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Bioreduction of chloroauric acid ($HAuCl_4$) for the synthesis of gold nanoparticles (GNPs): A special empathies of pharmacological activity

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Abstract

Gold nanoparticles (GNPs) were prepared using plant extract a simple biological approach. The *Hygrophila spinosa* aqueous leaf extract has been carried out in the present study. The bioreduction of chloroauric acid ($HAuCl_4$) for the synthesis of gold nanoparticles with the plant extract *H. spinosa*. The plant extract is mixed with $HAuCl_4$, The reduction of auric chloride led to the formation of AuNPs within 10 min at room temperature (28°C), The size, shape and elemental analysis were carried out using X-ray diffraction, SEM-EDAX, DLS, FT-IR and UV-spectroscopy. The results showed that the leaf extract of *H. spinosa* is very good bioreductant for the synthesis of gold nanoparticles. The synthesized gold nanoparticles have more active against for human pathogens and also have good anticancer property.

Keywords: Biosynthesis, Gold nanoparticles, *Hygrophila spinosa*, antimicrobial activity, anticancer activity.

1.Introduction

Nanotechnology is mainly concerned with the synthesis of nanoparticles of variable sizes, shapes, chemical compositions and controlled dispersity and their potential use for biomedical applications. In general, particles with a size less than 100 nm are referred to as NPs. Entirely novel and enhanced characteristics such as size, distribution and morphology have been revealed by these particles in comparison to the larger particles of the mass material that they have been prepared from. NPs of noble metals like gold, silver and platinum are well recognized to have significant applications in electronics, magnetic, optoelectronics and information storage.

As an alternative to toxic and expensive physical and chemical methods for nanoparticles fabrication, using microorganisms, plants and algae will help a lot to synthesize the materials in the nano range and in addition, the toxicity of the by-product would be lesser than the other synthetic methods. Among various synthetic methods, the solution phase synthesis involving the reduction of Au (III) to Au (0) by plant extracts has gained profound significance in recent years because of the renewable and nontoxic nature of the plant extracts, eco-friendly aqueous medium, and mild reaction condition [1]. Moreover, this method becomes more advantageous over other synthetic methods since the plant extract itself acts as a stabilizer, and no additional stabilizers or capping agents are needed. Stabilization of gold nanoparticles using phyto-synthesis method is an emerging area in the field of advanced nanoparticles synthesis. Several plants and plant products have been successfully used for efficient and rapid extracellular synthesis of gold nanoparticles.

In this study we synthesized the optimized gold nanoparticles by using medicinal plant extract *H. spinosa*. For the optimized production of gold nanoparticles, leaf extract was added with gold chloride solution. After the synthesis, the nanoparticles were confirmed by UV–Vis spectroscopy, FT-IR, Scanning Electron Microscopy with energy dispersive x-ray spectroscopy analysis, stability of nanoparticles also measured by DLS and its antimicrobial and anticancer properties were tested.

2. Materials and methods

2.1 Study area and sampling

The plant materials were collected from Koothaippar village of Tiruchirappalli district, Tamil Nadu during summer 2015. The *Hygrophila spinosa* was washed several times with water to remove the dust particles and then shade dried to remove the residual moisture and grinded to form powder. Then plant extract was prepared by mixing 1% of plant extract with deionized water in a 250ml of (Borosil, India) conical flask. Then the solution was incubated for 30 min. and then subjected to centrifuge for 30 min at room temperature with 5000 rpm. The supernatant was separated and filtered with (mm filter paper) filter paper with the help of vacuum filter. Then the solution was used for the reduction of gold ions (Au^{+}) to gold nanoparticles (Auo).

2.2 Biofabrication of nanoparticles

Biofabrication of gold nanoparticles, gold chloride prepared at the concentration of 10^{-3} M with pre-sterilized Milli Q water. A quantity of 10 ml plant extract was mixed with 90 ml of 10^{-3} M gold chloride for the synthesis of gold nanoparticles. Gold chloride has taken in similar quantities without adding plant extracts to main respective controls. The saline bottles were tightly covered with aluminium foil in order to avoid photo reduction of gold ions, incubated at room temperature under dark condition and observations were recorded.

