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In-vivo Genotoxicity of Synthesized Phyto-chemical and Chemical Silver Nanoparticles – A comparative study

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ABSTRACT

The present study was designed to synthesize and characterize the phyto-chemical and chemical AgNps (Silver Nanoparticles) and their in vivo genotoxicity assessment along with silver ion in Swiss albino mice. Phytosynthesis (Green synthesis) of AgNps was achieved by using the hydro extract of A.indica leaves, whereas chemical synthesis of AgNps was achieved by reduction of sodium citrate. The AgNps were characterized by advanced analytical methods like DLS, TEM and FT-IR spectroscopy. Toxicity of AgNps on gene was assessed by the alkaline comet assayand the Chromosomal Aberration (CA) assay. It was observed that there was a significant impairment in nuclear DNA and chromosomal aberrations which indicate AgNps interaction with DNA. Bone marrow cells exhibit diverse susceptibility towards genotoxicity mediated by both investigated green and chemically synthesized AgNps. Chemical AgNps possess high susceptibility to induced DNA break, genome instability and more toxic in low dose at 40 mg/kg body weight than green AgNps. From the results of present study, it can be concluded that the chemical AgNps have potential genotoxicity than that of phyto-synthesized green silver nanoparticles. Thus, green AgNps can be preferred in anticancer activities and possible health aspects of AgNps can be monitored.

Keywords: Silver nanoparticles, Phyto-synthesis, Nanotoxicology, Genotoxicityand Comet assay.

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INTRODUCTION

Nanotechnology is solitary of the rapidly increasing interdisciplinary areas of science and technology. This technology has extensive applications in the fields including electronics, pharmaceutical industry and cosmetics preparation. The application of nanomaterials in various medicinal formulations and industrialized products is increased with a due concern for theirpossible toxic effects on biological systems. The reasons which might have acollision on interactions between the metal nanoparticles and bio-molecules have remarkably increased though not solved [1]. It is reported that AgNps exhibit potential antimicrobial activity [2,3] and because of this effect these were used in consumer products of textiles and shoes including, personal care, cosmetics, clothing, food storing products and skin cares as subcategories, which gets exposed to the human beings by dermal application or by oral administration [4]. Furthermore, silver nanoparticles were instantaneously used for various medicinal applications such as surgical sutures and silver-coated medical diagnostic devices [5] however at the point of time literature supposed that silver nanoparticles produce cytotoxicity and genotoxicity *in-vitro* [6]. Phytochemicals includes first and secondary metabolized products from plant physiology, such as, flavonoids, flavones, isoflavones, alkaloids, anthrocyanids, steroids, carotenoids, carbohydrates and polyphenols which arepotential materials for biological activities and are recognized as essential natural resources for the synthesis of metallic nanoparticles. Hence, Green synthesis of nanomaterials are harmless to the environment and also one can obtain stable nanoparticles [7]. The plant leaf extract of *Cinnamomum camphoraI*[8],*Emblica offcinalis*[9], *Aloe vera* [10] and root extract of *Alfalfa* [11] were considered as a

reducing agent for the preparation of metal nanoparticles. Large number of active substances present in *Azadirachta indica* played an important role as reducing agent and capping agent. The important considerations in green synthesis are utilization of non-toxic chemicals, use of eco-friendly solvents and use of renewable materials. In recent years, biocompatible metal nanoparticles synthesized by green method are gaining considerable attraction in the field of biomedicine, due to utilization of natural resource, rapid rate of synthesis and being eco-friendly and safer method of nanoparticle synthesis. Therefore, it would be of great interest to take an advantage and explore these plants for green synthesis of silver nanoparticles [12]. From the literature phyto-chemical AgNps were observed to be safe and less toxic when compared with the chemical silver nanoparticles in cellular studies [13-16]. To date comparative *in vivo* genotoxicity of green silver nanoparticles with chemical silver nanoparticles was not yet proved. Thus, in this study, the phyto-chemical and chemical AgNps were synthesized, characterized and there *in vivo* genotoxicity was assessed along with silver ion in Swiss albino mice for the safety, efficacy and application of the green silver nanoparticles.

