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ANTIMICROBIAL AND CYTOTOXIC ACTIVITY OF BIOSYNTHESIZED GOLD NANOPARTICLES

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ABSTRACT

We present a simple and eco-friendly biosynthesis of gold nanoparticles (AuNPs) using naturally available *Malus domestica* fruit extract as a reducing, capping and stabilizing agent. The reaction process was simple and safe for the formation of highly stable AuNPs at room temperature by using the fruit extract. The surface plasmon resonance (SPR) band in UV-vis spectrum showed the reduction of auric acid to gold nanoparticles at 533 nm. The morphology of the nanoparticles was determined from high resolution transmission electron microscopy (HRTEM), selected area electron diffraction (SAED) and X-ray diffraction (XRD). The HRTEM images showed the mixture of spherical and triangular shapes of biosynthesized AuNPs having size ranging between 5 and 20 nm. Thermogravimetric analysis (TGA) was employed to determine the thermal stability of the AuNPs. They were also characterized by Fluorescence, Raman and Infrared (IR) spectroscopy. The biosynthesized AuNPs showed excellent antibacterial and anticancer activity. The gold nanoparticle synthesized using apple extract showed higher activity against *Aspergillus flavus* (MIC: 0.31 mg/mL) followed by, *Staphylococcus aureus* (MIC: 0.34 mg/mL), *Salmonella typhi* (MIC: 0. 63 mg/mL) and *Candida albicans.* Very low activity was found against *Vibreo cholerae* (MIC: 2.50 mg/mL). MTT assay was used to determine the cytotoxicity of biosynthesized gold nanoparticles on HeLa cancer cells.

KEYWORDS: ***Gold nanoparticles, Malus domestica, Green synthesis, Antimicrobial activity, Cytotoxicity***

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INTRODUCTION

A number of physical and chemical processes were developed for the synthesis of metal nanoparticles considering their applications in various fields such as catalysis, medicine, detection etc.[1,2,3] Recently researchers are attracted by nanoparticles for their unique physical and chemical properties, and showed much interest in the search of benign methods for the development of nanoparticles. The energy efficient, low-cost and eco friendly green method can eliminate the various problems faced by the other other physical and chemical methods. Various plant groups from algae to angiosperms possess different phyto-chemicals. There are several reports on the use of, *Basella alba*, *Helianthus annus, Saccharum officinarum*, *Sorghum bicolour*, *Zea mays*, *Medicago sativa* (Alfa alfa), *Aloe vera*, *Capsicum annuum*, *Azadirachta indica* (Neem), *Emblica officinalis* (Amla), *Magnolia kobus*, Coriandrum sp., Geranium sp., for the synthesis of various metal nanoparticles.[4,5,6] *Malus* domestica belongs to Rosaceae family , is commonly known as apple, the fruit extract of it is a rich source of highly potent antioxidants and is widely used in several traditional medicinal systems for the treatment of cancer and other diseases. Apple extract is primarily composed of a variety of phenolic phytochemicals. such as quercetin, catechin, phloridzin and chlorogenic acid. The antioxidant activity of phenolic compounds play a vital role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. Epicatechin is a naturally occurring phenolic compound in apple with antioxidant properties. Antioxidants are substances which can offer resistance against oxidative stress by scavenging the free radicals. The biocompatibility and non-toxic property of biosynthesized gold nanoparticles have shown their major role of applications in drug delivery, antimicrobial and anticancer activities.[7] Due to several advantages, the biosynthesized gold nanoparticles has considered as an important biomedical tool for cancer researchers for detecting cancer disease in a very small volume of tissue or cells and interacting with DNA, proteins, enzymes and cell receptors.[8,9] Gold nanoparticles have been developed for fuel cell applications and they showed their enormous potential as catalysts in a number of chemical reactions.[10] Gold nanoparticles, as colorimetric sensors, can identify if foods are suitable for consumption. They are designed for use as conductors from printable inks to electronic chips.[11] The effect of antimicrobial activity of the biosynthesized gold nanoparticles was investigated using various microorganisms such as *Staphylococcus aureus* (SA), *Staphylococcus typii* (ST), *Vibreo cholerae* (VC), *Candida albicans* (CA) and *Aspergillus flavus* (AF). The Minimum inhibitory concentration (MIC) was determined by Resazurin microtitre assay (REMA).[12] The cytotoxic effect of gold nanoparticles was studied in HeLa cell lines.[13] In the present study, we have explored an inventive contribution for the synthesis of gold nanoparticles using apple extract. The procedure for the development of stable gold nanoparticles is simple and viable. The biosynthesized gold nanoparticles were characterized by various methods, such as UV-Vis, IR, Flourescence, Raman spectroscopy, TGA, HRTEM, XRD and SAED. The biosynthesized gold nanoparticles showed excellent antimicrobial activity towards various bacteria and fungus. They showed an excellent efficacy towards HeLa cancer cell (cervical cancer, caused by Human Papilloma Virus) lines at different concentrations using MTT assay method.

