

BIOGENIC GOLD NANOPARTICLES SYNTHESIS THROUGH *ADATHODA VASICA* AND ITS ANTIMICROBIAL AND ANTICANCER EFFICACY

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ABSTRACT

The exploitation of bio (plant) materials for the biosynthesis of nanoparticles is considered a green technology as it does not involve any harmful chemicals. The present study reports the synthesis of gold (Au) nanoparticles from HAuCl_4 using the powder of novel *Adathoda vasica*. The secondary metabolites were responsible for the reduction of gold metal to nano-sized Au nanoparticles. The different techniques such as UV-vis, FTIR, SEM and DLS results confirmed the presence of nano-crystalline Au particles. In antimicrobial activity, the test sample against bacterial strains were most effective on *Micrococcus luteus* B3 while smaller effect was noticed from *Salmonella typhimurium* B4. In fungi, this was effective against *Trichophyton rubrum* F4 whereas smaller effect was observed in *Cryptococcus* sp. F2. The 50% (IC_{50}) value of cytotoxic activity was observed in 50 μL concentration of sample and was enough to control

the cancerous HeLa cells.

KEYWORDS: *Adathoda vasica*, Gold nanoparticles, Antimicrobial activity, Anticancer activity.

INTRODUCTION

The World Health Organization estimates that 80% of the people in developing countries of the world rely on traditional medicine for their primary health care, and about 85% of traditional medicine involves the use of plant extracts. This means that about 3.5 to 4 billion people in the world rely on plants as sources of drugs (Farnsworth 1988). Natural products

once served humankind as the source of all drugs, and higher plants provided most of these therapeutic agents. Last two decades, the combination of natural and chemical materials are prepared for the modern diseases. Now a days, the scientists are interested in preparing the nanoparticles synthesis in low cost and is used for all the purposes. The 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 be used to generate new techniques and devices (Schmid et al, 2009).

Recently, people are interest in the preparation and characterisation of nanostructured materials has dramatically increased due to their extraordinary electronic, magnetic, catalytic and optical properties, which give rise to versatile application possibilities in catalysis as well as in medical, optical and electronic fields (Vignesh et al., 2014). Depending on particle size, material or chemical structure a large variety of properties can be controlled and tailored depending on the needs of a specific application. Nanoparticles (NPs) can be made from almost any material including metals (Au, Ag, Pt, etc.), semiconductors (quantum dots e.g. from CdS, CdSe), insulators (e.g. SiO₂) or magnetic materials (e.g. super paramagnetic iron oxide nanoparticles, called SPIONs). All these classes of particles enrich specific fields of application depending on their unique properties. The materials can include nanoparticles with dimensions of less than 100 nm as well as patterned surfaces and more sophisticated assemblies. 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 (Pandiyarajan et al., 2013).

Gold nanoparticles have aroused great interest in the field of medical applications due to the inert nature and the biocompatibility of gold as well as the well-known chemistry of nano-gold (Wang 1991). One example in the field of therapeutics is the nano enabled hypothermia treatment, in which the nanoparticles are bound to specific target cells or proteins and irradiated with laser light. The gold nanoparticles convert the light to highly localized heat, which leads to damage of the tagged cells exclusively, thus enabling the destruction of specific cells with high selectivity and efficiency. Many diseases are considered treatable by this technique, including cancer which is one of the leading causes of mortality in the world (Colvin and Alivisatos 1994; Wang 1991). Inorganic nonmaterial have been widely used for cellular delivery due to their versatile features like wide availability, rich functionality, good compatibility, and capability of targeted drug delivery and controlled release of drugs

(Xu et al, 2006). The main object of the present study is to synthesize the Au nanoparticles by using green plants (*Adathoda vasica*) with low cost. And also to understand the antimicrobial/ anticancer efficacy of biosynthesized Ag nanoparticles.

MATERIALS AND METHODS

Plant extract preparation

Fresh leaves of *Adathoda vasica*, 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 (Figure 1). Then plant extract was prepared by mixing 1% of plant extract with deionized water. 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 (Au^0).