MATERIAL AND METHODS:

Materials

Silver nitrate ≥ 99% and Colchicine 95% were purchased from Sigma Aldrich, Triton X-100 and Ethidium bromide were purchased from Himedia, Sodium citrate and other analytical liquids, de-mineralized water were purchased from Fisher Scientific, India.

Preparation of Silver Nanoparticles

Silver nanoparticles were prepared with slight modifications in chemical synthesis [17]. 500 mL of sodium citrate (1% w/v) was heated and when it starts boiling 2mM silver nitrate (50 mL) was added drop wise. For green synthesis of AgNps, extract of *Azadirachta indica* leaves was prepared by decoction method, where 15g of dried leaves powder was boiled with 100 mL of de-mineralized water. The purified and supernatant extract of 500 mL was slowly added drop by drop to 100 mL of 2 m$MAgNo_3$ solution. Formation of AgNps were observed by visible color change, precipitation and also confirmed by UV spectroscopy (Hitachi Inkarp 2300 SICAN, Japan). The AgNps solution was filtered and finally purified by frozen centrifuge (Kubota 6500, Japan) at 4 °C with 17000 rpm (36,873 RCF) for 20 min. The pellets of AgNps were re-dispersed in a fresh de-ionized water for purification and repeatedly centrifuged for two times to separate the unwanted material of plant extract and excess of coating agent. Silver nanoparticles prepared by green synthesis method were referred as Green AgNps (G.AgNps) and those prepared by chemical method were referred as Chemical AgNps (C. AgNps).

Characterization of Silver Nanoparticles

Surface Plasmon resonances of synthesized Silver nanoparticles were analyzed by UV-Vis spectrophotometer. Suitably diluted AgNps were scanned between 350 to 500 nm. Nanotrack was used to measure the particle size and particle size distribution. Morphology of Synthesized AgNps was studied by TEM (Philips-CM 200, Tokyo, Japan), for this drop of AgNps was placed on the coated grid copper and subjected to TEM analysis. FTIR (Shimadzu-0815, Japan**)** was carried out to determine functional group adsorbed and bonded onto the shell of AgNps. The spectra were recorded over the wavelength region from4000 to 400 cm−1 at ambient temperature by KBr pellet method [18-19].

Animals and Conditions

Mice animals were procured from enterprises of Sri Venkateshwara, Bangalore. Ethical clearance was obtained from animal ethical committee of the institute with Resolution No. KLECOP/IAEC/Res.17-31,34 /08/2014 and lodged in individual cages with well environmental conditions (20 to 22˚C, humidity 50 to 60%, 12 hlight and dark photoperiod), after 10 days of acclimatization the animals were divided in to six groups each group containing five male mice. Every group involve specific treatment group with single dose administered by intra-peritoneal route. Group i: chemical AgNps 20 mg/kg; Group ii: chemical AgNps 40 mg/kg; iii: Group chemical AgNps 80 mg/kg; Group iv: green AgNps 20 mg/kg; Group v: green AgNps 40 mg/kg; Group vi: green AgNps 80 mg/kg; Group vii: Silver ion 20 mg/kg and Normal group., after 18 hr scarified respective groups.

Chromosome Aberration Assay (CA)

For chromosomal aberration assay, after 16.5 hr of induction the test compounds were injected with 0.04% of colchicine intraperitoneally to the animals based on individual body weight (1mL $100g^{-1}$) and subsequently, after 90 min mice were sacrificed. By using phosphate buffer (pH 7.4) samples of bone marrow were collected after 18 hr exposure to AgNps, when the cells were in their first metaphase [20,21]. Mice femur bones were flushed with PBS and transferred in to two centrifuge tubes, assessed each for comet assay and CA assay. Centrifuged cells were incubated in 0.075 M KCl at 37^0C for about 30 min. Subsequently they were fixed in ice-cold which was prepared with admixture of 3:1methanol: glacial

acetic acid respectively, for10 minutes. Incubated sample was cleaned two times in fixative, prepared slides were air dried and stained with 8% Giemsa in Sorenson's phosphate buffer pH 6.8.

