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Cultured Cell Lines Influenced by the Sizes and Concentrations of the Cytotoxic Gold Nanoparticles.

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ABSTRACT

Optical nanoparticles have the potential to provide new tool for diagnosis and treatment of human diseases. Gold nanospheres of different concentrations and particle sizes were prepared by using laser ablation technique. The particles were applied on two types of cultured cell line (*Human cervical cancer Hela cells* and *Human B cells Lymphoma*) to estimate the cytotoxic effect of the prepared gold nanoparticles on these cancer cells by performing nanotoxicity test. The inhibition rate was investigated through micro culture tetrazolium test, by using methyl thiazolyl tetrazolium (MTT) solution, where added to cancer cells after exposure to GNPs. It was found that NPs' toxicity was highly correlated to different factors related to the gold nanoparticles (size, concentration), and type of cell line.

Keywords: laser ablation, gold nanoparticles, Human cervical cancer, Human Lymphoma.

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INTRODUCTION

Nanomaterials display unique, distinct characteristics that are unavailable in conventional macroscopic materials. Their uniqueness arises specifically from higher surface to volume ratio. They represent an important class of materials in the development of novel devices that can be used in various physical, biological and biomedical aspects [1]. Nanoparticles have the ability to carry and bind compounds like small molecule drugs, proteins, DNA, RNA, and probes with high efficiency. They have high carrier capacity, high stability, and compatibility with different administration routes because of their shape, tunable size, and surface characteristics, thereby making them highly attractive in many aspects of oncology. Also in various applications such as: photo-thermal therapy, carrier systems for drug delivery, surface-enhanced Raman spectroscopy, imaging [2], diabetic healing [3] and antibacterial agent [1].

In order to obtain noble metal nanoparticles, laser ablation synthesis in solution emerged as a reliable alternative to the traditional chemical reduction methods in the past years. Laser ablation method gives the possibility to produce nanoparticles in a controllable, contamination-free environment, with direct formation of the colloidal solution[4].

MATERIAL AND METHODS

Pulsed Nd:YAG laser was used to synthesize GNPs through ablation of a piece of gold metal plate (with purity of99.99%) placed on the bottom of Pyrex tube containing 3.6 ml of deionized water. The spot size of the laser beam on the surface of the metal plate was 1 mm in diameter, this size was obtained by changing the distance between the focusing lens and the metal plate. The ablation was performed by using a focused beam output of pulsed laser at 1064 nm and different laser energies (500,600, and 1000) mJ respectively, with a repetition rate of 6 Hz per second and pulse width of 10 ns.

Absorption spectra of colloidal gold nanoparticles was measured at room-temperature by uv-visible recording spectrophotometer instrument shimadzu uv-160a model for a range of (300-800) nm.

A few drops of each samples were dried on slides at room temperature, then the Atomic force microscopy (model A3000 advanced angestrum inc., (USA)) was used to obtain the average size, surface roughness and the distribution of nanoparticles.

Atomic absorption spectrophotometer measurement was carried out for the prepared samples using AAS model novAA 350, Germany to determine the concentration of the GNPs samples.

Human cervical cancer (Hela) cells and Human B cells Lymphoma (SR) were cultured in RPMI 1640 medium supplemented with 15%Fetal Calf Serum FCS (Sigma), the cell lines were incubated in tissue culture flasks at 37°C, 5% CO_2 for 24 h in a humidified atmosphere.

Cell lines were sub cultured when monolayer was confluent. The growth medium was decanted and the cell sheet washed once with 2mL trypsin-versene solution. Two to three mL of trypsin-versene were added to the cell sheet then the flask rocked gently, part of it will decanted again to obtain about one milliliter of trypsin-versene solution covering cell surface then the cells incubated at 37ºC until they had detached from the inner wall of the flask for 1-2 min. Growth medium were added to the flask and re-incubated at 37ºC. A microtiter plates with 96 wells were seeded with the cell suspension, about 10^4-10^5 cells/200µL/well are covered with plate lid and sealed with adhesive parafilm then the plate gently shocked and returned to the incubator, 37˚c until the cells reached full growth for 24-48 h (i.e., vary according to the type of cell line).

When the cells are in full of its activity after 24 h(depending on the growth curve of each cell lines), were exposed to six concentrations of each GNPs extract (25, 5, 1, 0.1, 0.01, and 0.001) µg/mL were prepared for SR and HeLa cells. 200µL of each concentration were pipetted into each well (three replicate for each extract tested concentration) while 200µL of maintenance medium were added to each well of control group. The plates were sealed with adhesive parafilm then returned to the incubator at 37ºC, 5% CO_2in humidified atmosphere. The photo pictures were taken after 24 h, while the evaluation of cytotoxicity was carried out after exposure of the cells to extracts for 48 h.

