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CYTOTOXIC EFFECT OF SILVER NANOPARTICLES PRODUCED FROM A TYPE OF PATHOGENIC BACTERIA ISOLATED FROM CLINICAL SAMPLES IN IRAQ

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Abstract:

This work has been done to produce a silver nanoparticles by using a type of bacteria which considered a pathogenic bacteria , this bacteria was isolated from clinical samples in Iraq (vagina), the isolates which previously identified as *Proteus vulgaris* by using PCR technique. Then the cytotoxic effect of the bacteria against MCF-7 cell line was studied. The cytotoxic effect of *Proteus vulgaris* against MCF-7 cell line using 400 µg/ml reached 58.67 % from a cytotoxicity standpoint, this level of growth inhibition is tolerable. In vitro cytotoxic activity of Ag NPs against the MCF-7 cell line was achieved using 400g/ml ,growth suppression was 64.59 percent when nanoparticles were coupled with growth of bacteria (*Proteus vulgaris*) at the same concentration of AgNPs, the growth inhibition of MCF-7 cells increased to 73.55 percent.

Keywords: AgNPs, Cell line, XRD, AFM, TEM

INTRODUCTION

The problem of increasing multiple antibiotic-resistant microorganisms and failure to treat (specially infectious diseases) may be considered a serious problem in the medical field, therefore, many scientists research aim to produce anew efficient agents that diminish the resistance of these microorganisms and are also cost-effective [(1)] .Metal nanoparticles showed an obvious activity against microorganisms. They possess dimensions of (100 nm) or less. the large surface-area to-volume ratio is the most important property of nanoparticles materials , AgNPs have attracted significant interest in a number of applications , Silver is traditionally used as nonmaterial in shopper products [(2)]. The use of AgNPs in the treatment of wounds and burns infection is the most important application of in pharmaceutical manufacturing field [(3)]. It has been found that AgNPs are non-noxious to humans and are effective against bacteria at low-concentration, thus they don't having any side impacts to human, many studies proposed that AgNPs link to the cell membrane surfaces dispersion permeability and respiration behavior of the cell [(4)].

The genus Proteus bacteria are widely distributed in the natural environment. They can be found in the soil and manure ,they are [(5,6)]. *P. vulgaris* is human opportunistic pathogens it is the main cause of urinary tract infection after *E. coli and Klebsiella* cause of urinary tract infection and its complications, it is infection may lead also to formation of urinary bladder and kidney stone also this bacteria may be responsible for formation of crystalline biofilms on the inner and outer surface of indwelling urinary catheter , *P. vulgaris* may cause opportunistic infection to the wound , burns ,eyes, ears , nose , throat , when food and meat contaminated with *P. vulgaris* consumed this will lead to gastroenteritis gastro enteritis resulting from the consumption of contaminated meat or other food [(7)].this in turn lead for new antimicrobials agent like Ag NPs as an alternative to antibiotics Therefore, microbes establish a huge population in the living world. They have extra and intracellular vital products such as antibiotics, enzymes, toxins, biopolymers and pigments. More than 10,000 active broad-spectrum

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metabolites have been isolated from these microbes with a significant medicinal properties [5]. However, most of the microbial worlds remain unexplored owing to its vastness. Recent studies confirmed that only <0.1% of microbial world has been investigated till date [8]. Silver NPs may have a Cytotoxic effect due to its biological and chemical interaction with the the Deoxyribonucleic acid and cell proteins. Studies have shown that the cytotoxicity might also be due to initiation of apoptosis activated by the caspase-3 enzyme [9]. [10] and the oxidative stress is one of the main mechanisms of the cytotoxic effects induced by silver NPs. both shape and size are basic characteristics that determine the toxicity of silver NPs by induce reactive oxygen species [11]. Some of Ag NPs are decomposed due to the action of lysosome causing liberation of Ag ions leading to oxidative stress in the cell . because of oxidatively properties of the reactive oxygen that formed which cause damaging to DNA and cell protein and apoptosis leading to genetic defect and even up regulation of p53 gene [10], for that reason NPs may be used in the Medical application as anticancer , as up regulation of p53 gene initiate apoptosis [12]. On our work we aimed to use a pathogenic bacteria (*Proteus vulgaris*) that from women vagina to synthesize a nanoparticles and testing its cytotoxic effect against cell line.