MATERIALS AND METHODS

Materials

Apple fruits were collected from the local market which was used for the synthesis of gold nanoparticles. The chloroauric acid ($HAuCl_4.3H_2O$) (99.9%) was purchased from Sigma Aldrich India Minimal Essential Media supplemented (MEM) was purchased from Hi Media Laboratories and Fetal bovine serum (FBS) was purchased from Cistron laboratories, Dubai. Dimethyl sulfoxide (DMSO), Methylthiazolyldiphenyl- tetrazolium bromide (MTT) and Trypsin were purchased from Sisco Research Laboratory chemicals, Mumbai. The bacterial cultures of *Staphylococcus aureus* (ATCC 25175), *Salmonella typhi* (ATCC 14028), *Vibrio cholerae* (ATCC 14035), *Candida albicans* (ATCC 10231), *Aspergillus flavus* (ATCC 10124) were obtained from National Center for Cell Science (NCCS), Pune. Resazurin (redox) dye was obtained from Sigma Aldrich chemicals., India. A HeLa cell line for cytotoxic study was obtained from King Institute, Guindy, Chennai.

Synthesis of gold nanoparticles

An apple was thoroughly washed, crushed and filtered through Whatman No.1 filter paper. The aqueous solution of 0.01M $HAuCl_4$ was prepared and added to the aqueous solution of apple extract. The color change in the solution indicated the reduction of auric chloride to gold nanoparticles. To avoid the agglomeration of biosynthesized stable gold nanoparticles lyophilisation process was used.

Characterization of gold nanoparticles

Spectral analysis of nanoparticle formation was observed by UV–Vis spectroscopy using a Perkin-Elmer UV-Visible spectrophotometer of model 1800. Bruker FTIR spectrophotometer was employed for analyzing the functional groups present in biosynthesized gold nanoparticles. High resolution transmission electron microscope of model FEI-TECNAI-30 was used for the analysis of size and shape of developed nanoparticles. 3 µL of the sample solution was placed on copper grid making a thin film of sample on the grid for HRTEM measurements. To determine the phase and crystal structure of gold nanoparticles, XRD measurements were done using a model 3000 from Rich Siefert, Germany and was used with Cu-$K_{\alpha 1}$ radiation using λ value of 1.54056 Å. A model TGA Q50 V20.13 was used to determine the moisture absorption content and degradation temperatures of gold nanoparticles. Cary fluorescence spectrophotometer of model FL1201M002 was employed to carry out fluorescence measurements. The Raman measurements were carried out using BRUKER RFS 27: Stand-alone FT-Raman spectrometer equipped with Nd: YAG 1064 nm as an excitation source.

RESULTS AND DISCUSSION

UV-Vis analysis

Equivalent amounts of the suspension were diluted in a constant volume of distilled water and subsequently analyzed at room temperature. The progress of the reaction between metal ions and the fruit extracts were shown by the colour change from yellow to black (Inset Fig 1). UV–visible spectrum of biosynthesized gold nanoparticles in aqueous solution was recorded at different reaction times. At the initial stage, no characteristic SPR peak was observed .[14,15,16] The SPR peak started to appear in the visible range at 533 nm after 12 h of time. (Fig 1).The polyphenols present in the apple fruit extract act as a reducing and stabilizing agent in the synthesis of gold nanoparticles. The UV-Vis spectrum was recorded after 3 months and it showed an absorption peak at the same wavelength at 533 nm to confirm the stability of gold nanoparticles.

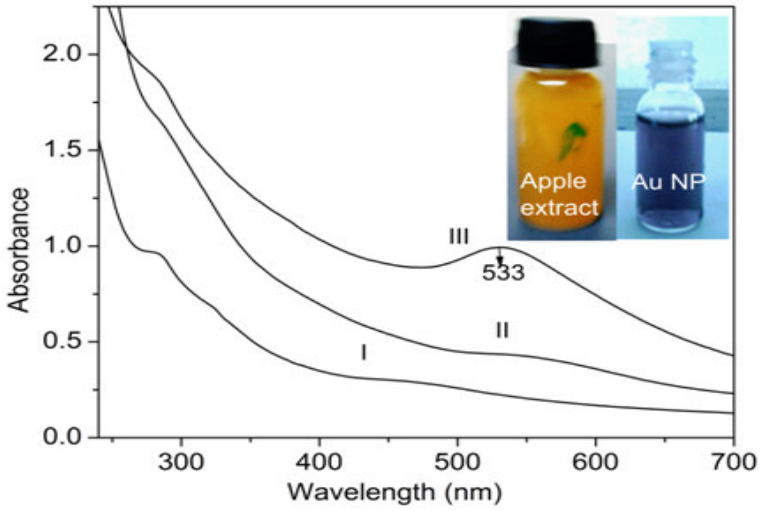


Figure 1

UV- Vis spectra of biosynthesized gold nanoparticles (I) Pure apple extract, Immediately after the addition of $HAuCl_4$ to apple extract (II) 2 h and (III) 12 h.

