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Toxicity assays of Silver nanoparticles in different diameters on L929 mouse fibroblasts

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ABSTRACT

Silver nanoparticles (AgNPs) are one of the most important nanoparticles which have various biomedical applications. For example, as antifungal, antibacterial, anti-cancer and anti-inflammatory agents. Skin infection caused by *Trichophyton rubrum* and some opportunistic fungi such as *Candida albicans* and *Aspergillus fumigatus* are sometimes difficult to be treat. Although silver nanoparticle has long been used as effective inorganic antifungal agent; the antifungal activity of nano-Ag in different size has not been investigated yet. Anti-cancer and antifungal effects of spherical silver nanoparticles (nano-Ag) were investigated in this study and we decided to determination toxicity of Nano-Ag in different diameters (10, 20 and 40 nm) on L929 mouse fibroblasts. TEM microscope has been used to evaluate nanoparticles size and morphology. Nano-Ag's toxicity was evaluated by MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. This study showed that the toxicity of Nano-Ag in low concentration (16-32µg/ml) are sustainable and Silver nanoparticle effects are size dependent.

1. Introduction

Silver nanoparticles (AgNPs) are used in various fields, including food, medical and industrial. Due to their unique physical and chemical properties, they have several applications such as antifungal, antibacterial, anti-cancer and anti-inflammatory agents (Zhang, et al., 2016; Villaverde, 2010) Recently, many fungus resistances to the main antifungals. Hence, new alternative is need to combat resistance. AgNPs play essential role as anti-fungal agents against fungi especially opportunistic fungi such as *Candida albicans* and *Aspergillus fumigatus* which caused various diseases (Cornistein et al., 2013). Recent studies have confirmed that Nano-Ag have good antibacterial activity (Sadeghi et al., 2015; Van Dong et al., 2012). The major mechanism of the antifungal and antibacterial properties of silver nanoparticles is by anchoring and penetrating of silver particles to the cell wall; once inside the cell, silver nanoparticles modulate the cellular signaling by dephosphorylating putative key peptide substrates, which are critical for cell viability and division (Gajbhiye et al., 2009;

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Piao et al., 2011). Nano-Ag breaks down the membrane permeability barrier of *C. albicans*, it is possible that nano-Ag perturbs the membrane lipid bilayers, causing the leakage of ions and other materials as well as forming pores and dissipating the electrical potential of the membrane (Van Dong et al., 2012). Silver nanoparticles have already been used in antibacterial clothing and burn ointments and as coating for medical devices (Navalakhe et al., 2007; Nasrollahi A., 2011).

Chemotherapeutic agents are currently used for cancer therapy that they have side effect or difficult process. AgNPs was found as useful alternative for chemotherapeutic agents (Burdu□el et al., 2018) Furthermore, eco-friendly method for producing Nano-Ag investigated and will be good alternative for chemical method which used for synthesizing chemotherapeutic drugs and may pollute environment (Sadeghi et al., 2015; Kumar et al., 2018). In high concentration, silver is toxic to human beings, whereas in low concentrations it is nontoxic (Sastry et al., 2003). A few researchers have investigated the toxicity of AgNPs in terms of cell death, little is known about the mechanisms of AgNPs-mediated toxicity (Tang et al., 2016). There is no doubt that nano materials will play a key role in many technologies of the future (Durán et al., 2007).

Based on studies and the importance of silver nanoparticles as an agent without side effects, in this study we focused on the concentration of silver nanoparticles as an antifungal agent and Nano-Ag's toxicity with different diameters on L929 mouse fibroblasts.

2. Materials and Methods

2.1. Microorganisms and culture conditions

Tricophyton rubrum [PTCC 5143(RI 613)] (1-4×10^4 cells/ml), *Aspergillus fumigatus* (PTCC 5009) (1.5×10^4 cells/ml) and *Candida albicans* (ATCC 10231) (1-4×10^6 cells/ml) were cultured in Sabouraud dextrose agar (SCC) containing chloramphenicol and cycloheximide, Sabouraud dextrose broth (SDB) respectively in 48 hours, 24 hours and one week of incubation at 28ºC.

