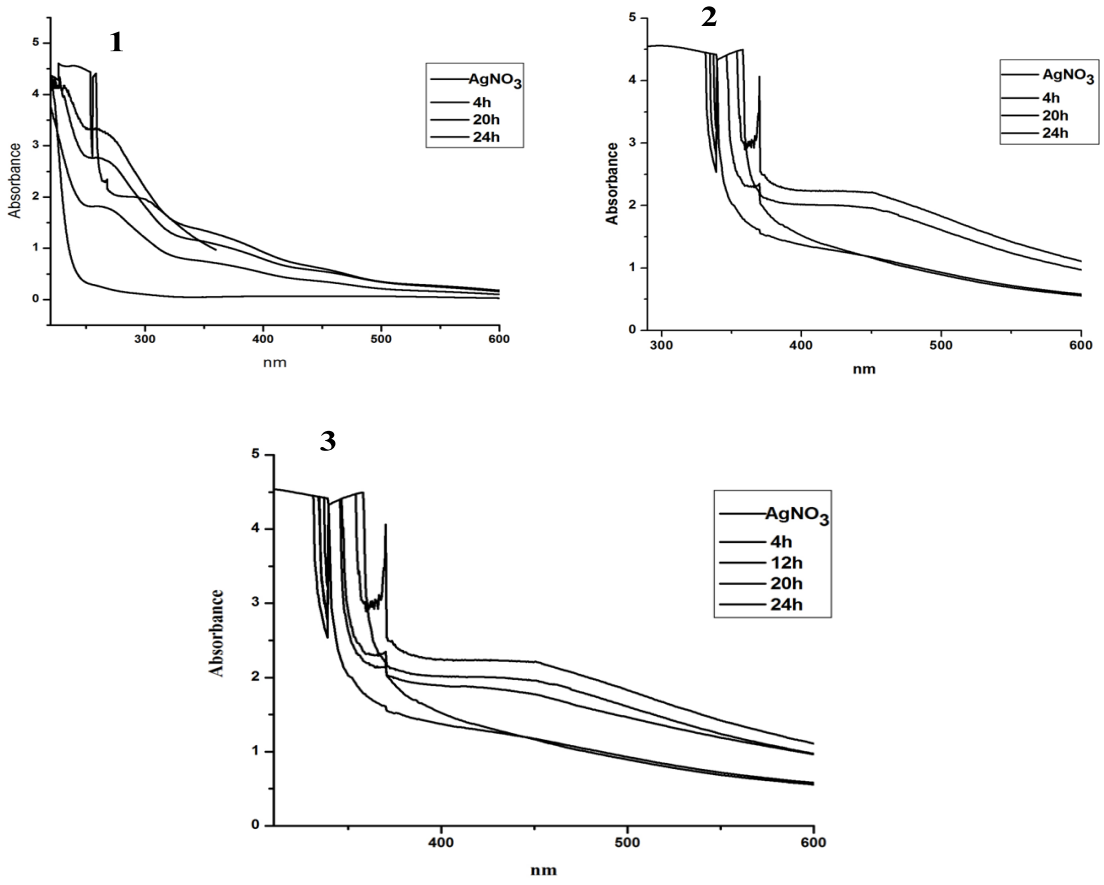


Fig. 1. UV–visible spectra of the synthesized AgNPs by supernatants of growing *F. oxysporum* on different culture media: 1. potato dextrose broth medium, 2. Sabouroud broth medium and 3. Malt yeast broth medium.

TEM analysis

The delegate TEM pictures obtained from the silver nanoparticle film placed on a carbon coated copper TEM grid is shown in Figure (2). These pictures show individual silver nanoparticles as well as a number of aggregates. Nanoparticles observed from the micrograph majority are spherical with a small percentage of elongated particles. Under examination of such descriptions, these assemblies were found to be aggregates of silver nanoparticles in the size range 9–24 nm (Santhosh *et al.*, 2015). The separation between the silver nanoparticles seen in the TEM pictures could be due to capping by proteins and would explain the UV-Vis spectroscopy measurements, which is characteristic of well-dispersed silver nanoparticles.

