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Silver nanoparticles produced by green synthesis using *Citrus paradise* peel inhibits *Botrytis cinerea* in vitro

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ABSTRACT

Purpose: Our objective was to undertake the green synthesis of silver nanoparticles using Grapefruit *(Citrus paradise)* peel extract and evaluate the effects of silver nanoparticles on *Botrytis cinerea.* **Research method:** The silver nanoparticles formation was evaluated at different temperatures and concentrations of $AgNO_3$. The experiment was conducted during 2015 at Science and Research Branch, Islamic Azad University, Tehran, Iran. **Main findings:** Silver nanoparticles were successfully synthesized by Grapefruit's peel through a simple green and eco-friendly route. Aqueous extract of Grapefruit's peel was used synthesize nanosilver. The size of nanoparticle was determined at 5-65 nm, with SPR absorption at 420 nm in UV-Vis spectroscopy. Transmission electron microscopy (TEM) and X-ray diffraction spectroscopy (XRD) revealed that the synthesized nanoparticle was face centered. The silver nanoparticles characterized for their size and shape using scanning electron microscopy and TEM, respectively. XRD was used to determine the concentration of metal ions. Result indicated that nanosilver reduced the growth of *Botrytis cinerea* inviro culture. The highest antifungal effect was seen in the treatment with 40g/l nanosilver. In the other hand, the effect of nanosilver and time on diameter growth of *Botrytis cinerea* was not significant, individually (p≤1%). **Limitations:** No limitations were founded. **Originality/Value:** Green Synthesis of Nano is a reliable method for the nanoparticles synthesis and environmentally friendly approach.

INTRODUCTION

Plant mediated synthesis of nanoparticles has been considered as green route and a reliable technique for the synthesis of nanoparticles due to its eco-friendly approach. Over the past decade, it has become clear that nano-scale materials have useful physicochemical, optical and electrical properties. Nanoparticles synthesis is emerging as one of the fastest growing field due to their physical, chemical and biological properties. Consequently, a steadily increasing number of consumer products employing nanotechnology have become available for purchase (Biswas & Wu, 2005). In green procedure for synthesis of metal nanoparticles, plant extract may act as both reducing and capping agents, due to their specific structures and functional groups (Kouvaris et al., 2012). Nanoparticles defined as considered as particles with a maximum size of 100 nm. They display completely improved properties, which are quite different from those of when particles. These characteristics depend on the size, shape, and surface compared to the larger particles of the bulk material (Gurunathan et al., 2009).
Nano sized silver particles have found tremendous applications in the different field such as high sensitivity, bio molecular detection, antimicrobials, antioxidants, therapeutics and catalysis (Tada et al., 2000). There are three methods for preparation of nanoparticles containing; Physical, chemical and biological. In fact, Nanoparticles can be synthesized by various approaches like chemical and photochemical reactions in reverse micelles, microwave assisted, thermal decomposition, electrochemical, sonochemical process and green synthesis methods (Maribel et al., 2009). The green synthetic approach for nanoparticles formation, using bacteria (Mukherjee et al., 2008), fungi (Mohanpuria et al., 2008) and plants (Dhillon et al., 2012) has already been reported.

The physical and chemical processes for nanoparticles synthesis are very costly; because of that reason, researchers have found the cheapest way by using microorganisms and plant extracts for nanoparticle synthesis. The uses of microorganisms in biological nanosynthesis have been widely established and it is a kind of bottom up approach where oxidation/reduction reaction is the main reaction (Gericke & Pinches, 2006; Kaler et al., 2011). Three procedures of green synthesis are: (a) use of microorganisms like fungi, eukaryotes and Prokaryotes, (b) Use of plant extracts or enzymes and (c) Use of templates like DNA, membranes, viruses and diatoms. .
Metal nanoparticles, such as Ag, Au, and Pt are applied in products that directly are exposed to the human health, such as household items like detergents, soaps, shampoos, cosmetic products, and toothpaste. They find applications in the pharmaceutical and medical area (Ankanna et al., 2010). Therefore, green synthesis of nanoparticles is gaining importance due to its simplicity, cost effective and eco- friendliness (Yogeswari et al., 2012; Farooqui et al., 2010).