2.2. Preparation of Nano-Ag

Nano–Ag colloidal solution was prepared based on the reduction reaction of silver nitrate by sodium borohydride. Trinatrium citrate was used as stabilizing agent Nano -Ag in size of 10nm, 20nm, and 40nm was purchased from Nanozino Co. Ltd. (ZSA, Roshd, Iran). The Dynamic Light Scattering (DLS) of nanoparticles was shown in figure 1. The micrograph of the nano –Ag was obtained using TEM (figure 2).

2.3. RPMI medium

RPMI 1640 medium purchased from Invitrogen Corp. /GIBCO. 10gr of RPMI 1640 medium was mixed with 2gr Isobicarbonate, 100 unit/ml penicillin and 100mg/ml streptomycin. Then 5ml FBS (Fetal Bovine Serum) was added to 45 ml RPMI and RPMI 10% was prepared (Liao et al., 2019; Saino et al., 2011).

2.4. Preparation of Nano-Ag and serial dilutions

6 sterile falcons containing RPMI 1640 medium with different concentration (128, 64, 32, 16, 8, 4 µg/ml) of Nano-Ag were prepared. 128 µg/ml Nano-Ag were added to the first falcon, after shaking, 500 µl of the first falcon was poured to the second and repeated until last, and then 500 µl of the later was discarded.

2.5. Fibroblast Cell line L929

Fibroblast cell line L929 was used to evaluate Nano-Ag cytotoxicity. The cells were cultured in flask containing RPMI 10% for 48h with once replacement medium. The murine cell line was prepared by cell culture laboratory in Karaj Islamic Azad University. Cells were checked out with invert microscope as the cells attached to bottom of flask was observed (figure 3). To separate attached cells, 2-3 ml of dissolved trypsin in EDTA (Amino tetra-acetate, ethylene dichloride) was added and Incubated in co_2 incubator at 37ºc for 2 min. After recheck with invert microscope and observe segregated cells, 3 ml FBS were added and then centrifuged at 1500 rpm for 5 min after disposed the supernatant embrocating with RPMI 10%. Then, RPMI 10% was added up to the falcon and up and down for a few times.

2.6. Transport to a 96-well plate

Amount of 150 µl of each falcon solution were added into wells and incubated for 24 h at 37 °C. After evacuating every well slowly with Pasteur pipette, 100 µl of the drug were distributed inside the wells and incubated for 24 h.

2.7. MTT assay

After discharging wells contents, 100 µl MTT was added and incubated the plate for 5 hours at 37 °C in 5% CO2 incubator. The MTT test based on mitochondrial dehydrogenase enzyme, viable cells change yellowish color tetrazolium to purple at the result of formazan. After 5 h MTT was removed and 5 µl Isopropranul was added in entire wells then plate was placed on a shaker for 10 min until all formosans came out of the cells.

2.8. ELISA reader

At the end of the culture period, in order to evaluate the amount of the extracellular matrix constituents on the film surface, the samples were washed extensively with sterile PBS to remove the culture medium, and then incubated for 24 h at 37 ″C with 1 mL of sterile sample buffer (20 mM Tris-HCl, 4 M GuHCl, 10 mM EDTA, 0.066% (w/v) SDS, pH 8.0). At the end of the incubation period, sample buffer aliquots were removed, and then the films were centrifuged at 4000 rpm for 15 min, then micro plate was placed in shaker for 3 min. The colorimetric is measured by ELISA reader at 400-550 nm.

2.9. Stain with Crystal violet

100 µl of crystal violet was poured into the empty wells and after 30-60 s micro plate was seen by invert microscope (figure 4). Cell lines samples with varying degrees of damage was observed after testing with different concentrations of nanoparticles (figure 5). Average the duplicate reading for each sample was done. The toxicity percentage was obtained using the following equation: % Cytoxicity = [100 x (control - sample)].