Inset Figure Digital photograph of apple fruit extract and gold nanoparticles

FTIR spectroscopy

The FTIR spectrum of gold nanoparticles using apple extract shows absorption bands at 3450, 2904, 2851, 1718, 1632, 1385, 1029 and 637 cm^{-1} (Fig 2). A strong band at 3450 cm^{-1} assigned for broad O-H stretching of water, phenols or alcohols. A peak at 2904 cm^{-1} showed the presence of O–H stretch in carboxylic acid group. The band positioned at 2851 cm^{-1} is due to the presence of aldehyde group. The peak at 1718 cm^{-1} corresponds to C = O stretching vibration for the presence of carbonyl group. The peak located at 1632 cm^{-1} could be attributed to amide group and 1385 cm^{-1} showed the presence of C-O groups from polyols which act as both reducing and capping agent for the stability of the bioreduced gold nanoparticles. [17,18] The peak at 1029 cm^{-1} corresponds to C–OH stretching vibration. It is a well known fact that proteins can bind to gold nanoparticles through free amine groups. The band at 637 cm^{-1} confirmed the presence of alkyl halides.

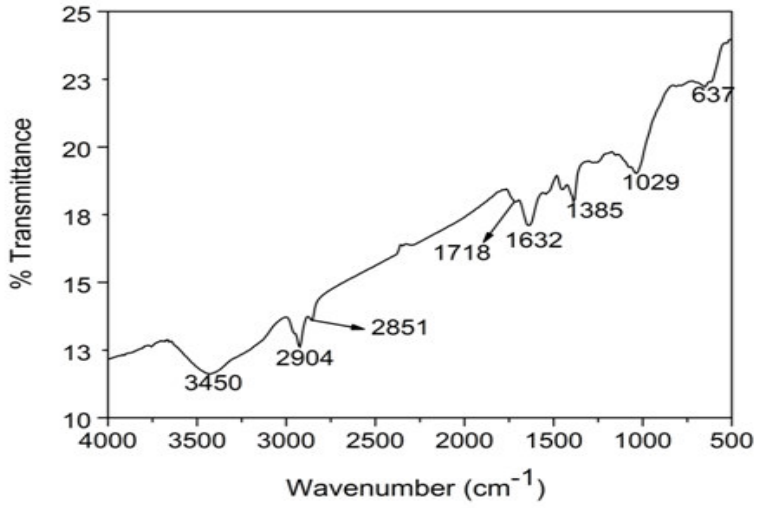


Figure 2

FTIR spectrum of biosynthesized gold nanoparticles

Raman spectroscopic analysis

The Raman spectrum shows bands at 525, 609, 649, 692, 724, 845,891, 989, 1027, 1079, 1154, 1254, 1313 and 1390 cm^{-1} (Fig 3). The peak at 525 cm^{-1} is due to C-N-C stretching vibration. The peaks at 609, 649 and 692 cm^{-1} assigned to the stretching vibrations of C– S–C. The peak at 724 cm^{-1} is due to the CH_2 rocking vibration mode. [19] The peaks at1154 cm^{-1} and 1254 cm^{-1} is assigned to C-C stretching vibration. The peaks at 1254; 845 and 891 cm^{-1} have come from the C–H in plane bending and out of plane wagging, respectively. The band at 989, 1027 and 1079 cm^{-1} is assigned to C-O stretching vibration. The peaks at 1313 and 1390 cm^{-1} are due to asymmetric C=O stretching vibrations of carboxylate group.