Among all the noble metals, silver nanoparticles are most important product in the field of nanotechnology which has gained boundless interests because of their unique properties; such as chemical stability, good conductivity, catalytic antibacterial, anti-viral, antifungal and anti-inflammatory activities. They can be incorporated into composite fibers, cryogenic superconducting materials, cosmetic products, food industry and electronic components (Ahmad et al., 2003; Klaus-Joerger et al., 2001).

Some reports have pointed out to the promising prospect of nano-silver by using various plants. Krishnaraj et al. (2010) synthesized nanosilver by *Acalypha indica* in spherical shape and diameter of 20-30 nm. Green synthesis of silver nanoparticles is currently being considered using *Allium sativum* in spherical shape and 4-22 nm. Chandran et al. (2006) studied one and remarkable effort were spent to develop Green synthesis of silver

nanoparticles by *Aloe vera* in spherical and triangular shape and size of 50-35nm. *Azadirachta indica* is extensively used for Silver nanoparticles synthesis in spherical, tringular and quasispherical shape and size of 7.5-65nm by Kasthuri et al. (2009).

Nanoparticles can be characterized by their size, surface area, and shape and dispersive (Jiang et al., 2009). Some common techniques shown their characterization such as, UV-visible spectrophotometer, scanning electron microscopy (SEM), Transmission Electron Microscopy (TEM), X-ray diffraction (XRD), Fourier transforms infrared spectroscopy (FTIR) and energy dispersive spectroscopy (EDS) (Feldheim & Foss, 2002; Sepeur, 2008; Shahverdi et al., 2011). UV-Vis spectrophotometer allows identification, characterization and analysis of metallic nanoparticles. Generally, 300-800nm light wavelength is used for the characterization of size range from 2 to 100nm (Feldheim & Foss, 2002). Electron microscopy is a common method for surface and morphological characterization. SEM and TEM are used for the morphological characterization at the nanometer to micrometer scale (Schaffer et al., 2009). SEM can provide morphological information on the submicron scale and elemental information at the micron scale; but TEM has a 1000 fold higher resolution compared with the SEM. Characterization of nanoparticle using FTIR is very useful for the understanding of the surface chemistry because the organic functional groups which are attached to the surface nanoparticles can be determined (Chitrani et al., 2006). XRD is used to examine the overall oxidation state of the particles as a function of time, i.e. phase identification and characterization of the crystal structure of the nanoparticles (Sun et al., 2000). EDS is used to determine the elemental composition of metal nanoparticles (Strasser et al., 2010).

Botrytis cinerea is considered as one of the serious diseases of cucumber plants. It can attack several plant tissues and many greenhouse crops, such as pepper, sweet basil strawberry, sweet basil tomato and strawberry. In vegetables, it may infect fruits, stems and leaves (De Cremer et al., 2013). In addition, it causes pre- and post-harvest diseases in at least 200 plant species (Jarvis, 1977). Infection resulting from growth through the infection or petiole of wounds may cause plant death. *B.cinerea* is one of the most comprehensively studied fungal plant pathogens in greenhouses (Van Kan, 2006). When *B. cinerea* infects the host, it can destroy the cell walls by secreting diverse enzymes and proteins (Zhang & Van Kan, 2013). The symptom of Botrytis infection of fruits appears as a gray rot. In the spring, the fungus germinates from small and dark-colored, over-seasoning structures known as sclerotia. Then the fungus produces asexual spores that caused spread the disease.

MATERIALS AND METHODS

Materials

All chemical materials were purchased from Merck Company, Germany. The *Citrus paradise* fruits were collected from Sari, Province of Mazandaran, with longitude 53˚ 5'Eand latitude 36 ◦ 4'Nat 132m above sea level and an average annual temperature of 15°C, which has a temperate climate Caspian. Plant material was identified in Citrus and subtropical research center of Iran. The fruit peel was cut and dried in shade under a stream of air in a chamber room. The dried peel was ground and stored at 4˚C.

Instruments

UV-Vis studies were carried out using a Varian Cary 300 UV-Vis spectrophotometer. Crystallographic studies were carried out using a 3003 PTS Seifert (Germany) X-ray diffraction (XRD) instrument. Morphology the synthesis of silver nanoparticles were performed by transition electron microscopy (TEM) using a PHILIPS EM 208 instrument

at100 Kv. FTIR experiment was used to determine the active functional groups in plant extract using a Nicolet NEXUS 870 FT-IR spectrophotometer.