Synthesis of gold nanoparticles (AuNPs)

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

Characterization of gold nanoparticles

UV-vis analysis

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

FTIR analysis

The chemical composition of the synthesized gold nanoparticles was studied by using FTIR spectrometer (Perkin-Elmer LS-55- Luminescence spectrometer). The solutions were dried at 75°C and the dried powders were characterized in the range $4000 - 400\text{ cm}^{-1}$ using KBr

pellet method.

SEM analysis

The morphological features of synthesized gold nanoparticles from *A. vasica* plant extract were studied by Scanning Electron Microscope (JSM-6480 LV). After 24Hrs of the addition of H₂AuCl₄ the SEM slides were prepared by making a smear of the solutions on slides. A thin layer of platinum was coated to make the samples conductive. Then the samples were characterized in the SEM at an accelerating voltage of 20 KV.

DLS and Zeta potential analysis

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

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), *Microsporium 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 disc diffusion method (Vignesh et al. 2012a; Vignesh et al., 2015a). This method was 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 (Vignesh et al., 2013; Vignesh et al., 2015b). A sterile cotton swab was 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 was used for control study by taking sterile triple distilled water (without test sample). The plates were incubated at 37±1°C for 24–48 h (for bacteria) and 25 ±1°C for 48-72 h (for fungus) (Vignesh et al., 2012b). After incubation, the zone of inhibition was measured with ruler/HiAntibiotic ZoneScale-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 (Vignesh *et al.*, 2014). All the media, standard discs and HiAntibiotic ZoneScale-C were purchased from Hi-Media (Mumbai, India).

Testing of anticancer activity

For anticancer study, an in-vitro and AuNPs samples were dissolved in DMSO, diluted in culture medium and used to treat the chosen cell line (Hep G2) (obtained from NCCS) 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. The 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 standard procedure (Lakshmi praba *et al.*, 2013). To each well, 20 µl of 5 mg/mL MTT in phosphate-buffer (PBS) was added and wrapped with aluminum foil, and incubated for 4 h at 37 °C. The purple formazan product was 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 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.

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

The IC₅₀ value was determined as the complex concentration that is required to reduce the absorbance to half that of the control.

RESULTS AND DISCUSSION

UV-Vis spectrophotometer analysis

Reduction of gold salt into gold nanoparticles during exposure to plant extracts was observed as a 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 were observed around 540 nm in case of *A. vasica*. From different literatures it was found that the gold nanoparticles show SPR peak at around 540 nm. From our studies we found the SPR peak for *A. vasica* at 540 nm. So we confirmed that *A. vasica* leaf extract has more potential to reduce Au ions into

