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Comparative studies of anticancer and antimicrobial potential of bioinspired silver and silver-selenium nanoparticles

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ABSTRACT

The antimicrobial and anticancer potential of synthesized silver nanoparticles by various medicinal plants and silver-selenium nanoparticles by phytochemicals (quercetin and gallic acid) are reported here. Medicinal plants such as *Syzygium cumini*, *Azadirachta indica* and *Catharanthusroseus* and quercetin-gallic acid as phytochemicals were selected after screening of various plants and phytochemicals in terms of the ability of nanoparticle synthesis. The synthesized nanoparticles were characterized using various analytical instrumentation techniques. All the nanoparticles are having less than 40 nm in size as confirmed by electron microscopy. Bactericidal action of the nanoparticles was determined using broth microdilution method on two microbial strains (*Escherichia coli* and *Bacillus subtilis)*. The results specified that all types of nanoparticles exhibited comparable bactericidal action against the different strains at 100 µg/ml, when compared to the standard drug, chloramphenicol (50 µg/ml). Additionally, the anticancer potential of the nanoparticles was evaluated using MTT assay on various cancer cells (HeLa, Hek-293 and MCF-7). The results concluded that *Syzygium cumini* silver nanoparticles were highly active at the minimum concentration of 10 µg/mL against all type of cells. The synthesized nanoparticles destroy the bacterial and tumour cells in a dose-dependent manner.

Keywords: Anticancer, Antimicrobial, Silver nanoparticles (AgNPs), Plant extract

INTRODUCTION

Nanotechnology is an emerging field for the development of novel nanocomposites into a new range of products and development of their applications.[1] It deals with manipulation of materials at atomic and molecular level.[2] In the field of nanomaterials, metals having a significant application in the field of pharmaceutical and medical sciences.[3] Increasing impact of metallic nanoparticles in biology has stimulated for the development of new synthesis methods with multiple applications.[4-7] The importance and interest of these nanomaterials have increased due to their distinctive antimicrobial and anticancer activities and applications in biomedical and biocatalytic reactions.[8] Metallic nanoparticles possess higher level of therapeutic activity with lower toxicity rather than their counterparts (metal salts). Nanoparticles can be synthesized through a varieties of chemical and physical methods, however, increasing alertness towards green chemistry, biological approaches have been developed which are also environment-friendly and non-toxic in terms of applications.[9-11] Many biological agents, such as bacteria, fungi, actinomycetes and plants have been utilized for nano-material synthesis.[10-12] The process takes place at normal temperature and pressure conditions, without requiring any other additives.

Silver nanoparticles (AgNP) having unique properties such as larger surface area to volume ratio, absorption in the visible spectrum region (400-450 nm) and cytotoxic against microbes and tumor cells.[13-16] These properties make them valuable for the development of biomedical applications like treatment of multidrug resistant microorganisms, development of various types of cancers and drug delivery system. Silver nanoparticles are currently being used for preventing infections as coatings of medical equipment, food packaging materials, wound dressings bandages, gloves and various wound healing gels.[17] Silver nanoparticles exhibit strong bactericidal action by affecting the cellular an enzymatic machinery of host and deposition silver ions on the microbial cell membrane.[13,17] It was also reported

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that silver nanoparticles are efficient to penetrate the bacterial cell wall and affect the permeability of cells.[18]

Nanomedicine field had visualized the next door for the ongoing development of cancer theranostic system. It is also expected that new strategies encompassing the use of functionalized nanomaterials can provide the early detection, diagnosis and therapy of the disease.[19] Various metal nanoparticles such as selenium, gold, platinum and silver are already reported as anticancer agents and being studied in various applications such as labelling, imaging, sensing of tumour cells, biosensor development and DNA detection.[11] Currently the metallic nanoparticles are the novel tools for targeting cancer cells and nanoparticle material in conjugation with anticancer drugs enhance their efficacy.[19]

In view of the wide-ranging potential uses of nanoparticles in pharmaceutical sciences, their biomedical applications are also recognized. The present study reports the synthesis of silver nanoparticles and silver-selenium (Ag-Se) nanoparticles, their characterization and evaluation of their effects on the biological system, especially against bacterial cells and several cancer cell lines.

