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Research Article

Evaluation of Chemopreventive Potential and Regulated Toxicity of a Phyto-fabricated Silver Nanoformulation using Bark Extract of *Saraca asoca* in Acute Myeloid Leukemia *in vivo* and *in vitro*

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Abstract

Background: Acute myeloid leukemia (AML) results impaired production of myeloid blood cell which leads to the reduction of erythrocytes, lymphocytes, and platelets. In contrast to the conventional treatment like chemotherapy, radiotherapy and stem cell transplantation, plantphytochemicals can be used as nontoxic chemopreventive agents to provide long-term therapeutic effect. In this study, we have analyzed the efficacy of the phyto-fabricated silver nanoparticle using the bark extract of *Saraca asoca* as a reducing agent against the secondary AMLmice model both *in vivo* and *in vitro* in comparison to common anticancer drug doxorubicin. **Methods:** The efficacy of silver nanoformulation was tested *in vivo* using secondary model of AML by survivability assay in comparison with control and standard chemotherapeutic drug doxorubicin. The cytotoxic and genotoxic potential of the nanoparticle *in vitro* was studied in leukemic cells in comparison to normal lymphocytes. The level of cytotoxicity was measured using cell viability assay, generation of percentage of nitric oxide, reactive oxygen species and mitochondrial membrane potential. The genotoxic potential was evaluated by using DAPI staining, comet assay, and DNA fragmentation assay and quantification of percentage of apoptosis and necrosis. The damage of leukemic cells was evaluated by transmission electron microscopy. **Results:** The percent survivability was increased maximum by 50% in comparison to that of untreated group of AML mice whereas mice treated with standard drug doxorubicin showed 83%survivability. But AgNPs were selectively cytotoxic towards leukemic lymphocyte cells through oxidative damage unlike the standard chemotherapeutic drug as doxorubicin induce general impact of DNA damage inhibiting cell proliferation, impairing mitochondrial function and cell death in normal cellular system too. **Conclusions:** This finding opens a new avenue for the biomedical application of phyto- fabricated AgNPs as nontoxic agents in chemoprevention of AML.

Keywords: Acute myeloid leukemia; Silver nanoparticle; Chemoprevention; Genotoxicity; Cytotoxicity; Doxorubicin; Regulated toxicity; Sustainable therapy

Background

Acute myeloid leukemia (AML) is one of the most complex types of leukemia that needs newand advanced treatment. The impaired production of myeloid blood cells or pancytopenia of AML leads to the reduction of erythrocytes, lymphocytes, and platelets. AML occurs due to exposure to agents like smoking, certain chemicals like alkylating agents, platinum and benzene,

certain blood diseases, genetic disorders, exposure to radiations, *etc*.

In contrast to the conventional treatment like chemotherapy, radiotherapy and stem cell transplantation, plant phytochemicals can be used as nontoxic chemopreventive agents to provide long term therapeutic effect [1-9].

Several reports are now available on the anticancer activity of the silver nanoparticles synthesized by green chemistry method. According to Adebayo et al (2020) Boswellia dalzielii-mediated silver nanoparticles inhibited acute myeloid leukemia (AML) Kasumi-1 cells by inducing cell cycle arrest.[10]. Prabhu *et al.*[2013], green-synthesized AgNPs by methanolic extract of *Vitex negundo* L. showed 50% inhibition of cell viability of human colon cancer cell lines (HCT 15) .[11]. The cytotoxic properties of biologically synthesized silver nanoparticles by *Acalypha indica* was observed in human breast tumor cells, MDA-MB-231[12, 13]. AgNPs (silver nanoparticles) synthesized by *Andrographis echioides* inhibited the growth ofhuman breast adenocarcinoma cells [14]. Additionally, biofunctionalized green-synthesized AgNPs exhibited potential cytotoxic activity against HT29 human colon adenocarcinoma cells [15]. AgNPs bioformulated by using *Premna serratifolia* leaves extract displayed significant anticancer activity on liver cancer of Swiss albino mice [16]. Sre *et al.*[2015] reported the cytotoxic activity of phytochemically synthesized AgNPs by *Erythrina indica* on MCF-7 (breast cancer) cells and HepG2 (hepatocellular carcinoma) cells.[17]. Another group of scientists reported the AgNPs, synthesized using the latex of *Euphorbia nivulia,* exhibited potentiallycytotoxic effects in a dose-dependent manner against human lung carcinoma (A549) cells [18]. *Annona squamosa* seed extract displayed good anticancer activities against human hepatoma and breast cancer cells both *in vitro* and *in vivo* [19,20] The anticancer efficacy of *Allium sativum* (garlic) exerted the protective effect against gastrointestinal cancers [21] and administration of garlic enhanced the efficacy of natural killer cells in patients with advanced digestive system cancer [22]. Curcumin, a polyphenol (diferuloylmethane) derived from the rhizome of turmeric (*Curcuma longa* Linn), is a strong anticancer agent and showed its multiple actions on mutagenesis, cell cycle progression, apoptosis, oncogene expression, and metastasis [23, 24]. According to the study of Dhillon *et al.* [2008], curcumin showed positive results in pancreatic bladder cancer patients too [25]. So it is quite evident that the silver nanoparticles capped with phytocomponents of medicinal value have a great prospect as a new treatment for acute myeloid leukemia. A few reports are available on the anti-leukemic activity of silver nanoparticles in acute myeloid leukemia cells [10, 26, 27].

The plant *Saraca asoca* is a rain-forest tree belonging to the family Caesalpinaceae. It is a native of Asia and South America. In India, it is distributed throughout central areas of Deccan plateau and the Western Ghats of the subcontinent. The bark of *Saraca asoca* has natural detoxification properties and is rich in different phyto-compounds that are used in the treatment of health issueslike bleeding disorders, menorrhagia, and diarrhea, even in control of obesity. In this study, we have analyzed the efficacy of the phyto-fabricated silver nanoparticle using the bark extract of *Saraca asoca* as a reducing agent against the secondary AML mice model both *in vivo* and *in vitro* incomparison to common anticancer drug doxorubicin in the cytotoxicity and genotoxicity effect specifically on cancerous cell.

Methods

Chemicals

Osmium tetroxide, Glutaraldehyde (electron microscopy grade), 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI), 2,7-dichlorofluorescein di-acetate (DCFH-DA) dye, Histopaque, doxorubicin (purchased from Sigma Aldrich Chemical Company, Mumbai, India). Annexin V- FITC apoptosis detection kit was purchased from Invitrogen, Thermo Fisher Scientific (Mumbai, India).

Animals

All the animal experimentations were performed strictly adhering to the recommendation of Institutional animal ethics committee of the institution (Registration no: 892/GO/Re/S/01/CPCSEA). A total of 30 Swiss albino male mice (*Mus musculus*) aged 6-8 weeks from specified strain were purchased from a registered supplier. All the animals were maintained in stainless steel wire cages (Tarsons, India) under 12-hour light-dark cycle. Pellet diet (West Bengal Diary and Poultry Development Corp. Ltd., Kalyani Industrial Area, Kalyani, WB India) was provided and water was supplied *ad libitum* automatically through the tubing during the study period.