METHODS

Synthesis of silver nanoparticles: Silver nitrate (AgNO3, 99%) (Aldrich/Germany) was used in the preparation of the silver nanoparticles from pathogenic bacteria. Silver nanoparticles were synthesized according to a method described by [13]. Method of synthesis was done by two solutions:

-Solution (A) is prepared as follows: 0.02 gm of AgNO3 (0.1 mM) was dispersed by ultra-sonication in 20 ml deionized water (DI) for 2 minutes. "The interaction and production of nanoparticles to be done, need for reducing agent and stabilizer to prevent aggregation. Solution (B) which consists of (*Proteus vulgaris*) suspension that extracted from local isolate, acts as capping stabilizer and reducing agent. The two solutions (A and B) are mixed by magnetic stirrer and exposede to the direct sunlight for about 5 min at pH 5. The solution is converted to suspension contain silver nanoparticles, was separated and concentrated by centrifugation at 10,000 rpm for 15 min and washed twice by DI water and precipitated by centrifugation at 10,000 rpm for 15 min. The biosynthesis of silver nanoparticles by this method was optimized with different parameters such as pH, temperature, time of reaction and concentrations for AgNO3 and suspension of bacterial growth (*Proteus vulgaris*) , then dried in the oven at 60°C for 30 minutes to obtain a brownish black powder, and kept in dark vial for further characterization and applications

Characterization of AgNPs: Nanoparticle behavior, biodistribution, safety, and efficacy are all influenced by their physicochemical qualities. As aresult, AgNP characterisation to assess the functional properties of the particles created. A range of analytical techniques were used to characterize the samples, Microscope with atomic force: The SPM-:including AA300 of angstrom advanced Inc. USA was utilized to determine the surface topography of the silver nanoparticles utilizing AFM contact mode. [14]. The experiment was done in the CAC center / Baghdad.

Transmission Electron Microscopy: Transmission Electron Microscopy (TEM) analysis was carried out to investigate the size, shape, and morphology of Ag NPs, Finally, c the XRD data should be examine by looking at the interface between the polymer and the fillers. whereas the device was manufactured by Philips CM 10, Netherland [15]. The experiment was done in the CAC center / Baghdad.

The biological activity of silver nanoparticles against cell line

(The Cytotoxic Effect of Nanoparticles from *Proteus vulgaris*): In *vitro* method was performed to investigate the possible cytotoxic effect of suspension of bacterial growth (Proteus vulgaris) is isolated from wound and biosynthesized nanoparticles on two cell lines on of them is human breast cancer cell line (MCF-7) and another is a normal cell line such as human normal liver cell line (WRL 68).

Cell culture maintenance: MCF-7 and WRL 68 cells were grown in RPMI-1640 with 10% fetal bovine serum, 100 units/mL penicillin, and 100 g/mL streptomycin added. Cells were passaged twice a week with Trypsin-EDTA, reseeded at 80% confluence, and incubated at 37 degrees Celsius [16]. various concentrations The MTT cell viability assay was used in 96-well plates to examine the cytotoxic effect of suspension of bacterial growth (Proteus vulgaris), as well as the effect of a mixture of both suspension of bacterial growth and nanoparticles. MCF-7 and WRL 68 cell lines were planted at 1 104 cells per well. Cells were treated with investigated substances at varied concentrations (12.5, 25, 50, 100, 200, and 400 g/ml) after 24 hours or when a confluent monolayer was established. . After 24 hours of treatment, cell

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viability was determined by removing the medium, adding 28 liters of a 2 mg/mL MTT solution, and incubating the cells for 2.5 hours at 37 °C. After removing the MTT solution, the crystals in the wells were solubilized by adding 130 L of DMSO and incubating for 15 minutes at 37 °C with shaking , The absorbency was measured at 492 nm using a microplate reader, the experiment was carried out three times. The rate of cell growth was calculated using the following formula. [17].