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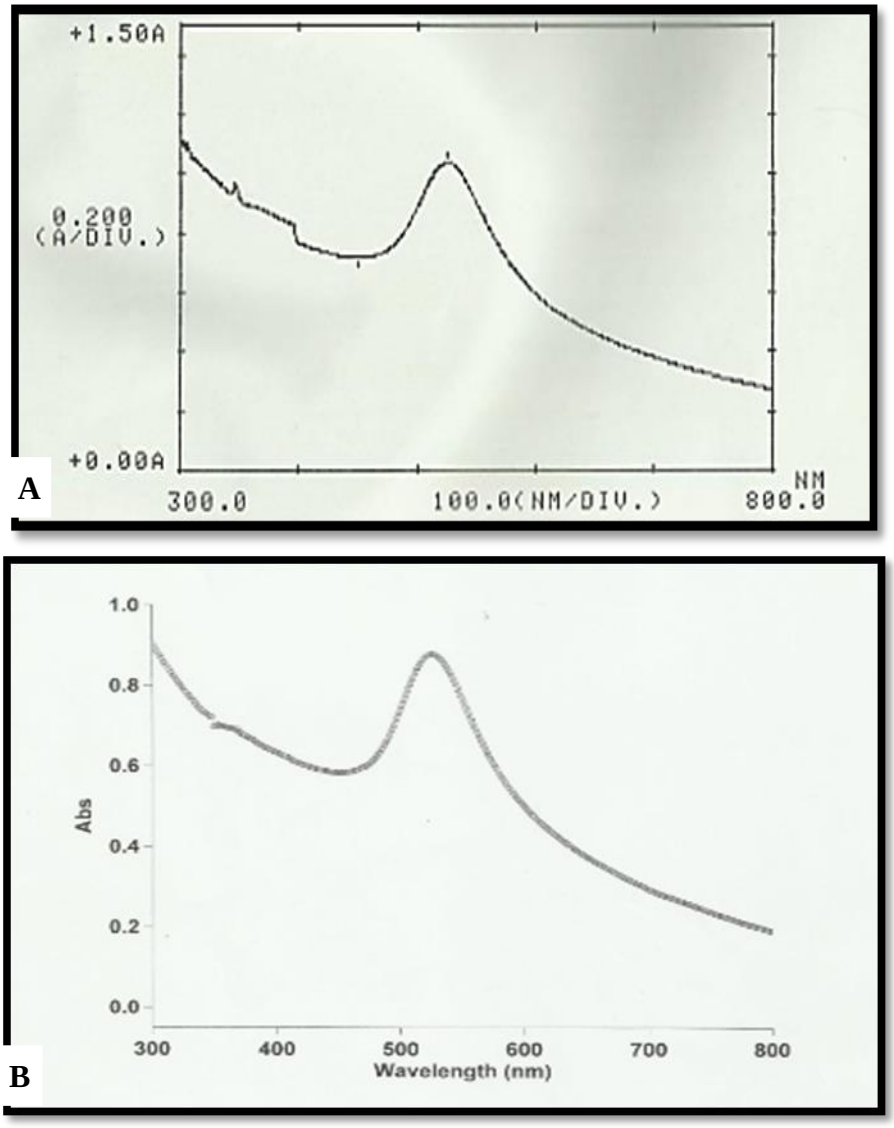
Cell viability which reflects the efficiency of eliminating cancer cells by GNPs, was measured after 48 h of exposure by removing the medium, adding 2 µl of 5 mg/ml solution of MTT and incubating for 4 h at 37°C. After removing the MTT solution, the crystals remaining in the wells were solubilized by the addition of 200 µL of Dimethyl Sulphoxide (DMSO) followed by incubation in 37°C for 15 min with shaking. The absorbance was determined by the microplate reader at wavelength of 620 nm. The inhibiting rate of cell growth was calculated according to Betancur- Galvis *et al*., in 1999 [5] and Gao *et al*., in 2003 [6] as follows:

$$\text{Inhibition rate} = \frac{mean\ of\ control - mean\ of\ treatment}{mean\ of\ control} \times 100$$

RESULTS AND DISCUSSION OF GOLD NANOPARTICLES

An important parameter that effects on the formation of gold nanoparticles is the laser energy. As shown in Fig. 1(A, B, and C)the UV spectra of Au samples was prepared at different laser ablation energies. Laser ablation listed for 3 min and 30 sec and the solution gradually became colored with the increase of the number of laser pulses. The solution was colored pink in small size and changed to faint purple at larger size particles.



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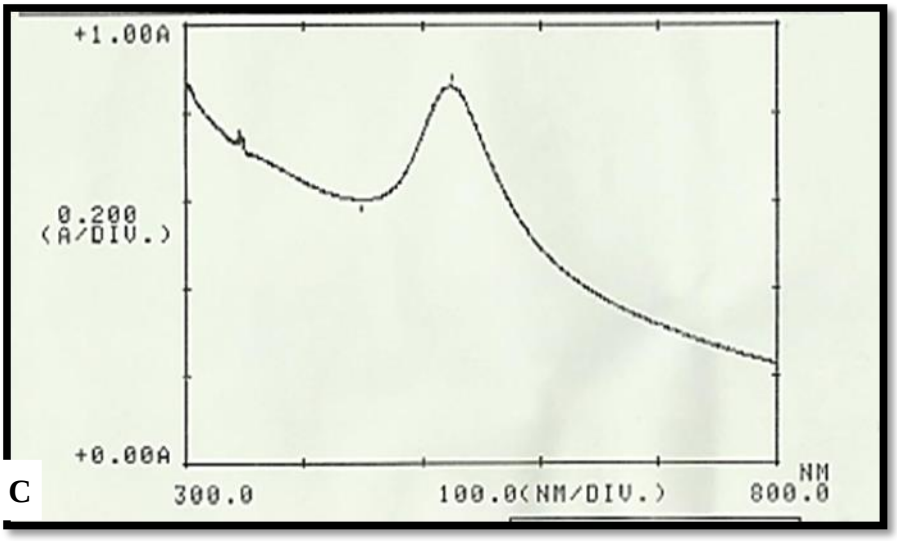

Figure 1: Absorption spectra of the colloidal gold nanoparticles at laser energy (A) 1000mJ (B) 600mJ and (C) 500mJ.

The concentration of metals nanoparticles formed in solution during the ablation process were increased. The height and the width of the absorption spectra peaks were found also to be dependent upon the laser energy.

The peak intensity was around 523-525 nm that indicates the production of gold nanoparticles.

The AFM pictures and size distributions of gold nanoparticles, produced by laser ablation of metal plates immersed in DIW as shown in figure 2 (A, B, and C). The produced nanoparticles have the average diameters of 29, 34 and 58 nm at the laser energies of 1000, 600, and 500 mJ respectively. That means the size of GNPs is inversely proportional to laser energies and this is in agreement with Imam et. al. in 2012, they reported that the decrease in the average size of gold nanoparticles can be attributed to a large energy which excited the gold nanoparticles in a solution, the photon energy is readily converted to the internal modes of the nanoparticles as during a single laser pulse, the formed gold nanoparticle absorb consecutively more than one thousand photon and it's temperature rises significantly so that the nanoparticle starts to fragment. After the single laser pulseis diffused into the solution, the temperature of nanoparticles returns to room temperature before the next one is diffused. The heating and cooling of nanoparticles occur in every laser pulse [7].

