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Green Synthesis of Silver Nanoparticles from *Caesalpinia pulcherrima* Leaf Extract and Evaluation of Their Antimicrobial, Cytotoxic and Genotoxic Potential (3-in-1 System)

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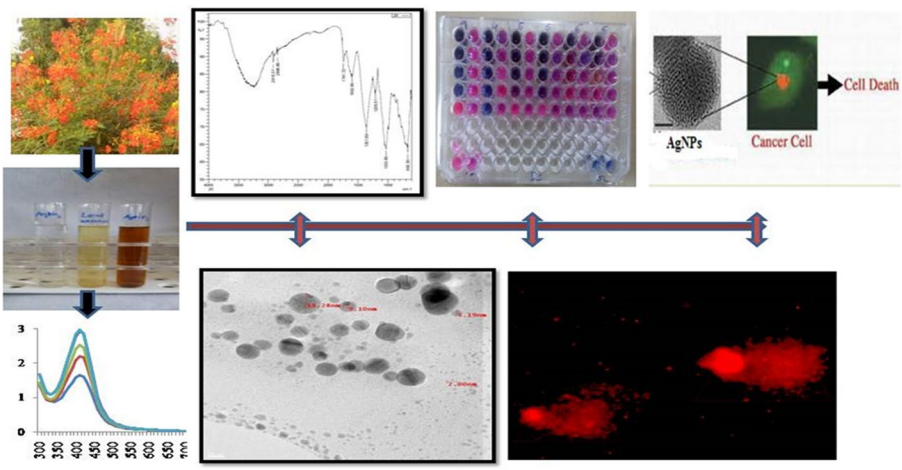
Abstract

Green synthesis of silver nanoparticles using plant extracts is popular because of its ease, simplicity and wide applications. Here we report the synthesis of silver nanoparticles (AgNPs) by *Caesalpinia pulcherrima* leaf extract. Optimization of various parameters like boiling time for plant extract preparation, concentration of plant extract and silver nitrate, pH and incubation time of reaction mixture were standardized. AgNPs were synthesized using standardized and optimized parameters. The characterization of synthesized AgNPs was done by various spectroscopic methods. UV–Vis spectroscopy showed characteristic peak at 410 nm, FTIR analysis indicated the presence of alcohols, carboxylic acids, esters and ethers. XRD confirmed the crystalline nature of AgNPs while TEM revealed the particles to be spherical in shape with an average size of 9 nm. The synthesized AgNPs showed good antimicrobial activity especially more against Gram negative bacteria. The cytotoxicity effect was dose dependent and genotoxic study revealed non toxic nature at lower concentration.

Graphic Abstract



Keywords Green synthesis · silver nanoparticles · Characterization · Antimicrobial activity · Cytotoxicity

1 Introduction

Nanotechnology is an exciting and important interdisciplinary science which brings together diverse scientific disciplines such as biology, chemistry, material science and physics. It is the most revolutionary technological

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innovations of twenty-first century. Particles with size up to 100 nm are considered as nanoparticles; they have unique physical and chemical properties because of their high surface area to volume ratio. They exhibit entirely new properties other than that of the bulk material of which they are made up of. Their unique optical, electronic, mechanical, magnetic and chemical properties are significantly different from those of bulk materials. Nanoparticles can be synthesized from various metals like gold, silver, zinc, copper, palladium, titanium, etc. but silver nanoparticles have received much attention due to its wide applications even in ionic and atomic states.

Silver nanoparticles (AgNPs) can be synthesized by different physical, chemical and biological methods. Some of the physical and chemical methods are microwave irradiation [1], reversed micelle processes [2], electrochemical reduction [3], radiation [4], Langmuir–Blodgett [5], etc. They are numerous in number but there are many disadvantages for eg. They are expensive, complex, time consuming, make use of expensive and toxic chemicals, require vigorous processing, not environmental friendly, produce hazardous by products which add to pollution, require expensive and highly complex equipments, requires addition of reducing and stabilizing agents and hence not much favoured. The alternative and much favoured method is biological method which makes use of plants and microorganisms**.** It is simple cost effective, eco-friendly and does not require high pressure, energy, temperature or toxic chemical reagents; on the other hand, requires very low maintenance and natural phytochemicals present in various parts of the plants like enzymes, proteins, phenols, flavonoids, act as reducing and stabilizing agents, and produce highly biocaompatible and stable nanoparticles [6].

Silver nanoparticles have been synthesized using leaves of different plants for eg. *Saccharum officinarum* [7], *Dolichos lablab* [8], *Berberis vulgaris* [9], *Dodonaea viscose* [10], *Eriobotrya japonica* [11], *Tragopogon collinus*, [12], etc. Because of their unique properties, AgNPs find many applications like in medical devices, health care, diagnostic and drug delivery, cosmetics, electronic and house hold appliances, catalysis, waste water treatment, bio sensing, agriculture, etc. [13, 14]. Silver nanoparticles possess properties like antiproliferative [15], wound healing [16], antidiabetic [17], antimycotic [18], antileishmanial [19], hepatoprotective activity [20], anticancer [21], antioxidant ([22], larvicidal [23], photocatalytic [24], etc. The antibacterial property of AgNPs have been reported by many researchers. Qidwai et al. [25] synthesized AgNPs from *Phoenix sylvestris* seed extract which showed high antibacterial activity with high time-kill kinetics against *P. acnes* and *S. epidermidis* while seed extract of *Pisum sativum* showed good antimicrobial activity against *Escherichia coli* and *Candida albicans* [26]. Golabiazar et al., [27] synthesized AgNPs using *Pistacia atlantica* leaf extract as a reductant, stabilizer, and capping agent which showed antibacterial activity against Gram negative (*Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa* and *Salmonella paratyphi* B) and Gram positive (*Staphylococcus aureus* and *Streptococcus pyogenes*) bacterial strains. Siddiqi et al., [28] reported antibacterial activity of AgNPs against nine bacterial strains synthesized from dried leaf extract of *Diospyros montana*. Maximum activity of AgNPs was against *K. pneuomoniae* and *E. coli* while moderate efficacy was against *S. mutans, S. aureus, S. pyrogenes, S. viridians, P. aeruginosa, C. diphtheriae* and *C. xerosis*. Ajitha et al. [29] synthesized stable flower-shaped AgNPs which showed prominent bactericidal activity against *Escherichia coli, Pseudomonas spp., Bacillus spp*. and *Staphylococcus spp*. and fungicidal activity against *Aspergillus niger, Aspergillus flavus* and *Penicillium* spp.