Metaphase cells were analyzed from each animal as per the treatment randomly. Aberrations were marked and recorded strictly following the standard method. The metaphase cells were graded at high magnification; each chromosome aberrations were showing the following characteristics like chromatid and isochromatid gaps and chromatid rearrangement. Results were calculated as per the damaged metaphase cell (% DC) and the number of aberrations per cell.

Comet Assay

Alkaline comet assay was performed to determine the DNA damage by AgNps in lymphocytes with minor modifications [22]. The slides were prepared by combining around 100 µL of cells (2X 10^4) to 100µL of LMP (low melting point) agarose. About 100 µL of this cell suspension was roofed onto pre-coated normal melting point (NMP) agarose on roughed glass slides and then immediately cover slip was placed. The agar slide was kept on ice for solidification. Then the agar slides were immersed vessel containing freshly prepared lysis solution [10 mM Trizna, 100 mM EDTA, 2.5 M NaCl, 1%Triton-X-100, 10% (v/v) DMSO] in the refrigerated for 2h. The lysed cells were subjected to electrophoresis in buffer (pH > 13) for 30 min. Initially, electrophoresis was kept at 25 V for 30 mins. Subsequently pH of agar slides was dropped down to pH 7.0 by using 0.04 M Tris–HCl with pH 7.5 and slides were kept aside for10 min at attain the required pH. Just prior to analysis of image, the slides were stained with ethidium bromide (2µg/mL) and DNA relocation was retrieved by fluorescence microscope (Olympus, CKX41, and Japan, excitation filter 515–560 nm and filter of 590 nm). The pictures were analyzed by auto-image analysis using software Komet5.5 copy version. The DNA damage and the median values of comet parameter in tail DNA (%) were processed. Images of 150 (50×3) cells of each concentration were calculated.

RESULTS

Preparation of Silver Nanoparticles

AgNps prepared by green chemistry method showed change in colour from pale yellow to brown visually. It indicates that AgNps were successfully synthesized using an *A.indica* leaves extract as a reduction and coat formation agent. Whereas in chemical method, where sodium citrate was used as reducing agent showed colour change from colourless to greenish grey.

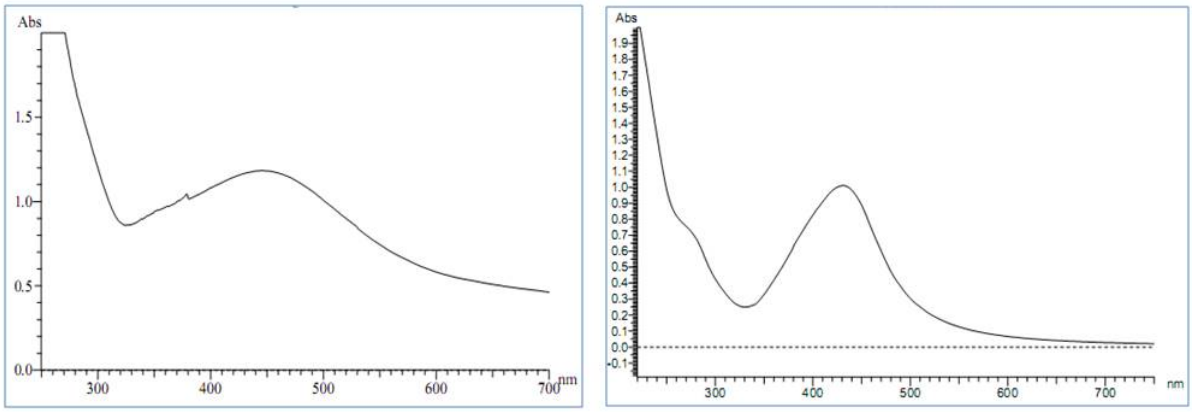


Figure 1a **Figure 1b**

Fig. 1 UV–vis absorption spectrum of silver nanoparticles. From (Figure 1a) *A. indica* plant extract (Figure 1b) Tri sodium citrate