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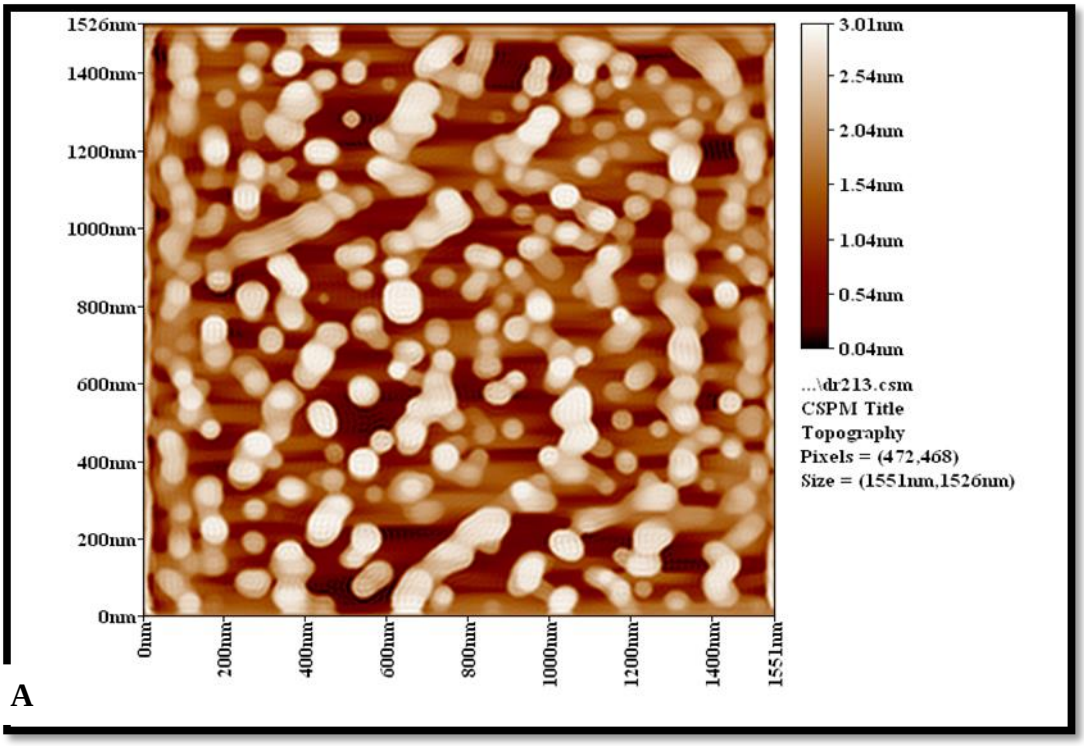
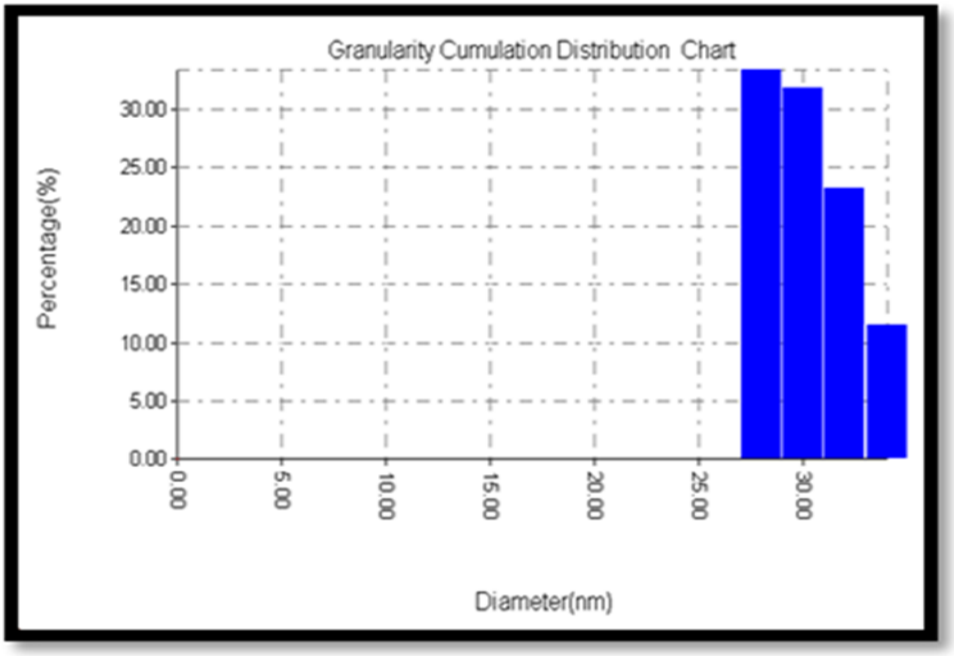