Au nanoparticles, which lead us for further research on synthesis of gold nanoparticles from *A. vasica* leaf extracts. The intensity of absorption peak increases with increasing time period. This characteristic color variation is due to the excitation of the SPR in the metal nanoparticles. The reduction of the metal ions occurs fairly 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 were 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 was cleared that reduces metal ions. So the further characterizations were carried out with *A. vasica* (Figure 2). The UV-Vis absorption spectroscopy is one of the main techniques followed to examine size and shape of the nanoparticles in the aqueous suspensions (Wiley et al. 2006). Huang et al. (2007) reported formation of gold nanoparticles when constant aqueous HAuCl₄ 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 were 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 were recorded in the region between 4000 and 400 cm⁻¹. The FTIR spectra of AuNPs and plant extracts are shown in Figure 3a and 3b. In *A. vasica* extract, the main peaks at around 3270, 2900, 1616, 1388, 766, 672 cm⁻¹ whereas, the gold nanoparticles in the presence of *A. vasica* extract shows Figure 3a the major peaks at 3434, 2361, 2076, 1636, 1403.45, 1113.74 and 670.92 cm⁻¹. *A. vasica* extract shows the peak at 3270 cm⁻¹ can be assigned to O-H stretch and peak at 2900 cm⁻¹ corresponds to C-H stretch. The band at 1616 cm⁻¹ is assigned to C=O stretch. The band found at 1388 cm⁻¹ can be assigned to C-O-C stretch. Another band at 766 cm⁻¹ and 672 cm⁻¹ assigned to C-H bend and C-H bond respectively. After synthesis the gold nanoparticles by the *A. vasica* extracts have the broad peak at 3434 cm⁻¹ assigned to O-H stretch and also peak at 2361 cm⁻¹ assigned to O-H stretch. The band at 2076 cm⁻¹ and 1636 cm⁻¹ are assigned to C-H stretch and C=O stretch respectively. The band found at 1403.45 cm⁻¹ and 113.74 cm⁻¹ are assigned to C-O-C stretch. Another band found at 670.92 cm⁻¹ assigned to C-H bond. Therefore the synthesized nanoparticles were surrounded by proteins and metabolites having functional groups. 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 (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 adsorbed on the surface of metal nanoparticles. Flavanones or terpenoids could be 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. The presence of reducing sugars in the solution could be responsible for the reduction of metal ions and formation of the corresponding metal nanoparticles. It is also possible that the terpenoids play a role in reduction of metal ions by oxidation of aldehydic groups in the molecules to carboxylic acids. These issues can be addressed once the various fractions of the plant extract are separated, identified and individually assayed for reduction of the metal ions. This rather elaborate study is currently underway.

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 was more than the desired size as a result of the proteins which were bound in the surface of the nanoparticles (Figure 4).

DLS 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 was of 73nm is very appropriate since it gives lowest average size of nanoparticles (Figure 5).

Zeta potential analysis

The Figure 6 shows the zeta potential (ζ) is a measure of the electrostatic potential on the surface of the nanoparticles and is 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. So 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 6).

Antibacterial and antifungal screening

Gold nanoparticles were tested in triplicates for antimicrobial activity. The values were recorded and averaged (Tables 1). *A. vasica* 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 were showed significant sensitivity against the biosynthesized nanoparticles. The antimicrobial activity of test sample was examined with various pathogenic microorganisms using the (measure the inhibition zone) Disc diffusion test. The results of the antimicrobial activities are summarized in Plates 1-2. In the present study, higher (30 $\mu\text{L}/\text{disc}$) concentration of sample got greater sensitivity than (15 $\mu\text{L}/\text{disc}$) 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. 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/ sulphur containing DNA and its replication (Anitha et al., 2011). 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) and he concluded that the silver materials are an efficient alternative to antibiotics for the treatment. This nanoparticles release silver ions in the bacterial cells, which enhance their bactericidal activity (Hussain Beevi et al., 2012). 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.

Anticancer activity

The cytotoxic effect of the AuNPs were examined on human cell lines (HeLa cells) for 24 h and 48 h (Sample conc. = 0.1 – 50 μL). The cytotoxicity effect is very high in biosynthesized AuNPs against HeLa cell lines (Figure 7). The AuNPs inhibited the growth of the cancer cells significantly, in a dose and duration dependent manner. The cytotoxic activity was finding according to the dose values of the exposure of the complex required to reduce survival to 50% (IC_{50}), compared to untreated cells. In AuNPs, the 50 μL sample is enough to control cancerous cell (Figure 8). 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 tumours and also alter hypoxia status in the cancer cell. The cytotoxicity effect was compared with the standard anticancer drug 5-FU against HeLa cells and their LC_{50} value was observed (Lokina and Narayanan, 2013). Similarly cytotoxicity of chemically synthesized AuNPs was reported against HeLa cells by Miura and Shinohara. A large number of in vitro studies indicate that AuNPs are toxic to the mammalian cells. Interestingly, some studies have shown that AuNPs has the potential to intervene genes associated with cell cycle progression, also induce DNA damage and apoptosis in cancer cells. Indeed, the results of present study provide conclusive evidence for cytotoxic effect of AgNPs on cancer cell lines rather than normal cell lines.