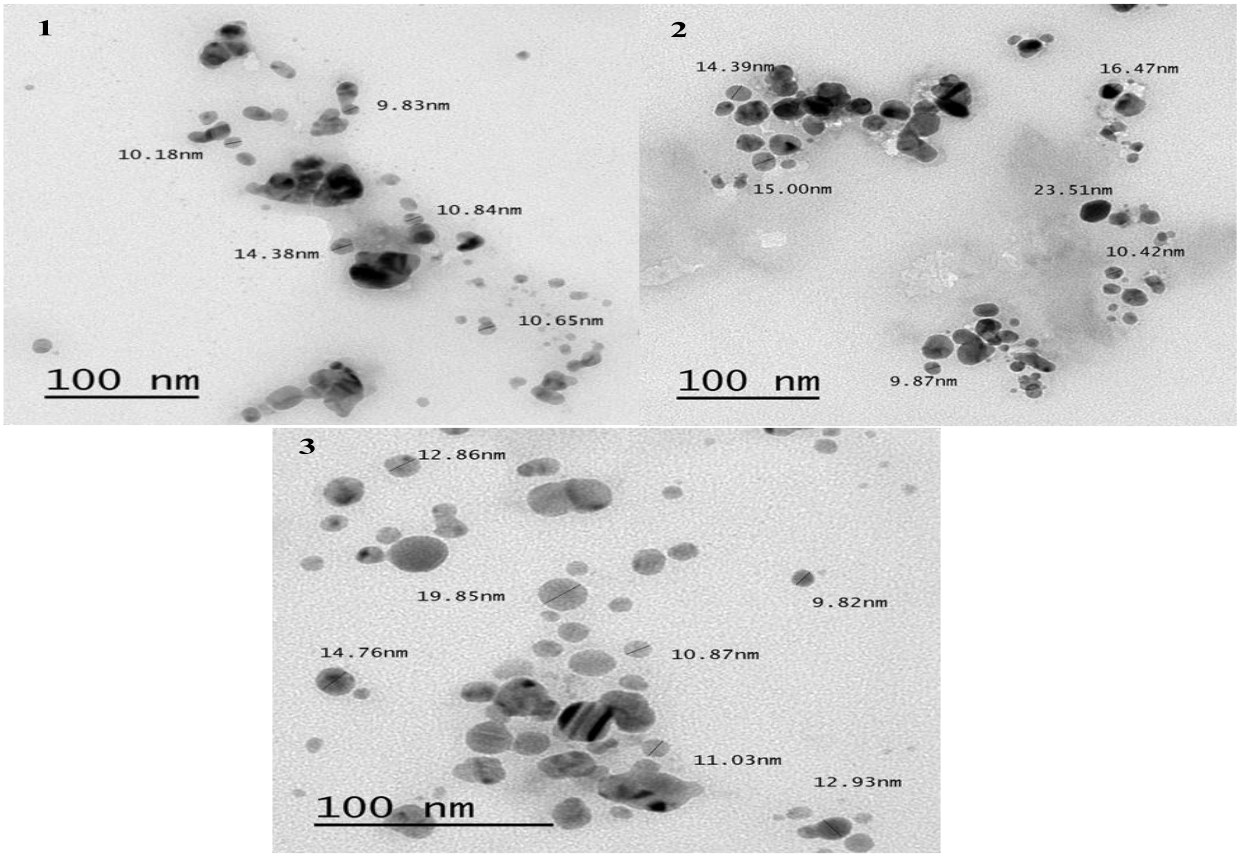


Fig. 2. SEM image of spherical synthesized AgNPs by supernatants of growing *F. oxysporum* on different culture media: 1. potato dextrose broth medium, 2. Sabouroud broth medium and 3. malt yeast broth medium.

Zeta potential

Zeta potential values reveal information regarding the surface charge and stability of the synthesized AgNPs. The zeta potential values of silver nanoparticles synthesized using supernatants of growing *F. oxysporum* on different culture media potato dextrose broth (1), sabouroud broth (2), malt yeast broth (3) were -22.2, -26.1 and -18.3mV, respectively (Fig. 3). The negative values strongly supporting long time stability of AgNPs (AgNPs are stable up to three month without agglomeration) (Goldstein and Greenlee, 2012). The estimation of zeta potential is based on the direction of velocity of particles under the influence of known electric field (Gengan *et al*., 2013).

