Cytotoxic attribute of extracellular gold nanoparticles mediated by Streptomyces tuirus DBZ39

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Abstract

Streptomyces tuirus DBZ39, a novel isolate obtained from limestone quarry, was proved to be efficient strain for the synthesis of extracellular gold nanoparticles. The present investigation reveals the enhanced production of extracellular gold nanoparticles and its cytotoxic attributes. An upstream bioprocess was optimized for the synthesis of controlled size gold nanoparticles with solitary monodispersal pattern in aurium chloride solution. Inoculum size (biomass) of 1.5 g, 1mM substrate concentration (aurium chloride) at 40 ^{0}C temperature and pH 8.0 were observed as optimum conditions for achieving 22 nm size gold nanoparticles. Gold nanoparticles obtained was illustrated by Scanning and Transmission Electron Microscopy, Energy Dispersive X-ray analysis (EDAX) and X-ray diffraction (XRD) analysis. Scanning and Transmission electron micrographs of gold nanoparticles reveal an average size of about 22nm which were dispersed uniformly. An X-ray diffraction pattern shows two major characteristic peaks at the range of 2 theta correspondingly and the crystal phases of gold nanoparticles. EDAX confirms an elemental occurrence of AuNPs showing maximum peak with better cycle per second (CPS) values. The cytotoxicity of biosynthesized and commercial gold nanoparticles against brine shrimps are reported by survival and mortality of shrimps after exposure time of 6, 12 and 24 h.The biosynthesized gold nanoparticles exhibited highest cytotoxic activity with LC50 value of 0.40 µg/ml compared to commercial gold nanoparticles with LC50 value of 0.60µg/ml.

Keywords: Streptomyces, Extracellular gold nanoparticles, Influencing parameters, cytotoxicity

1. Introduction

Nanoparticles have tremendous and wide range of applications [1, 2, 3]. Antimicrobial properties, anticancer activities, genotoxicity, diagnostic devices, drug delivery systems, detection of pathogens and nano drugs are some of the important and potential applications [4,5]. Among all the nanoparticles, metallic nanoparticles were reported to have attractive applications. The synthesis of nanoparticles employing toxic chemicals are not eco friendly. For the synthesis of nanoparticles there is need for developing green processes

that do not use toxic chemicals in the production protocol [6]. The importnant aspect of nanotechnology is to develop a reliable and ecofriendly process for the synthesis of nanomaterials. In the present investigation an efforts made to develop green technology for the synthesis of nanomaterials which is of considerable importance [7].

The exploitation of biological systems for the synthesis of metallic nanoparticles was a non toxic and unconventional method. A unique method for the synthesis of gold nanoparticles with microbial cell have been used. Microorganisms and plants were used as a wide range of resources for the synthesis [7, 8, 9]. Among microorganisms, especially actinomycetes are very potential for the synthesis of several nanoparticles with much improved controlled size, shape and composition of nanoparticles. Recently, the genus Streptomyces has been studied as potential producer of certain bioactive molecules with diverse, chemical structures and biological activities. The basic steps for gold nanoparticles biosynthesis include the microbial growth and the metal reduction process, which takes place by intra or extracellular reduction [10]. In actinomycetes, reduction of metal ions occurs on the surface mycelia along with cytoplasmic membrane leading to the formation of nanoparticles [7, 10].

The advent of nanotechnology has resulted in increased use of nanomaterial based products in daily life. However, major and simultaneous outcome of this rapidly developing field of nanotechnology have adverse human health effects resulting from exposure to commonly used nanomaterial [11]. Nanogold is being used in a wide range and number of products which are being consumed by the human beings. The irregularity in dose response could however be owing to the formation of agglomerates with increase in treatment concentration. It is a vital part of regulatory norms as damage to the genome may promote carcinogenicity or have an impact on reproduction.Gold in similar way as that of silver does not negatively affect the human body when used in appropriate concentrations. Gold nanoparticles have been employed in biomedicine because of their attractive properties [12]. Although, synthesis of gold nanoparticles was mediated by several bacteria [13, 14], fungi [15,16], few actinomycetes [17,18] and yeasts [19,20], with defined dimensions and distinct monodispersity which is a challenging one. The most infectious pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumonia* and several others were affected by reaction of metal nanoparticles [21]. Keeping this in view, an attempt was made to study the cytotoxicity of microbial synthesized gold nanoparticles. An efficient isolate *Streptomyces tuirus* DBZ39 discovered from Shahabad limestone quarry was potential to synthesize both tyrosinase and gold nanoparticles for environmental application was a striking feature [22, 23]. Hence, the goal of the current work is to provide an update of the cytotoxic effects of *Streptomyces tuirus* DBZ39 mediated gold nanoparticles using brine shrimp as a model.