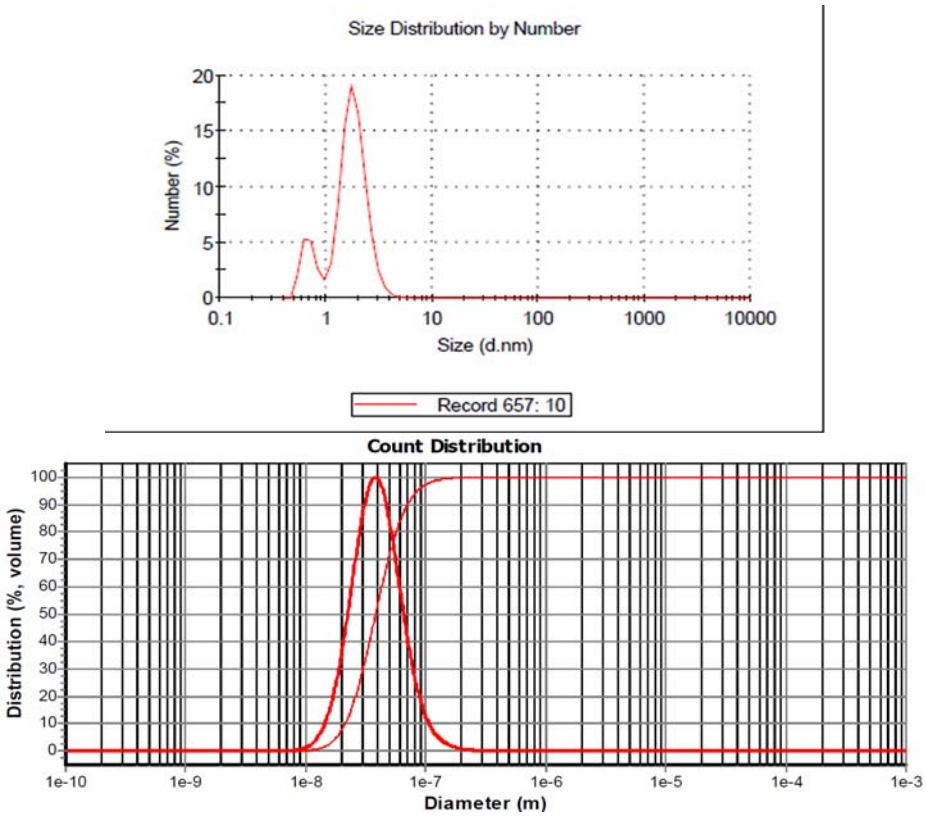


Figure 1. Dynamic Light Scattering (DLS) of Nano-Ag

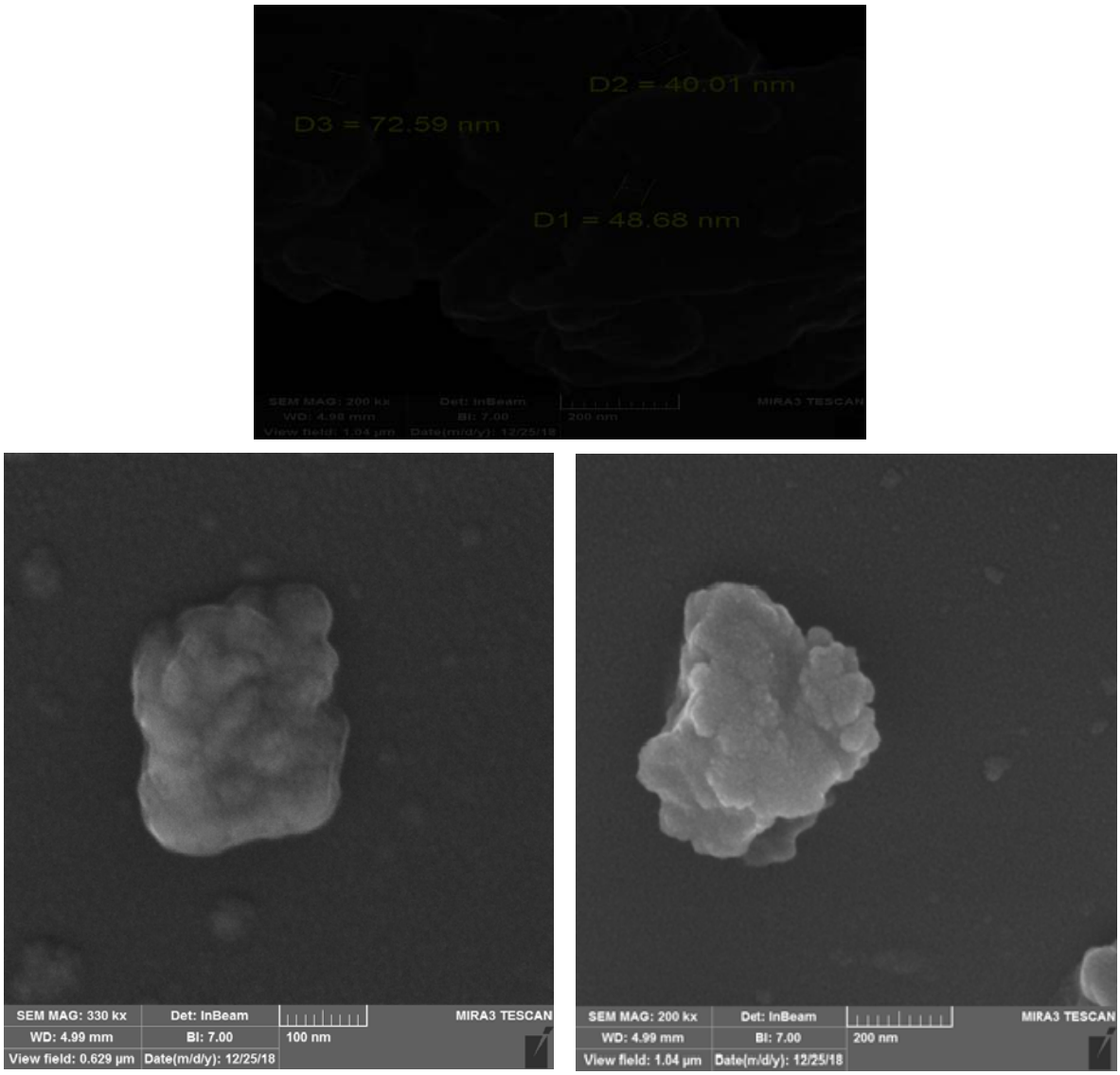


Fig 2. The morphology of Nano-Ag.