Secondary AML mice model

The secondary AML mice model was established following the methodology of Saha *et. al.*[2012] [28]. Liquid benzene (HPLC grade) (MERCK, India) was vaporized by heating at 16℃ and then the vapor was channeled into the inhalation chamber (1.3 m^3). A total of 24 mice were exposed to 300 ppm benzene (in vapor form) for 6 h/day, 5 days/week for 2 weeks. Cumulative exposure= ppm × Number of hours × Number of days. Temperature and humidity were maintained automatically at (24±1)℃ and (55±10)%, respectively inside the chambers.

Green phyto-fabricated silver nanoparticle

Green phyto-fabricated silver nanoparticles were synthesized using aqueous bark extract of *Saraca asoca* as the reducing agent [29]. The taxonomic identification of plant material was confirmed by Dr. GG Maity, Professor of Taxonomy, Taxonomy, and Plant

Systematic Unit, Department of Botany, University Of Kalyani. The voucher specimens (Deb.kly-60) was deposited and preserved in the Department of Botany. The LD50 of the synthesized silver nanoparticle was 2 165 mg/kg body weight (Table 1). Two selected doses (for *in vivo*) were 43 mg/kg b.w and 86 mg/kg b.w. which were actually the 1/50th and 1/25th fraction of the determined LD50 of the green silver nanoparticle**.** Doses were selected as per the guidelines of OECD (2001) [30].

Table 1. LD5 to LD90 values of synthesized silver nanoparticle

Percentile	Probit	Dose (mg/kg) Mean ± SEM
5	3.36	720.02± 330.51
10	3.72	908.05± 358.37
20	4.16	1142.45 ± 398.48
30	4.48	1403.09 ± 412.11
40	4.75	180936 ± 421.15
50	5.00	2165.26 ± 452.17
60	5.25	2673.28 ± 491.44
70	5.52	3113.51 ± 615.91
80	5.84	3768.40 ± 903.93
90	6.28	5322.89± 1647.36

Study of anti-leukemic activity of phyto-fabricated silver nanoparticle in secondaryAML mice model

Experimental design

The treatments (the synthesized silver nanoparticle and the standard drug) were given by injection *i.p.* every second day up to 30 days. Overall survival was recorded on the 35th day ofthe study. The groups included Group Ⅰ: the control group normal healthy mice without any disease and received no treatment; Group Ⅱ: AML mice received no treatment; Group Ⅲ: AML mice treated with phyto-fabricated AgNPs at 43 mg/kg b.w.; Group Ⅳ: AML mice treated with phyto-fabricated AgNPs at 86 mg/kg b.w.; Group Ⅴ: AML mice treated with doxorubicin at 2 mg/kg b.w.

Survivability assay

The study was conducted for a period of 35 days. Kaplan-Meier [1958] [31] analysis was performed to determine the *in vivo* survival distributions. The log-rank method was used to compare the survival curves among groups.

Cytotoxicity and genotoxicity of the phyto-fabricated silver nanoparticle

Cell sample preparation

Lymphocytes were isolated from the bone marrow of the secondary AML mice using Histopaque1077 (Sigma–Aldrich, St. Louis, MO, USA) following the methodology of Thorsby [32] with modifications. The isolated leukemic lymphocytes were incubated with different concentrations of the phyto-fabricated AgNPs (40 µg/mL and 80 µg/mL) and standard drug doxorubicin (at a concentration of 1 µM) in RPMI-1640 media for 3 hours at 37℃. Normal lymphocyte cells treated with phyto-fabricated AgNPs at the highest concentration were the control system Concentration at 10g/mL and 20 g/mL of silver nanoparticle when tested *in vivo* (survivability assay) showed insignificant improvement in comparison with the standard drug Doxorubicin. Thus for the *in vitro* experiments with AML cells 40µg/mL and 80µg/mL concentration of AgNPs were tested.

Cell viability assay

Cell viability was checked by the trypan blue exclusion method described by Tennant [1964] [33]. Trypan blue at 0.4% solution in a buffered isotonic salt solution, pH 7.2 to 7.3 (i.e., phosphate-buffered saline) was added to 1 mL of cell suspension (10^6 cells/mL). Cell viability should be at least 95% for a healthy log-phase. The percent of viable cells was calculated using the formula: % of viable cells = (1.00 – Number of blue cells/ Number of total cells) × 100 and percent Viability reduction = [Total Viable cells before treatment (Unstained) - Total Viable cells after treatment (Unstained) / Total cells (Viable +Dead)] X 100.

Determination of nitric oxide (NO)

Nitric oxide basically is generated from sodium nitroprusside and it is measured by the Griess reaction [34]. The incubation time was five hours so that the part of the generated nitric oxide was scavenged. The amount of leftover nitric oxide was calculated. The absorbance of the chromophore was then measured at 546 nm. Percentage of scavenging activity = (OD of control /OD of the test) × 100

Reactive oxygen species (ROS) generation by DCFDA assay

The generation of ROS was detected by DCFH-DA fluorescence. The cell suspension containing approximately 10^6 cells per tube was taken for the flow cytometric analysis. The generation of ROS was detected following the methodology of Roy *et al*.[2008] [35]. The samples were normal cells treated with highest concentration of AgNPs (80µg/ml); Leukemic lymphocytes treated with two different concentrations of AgNPs

(40µg/ml and 80µg/ml); leukemic lymphocytes treated with standard drug (1 µM). DCF was excited by the 488 nm laser and detected at 535 nm (typically FL1). Ideally, 10 000 cells were analyzed per experimental condition. Mean fluorescent intensity change was tested among the treated normal control, positive control and treated diseased cells samples.

Determination of mitochondrial membrane potential (MMP)

Change in the MMP was determined following the methodology of Dash *et al*.[2013] [36] by flow cytometry at the single-cell layer.Four groups of animals were tested for MMP. **Group 1:** Normal lymphocytes treated with phyto-fabricated AgNPs at 80µg/mL concentration (control). **Group: 2** AML cells treated with phyto-fabricated AgNPs at a concentration of 40µg/ml.**Group: 3** AML cells treated with phyto-fabricated silver nanoparticle at a concentration of 80µg/ml. **Group: 4** AML cells treated with standard drug doxorubicin at a concentration of 1 µM.

The cells were stained with the dye DiOC6 by incubating for 15 min at 37℃ in the complete dark in phosphate buffer saline. Loss of DiOC6 fluorescence indicates the disruption of the mitochondrial inner transmembrane potential. The fluorescence was detected at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The fluorescence intensity was measured on a FACS Calibur (Becton Dickinson), and data were analyzed using WINMDI 2.9 software, representing the mean fluorescence intensity.

DAPI

Analysis of the nuclear membrane integrity of four groups of cells as mentioned above was performed by DAPI following the methodology of Mollick *et al*. [2014] [37]. Cells were exposed to 300 nM DAPI stain solution. Cells were imaged under a fluorescence microscope at 340 nm.

Analysis of apoptotic and necrotic cells

Three groups of cells were teated for testing Apoptosis. **Group: 1** Normal lymphocytes treated with phyto-fabricated AgNPs at 80µg/mL concentration. **Group: 2** AML cells treated with phyto-fabricated silver nanoparticle at a concentration of 80µg/ml. **Group: 3** AML cells treated with standard drug doxorubicin at a concentration of 1µM.