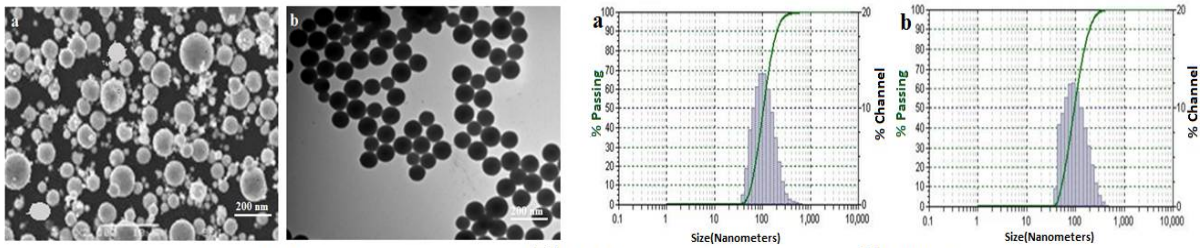
Characterization of Silver Nanoparticles

UV-Visible spectroscopic method also used to confirm synthesis of AgNps. Sharp peaks were obtained at 420 and 430 nm for G.AgNps and C.AgNps respectively (data not showed), which confirms AgNps were formed in both methods of preparation. The average size distribution of G.AgNps and C.AgNps were found to be 94nm and105nm respectively (Figure 1). Zeta potential values (Data not shown) indicate that the phyto-synthesized (green synthesized) AgNps have a reasonable stability. The diameter of the G.AgNps nanoparticles was found to be 20 nm to 60 nm with few particles having greater diameter (~90 nm); while C. AgNps with spherical shape showed average particle diameter of about 90 nm (Figure 2).

In the (Figure 3a. (i-iii) FTIR spectrum of G.AgNps O-H stretching, phenolic groups, and H-bonded alcoholics are represented by peaks at 3490-3500 cm−1. The presence of (N-H) aromatic secondary amine and N-H stretching accounts for the peak at 1400-1550 cm−1. The stretching vibrations of (–C=O) and (–C=C) are represented by the bands (medium intensity) at 1450 and 1500 cm1, respectively. Finally,

the yield of the green AgNps was observed to be 152 mg from the 150 g of *Azadirachta indica* leaves and 240 mg of silver nitrate. FTIR spectra of C. AgNps (Figure 3b (i-iii)), shows bands at 3224 and 3298 cm^{-1}corresponds to OH stretch denoting carboxylic acid group. A sharp band at 1591 cm^{-1} corresponds to stretching of (C–C) aromatic and (N–H) bend of primary amines.

Fig. 2 Size distribution and Morphology of: a: green synthesized AgNps and b: chemically synthesised AgNps by TEM and Nanotrac.



TEM Figure 2 Nanotrac

Fig. 3a FT-IRspectrum of: i: Phyto-mediated AgNps; ii: supernatant of leaves extract of *A indica*and iii: AgNO3.