The increased emergence of multi drug-resistant microbes and incidence of various types of cancer has become an increased cause of worry and stands as major burning problems worldwide affecting people to a great extent. The earlier treatments which were effective, have now become useless. The existing antibiotics are not able to bring relief from infectious menace. Cancer is a deadly disease affecting people in developing and developed countries and is one of the major health burden in the world. There are many synthetic chemotherapeutics to treat cancer but are associated with many disadvantages and best cure is still from nature. Majority of the anticancer drugs in use are derived from medicinal plants. The need of the hour is new ways and novel approaches to combat this life threatening diseases. Recently the attention has turned towards green synthesized metal nanoparticles especially AgNPs which showed promising anticancer activity. Apparently, nanobiotechnology has been a boon and novel strategy for the treatment and diagnosis of cancer. In vitro anticancer activity of AgNPs has been reported against an array of cell lines such as HeLa (cervical cancer cell line), MCF-7 (breast cancer cell line), COLO-205 (colon ceancer cell line), A-549 (lung cancer cell line) PC-3 (prostrate cancer cell line), A-431 (epidermal cancer cell line), Hep-G2 (hepatic cancer cell line), etc. [13].

Caesalpinia pulcherrima (Linn.) SW. is an ornamental plant belonging to Caesalpinaceae family with several medicinal properties. The leaves are reported for antimicrobial, antioxidant, antiulcer properties [30–32]. However, the efficacy of *C. pulcherrima* leaves to synthesize AgNPs is yet to be studied. Hence, the aim of the present investigation was (i) to synthesize AgNPs using *C. pulcherrima* leaf extract as a bioreductant and stabilizing agent, (ii) characterization of green synthesized AgNPs by various spectral techniques (iii) evaluation of antimicrobial potential of synthesized

AgNPs (iv) evaluation of cytotoxic potential and genotoxic properties.

2 Materials and Methods

2.1 Plant Extract Preparation

The fresh leaves of *C. pulcherrima* were collected from Rajkot, Gujarat, India. They were thoroughly washed with tap water, followed by double distilled water and cut into small pieces. Five grams of cut leaves were boiled in 100 ml ultra pure water and filtered through Whatmann No. 1 filter paper. The filtrate was cooled to room temperature and used as bioreductant for the synthesis of AgNPs.

2.2 Biosynthesis of Silver Nanoparticles

The general procedure for AgNPs synthesis was as follows: 3 ml of leaf extract was added to 40 ml of aqueous $AgNO_3$ (1 mM) solution at room temperature (25 °C + 2 °C); the colourless solution turned to brown indicating the formation of AgNPs. This solution was incubated in dark for 24 h. The nanoparticles solution was purified by repeated centrifugation at 10,000 rpm for 10 min followed by redispersion of the pellet of nanoparticles in acetone. They were air dried and then stored at 4 °C for further analysis. Various parameters like boiling time of plant extract, extract amount, concentration of silver nitrate, pH (6–10), and incubation time were optimized and finally AgNPs were synthesized using optimized parameters.

2.3 Optimization of Different Parameters

AgNPs were synthesized using *C. pulcherrima* leaf aqueous extract. The reduction of the silver ions in solution was monitored by UV–Vis spectrophotometer (Shimadzu UV-1601, Shimadzu Corporation, Kyoto, Japan) in 350–700 nm range operated at an interval of 10 nm. Various parameters optimized were boiling time of plant extract (5 min, 10 min, and 15 min), extract amount (1.5 ml, 3 ml, 6 ml, 9 ml, and 12 ml), concentration of silver nitrate (0.5 mM, 1 mM, 1.5 mM, and 2 mM), pH (6–10), and incubation time (0 min–24 h).

2.4 Characterization Techniques

2.4.1 UV–Visible Spectroscopy

Synthesis of silver nanoparticles was observed by UV–Vis-spectroscopy. The reduction of the Ag + ions in solution was monitored by periodic sampling of aqueous component and measuring the UV–Vis spectra of the solution. UV–Vis spectra of these aliquots were monitored as a function of time of reaction on a spectrophotometer (Shimadzu UV-1601) in 400–700 nm range operated at a resolution of 10 nm.

2.4.2 FTIR Analysis

The FTIR spectrum was recorded in the range of 400–4000 cm^{-1} Nicolet IS10 (Thermo Scientific, Waltham, MA). Various modes of vibrations were identified and assigned to determine the different functional groups present in *C. pulcherrima* leaf extract.

2.4.3 XRD Analysis

The structure and composition of synthesizd AgNPs was analyzed by XRD. The formation of AgNPs was determined by an X'Pert Pro X-ray diffractometer (PAN analytical BV) operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation in **θ–2θ** configurations. The crystallite domain size was calculated from the width of the XRD peaks, assuming that they are free from non-uniform strains, using the Scherrer formula. D¼0.94 λ/ β cos **θ**, 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.

2.4.4 Thermal Gravimetric Analysis

Thermal stability and surface weight loss of AgNPs was determined by Thermogravemeteric (TGA) analysis using DTG-60H instrument (Shimadzu Corporation, Kyoto, Japan). TGA spectra was recorded under a nitrogen gas flow of 100.0 ml min^{-1} and at heating rate of 10 °C min^{-1} to 1000 °C.

2.5 TEM Analysis

TEM analysis was done to visualize the shape as well as to measure the size of green synthesized AgNPs. The sample was dispersed in double distilled water and a drop of thin dispersion was placed on a "staining mat". Carbon coated copper grid was inserted into the drop for 10 min, air dried and then screened in JEOL JEM 2100 Transmission Electron Microscope (JOEL Corp, Tokyo, Japan).

2.5.1 Biological Activity of Synthesized Silver Nanoparticles

The green synthesized AgNPs were evaluated for their antimicrobial, cytotoxic and genotoxic potential. The antimicrobial activity was measured by measuring the minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of AgNPs [33, 34] and growth curve assay [35] against eleven microorganisms. The cytotoxic activity was evaluated by MTT assay against HeLa cell line. The genotoxicity was evaluated by comet assay. The procedure followed is as described earlier [36].