Cytotoxicity = A-B/A *100

, where A and B are the control and test optical densities, respectively. 200 L of cell suspensions were planted at a density of 1x104 cells mL-1 in 96-well micro-titration plates and cultured for 48 hours at 37°C to observe cell morphology under an inverted microscope. Then the medium removed and added suspension of bacterial growth (*Proteus vulgaris*), and effect this suspension mix with nanoparticles After 24 hours, 50 ml of Crystal violet was used for staining then the plates should be introduce for incubation at 37 °C for 15 minutes, after that carefully rinsing to the stain by tap water should be done in order to get rid from the dye . The cell was viewed using a 100x magnification inverted microscope and taken with a digital camera. [18].

Statistical Analysis): Data that obtained were statistically analyzed analyzed by Graph Pad Prism 6 and an unpaired t-test. " The results were provided as the mean SEM of three independent measurements [19] .

RESULTS

UV–Visible analysis of AgNPs: because of their noble metal NPs (plasmonic) they can be distinguished from other nanoparticles such as semiconductor quantum dots, polymeric, and magnetic nanoparticles by. SPR dominates the optical absorption spectra of AgNPs, with a shift toward the brown or yellow end depending on particle size, aggregation state, shape, and the dielectric medium.. The formation of AgNPs was confirmed by changes in the solution color from colorless to yellow brown figure (1). Samples are examined by using the UV–V is spectroscopy and the application of the Nano product as an anticancer agent.

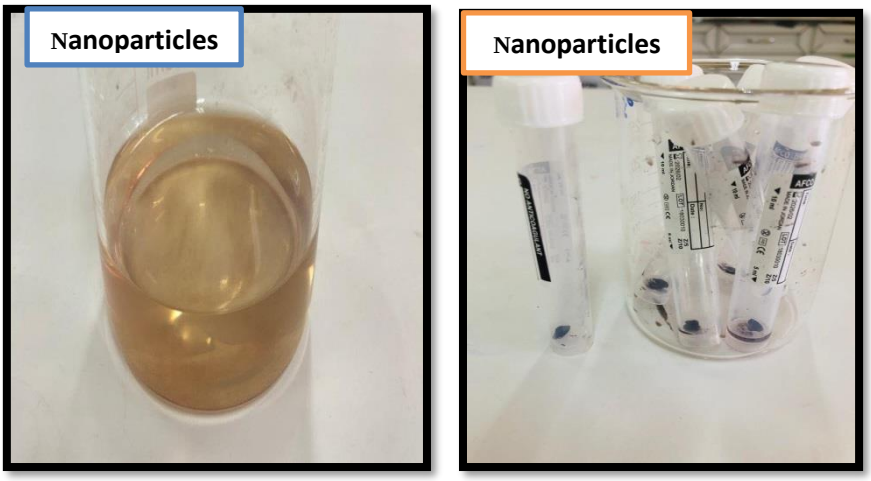

Figure (1) The steps of AgNPs formation, confirmed by changes in the solution color from colorless to yellow brown.

CHARACTERIZATION OF SILVER NANOPARTICLES

Atomic Force Microscope (AFM)

Surface analysis (AFM) requires good attention because of factors that effect on results such as pollutions. The size of silver nanoparticles was estimated by using AFM-SPM shown in table (1). The result shows that the average size of AgNPs was 54 nm figure (2).

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Table (1) Estimation size of AgNPs.

SPM Scanning Probe Microscope

Granularity Cumulation Distribution Report

Sample: AA	**Code: Sample Code**
Line No.: lineno	**Grain No.:**241
Instrument: CSPM	**Date:**2021-10-06
Avg. Diameter:54.06 nm	**<=10% Diameter:0 nm**
<=50% Diameter:51.00 nm	**<=90% Diameter:60.00 nm**

Diameter (nm)<	Volume (%)	Cumulation (%)	Diameter (nm)<	Volume (%)	Cumulation (%)	Diameter (nm)<	Volume(%)	Cumulation (%)
46.00	14.48	14.48	56.00	23.70	65.54	66.00	13.77	100.00
51.00	29.37	42.84	61.00	23.70	88.23			