Figure 1: *Adathoda vasica* plant and their powder form.

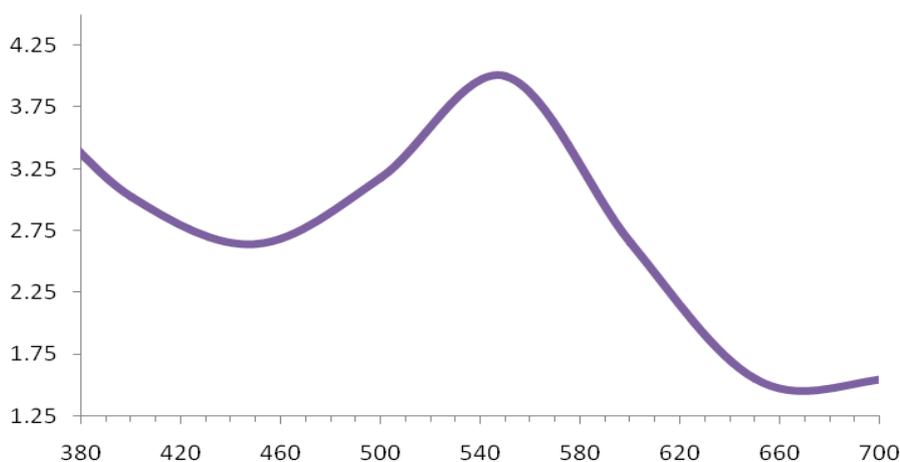


Figure 2. UV-VIS spectral analysis of Au nanoparticles.

