WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 6.805
Volume 5, Issue 4, 1072-1085. Research Article ISSN 2277– 7105

GREEN SYNTHESIS OF GOLD NANOPARTICLES (Au-NPs) USING *Barleria cristata* AND STUDY THEIR PHARMACOLOGICAL APPLICATIONS

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Article Received on
26 Jan 2016,
Revised on 22 Feb 2016,
Accepted on 13 Mar 2016
DOI: 10.20959/wjpr20164-5894

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ABSTRACT

Nano revolution is imperative to integrate nanoscience and medicine. Metal nanoparticles have several applications such as optics, biomedical sciences, drug delivery, catalysis and electronics. The present investigation deals with the green synthesis of gold nanoparticles (AuNP) using leaves extract of Barleria cristata for some pharmacological experiments. Several human pathogens were used to screen the antimicrobial properties and biological mechanism of gold nanoparticles (AuNPs) was studied with Hela cell line for their anticancer potentiality. Aqueous extract (pH 7.4 - inherent pH of the extract) was reacted with 1mM Chloroauric acid (HAuCl4.3H2O) and kept at room temperature. The immediate change in colour from pale yellow to pink indicated the reduction of Au 3+ ions to Au 0. The synthesized AuNP's were monitored using UV-Visible spectrophotometer. X-ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Scanning electron microscopy (SEM), and Dynamic Light Scattering (DLS). The in-vitro antimicrobial properties were confirmed by disc diffusion method on some human pathogens and their anticancer activity confirmed by MTT assay on the cell lines of Hela carcinoma cells showed IC_{50} values of extract at 50 µg/mL.

KEYWORDS: Barleria cristata, Anticancer activity, Antimicrobial activity, Gold nanoparticles

INTRODUCTION

Metal nanoparticles are of great importance due to their high surface area and a high fraction of surface atoms. The researchers show much interest in synthesizing nanoparticles and study their size with various applications because of their unique physicochemical characteristics (Beevi et al., 2012). The scientific and technological significance of metal nanoparticles has made them the subject of intensive research, given their special chemical and physical properties. The new or modern nanotechnology embraces all the medical clinical application, green synthesis technique and improvements made in the new nanotechnological process in particular, gold nanoparticles are employed in many fields: biosensing, catalysis, electronics, enzyme electrodes, super conductors and cancer therapy among others (Vignesh et al., 2014).

In recent years, numerous methodologies are developed to synthesize noble metal nanoparticles of particular shape and size depending on specific requirements. Biosynthesis of nanoparticles has an emerging highlight of the intersection of nanotechnology and biotechnology which has received increased attention to a growing need to develop environmentally benign technologies in material syntheses. Biomolecules as reductants are found to have significant advantage over chemical reductants due to their non-biocompatible nature. Synthesis of nanoparticles by using plants is gaining importance due to its effortlessness and eco-friendly (Huang et al. 2007a).

The remarkable antimicrobial effect of metallic nanoparticles is of interest for researchers due to the growing microbial resistance against the antibiotics and development of resistant strains. Uncontrolled growth and spread of abnormal cells lead to cancer and finally results in death. Ethno-pharmacological process on the synthesis of nanoparticles is an amazing technology beneath construction symbiosis between nanoscience and medical sciences. In this regard, the idea of functionalizing gold nanoparticles for antidiabetic nanomaterial by synthesizing pharmacologically key plant materials often been considered. Advances beneath nanotechnology have identified possible candidates for biological and biomedical programs on pharmaceutics, for novel diagnostics and medical agents. The nanoparticle drug delivery system has the advantages of accumulating large amounts of therapeutic drugs in the tumour tissues through the passive and active targeting approach (Anitha et al., 2011). Now a day, the nmaoparticles can used in the water treatment and antifouling agents (Muthukumar et al., 2015).

The green synthesized, characterized and bio-functionalized gold nanoparticles from Barleria cristata were tested for in vitro anticancer activity against Hela carcinoma cells. Our present findings clearly demonstrated that it is indeed possible to have a much greener way to synthesize Au-NPs without compromising their medicinal properties and thus plant extracts may prove to be a good alternative to obtain Au-NPs with improved antimicrobial and anticancer properties.