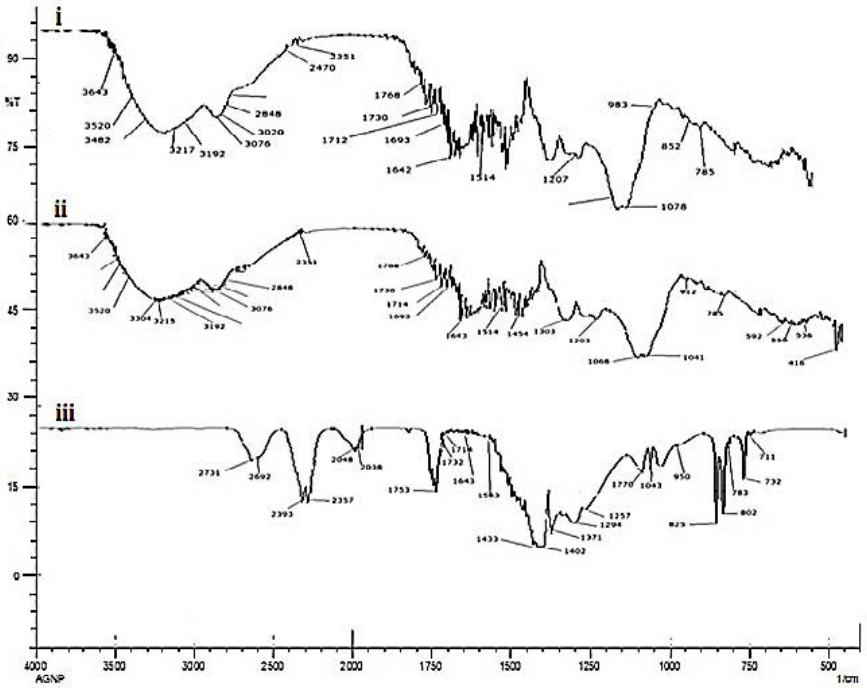


Fig. 3b FT-IR spectra of i: Chemically synthesized AgNps; ii: Tri sodium citrate and iii: $AgNO_3$.

Comet Assay

The increased DNA damage in AgNps and silver ion treated groups was clearly observed. The tail intensity (% tail DNA) was higher in the silver ion group as well as in chemical AgNps treated groups when compared control. The comet (% tail DNA) of chemical AgNps (20 mg kg^{-1} and 80 mg kg^{-1}) increases gradually when the concentration increases. In case of green AgNps the tail intensity was sharp at high concentration only (Figure 4). Responses were statistically significant (*P≤0.05*) at concentrations 20, 40 and 80 mg $kg^{-1}$of chemical AgNps, 80 mg $kg^{-1}$of green AgNps and in silver ion (20 mg kg^{-1}).

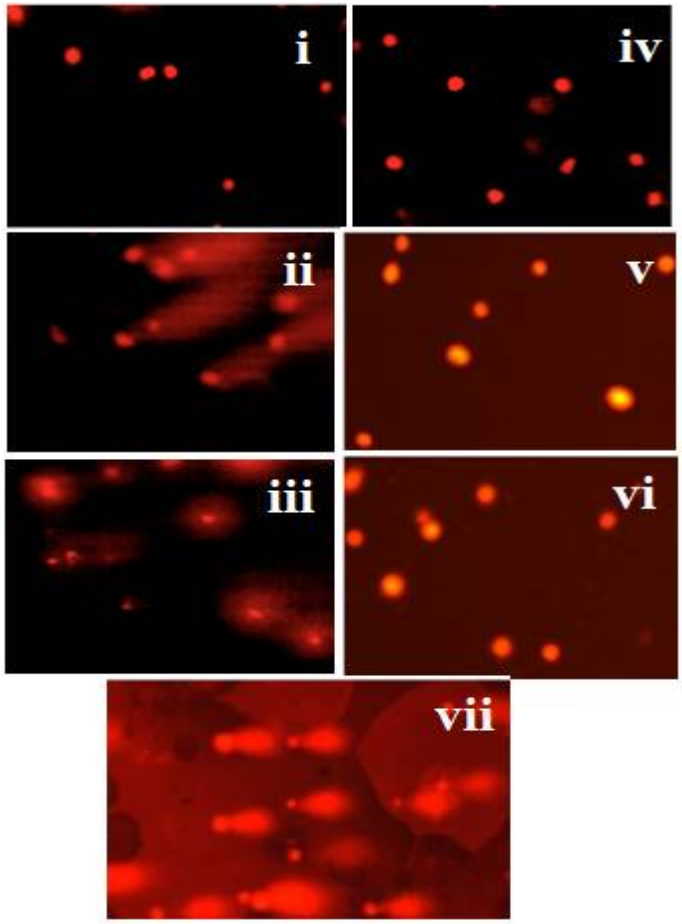


Fig. 4 Photomicrographs of Comet assay: i: chemical AgNps 20 mg/kg;ii: chemical AgNps 40 mg/kg; iii: chemical AgNps 80 mg/kg; iv: green AgNps 20 mg/kg; v: green AgNps 40 mg/kg; vi: green AgNps 80 mg/kg; vii: Silver ion 20 mg/kg.