2. Materials and Methods

2.1.*Synthesis of extracellular gold nanoparticles*

Streptomyces tuirus DBZ39 isolated from limestone quarry soil [23] in our A-DBT (Actinomycetes-Diversity and Bioprocess Technology) research laboratory was employed for the synthesis of extracellular gold nanoparticles, as per the standard protocol [7, 24]. A loopful of three days old culture of *Streptomyces tuirus* DBZ39 was inoculated into starch casein broth containing Starch-1g, casein-0.003 g, KH_2PO_4- 2.0 g, KNO_3-2.0 g, NaCl-2.0 g, $MgSO_4$-0.002 g, $FeSO_4$-0.001 g, $CaCo_3$- 0.001 g and incubated at 40^0 C for 5 days on rotatory shaker (200 rpm). After incubation, the broth culture was centrifuged at 8000 g. The biomass obtained was suspended in $AuCl_4$ (0.5 mM) solution and kept for incubation at 37^0 C on shaker (200 rpm) for three days. The gold nanoparticles synthesized were confirmed by the development of deep purple color asvisual observation and UV-visible absorption spectrum in the range of 500-550nm using Systronics 2201 double beam UV-VIS spectrophotometer.

2.2.Influence of process variables

Submerged bioprocess in Starch Casein broth was standardized for the production of extracellular gold nanoparticles using *Streptomyces tuirus* DBZ39, employing important process variables as per the standard protocol [10].The influence of inoculum size (0.5, 1.0, 2.0, 2.5 and 3.0 g/100 ml), substrate concentration (0.5, 1.0, 1.5, 2.0 and 2.5 mM), temperature (30, 35, 40, 45 and 50 ^{0}C) and pH (7.0, 7.5, 8.0, 8.5 and 9.0) were optimized. The size of gold nanoparticles obtained at each optimized parameters were characterized by Scanning Electron Microscopy.

2.3.Characterization of gold nanoparticles

The major compositions of gold nanoparticles mainly including shape, size and dispersion at all optimized parameters were determined by Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Energy Dispersive X-Ray analysis (EDAX) and X-Ray Diffraction (XRD). Scanning electron microscopic photographs were observed to detect the gold nanoparticles [25]. A smear of solution carrying gold nanoparticles was prepared and air dried on a small thin aluminum foil. The smear was treated with 0.2% gluteraldehyde. Then a smear was scanned for gold nanoparticles under electron microscope (Zeiss).

A beam of electrons was transmitted through an ultrathin specimen, prepared as per the standard protocol [26]. An image of gold nanoparticles was formed from the interaction of the electrons transmitted through the specimen. The image was magnified and focussed on to an imaging device. The pattern were recorded on a carbon coated copper grid on a Phiplips (CM-200) machine. The EDAX of gold nanoparticles was carried out by the JEOL mode JED-2300 equipment as per the standard protocol prescribed [27]. The size of the gold nanoparticles were determined by XRD analysis using PHILIPS PW 1051 model devise [28]. The average crystllite size of gold nanoparticles was calculated using the scherrers formula as mentoined below

$B(FWHM)=K\lambda / L \cos\theta$

B (FWHM): full width at half maxmimum or integral breadth K

: Constant -0.94

λ	**:** Wavelength of X-ray
L	: Average crystallite size
$\cos\theta$	: Observed θ values (peak values)

Different concentration of gold nanoparticles ranging from 1, 2, 4, 8, 16, 32 and 64 µg/ml was used to test the cytotoxicity.

2.4.Cytotoxicity of gold nanoparticles

The cytotoxicity of extracellular gold nanoparticles was determined by following the standard protocol [29]. Seawater water was prepared artificially by dissolving 20 g of NaCl in 100 mL of distilled water and the pH was adjusted to 8.5 using 0.1 M Na_2CO_3. 1g of brine shrimp eggs were added in the seawater and incubated for 48 h at 28^0C in the constant air supply and light. The hatched brine shrimps were collected and rinsed in fresh seawater. Extracellular gold nanoparticles from 1, 2, 4, 8, 16, 32 and 64 µg/ml concentration were diluted in 5 ml seawater in tubes separately. A control was maintained with zero concentration. The mortality of brine shrimps was recorded for 24 h at every 6 h intervals and the percentage of mortalityLC50 (µg/ml) was evaluated. The end point of mortality was confirmed by the absence of controlled forward motion during 30 seconds and the concentration that killed 50% of brine shrimps was LC50 [30]. A commercially available gold nanoparticle was used as control to compare the cytotoxicity of biosynthesized gold nanoparticles.

