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THE BIOSYNTHESIS OF *CATHARANTHUS ROSEUS* LEAVES BASED GOLD NANOPARTICLES (AUNPS) AND THEIR ANTIMICROBIAL AND ANTICANCER APPLICATIONS

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ABSTRACT: A bioreductive approach to the synthesis of gold nanoparticles in the leaves of *Catharanthus roseus* demonstrates the formation of circle-shaped gold nanoparticles to the aqueous solutions in respective ranges. The marked difference in the shape of the gold nanoparticles has attributed to the comparative advantage of protective biomolecules and reductive biomolecules. The results were verified using UV-Vis spectroscopy, FTIR, SEM DLS-SIZE and Zeta measurements. The AuNPs were monodisperse and found to be 10-100 nm in size. The AuNPs had challenged against certain pathogenic microbial strains and human cancer cell line. In antimicrobial activity, the AuNPs has most effective against *Salmonella typhimurium* while smaller effect has noticed from *Micrococcus luteus*. The cytotoxic of AuNps showed highly effective against HeLa cells.

KEYWORDS: *Catharanthus roseus*, Antimicrobial activity, Gold Nanoparticles, Anticancer activity.

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1. INTRODUCTION

The term “nanotechnology” describes the field of developments in which size-dependent properties of materials in the nanometre regime play a dominant role, and where these properties can used to generate new techniques and devices [1]. The materials can include nanoparticles with

dimensions of less than 100nm as well as patterned surfaces and assemblies that are more sophisticated. Nanotechnology is an important field of modern research dealing with design, synthesis, and manipulation of particles structure ranging from approximately 1-100 nm in one dimension. Nanotechnology is an interdisciplinary Field with contributions from physics, chemistry, biology, materials science, medicine and other disciplines. Remarkable growth in this up-and-coming technology has opened novel fundamental and applied frontiers, including the synthesis of nanoscale materials and exploration or utilization of their exotic physicochemical and optoelectronic properties. The major advantage of using plant extracts for gold nanoparticles synthesisis that they are easily available, safe, and nontoxic in most cases, have a broad variety of metabolites that can aid in the reduction of Gold ions, and are quicker than microbes in the synthesis. The main mechanism considered for the process is plant-assisted reduction due to phytochemicals. The main phytochemicals involved are terpenoids, flavones, ketones, aldehydes, amides, and carboxylic acids. Flavones, organic acids, and quinines are water-soluble phytochemicals that are responsible for the immediate reduction of the ions. Cytostatic drugs used nowadays in chemotherapy have several disadvantages that have to solve for a better treatment of patients. One huge disadvantage is the general toxicity of most cytostatic drugs, thereby leading to severe side effects during cancer therapy such as depression of the immune system, fatigue, nausea and others. Therefore, anticancer drugs need urgently to be improved and more specific. Thus, targeting molecules can attached to the NP surface, leading to a specific interaction with cells having suitable receptors. Appropriate target molecules include peptides, proteins, enzymes and antibodies, depending on the aspired application [2], [3]. NPs with active targeting functionalities has classified as third generation NPs and are capable of specifically recognizing their target

2. MATERIALS AND METHODS

Fresh leaves of *C. roseus,* were collected from in Tiruchirappalli district, Tamil Nadu, and 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 has incubated for 30 min. and then subjected to centrifuge for 30 min at room temperature with 5000 rpm. The supernatant has separated and filtered with (mm filter paper) filter paper with the help of vacuum filter. Then the solution has used for the reduction of gold ions (Au^{+}) to gold nanoparticles (Au^{o}).

Synthesis of gold nanoparticles

For the synthesis of Gold nanoparticles, gold chloride prepared at the concentration of 10^{-3} M with pre-sterilized Milli Q water has used. A quantity of 1.5 ml of each extract has mixed with 30 ml of

10^{-3} M of gold chloride for the synthesis of gold nanoparticles. Gold chloride has taken in similar quantities of 1.5 ml each without adding plant extracts to main respective controls. The saline bottles has tightly covered with aluminium foil in order to avoid photo reduction of gold ions, incubated at room temperature under dark condition and observations has recorded.