2. MATERIALS AND METHODS

2.1 Chemicals, cells lines and microorganisms

Silver nitrate and sodium selenite, 3-(4,5-dimethylthiazole-2-yl)-2,5- diphenyltetrazolium bromide (MTT), ammonium thiocyanate (99.99%), silver nano-powder (<100 nm), gallic acid and quercetin dehydrate were purchased from Sigma-Aldrich Ltd. For cell culture, RPMI-1640 culture medium and fetal calf serum (FCS) were purchased from HiMedia, Mumbai, India and Invitrogen, USA. All other chemicals were purchased either from HiMedia, Mumbai, India or SRL, Mumbai, India. The bacterial strains, *Escherichia coli* (MTCC 433) and *Bacillus subtilis* (MTCC 441) were procured from the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. The cell lines such as HeLa, Hek-293 and MCF-7 were obtained from National Centre for Cell Science, Pune, India. The plant parts of *Azadiricta indica* (Neem) and *Catharanthus roseus* (Vinca) were collected from the NIPER campus, SAS Nagar and fruit powder of *Syzygium cumini* was collected from the Department of Natural Products, NIPER, SAS Nagar, India. Plant extract and synthesized nanoparticles were lyophilized using freeze drier (Allied-Frost, New Delhi, India). The average size and charge of nanoparticles were estimated by Zeta-sizer Nano (Malvern Instruments, USA). The nanoparticles were harvested by centrifugation at 15,000 x g for 30 min (Eppendorf, USA). The transmission electron microscopic study was performed on FEI HR-TEM to know the exact particle size. The antimicrobial and cytotoxicity (MTT) assays were performed in 96-well plate and absorbance was measured on microplate reader (Multiskan, Thermo Scientific).

2.2 Preparation of plant extract

For the preparation of *Azadiricta indica* (Neem) extract, leaves were thoroughly washed and dried completely under the shade and powdered in a grinder. Dried powder (100 g) was loaded to Soxhlet apparatus (1000 mL), defatted by hexane and extracted with 1:1 deionised water and methanol for 48 h. The extract was filtered, methanol was evaporated in rota-vapour and water was removed using lyophilizer. The resulting dried powder was then mixed with 100 ml deionized water and stored at 4°C for further experiments. For the preparation of plant extract from *Catharanthus roseus* (Vinca) flower, the parts were completely dried. The dried parts were powdered in a grinder and 50 g of this dried powder was extracted in Soxhlet apparatus with 1:1 deionised water and methanol for 48 h. The extract was filtered and dried using rotavapour and lyophilizer. The powder was mixed with 100 ml deionized water and stored at 4°C for further experiments. The *Syzygium cumini* fruit extract was prepared according to our previously reported method[20]. Further, the resultant dried extract was suspended in 100 ml deionized water and stored at 4°C for further experiments.

2.3 Synthesis of nanoparticles using various sources

For the synthesis of nanoparticles, various concentrations of extract or phytochemicals and metal salts were reacted in an aqueous medium and incubated in dark at room temperature with shaking (200 rpm) for 6 h or until the color change was observed. The nanoparticle synthesis was performed according to previously published article by our group[21-23]. All the reactions were performed in deionized water and various parameters (concentrations of metal salts and reducing agents, pH, temperature and reaction time) were optimized to control the shape and size of the nanoparticles.

2.4 Characterization of nanoparticles

The synthesized nanoparticles centrifuged at 15,000 x g for 30 min and the pellets were washed thoroughly with deionized water and completely dried in a freeze dryer. The reduction of Ag^+ ions to AgNPs in a colloidal solution was monitored at 300-700 nm by measuring the UV-Vis spectrum of the reaction medium after every 3h of reaction. The average size and shape of the nanoparticles was determined using the transmission electron microscope (HR-TEM). Samples were prepared by drop coating onto carbon coated copper TEM grids. The microscopic measurements were taken on FEI HR-TEM operated at an accelerating voltage (100-200 keV). The presence of same elemental metal was confirmed through energy dispersive X-ray (EDX) attached with TEM instrument.

2.5 Antibacterial assays

The antimicrobial potential of the synthesized nanoparticles was found by performing the antimicrobial assay. The experiments were performed on common laboratory bacterial strains *Escherichia coli* MTCC 433 (Gram-negative) and *Bacillus subtilis* MTCC 441 (Gram-positive). The activity was determined by broth microdiluation assay on 96 well plates. Nutrient broth (NB) was used as culture medium. The suspension of bacteria's equivalent to 0.5 McFarland's Standard was pipetted into a 96 well-plate and optical density for 24 hours at 37 °C was recorded using automated microplate reader at a wavelength of 600 nm.[24] The nanoparticles were dispersed in the water with the stock concentration of 100 µg/ml and made twofold serial dilutions to from 50, 25 µg/ml. The autoclaved water and Eppendorf tubes were used for the making

the dilutions. Chloramphenicol and silver nitrate were used as positive controls and broth without any treatment was used as negative control.