3 Results and Siscussion

3.1 Optimization of Different Parameters

Silver nanoparticles formation in the reaction mixture is easily detected by the change in colour from pale yellow to dark brown which is due to excitation of surface plasmon vibration of the silver nanoparticles as also reported by other researchers [37, 38]. UV–Vis spectroscopy is a fundamental technique to determine the formation of metal nanoparticles in aqueous medium. Boiling time of plant extract, plant extract concentration, silver nitrate concentration and pH of reaction mixture are some of the key factors that determine the formation and size of synthesized AgNPs. It is generally reported that the UV absorption peak reflects the size of the nanoparticles i.e. larger particles show broader peak and smaller particles show narrow absorption peak and the absorption intensity directly indicates the number of nanoparticles formed [39]. Thus, this criterion was used for selection of the correct parameter for synthesis of AgNPs.

In order to evaluate the effect of boiling time for the leaf extract preparation, the leaves were boiled for 5, 10 and 15 min. AgNP formation was faster when 5 min boiled leaf extract was added than when 10 and 15 min boiled leaf extract was added (Fig. 1a). The absorption intensity was also slightly higher in 5 min boiled leaf extract as compared to 10 and 15 min boiled leaf extract. Hence, 5 min boiling time was finalized for the preparation of the leaf extract.

In order to evaluate the effect of leaf extract concentration for the formation of AgNPs, different aliquots (1.5, 3, 6, 9 and 12 ml) of 5 min boiled leaf extract was added to 40 ml 1 mM $AgNO_3$ solution. The synthesis of particles occurred faster when 6 ml extract amount was added to the reaction mixture than when 1.5, 3, 9, 12 ml extract was added (Fig. 1b). The narrow absorption peak indicates the smaller size [40] and increase in absorption peak indicates formation of more AgNPs [41]. When 6 ml extract was added, the absorption peak was narrow and absorption intensity was high. Hence, addition of 6 ml extract was finalized for the synthesis of AgNPs.

In order to evaluate the effect of silver nitrate concentration for the formation of AgNPs, 6 ml 5 min boiled leaf extract was added to 40 ml different concentration of silver nitrate solution (0.5, 1, 1.5, 2 mM). 0.5 mM $AgNO_3$ containing reaction mixture developed light brown color while 1.0, 1.5 and 2.0 mM $AgNO_3$ containing reaction mixtures developed darker brown colour. 1 mM of $AgNO_3$ concentration supported rapid formation of dark brown colour for AgNPs (Fig. 1c). Hence, 1 mM $AgNO_3$ concentration was finalized for the synthesis of AgNPs. Upon increasing the concentration of $AgNO_3$, the intensity of the SPR bands increased due to the enhancement in the nuclei formation indicating larger particle size [42, 43].

In order to evaluate the effect of pH for the formation of AgNPs, 6 ml 5 min boiled leaf extract and 40 ml 1 mM silver nitrate reaction mixture was adjusted with different pH (6, 7, 8, 9, 10). At pH 6 and pH 7, the absorbance band was broad indicating formation of larger nanoparticles (Fig. 1d). At pH 8 absorbance band was narrow as compared to pH 9 and pH 10. Hence, pH 8 was finalized for the synthesis of AgNPs. As already stated above, narrow SPR band indicates smaller particles size while broad SPR band indicates large particles size. Use of alkaline pH for synthesis of AgNPs is also reported by Ganesh Kumar and Poornachandra [44] and Verma and Mehata [45].

The UV–Vis spectra were recorded at different time intervals (30 min, 60 min, 2 h, 24 h); however no change in peak intensity was found (Fig. 1e). The absorption maxima peak was observed at 410 nm which is due to surface plasmon vibrations. The AgNPs synthesized using bagasse extract also showed an absorption peak at 410 nm [46] while AgNPs synthesized using apple peel and grape fruits extracts showed an absorption peak at 420 nm [24]. Therefore optimum conditions for green synthesis of AgNPs by *C. pulcherrima* leaf extract was 5 min boiling time for leaf extract preparation, 6 ml leaf extract addition to reaction medium, 1 mM silver nitrate concentration, pH 8 of reaction medium and reaction time for synthesis of AgNPs was 24 h. AgNPs were synthesized using above optimized conditions. Seifipour et al. [12], also optimized various parameters for AgNPs synthesis.

3.2 FTIR Analysis

FTIR analysis is generally done to recognize the functional groups involved in silver nanoparticles formation. FTIR spectrum of AgNPs, recorded in the range of 500–4000 cm^{-1}, showed prominent peaks at 3512.37, 2891.30, 1591.27, 1323.17, 1147.65, 983.70 and 704.02 cm^{-1} (Fig. 2a). The peak at 3512.37 cm^{-1} corresponds to O–H stretch of alcohols and phenols.