MATERIALS AND METHODS

Plant collection and leaf extractions

The plant material, B. cristata leaves were collected in the month of January 2016 from Tiruchirappalli district of Tamilnadu. The leaves were washed thoroughly thrice with distilled water, shade-dried up to 5 days and prepared fine powder by grinding. The fine powder of the plant material was sterilized at 121°C for 15 min and weighed. Sterilized fine powder, 20 g each was taken, mixed with 200 ml of Milli Q water and kept in boiling water bath at 60°C for 10 min. The extracts were filtered with Whatman 1 filter paper and the filtered extracts were stored in a refrigerator at 4°C for further studies to avoid microbial contamination.

Biosynthesis of nanoparticles

Biosynthesis 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.

Characterization of nanoparticles

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.

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 analysed.

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 Ag sample was done by the SEM (JEOLJSM 5800) machine. The EDS normally reveals the presence of phases.

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.

Antimicrobial screening

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 disc diffusion method (Pandiyarajan et al., 2013; Lakshmi praba et al., 2013; Vignesh et al., 2012a). 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., 2012b; Vignesh et al., 2013). 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) (Koperuncholan et al., 2010). The plates were 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 was measured with ruler/HiAntibiotic ZoneScale-C (Vignesh et al., 2015a). 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., 2015b). All the media, standard discs and HiAntibiotic ZoneScale-C were purchased from Hi-Media (Mumbai, India).

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 (Hela) (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 (Sinthiya and Koperuncholan, 2015) 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 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 (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,

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

RESULTS AND DISCUSSION

Chloroauric acid was selected for the study because of their high antimicrobial property and commercially more viable. Leaves of B. cristata which is considered as one of the important medicinal plants in the Indigenous Systems of Medicine and one of the food ingredients used as digestive in India, was chosen to study the biosynthesis of gold nanoparticles. The positive results achieved upon intra-articular introduction of colloidal gold into rats with collagen-induced arthritis were described (Koperuncholan, 2015). The authors attribute the positive effect to an increase in anti-angiogenic activity due to the binding between GNP and the vascular endothelial growth factor and, therefore, the decrease in macrophage infiltration and inflammation. Similar results were obtained upon subcutaneous introduction of gold nanoparticles into rats with collagen- and pristan-induced arthritis (Ramesh et al. 2014)

Biosynthesis of gold nanoparticles by B. cristata leaves

After incubation, biosynthesis of nanoparticles was indicated by the change of colours from light yellow to pink for gold nanoparticles Spectroscopic data were analysed to characterize gold nanoparticles.

UV-VIS spectroscopy

The UV-VIS spectroscopic studies revealed the presence of beard peaks at 545 nm. The plasmon resonance of the gold nanoparticles was recorded. When the precursor chloroauric acid solutions was mixed with the plant extracts they were reduced into gold (Au) nanoparticles. When the leaf extract of B. cristata was mixed at 0.1% concentration of the respective chloroauric acid ($HAuCl_4$) aqueous solutions the solutions changed their colour from light yellow to pink for gold nanoparticles. The change in colour is due to the excitation of surface Plasmon vibration, which is indicated by the formation of gold nanoparticles (Figure 1).

Fourier transform infra-red spectroscopy

The FTIR spectrum of AuNps derived from leaf extract is given wherein some pronounced absorbance was recorded in the region between 4000 and 400 cm^{-1}. They include 3356 (secondary amine, free, N-H asymmetric stretching), 2729.17 (alkyl ethers for C-H stretching 1696.91 (β-dikeone, enolic form, C=O) (Figure 2).