Preparation of Grapefruit peels extract

Twenty (20) gr of *Citrus paradise* fruit peel was extracted using 100 ml deionized water for 30 min in an 80˚C water bath. The extract was filtered via a No.1 whatman filter paper. The volume of solution was adjusted to 100 ml in a volumetric flask. The concentration of extract in solution was 0.025gr/ml. The solution was stored in 4˚C for further experiments.

Plant mediated synthesis of Ag nanoparticles

Twenty (20) ml of *Citrus paradise* fruit peel extract solution was added to 80 ml of 0.001M solution of $AgNO_3$ at room temperature (25° C). The mixture was shaken in darkness at 200 rpm using a IKA orbital shaker in four pH (4, 7, 8 and 10) and three temperatures (40, 50 and 60° C). The color change of solution from pale yellow to reddish brown after 30 min showed the reduction of Ag^+ ions to Ag^0 nanoparticles.

Characterization of synthesized Ag nanoparticles

The plant mediated synthesized Ag nanoparticles, structurally, was investigated by X-ray diffraction, transmission electron microscopy (TEM) and Ft-IR instrument. The nanoparticles were precipitated by centrifugation at 12000 rpm for 20 min. The obtained pellets were washed twice with deionized water, was further washed with ethanol. It was dried at 60˚C in vacuum oven for 5h. Ft-IR experiments were done by KBr disk method. A 0.5 mg of the sample was ground with KBr in an agate mortar and 15 mm disks were made using 10-ton pressure. The FTIR spectra was achieved using a Nicolet NEXUS 870 FTIR spectrophotometer from 4000-400 cm^{-1}.

XRD analysis was done to determine the structure of synthesized Ag nanoparticles. XRD studies were done by a Sifert XRD 3003 PD (Germany) with a Cu-Kα_1 X-ray tube with λ of 1.54 A° in the range of 2θ from 20° to 90° using dried powdered of Ag nanoparticles. Calculation of particles size of synthesized Ag nanoparticles was done using Debye-Scherer's equation from XRD parameters as shown below:

$$D = K\lambda / ß \cos\theta$$

Where, D is particle size, K is a dimensionless shape factor, with a value close to unity (0.9), λ is wavelength of X-Ray in angstrom (A°), ß is the full width at half the maximum intensity (FWHM) in radian and θ is the diffraction Braggs angle. Elimination of additional instrumental broadening the ß value should be corrected according to blow formula using the FWHM from a large grained silicon sample, which was calculated as 0.15868 A° in our XRD instrument (Ghosh et al., 2012).

$$ß \text{ corrected} = [(FWHM_{sample})^2 - (FWHM_{Si})^2]^{0.5}$$

Morphology of Ag nanoparticles was performed using transmission electron microscopy (TEM) image by means of a Philips EM 208 instrument with 100 kV acceleration voltages. 10-µL solution of dried Ag nanoparticles that were dispersed in deionized water using ultrasonic bath, was located on a carbon coated TEM grid and subjected to TEM instrument.

Preparation of fungal culture and inoculums

The pathogen, *Botrytis cinerea*, was obtained from Plant Pathology laboratory in Islamic Azad University, Science and Research Branch, Tehran, Iran. The cultures were cultured on potato dextrose agar (PDA) medium at 4°C; and fresh cultures were grown on PDA plates at 25°C.

RESULTS AND DISCUSSION

UV-Vis spectral observation

Color change of mixture solution of *Citrus paradise* fruit peel extract and 0.001M $AgNO_3$ from pale yellow to reddish brown was due to the formation of Ag nanoparticles (Fig. 1).

The UV-Vis spectral studies at different time intervals 30 min, 180 min, 24 h and 48 h) as shown in Figure 1. The Surface Plasmon Resonance (SPR) absorption in 430 nm appeared due to colloidal Ag nanoparticles (Fig. 2).

The FTIR spectra of *Citrus paradise* peel extract with three strong absorption peaks at 3410,2929 and 1637cm^{-1} (Fig. 3, A) which related to stretching frequency of OH, C-H and C=O, respectively showed that the active biomaterials such as flavonoids, carbohydrates and phenolic compounds are responsible for reduction and stabilization Ag^{+} ions to Ag^{0} nanoparticles. This could be seen by a reduction in intensity of main peaks after decreasing reduction of Ag+ ions (Fig. 3, B) observed (Fig. 2).