Alexa Fluor® 488 annexin V and propidium iodide (PI) (Invitrogen) assay was performed according to the manufacturer's protocol to quantify the number of apoptotic and necrotic cells. After staining with Alexa Fluor® 488 annexin V and PI the apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence. The cells were washed in cold phosphate-buffered saline. Finally, 5µL Alexa Fluor® 488 annexin V and 1 µL 100µg/mL of PI working solution was added to each 100µL of cell suspension. The stained cells were analyzed by flow cytometry at an excitation wavelength of 488 nm, the fluorescence was measured at the emission wavelength of 530 nm and 575 nm (or equivalent).

Comet assay

Analysis of the extent of DNA damage was assessed in three groups as mentioned above by comet assay following the methodology of Sing et al [1988] [38]. Following unwinding, the DNA is electrophoresed and stained with a fluorescent dye. DNA migration was analyzed by fluorescence microscopy (excitation filter 515–560 nm and a barrier filter of 590 nm) attached with a CCD camera. The images were analyzed by the Komet version 5.5 auto image analysis software. Quantitative and qualitative analysis of DNA damage was determined by measuring the length of DNA migration and the percentage of migrated DNA in the cells to calculate the tail length.

DNA fragmentation assay

DNA was extracted from both the leukemic lymphocyte cells and control cells in three groups ofcells as mentioned above following the protocol suggested by Paul *et al*.[2011] [39]. After the treatment, the cells were lysed and DNA was extracted with a mixture of phenol, chloroform,and isoamyl alcohol (25:24:1) and electrophoretically separated on a 2% agarose gel containing 1 µg/mL ethidium bromide and visualized under ultraviolet transillumination.

Study of cellular uptake and accumulation by transmission electron microscope (TEM)

A modified cytotoxicity test with AML cells treated with phyto-fabricated AgNPs was carried out by TEM imaging following the methodology of AshaRani *et al*. [2009] [40] for the qualitative assessment. AML cells incubated with phyto-fabricated AgNPs (80 µg/mL) were evaluated for structural alterations and phyto-fabricated AgNPs accumulation. Embedded samples were fixed with 2% glutaraldehyde and then post fixed with 2% osmium tetroxide. Ultrathin sections were cut with glass knives and examined in a Zeiss EM910 transmission electron microscope at an acceleration voltage of 80 kV.

Statistical analysis

The log-rank method was used to compare the survival curves among groups. All data are expressed as means ± SD (Standard daviation of the Mean) and were compared with the ANOVA (n=5), $p < 0.05$ was taken as the level of significance after doing T test.

Results

Survivability assay

Figure 1A represents the survivability percentage of different groups. At the end of 35 days of the study period, the survivability was significantly increased (P<0.05) in the group treated with 43 mg/kg b.w and 86 mg/kg b.w. of phyto-fabricated AgNPs. The normal control group showed 100% survivability whereas the untreated AML group showed only 17% survivability. Mice treated with phyto-fabricated AgNPs at 43 mg/kg b.w. revealed an increase of 33% and 50% at 86 mg/kg b.w. in comparison to that of the untreated group. Mice treated with standard drug at 2 mg/kg b.w. showed the highest survivability (Figure 1A)

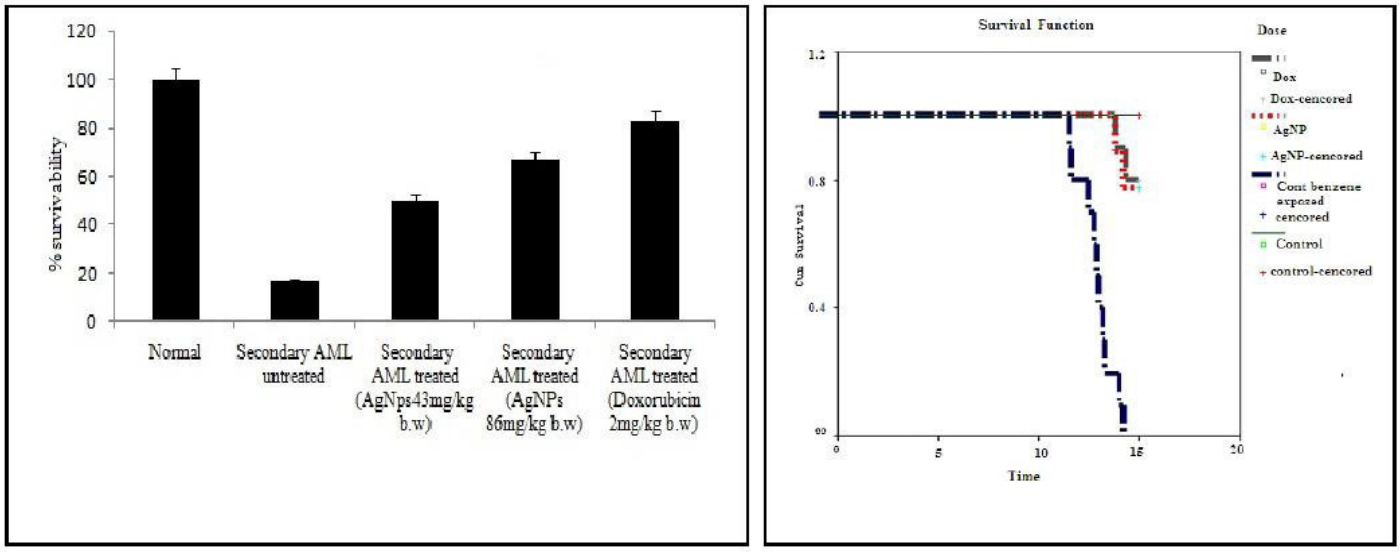

Figure 1: Survivability time. A : Survivability percentages of different groups of animals. B.Survival curve.

Cytotoxicity and genotoxicity of phyto-fabricated silver nanoparticles in leukemiclymphocytes

Cell viability test by trypan blue exclusion method

Figure 2A shows the cell viability of the leukemic lymphocyte cells treated with different concentrations (10 µg/mL, 20 µg/mL, 40 µg/mL and 80 µg/mL) of phyto-fabricated AgNPs and treated with standard drug doxorubicin at 1 µM. The viability of leukemic lymphocyte cells was reduced by 89.14% in the treatment of phyto-fabricated AgNPs at 80 µg/mL, and by 91.23% in doxorubicin treated leukemic lymphocytes. The viability of leukemic lymphocytes was significantly reduced when treated with different concentrations of phyto-fabricated AgNPs in a dose-dependent manner (P<0.05).