2.6 Cytotoxicity test (MTT assay)

The cytotoxicity of synthesized NPs was tested using on HeLa (cervical cancer), Hek-293 (kidney cancer) and MCF-7 (breast cancer) cell lines by MTT dye reduction assay. The cells were cultured at the density of 1×10^6 cells/mL and then harvested. The cells were treated with the increasing concentrations of nanoparticles (5, 10 and 25 µg/mL) and incubated for 24 and 48 h in 5 % CO_2 at 37 °C. Each experiment was performed in triplicate with duplicate plates. MTT solution (10 µL) was added to each well and the plates were further incubated for 24 and 48 h at 37 °C in 5 % CO_2. The resulting blue coloured product was dissolved in 200 µL dimethyl sulfoxide (DMSO) and the absorption was read at 595 nm. The absorbance of untreated cells was used as control (100% viable).

3. RESULT AND DISCUSSION

3.1 Biosynthesis of nanoparticles

The present study revealed that the biological sources such as plant extract and phytochemicals could be potential agents to reduce silver or selenium ions and form nanomaterials in the reaction medium. It is already reported[13, 25] that metallic nanoparticles exhibit color changes after the synthesis, due to excitation of surface plasmon vibrations in a colloidal solution. The change in color of reaction mixture ensured the formation of nanoparticles.

3.2 Characterization of nanoparticles

The synthesis of nanoparticles was further confirmed by UV–Vis spectroscopy with the characteristic peak[20, 26]. Figure1 shows the UV-Vis absorption characteristic peak of all the synthesized nanoparticles. All the nanoparticles show absorption in the range of 400-450 nm.

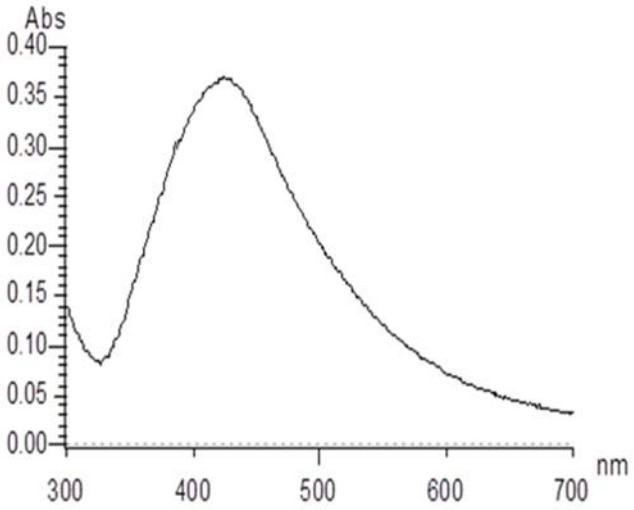

Figure 1. UV-Visible absorption spectrum of colloidal solution of nanoparticles synthesized using green method

Further characterization using transmission electron microscopy (TEM) showed that all the nanoparticles synthesized by various biological sources are having size less than 40 nm (Figure 2). Figure 2 a-d showed that all the synthesized nanoparticles are having size ranges between 5-40 nm with spherical in nature. Results of EDX showed the presence of same element on the respective nanoparticles. Based on UV-Vis spectroscopy, various reaction parameters (concentration of plant extract and metal ions, reaction temperature, pH and reaction time) were optimized to enhance the yield of nanoparticles (Figure S1 to S4in the electronic supplementary material). Metal salts (1 to 5 mM) were able to produce maximum yield of nanoparticles with controlled properties. All the plant extract- and phytochemical-mediated nanoparticle synthesis showed the maximum yield at 45 ºC and pH 8 except *Syzygium cumini* (35 ºC). The optimum time required for the maximum nanoparticle synthesis by *Azadirachta indica, Catharanthus roseus, Syzygium cumini* anda mixture of gallic acid-quercetin were found to be 6, 15, 12 and 8 h, respectively. The detailed results of optimization parameters for the synthesis of various nanoparticles were previously reported by our group.[20,22] The synthesized nanoparticles showed good stability over the period of three month in the form of colloidal solution and one year in the form of lyophilized powder. The UV-Vis spectra of stored nanoparticles (colloid solution and powder form) did not show significant changes (three month and one year) in absorbance pattern indicating stable particle size and size distribution.