2.3 Characterization of nanoparticles

2.3.1 UV-VIS spectroscopy

The Au nanoparticles were characterized in a Perkin-Elmer UV-VIS spectrophotometer, Lambda-19 to know the kinetic behaviour of Au nanoparticles. The scanning range of the samples was 200-800 nm at a scan speed of 480 mm/min. Baseline correction of the spectrophotometer was carried out by using a blank reference.

2.3.2 Scanning electron microscope (SEM) and energy dispersive spectroscopy (EDS)

In this research work, Joel JSM-6480 LV SEM machine was used to characterize the mean particle size and morphology of nanoparticles. Compositional analysis on the sample was carried out by the energy dispersive X-ray spectroscopy (EDS) attached with the SEM. The EDS analysis of Au sample was done by the SEM (JEOLJSM 5800) machine. The EDS normally reveals the presence of phases.

2.3.3 Dynamic Light Scattering *Particle size analyzer*

In order to find out the particles size distribution the Au powder was dispersed in water by horn type ultrasonic processor [Vibronics, model: VPLP1]. Then experiment was carried out in computer controlled particle size analyzer [ZETA Sizers Nanoseries (Malvern Instruments Nano ZS)] to find out the particles size distribution.

2.3.4 Dynamic Light Scattering *Zeta Potential Measurement*

Zeta potential describes the electrical potential in the double layer of ions surrounding a particle at the boundary of the particle surface and the adsorbed ions in the diffuse layer [2]. Zeta potentials were determined with a Zetaphorementer IV (CAD, France).

2.3.5 Fourier transform-infra red (FT-IR) spectroscopy

The analysis of bio-reducing agent present in each of the extracts was measured by FT-IR. After the reaction, a small aliquot of the concentrated reaction mixture was measured in the transmittance mode at 400 to 4000 cm^{-1}. The spectra of the extracts taken after the biosynthesis of nanoparticles were analyzed.

2.3.6 X-ray diffraction method

The phase evolution of calcined powder as well as that of sintered samples was studied by X-ray diffraction technique (Philips PAN analytical, The Netherlands) using Cu radiation. The generator voltage and current was set at 40 KV and 30 mA respectively. The Au sample was scanned in the range 10.0000 - 90.0000^{o} in continuous scan mode. The scan rate was 0.60/sec.

2.3.7 Testing of antimicrobial activity

The test strains were: *Aeromonas liquefaciens* MTCC 2645 (B1), *Enterococcus faecalis* MTCC 439 (B2), *Klebsiella pneumonia* NCIM 2883 (B3), *Micrococcus luteus* NCIM 2871 (B4), *Salmonella typhimurium* NCIM

2501 (B5), *Vibrio cholerae* MTCC 3906 (B6), *Candida albicans* MTCC 1637 (F1), *Cryptococcus* sp. MTCC 7076 (F2), *Microsporum canis* MTCC 3270 (F3), *Trichophyton rubrum* MTCC 3272 (F4). The cultures were obtained from MTCC, Chandigarh and NCIM, Pune, India. Microbial strains were tested for antimicrobial sensitivity using the plate diffusion method. [3][4]. The antibacterial and antifungal activities of test samples were analyzed against certain microorganisms on muller hinton agar (MHA) and potato dextrose agar (PDA), respectively. A sterile cotton swab was used to inoculate the bacterial suspension on surface of agar plate. The two different concentrations (50 and 100µl) of gold nano samples were poured into well (1 cm in diameter and 4 mm in depth) of the agar plates, separately. The plates were incubated at 37±1°C for 24–48 h (for bacteria) and 25 ±1°C for 48-72 h (for fungi). After incubation, the zone of inhibition was measured with ruler/ antibiotic zone scale-C. The assays were performed in triplicate and the average values are presented. Methicillin – 10mcg (for bacteria) and Itraconazole – 10mcg (for fungus) was used as positive control. All the media, standard discs and sterile disc were purchased from Hi-Media (Mumbai, India).