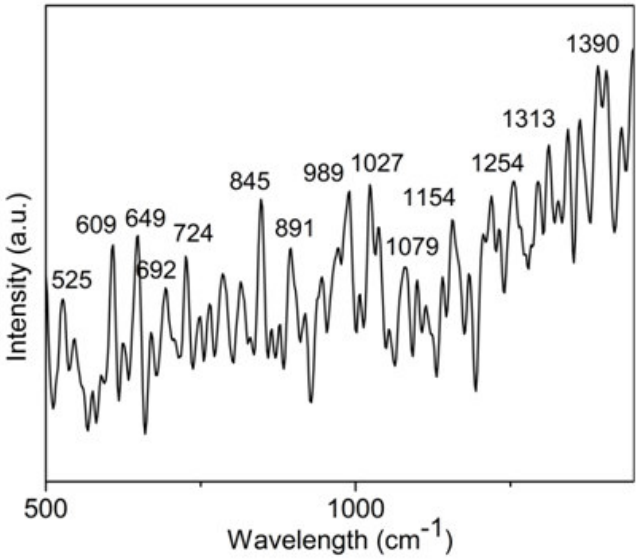


Figure 3
Raman spectrum of biosynthesized gold nanoparticles

Fluorescence spectroscopy analysis

Photoluminescence of apple mediated gold nanoparticles were examined to know its fluorescence property. They were excited at 450 nm and emission of fluorescence was obtained at 546 nm (Fig 4). The origin of the fluorescence can be attributed to the promotion of d-band electrons of the gold nanoparticles on absorption of the incident photon energy, to higher electronic states in the sp-band.

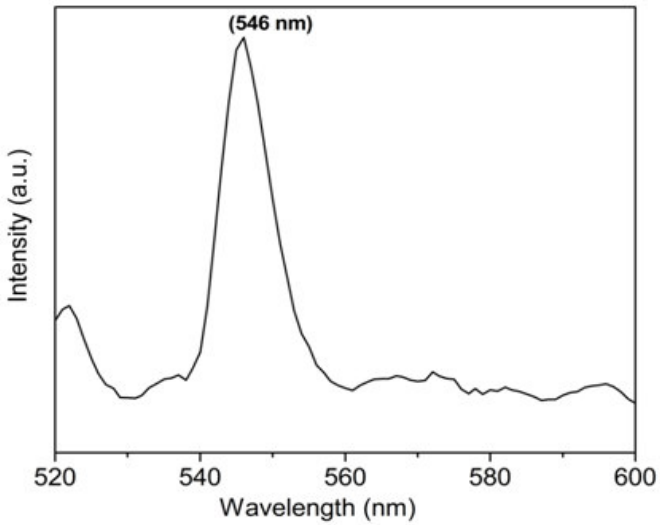


Figure 4
Fluorescence spectrum of biosynthesized gold nanoparticles

TGA

The thermal stability of the biosynthesized gold nanoparticles was recorded by TGA analysis at a heating rate of 20 °C/min in flowing nitrogen. The thermal decomposition process is represented by TGA curves where the gold nanoparticles were subjected to a heating from 50 to 800°C. In thermogram, it showed multiple falls in weight over the temperature ranges from 50 to 200 °C, 200 to 320 °C, 320 to 490 °C, 490 to 800 °C (Fig 5). The weight loss between 50 to 200 °C was attributed to residual water evaporation, whereas the rest are due to the decomposition of organic contents.[20] The residual mass of 41% indicates that high amount of organic contents were present in the biosynthesized gold nanoparticles.

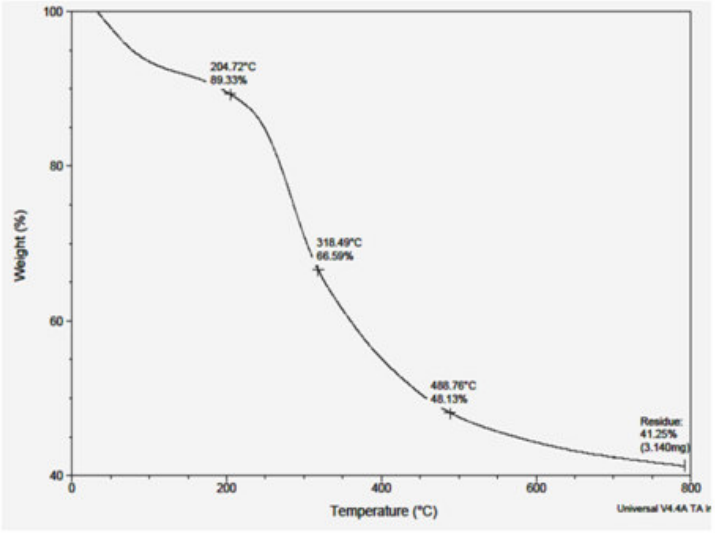


Figure 5
TGA of biosynthesized gold nanoparticles

XRD analysis

The X-ray diffraction (XRD) pattern of the prepared gold nanoparticles is shown in Fig 6. In the XRD pattern of gold nanoparticles, diffraction peaks at 38.0°, 44.3° and 64.3° can be assigned to face-centered cubic (fcc) metallic gold corresponding to the (111), (200) and (220) of the gold crystals. The peaks were well matched with the standard gold particles with JCPDS No-03-065-2870. The interplanar spacing, *d* values are 2.36, 2.04 and 1.44 Å for (111), (200) and (220) planes respectively. The results are in good agreement with the reported literature. [21, 22] The crystallite size of biosynthesized gold nanoparticles was calculated from the width of the XRD peaks, assuming that they are free from non-uniform strains, using the Scherrer formula, $D = 0.94\ \lambda / \beta \cos\theta$, where D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the full width at half maximum (FWHM), and θ is the diffraction angle.