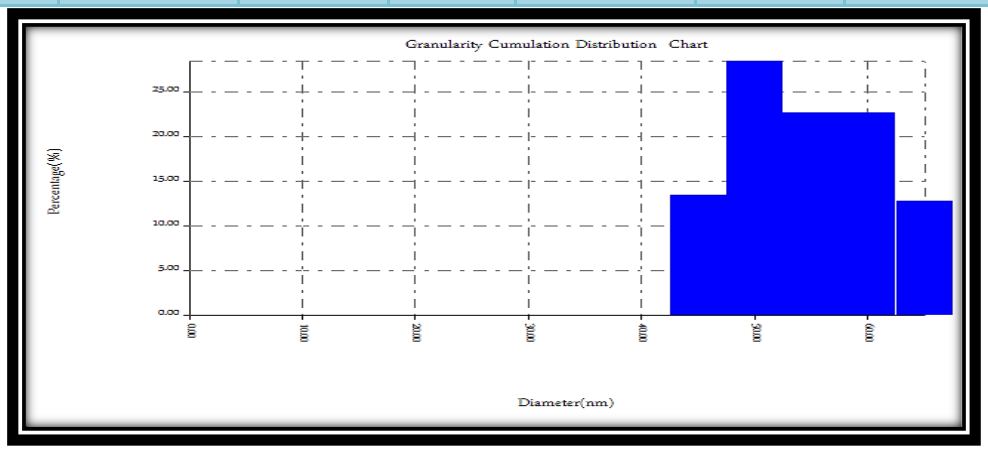

Figure (2) Average size of silver nanoparticles.

Transmission electron microscopy (TEM) analysis of silver nanoparticles (AgNPs)

TEM is a valuable tool to analyze the size and morphology of nanoparticles. TEM images of AgNPs as figure (3) showed distributed spherical shaped particles with numerous sizes ranging from 20 to 300 nm.

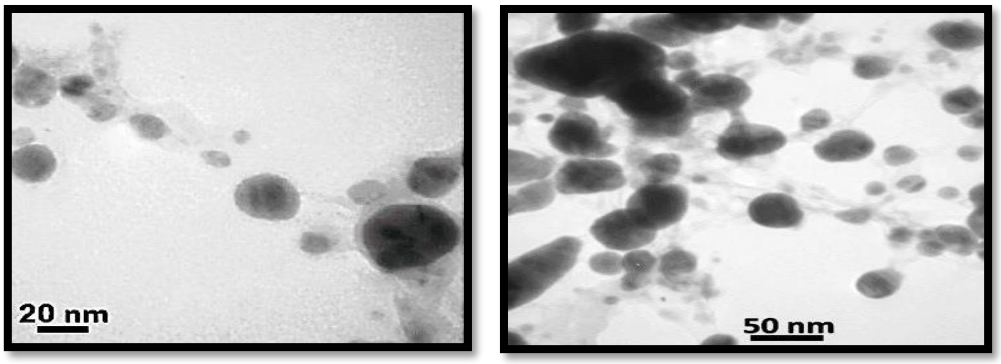

Figure (3) TEM image of silver nanoparticles prepared by pathogenic bacteria (*Proteus vulgaris*).

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The biological activity of suspension of pathogenic bacteria (*Proteus vulgaris*) against cell line.

Cytotoxic Effect of synthesized nanoparticles

Results in table (2) and figure (4) reveal that the REF cell line's growth inhibition decreased over time when suspension of bacterial growth (*Proteus vulgaris*) concentration increased. suspension of bacterial growth (*Proteus vulgaris*) has considerable cytotoxic impact variations on MCF-7 cell line (P≤0.05), 36.68, 57.675 and 64.597 % suppression of growth was showed at different concentratoions 100 , 200, & 400 µg/ml respectively in nanoparticles produced from pathogenic bacteria (*Proteus vulgaris*).

Table (2) The effect of different concentration of nanoparticles produced from *Proteus vulgaris* on cell inhibition of MCF – 7 cell line.