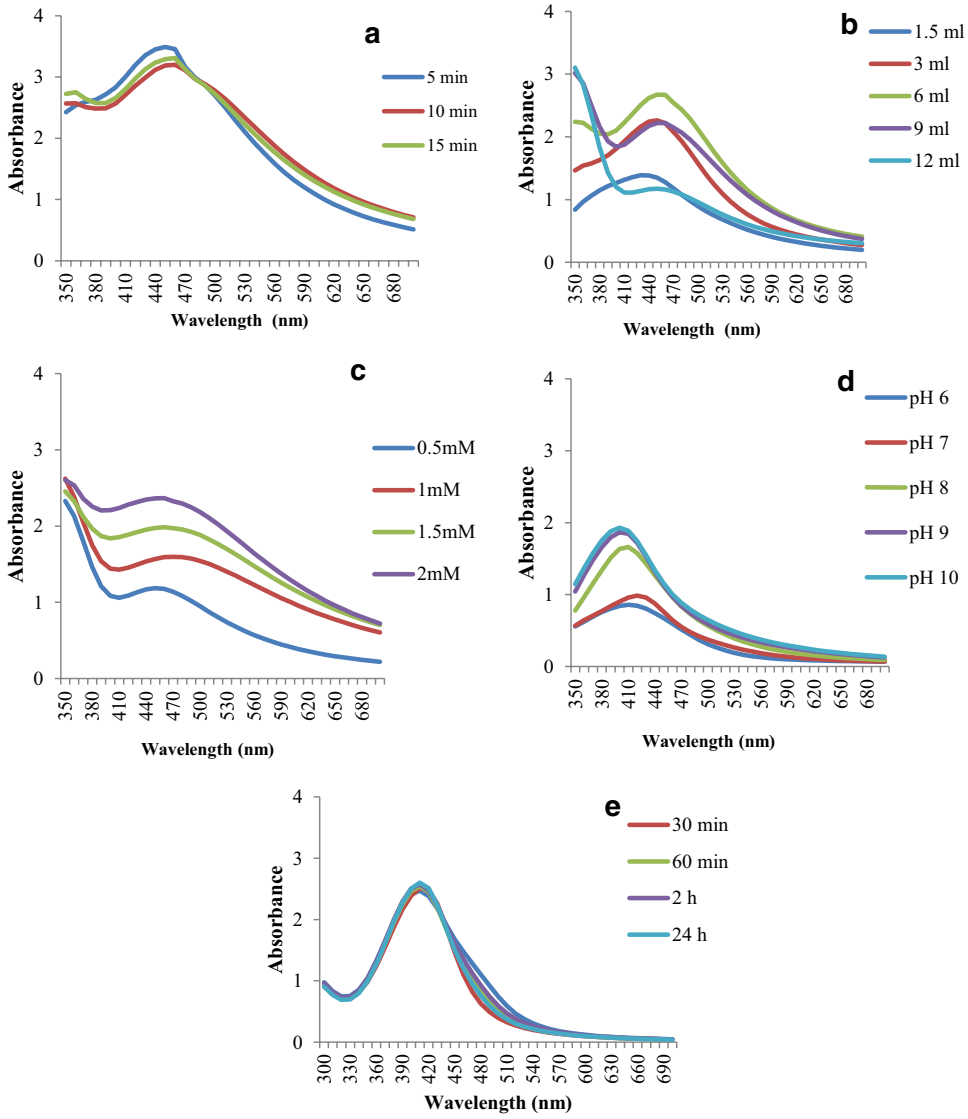

Fig. 1 **a** Effect of boiling time. **b** Effect of extract amount. **c** Effect of silver nitrate concentration. **d** Effect of pH. **e** UV–Vis spectra at different time interval

2891.30 cm^{-1} peak is due to the C–H stretch of alkanes while 1591.27 cm^{-1} peak is due to N–H bend of primary amines. 1323.17 corresponds to N–O symmetric stretch of nitro compounds. 1147.65 cm^{-1} is assigned to the C–N stretching of aliphatic amines. 983.70 cm^{-1} indicate the C–H bend of alkenes group while 704.02 cm^{-1} is assigned to the C–Cl stretching of alkyl halides. Similar peaks were reported by other researchers. 3419 cm^{-1} corresponds to O–H stretching of alcohols and phenols and 1648 cm^{-1} corresponds to N–H bend of primary amines [47]. 2886 cm^{-1} is due to C–H stretching of alkanes [48] while 1384 cm^{-1} is due to nitro N–O bending [49], 1218 cm^{-1} stretching vibrations is of C–N of aliphatic amines [50]. 821.96 cm^{-1} is assigned to C–Cl stretching of alkyl halides [51]. It is suggested that different functional groups such as alcohols, carboxylic acids, esters, ethers etc. are responsible for reduction of silver ions and also prevent the agglomeration of AgNPs.

3.3 TGA Analysis

The TGA curve of green synthesized AgNPs is shown in Fig. 2b. The initial weight loss of about 4% at the temperature of 100 °C was due to loss of water molecules adsorbed on the surface of AgNPs. The second weight loss of about 34% was in the temperature range of 300–400 °C and third weight loss was about 51% in the temperature range of 500–600 °C. This weight loss is attributed to the degradation of bioorganic molecules biocapped on the surface of the nanoparticles. At the temperature above 800 °C there was no weight loss in TGA curve, indicating that the synthesized AgNPs were stable within this temperature range. Similar decomposition of AgNPs was reported by Ahmad et al. [52].