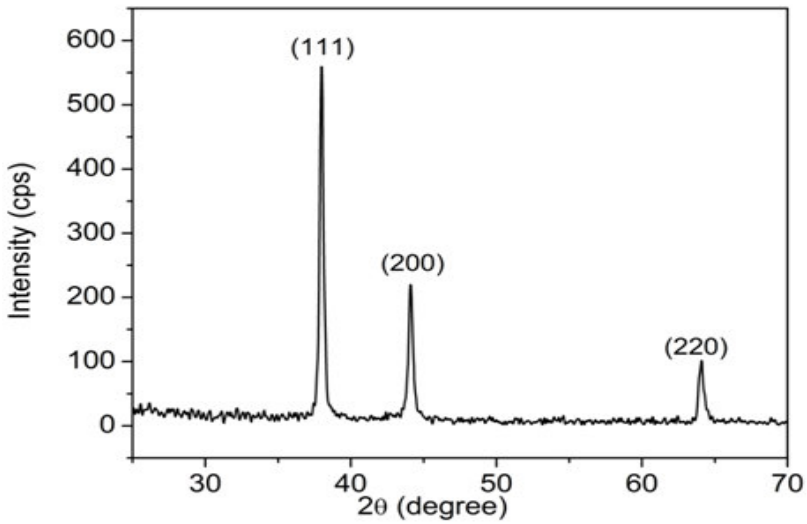


Figure 6
XRD pattern of biosynthesized gold nanoparticles

Morphological analysis

The morphology and size of the biosynthesized gold nanoparticles were examined by FE-SEM (Fig 7a). A typical bright-field HRTEM micrographs showed the mixture of nanoprisms, nanorods and nanospheres of biosynthesized gold nanoparticles (Fig 7b). [23,24,25] These images suggest that the nanoparticles are polydisperse and are mostly spherical in shape. It is evident that there is variation in particle sizes and the average size estimated was 5 nm gold nanoparticles (Fig 7c). The spot in SAED pattern reveals that the biosynthesized gold nanoparticles are crystalline in nature (Fig 7d). These rings can be attributed to the diffraction from the (1 1 1), (2 0 0) and (2 2 0) planes of fcc gold. [26, 27]

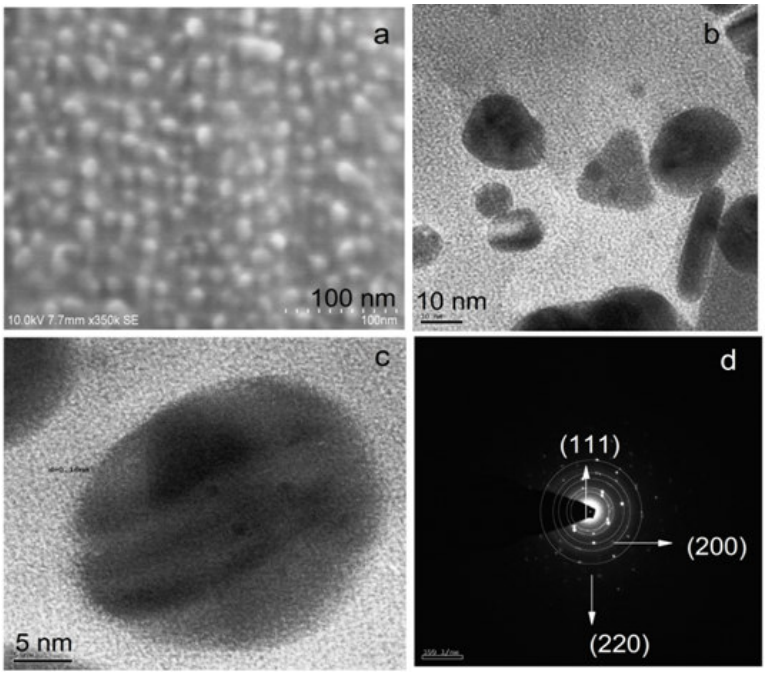


Figure 7
(a) FESEM image and HRTEM images of biosynthesized gold nanoparticles at (b) 10 nm scale and (c) 5 nm scale, (d) SAED pattern showing the characteristic crystal planes of elemental gold