A



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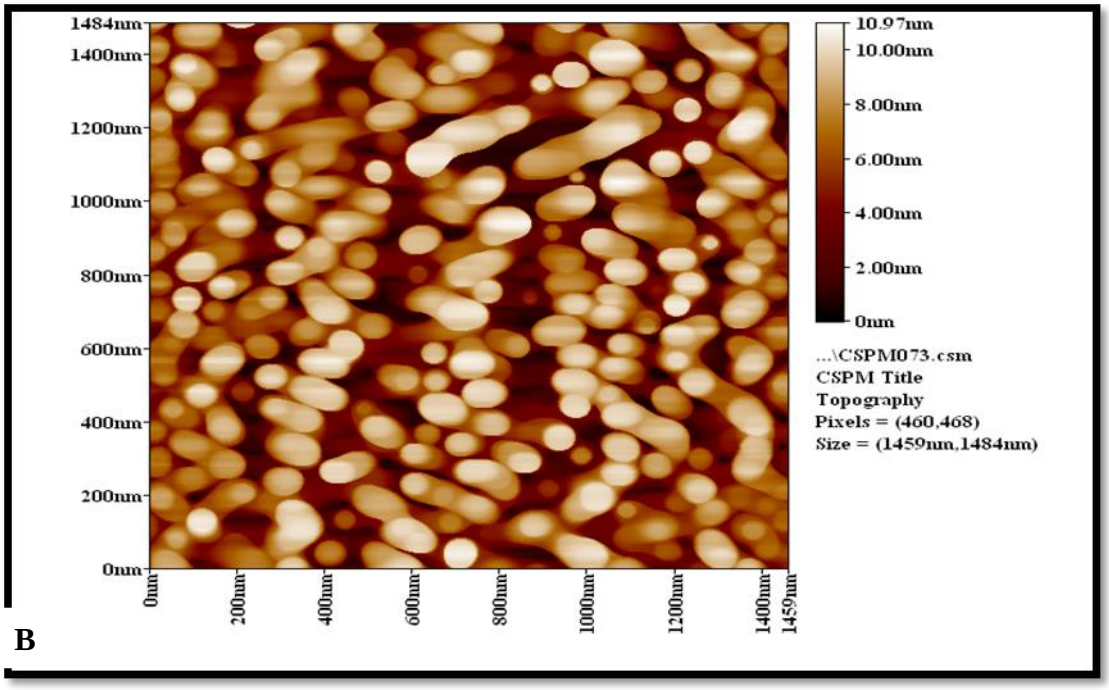

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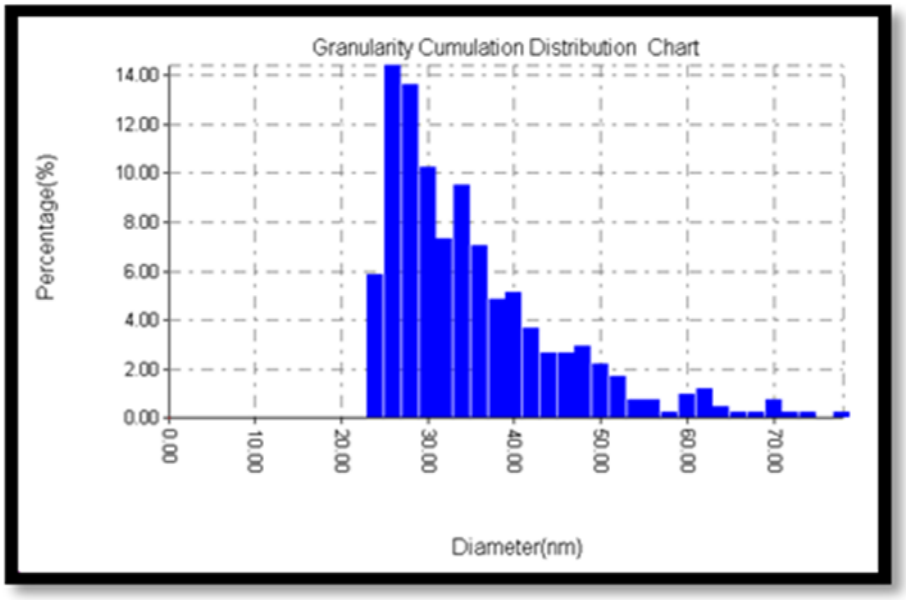

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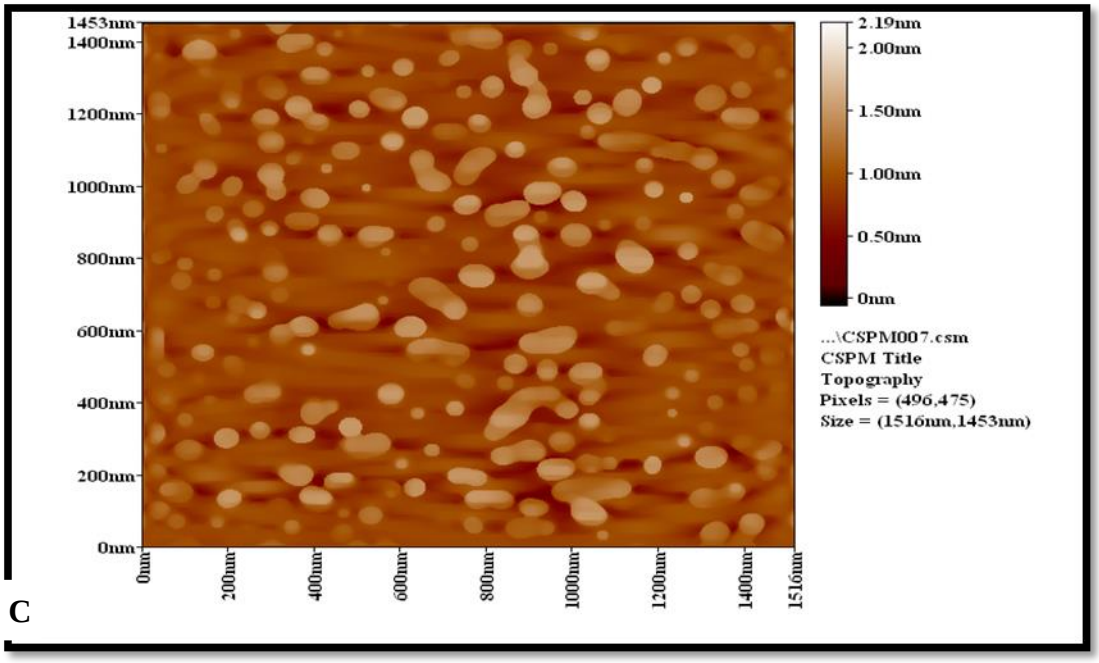