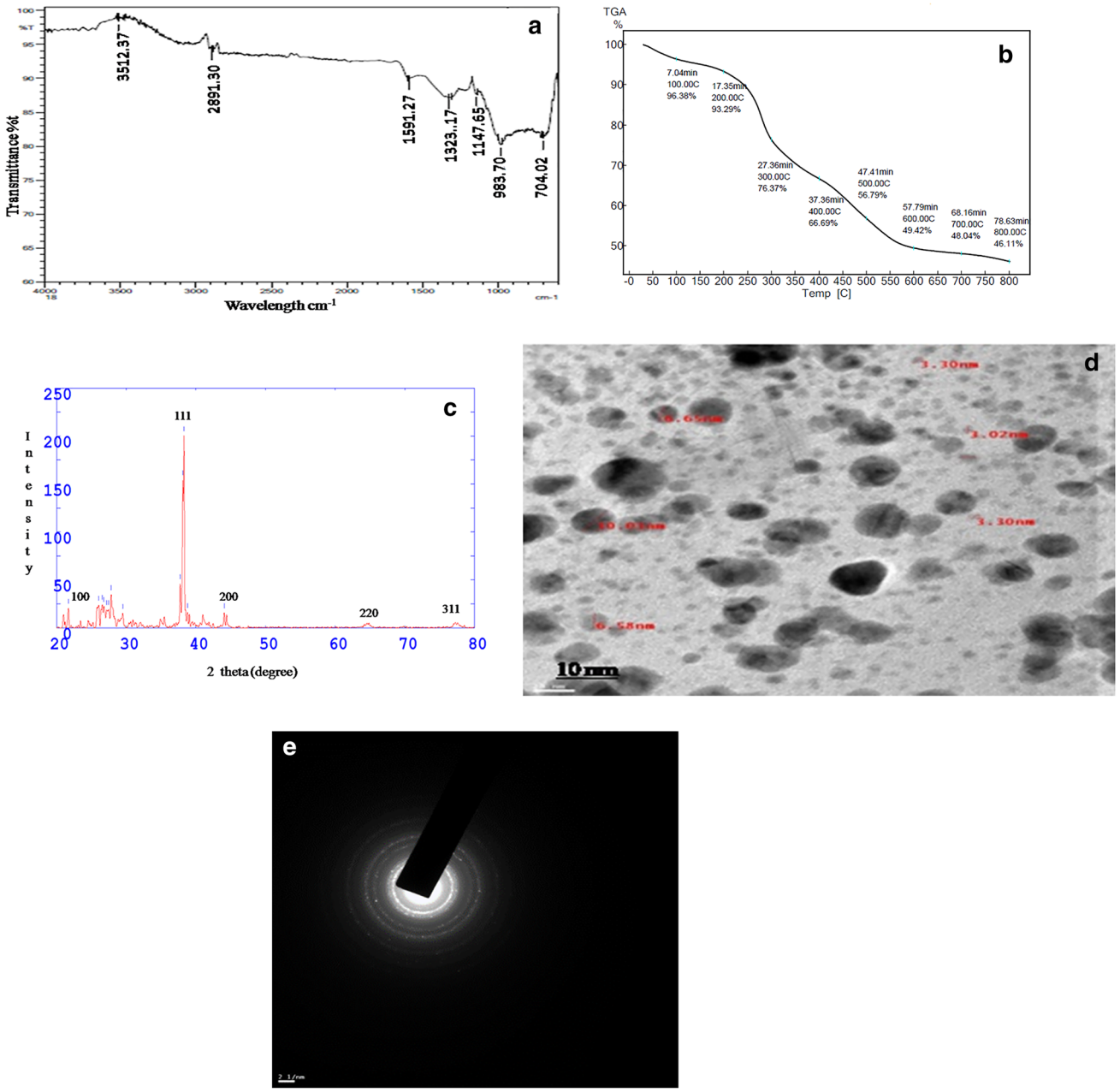

Fig. 2 **a** FTIR spectrum of AgNPs. **b** TG curve of AgNPs. **c** XRD spectrum of AgNPs. **d** TEM images of AgNPs. **e** SAED patterns of the AgNPs

Further there was no weight gain which is an indication that the synthesized AgNPs remained intact without any oxidation because of inert nitrogen atmosphere. Yallappa et al. [53] reported similar behavior of AgNPs synthesized from *J. sambac* leaf extract.

3.4 XRD Analysis

The crystalline nature of synthesized AgNPs was confirmed by X-ray diffraction (XRD) analysis (Fig. 2c). The observed peaks clearly demonstrated the crystalline nature of synthesized AgNPs. Five prominent diffraction peaks were obtained 21.71°, 38.29°, 44.08°, 64.64° and 78.69° which represent (100), (111), (200), (220) and (311) planes of a face center cubic (fcc) lattice of silver crystal (Fig. 2c). The observed XRD pattern was compared and matched with the JCPDS data file No. 4.0783. The XRD pattern found in the present study are similar to those reported for other AgNPs [25, 27].

3.5 TEM Analysis

Morphology and particle size of AgNPs was characterized using TEM (Fig. 2d). TEM images revealed the shape of the particles to be spherical and the size ranged from 2 to 24 nm; average size was 9 nm. The AgNPs synthesized from *S. vulgare* and *L. chinensis* also possessed average particle size of 10 nm [54, 55] while those synthesized from leaf extract of *D. montana* possessed an average size of 11.65 nm [28]. Rautela et al. [56] synthesized AgNPs from *Tectona grandis* seed extract and their size was in the range of 10–30 nm. The selected area electron diffraction (SAED) pattern of AgNPs suggests the crystalline nature of AgNPs (Fig. 2e) which is in good agreement with the XRD results. The characteristic bright circular fringes can be indexed to (111), (200), (220) and (311) of the pure face centered cubic (fcc) lattice structure [57]. Presence of no other peak than silver suggests the purity of the green synthesized AgNPs. Similar results are reported by Roy et al., [58] in *Petroselinum crispum* leaf extract synthesized AgNPs.

3.6 Antimicrobial Activity of Silver Nanoparticles

The antimicrobial potential of green synthesized AgNPs was evaluated by determining their MIC and MBC values and growth curve studies. The MIC and MBC of synthesized AgNPs was evaluated against four Gram positive, four Gram negative and three fungi using different concentrations (0.019–10 mg/ml) by Resazurin assay (Table 1). Chloramphenicol and Amphotericin B antibiotics were used as positive control for bacteria and fungi respectively. Different microorganisms showed different level of MIC and MBC values. The MIC values ranged from 0.078 mg/ml to 1.25 mg/ml while MBC values ranged from 1.25 mg/ml to 10 mg/ml. The MIC value of standard antibiotic Chloramphenicol ranged from 1.25 to 5 mg/ml while MBC values ranged from 5 mg/ml to 10 mg/ml. The MIC and MBC values of the antifungal antibiotic Amphotericin B was > 10 mg/ml. The lowest MIC value was found against *K. pneumonia* (0.078 mg/ml) followed by *E. coli* (0.156 mg/ml). Both these organisms had lowest MBC values. *K. pneumonia* was the most susceptible organism. It's MIC and MBC values were less than that of standard antibiotic.

AgNPs synthesized using *Avicennia marina* seed extract showed antibacterial activity against *K. pneumoniae* (ATCC 700,603), *E. coli* (ATCC 35,218), *S. aureus* (ATCC 43,300), *E. faecalis* (ATCC 5129), and *P. aeruginosa* (ATCC 27,853). *E. coli* was the most sensitive strain with MIC value of 6.25 µg/mL followed by *K. pneumoniae* and *P. aeruginosa* (MIC 12.5 µg/mL) while *E. faecalis* was the resistant strain [38]. *Arnebia hispidissima* mediated synthesized AgNPs showed strong anti-microbial activity against *Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumonia, Escherichia coli, Candida albicans, Candida tropicalis* and *Geotrichum candidum* [59]. Antibacterial activity against *B. subtilis, B. vallismortis* and *E. coli* by AgNPs synthesized using plant extract of *Salvia spinosa* is reported by Pirtarighat et al. [60]. Bagherzade et al. [61] reported that biosynthesized Ag NPs using *Crocus sativus* wastages showed significant antibacterial effect against *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Shigella flexneri* and *Bacillus subtilis*.

The green synthesized AgNPs showed better antimicrobial activity against Gram negative bacteria than Gram positive bacteria**.** This is in agreement with previous studies [28, 62] wherein Gram positive bacteria were less susceptible than Gram negative bacteria**.** This is attributed to change in cell wall composition of both these bacterial strains. The simple reason may be the peptidoglycan cell wall nature of the Gram negative bacteria. The Gram negative bacteria

Table 1 MIC and MBC values (mg/ml) of AgNPs against Gram positive, Gram negative bacteria and fungi