Characterization of gold nanoparticles

UV-vis analysis

The optical property of AuNPs was determined by UV-Vis spectrophotometer (Perkin-Elmer, Lamda 35, Germany). After the addition of $HAuCl_4$ to the plant extract, the spectra's has taken different time intervals up to 24hrs between 450 nm to 540 nm. Then the spectrum has taken after 24hrs of $HAuCl_4$ addition.

FTIR analysis

The chemical composition of the synthesized Gold nanoparticles has studied by using FTIR spectrometer (perkin-Elmer LS-55- Luminescence spectrometer). The solutions has dried at 75^o C and the dried powders has characterized in the range 4000–400 cm-1 using KBr pellet method.

SEM analysis

The morphological features of synthesized gold nanoparticles from *C. roseus* plant extract has studied by Scanning Electron Microscope (JSM-6480 LV). After 24Hrs of the addition of $HAuCl_4$, the SEM slides were prepared by making a smear of the solutions on slides. A thin layer of platinum has coated to make the samples conductive. Then the samples has characterized in the SEM at an accelerating voltage of 20 KV.

DLS- Particle size distribution and Zeta potential analysis

Dynamic light scattering (DLS) which has based on the laser diffraction method with multiple scattering techniques has employed to study the average particle size of gold nanoparticles. The prepared sample has dispersed in deionized water followed by ultra-sonication. Then solution has filtered and centrifuged for 15 min. at 25^0C with 5000 rpm and the supernatant has collected. The supernatant has diluted for 4 to 5 times and then the particles distribution in liquid has studied in a computer controlled particle size analyzer (ZETA sizer Nanoseries, Malvern instrument Nano Zs).

Testing of antimicrobial activity

The test strains: *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 has obtained from MTCC, Chandigarh and NCIM, Pune, India. Microbial strains has tested for antimicrobial sensitivity using the disc diffusion method

(Bauer et al 1966). This method has used to evaluate in vitro antibacterial and antifungal activity of test sample against certain human pathogenic microorganisms on Muller Hinton agar (MHA) and potato dextrose agar (PDA), respectively. A sterile cotton swab has used to inoculate the standardized bacterial suspension on surface of agar plate. The 15 and 30 µL of test solutions were poured in each disc (6 mm diameter), separately. One separate disc has used for control study by taking sterile triple distilled water (without test sample). The plates has incubated at 37±1°C for 24–48 h (for bacteria) and 25-±1°C for 48-72 h (for fungus). After incubation, the zone of inhibition has measured with ruler/HiAntibiotic ZoneScale-C. The assays has performed in triplicate and the average values has presented. Methicillin – 10mcg (for bacteria) and Itraconazole – 10mcg (for fungus) has used as positive control. All the media, standard discs and HiAntibiotic ZoneScale-C has purchased from Hi-Media (Mumbai, India).

Cytotoxicity assay (MTT assay)

The biosynthesized Au nanoparticles has dissolved in DMSO. Diluted in culture medium and used to treat the chosen cell line (HeLa) over a sample concentration (10 different concentrations - 0.6, 1.2, 2.5, 5, 7.5, 10, 15, 20, 25 and 30 µg/ml) range of 0.6 - 30 µg/ml for a period of 24 h and 48 h. DMSO solution has 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) has carried out according to the method described by [4],[5]. To each well, 20 µl of 5 mg/ml MTT in phosphate-buffer (PBS) has added. The plates has wrapped with aluminium foil and incubated for 4 h at 37^0C. The purple formazan product has dissolved by addition of 100 µl of 100% DMSO to each well. The absorbance was monitored at 570 nm (measurement) and 630 nm (reference) using a 96 well plate reader (Bio-Rad, Hercules, CA, USA). Data has collected for four replicates. Each and used to calculate the respective means. The percentage of inhibition has calculated, from this data, using the formula:

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

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

3. RESULTS AND DISCUSSION

Characterizations of AuNPs

UV-Vis spectrophotometer analysis

Reduction of gold salt into gold nanoparticles during exposure to plant extracts has observed as result of the colour change. The colour change is due to the Surface Plasmon Resonance (SPR) phenomenon. The metal nanoparticles have free electrons, which give the SPR absorption band,

due to the combined vibration of electrons of metal nanoparticles in resonance with light wave. The sharp bands of gold nanoparticles has observed around 540 nm in case of *C. roseus*. From different literatures, it has found that the gold nanoparticles show SPR peak at around 540 nm. From our studies, we found the SPR peak for *C. roseus* at 540 nm. Therefore, we confirmed that *C. roseus* leaf extract has more potential to reduce Au ions into Au nanoparticles, which lead us for further research on synthesis of gold nanoparticles from *C. roseus* leaf extracts. The intensity of absorption peak increases with increasing time. This characteristic colour variation is due to the excitation of the SPR in the metal nanoparticles. The reduction of the metal ions occurs rapidly; more than 90% of reduction of Au+ ions is complete within 2 hrs. after addition of the metal ions to the plant extract. The metal particles has observed to be stable in solution even 4 weeks after their synthesis. By stability, we mean that there was no observable variation in the optical properties of the nanoparticles solutions with time. On the behalf of UV-vis data, it has cleared that reduces metal ions. Therefore, the further characterizations has carried out with *C. roseus* (Figure. 1). The UV-Vis absorption spectroscopy is one of the main techniques followed to examine size and shape of the nanoparticles in the aqueous suspensions [6]. Huang [7] reported formation of gold nanoparticles when constant aqueous $HAuCl_4$ at 50 ml, 1 mM with 0.1 g biomass produced gold nanoparticles as indicated by sharp absorbance at around 540 nm in *Cinnamomum camphora*.

FTIR analysis

FTIR measurements has carried out to identify the biomolecules for capping and efficient stabilization of the metal nanoparticles synthesized. The FTIR spectrum of gold nanoparticles wherein some pronounced absorbance has recorded in the region between 4000 and 400 cm^{-1}. The FTIR spectra of AuNPs shown in Table.1, Figure 2. The main peaks at around 3435, 2334, 2050, 1641 and 658 cm-1. After synthesis, the gold nanoparticles by the *C. roseus* extracts have the broad peak at 3435 cm-1 assigned to O-H stretch also peak at 2334 cm-1 assigned to O-H stretch. The band at 2050 cm-1 and 658 cm-1 are assigned to C-H stretch and The band at 1641 cm-1 are assigned to C=O stretch respectively (Figure. 2). Therefore, proteins and metabolites having functional groups surrounded the synthesized nanoparticles. From the analysis of FTIR studies we confirmed that the carbonyl groups from the amino acid residues and proteins has the stronger ability to bind metal indicating that the proteins could possibly from the metal nanoparticles[8], [9], [10] (i.e.; capping of gold nanoparticles) to prevent Agglomeration and thereby stabilize the medium. This suggests that the biological molecules could possibly perform dual functions of formation and stabilization of gold nanoparticles in the aqueous medium. Carbonyl groups proved that flavanones or terpenoids absorbed on the surface of metal nanoparticles [11], [2], [13]. Flavanones or terpenoids has adsorbed on the surface of metal nanoparticles, possibly by

interaction through carbonyl groups or π-electrons in the absence of other strong ligating Agents in sufficient concentration [14], [15]. The presence of reducing sugars in the solution could be responsible for the reduction of metal ions and formation of the corresponding metal nanoparticles [16], [17]. It is also possible that the terpenoids play a role in reduction of metal ions by oxidation of aldehyde groups in the molecules to carboxylic acids [18], [19]. These issues has addressed once the various fractions of the plant extract has separated, identified and individually assayed for reduction of the metal ions. This rather elaborate study is currently underway [20], [21].