3. Results and discussion

3.1.*Synthesis of Streptomyces mediated gold nanoparticles*

*Streptomyces tuirus*DBZ39isolated from limestone quarry soil as novel isolate [22,23], was proved to be most efficient isolate for the extracellular synthesis of gold nanoparticles compared to the isolates reported from our research laboratory. Development of deep purple color (Fig.1 A) in treated solution when compared to substrate and biomass control indicates the synthesis of gold nanoparticles by *Streptomyces tuirus*DBZ39.The change in development of wine red color in the test solution reveals the synthesis and strong physiological capability of *Streptomyces tuirus* DBZ39 for the better synthesis of extracellular gold nanoparticles.The presence of extracellular gold nanoparticles in the solution was confirmed by UV-vis analysis (Fig. 1B) at 520 nm [10].

A large number of reports are available on the synthesis of gold nanoparticles by biological entities, especially from plants, bacteria and fungi. However, there are very few reports on the synthesis of extracellular gold nanoparticles by actinomycetes [31, 24, 32, 33]. Microbes affect the redistribution of metal by oxidation, reduction or biosorption. Microbes may solubilize the metals in case of uranium, or reduce themin case of Iron and Manganese. Microbial biomass can retain relatively high quantities of metal by biosorption (passive mode) or by bioaccumulation (actively by viable cells) [34]. Bioreduction of metal nanoparticles was regarded as an organism's survival mechanism against toxic metal ions [25]. During the synthesis of gold nanoparticles, Au^{+} ions were trapped on the surface of cells by electrostatic interaction between Au^{+} and negatively charged carboxylate group present in NADH- dependent reductase enzyme of the cell wall leading to the synthesis of gold nuclei at nanoscale [35].

Bacterial cells constantly exposed to stressful situations have an ability to resist those stresses for their survival. The ability of microorganisms to grow in presence of high metal concentrations might have resulted from specific mechanisms of resistance. Such mechanisms include: efflux systems; alteration of solubility and toxicity by changes in the redox state of the metal ions; extracellular complexion or precipitation of metals; and the lack of specific metal transport systems [36].

3.2.Optimization of bioprocess

Important physicochemical parameters areInoculum size of 1.5 g (Fig. 2A), substrate concentration of 1mM (Fig. 2B), temperature 40^{0}C (Fig. 3A) and pH 8.0 (Fig. 3B) were proved to be optimum for the quantity and

quality enhanced production of gold nanoparticlesunder submerged bioprocess as mentioned earlier. A principle of operating one variable at a time keeping others constant [37] was followed to record the optimum conditions. Optimized production of extracellular gold nanoparticles after the manual process of optimization was carried out by following automated statistical optimization based on maximum absorption peak and size of nanoparticles obtained at the optimized parameters. The enhanced production of gold nanoparticles at the optimum conditions was illustrated by Scanning Electron Microscopic images (Fig. 2 and 3). Physicochemical conditions are the most important factors considered to influence the level of production of nanoparticles. Efforts were made to develop a submerged bioprocessby optimizing physicochemical components for the maximum production of nanoparticles by *Streptomyces tuirus* DBZ39.Several investigations have revealed the synthesis of nanoparticles by microorganisms in a suitable growth medium regulated by the pH and temperature. The physical parameters such as pH, temperature, inoculum size and substrate concentration will play a vital role in the production of bioactive molecules by actinomycetes [38]. Initial pH of the medium is one of the crucial factors for the successful production of nanoparticles. In most of the industrial fermentation it is essential to control pH of the medium for achieving maximum product formation [39]. Inoculum size plays an important role in enzyme production under any bioprocess. An increase in inoculum generally improves the growth and growth activities of the organism up to a certain level and with further increase, there could be a reduction in microbial activity due to nutrient limitations. Due to lower inoculum size, a longer time is required for the organism to grow up to optimum number to utilize the substrate and form the desired product [40]. Temperature is also an important factor that governs the process of fermentation and recovery of desired product. Transformation of substrate into product is under the influence of temperature for biochemical conversion of nutritional reactants to products. Influence of higher range of temperature on actinomycetes for the maximum production of nanoparticles is well established. As such efforts have been made in the present investigation to understand the influence of all these factors on the maximum production of nanoparticles. These observations are in confirmation with the findings of several researchers [41, 7].Relatively, a less extremophilic conditions such as pH 8.0 and temperature 40^0C, lead the novel isolate, *Streptomyces tuirus* DBZ39, in the present investigation for the synthesis of a controlled size, spherical shape extracellular gold nanoparticles with solitary mono dispersion in the solution.

A strategic approach for the synthesis of extracellular gold nanoparticles is an important criterion to obtain highly controlled size gold nanoparticles. Murali *et al.*[25] presented a research account on the integration of various process variables, as mentioned above for the extracellular synthesis of gold nanoparticles by an isolate of actinomycete, *Thermomonospora sp*. Novel alkalothermophilic actinomycetes, *Thermomonospora sp*., isolated from self-heating compost was reported to have pH 9.0, temperature 50^0 C and 1mM substrate

concentration as ideal conditions for the extracellular synthesis of gold nanoparticles. It was also reported that, the use of extreme biological conditions in the synthesis could be a contributory factor in the size and mono dispersal control using actinomycetes as biological source [7].