Scanning electron microscope with Energy dispersive spectroscopy (SEM/EDS)

SEM absorption of the products was recorded as synthesis of nanoparticles spherical rod, triangle and circular in structure of about 40 nm in diameter (Figure 3). The energy dispersive spectroscopy is an analysis or chemical characterization of a sample. Leaf extract of B. cristata is a promising one for the development of gold nanoparticles. SEM studies showed rod, round and triangular-shaped gold nanoparticles at 40 nm in higher densities. EDS revealed the presence of pure gold nanoparticles in higher percentages. (Figure 4)

Antimicrobial screening

The antimicrobial activity of test sample was examined with various pathogenic microorganisms using the (measure the inhibition zone) disc diffusion test (Anitha et al. 2011). Found that the Au nanoparticles have exhibited considerable activity against some human pathogens. The antimicrobial property of gold is found to be the best among different metals in the following order Au>Zn>Fe>Mn>Mo>Sn (Koperuncholan et al. 2010). The

results of the antimicrobial activities are summarized in Table 1. In the present study, higher (30 µL/disc) concentration of 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.

Koperuncholan and Ahmed john (2011) reported the antimicrobial activity of ethanol extract of leaf of Myristica dactyloides, which showed the maximum activity against Shigella dysenteriae and Salmonella typhi. Suresh et al. (2008) reported the best antimicrobial activity of ethanol extract obtained from Rauvolfia tetraphylla, which showed maximum activity against E. coli and Enterobacter aerogenes, and various tested fungi such as A. niger and Penicillium spp, were found to be more sensitive to crude extract when compared to others. Several phytoconstituents such as terpenoids (Ahmed John and Koperuncholan, 2012), flavonoids (Ahmed John and Koperuncholan, 2012a) and tannins (Fazal Mohamed et al., 2011) are effective antibacterial against a wide range of microorganisms. The results of the present investigation clearly demon-strate the antibacterial and antifungal activities of the ethanol, methanol, acetone, chloroform and petroleum ether extracts of the leaves.

Anticancer activity

The cytotoxic effect of the AuNPs were examined on human cell lines (HeLa cells) for 48 h (Sample conc. = 0.1 – 50 µL). The cytotoxicity effect is very high in biosynthesized AuNPs against HeLa cell lines (Graph 1). 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% (IC50), compared to untreated cells. In AuNPs, the 50 µL sample is enough to control cancerous cell. 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 LC50 value was observed. 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 AuNPs on cancer cell lines rather than normal cell lines.

The antiangiogenic properties of GNP (Koperuncholan and Ahmed John 2011) were observed in vitro and in vivo. It turned out that GNP interact with heparin-binding glycoproteins – vascular permeability factors, growth factors of cardiac endothelium and fibroblasts. These agents mediate angiogenesis, including that in tumor tissues; therefore, GNPs inhibit their activity. Since intensive angiogenesis (the process of formation of new blood vessels in organs or tissues) is considered as one of the main tumor growth factors, the existence of antiangiogenic properties in GNPs could make them promising for tumor therapy. It was also demonstrated by the same researchers that gold nanoparticles enhance the apoptosis of the chronic lymphocytic leukemia cells that are stable to programmed death (Koperuncholan and Ahmed John 2011a) and suppress the proliferation of multiple myeloma cells (Koperuncholan and Manogaran, 2015).

Table 1. Antimicrobial activity of AuNps derived from B. cristata leaves.

S.No	Test Microorganisms		AuNPs (µl/disc)		PC	Diseases	Route of Transmission
Bacteria			15	30	10 mcg		
1.	Aeromonas liquefaciens	B1	12	15	14	Wound Infections / Gastroenteritis	Water / Food
2.	Enterococcus fecalis	B2	13	14	8	Endocarditis / Epididymal Infections	Water / Food
3.	Klebsiella pneumoniae	B3	12	16	28	Acute diarrhoea / Dysentery	Water / Food
4.	Micrococcus luteus	B4	11	13	38	Skin & Pulmonary infections	Soil / Water / Air / Food
5.	Salmonella typhimurium	B5	13	17	0	Typhoid	Water / Food
6.	Vibrio cholarae	B6	12	15	16	Cholera	Water / Food
Fungi							
7.	Candida albicans	F1	12	16	10	Skin infection / Gastrointestinal tract Infection	Air / Wound / Soil / Water
8.	Cryptococcus sp.	F2	10	12	9	Bronchiectasis / Endophthalmitis.	Air / Wound / Soil / Water
9.	Microsporum canis	F3	16	16	9	Tinea capitis /Ringworm	Air / Wound / Soil / Water
10.	Trichophyton rubrum	F4	12	14	7	Tinea corporis / Tinea pedis	Air / Wound / Soil / Water