SEM analysis

SEM provided further insight into the morphology and size details of the gold nanoparticles. Comparison of experimental results showed that the diameters of prepared nanoparticles in the solution have sizes several nano meters i.e. between 1-100 nm. The size has more than the desired size as result of the proteins, which has bound in the surface of the nanoparticles (Figure 3).

DLS - Particle Size Distribution (PSD) analysis

The particle size distribution (PSD) of synthesized gold nanoparticles, it was found that Au nanoparticles size were in the range of 80-120nm. However, beyond 100 nm range the percentage of nanoparticles present is very less. The highest fraction of AuNPs present in the solution has of 73nm is very appropriate since it gives lowest average size of nanoparticles (Figure 4).

Zeta potential analysis

The Figure 5 shows the zeta potential (ζ) is a measure of the electrostatic potential on the surface of the nanoparticles and has related to the electrophoretic mobility and stability of the suspension of nanoparticles of the nanogold. The overall absorbance of Zeta Potential revealed the energetically very unstable. Therefore, the particles undergo Agglomeration/Aggregation to stabilize themselves. Therefore, there were some potential charges on the surface of the nanoparticles, which makes them stable. These charge potential we got from this analysis. Zeta potential (Surface potential) has direct relation with the stability of a form/structure as mentioned below (Figure 5).

Antimicrobial activity

Gold nanoparticles has tested in triplicates for antimicrobial activity. The values has recorded and averaged (Tables 2). *C. roseus* has tested and recorded the results for the gram-positive, gram-negative bacteria and fungi. The gram-positive were highly sensitive than gram-negative bacteria. Selected microorganisms has showed significant sensitivity against the biosynthesized nanoparticles. The antimicrobial activity of test sample has examined with various pathogenic microorganisms using the (measure the inhibition zone) Disc diffusion test. In the present study, higher (30 μL/disc) concentration of sample got greater sensitivity than (15 μL/disc) lower

concentration in all the tested microorganisms. In this study, all the pathogens has fairly affected and nil effect has not observed in the test samples. The gold nanoparticles not only interact at the surface of cell membrane, but also enter inside the bacteria and cause damage of the cells by interacting with phosphorus/ sulfur containing DNA and its replication. In bacteria, the test sample was most effective against B5 while smaller effect has noticed from B4. In fungi, this was effective against F4 whereas smaller effect has observed in F2. All the microbial strains depict higher sensitivity to the higher concentration (30 μL) and he concluded that the gold materials are an efficient alternative to antibiotics for the treatment. This nanoparticles release gold ions in the bacterial cells, which enhance their bactericidal activity. There is no antimicrobial activity in solution devoid of sample used as a vehicle control (sterile triple distilled water), reflecting that antimicrobial activity was directly related to the sample.

MTT assay

The cytotoxic effect of the biosynthesized Au nanoparticles has examined on cultured HeLa cells by exposing cells for 24 h and 48 h to medium containing the complex at 0.6 – 30 g/ml concentration. The Au nanoparticles inhibited the growth of the cancer cells significantly, in a dose and duration dependent manner. The cytotoxic activity has determined according to the dose values to exposure of the complex required to reduce survival of 50% (IC_{50}), compared to untreated cells Figure 6. The Au nanoparticles showed highly effective cytotoxic activity against HeLa cells. The cytotoxic effect of the sample may be interpretable as due to its amphiphilic nature[22], [23], [24] and, hence, would penetrate the cell membrane easily, reduce the energy status in tumours and also alter hypoxia status in the cancer cell micro environment, which are factors that would influence the antitumor acidity[25], [26], [27]. It has known that biosynthesized Au nanoparticles have a wide range of biological activities such as antitumor, apoptosis, interaction with DNA thereby inhibiting replication, transcription, and other nuclear functions also arresting cancer cell proliferation to arrest tumour growth [28], [29], [30].