Antimicrobial activity of gold nanoparticles synthesized by using apple extract

Microorganisms and culture medium

All the microorganisms were incubated at 37 ^{o}C for 24 h in nutrient broth. The fungus *C. albicans* was incubated in Sabouraud dextrose broth at 37^{o}C for 48 h. The culture suspensions were prepared and adjusted by comparing against 0.4–0.5 Mc Farland turbidity standard tubes. 20 mL of Sabouraud dextrose and Nutrient agar (20 mL) were poured into each sterilized petri dish with 10 mm×100 mm diameter. Then they were allowed to solidify and the bacterial culture and *C. albicans* were swabbed in nutrient agar plates and Sabouraud dextrose plates respectively.[28] The gold nanoparticles were dispersed in dimethyl sulfoxide (DMSO) to a final concentration of 100 µg/mL and sterilized by filtration through a 0.22 µm membrane filter for the investigation of the antibacterial and antifungal activity. Each sample was directly filled into the agar plates wells. [29,30] Plates swabbed with the bacteria culture and the fungus were incubated at 37 ^{o}C for 24 h and 37 ^{o}C for 48 h respectively. Inhibition zones formed on the medium were measured at the end of the incubation period. The inhibition zones were compared with those of reference discs and inhibitory activity of DMSO was also tested. [31] Reference values used from reference disc as a control were Tetracycline (30 µg) and Ampicillin (10 µg). All studies were done in triplicate. The cup plate method using nutrient agar was employed to test the antimicrobial activity of the gold nanoparticles. The radial growth of the colony was recorded on completion of the incubation of the bactericidal growth and the mean diameter for each at a concentration of 100 µg/mL was recorded. The average percentage inhibition was compared using the Vincent equation: I = 100(C-T)/C, where I= percentage inhibition, T= average diameter of the bacterial growth on the tested plates and C=average diameter of the growth on the control plates. [32]

Determination of minimum inhibitory concentration (MIC) using Resazurin Microtitre Assay (REMA)

Preparation of Resazurin solution

Isosensitest medium was used to carry out the test to determine the MIC of the gold nanoparticles. The biosynthesized gold nanoparticles showed similar results for most of the tested bacterial strains.[33,34] The resazurin solution was prepared by dissolving 270 mg in 40 mL of sterile distilled water. A vortex mixer was used to obtain the homogenous solution. The test was carried out in a sterile 96 well plates. Resazurin indicator (10 µL) solution and 3. 3 × strength isosensitised broths (30 µL) were added to each well. 5 × 10^{6} cfu/mL of the bacterial suspension was added to each well. The triplicate plates were placed in an incubator for 18–24 h at 37 °C. The colour change from purple to pink or colourless was recorded as positive.

MIC of gold nanoparticles synthesized by using apple extract

The MIC study was conducted to investigate the antimicrobial activity of biosynthesized gold nanoparticles against bacterial strains such as *S. aureus*, *S. typhi*, *V. cholerae* and against the funguses *C. albicans* and *A. flavus.* The colour change from purple to pink or colorless was recorded as positive. [35] The MIC value was taken from the lowest concentration and the average of the three values was calculated. Fig 8 compared the MIC of gold nanoparticles against various microorganisms. [36] The MIC of the gold nanoparticles synthesized using apple extract was summarized in Table 1. The gold nanoparticle synthesized using apple extract showed higher activity against *A. flavus* (MIC: 0.31 mg/mL) followed by, *S.aureus* (MIC: 0.34 mg/mL), *S. typhi* (MIC: 0. 63 mg/mL) and *C. albicans.* Very low activity was found against *V. cholerae* (MIC: 2.50 mg/mL).

Table 1
MIC of biosynthesized gold nanoparticles synthesized using apple extract against microorganisms

S.No	Microorganisms	MIC (mg/mL)*
1	*C. albican*	1.25
2	*A. flavus*	0.31
3	*S. aureus*	0.34
4	*S. typhi*	0.63
5	*V. cholerae*	2.50