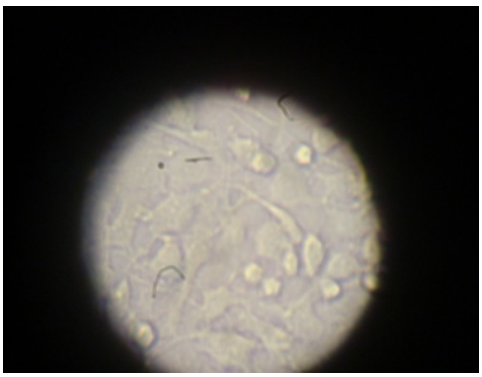


Fig 3. the cell line L929

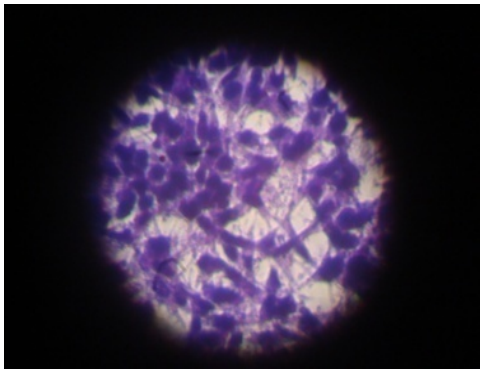
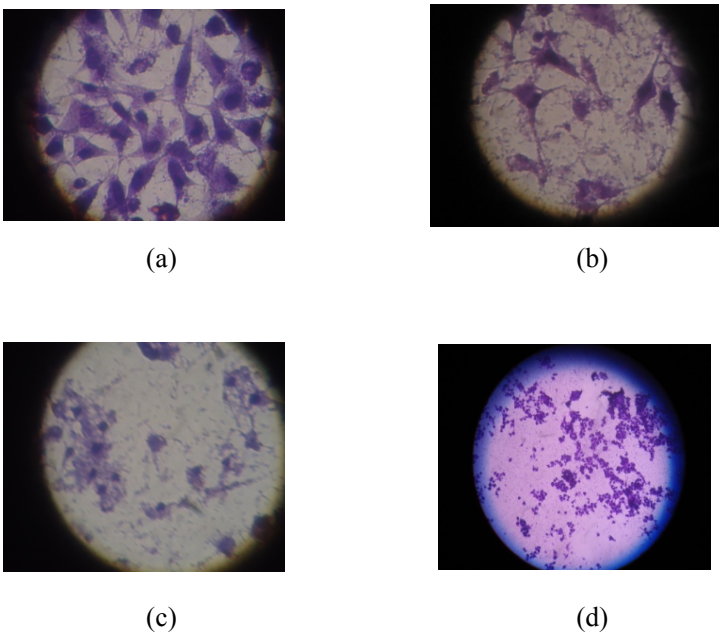