3.3.Properties of extracellular gold nanoparticles

The characteristic properties of any nanoparticles are very important and critical from the point of their applications in various fields of biotechnology. The transmission electron microscopy (Fig. 4A) reveals accurate size, shape, arrangements and distribution pattern of gold nanoparticles in the solution. The spectrum of EDAX (Fig.4B) confirms the purity of gold nanoparticles, whereas the spectrum of XRD (Fig.5) exhibits the crystal phasic appearance of gold nanoparticles.

Nanoparticles have been investigated extensively in recent years because of their potential applications [42-44]. The determination of accurate size of nanoparticles with the computational system of TEM is more accurate and authenticated when compared to scanning electron microscopy. The energy dispersive X-ray analysis (EDAX) confirms the presence of a specific metallic element and also indicates the quantum of the element present. In addition to the above said properties, structural features and nature of the nanoparticles are also equally important to understand the biocompatibility of these nanoparticles, from the point of biomedical applications. X-ray diffraction analysis is expected to reveal structural lattices and crystalline or amorphous nature of the nanoparticles. The gold nanoparticles synthesized by *Streptomyces tuirus* DBZ39 was subjected for the X-ray diffraction analysis.

3.4.*Cytotoxicity of extracellular gold nanoparticles against brine shrimps*

Exploration of substrates for cytotoxic properties is an ever encouraging field of medical biotechnology. The cytotoxic property of biosythesized gold nanoparticles and commercially available gold nanoparticles against brine shrimps was revealed for the first time as per the literature available and the inferences drawn are as presented in Fig. 6a and 6b.

The number of shrimps survived and the percentage of mortality for 6, 12 and 24 hours was plotted in graph, concentration (µg/ml) verses percentage of mortality (Figure. 6 a and 6b) for biosynthesized and commercial gold nanoparticles respectively. After 24 hours, the total mortality was 100 % in the highest concentration of biosynthesized gold nanoparticles and 93% mortality was observed in commercial gold nanoparticles. The LC_{50}

value was 0.40µg/ml for biosynthesized gold nanoparticles and 0.60µg/ml for commercial gold nanoparticles (Table 1).

The interaction between metals and microorganisms has been well documented [45,46]. Among microorganisms, actinomycetes are considered as best nanofactories as they possess dual characteristics of bacteria and fungi. Several actinomycetes are known to produce various bioactive molecules such as enzymes [47], antibiotics [48,49] and other variety of secondary metabolites [7,23,41]. Recently, they are regarded as producers of bionanomaterials. The genus Streptomyces being one of the major antibiotic producers among the actinomycetes, is a wide and major known organism. Assessment of cytotoxicity of chemicals using cell lines is not an uncommon procedure and is accurately correlated with the assessment of cytotoxicity using brine shrimps [50]. The brine shrimp assay method is considered as an excellent alternate option to assess the cytotoxic activity of the biological product [51]. From the beginning of its introduction to standardization [52], this *in vivo* test had successfully been adopted for the bioassay of active cytotoxic and antitumor agents [53]. Further the lethal concentration of brine shrimp can be correlated with the lethal dose in mice and was explained using medicinal plants earlier [54, 55].

4. Conclusion.

Synthesis of extracellular gold nanoparticles in the commercial scale is at most necessary to fulfill the demand, because of its application in different fields and especially in medical use. The microbial production of gold nanoparticles is an alternative to the chemical processes which is more economic and safer. In the present investigation *Streptomyces tuirus* DBZ39was proved to be prominent for gold nanoparticles synthesis. Further, it is essential to develop a suitable downstream process for the synthesis of controlled size (22 nm) gold nanoparticles, which would establish a substitution for chemically synthesized nanoparticles. This investigation reveals notably high cytotoxic activity of gold nanoparticles in less concentration and promising natural agent against tumor cells.

Conflict of interest

The authors declare there is no conflict of interests regarding the publication of this paper.