Concentration of nanoparticles µg/ml	Cell viability (%) by MCF-7 Mean ± SD	No. of dead cells (%)	Cell viability (%) by WRL68 Mean ± SD	No. of dead cells (%)
12.5	95.175 ± 1.260	4.825	94.948 ±1.027	5.052
25	94.618 ± 0.710	5.382	95.213 ± 0.621	4.787
50	86.502 ± 3.182	13.498	95.322 ± 1.173	4.678
100	63.320 ± 3.131	36.68	91.337 ± 1.513	8.663
200	42.325 ± 5.155	57.675	84.645 ± 2.732	15.355
400	35.403 ± 3.123	64.597	79.186 ± 2.124	20.814

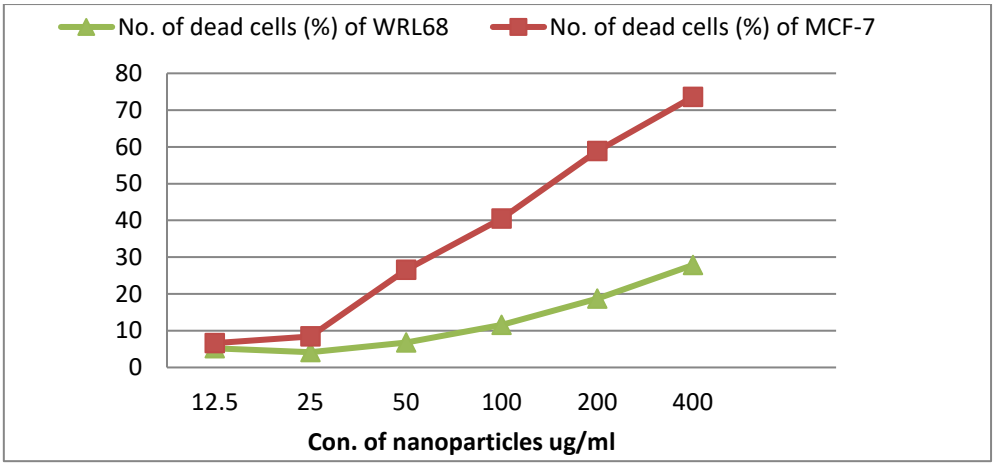

Figure (4) Cytotoxic effect of different concentrations of Nanoparticles produced from *Proteus vulgaris* on MCF-7 cell line after 24 hr. compared with the control (WRL68).

Cytotoxic Effect of pathogenic bacteria

A cytotoxic effect was found to be significant (P< 0.05).was observed on the growth of MCF-7 at the concentrations of the cell line of 100, 200 & 400 µg/ml having a proportion of growth inhibition 23.929%, 40.761% and 58.672 % respectively, as shown in the table (3) and figure (5).

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Table (3) The effect of different concentration of growth of *Proteus vulgaris* on cell inhibition of MCF – 7 cell line.

Concentration of growth of *Proteus vulgaris* µg/ml	Cell viability (%) by MCF-7 Mean ± SD	No. of dead cells (%)	Cell viability (%) by WRL68 Mean ± SD	No. of dead cells (%)
12.5	93.587± 0.836	6.413	94.817 ± 2.189	5.183
25	94.588 ± 2.156	5.412	96.732 ± 1.132	3.268
50	87.821 ± 2.272	12.179	96.190 ± 1.242	3.81
100	76.071 ± 1.619	23.929	91.120 ± 1.427	8.88
200	59.239 ± 4.323	40.761	85.242 ± 0.898	14.758
400	41.328 ± 1.685	58.672	81.088 ± 2.217	18.912