Evaluation the antimicrobial effect

Antibacterial effect

Because of the growing problem of antibiotics overuse and resistance, nanoparticles are being tested as alternatives to the conventional antibiotics. The inhibitory activity of the silver nanoparticles of *F. oxysporum* was compared favorably with silver nitrate solution and standard antibiotics (Norfloxacin and Ofloxacin for *E. coli*, Erythromycin and Oflaxacin for each *Staphylococcus aureus* and *B. cereus*) as shown in Figure (4). Obtained results also showed that the diameters of inhibition zone were proportional increment by increasing the synthesized AgNPs volume. Moreover, the synthesized AgNPs at volume 100 µl exhibited more activity than pure silver nitrate and standard antibiotics. The synthesized AgNPs using supernatant of media (3) gave maximum inhibition zone. The maximum antibacterial effect of AgNPs was shown against *S. aureus* (19 mm), followed by *E. coli* (17 mm) and *B. cereus* (15 mm). Our findings corroborate the report of Saraniya *et al*.,(2014) who reported that *S. aureus* had the maximum sensitivity to silver nanoparticles, while *E. coli* was less sensitive, whereas the opposite results were reported in (Kim *et al.*, 2007). The mechanism of species specific efficiency of silver nanoparticles and possible differences from strain to another strain are currently unknown and the reason for their antibacterial effects requires further researches.

Gram-positive and Gram-negative bacteria are different in their membranes structure. The nanoparticles accumulate in or near the membranes with some particles pass through it. The permeability of these membranes and the ability of releasing silver ions into the intracellular medium may influence the degree of antibacterial effects of AgNPs (Rayikumar *et al*., 2010). There is a small difference between the antibacterial effects of three supernatants; this may depends upon the differences between the size and surface area of nanoparticles. For example, AgNPs with smaller size (having the large surface area available for interaction) would have better bactericidal effect and vice versa. Morones *et al*., (2005) reported that AgNPs with size range from 1 to 10 nm, adhere to cell membrane surface and destroy its proper function as respiration and permeability.