PC - Positive Control (Using antibiotic disc; Bacteria – Methicillin (10mcg/disc); Fungi – Itraconazole (10mcg/disc)

Samples – 2.5, 5, 10 mg/ml (well)

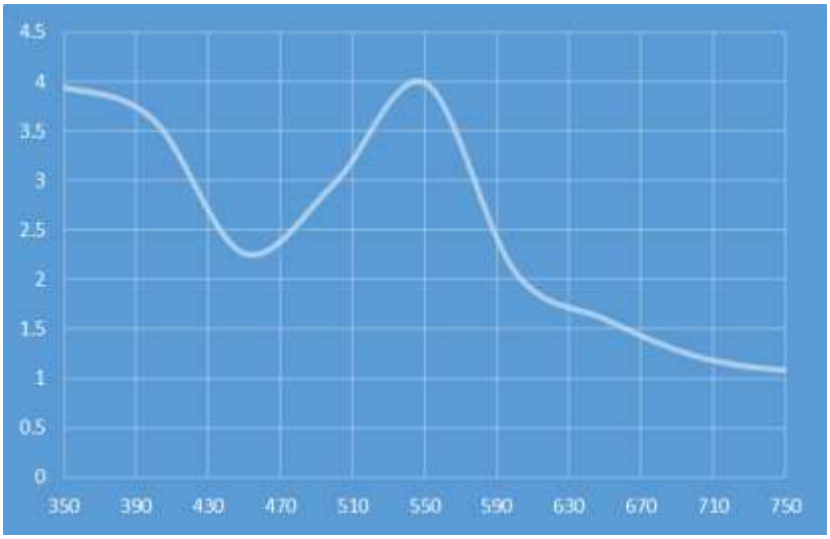

Figure 1: UV-Spectrum of AuNPs

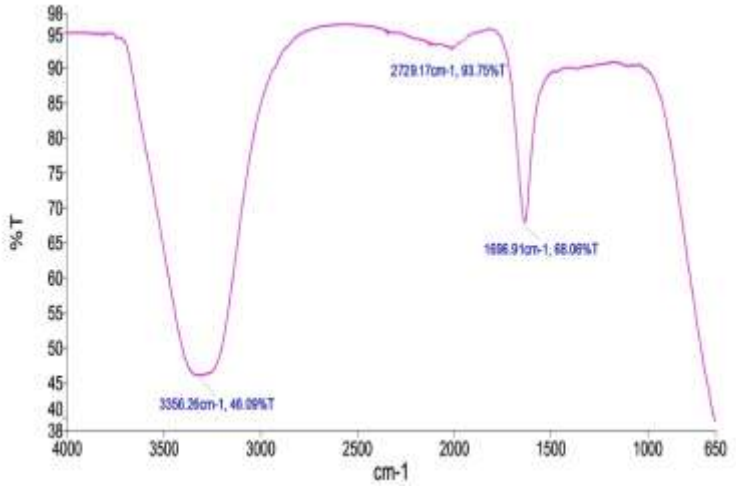

Figure 2: FTIR-Spectrum of AuNPs

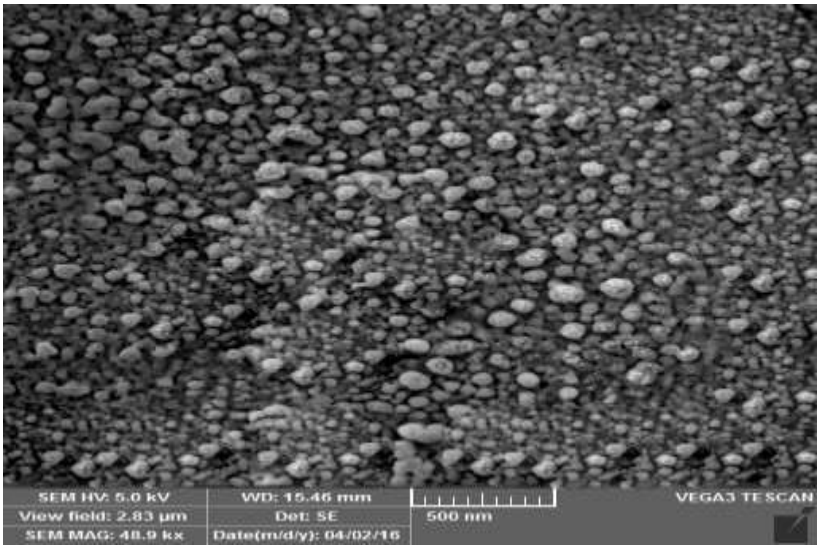

Figure 3: SEM Image of AuNPs

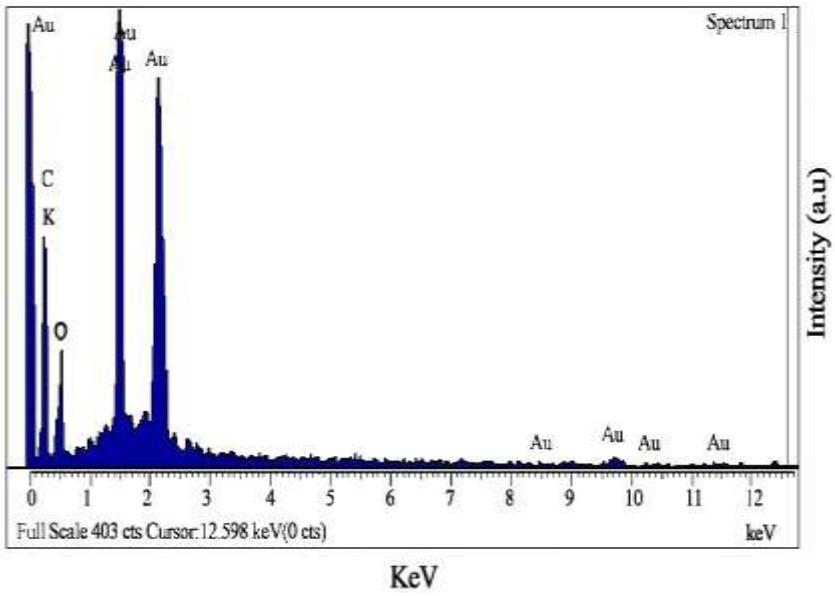

Figure 4: EDAX spectrum of AuNPs

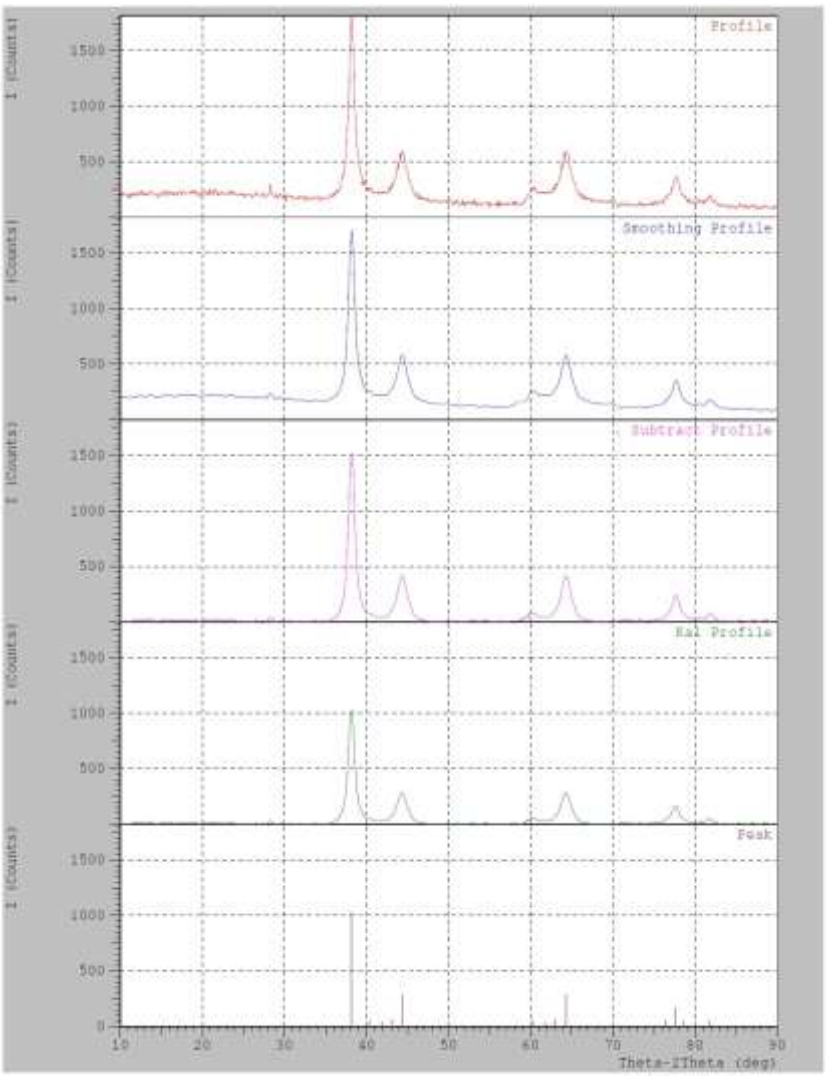