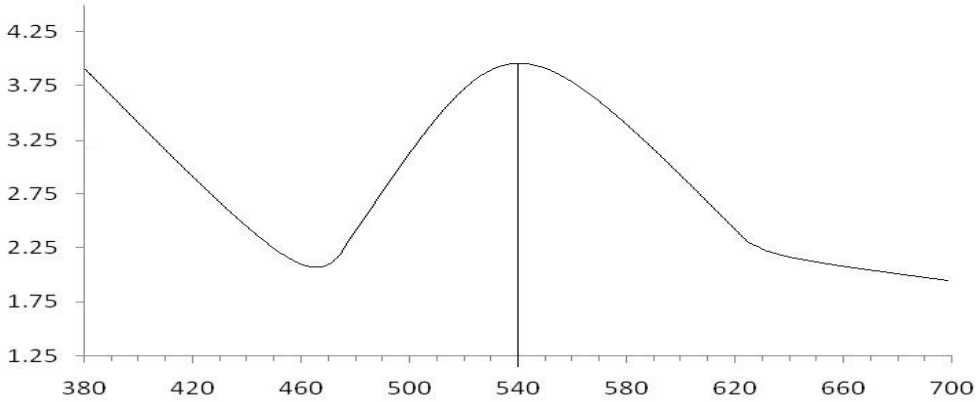


Figure 1. UV-VIS spectral analysis of Au nanoparticles