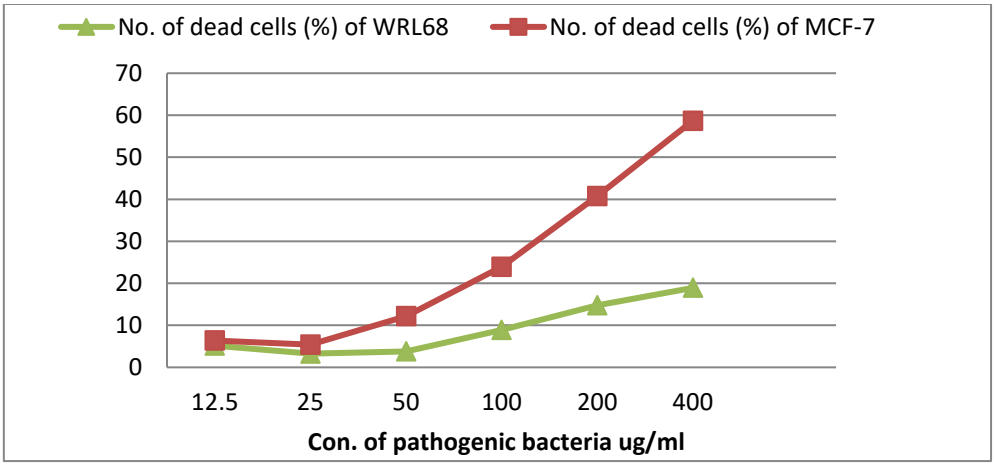

Figure (5) Cytotoxic effect of different concentrations of pathogenic bacteria (*Proteus vulgaris*) on MCF-7 cell line after 24 hr. compared with the control (WRL68).

Combine Cytotoxic Effect of nanoparticles with suspension of pathogenic bacteria

MCF-7 cell line treated with suspension of bacterial growth (*Proteus vulgaris*) mix with nanoparticles at the same concentrations of 100, 200, and 400 µg/ml and indicated a proportion of growth inhibition of 40.484, 58.859 and 73.553% respectively when used Nano particles with pathogenic bacteria (*Proteus vulgaris*), as show in figure (6) and table (4). Growth inhibition of MCF-7 cell line was increased gradually with the increase of with suspension of pathogenic bacteria (*Proteus vulgaris*) mix with nanoparticles concentration and when compared to the control, there was a significant cytotoxic effect (P 0.05) between the concentrations (WRL68).

Table (4) The effect of different concentration of AgNPs mix with same concentration of suspension of pathogenic bacteria (*Proteus vulgaris*) on cell inhibition of MCF – 7 cell line.

Concentration of AgNPs mix with suspension bacteria µg/ml	Cell viability (%) by MCF-7 Mean ± SD	No. of dead cells (%)	Cell viability (%) by WRL68 Mean ± SD	No. of dead cells (%)
12.5	93.380 ± 1.863	6.62	94.817± 2.177	5.183
25	91.588 ± 2.166	8.412	95.852 ± 1.192	4.148
50	73.445 ± 1.573	26.555	93.190 ± 1.152	6.81
100	59.516 ± 4.824	40.484	88.473 ± 2.331	11.527
200	41.141 ± 4.492	58.859	81.257± 0.580	18.743
400	26.447 ± 4.634	73.553	72.163 ± 2.178	27.837

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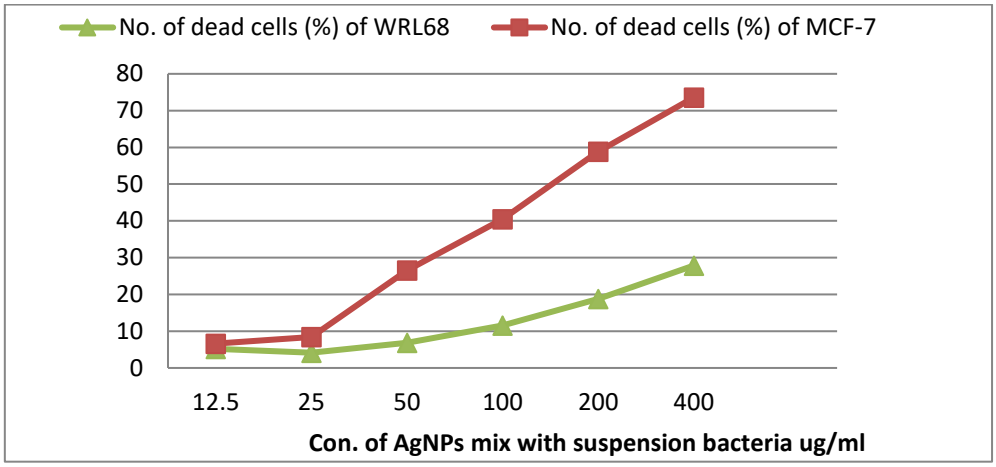

Figure (6) Cytotoxic effect of different concentrations of AgNPs mix with suspension bacteria *(Proteus vulgaris)* on MCF-7 cell line after 24 hr. compared with the control (WRL68).