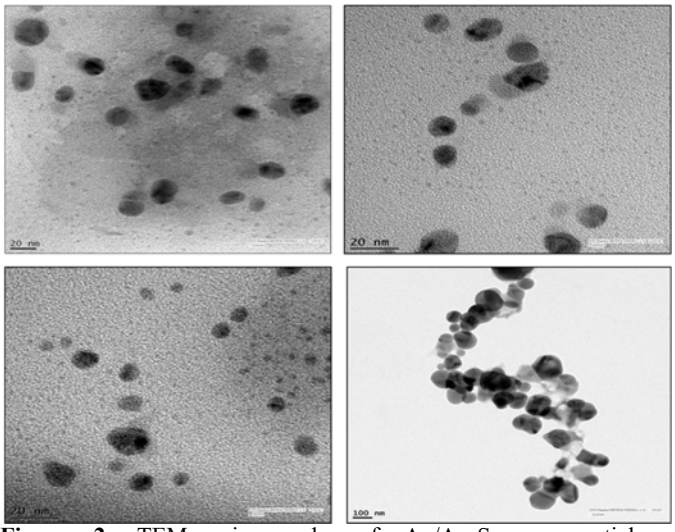

Figure 2. TEM micrographs of Ag/Ag-Se nanoparticles synthesized using various biological souces (a) *Azadiricta indica* (Neem) leave extract; (b) *Catharanthus roseus* (Vinca) flower extract; (c) *Syzygium cumini* fruit extract and (d) Phytochemicals quercetin dihydrate and gallic acid

3.3 Antimicrobial effect of bioinspired nanoparticles

Metal nanoparticles have gained more importance due to their excellent antimicrobial activity.[17] The main advantages of nanoparticles is the weaker ability of bacteria to develop resistances and nontoxic effect at lower concentration in human cells.[27] The present study was performed to see the growth inhibitory effect of biosynthesized nanoparticles on common laboratory microorganisms. Broth microdilution method was

used and inhibition was measured on the basis of cellular absorbance. All the synthesized nanoparticles showed bacterial growth inhibition (30-40 %) at 25 µg/mL and it was more (50-70 %) when nanoparticle concentration was increased upto 100 µg/mL. Chloramphenicol standard drug exhibited 60 % growth inhibition at 50 µg/mL while silver salt showed 40 % only at same concentration. The results of broth mircodilution method are shown in Figures 3 and 4.

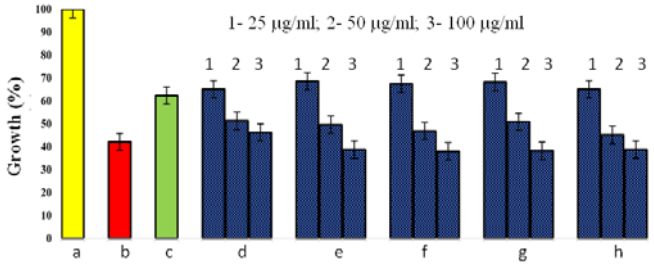

Figure 3. Antibacterial action of various plant-inspired nanoparticles on *Escherichia coli*. *a-Escherichia coli* (control); b-Chloromphenicol (50 µg/ml); c-Silver nitrate (50 µg/ml); d-silver nanoparticles procured from sigma chemical; e- *Azadirachta indica* AgNPs; f-*Catharanthus roseus* AgNPs; g-*Syzygium cumini* AgNPs; h-Quercetin-gallic acid Ag-SeNPs.

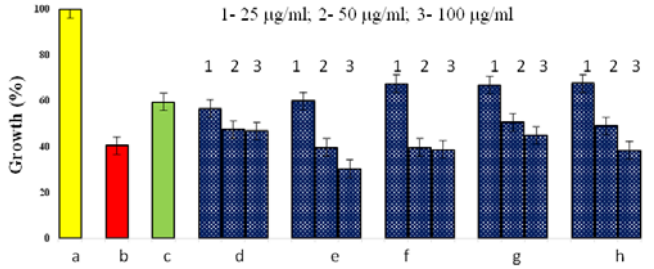

Figure 4. Antibacterial action of various plant-inspired nanoparticles on *Bacillus subtilis*. *a-Bacillus subtilis* (control); b-Chloromphenicol (50 µg/ml); c-Silver nitrate (50 µg/ml); d-silver nanoparticles procured from sigma chemical; e- *Azadirachta indica* AgNPs; f-*Catharanthus roseus* AgNPs; g-*Syzygium cumini* AgNPs; h-Quercetin-gallic acid Ag-SeNPs.