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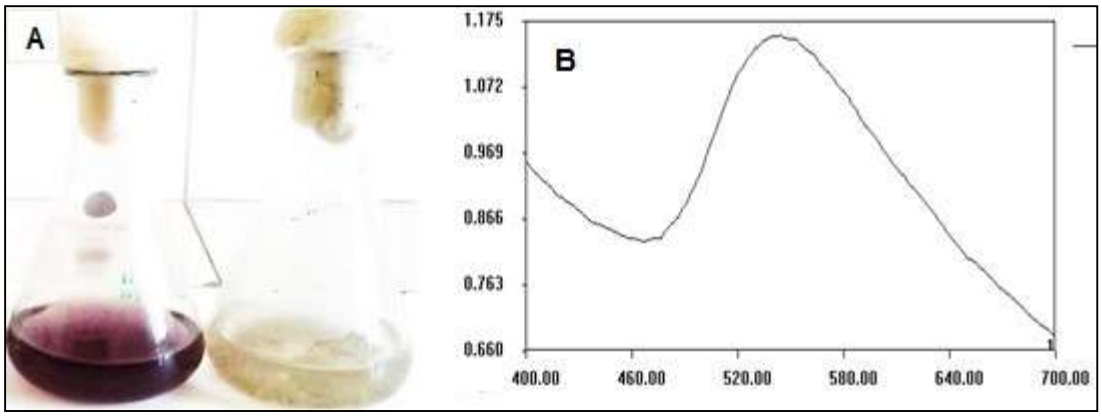
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B
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700.00

Fig. 1

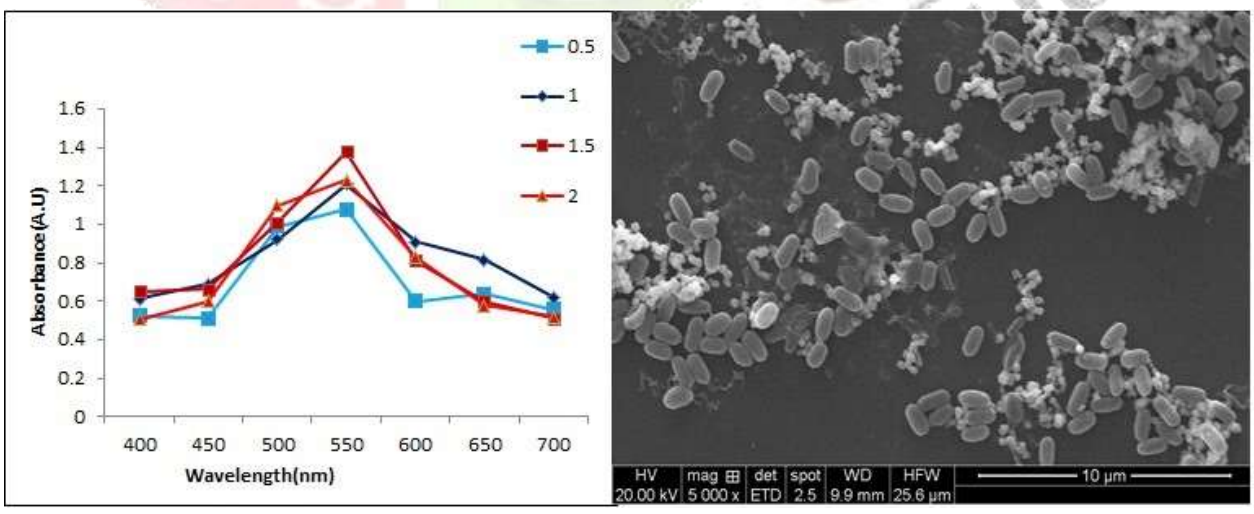
0.5
1
1.5
2
Absorbance (A.U)
Wavelength(nm)
400
450
500
550
600
650
700
HV
20.00 kV
mag
5 000 x
det
ETD
spot
2.5
WD
9.9 mm
HFW
25.6 µm
10 µm

Fig.2a

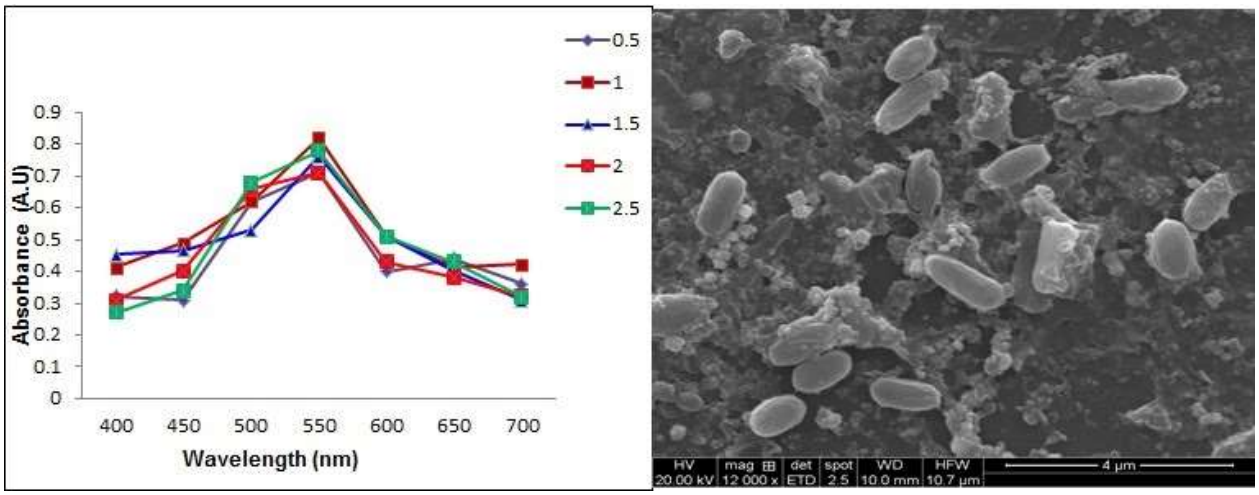
0.5
1
1.5
2
2.5
Absorbance (A.U)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
400 450 500 550 600 650 700
Wavelength (nm)
HV 20.00 kV
mag 12 000 x
det ETD
spot 2.5
WD 10.0 mm
HFW 10.7 µm
4 µm