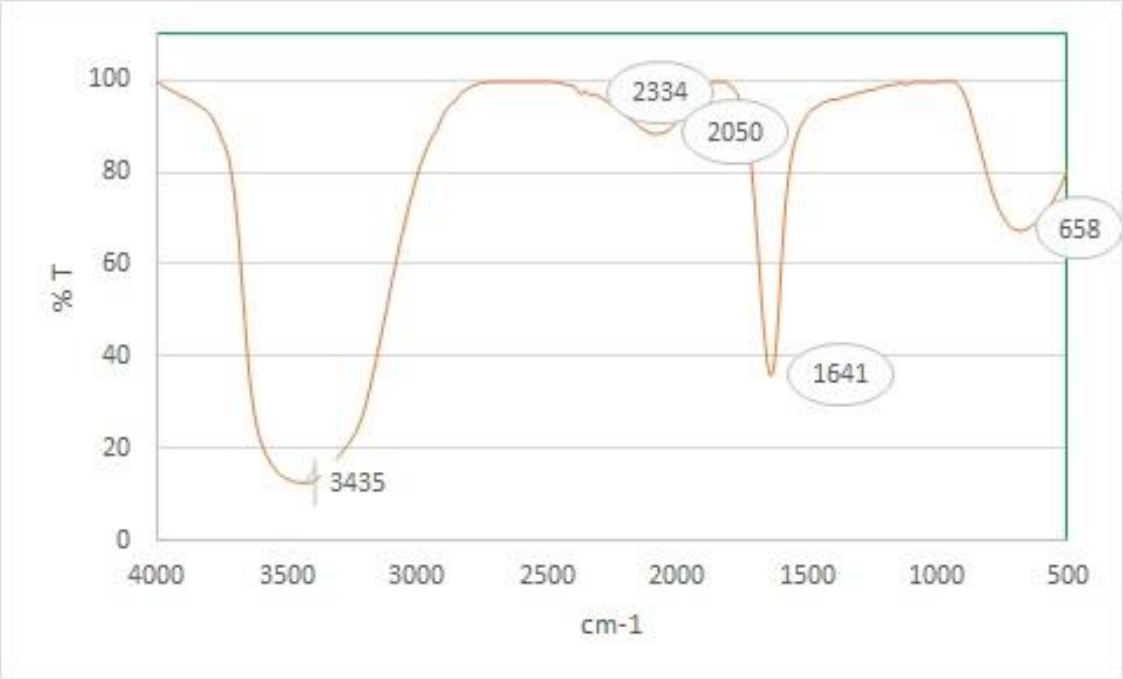


Figure 2. FTIR analysis of vibration modes and function groups of AuNPs