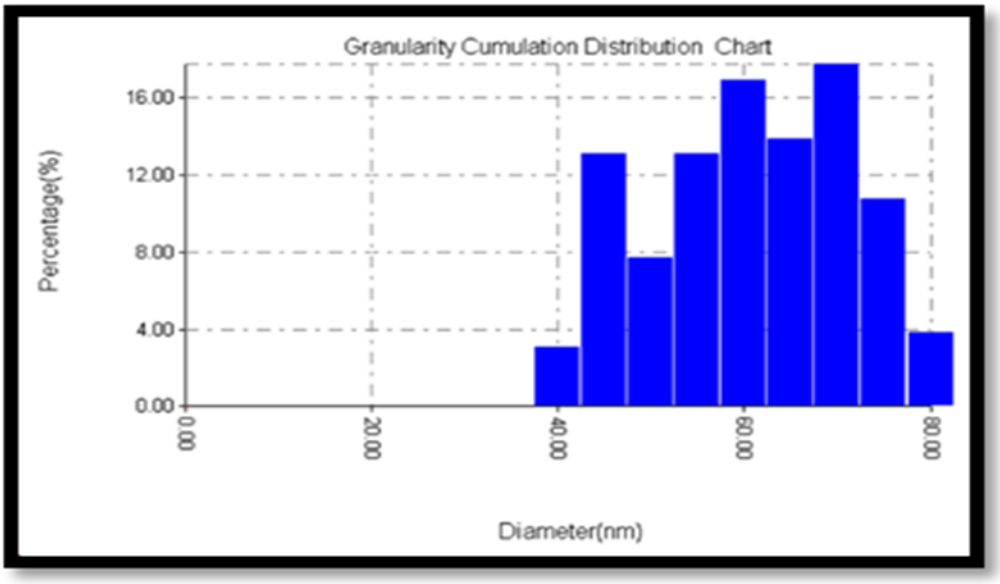

Figure 2: Shows AFM images and its size distributions of the gold nanoparticles depending on the laser energies; 1000 mJ (A), 600 mJ (B), and 500 mJ (C) with (λ=1064 nm, P.R.R. =6 Hz).

Also Sasohet. al. in 2005 and Nichols et. al. in 2006 reported that: the difference in size distributions of the gold nanoparticles can be attributed to:

1) Target surface vaporization;
2) Explosive ejection of molten droplets directly from the target. And this leads to abroad size distribution.

The explosive ejection occurs when the temperature approaches the thermodynamics critical temperature, thermal fluctuation is amplified and the rate of homogenous bubble nucleation rises dramatically and the target makes rapid transition from superheated liquid to a mixture of vapor and equilibrium liquid droplet. At this fluence, the momentum of a plume allows it to expand further out into the liquid, increasing the plasma life time and this results in an increase of screening of laser light from surface of bulk Gold target [8, 9].

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EFFECT OF GOLD NANOPARTICLES ON THE VIABILITY OF SR AND HELA CELL LINES:

The effect of different GNPs concentrations on the HeLa and SR cells proliferation was mentioned as shown in Fig. 3 and table 1. The two cell lines were treated with (25, 5, 1, 0.1, 0.01 and 0.001) µM of 58 nm gold nanoparticles for 48 h.

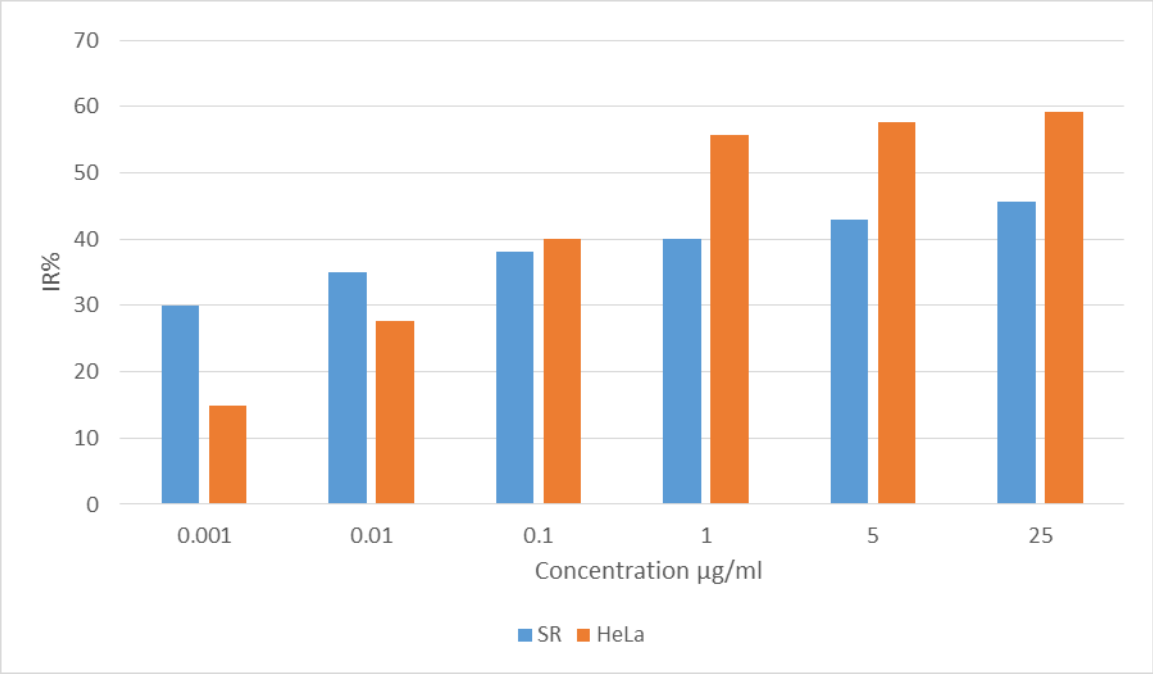

Figure 3:Effect of different concentrations of gold NPs on cell viability in SR and Hela cells. The data plotted are mean, n = 3. *p < 0.05 vs. control group.