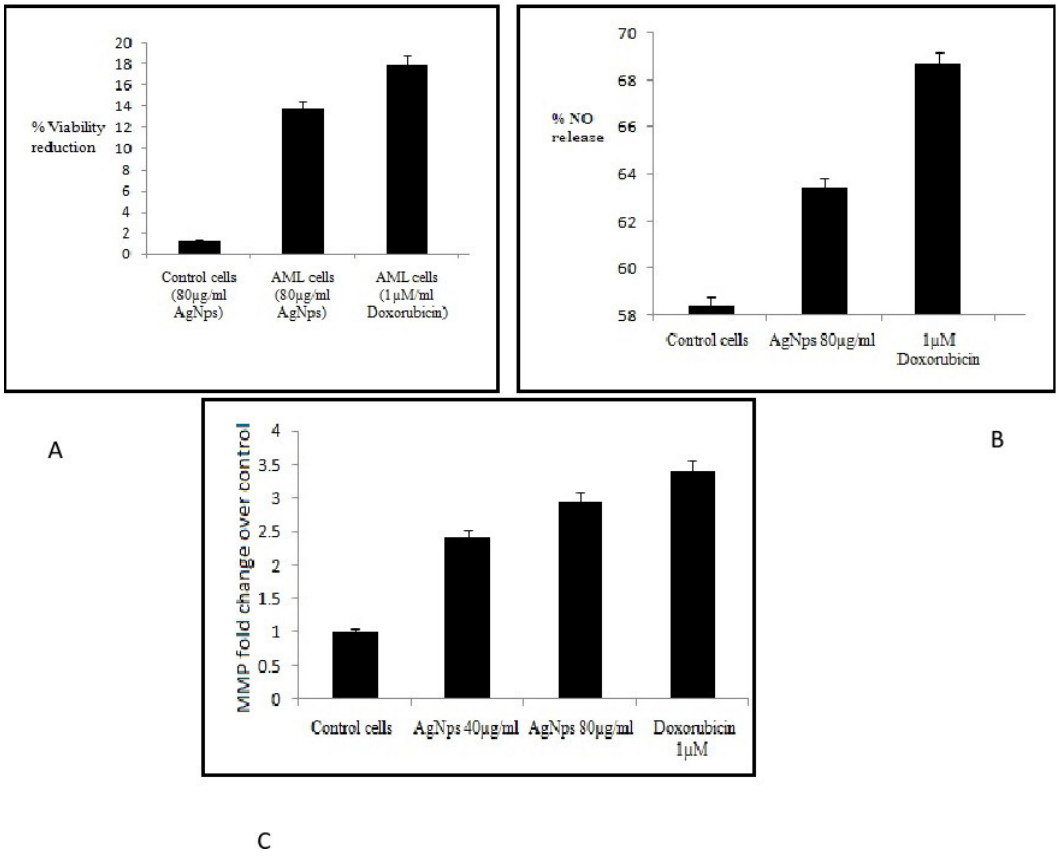

Figure 2: Cell viability (A), NO release (B) and mitochondrial membrane potential (C)

NO release level

Phyto-fabricated AgNPs at 80 µg/mL significantly (P<0.05) increase NO level to 63.4% in the leukemic lymphocyte cells as compared to the control and the value was close to the level of NO (68.7%) in the leukemic lymphocytes treated with doxorubicin (Figure 2B). No significant increase in the level of NO was found in the normal cells treated with the highest concentration of phyto-fabricated AgNPs.

Alteration in MMP

In this study, the MMP of the leukemic lymphocytes treated with phyto-fabricated AgNPs was depleted significantly in comparison to the control lymphocytes; similar depletion was alsofound in the doxorubicin treated leukemic lymphocyte cells. The percentage of MMP wasdecreased in a dose-dependent manner. Phyto-fabricated AgNPs at 80 µg/mL induced a significantly higher decrease in MMP by 72.7% (P<0.05) in comparison to the control (Figure 2C).

ROS generation

The intracellular concentration of ROS (Figure 3) was significantly (P<0.05) higher in the leukemic lymphocytes treated with phyto-fabricated AgNPs at 80 µg/mL when compared to the control. In phyto-fabricated AgNPs at 80 µg/mL treated cells, the ROS was elevated by 53.7% (2.26 fold compared to control or normal cells treated with AgNPs at 80 µg/mL) and in doxorubicin treated cells the ROS was elevated by 66.76% (2.81 fold compared to control).

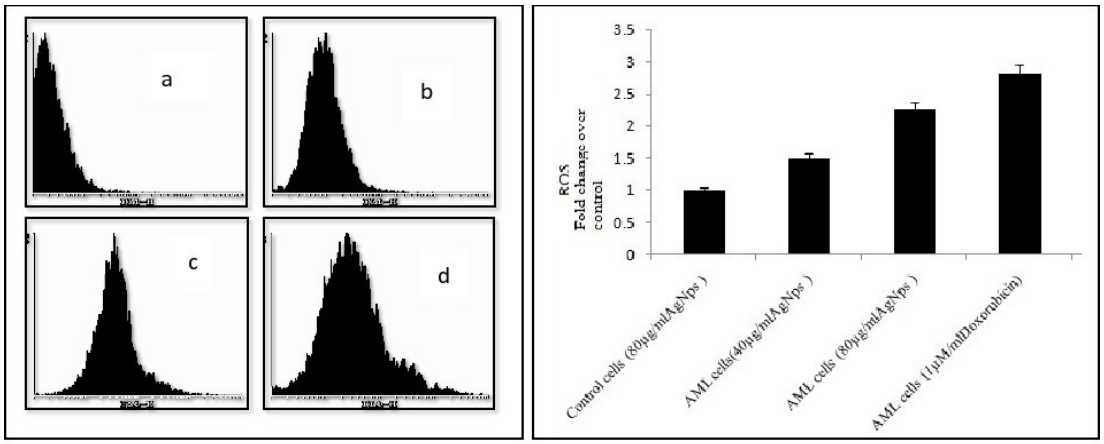

Figure 3. Flow cytometric analysis (A) and fold change (B) of ROS generation. (a): control cellswith AgNP at 80 µg/mL; (b) leukemic lymphocytes treated with AgNPs (40 µg/mL); (c) leukemic lymphocytes treated with AgNP (80 µg/mL); (d) leukemic lymphocytes treated with standard drug doxorubicin (1 µM).

Fluorescence study by DAPI staining

Imaging of cells after staining with DAPI revealed chromatin condensation and fragmentation in leukemic lymphocytes treated with phyto-fabricated AgNPs at 80 µg/mL (Figure 4B). A similar change in the nuclear material was also observed in leukemic cells treated with doxorubicin (Figure 4C). But normal lymphocytes treated with the highest concentration of phyto-fabricated AgNPs showed no typical characteristics of apoptosis such as nuclear condensation or cell blebbing (Figure 4A).

We also found a significant (P<0.05) increase in both apoptosis and necrosis in the leukemic lymphocyte cells treated with phyto-fabricated AgNPs (Figure 5A). The result was similar in the case of the doxorubicin treated leukemic lymphocyte cell population (Figure 5A). A significant increase in the number of necrotic cells was observed at the highest concentration of phyto- fabricated AgNPs (Figure 5B).