2.3.8 Cytotoxicity/ MTT assay

The biosynthesized Au nanoparticles was dissolved in DMSO, diluted in culture medium and used to treat the chosen cell line (HeLa) over a sample concentration (5 different concentrations 1, 5, 10 25 and 50 µg/mL) range of 1 - 50 µg/ml for a period of 24 h and 48 h. DMSO solution was used as the solvent control. A miniaturized viability assay using 3-(4, 5-di-methylthiazol-2-yl)-2,5-diphenyl-2H-tetra-zolium bromide (MTT) was carried out according to the method described by [5][6]. To each well, 20 µl of 5 mg/ml MTT in phosphate-buffer (PBS) was added. The plates were wrapped with aluminum foil and incubated for 4 h at 37^0C. The purple formazan product was dissolved by addition of 100 µl of 100% DMSO to each well. The absorbance was monitored at 570 nm (measure-ment) and 630 nm (reference) using a 96 well plate reader (Bio-Rad, Hercules, CA, USA). Data were collected for four replicates. Each and used to calculate the respective means. The percentage of inhibition was calculated, from this data, using the formula:

$$\frac{\text{Mean absorbance of untreated cells (control) – mean absorbance of treated cells (test) x 100}}{\text{Mean absorbance of untreated cells (control)}}$$

The IC_{50} value was determined as the complex concentration that is required to reduce the absorbance to half that of the control.

3. Result and discussion

3.1 Green synthesis of Au nanoparticles

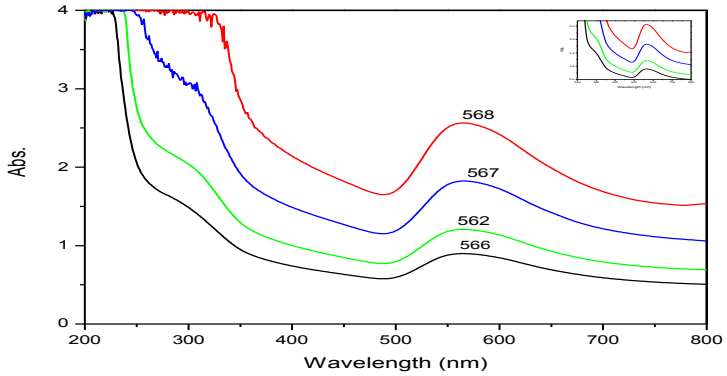
The plant aqueous solution and chloroauric acid solutions were developed individually. A quantity of 10 ml plant extract was mixed with 90 ml of 10^{-3} M gold chloride for the synthesis of gold nanoparticles for the synthesis of gold nano particles. During gold nanoparticles synthesis, the change of color from pale greenish to pink color suggested the formation of gold nanoparticles.

3.2 UV-Vis, SEM/EDS, DLS, FTIR and XRD studies

UV-Vis spectra recorded at different time intervals for the reaction with aqueous chloroauric acid solution showed an initial increase in the absorbance, which later decreased with higher incubation period and became constant giving a maximum absorbance at 560.00 nm at one hours of incubation. The appearance of the lower color confirms the formation of gold nanoparticles in the reaction mixture and efficient reduction of the Au^{3+} to Au0 (Figure 1).

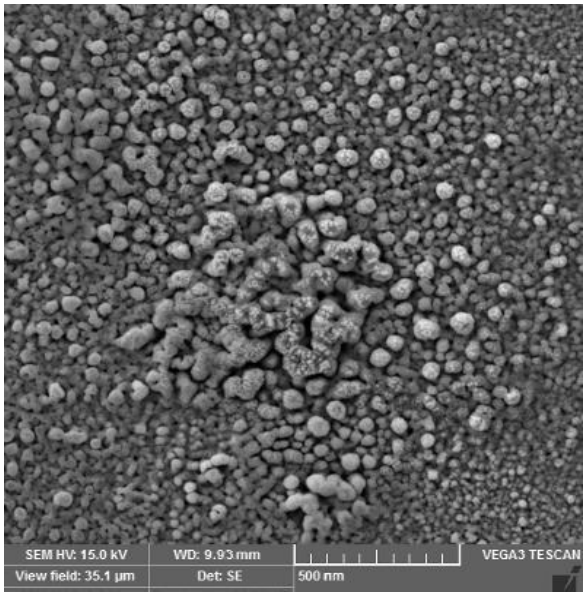
Colored solution allowed measuring the absorbance against distinct wavelength to confirm the formation of AuNPs. UV-vis spectra recorded at different time intervals for the reaction with aqueous solution showed appearance of absorbance peak at 560-570 nm after 1 hour. In order to determine the rate of AuNPs formation the kinetics of the reaction with respect to time was studied with the help of UV-vis spectroscopy. The corresponding UV-vis spectra recorded from $HAuCl_4$ plant extract reduction at various time intervals is shown in Figure 1. On reduction of $HAuCl_4$ by leaf extract for various time intervals shows a decrease in the intensity of Au^+ at 500 nm bands and appearance of absorbance band at about 560 nm. A gradual increase in the intensity of absorbance band without any shift with increasing time from spectra indicates the slow reduction of Au^{3+} to Au0. No significant change in the intensity from spectra also suggests that the reduction is going over upto 1 hrs.