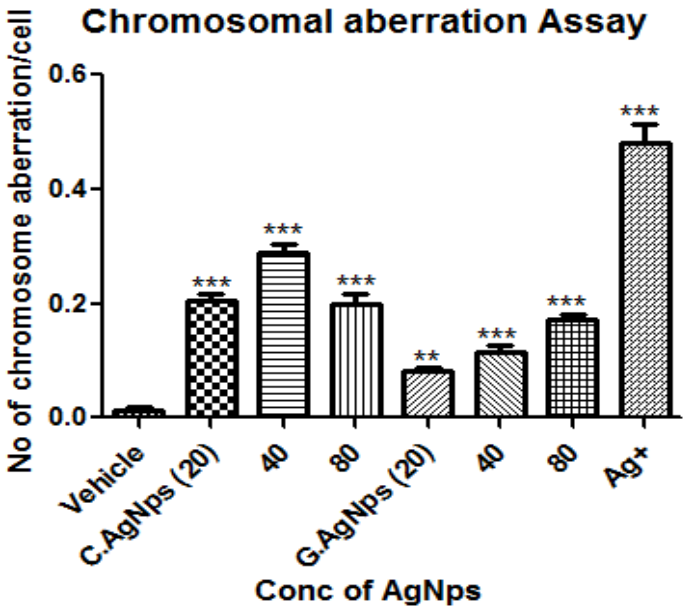


Fig. 5 Effect of green and chemical silver nanoparticles on Number of chromosome aberration/cell i: chemical AgNps 20 mg/kg; ii: chemical AgNps 40 mg/kg; iii: chemical AgNps 80 mg/kg; iv: green AgNps 20 mg/kg; v: green AgNps 40 mg/kg; vi: green AgNps 80 mg/kg; vii: Silver ion 20 mg/kg.

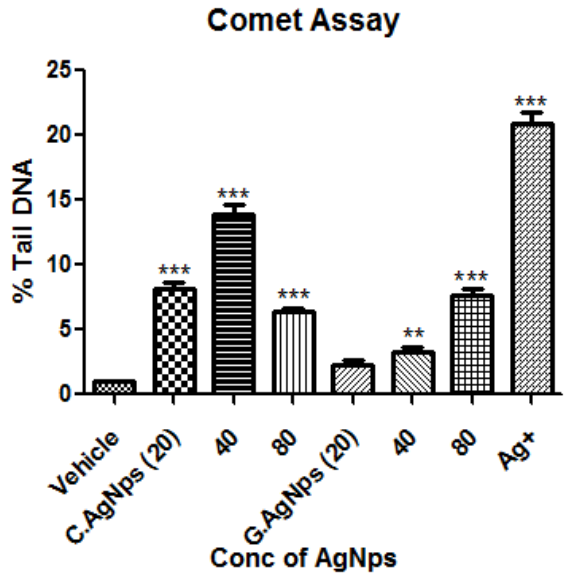


Fig. 6 Effect of green and chemical silver nanoparticles on % Tail DNA i: chemical AgNps 20 mg/kg; ii: chemical AgNps 40 mg/kg; iii: chemical AgNps 80 mg/kg; iv: green AgNps 20 mg/kg; v: green AgNps 40 mg/kg; vi: green AgNps 80 mg/kg; vii: Silver ion 20 mg/kg.

DISCUSSION

The silver ion bonded with trisodium citrate according to the reaction expressed below [22]. Negative charge of nanoparticles and repulsion forces prevent aggregation and their mutual electrostatic repulsion. Therefore, particles were stable without stabilizing agent. While in phyto-mediated silver nanoparticles, the extract act as a capping agent.

$C_6H_5O_7Na_3 + 4Ag^+ \quad 2H_2O \rightarrow C_6H_5O_7H_3 + 4Ag^0 + 3Na^+ + H^+ + O_2\uparrow$