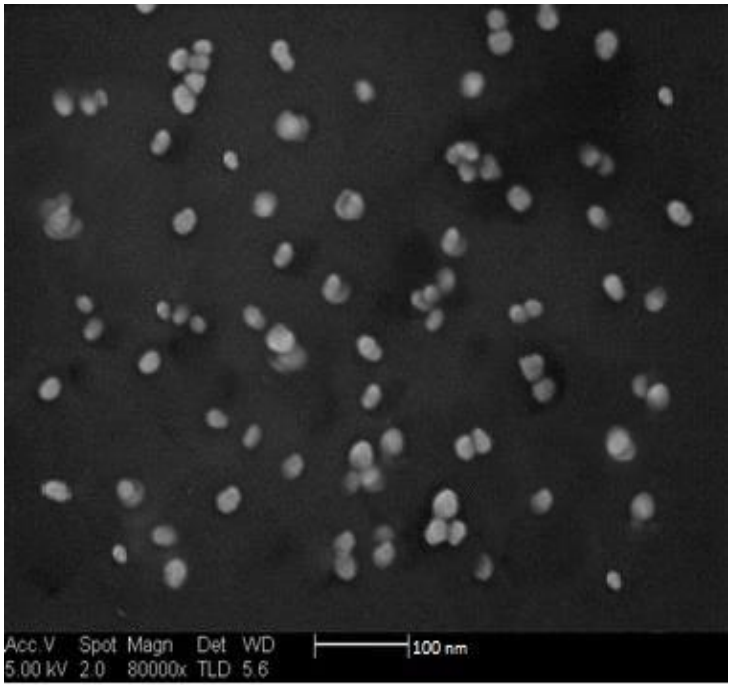
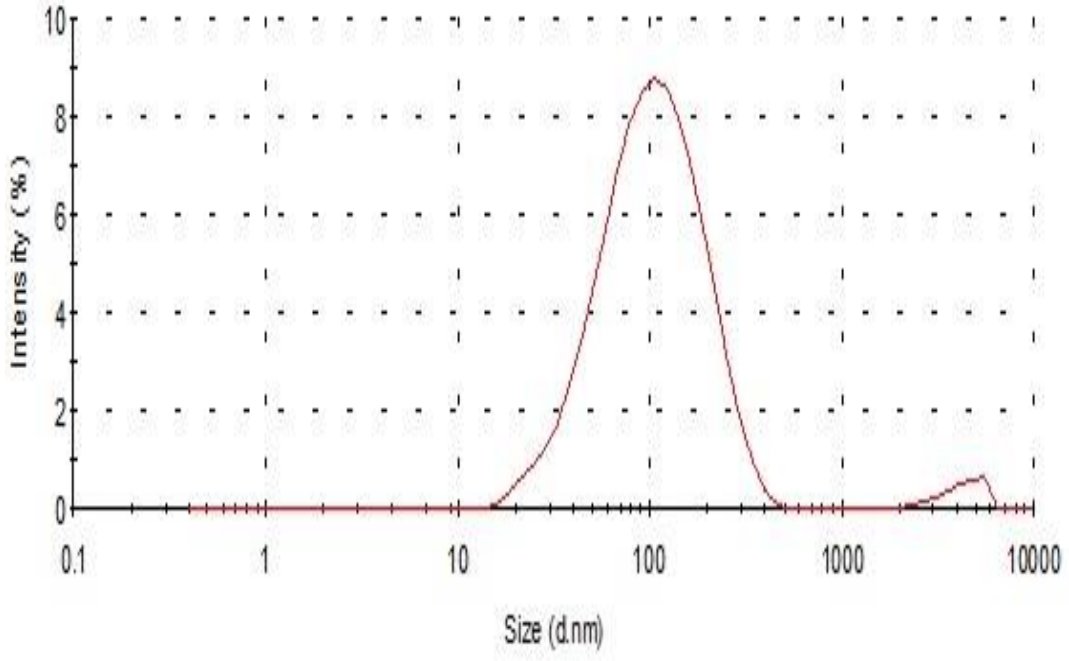
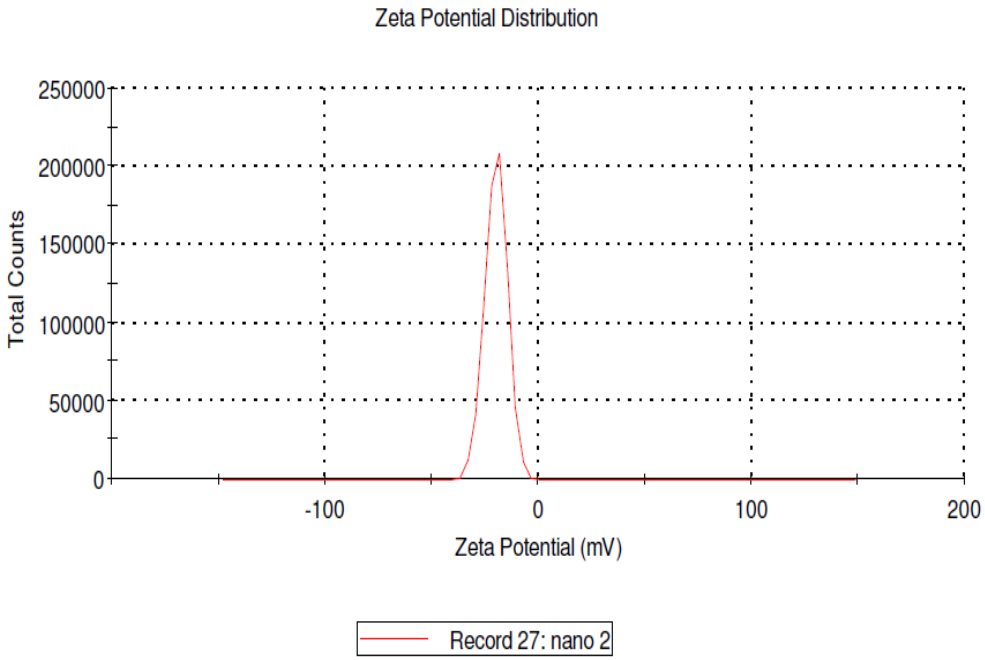