DISCUSSION

The present study demonstrated that the viability of cells decreased at high concentrations of nano silver, stated that Nano-Ag exhibited lower cytotoxicity toward normal cells (M-Stem cell and human fibroblasts (HF2), also, [20,21] study which evaluated the toxicity of AgNPs on human hepatocytes found that nano silver does not has toxicity at low concentrations, but it has toxic effects on human hepatocytes at high concentrations. Also another study performed by [22], found that viability of human gingival epithelial cells was significantly decreased at high concentrations of AgNPs under in-vitro conditions. Different toxic concentrations reported by previous studies may be due to different preparation method of silver nanoparticles or size of the particles. A direct prediction and comparison in activity (on bacteria) and toxicity (versus human cell lines) is possible [23].

Clarified that silver with a lattice parameter [24] of a = 4.0862 Å were in good agreement with reference of the face-centered cubic (fcc) crystal lattice of metallic silver, the size of the AgNPs calculated by the Debye-Scherrer equation ($D = 0.94\lambda/d \cos\theta$), and concluded the size of the AgNPs was 38 nm.

To obtain three dimensional feature Atomic force microscope (AFM) was done for measuring t surface morphology the and for topography determination [25].

(AFM) used to measure the average of particle diameter was calculated in nanoscale size.

Some studies using TEM images Showed size of 30-60 nm in a polymeric spherical shaped shaped NP that obtained from Cs-HK fungal cultures, Streptomyces spp, MBR-91 and *B. subtilis* MSBN17 [26]. Ag NPs AgNPs with nanoscale of between 3O-5O nm was considerable reported for b bacterio cidal activity against pathogens [27]. And the maximum Polymeric NPs in nanoscale between 20-300 nm potential for –in vivo application [28].

New studies revealed to a that the unfamiliar method in the therapeutic application can be manufactured [27] , depend on the properties of biosurfactant which cause fluidize in the cancerous tissue by ultering its lipid componant can alter lipid content to fluidize rigid cancerous tissues and to modulate interfacial properties. While [29] found that the biosurfactant's potential to rupture cell membranes, resulting in decomposition, enhanced membrane permeability, and metabolite leakage, has also been suggested as a possible mechanism of anticancer action. [30] facts reveals that the action of surfactant cause liberation of reactive oxygen which lead to which activate apoptosis process and cell death. Because of the effect of apoptosis that cause low level of fatty acid due to obvious decrease of unsaturated fatty acid in Bcap-37 cell., enhancing the fluidity of membrane [31]. The inhibitory activity against pathogenic bacteria was used to investigate the antimicrobial effects of biosrfactant generated by chosen islate (*L. plantarum*) ADK2. According to Figure 21, biosurfactant has an inhibitory impact on harmful bacteria such as *S. aureus* and *P. aeruginosa*. Supernatant from ADK2 isolate demonstrated a high inhibitory effect against *S. aureus* and *P. aeruginosa,* with 34.18 and 38.43 mm inhibitory effect, respectively. [32].

Ag NPs concentration that cause inhibition in the growth of bacteria and does not lead to the body

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cells defect cells was(100 µg /ml [33]., The MCF-7 cause inhibition to the growth of cell line which was increased proportionally with the bio surfactants that add to NPs concentration with a considerable cytotoxic result (P≤ 0.05) within the concentrations that compare to the control (WRL68) [36].

CONCLUSION

Biosynthesis of AgNPs using produced pathogenic bacteria (*Proteus vulgaris*) is efficient to convert $AgNO_3$ to spherical shaped particls with numerous size ranging 20 – 100 nm. The result of Cytotoxic effect showed that the potent effect was seen obviously in MCF-7 cell line than WRL68 when used suspension of *Proteus vulgaris* and / or AgNPs.

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