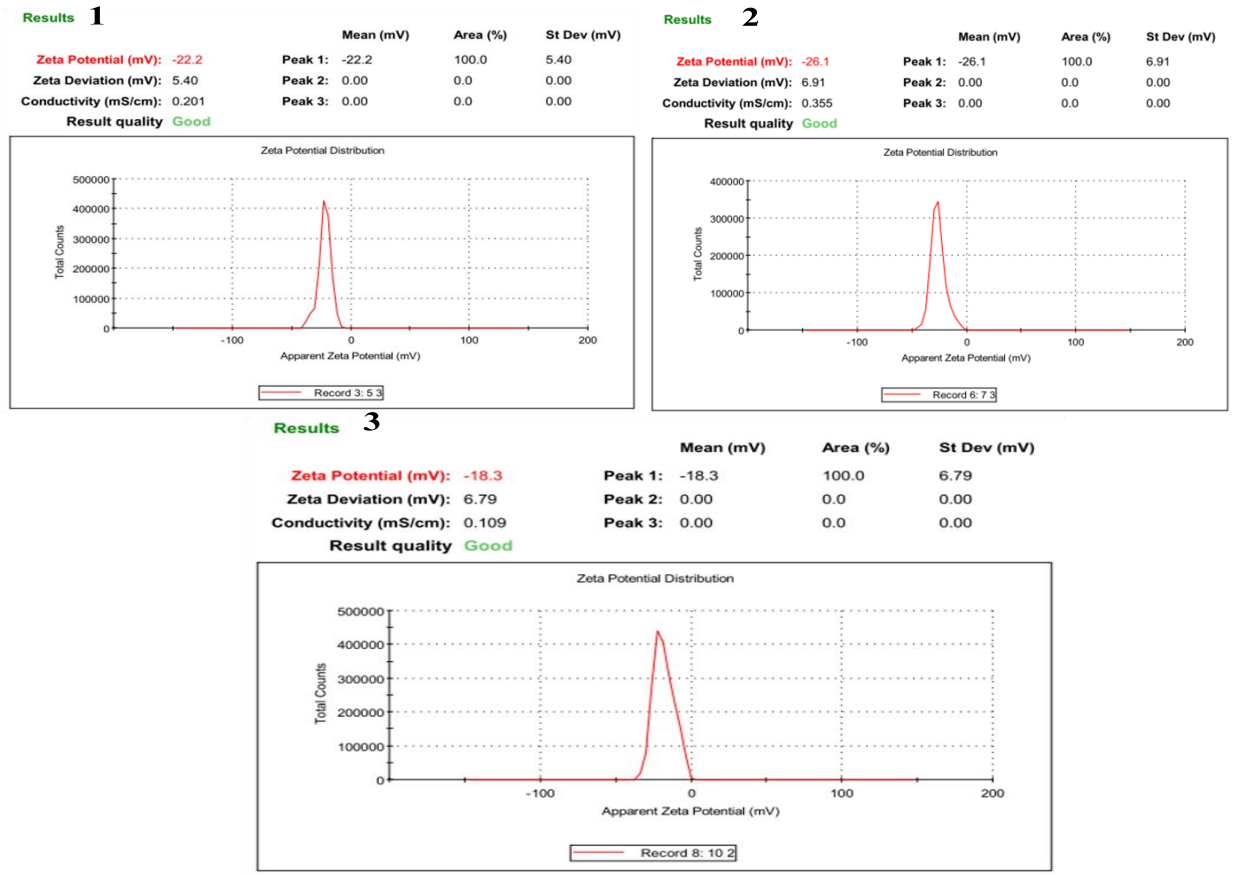


Fig. 3. Zeta potential of the synthesized AgNPs by supernatants of growing *F. oxysporum* on different culture media: 1. potato dextrose broth medium, 2. Sabouroud broth medium and 3. malt yeast broth medium.

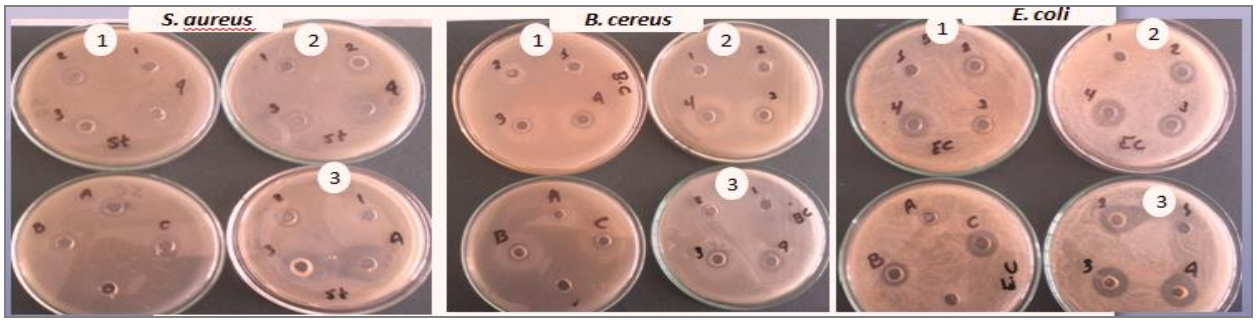


Fig.4. Antibacterial activity of different volumes of AgNPs synthesized using supernatants of growing *F. oxysporum* on different culture media The numbersof wells 1, 2, 3 and 4 represents the volume of AgNPs 10, 20, 50 and100 µl – A: a pure $AgNo_3$, B: Erythromycin and C: Oflaxacin for *each S. aureus* and *B. cereus* and B: Norfloxacin, C: Ofloxacin for *E. coli.*