Table (1): Mean values of inhibition rate percentage (IR %) for SR and HeLa cell lines by aqueous solution of GNPs after 48 h of exposure.

Con. µg/mL	GNPs of 58.49nm	
	IR% for SR cell line	IR% for HeLa cell line
25	45.6±0.924a	59.2±0.462a
5	43.0±2.078ab	57.6±0.462a
1	40.0±0.924abc	55.6±1.155b
0.1	38.0±1.617bc	40.0±0.462c
0.01	35.0±3.695cd	27.6±0.231d
0.001	30.0±0.924d	14.8±0.231e

Note: Data are expressed as mean ± S.E.; values with different small letters superscript (a, b, c, and d) on the same column differ significantly (P≤ 0.05).

The GNSs with 58 nm showed significant (p≤0.05) reduction of HeLa cells proliferation, the IR of cell growthwas14.8% in 0.001µM, 55.6% in 1 µM and 59.2%in 25µM comparison with the control group. However, the significant (p≤0.05) reduction of cell survival rate in SR cell culture, the IR of cell growth was 30 % in 0.001 µM, 40 % in 1 µM and 45.6% in 25 µM in comparison with the control group. The inhibition rate increased as the concentration of GNPs was increased. This is possible interpolated as the presence of abundant GNPs in the solution, receptors will be able to receive nanoparticles faster and easier, leading to shorter wrapping times and higher uptake by the cell. Hence, higher uptake of gold nanoparticles caused high inhibition rate at the same particle size. And this is in agreement with Trono JD*et al.* in 2011they demonstrated that the fewer gold nanoparticles are in the solution, the lesser chance for a receptor to receive gold nanoparticle; membrane wrapping time is longer, resulting to lower uptake by the cell and less chance for inhibition. Also, at lower

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concentrations, increasing the incubation time will not increase the gold nanoparticle uptake by the cell because of fewer nanoparticles reaching the receptors [10].

Microscopically the morphology of incubated cells with nanoparticles for 24 h revealed cellular death due to apoptotic mechanisms which is evident by changes in membrane integrity, inhibition of cell growth and cell shrinkage as shown in fig. (4). Shrinkage cell and cell debris were observed in *SR cells* treated with 25, 1, and 0.001µM GNSs, which cellular morphology was greatly different from the control sample.

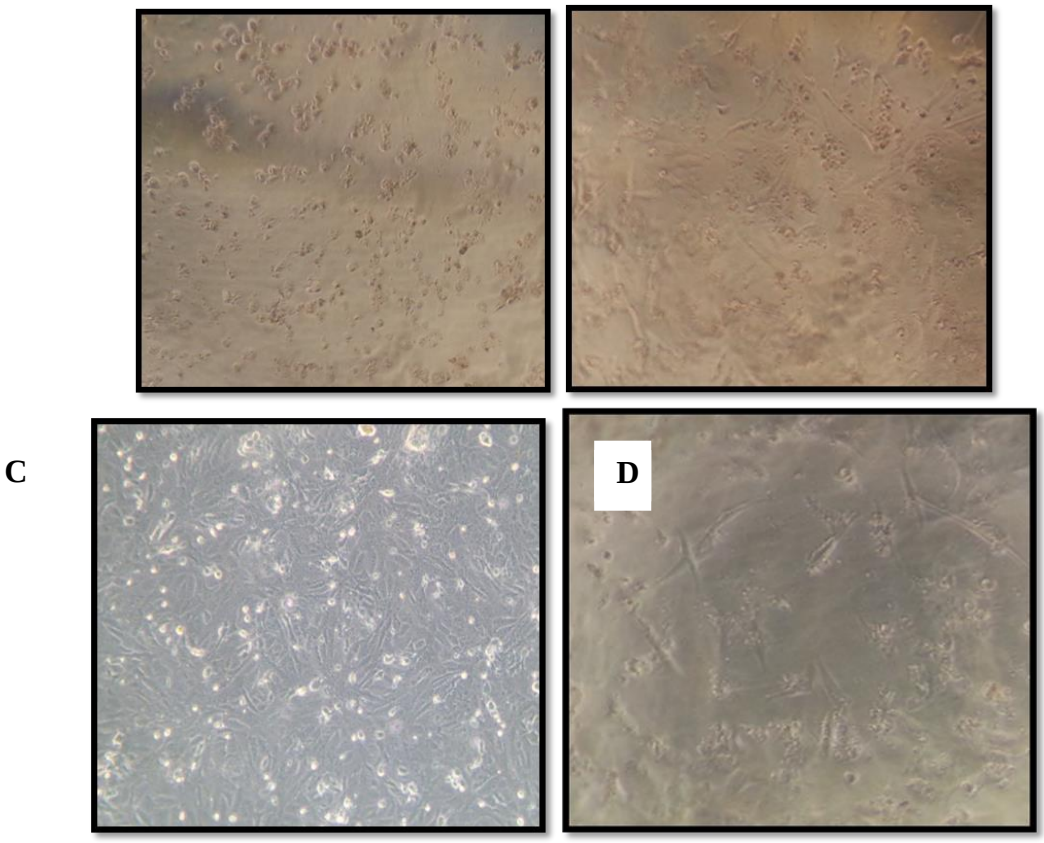

Figure 4: Effects of gold NPs on SR cells morphology after incubation for 24 h at 37ºC, with different concentrations of the same size (58.49nm). The cell viability of control cells were set arbitrarily to 100 %. (A) Control (B) 25µg/ml (C) 1 µg/ml (D) 0.001 µg/ml.

Cells were treated with 0.001 µM gold nanoparticles of various sizes for 48 hours. The inhibition rate versus different sizes of the nanoparticles as shown in Fig.5 which manifest the cytotoxic effect is highly dependent on size. The gold nanoparticles of 29 and 34 nm size have significantly lower cytotoxic effect on *HeLa cell line* growth in comparison to 58 nm, this is in agreement with Chithrani et al in 2006 whom reported that 50 nm has the highest uptake [11].It was shown that 29 nm GNSs did not influence cell growth in *HeLa cell line*, however, 58 nm GNSs showed the reduction of *HeLa cells* proliferation ratio by 14.8% and 2% in 34 nm in comparison with the control group. While SR cells showed more cytotoxic effect of particles' sizes (29, 34, and 58)nm which reflects the elevation of inhibition rate of cell growth respectively, this agrees with Albanese et. al. in 2012 whom reported that the cell type is one of the determining factor in the bio-nano interaction [12].