Fig 4. Experimental cell line before testing



(a) (b)

(c) (d)

Figure 5. Varying degrees of damage after testing with different concenteation of nanoparticles

3. Results

The viability cells versus silver nanoparticles in concentration 128-4 µg/ml for Nano-Ag10 is 65-100% and cytotoxicity is 0-0.35 % (Table & diagram 1), for NanoAg20 the viability is 49-100% and the cytotoxicity is 0-51 % (Tables & diagram 2) and for Nano-Ag40 the viability is 39-100 and the cytotoxicity is 0/61-0% (Table & diagram 3). The Nano-Ag are compared in diagram 4, according to it, Nano-Ag10 has the lowest toxicity specially in low concentration 16-4 µg/ml and has high viability in this concentration and afterwards Nano-Ag 20 and Nano-Ag40, respectively.

Table 1. Cytotoxicity of Nano^{10} –Ag

Nano^{10}–Ag Concentration	OD(average)	Cytotoxicity	Viability
128	0/20	0/35	**65%**
64	0/22	0/29	**71%**
32	0/28	0/09	**91%**
16	0/3	0/03	**97%**
8	0	0	**100 %**
4	**0**	**0**	**100 %**

Table 2. Cytotoxicity of Nano20 –Ag

Nano^{20}–Ag Concentration	(average) OD	Cytotoxicity	Viability
128	0/15	0/51	**49%**
64	0/13	0/32	**68%**
32	0/25	0/19	**81%**
16	0/28	0/09	**90%**
8	0	0	**100 %**
4	**0**	**0**	**100 %**

Table 3. Cytotoxicity of Nano^{40} –Ag

Nano^{40}–Ag Concentration	(average) OD	Cytotoxicity	Viability
128	0/12	0/61	**39%**
64	0/16	0/48	**52%**
32	0/21	0/32	**68%**
16	0/26	0/16	**84%**
8	0	0	**100 %**
4	**0**	**0**	**100 %**

Diagram (1) comparison of various Nano-Ag in cytotoxicity and viability of cell line
*P<0.05 ،**P<0.01 ،***P<0.001) Nano-Ag 20 and 40 in comparison with Nano-Ag 10

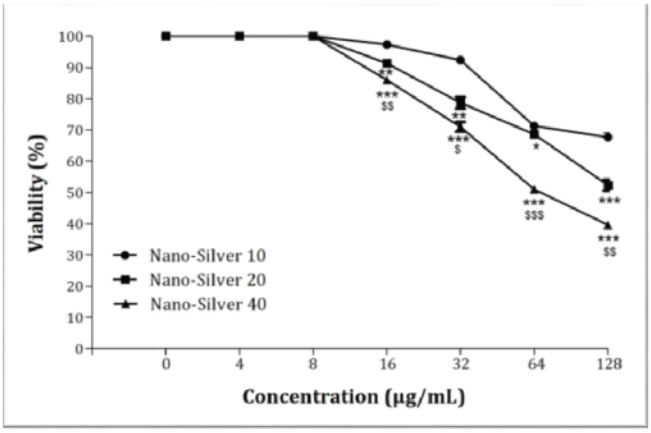


Diagram (2) evaluation viability of cells to Nano –Ag 10
*P<0.05 ،**P<0.01 ،***P<0.001) Nano-Ag 20 and 40 in comparison with Nano-Ag 10