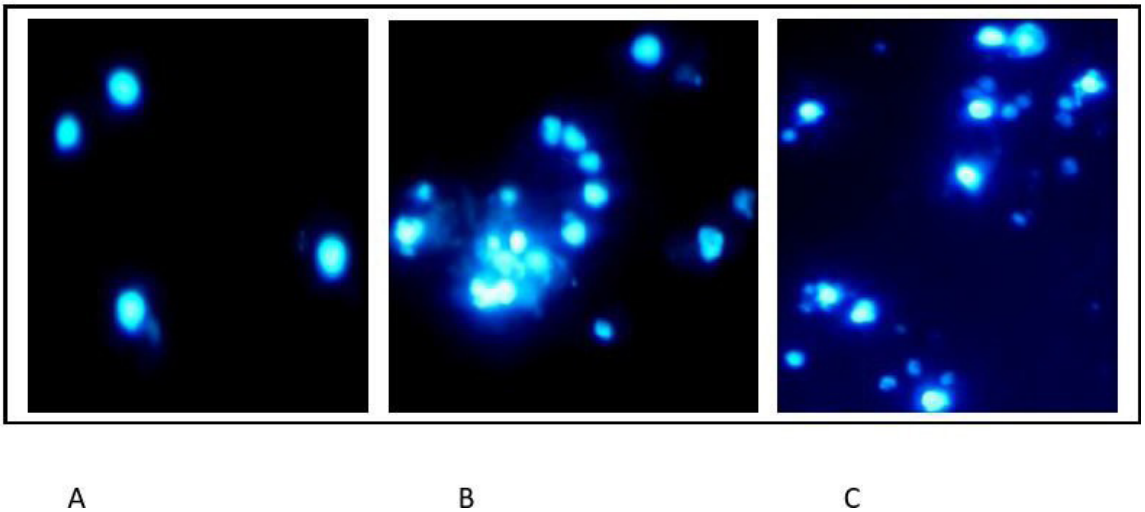

Figure 4. Tests of cytotoxicity by staining with DAPI. A. Control cells treated with phyto- fabricated AgNPs; B: leukemic lymphocytes treated with phyto-fabricated AgNPs; C: leukemic lymphocytes treated with doxorubicin.

Estimation of DNA damage by Comet and DNA fragmentation assay

After the treatment with phyto-fabricated AgNPs, the damaged DNA of the leukemic lymphocytes was observed by comet assay (Figure 6A&B). The quantification of the damaged DNA was determined by comparing the tail length of the comet between the leukemic lymphocyte cells treated with phyto-fabricated AgNPs with the control cell population. A significant (P<0.05) increase in DNA damage was observed in both phyto-fabricated AgNPs at 80µg/mL and doxorubicin treated leukemic lymphocytes (Figure 6C). In the phyto-fabricated AgNPs treated AML cells, the tail length of DNA was approximately 11 fold higher whereas in doxorubicin treated cells it was 14 fold higher than the value of comet obtained in the control cells (normal lymphocytes treated with phyto-fabricated AgNPs) (Figure 6C).

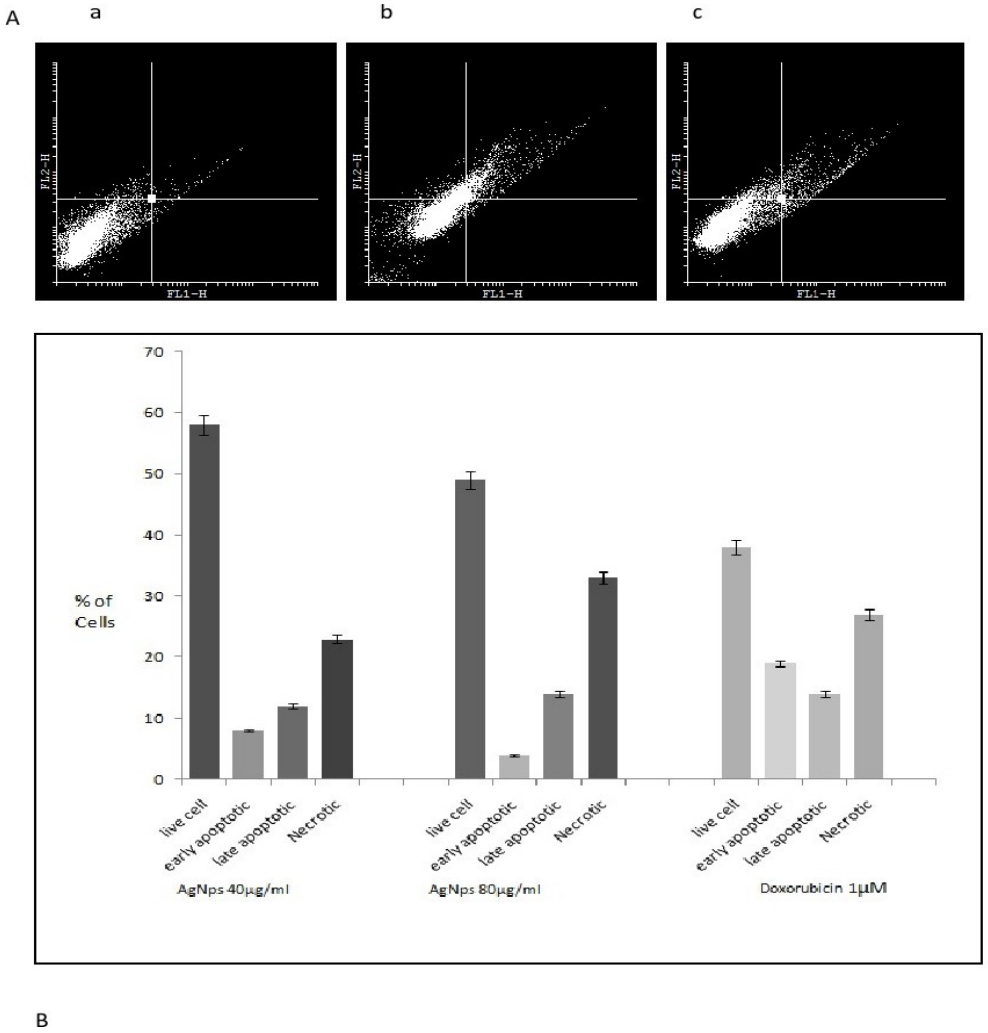

Figure 5. Assessment of apoptosis and necrosis. A: Flow cytometric analysis (a) Control cell (with AgNPs); (b) after treatment with AgNPs(80µg/ml); (c) leukemic lymphocytes treated with standard chemotherapeutic drug doxorubicin.; B: Comparison among the percentage of leukemiclymphocytes at different phages of cell death.