Figure 1. UV-Vis spectrum of plasmon resonance of gold nanoparticles reduced by leaves in *H.spinosa*



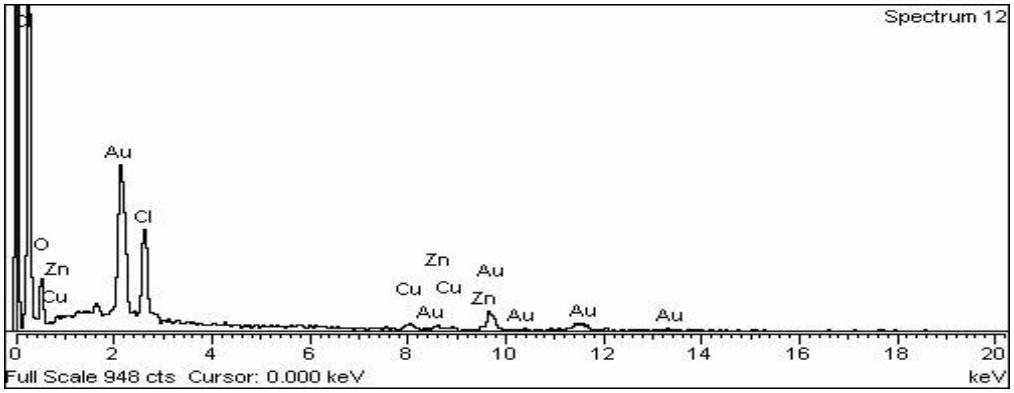
SEM images in (Figure 2) showed aggregation of AuNPs formed with diameter range from 50 nm to 80 nm. Analysis of the bio reduced green synthesized AuNPs s by SEM confirmed that they were in the nano range and of triangular and spherical shape. The triangular shaped AuNPs formed were nano dispersed with large surface area. A large quantity of AuNPs was with thin smooth ends on the exterior of the nanoparticles with a range of 150 nm (Triangular shaped) in diameter at the highest resolution and the spherical shaped were of 80 nm.

Figure 2: SEM analysis of *H.spinosa* mediated AuNPs



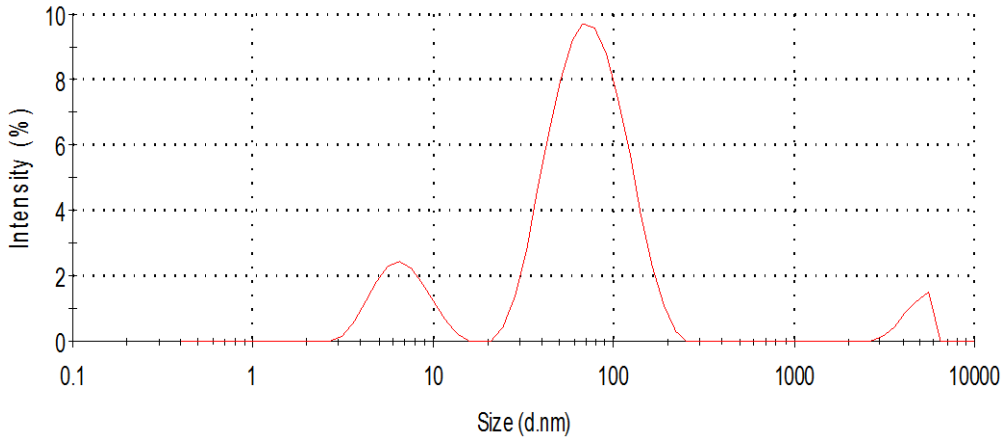
EDS revealed the presence of pure gold (Figure 3) nanoparticles in higher percentages. Gold peak is higher than other peak. The EDX reading proved that the required phase of gold (Au) is present in the sample. This is probably due to the presence of substrate over which the NP sample was held during SEM microscopy.