Antifungal effect

In current study, the antifungal effect of AgNPs synthesized using supernatant of growing *F. oxysporum* on different culture media (1, 2 and 3) against *Fusarium oxysporum, Aspergillus oryzae* and *Aspergillus niger* were investigated. Silver nitrate and antifungal drug-Nystatin were used as comparable control. The toxicity was assessed through wells diffusion method with 100 µl of each AgNPs solutions. AgNPs showed more powerful antifungal effects than in case of silver nitrate and Nystatin. The synthesized AgNPs using supernatant of media (3) showed higher antifungal effect with inhibition zones of 18, 17 and 17mm for *Aspergillus oryzae*, *Aspergillus niger* and *Fusarium oxysporum*, respectively (Fig 5). It has been clear that antimicrobial effects of AgNPs may be correlated with the decrease in their size and shape leading to increased their surface area that enhances their antimicrobial effect. The biosynthesized silver nanoparticles exhibited strong antifungal activity against the phyto pathogenic fungi, *Fusarium oxysporum* (Gopinath and Velusamy 2013), *Alternaria alternata, Sclerotinia sclerotiorum, Macrophomina phaseolina, Rhizoctonia solani, Botrytis cinerea, Curvularia lunata* (Krishnaraj *et al*. 2012), *Aspergillus niger* (Pinto *et al.* 2013). Kim *et al*. (2007) claimed that spherical AgNPs had more powerful effect against *Candida albicans* than in case of commercially available antifungal agents. Using conventional antifungal drugs as Amphotericin B and Nystatin

represents a major health problem due to their serious side effects as renal and liver dysfunction (Hoeharner *et al*., 2010). Biosynthesis of silver nanoparticles by *Fusarium oxysporum* is economic and eco-friendly.

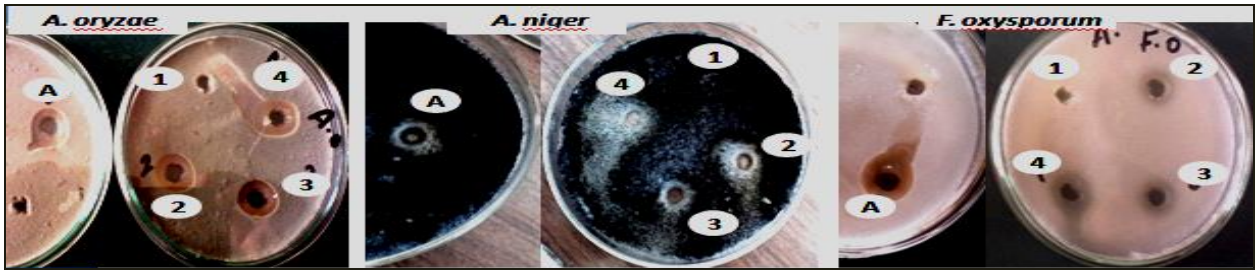


Fig. 5. Antifungal activity of the synthesized AgNPs. (1) represents $AgNO_3$, Numbers 2, 3 and 4 represent AgNPs synthesized using supernatant of media 1, 2 and 3, respectively- (A). Antifungal drug nystatin.

CONCLUSION

In our study, AgNPs were synthesized extracellulary by *Fusarium oxysporum* grown on different media. The study showed that the spherical Ag NPs were stable and their size ranged from 9 to 24nm and had good antimicrobial effects against a variety of bacteria and fungi. The green synthesis of AgNPs as potent antimicrobial agents using *Fusarium oxysporum* is likely to be a promising science achievement.

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التخليق الاخضر لمركبات الفضة النانومترية باستخدام فطر *Fusarium oxysporum* و تقيم نشاطها المضاد للميكروبات

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من الإنجازات المبشرة بالخير في المستقبل القريب هي استخدام الكائنات الحية الدقيقة والأنظمة الحيوية في تخليق المركبات النانومترية. و فى الآونة الأخيرة أصبحت الفطريات أحد أهم الوسائل الحديثة في تحضير المركبات النانومترية. وفي هذا الإطار تم استخدام راشح بيئة فطر الـ *Fusarium oxysporum* المنمي علي بيئات مختلفة في اختزال محلول نترات الفضة (0.1 mM) للتخليق الأخضر لدقائق الفضة النانومترية. وقد أوضحت النتائج أن شكل دقائق الفضة النانومترية كروي متجانس وذات أحجام تتراوح ما بين 24.9 nm. كما تم تقيم النشاط المضاد لثلاث سلالات بكتيرية ممرضة (*Staphylococcus aureus*, *Bacillus cereus* and *E. coli*) وثلاث فطريات *Aspergillus niger*, *A. oryzae* and *Fusarium oxysporum*. وعليه أوضحت الدراسة أنه يمكن استخدام راشح فطر *Fusarium oxysporum* في تحضير دقائق الفضة النانومترية بطريقة صديقة للبيئة وأن هذه الدقائق لها تأثيرفعال في النشاط المضاد للميكروبات.