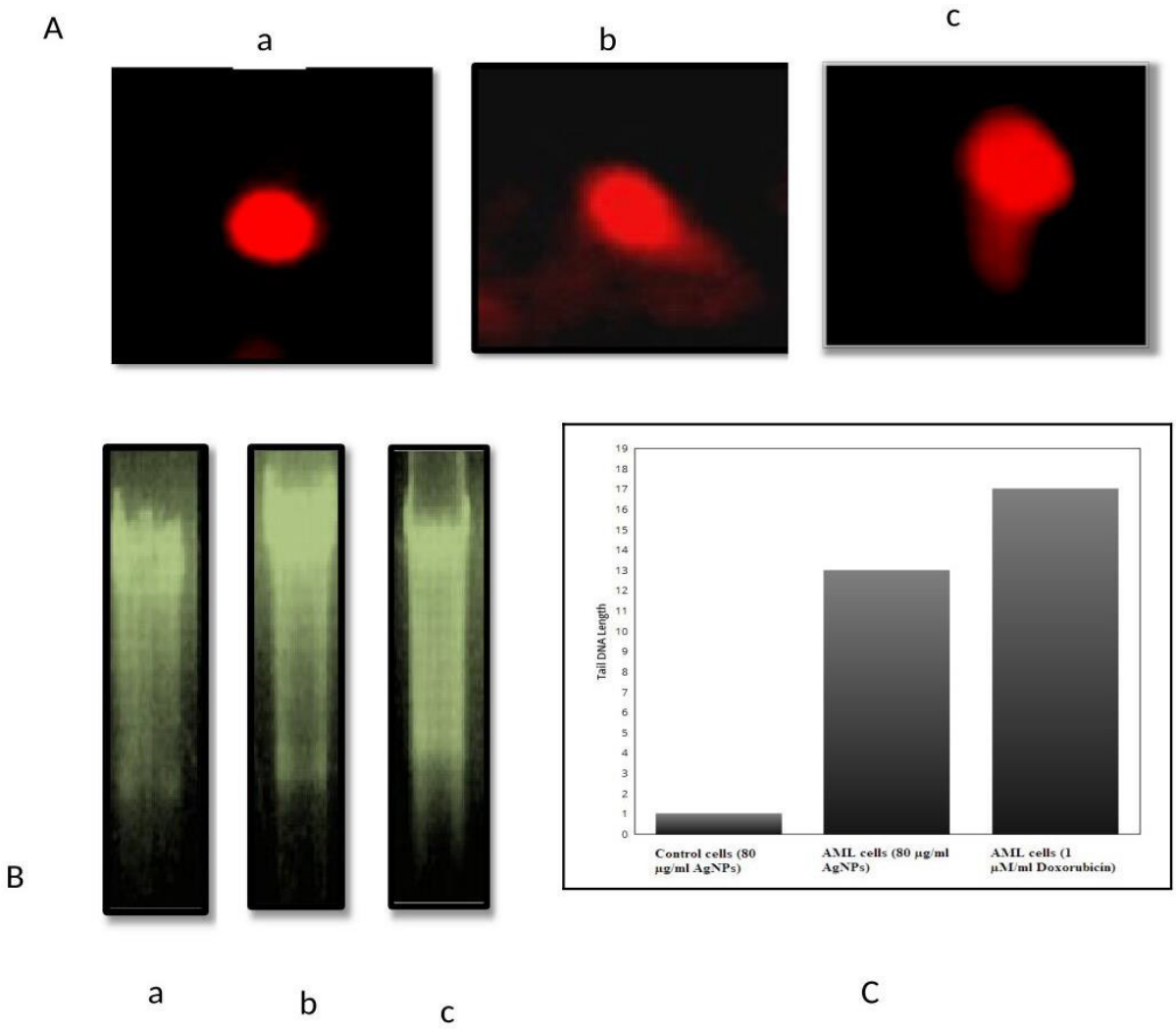

Figure 6. Quantitative (A) and qualitative (C) analysis of comet assay B. Fragmentation of DNA.

Uptake and structural alterations of AML cells by TEM

AML cells incubated with phyto-fabricated AgNPs were characterized by different degrees of deformities like damaged cell membranes, extensive vacuolation, and vesicular degeneration. Features like loss of nuclear organization with the ruptured plasma membrane and shrinkage of the protoplast (Figure 7A) were observed AML cells treated with phyto- fabricated AgNPs. The localization of nanoparticles was observed in the vesicles (Figure 7E) of the cells. Extensive vacuole formation (Figure 7D) and lysosomes with partly degraded content (Figure 7C) were also observed.

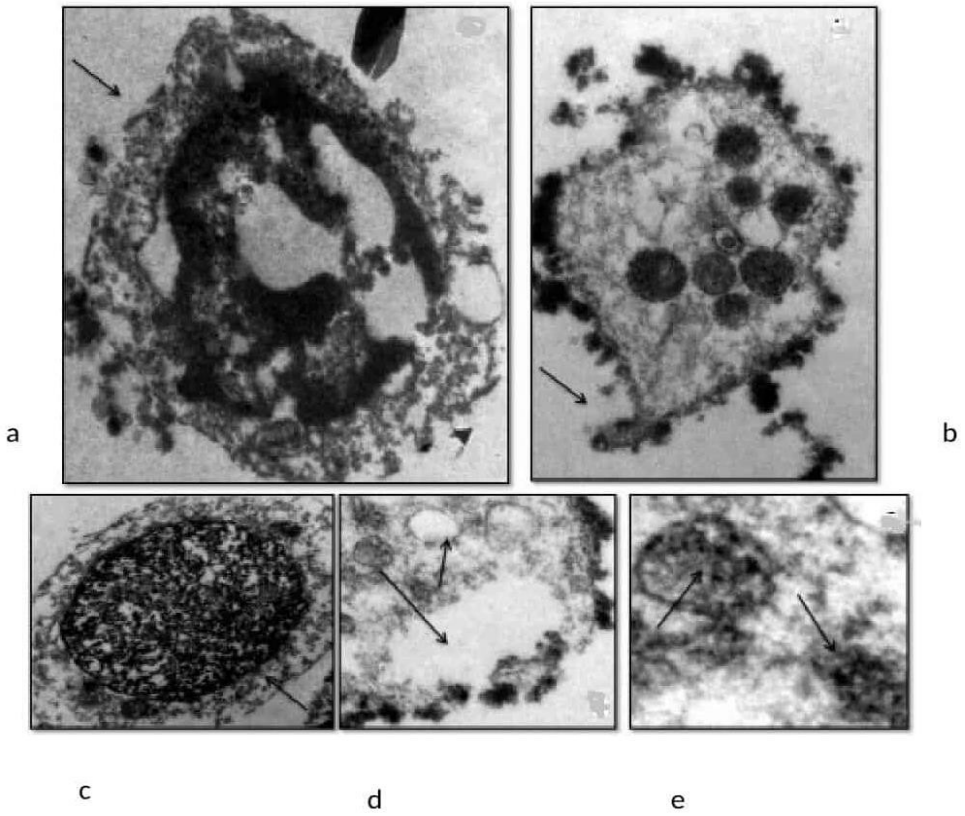

Figure 7. Structural alteration of AML cells treated with silver nanoparticles (a) treated AML cells with loss of nuclear organization, ruptured plasma membrane and degraded protoplast; (b) AML cells with pseudopodia; (c) lysosome with partly degraded content; (d) extensive vacuolation in of the cell; (e) localization of nanoparticles inside the vesicles.

Discussion

Very few reports are available on the efficacy of green silver nanomaterials in the treatment of AML cells [26, 41]. We have developed eco-friendly, phyto-fabricated silver nanoparticles using the aqueous bark extract of the plant *Saraca asoca* following the green chemistry method [29]. The average range of particle size calculated using dynamic light scattering measurements was 3-10 nm [29]. AFM analysis showed the presence of almost spherical shaped particles within the size range of <5 nm. FTIR analysis indicated the involvement of carboxyl (-C=O), hydroxyl (- OH) and amine (-NH) functional groups of the phytochemicals in capping and stabilizing silver nanoparticles. The capping and/or stabilizing materials are phyto-compounds like 1, 2, 4-triazole and diethyl acetylenedicarboxylate with relatively higher abundance (data not shown). The rapid electrokinetic behavior of the silver was evaluated using zeta potential (approx -23.2 mV) to confirm its stability [29].