The morphology of AgNps was observed by TEM (Figure 2). It reveals that the phyto-chemical AgNps were monodispersed and spherical shape. Morphology and structure of AgNps were due to numerous reasons, such as pH, concentration of reducing agent, concentration of Ag ion, incubation time, the method of preparation [23,24] and categorization [25,26]. In FTIR, bands corresponding to (–C=C) and (–C=O) bonds are derived from flavonoids and terpenoids that may be held responsible for efficient capping and stabilization of obtained green AgNps. Earlier reports were also evidenced the stability of *C. Camphora* leaf extract AgNps due to the presence of proteins which coated the surface of AgNps [27,12]. In our previous study, the green AgNps effect on apoptosis in fibroblast cells were studied and observed apoptotic cells and viable cells in the microscopic fields and subsequently % apoptotic cells were calculated [12]. A number of previous studies employing various kinds of cultured cells and animal models suggest that both genotoxicity and apoptosis are important mechanisms for AgNps induced toxicity. In the current study genotoxicity of AgNps was evident in bone marrow cells by comet assay. Statistically high significant nucleic acid damage was found at high dose level in cells during exposure period. These results are in concomitance with other studies showing molecular and biochemical changes linked to genotoxicity initiated by AgNps in cultured cells. AgNps induced DNA breakage was detected in cell lines using the DNA comet assay. Furthermore, DNA double-stranded breaks, similar results were found in AgNps treated human hepatoma cells, dermal cells, fibroblasts and stem cells. [28,29]

The integrity of genomic DNA is always under threat, even in healthy cells. Evidence of present study and comparable previous studies indicate AgNps possesses the potential to interact with DNA and cause alterations in biological cells. Dangerous alterations within the genetic material include chromosomal aberrations and mutations involving an amendment in an exceedingly single base. Errors in replication or recombination, as well as environmental toxicants can also damage cellular DNA [29]. The main molecular mechanism of nanomaterials induced DNA damage is considered to be the induction of oxidative stress resulting from the generation of ROS. Silver nanoparticles have capable of develop reactive oxygen species when they reacted with cell components *in vitro* [30] and *in vivo* studies [31,32].

Metal nanoparticles increased the production of Rad51, a DNA damage repair super protein [33]. Damaged DNA activates a signaling network that controls cell cycle checkpoints and DNA repair. Damaged DNA accumulates in cells during the gap 1 (G1), DNA synthesis (S), or gap 2/mitosis (G2/M) phases. In the sub-G1 phase, DNA damage causes an accumulation of apoptotic cells. Silver nanoparticles concentration and time dependent increase in the damage of DNA is reported with the A549 cells. [34,35]

According to an ANOVA test (Figure 5), the frequency of aberrant cells and the number of breaks per cell were significantly higher ($P \leq 0.05$) than in the control group.

We performed *In vivo* genotoxicity of AgNps in mouse bone marrow cells. Compared to control, the percentage of aberrant cells induced by Chemical AgNps (20-80 mg/kg body weight) and green AgNps (80mg/kg) were significantly higher Figure 5. The chromosome aberrations were calculated that indicates strand breaks. Therefore, AgNps may be classified as a clastogen. According to the Kim *et al*. Genotoxicity was negatively reported in Sprague-Dawley rat bone marrow cells after exposing twenty-eight days and these results didn't effect on polychromatic erythrocytes. An increase DNA damaging effect of AgNps was observed at certain concentrations (20 mg kg^{-1}) of chemical AgNps as well as phyto-synthesized AgNps (80mg kg^{-1}). Previous studies in an *A. cepa* have revealed that AgNps might enter plant system, have an effect on cellular separation exhibiting cyto-toxic response and cause aberrations [36,37]. Numbers of studies have co-jointly shown that AgNps enhance and break the DNA damaging on aquatic organism [37,38] and plant cells [36] with impairment of cellular division.

CONCLUSION

Present study revealed that synthesis, characterization and genotoxicity of biosynthesized AgNps from *A. indica* against *in vivo* models. We have noticed that significant impairment in nuclear DNA and chromosomal aberrations indicating that AgNps interacted with DNA. Bone marrow cells exhibit diverse susceptibility toward genotoxicity mediated by both investigated green and chemically synthesized AgNps. Chemical AgNps were highly susceptible to induced DNA break, genome instability, and Chemical AgNps are more toxic at dose of 40and 80 mg kg^{-1} body weight than green AgNps. Collectively, our data suggests that C. AgNps possess superior cytotoxic activity and genotoxicity compared to the G.AgNps from *A. indica* aqueous extract. Present study also suggests that at the dose of 20 mg kg^{-1} green silver nanoparticles can be safely used in anticancer and antimicrobial and antiviral therapy.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest concerningthis article.

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