Microorganisms	MIC of AgNPs	MBC of AgNPs	MIC of chloramphenicol	MBC of chloramphenicol	MIC of amphotericin B	MBC of amphotericin B
B. cereus	1.25	5	1.25	5	NA	NA
B. subtilis	0.625	10	1.25	5	NA	NA
S. aureus	0.625	10	1.25	5	NA	NA
C. rubrum	1.25	10	2.5	10	NA	NA
E. coli	0.156	1.25	2.5	10	NA	NA
P. aeruginosa	0.625	5	2.5	10	NA	NA
S. typhimurium	0.312	2.5	1.25	5	NA	NA
K. pneumoniae	0.078	1.25	2.5	10	NA	NA
C. albicans	1.25	5	NA	NA	> 10	> 10
C. glabrata	1.25	5	NA	NA	> 10	> 10
C. neoformans	1.25	5	NA	NA	> 10	> 10

has thin peptidoglycan layer while Gram positive bacteria possesses a thick and rigid peptidoglycan layer. This difference hinders the penetration of AgNPs easily into the cell wall. The AgNPs can easily penetrate the thin peptidoglycan layer of Gram negative bacteria while prevent the entry of AgNPs in to the cell wall of Gram positive bacteria. Further it can be stated, that the positively charged AgNPs are greatly attracted to negatively charged Gram negative bacteria and rupture the cell wall which will destabilize the cell and plasma membrane which leads to depletion of intracellular ATP [63]. However there are reports where green synthesized AgNPs showed better activity against Gram positive bacteria [36, 43]. Thus, AgNPs have the potential to inhibit the growth of both Gram positive and Gram negative bacteria.

3.7 Growth Curve of Silver Nanoparticles

The growth curve assay was used to determine the microorganisms viability and to define the minimum time necessary to reach an inhibitory or bactericidal effect. AgNPs inhibitory effect was carried out against four Gram positive, four Gram negative and three fungi using the growth curves under AgNPs concentration equal to $1 \times$ MIC. For negative control only microorganisms were used and antibiotics Chloramphenicol and Amphotericin B were used as a positive control. The optical density at 600 nm (OD600) was measured at different time intervals to monitor the bacterial and fungal growth. Growth curve of synthesized AgNPs, Chloramphenicol and Amphotericin B showed similar antimicrobial trend that was observed in MIC results. AgNPs and Chloramphenicol treated culture showed suppressed growth of all the four Gram positive bacteria with respect to negative control (Fig. 3). The optical density was almost same over the time while in the negative control (only bacteria) the optical density increased with respect to time. All the four Gram positive bacteria exposed to AgNPs, showed complete growth inhibition over a period of time (24 h). The antibiotic Chloramphenicol also showed similar results i.e. complete growth inhibition. A slightly different trend was observed with the growth of Gram negative bacteria exposed to AgNPs. The green synthesized AgNPs inhibited the Gram negative bacteria more than that of positive control Chloramphenicol (Fig. 4). Chloramphenicol treated organisms reached stationary growth phase after 6 h while AgNPs treated organisms showed suppressed growth till 24 h; i.e. growth inhibition of Gram negative bacteria was better than that of positive control.

Growth curve of AgNPs and Amphotericin B against three fungi *C. albicans, C. glabrata* and *C. neoformans* is given in Fig. 5. All the three fungi, showed a remarkable growth inhibition effect; even better than that of positive control (Amphotericin B). Amphotericin B treated organisms reached stationary growth phase after 8 h while AgNPs treated organisms showed suppressed growth till 48 h like that of Gram negative bacteria. The optical density of all the three fungal cultures was almost same and steady with

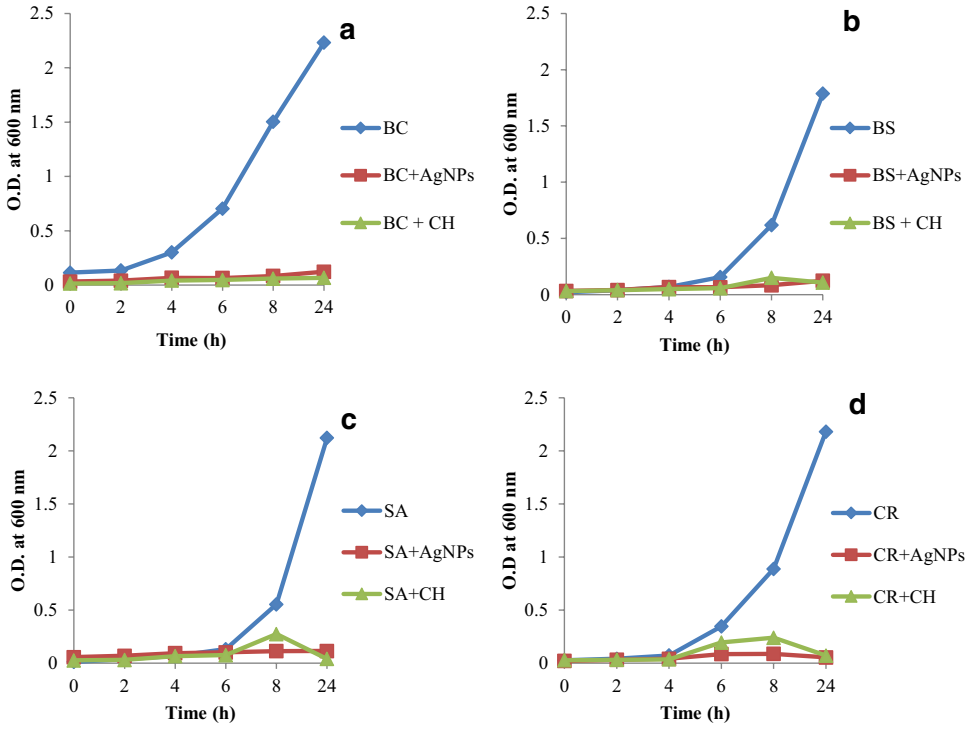

Fig. 3 Growth curve assay of AgNPs and chloramphenicol against **a** *B. cereus* (BC), **b** *B. subtilis* (BS), **c** *S.aureus* (SA) and **d** *C. rubrum* (CR)