As well as, there is a relation between the particle size and the cell uptake efficiency. Factors such as ratio of adhesion and membrane stretching, the membrane's bending energy, may also affect size selectivity and this agree with Gao *et al*. in 2005 suggested that the wrapping time is dependent on particle size. Wrapping time describes how a membrane encloses a particle. For smaller particles, the receptors need to be filled-up before the nanoparticles are wrapped by the cell membrane and uptaken by the cell. If the receptors are empty, the signal for the cell to wrap around the nanoparticles is delayed, leading to longer wrapping time and hence to lower uptake. While larger nanoparticles, the uptake requires more receptors before the cell membrane can wrap itself around it, leading also to longer wrapping time, hence lower uptake[13].

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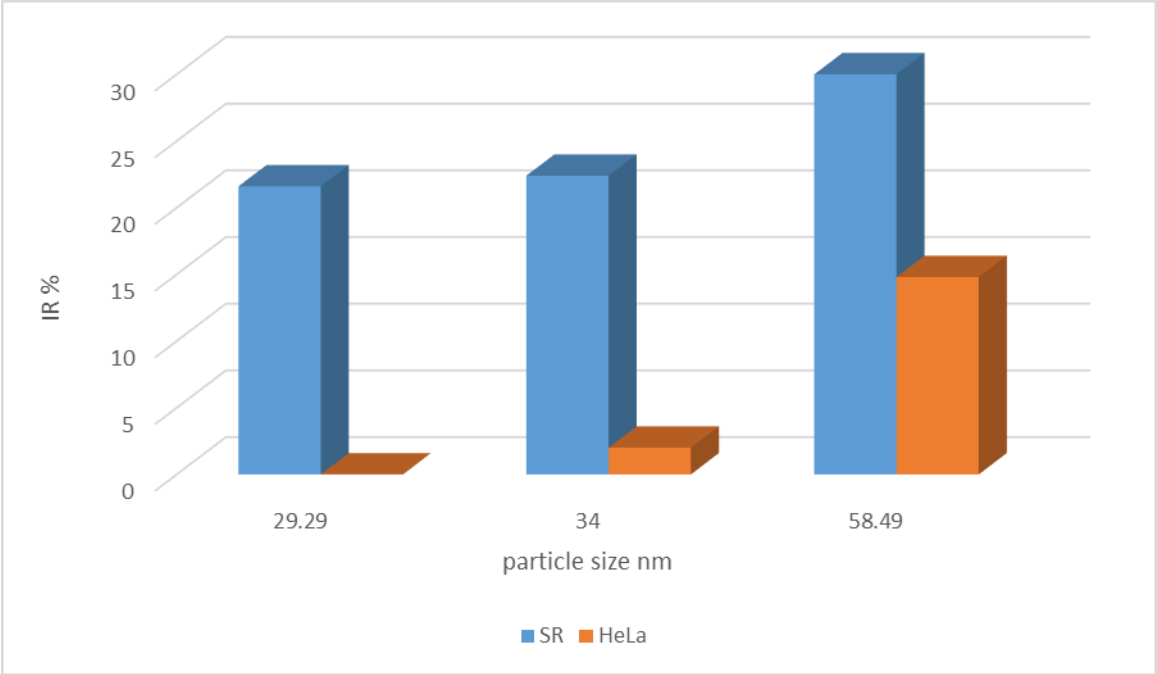

Figure 5:Effects of gold NPs on cell viability in SR and Hela cells. At 48 h-post treatment (exposure) with the same concentration of GNPs (0.001µm) and different particles' sizes (29.29, 34 and 58.49) nm.

In figure 5 noticed the cell viability was decreased when the particle sizes were increased which it is maybe related to surface charge regarding to the concentrations. Since the cell intake the large particle size more than the smaller one the toxicity of GNSs were increased by the size-dependent manner and this is in agreement with Zeng et al. in 2014and Tan et al. in 2010 they demonstrated that cellular endocytosis, cellular delivery, and subcellular targeting inducing nanotoxicity of a nanoparticle through a change in the particle's size, shape and/or surface functional group may lead to significant alteration in cellular interaction or a larger particle size and a higher surface charge would often induce high nonspecific cellular uptake of NPs [14 and 15], this is confirmed by Alkilany et. al.in 2009 and Hauck et. al. in 2008 whom reported that the larger particles were more toxic in compression with the smaller particles where they were in the same concentration, with the surface charge play a significant role in the process of cellular uptake[16and 17]. Also, Zeng et al. in 2014 treated HeLa cell line with GNPs, they demonstrated that the larger size and a higher surface charge NPs will cause a relative obvious interaction with the cell line and lead to a serious cell toxicity [14].

Microscopically the morphology of incubated cells (HeLa or SR) with different nanoparticles' sizes for 24 h revealed enhancing in cellular death due to apoptotic mechanisms which is evident by changes in membrane integrity, inhibition of cell growth and cell shrinkage in comparison to the control group as manifested in fig. 6, for HeLa cell line.

Also our results are in agreement with Kodiha et al. in 2014 whom reported that the size of gold nanoparticles can determine the severity of cellular damage and changed multiple nuclear parameters. GNPs redistributed stress-sensitive regulators of nuclear biology, altered the nuclear morphology, reorganized nuclear laminae and envelopes, and inhibited nucleolar functions. In particular, gold nanoparticles reduced the de novo biosynthesis of RNA in nucleoli. The small gold nanospheres, but not big gold nanospheres depends on the cell type, damaged the nucleus at normal growth temperature. Taken together, the toxicity of gold nanoparticles correlated with changes in nuclear organization and function. These results emphasize that the cell nucleus is a prominent target for gold nanoparticles of different morphologies[18]. The growth and proliferation of cancer cells is intimately connected to protein synthesis and thus relies on the ability of cells to produce ribosomes. Nucleoli are specialized compartments in the nucleus that transcribe ribosomal RNA genes and assemble ribosomal subunits [19].