Figure 3: EDS analysis of *H. spinosa* mediated AuNPs



Dynamic light scattering (DLS) is a technique used to determine the size, size distribution profile and poly disparity index of particles in a colloidal suspension. The Figure 4 shows the particle size of the nanoparticles samples. After analyzing data, it was found that Au nanoparticles size were in the range of 50-100nm. The highest fraction of Au-NP present in the solution was of 44.52nm. From the plot it was evident that the solution was consist of nanoparticles having various sizes which are indeed in agreement of the result obtained by SEM analysis.

Figure 4: DLS-particle size analysis of *H.spinosa* mediated AuNPs

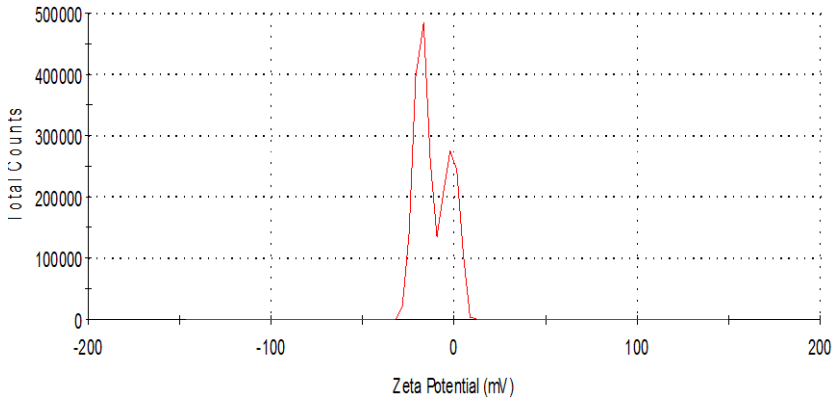


H= *H. auriculate*(Gold Nano particles)

Z-Average (d.nm): 44.52

Zeta potential measures the potential stability of the particles in the colloidal suspension. Gold nanoparticles generally carry a negative charge. The synthesized gold nanoparticles from the plant showed negative charge and were stable at room temperature. DLS-zeta potential showed negative charge (-11.5) which indicated that the sample is moderately stable at room temperature (Figure 5).

Figure 5: DLS-Zeta potential analysis of *H.spinosa* mediated AuNPs

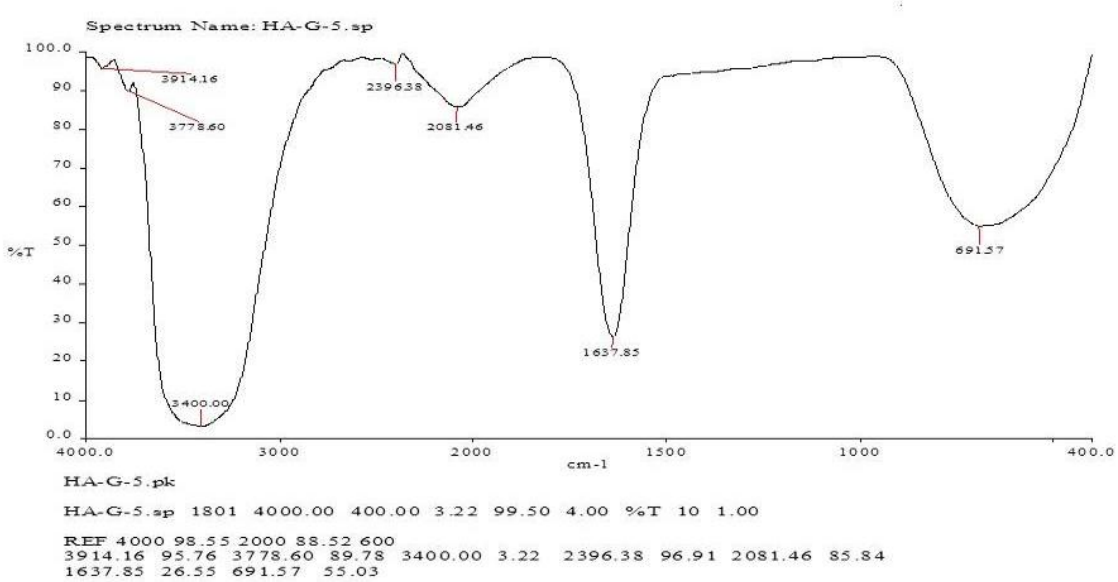


H= *H. auriculate*(Gold Nano particles)