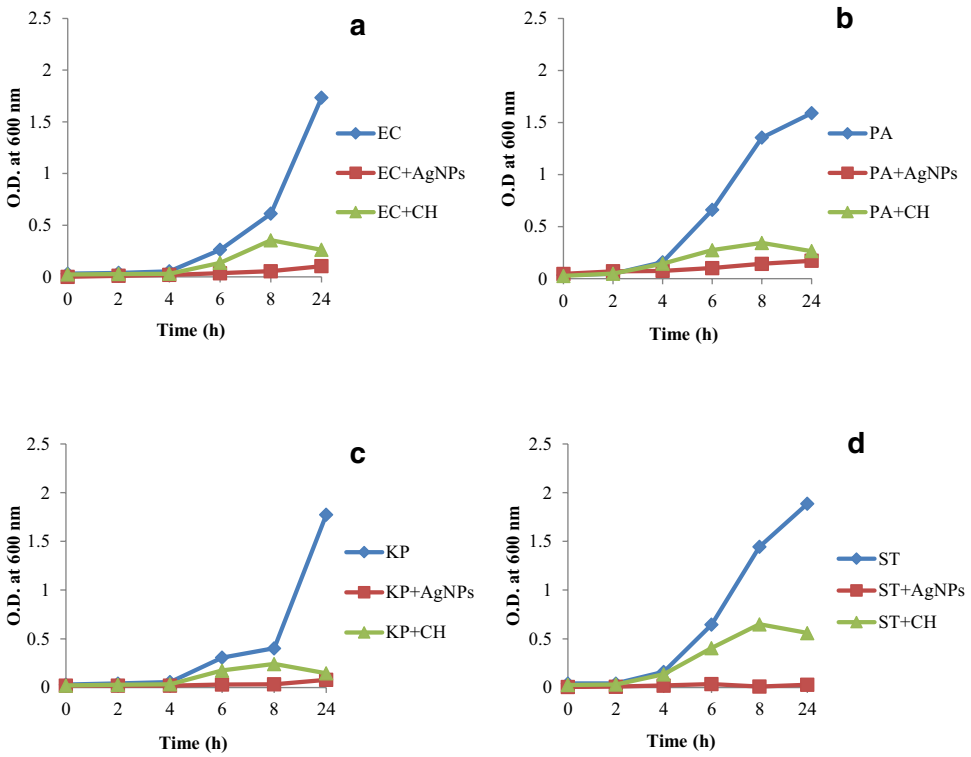

Fig. 4 Growth curve assay of AgNPs and chloramphenicol against **a** *E. coli* (EC), **b** *P. aeruginosa* (PA), **c** *K. pneumoniae* (KP) and **d** *S. typhymurium* (ST)

respect to time indicating complete inhibition of fungal growth. Antimicrobial effect of synthesized AgNPs was clearly envisaged. Dose and time dependant growth curve study against *E. coli* and *S. aureus* is also reported by He et al. [64] and Das et al. [65],while complete inhibition or suppressed growth against *E. coli* and *S. aureus* is reported by Elbeshehy et al. [66] and Wang et al. [67].

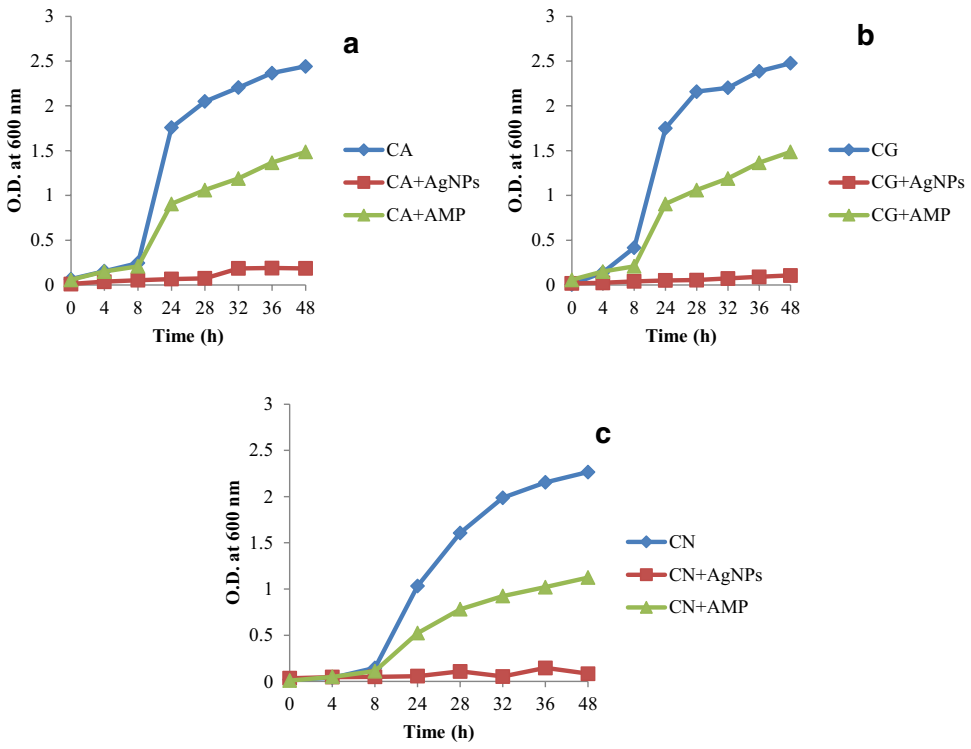

Fig. 5 Growth curve assay of AgNPs and Amphotericin B against **a** *C. albicans* (CA), **b** *C. glabrata* (CG) and **c** *C. neoformans* (CN)

3.8 Cytotoxicity Analysis of Silver Nanoparticles on Hela Cell Lines

In the present study, MTT assay was used to assess the effect of different concentrations of AgNPs (2 µg, 10 µg, 30 µg, 50 µg, 100 µg, 150 µg, 200 µg) on HeLa cell lines (Fig. 6). The cancer cell viability decreased with increasing concentration of AgNPs. At low concentration, 100% cell viability was found while at 50 µg/ml concentration, cell viability was 73% and at 200 µg/ml concentration, the cell viability was reduced to 34% i.e. a clear dose dependent effect was observed. Farah et al. [68] found IC_{50} value of 217 µg/ml of *A. obesum* leaves extract synthesized AgNPs against MCF-7 cells, while in the present work, a much lower IC_{50} value was found (70 µg/ml). AgNPs synthesized using aqueous root extract of *Arnebia hispidissima* showed dose-dependent cytotoxicity against HeLa cells (IC_{50} = 4.44 mg/mL) and were non-toxic towards normal L20 B cells (non-malignant mouse cell line) ([59]. Vivek et al. [69] synthesized spherical shaped Ag NPs with 20–100 nm size, using leaf extract of *Annona squamosa* for treatment of human breast cancer cell (MCF-7) IC_{50} value was 50 = g/ml after 24 h. *Datura inoxia* leaf extract mediated synthesized AgNPs inhibited proliferation of human breast cancer cell line MCF7 with an IC_{50} of 20 mg/ml at 24 h incubation [70]. *Andrographis paniculata* mediated synthesized AgNPs also showed anticancer activity against activity against HepG2 cells (27.01 µg/ ml) and PC3 cells (32.15 µg/ ml) [71]. Green synthesized AgNPs using *Artemisia turcomanica* leaf extract induced apoptosis and showed a cytotoxic and anti-cancer effect against gastric cancer cell lines in a dose- and time-dependent manner [72]. The observed cytotoxic effect may be via generation of reactive oxygen species via intracellular oxidative stress resulting in damage to cellular components like lipids, DNA and proteins which finally lead to cell death [73, 74]. Thus, the present study implicates that green synthesized AgNPs has good cytotoxic effect and it can be used as an alternative source for cancer chemotherapy.