Fig.2b

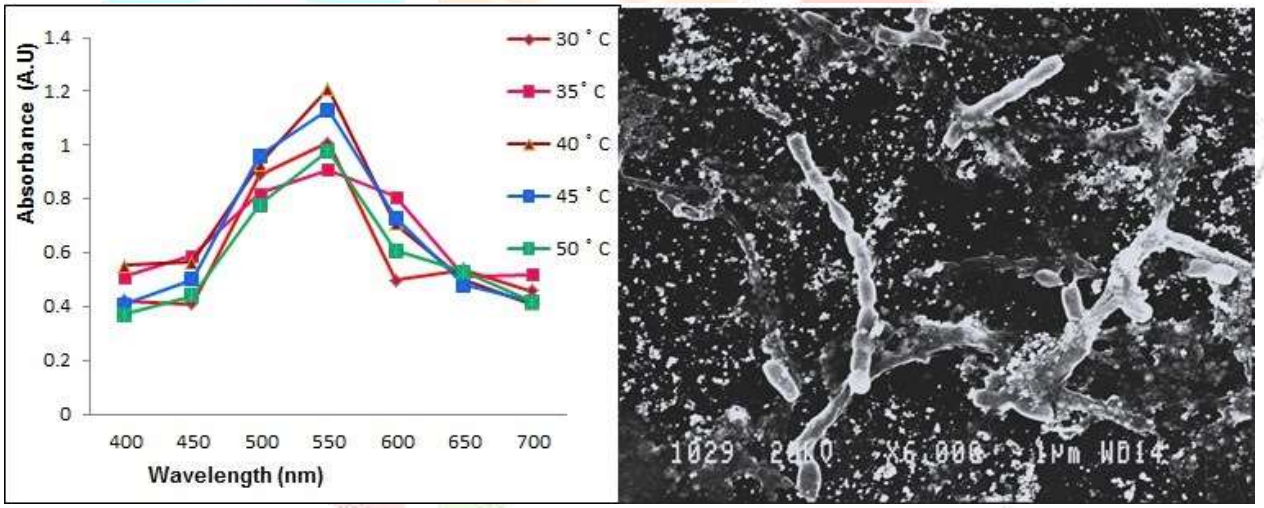
30 ° C
35 ° C
40 ° C
45 ° C
50 ° C
Absorbance (A.U)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
400 450 500 550 600 650 700
Wavelength (nm)
1029 20kV X6,000 1µm WD14

Fig.3a

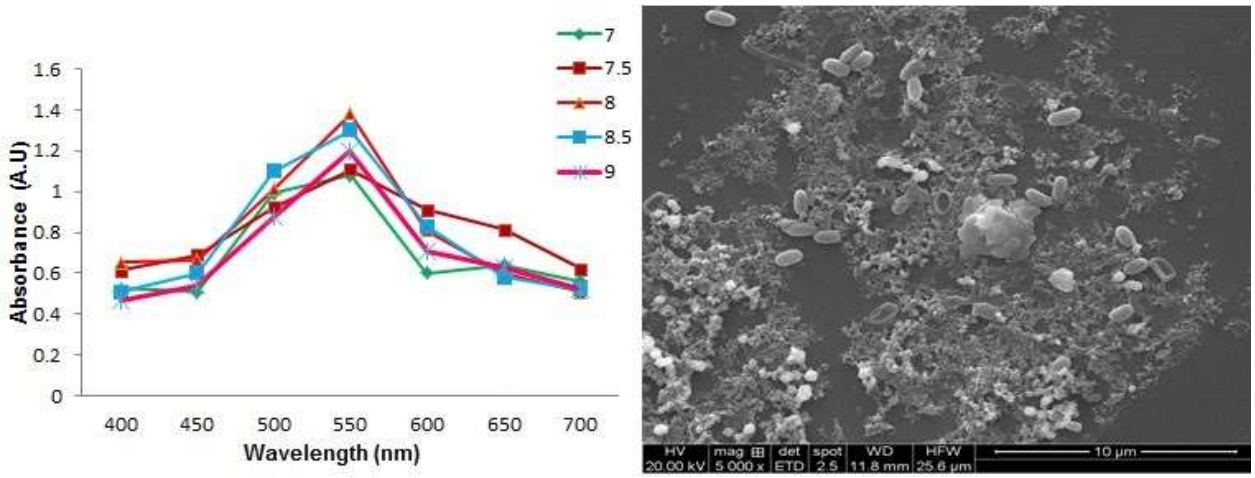
7
7.5
8
8.5
9
Absorbance (A.U)
Wavelength (nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
400
450
500
550
600
650
700
HV
20.00 kV
mag
5 000 x
det
ETD
spot
2.5
WD
11.8 mm
HFW
25.6 µm
10 µm