Zeta Potential (mV): -11.5

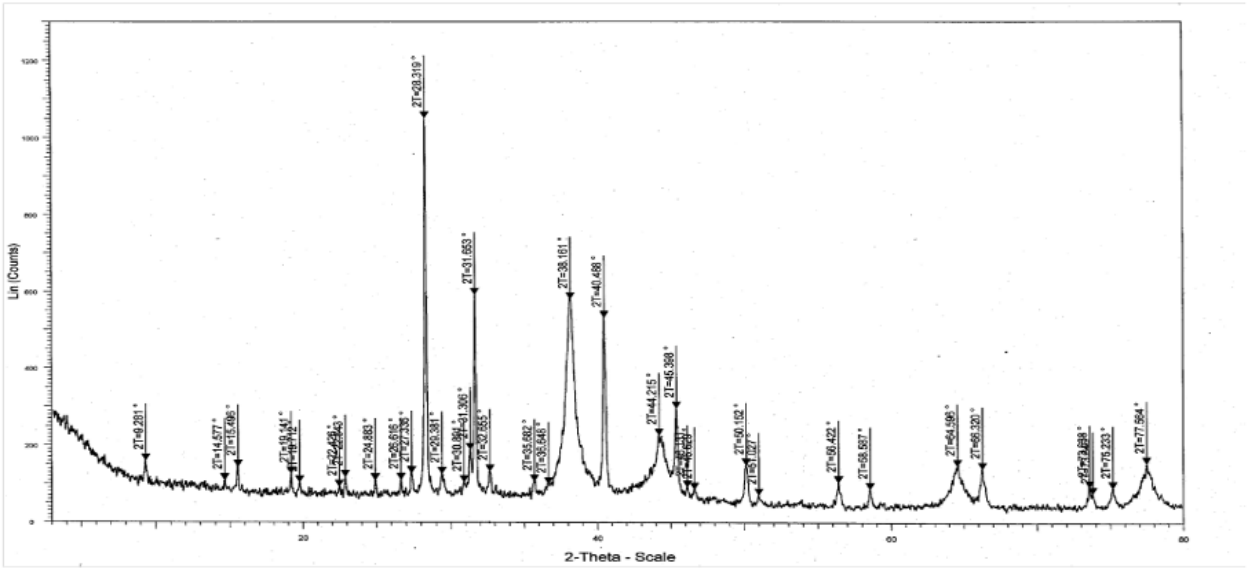
The FTIR spectra of AuNPs with absorption peaks at 3914, 3778, 3400, 2396, 2081, 1637 and 691 were observed (Figure 6). The spectra obtained to characterize the interaction between $HAuCl_4$ and plant extract has strong peak at 3400.0 shows the OH group (stretch H bonded, strong broad) along with the above mentioned peaks, some other peaks at 2396 and 2081were also seen which corresponds to C-H group (stretch strong), C-H group (variable), C-O group (strong), =C- H group (strong), C=C group (variable). The highest absorption peak 3400 reflects that the OH group might be responsible for the reducing property of the extract.

Figure 6: FTIR analysis of vibration modes and function groups of *H .spinosa* plant extract with gold chloride solution



XRD Analysis the XRD pattern shows the production of AuNPs by the reduction of Au^{3+} to Au0 using *H. spinosa*. The diffracted intensities have been recorded from 20° to 80° at 2 theta angles. The diffracted pattern in (Figure. 7) significantly corresponds to pure AuNPs. In the spectra clear peaks are not observed it indicates that the nanoparticles had a spherical structure. Broadening peak and noise were probably related to the effect of the nano particles as supported by SEM and the presence of various crystalline biological molecules in the plants extracts.

Figure 7: PXRD analysis of *H.spinosa* mediated AuNPs



3.3 Antibacterial and antifungal screening

The antimicrobial activity of test sample was examined with various pathogenic microorganisms using the (measure the inhibition zone) disc diffusion test [7]. Found that the Ag and Au nanoflakes have exhibited considerable activity against some human pathogens. The antimicrobial property of silver and gold are found to be the best among different metals in the following order Ag >Hg > Cu >Cd>Cr>Pb>Co>Au>Zn>Fe>Mn>Mo>Sn [4]. The results of the antimicrobial activities are summarized in Table 1.