The spectra characterizing the phytochemical fabricated Ag nanoparticles showed a XRD pattern with four main peaks at 37.8431°, 45.9587°, 64.1242° and 76.9911° in the range of 2θ from 20° to 90° related to (111), (200), (311) and (222) HKL values, respectively, which shown that the Ag nanoparticles were synthesized in a face center cubic (fcc) lattice system (Fig. 4).

Fig. 1. Color change of AgNO3 solution + *Citrus paradise* extract from pale yellow (A) to reddish brown (B)

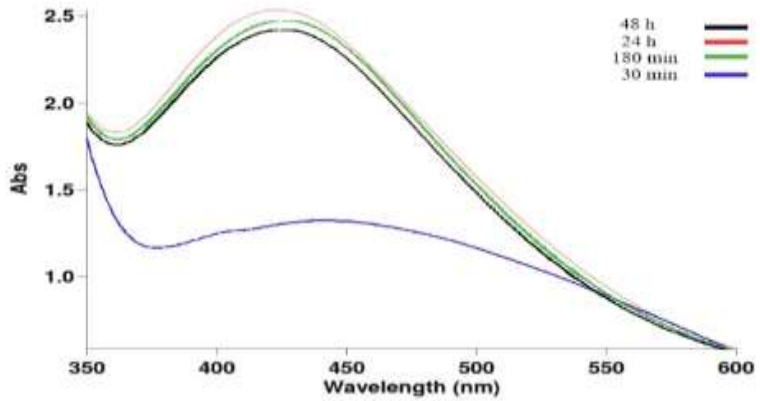

Fig. 2. UV.Vis spectra of plant mediated synthesized Ag nanoparticles

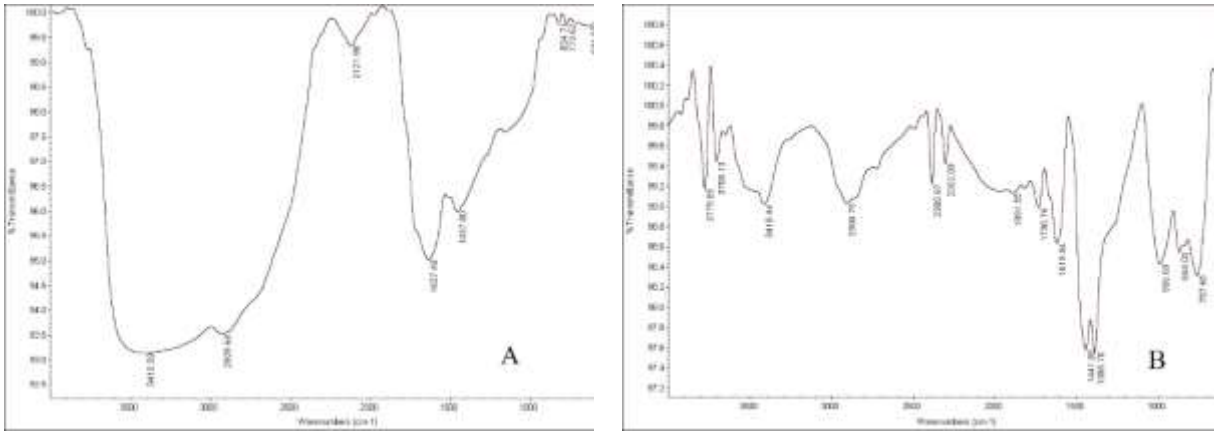

Fig. 3. FTIR spectra of *Citrus paradise* peel extract (A) and synthesized Ag nanoparticles (B)