Furthermore, the antibacterial effect of biosynthesized nanoparticles was compared with marketed nanopowder sold by Sigma, USA. These marketed nanopowder also showed significant bactericidal activity against selected bacterial strains at 25 µg/mL. At higher concentration (50 µg/mL) there is no significant difference on antibacterial action. This was due to poor solubility of nanopowder at higher concentration. The biosynthesized nanoparticles are having higher antimicrobial activity, might be due to their higher solubility (capping of polar groups of phytochemicals) and higher diffusion potential. Conclusively the results demonstrated that, biosynthesized nanoparticles exhibited good bactericidal activity at all concentrations. These biosynthesized metallic nanoparticles can be used for the next generation of antimicrobials.

Saxena et al. in 2012 reported the synthesis of silver nanoparticles by *Ficus benghalensis* leaf extract and effective bactericidal activity against *Escherichia coli*.[28] Though the exact mechanism for the microbicidal activity for AgNPs has not yet been reported in detail, there are few reports stating its possible mechanism.[29] Another mechanism is the inhibition of bacterial respiratory chain.[29] Furthermore, the interaction of Ag^{+} with the thiol groups in bacterial proteins or interference in DNA replication is well known.[30,31] The interaction of AgNPs with Gram-negative bacteria and disruption of cell membrane and penetration of nanoparticles into the cytoplasm, binding with DNA and ribosomes are well reported in the literature.[27]

3.4 Cytotoxicity of plant-inspired nanoparticles on various cancer cell lines

The cytotoxicity of all nanoparticles synthesized by different plant extracts or phytochemicals was evaluated on the HeLa, Hek-293 and MCF-7 cell lines using MTT assay. All the nanoparticles were able to reduce the viability of the all cell lines in a dose-dependent manner, as shown in Figures 5 to 7. The 50 % cytotoxic effect of *Azadirachta indica* silver nanoparticles was observed at 10 and 25 µg/mL after 24 h treatment on MCF-7 cell lines while it reached upto 90 % at 48 h (Figure 5). These nanoparticles did not show significant cytotoxicity against HeLa and Hek-293 cell lines.

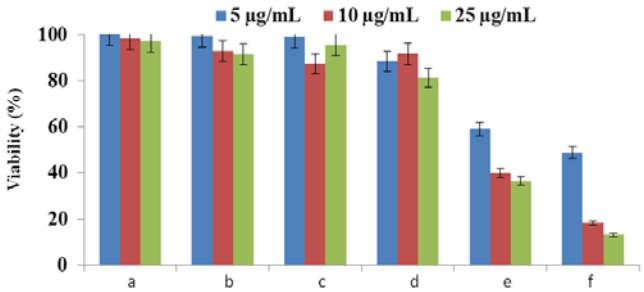

Figure 5. Cytotoxic effect of silver nanoparticles synthesized by the extract of *Azadirachta indica* on various cell lines. a-HeLa cells (24 h of treatment); b-HeLa cells (48 h of treatment); c-Hek-293 cells (24 h of treatment); d- Hek-293 cells (48 h of treatment); e-MCF-7 cells (24 h of treatment); f-MCF-7 cells (48 h of treatment).

Catharanthus roseus mediated silver nanoparticles did not show any significant level of toxicity on all the cell lines (Figure S5 in the electronic supplementary material). The silver nanoparticles synthesized by *Syzygium cumini* fruit extract inhibited the proliferation (up to 80 %) of HeLa and Hek-293 cell lines at 10 and 25 µg/mL after 24 and 48 h of incubation and the same percentage of inhibition was observed with MCF-7 cell lines at the same concentration after 48 h (Figure 6). At 5 µg/mL, *S. cumini* NPs did not show significant toxicity on any cell lines. Quercetin and gallic acid Ag-SeNPs exhibited the toxicity on HeLa and Hek-293 cells at higher concentration while it did not exhibit any toxicity on MCF-7 cells (Figure 7).