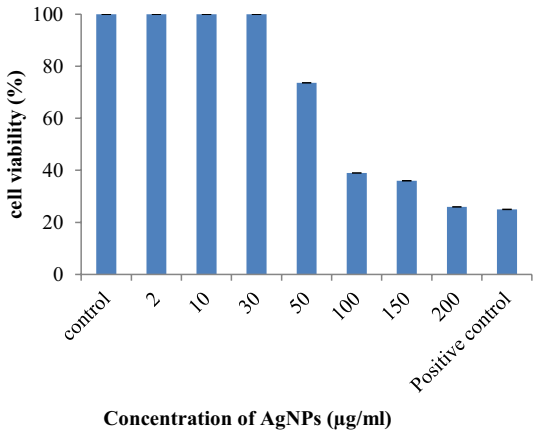

Fig. 6 In vitro cytotoxicity of AgNPs against HeLa cell line

3.9 Genotoxicity Analysis

The genotoxicity of AgNPs to damage DNA in the lymphocyte culture was evaluated by alkaline comet assay. The comet assay is a simple and reliable method to detect DNA damage and also used for assessment of DNA repair [75]. Lymphocyte culture was treated with three different concentrations of AgNPs (2 µg, 50 µg, 200 µg) and DNA damage was measured according to comet length or tail length (Fig. 7). In negative control, halo surrounding nuclei was clearly found (Fig. 7a). In positive control, cells were treated with Mitomycin C drug (Fig. 7b). 2 µg and 50 µg AgNPs treated cells showed intact and round nuclei without any fragmented DNA (Fig. 7 c, d) while 200 µg treated cells showed fragmented DNA (Fig. 7e). The comet tail length increased in a dose dependent manner and maximum length was with highest concentration of AgNPs (200 µg/ml) but it was less than that of positive control (Fig. 7f). The dose dependent genotoxicity is also reported in A549 cell line [76] and Swiss albino mice [77].

4 Conclusion

In this study, we report for the first time the antimicrobial, anticancer and genotoxic effect of silver nanoparticles synthesized from *C. pulcherrima* leaves extract. AgNPs showed characteristic peak at 410 nm. The green synthesized nanoparticles were spherical in shape and average size was 9 nm and exhibited excellent antimicrobial activity and higher effectiveness was found against Gram negative bacteria. The AgNPs also showed potential cytotoxicity on HeLa cancer cell line. In vivo genotoxic study demonstrated not toxic nature of AgNPs at lower concentration. The results suggest that green synthesized AgNPs can be used as a natural antimicrobial agent and also in cancer treatment.

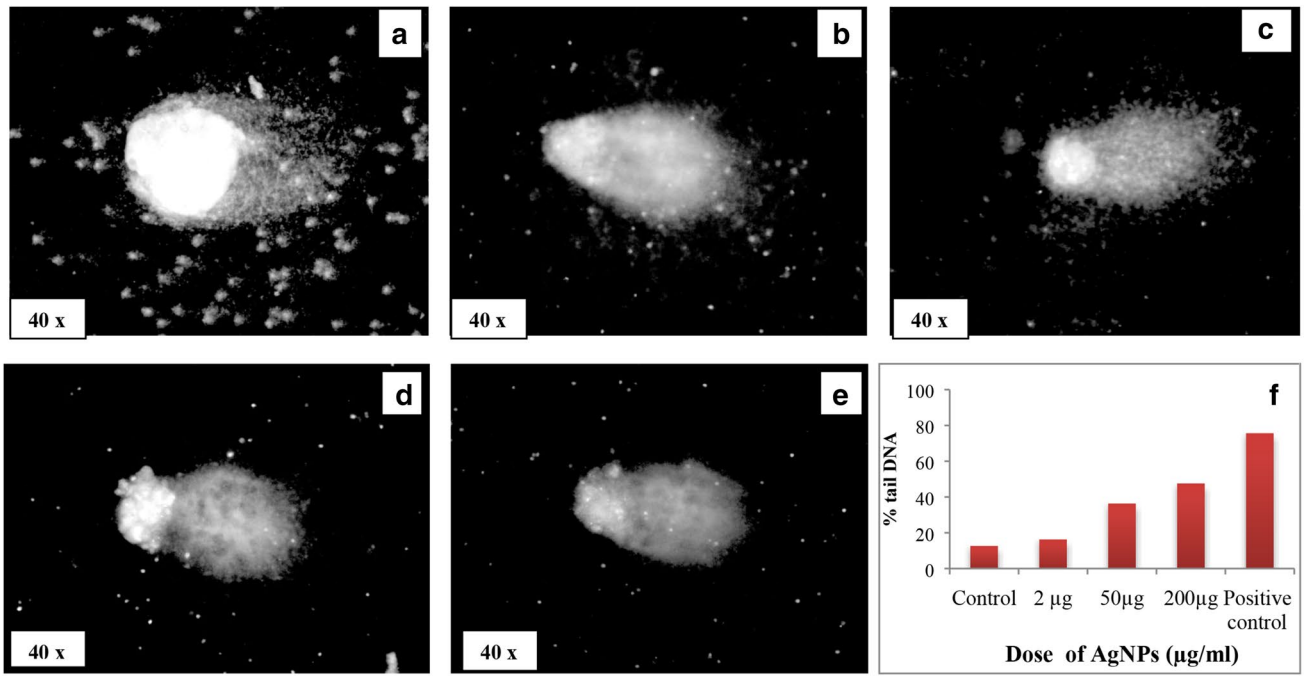

Fig. 7 DNA damage by comet assay **a** Control, **b** Positive control, **c** 2 µg of AgNPs, **d** 50 µg of AgNPs, **e** 200 µg of AgNPs, **f** DNA damage in cell after exposure of AgNPs (Magnification-×40)

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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