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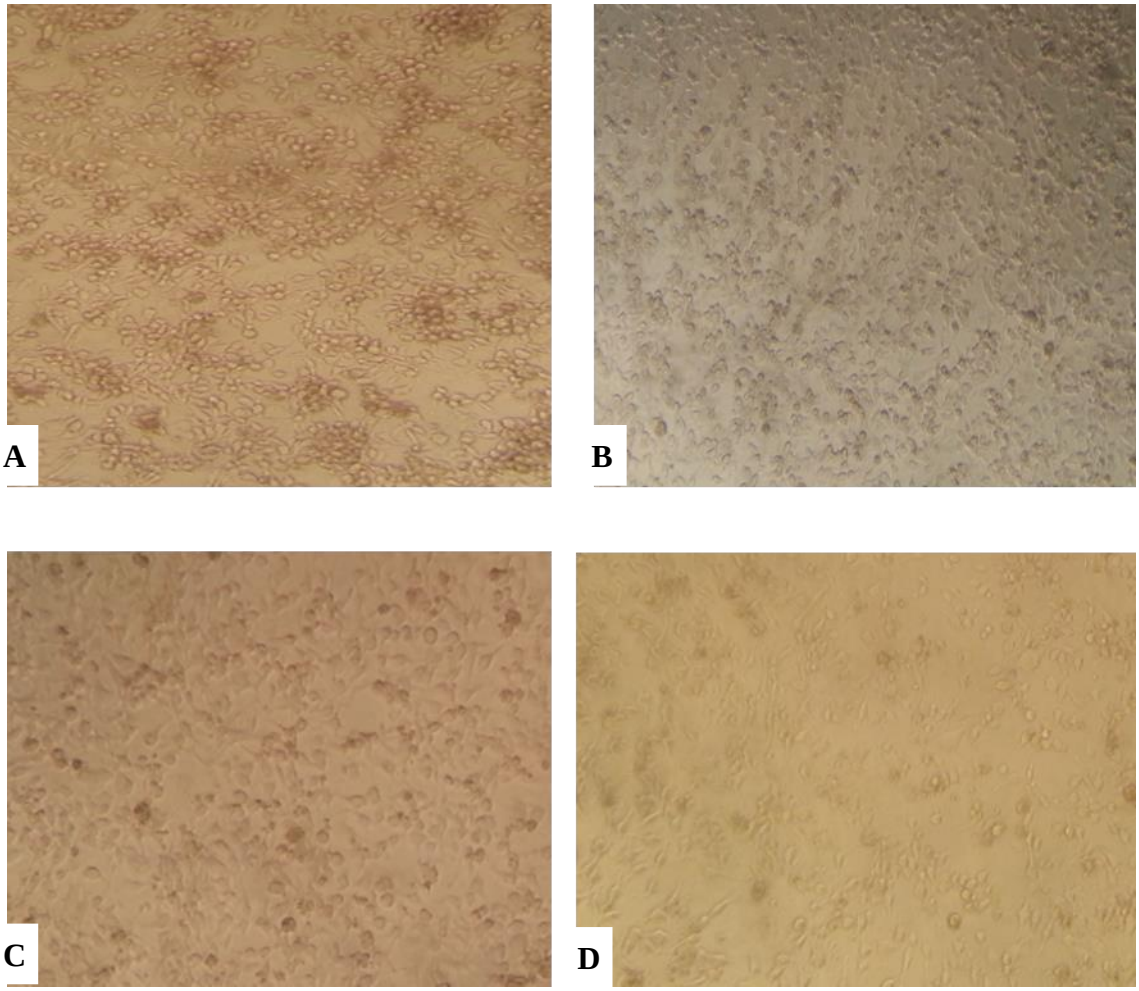

Figure 6: Effect of gold NPs on HeLa cells morphology at 0.001 µg/ml and 24h post treatment with different particles' sizes. The cell viability of control cells were set arbitrarily to 100 %. (A) Control, (B) 29 nm, (C) 34 nm, and (D) 58 nm.

According to Ashokkumar et al.in 2014 gold nanoparticles arrest all stages of the cell cycle and found to damage the DNA due to the Excessive reactive oxygen species (ROS) generation may play a dominant role in the newly synthesized gold nanoparticles induced cancer cells' apoptosis [20].

Furthermore, other studies reported that the production of intracellular reactive oxygen species (ROS), increased expression of cleaved caspase proteins and p53 activation were commonly involved in the mechanisms of apoptosis induced by NPs as demonstrated in [21, 22, 23].

However, the initiators and signaling pathways of apoptosis are different, which is depending on the special natures of NPs. In 2011Gao et al. reported that gold NPs caused hydrogen oxygen accumulation by cytosolic glutathione (GSH) depletion and subsequently activated mitochondrial apoptosis pathway [24]. While Kang et al. in 2010 stated that gold NPs localizing in cell nuclei would induce DNA damage and cytokinesis arrest [25].

CONCLUSION

Gold nanoparticles were synthesized with easier and cheaper method, with different energies in order to produce different sizes to evaluate their cytotoxic effect on the cultured cancer cells (HeLa and SR). As well as, determination of the appropriate size and concentration of GNPs making it promising agent for cancer therapy. Different cell lines show different responses toward GNPs regarding concentration and size, in SR cell

line the inhibition rate of cell growth increases proportionally with the increase in particle size, while the IR of HeLa cells growth increases proportionally with increase in GNPs concentration.

So the effect of GNPs depends not only on their size or concentrations but depends also on the type of the cultured cancer cells.

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