The therapeutic study of the synthesized phyto-fabricated silver nanoparticle [42,43] revealed that the survivability was increased by 50% in comparison to that of untreated AML mice whereas mice treated with doxorubicin (2 mg/kg b.w.) showed 83% survivability. This study demonstrated that phyto-fabricated silver nanoparticles could effectively prolong the survival time *in vivo*. The cytotoxicity and genotoxicity of the phyto-fabricated silver nanoparticle were compared with the normal lymphocytes and AML cells *in vitro* to confirm the target-specific toxicity of the capped nanoparticle as reported by Netchareonsirisuk *et al*. [2016] [44]. We also confirmed the dose-dependent toxicity of the synthesized phyto-fabricated silver nanoparticle in AML cells. Previous evidences were available stating that exposure to AgNPs at different concentrations can cause dose-dependent toxicity by inducing oxidative stress and DNA damage which ultimately leads to cell death [45].

The result of the trypan blue exclusion method also revealed the dose-dependent toxicity of the green AgNPs as the reduction in the viability of leukemic lymphocytes was increased from 50% to 89.14% at 80 µg/mL whereas normal lymphocytes treated at a concentration of 80µg/mL showed no significant reduction. This

finding confirmed the cell-specific cytotoxicity of the synthesized phyto-fabricated silver nanoparticle. Many researchers showed that silver nanoparticles can cause DNA damage and stimulate oxidative stress to those cells with a lack in their capacity to repair damaged DNA or other oxidative stress [45]. In our study, nanosilver with their phytochemical capping on its surface inhibited the viability of the cells by inducing oxidative stress. The presence of triazole is assumed to have induced the cytotoxicity of AgNPs as it has proven anti-cancerous activity against a wide range of cancerous cells [46]. So the potential vulnerability of the AML cells to the cytotoxicity of the synthesized green silver nanoparticle can be exploited to develop these green AgNPs as new therapeutic agents in AML treatment.

The elevated production of NO in the cell after exposure to silver nanoparticles is also crucial in asserting its cytotoxic ability because in reaction with superoxide NO usually produces more toxic peroxynitrite ($ONOO^-$) which attributes to severe oxidative damage leading to cell death [36]. In our study, NO level in the leukemic lymphocyte cells was 63.4% which was close to the value of doxorubicin treated AML cells where the value was 68.7%. No significant increase of NO was found in the control cells treated with the green AgNPs (data not shown). Thus the elevated level of NO in the green AgNPs treated leukemic lymphocytes contributed to the severe oxidative injury leading to the death of AML cells.

One possible mechanism to induce cell death is by promoting the generation of ROS. Normal cellular growth and survival are controlled by maintaining homeostasis in cellular ROS [47]. By estimating the amount of intracellular ROS the vulnerability of the cell to oxidative stress can be determined. In our study generation of ROS was elevated by 2.26 fold (53.7%) after exposure to the synthesized silver nanoparticle in comparison to the control where the production of ROS was moderate (23.76%). An excessive amount of ROS attributes to cell death by two pathways either apoptosis or necrosis. In our study, significant ($P<0.05$) elevation of ROS in AML cells after treatment with AgNPs is due to the cytotoxic efficacy of the synthesized green AgNPs. The toxic effect of nanoparticles is due to its small size (<5 nm) and the power of penetration of biological membrane barriers to reach different organs. On the other hand, surface functionalization by the phyto-components increased the chemical reactivity inside the cell leading to the excess generation of ROS [48].

The apoptotic cells can be identified by the loss of mitochondrial membrane integrity though in living cells the mitochondrial membrane integrity remains unchanged. The percentage of MMP decreased significantly ($P<0.05$) with the increase in the concentration of AgNPs. After treatment with the phyto-fabricated silver nanoparticle, the depletion in MMP was 2.4 fold at 40 µg/mL, 2.95 fold at 80 µg/mL and in doxorubicin treated cells it was 3.4 fold. The dissipation in MMP in the AgNPs exposed AML cells are assumed to be disrupted of their mitochondrial membrane.

The quantification of the percent of apoptosis and necrosis also revealed that the phyto- fabricated silver nanoparticles were toxic to the AML cells in a dose-dependent manner, green AgNPs at highest concentration increased the percent of apoptotic cells by 1.5 fold in comparison to control, and the percent of necrosis was higher than apoptosis in this group of cells. After exposure, the increase in ROS and subsequent decrease in MMP might be the reason for the induction of apoptosis and necrosis in the green AgNPs treated cells. DAPI staining and imaging of the AML cells showed condensed and fragmented chromatin as well as cell blebbing in AML cells after treatment with nanoparticles, a similar result was obtained when AML cells were treated with doxorubicin indicating that the synthesized phyto-fabricated silver nanoparticle was also able to induce death of leukemic cells like that of standard chemotherapeutic drug. The disruption in chromatin structure contributes to cell death by turning on the apoptotic process [37]. Comet tail length of the AML cells treated with AgNPs and doxorubicin in comparison with control cells concurrent with this observation. The value of tail DNA was approximately 11 fold higher than the control whereas in doxorubicin treated cells it was approximately 14 fold higher. The presence of comet tail was due to the oxidative attack causing DNA damage which was attributed by the cytotoxic effect of the green silver nanoparticle. The damage of the cellular architecture was shown by TEM images of the AML cells treated with phyto-fabricated silver nanoparticles. The damage was due to morphological alterations, extensive vacuolation, loss of nuclear organization, ruptured plasma membrane, partly degradation of lysosome contents, and shrinkage of the protoplast associated with apoptotic/necrotic cell death induced by the phyto- fabricated AgNPs after internalization into the cellular vesicles. These findings thus confirmed the uptake of green AgNPs by the AML cells and also the induced cytotoxicity due to oxidative damage.

Conclusions

We have demonstrated that the synthesized phyto-fabricated silver nanoparticle has a unique toxicity profile. The AML cells were vulnerable to the toxicity of the green silver nanoparticle in a highly selective manner which was in agreement with other published reports (49, 50). Furthermore, we found that the phyto-fabricated AgNPs were selectively cytotoxic to the AML cells at higher concentration but it showed no such effect on the non leukemic control lymphocytes. A recent study demonstrated that the cytotoxicity of AgNPs might be approx.2- fold more in acute myeloid leukemia cells in comparison to the normal bone marrow derived lymphocytes (26) in concurrence with our observations. To develop the phyto-fabricated silver nanoparticle as a new line of treatment of AML, it was necessary to determine the sensitivity

of both leukemic and non-leukemic cells towards the green AgNPs and also its comparative cytotoxic efficacy against standard chemotherapeutic drug. The analysis through the comparative study including multiple criteria like cell viability, release of NO, production of ROS, change in mitochondrial membrane potential, induction of apoptosis and necrosis, and DNA damage revealed that the synthesized phyto-fabricated AgNPs were selectively cytotoxic towards leukemic lymphocyte cells and its anti-leukemic efficacy was comparable to the standard chemotherapeutic drug and simultaneously it increased the percent survivability of secondary AML mice when administered *in vivo*. These findings definitely open a new avenue directed towards the biomedical application of green phyto-fabricated AgNPs in cancer cells.