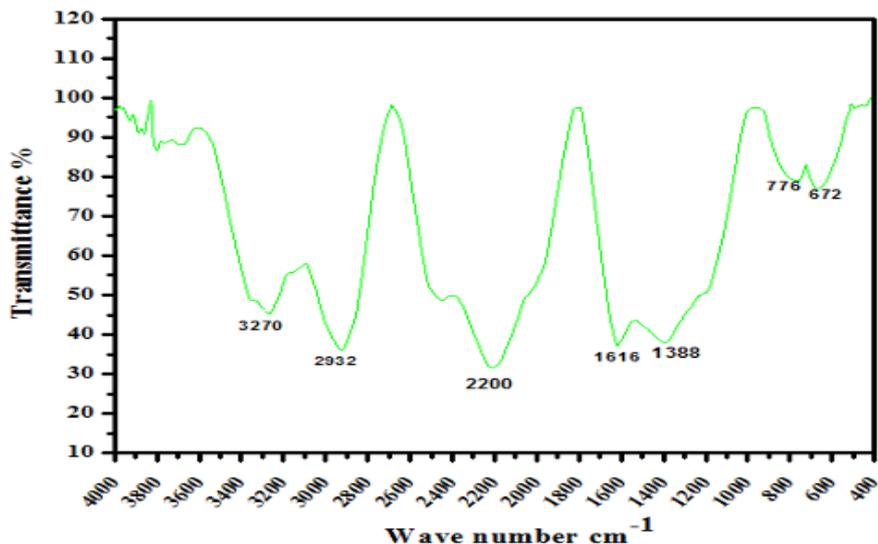


Figure 3a. FTIR analysis of vibration modes and function groups of *A. vasica*

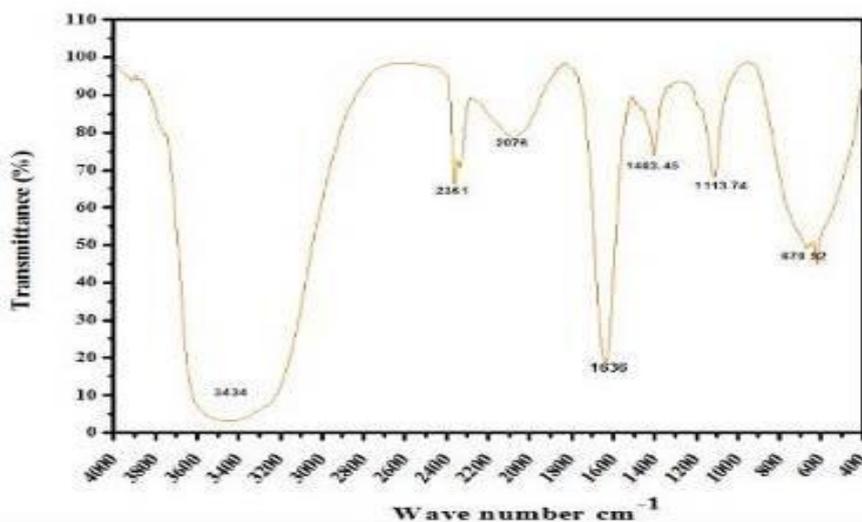


Figure 3b. FTIR analysis of vibration modes and function groups of AuNPs

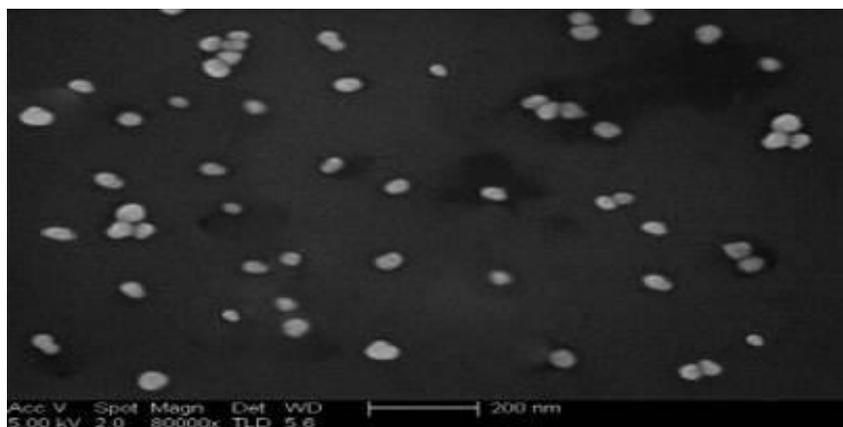
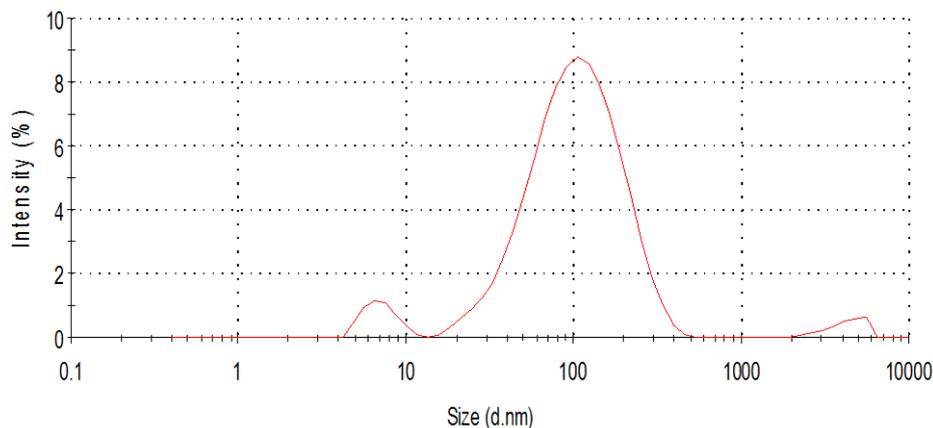
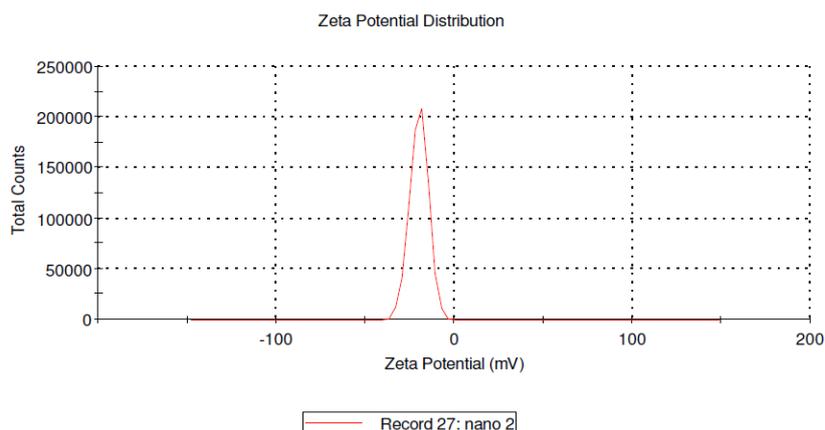