Fig.3b

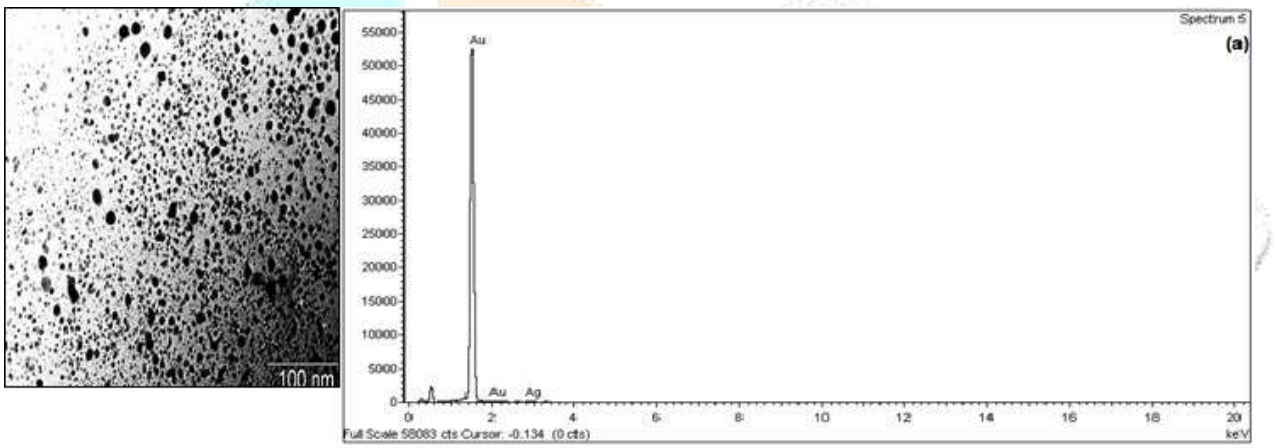
100 nm
Spectrum 5
(a)
Au
Au
Ag
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
0
2
4
6
8
10
12
14
16
18
20
keV
Full Scale 58083 cts Cursor: -0.134 (0 cts)

Fig.4

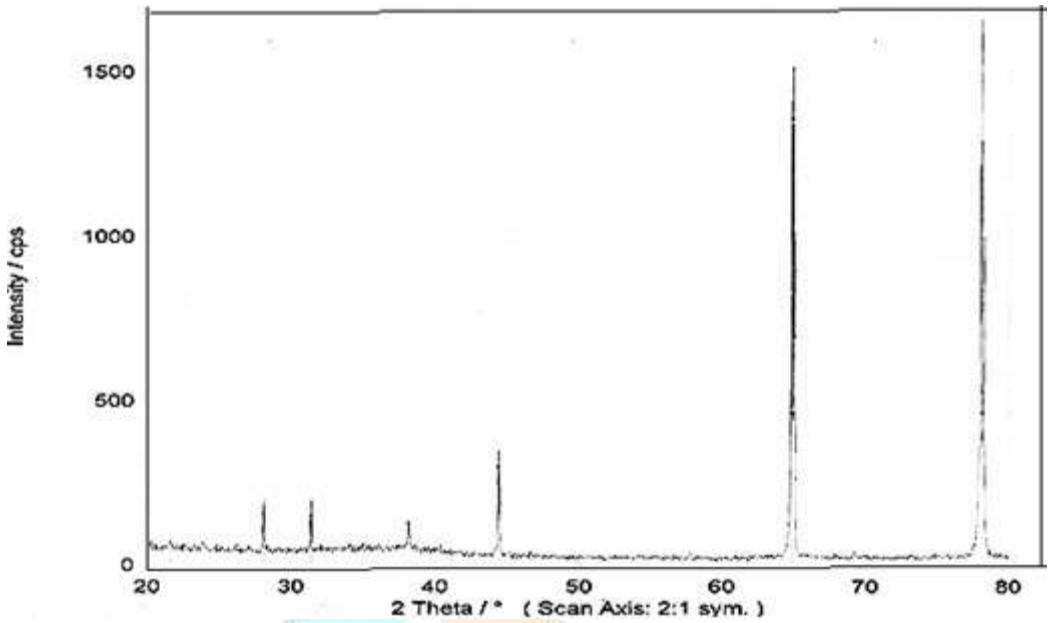
Intensity / cps
1500
1000
500
0
20
30
40
50
60
70
80
2 Theta / ° (Scan Axis: 2:1 sym.)