Figure 3. SEM –microscopic view of *C. roseus* reduced gold nano particles



C. roseus (Gold Nano particles); Z-Average (d.nm): 73.26

Figure 4. Dynamic Light Scattering of Particle Size Analyzer of Au Nanoparticles



Zeta Potential (mV): from ±10 to ±30 = Incipient instability

Figure 5. Zeta Potential Measurement of Au Nanoparticles

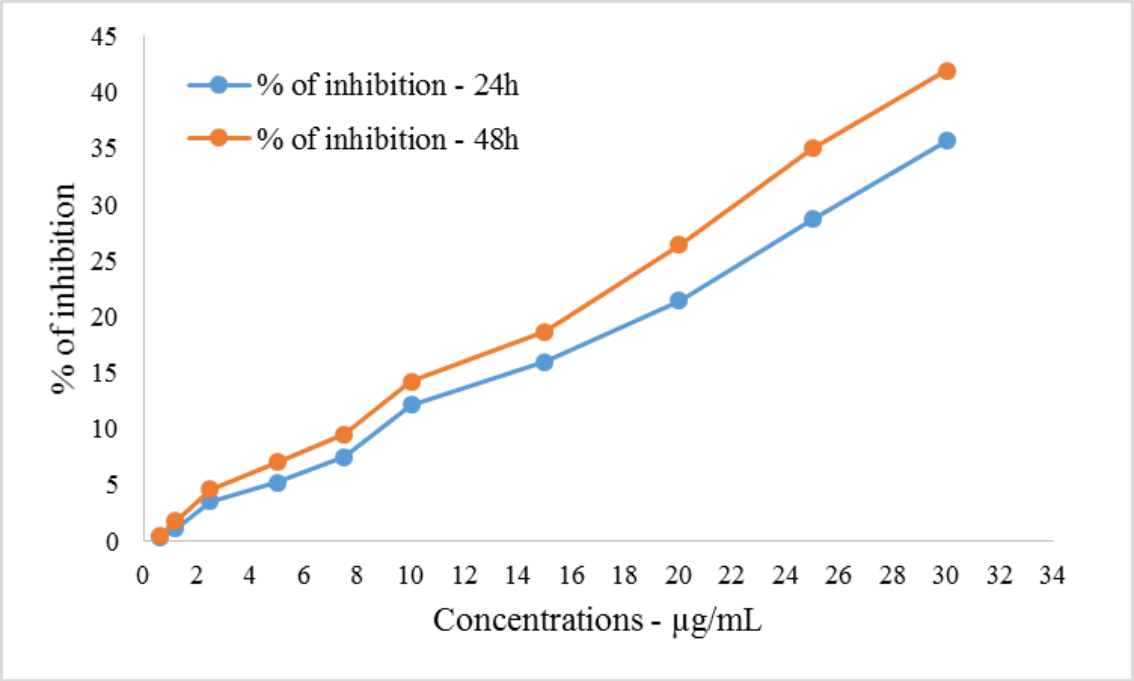


Figure. 6 Cytotoxicity of AuNPs (MTT assay)

Table 1. Vibration modes of synthesized NP's

Wave number (cm^{-1})	Vibration modes
3435	O-H Stretch
2334	O-H Stretch
2050	C-H Stretch
1641	C=O Stretch
658	C-H bond

Table 2. Antimicrobial screening of AuNPs derived by *C. roseus* leaves

S.No	Test Microorganisms	Zone of inhibition (mm) Sample (15 & 30) µL / disc				Diseases	Route of Transmission
		15 µL	30 µL	PC	Remarks		
	Bacteria						
1	*Aeromonas liquefaciens* B1	17	19	14	> PC	Wound Infections / Gastroenteritis	Water / Food
2	*Enterococcus fecalis* B2	14	16	8	> PC	Endocarditis / Bladder, Prostate, and Epididymal Infections / Nervous system Infections	Water / Food
3	*Micrococcus luteus* B3	12	17	38	< *PC*	Skin & Pulmonary infections / Septic shock / Pneumonia endocarditis	Soil / Dust / Water / Airways / Food
4	*Salmonella typhimurium* B4	13	14	0	> PC	Typhoid	Water / Food
	Fungi						
5	*Candida albicans* F1	13	15	10	> PC	Skin (Integument) Infections / Gastrointestinal tract Infection	Airways / Wound / Soil / Water
6	*Cryptococcus* sp. F2	11	12	9	> PC	Cryptococcal disease / Bronchiectasis / Endophthalmitis.	Airways / Wound / Soil / Water
7	*Microsporum canis F3*	12	13	9	> PC	Tinea capitis /Ringworm	Airways / Wound / Soil / Water
8	*Trichophyton rubrum* F4	14	16	7	> PC	Tinea corporis / Tinea cruris / Tinea pedis / Onychomycosis	Airways / Wound / Soil / Water

PC - Positive Control (Using antibiotic disc; Bacteria – Methicillin (10mcg/disc); Fungi – Itraconazole (10mcg/disc); Samples - 15 µL / disc & 30 µL / disc; > PC – greater than positive control; < PC – less than positive control

4. CONCLUSION

Instead of the boiled leaf broth method followed in the previous studies, leaves of *C.roseus* appear to be environmental-friendly and low-cost aspirant as a reductant for synthesizing gold nanoparticles. This procedure has extended to the synthesis of other nanoparticles from different chemical compositions. Synthesis of nanoparticles has many advantages by scale up of each process because of its economic viability, possibility of covering large surface area easily by suitable growth of the filaments, etc. Equally, the synthesis of metal ion reduction or reaction process in cellular metabolism explaining whether the nanoparticles formed as by-products of the process has role to play in a cellular activity. In this low cost procedure, effective synthesis of nanoparticles will have greater implication and application in biomedical research.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

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