Average of three experiments

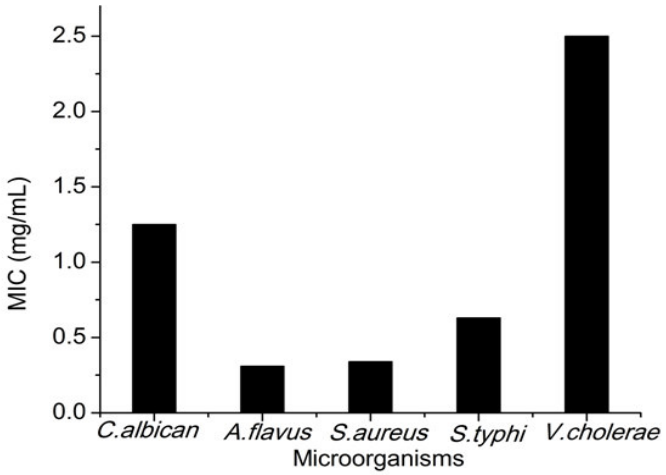
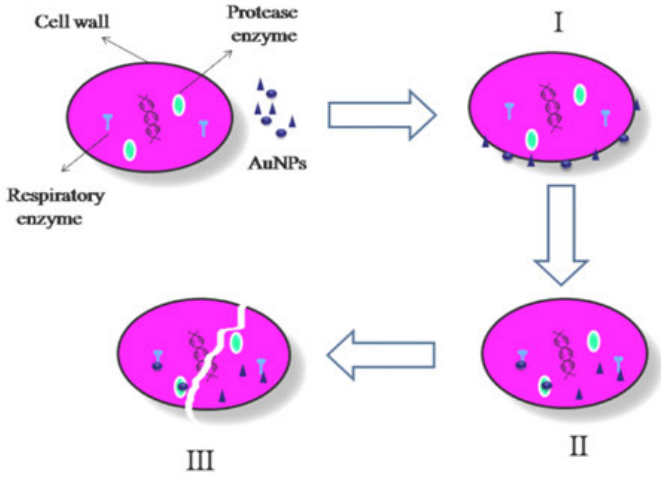


Figure 8
MIC of biosynthesized gold nanoparticles against various microorganisms

Mechanism of antibacterial activity of gold nanoparticle on bacterial cells

The biosynthesized gold nanoparticles easily entered into bacterial cell. They combined with respiratory enzyme, protease enzyme and DNAs of bacteria. The cell functions of bacteria were deactivated by releasing the gold ion which damaged the cell and lead to the death of bacterial cell (Scheme 1) [37].



Scheme 1

Mechanism of antimicrobial activity of biosynthesized AuNPs
(I) Bacterial cells take up biosynthesized AuNPs
(II) Interaction of AuNPs with respiratory and protease enzyme
(III) Destruction of bacterial cells by AuNPs

Cytotoxicity of gold nanoparticles against hela cancer cells

Cell culture and medium

HeLa cell lines were maintained in MEM and supplemented with 10% fetal bovine serum (FBS), streptomycin (100 µg/mL) and penicillin (100 µg/mL) in a humidified atmosphere of 50 µg/mL CO_2 at 37 °C.

MTT assay for cytotoxicity (In-vitro)

MTT assay was used to determine the cytotoxicity of biosynthesized gold nanoparticles on HeLa cancer cells.[38] Gupta *et al.,* were plated in 1ml of medium in 24-well plates (Costar Corning, Rochester, NY). The cell reaches the confluence after 48 h of incubation. Then

the cells were incubated in the presence of various concentrations of the samples in 0.1% DMSO for 48 h at 37°C. The sample solution was removed and washed with PBS of 200 µL/well (5 mg/mL) and pH 7.4. Isopropanol /HCl of 0.04 M were added after 4 h of incubation. Viable cells showed their absorbance at 570 nm which was measured with a UV- Spectrophotometer using wells without sample containing cells as blanks. The concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically. [39] The effect of the samples on the proliferation of HeLa was expressed as the % cell viability, using the formula, % cell viability = A_{570} of treated cells / A_{570} of control cells × 100%. The cell viability of HeLa cancer cells was assessed by MTT assay for 48 h after treatment with the gold nanoparticles. [40] The cytotoxicity results were shown in Table 2 for different concentrations (1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8 µg/mL) of gold nanoparticles. Viable cells were determined by the absorbance at 570 nm. The normal HeLa cells were shown in Fig 9a and the cytotoxicity effect of gold nanoparticles at various concentrations like 1000, 250, 62.5 and 31.2 µg/mL on HeLa cells were shown in Fig 9b-e. The results revealed that the % cell viability at high dose of 1000 µg/mL is 8.9. But, when the doses were decreased like 500, 250, 125, 62.5, 31.2, 15.6 and 7.8 µg/mL, then the percentage of viable cells was increased to 22.3, 35.8, 47.7, 56.7, 64.1, 68.6 and 79.6 respectively. 91.1% of cancer cells were dead at high dose of 1000 µg/mL. The results of present work demonstrate the IC_{50} of gold nanoparticles as 125 µg/mL for HeLa cervical cancer cell lines (Fig 10). The experimental results clearly proved the excellent anticancer activity of gold nanoparticles against the HeLa cell line.[41]

Table 2

Cell viability of biosynthesized AuNPs on HeLa cell lines

S.No	Concentration (µg/mL)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000.0	Neat	0.06	8.9
2	500.0	1:1	0.15	22.3
3	250.0	1:2	0.24	35.8
4	125.0	1:4	0.32	47.7
5	62.5	1:8	0.38	56.7
6	31.2	1:16	0.43	64.1
7	15.6	1:32	0.46	68.6
8	7.8	1:64	0.53	79.6
9	Cell control	-	0.67	100