Table 1: Antimicrobial screening of Au nanoflakes

S.No	Test Microorganisms	Zone of inhibition (mm)				Diseases	Route of Transmission
		AuNps		PC	Remarks		
		15	30				
	Bacteria						
1.	*Aeromonas liquefaciens* B1	15	17	14	> PC	Wound Infections / Gastroenteritis	Water / Food
2.	*Enterococcus fecalis* B2	12	14	8	> PC	Endocarditis / Bladder, Prostate, and Epididymal Infections / Nervous system Infections	Water / Food
3.	*Klebsiella pneumoniae* B3	14	16	28	< *PC*	Acute diarrhoea / Dysentery	Water / Food
4.	*Micrococcus luteus* B4	11	14	38	< *PC*	Skin & Pulmonary infections / Septic shock / Pneumonia endocarditis	Soil / Dust / Water / Airways / Food
5.	*Salmonella typhimurium* B5	15	18	0	> PC	Typhoid	Water / Food
6.	*Vibrio cholarae* B6	13	14	16	< *PC*	Cholera	Water / Food
	Fungi						
7.	*Candida albicans* F1	12	15	10	> PC	Skin (Integument) Infections / Gastrointestinal tract Infection	Airways / Wound / Soil / Water
8.	*Cryptococcus* sp. F2	11	14	9	> PC	Cryptococcal disease / Bronchiectasis / Endophthalmitis.	Airways / Wound / Soil / Water
9.	*Microsporum canis F3*	13	16	9	> PC	*Tinea capitis* /Ringworm	Airways / Wound / Soil / Water
10.	*Trichophyton rubrum* F4	14	18	7	> PC	*Tinea corporis / Tinea cruris / Tinea pedis* / Onychomycosis	Airways / Wound / Soil / Water

PC -Positive Control (Bacteria – Methicillin (10mcg/disc); Fungi – Itraconazole (10mcg/disc); > PC – greater than positive control; < PC – less than positive control

In the present study, higher (30 µL/disc) concentration of Ag & Au samples got greater sensitivity than (15 µL/disc) lower concentration in all the tested microorganisms. In this study, all the pathogens were fairly affected and nil effect was not observed in the test samples. In bacteria, the test sample was most effective against B5 while smaller effect was noticed from B4. In fungi, this was effective against F4 whereas smaller effect was observed in F2. All the microbial strains depict higher sensitivity to the higher concentration (30 µL) for the test sample when compared to the positive control except B3, B4 and B6.