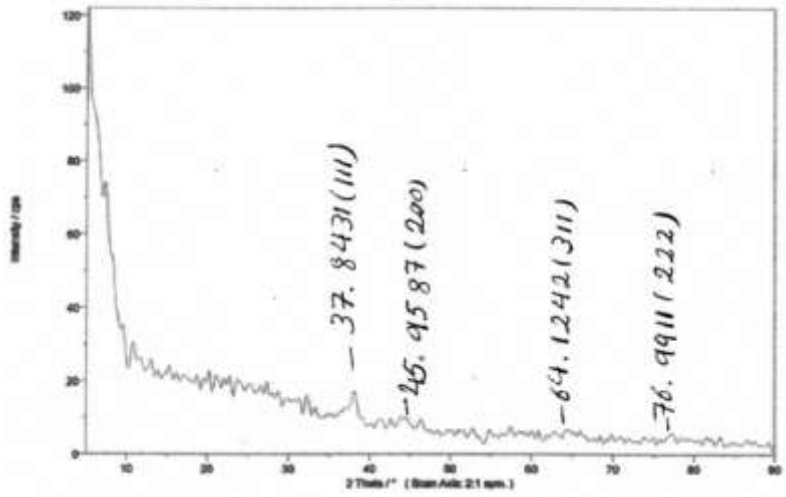

Fig. 4. XRD pattern of Ag nanoparticles using aqueous *Citrus paradise* peel extract

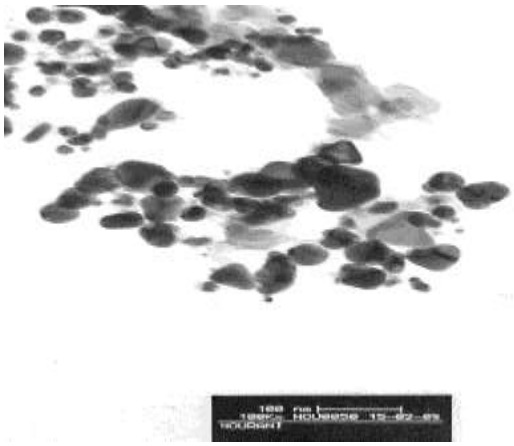

Fig. 5. TEM image of Ag nanoparticles using aqueous *Citrus paradise* peel extract

The average size of Ag nanoparticles was calculated as 55.02 nm according to Debye-Scherer's calculation at 2θ of 37.8431° (111) whit FWHM $_{sample}$ and FWHM $_{Si}$ were 0.2202 and 0.15868, respectively.

TEM image (Fig. 5) revealed that the aqueous *Citrus paradise* peel extract could be fabricated the Ag nanoparticles in a spherical shape with the diameter ranging from 5-65 nm.

The inhibitory effect of nanosilver on the growth of the *Botrytis*

According to Figure 6, the highest inhibitory effects on the growth of the *Botrytis* were related was at 40g/l nanosilver. Also 20 and 30 g/l nanosilver had significant inhibitory effects; nanosilver concentration decreased fungal growth. Increasing nanosilver from 20 g/l to 30 g/l or even up to 40 g/l significantly reduced the growth of microorganisms.

The effect of time on *Botrytis* growth had shown an increase in the diameter of the fungus over time. Figure 7 is shown the lowest *Botrytis* growth rate in the first day (0.92 cm). After 11 days, it reached to 6.81 cm, which was the highest growth.

Result indicated that, 40g/l nanosilver had the highest inhibitory effect. On the other hand, 30 and 20 g/l nanosilver had a considerable rate (Fig. 8).

It showed that the growth of *Botrytis cineara* is inhibited at different concentrations of silver nanoparticles. In addition, it presented in this investigation the inhibitory effect of nanosilver on growth of *Botrytis cinerea.* This agrees with other studies which stated, antimicrobial activity of silver was different depending on microbial species (Galeano et al., 2003).

Nanosilver can significantly delay mycelial growth in a different concentration in vitro (Aguilar-Mendez et al., 2011). It may directly stick to and enter the cell membrane to destroy spores, but this mechanism is not understood (Hwang et al., 2008).