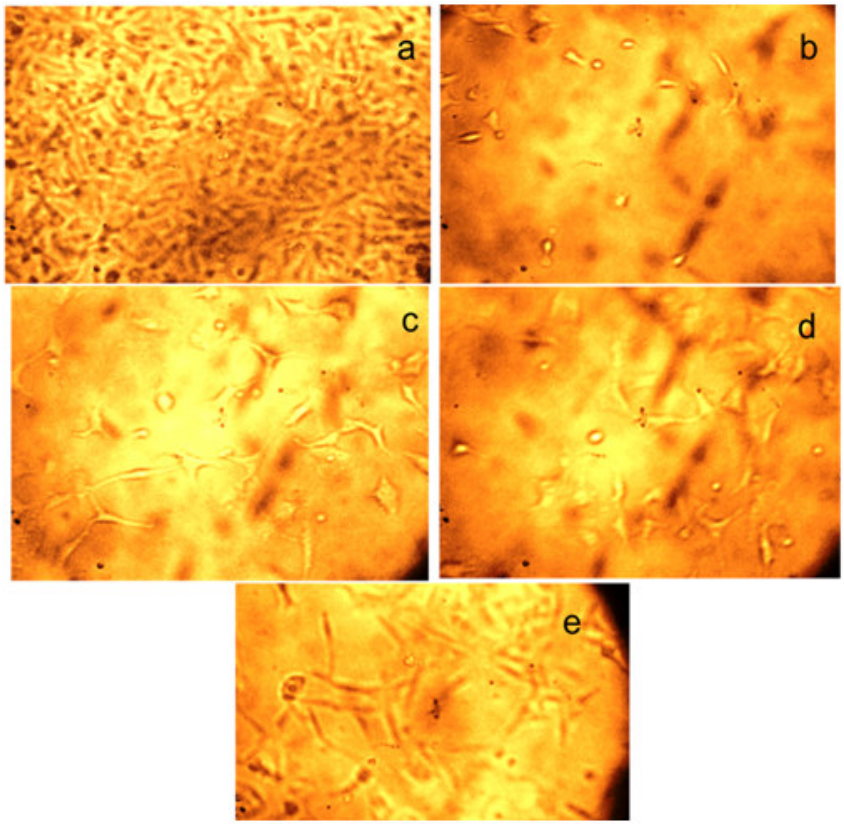


Figure 9

Microscopic images of HeLa cells (a) after 24 h incubation; cells without any treatment (b) at 1000 µg/mL (b) 250µg/mL (c) 62.5µg/mL (e) 31.2µg/mL of gold nanoparticles synthesized using apple extract.

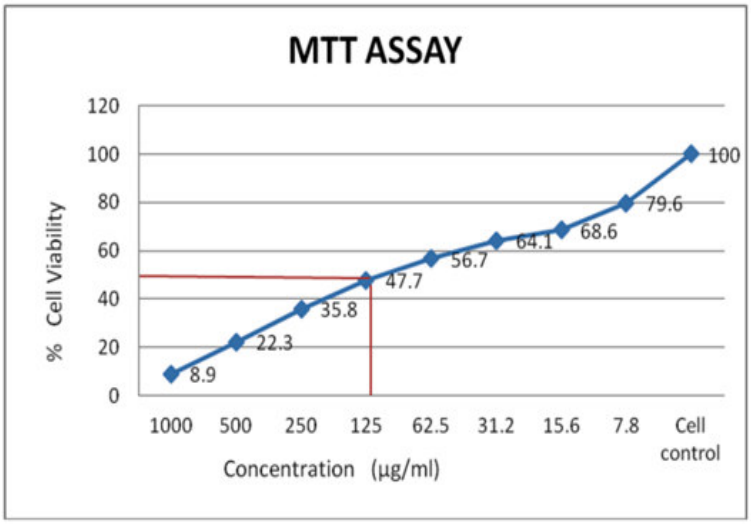


Figure 10
Cell viability of the biosynthesized gold nanoparticles at different concentration.

CONCLUSION

This simple and eco friendly method for the synthesis of gold nanoparticles played an important tool in the area of nanotechnology and green synthesis. In this work, apple fruit extract was prepared and successfully employed for the development of gold nanoparticles with spherical and triangular shapes. The polyphenols and flavanoids present in apple extract played a vital role in the reduction of auric acid and stabilization of gold nanoparticles. XRD study showed the face-centered cubic lattice of gold nanoparticles. The HRTEM images confirmed the crystalline nature of biosynthesized gold nanoparticles. SAED pattern showed the characteristic crystal plane of elemental gold. They showed their excellent potential in antimicrobial activity towards various microorganisms. The gold nanoparticles synthesized using apple extract showed significant cytotoxic effects against HeLa cancer cell lines.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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