The cytotoxicity of biosynthesized nanoparticles was compared with the marketed Sigma nanopowder. The marketed nanopowder did not show significant level of toxicity to all the cell lines at all concentrations (Figure S6 in the electronic supplementary material). The results demonstrated that biosynthesized metal nanoparticles exhibited cell type, dose and time dependent toxicity. These biosynthesized nanoparticles may be used for the effective therapy to kill various types of cancer tumour cells.

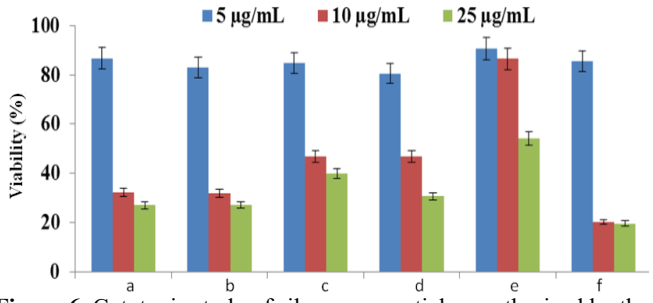

Figure 6. Cytotoxic study of silver nanoparticles synthesized by the extract of *Syzygium cumini* on various cell lines. a-HeLa cells (24 h of treatment); b-HeLa cells (48 h of treatment); c-Hek-293 cells (24 h of treatment); d- Hek-293 cells (48 h of treatment); e-MCF-7 cells (24 h of treatment); f-MCF-7 cells (48 h of treatment).

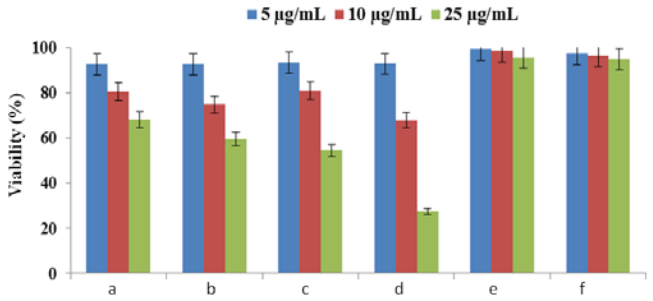

Figure 7. Cytotoxic study of Ag-SeNPs synthesized by gallic acid and quercetin on various cell lines. a-HeLa cells (24 h of treatment); b-HeLa cells (48 h of treatment); c-Hek-293 cells (24 h of treatment); d- Hek-293 cells (48 h of treatment); e-MCF-7 cells (24 h of treatment); f-MCF-7 cells (48 h of treatment).

Similarly Liu et al. in 2010 found the cytotoxicity of three different size nanoparticles (SNP-5, SNP-20 and SNP-50) on four different cell lines (A549, SGC-7901, HepG2 and MCF-7). They found the deleterious effect of AgNPs on the cell morphology and membrane integrity and the elevated levels of the ROS in the cells.[32] Sarkar et al. in 2011 investigated the toxicity of biogenic silver nanoparticles on human lymphocytes using comet assay showing no DNA damage at 50 mg/mL, while with 300 mg/mL AgNPs, DNA damage was observed.[33] Dipankar and Murugan in 2012 reported the biosynthesized silver nanoparticles, exhibiting a potent toxic effect on HeLa cancer cells with 88 % death after treatment with 300 µg/mL AgNPs.[34] Vivek et al. in 2012 reported the cytotoxicity of silver NPs synthesized by plant *Annona squamosal* and reported dose-dependent cytotoxicity with IC_{50} values of 50, 30 and 80, 60 µg/mL against MCF-7 and normal HBL-100 cell lines at 24 and 48 h of treatment, respectively.[35] Recently, Prabhu et al. in 2013 reported the growth inhibition of human colon cancer cell lines (HCT15) by the biocatalysed silver nanoparticles with an IC_{50} of 20 µg/mL.[36]

CONCLUSION

The synthesis of metal nanoparticles by plant extracts and phytochemicals is a simple, rapid and economical route. The method has many advantages such as, economic viability, higher rate of production, biocompatibility, higher solubility etc. The biosynthesis of nanoparticles using living material is the non-conventional and eco-friendly method compared to the chemical synthesis. The nanoparticles can be used for the treatment of multi-drug resistant bacterial strains, improving of wound healing, development of anticancer drug delivery system and much more. Furthermore, the nanoparticles synthesized via green route possesses the greater potential to kill only infected cells. These properties can be considered for the biomedical application and efficient drug delivery in near future.

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SUPPLEMENTARY MATERIAL

Cytotoxicity study details and results are provided as Supplementary material that can be downloaded from journal site.