Fig.5

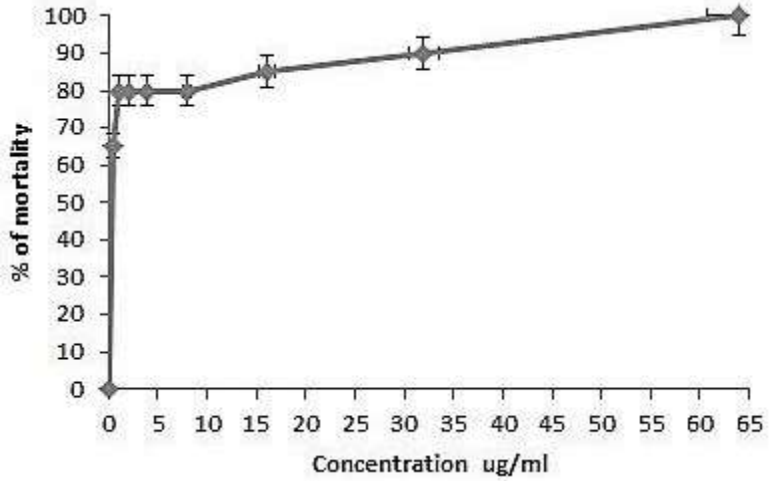
% of mortality
100
90
80
70
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65
Concentration ug/ml

Fig.6a

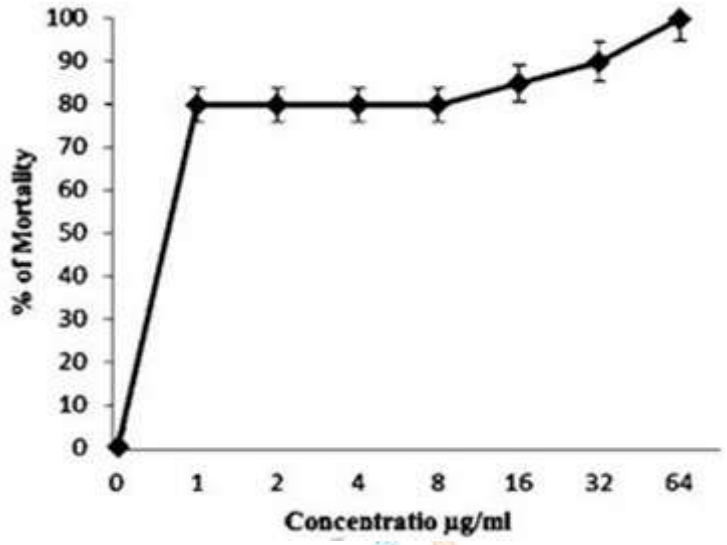

Fig.6b

Table 1. Cytotoxic attributes of biosynthesized and commercial gold nanoparticles showing percentage mortality and Lc50 value at different concentrations

Concentration of biosynthesized AuNps (ug/ml)	Log of concentration	Number of survival			Percentage of mortality			Lc 50 (ug/ml) at 24 h
		6 h	12 h	24 h	6 h	12 h	24 h	
00.5	-0.301	18	09	07	10	55	65	0.40
01.0	0	17	05	04	15	75	80	
02.0	0.301	17	05	04	15	75	80	
04.0	0.602	16	05	04	20	75	80	
08.0	0.903	16	05	04	20	75	80	
16.0	1.204	15	05	03	25	75	85	
32.0	1.505	15	04	02	25	80	90	
64.0	1.806	15	03	00	25	85	100	
Concentration of commercial AuNps (µg/ml)	**Log of concentration**	**Number of survival**			**Percentage of mortality**			**Lc 50 (µg/ml) at 24hours**
		6 h	12 h	24 h	6 h	12 h	24 h	
01.0	0	17	09	04	08	43	68	
02.0	0.101	15	09	05	13	43	63	
04.0	0.402	15	08	04	13	48	68	
08.0	0.703	15	07	02	13	53	78	0.60
16.0	0.804	14	07	02	19	53	78	
32.0	0.105	14	07	00	19	53	83	
64.0	0.606	14	05	00	18	63	93	