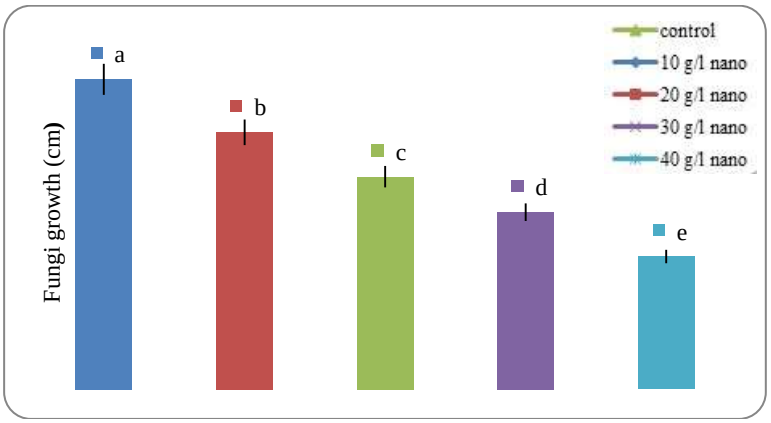

Fig. 6. Effect of treatment on *Botrytis* growth

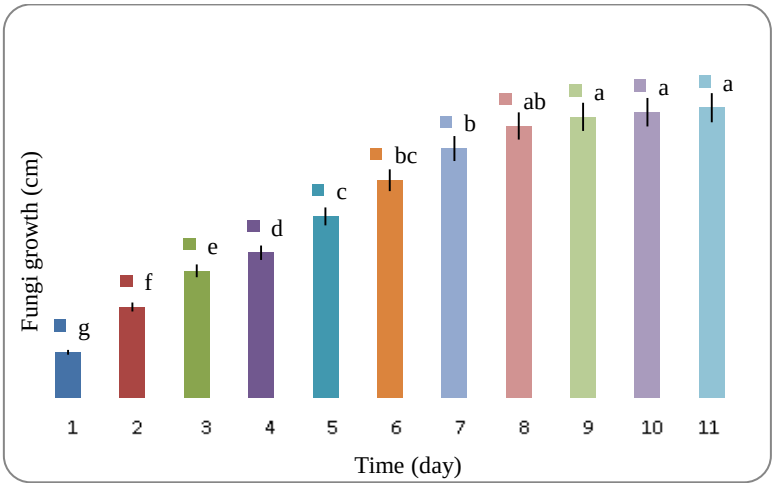

Fig. 7. Effect of time on *Botrytis* growth

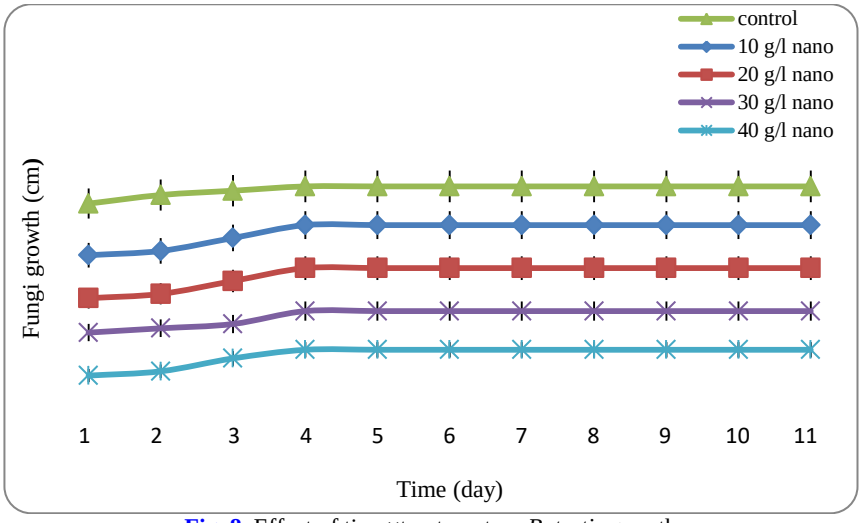

Fig. 8. Effect of time×treatment on *Botrytis* growth

Our results demonstrate that fungal growth was associated with amount of silver nanoparticles, which is consistent with research by Sahar and Ouda (2014), who indicated that nanosilver reduced the growth of microorganisms.

The highest antifungal effect was seen in the treatment with 40g/l nanosilver. It revealed that nanosilver damaged the *Botrytis cinerea* hyphae when compared with control. Qiu et al. (2014) reported that nanosilver caused deleterious effects, not only on fungal hyphae, but also on conidial germination. It showed bacteriostatic action against *Botrytis cinerea*. The principle of bacteriostasis is that nanosilver penetrates the cell membrane of *B. cinerea* and damages it.

CONCLUSION

This study indicated that Silver nanoparticles were successfully synthesized by Grapefruit's peel through a simple green and eco-friendly route. The highest antifungal effect was seen in the treatment with 40g/l nanosilver. In conclusion, results showed that fungi could not be grown on low concentration of nanosilver.

Conflict of interest

The authors declare that they have no conflict of interest.

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