Figure 4. SEM –microscopic view of *A. vasica* reduced gold nano particles.



***A. vasica* (Gold Nano particles); Z-AverAue (d.nm): 73.84**

Figure 5. Dynamic Light Scattering of Particle Size Analyser of Au Nanoparticles



Results

Zeta Potential (mV): -19.7	Mean (mV)	Area (%)	Width (mV)
Zeta Deviation (mV): 5.29	Peak 1: -19.7	100.0	5.29
Conductivity (mS/cm): 0.820	Peak 2: 0.00	0.0	0.00
Result quality : Good	Peak 3: 0.00	0.0	0.00

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

Figure 6. Zeta Potential Measurement of Au Nanoparticles



Control



0.1 (µl)

Table 1: Antimicrobial screening of AuNPs derived by *A. vasica* leaves.

S.No	Test Microorganisms	Zone of inhibition (mm) Sample (15 & 30) μ L / disc				Diseases	Route of Transmission
		15 μ L	30 μ L	PC	Remarks		
Bacteria							
1	<i>Aeromonas liquefaciens</i> B1	15	19	14	> PC	Wound Infections / Gastroenteritis	Water / Food
2	<i>Enterococcus faecalis</i> B2	17	18	8	> PC	Endocarditis / Bladder, Prostate, and Epididymal Infections / Nervous system Infections	Water / Food
3	<i>Micrococcus luteus</i> B3	18	21	38	< PC	Skin & Pulmonary infections / Septic shock / Pneumonia endocarditis	Soil / Dust / Water / Airways / Food
4	<i>Salmonella typhimurium</i> B4	14	16	0	> PC	Typhoid	Water / Food
Fungi							
5	<i>Candida albicans</i> F1	11	13	10	> PC	Skin (Integument) Infections / Gastrointestinal tract Infection	Airways / Wound / Soil / Water
6	<i>Cryptococcus</i> sp. F2	11	12	9	> PC	Cryptococcal disease / Bronchiectasis / Endophthalmitis.	Airways / Wound / Soil / Water
7	<i>Microsporum canis</i> F3	12	13	9	> PC	Tinea capitis / Ringworm	Airways / Wound / Soil / Water
8	<i>Trichophyton rubrum</i> F4	12	15	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)

> PC – greater than positive control; < PC – less than positive control

CONCLUSIONS

The rapid biological synthesis of gold nanoparticles using *A. vasica* leaves extract provides environmental friendly, simple and efficient route for synthesis of benign nanoparticles. The synthesized nanoparticles were of spherical and sheet shaped and the estimated sizes were 60-100 nm and other techniques were confirmed the reduction of gold nanoparticles. The cytotoxicity effect is very high in biosynthesized AuNPs against HeLa cell lines. In AuNPs, the 50 μ L sample is enough to control cancerous cell. In antimicrobial activity, the higher (30 μ L/disc) concentration of sample got greater sensitivity than (15 μ L/disc) lower concentration in all the tested microorganisms. Interestingly, the gram-positive were highly sensitive than gram-negative bacteria.

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