Figure 5: XRD Pattern of AuNPs

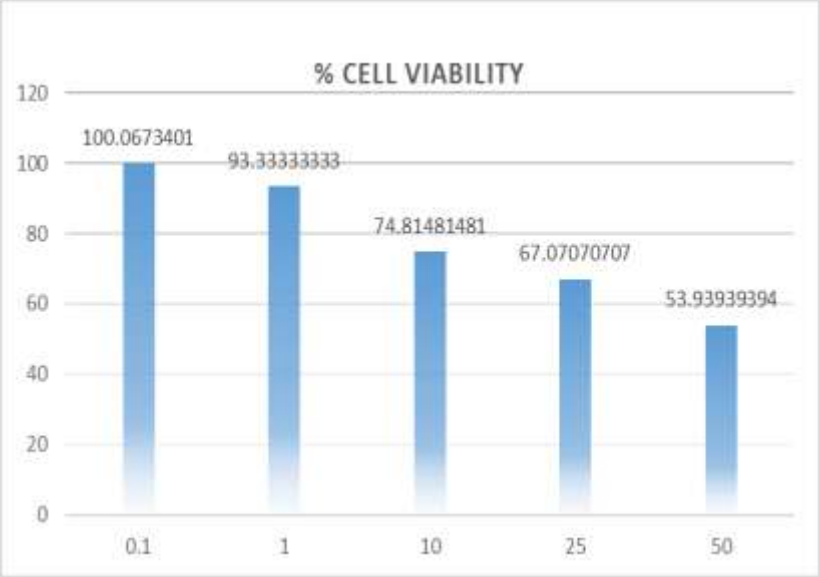

Figure 6: Anticancer activity of AuNps

CONCLUSION

In conclusion we introduce a simple, fast, and economical biological procedure to synthesize Au nanoparticles using Barleria cristata leaf extract. the biosynthesis of AuNPs were confirmed by the rapid colour change of plant extracts and characterized these nanoparticles using SEM, EDAX, XRD, and UV-visible, FTIR spectroscopic techniques. AuNPs biosynthesized from B. cristata leaves also exhibits great antimicrobial and anticancer activities against some microbes and human cancer cell cultures. These biosynthesised gold nanoparticles can potentially be used for different medical applications.

ACKNOWLEDGEMENT

The authors thank the Biospark Biotechnological Research Center (BBRC), Tiruchirapalli, Tamil Nadu, India for antimicrobial and anticancer studies.

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