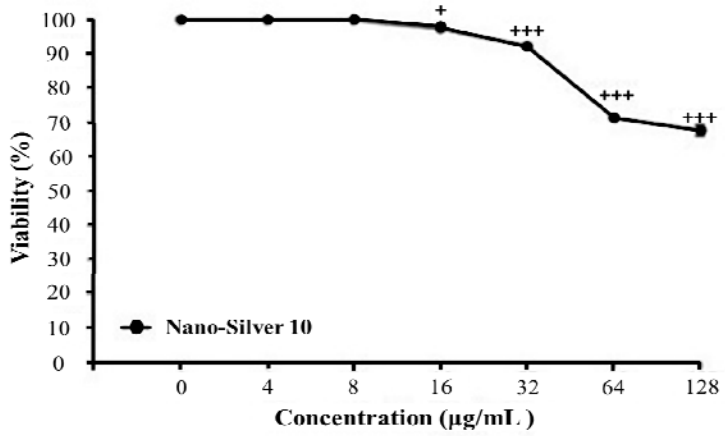


Diagram (3) evaluation viability of cells to Nano –Ag 20
*P<0.05 ،**P<0.01 ،***P<0.001) Nano-Ag 20 and 40 in comparison with Nano-Ag 10

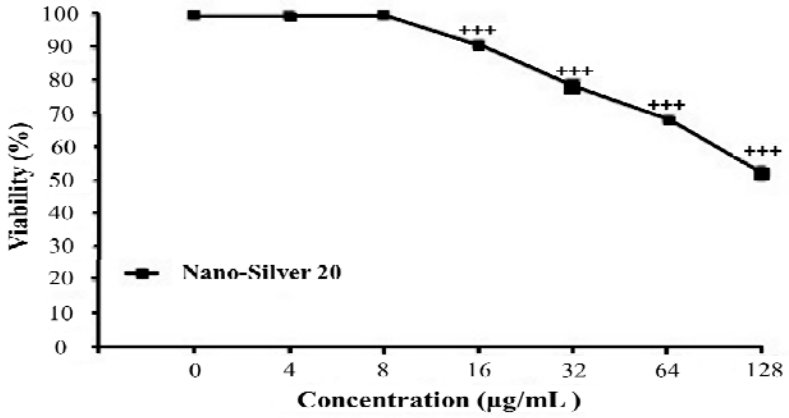
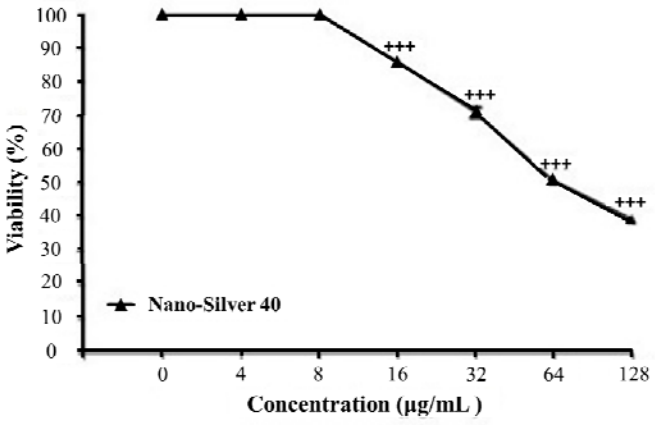


Diagram (4) evaluation viability of cells to Nano –Ag 40
*P<0.05 ،**P<0.01 ،***P<0.001) Nano-Ag 20 and 40 in comparison with Nano-Ag 10



4. Discussion

M. Gajbhiye et al. has mentioned, in high concentration silver is toxic to beings, whereas in low concentration is nontoxic (Sadeghi et al., 2015). We confirmed their result and added that cytotoxicity of nano-Ag is size dependent so that smaller particles have lower toxicity. Lara et al. reported that Ag nanoparticles are attractive because they are non-toxic to the human bodies at low concentration and have broad spectrum antimicrobial action (Lara et al., 2011), in our research that Ag nanoparticle have a good antifungal activity. Huang et al. remarked that the Nano-Ag have been shown to be cytotoxic at higher concentration than 6µg/ml (Huang, 2015). We focus on nano-Ag's size and compared cytotoxicity of silver nanoparticles in different diameters which is novelty of this research.

Conclusion

As we have noticed above the headmost of result is Nano-Ag in low concentration is low toxic and based to previous article, this concentration is too enough to kill pathogenic microorganism and secondly, the cytotoxicity of Nano-Ag has a direct outcome due to their size. The result of this study confirmed that Nano-Ag is a potent medicine to treat intense microbial infection. To the best of our knowledge, the present study is the first study about determination of cytotoxicity of Nano-Ag in various size as an antifungal drug.

Refereces

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