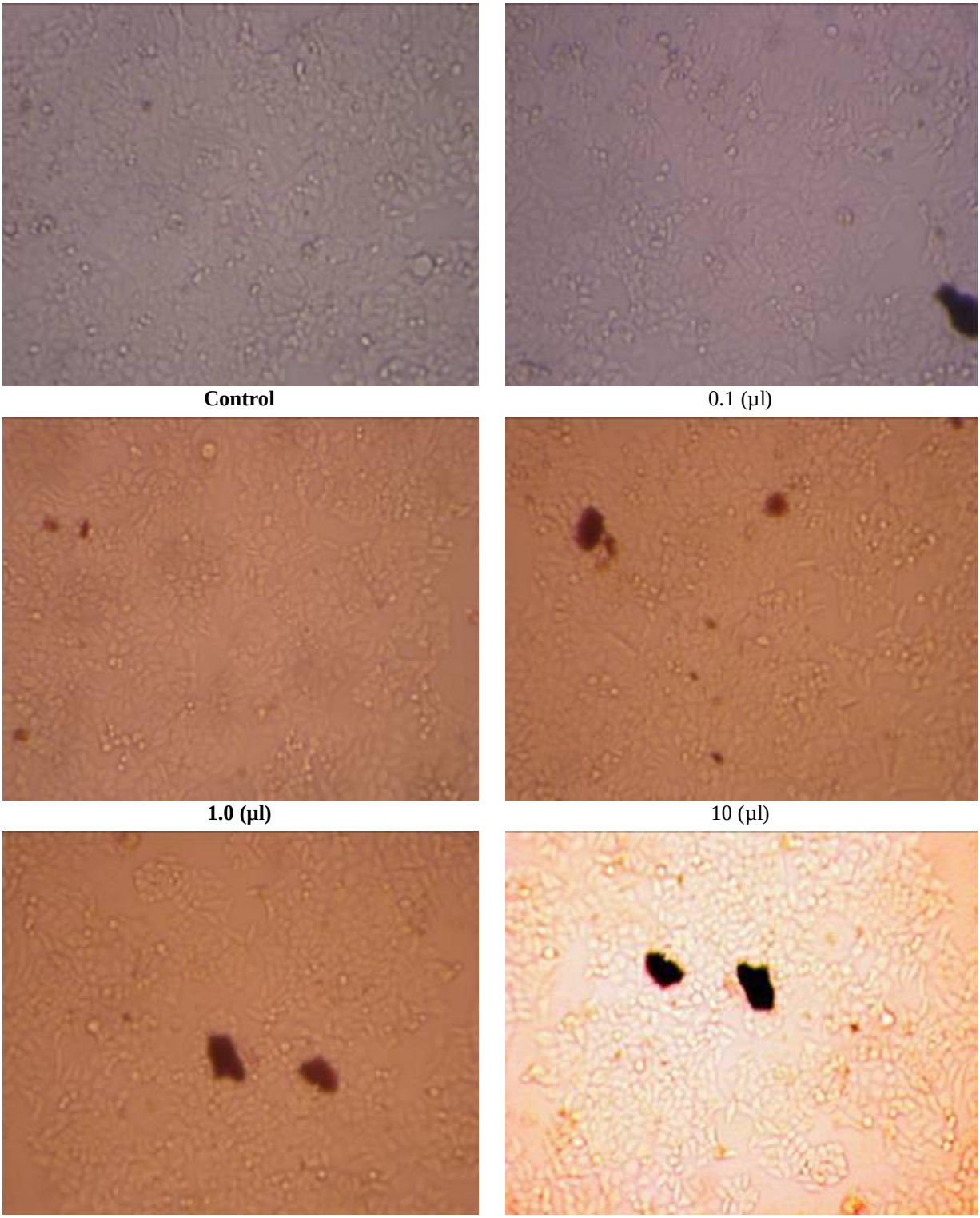
3.4 MTT assay

The cytotoxic effect of the biosynthesized Au nanoparticles was examined on cultured HeLa cells by exposing cells for 24 h and 48 h to medium containing the complex at 1 - 50 µg/ml concentration (Figure 8, Table 2).

Table 2: IC_{50} range of biosynthesized Au nanoparticles for HeLa cells

Cell line	Inhibitory concentration 50 (IC_{50}) µg/ml	
	24 h	48 h
HeLa cell	25 µg/ml	45 µg/ml

Figure 8: *In-vitro* Cytotoxicity of biosynthesized Gold Nanoparticles by *H. spinosa*



Control 0.1 (µl)

1.0 (µl) 10 (µl)

25(µl) 50(µl)

The Au nanoparticles inhibited the growth of the cancer cells significantly, in a dose- and duration dependent manner. The cytotoxic activity was determined according to the dose values of the exposure of the complex required to reduce survival to 50% (IC_{50}), compared to untreated cells. The IC_{50} values are given in Table 2. The Au nanoparticles showed highly effective cytotoxic activity against Hela cells at 48 h than 24 h in the treatment group. The cytotoxic effect of the sample may be interpretable as due to its amphiphilic nature and, hence, would penetrate the cell membrane easily, reduce the energy status in tumors and also alter hypoxia status in the

cancer cell micro environment, which are factors that would influence the antitumor acidity. It is known that biosynthesized Au nanoparticles have a wide range of biological activities such as antitumor, antifungal, apoptosis [8][9], interaction with DNA thereby inhibiting replication, transcription, and other nuclear functions and arresting cancer cell proliferation so as to arrest tumor growth.

4. Conclusion

Nanotechnology is a most promising field for generating new applications in medicine. The present investigation is highly warranted to through more light upon the Au nanoparticles from medicinal plants will helpful to investigate the active principle action for biochemical and molecular studies. At nanoscale, gold exhibits remarkably unusual physical, chemical and biological properties. Effective green synthesis of nanoparticles will have greater implication and application in biomedical research. In this study nanoparticles of 80 ± 90 nm were synthesized by using *H. spinosa*, as confirmed by SEM. These nanoparticles showed characteristic absorption peak at 540 nm in UV spectra. The possibility of protein as a stabilizing material in gold nanoparticles is revealed by FTIR analysis. The crystalline structure of gold nanoparticles was confirmed by XRD. The antimicrobial and anticancer study was confirmed that Au biosynthesized nanoparticles will act as an alternative antibiotic in future.

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