Summary Points

In **summary**, we demonstrated that the synthesized phyto-fabricated silver nanoparticle has a unique selective toxicity profile. Furthermore, we found that the phyto-fabrication reduced the toxicity of the nanoparticle itself on normal cells and capping materials imposed the chemopreventive potential on AgNPs in an environmentally sustainable pathway.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

References

1. Jain, A., Madu, C. O., & Lu, Y. 2021 Phytochemicals in Chemoprevention: A Cost-Effective Complementary Approach. J of Cancer, 12, 3686–3700.
2. Sheikh I , Sharma V R , Tuli H S, et al. 2021 Cancer Chemoprevention by Flavonoids, Dietary Polyphenols and Terpenoids. Biointerface Research in Applied Chem 11, 8502 – 8537.
3. Dave A, Parande F, Park EJ, et al. 2020 Phytochemicals and cancer chemoprevention. J Cancer Metastasis Treat 6:46.
4. Rizeq B, Gupta I, Ilesanmi J, et al. 2020 The Power of Phytochemicals Combination in Cancer Chemoprevention. J Cancer 11(15):4521-4533.
5. Hu ML. 2011 Dietary polyphenols as antioxidants and anticancer agents: more questionsthan answers. Chang Gung Med . 34: 449–460.
6. Scott EN, Gescher AJ, Steward WP, et al. 2009 Development of dietary phytochemical chemopreventive agents: biomarkers and choice of dose for early clinical trials. Cancer Prev Res (Phila) 2: 525-530.
7. Cragg GM, Newman DJ. 2005 Plants as a source of anticancer agents. J Ethnopharmacol100: 72–79.
8. Balunas MJ, Kinghorn AD. 2005 Drug discovery from medicinal plants. Life Sci 78: 431–441.
9. Liu RH. 2004 Potential synergy of phytochemicals in cancer prevention: mechanism of action. J Nutr. 134: 3479S–3485S.
10. Adebayo IA, Usman AI, Shittu FB, et al.2020 Boswellia dalzielii-Mediated Silver Nanoparticles Inhibited Acute Myeloid Leukemia (AML) Kasumi-1 Cells by Inducing Cell Cycle Arrest. Bioinorg Chem Appl. Sep 22;2020:8898360. doi: 10.1155/2020/8898360.
11. Prabhu D, Arulvasu C, Babu G, et al. 2013 Biologically synthesized green silver nanoparticles from leaf extract of *Vitex negundo* L induce growth-inhibitory effect on human colon cancer cell line HCT15. Proc Biochem 48: 317–324.
12. Krishnaraj C, Muthukumaran P, Ramachandran R, et al. 2014 Acalypha indica Linn: Biogenic synthesis of silver and gold nanoparticles and theircytotoxic effects against MDA-MB-231 human breast cancer cells. Biotech Rept 4: 42– 49.
13. Gurunathan S, Han JW, Eppakayala V, et al. 2013 Cytotoxicity of biologically synthesized silver nanoparticles in MDA-MB-231 human breast cancer cells.Biomed Res Int 96: 53-57.
14. Elangovan K, Elumalai D, Anupriya S, et al. 2015 Phyto mediated biogenic synthesis of silver nanoparticles using leaf extract of Andrographis echioides and its bio-efficacy on anticancer and antibacterial activities. J Photochem Photobiol B: Biol 151: 118–124.
15. Arunachalam KD, Annamalai SK, Arunachalam AM, et al. 2013 Green synthesis of crystalline silver nanoparticles using Indigofera aspalathoides-medicinal plant extract for wound healing applications As J Chem25: S311–S314.
16. Paul JAJ, Selvi BK , Karmegam N 2015 Biosynthesis of silver nanoparticles fromPremna serratifolia L leaf and its anticancer activity in CCl4-induced hepato-cancerous Swiss albino mice. ApplNanosci 5: 937–944.
17. Sre PRR, Reka M, Poovazhagi R, et al 2015 Antibacterial and cytotoxic effect of biologically synthesized silver nanoparticles using aqueous root extract of Erythrina indica lam. Spectrochimica Acta Part A: MolBiomol Spectroscop 135: 1137–1144.
18. Sukumar UK, Bhushan B, Dubey P, et al. 2013 Emergingapplications of nanoparticles for lung cancer diagnosis and therapy. IntNano Lett 3: 45-17.
19. Pardhasaradhi BVV, Reddy M, Ali AM, et al 2004 Antitumour activity of Annona squamosa seed extracts is through the generation of free radicals and induction of apoptosis. Ind J Biochem Biophys. 41: 167–172.
20. Chen WY, Chang FR, Huang ZY, et al. 2008 Tubocapsenolide: A novel withanolide inhibits proliferation and induces apoptosis in MDA-MB-231 cells by thiol oxidation of heat shock proteins. JBiolChem 283: 17184–17193.
21. Fleischauer AT, Poole C , Arab L. 2000 Garlic consumption and cancer prevention: meta-analyses of colorectal and stomach cancers. Am J Clinc Nutr. 72: 1047– 1052.
22. Ishikawa H, Saeki T, Otani T, et al. 2006 Aged garlic extract prevents a decline of NK cell number and activity in patients with advanced cancer J Nut 136: 816S–820S.
23. Zhou H, Ning Y, Zeng G, et al. 2021 Curcumin promotes cell cycle arrest and apoptosis of acute myeloid leukemia cells by inactivating AKT. Oncol Rep.45:11.

24. Wilken R, Veena MS, Wang MB, et al. 2011 Curcumin: a review of anti-cancerproperties and therapeutic activity in head and neck squamous cell carcinoma. Mol Cancer. 10(12): 1–19.

25. Dhillon N, Aggarwal BB, Newman RA, et al. 2008 Phase II trial of curcumin in patients with advanced pancreatic cancer. Clin Cancer Res. 14:4491–4499.

26. Guo D, Zhu L, Huang Z, et al. 2013 Anti-leukemia activity of PVP-coated silver nanoparticles via generation of reactive oxygen species and release of silver ions. Biomaterials 34:7884– 7894.

27. Roy I, Das B, Rahaman Mollick M, et al. 2016 Nanotherapy on human acute myeloid leukemia cells using RGO/Ag nanocomposites.RSC Adv. 6: 52403-52410.

28. Saha S, Mukhopadhyay MK, Ghosh PD, et al.2012 Effect of methanolic leaf extract of *Ocimum basilicum* L on benzene-induced hematotoxicity in mice. Evidence Based Compl Alt Med. 176-385.

29. Banerjee P, Nath D 2015 A Phytochemical Approach to Synthesize Silver Nanoparticles for Non-Toxic Biomedical Application and Study on their Antibacterial Efficacy. Nanosci Technol 2: 1-14.

30. OECD. 2001. Test guideline 425. Acute Oral toxicity test: the up and down procedure. *In*: OECD guidelines for the testing of chemicals. Paris, France: Organization for Economic Cooperation and Development. Organization for Economic Cooperation & Development (OECD).

31. Kaplan EL and Meier P 1958 Nonparametric estimation from incomplete observations. JAmer Statist Assn 53(282): 457-481.

32. Thorsby E, Bratlie A, 1970 A rapid method for preparation of pure lymphocyte suspensions. Histocompatibility Testing, Terasaki, P.I., ed., 665- 666.

33. Tennant JR 1964 Evaluation of the trypan blue technique for determination of cell viability Transplantation 2: 685–694.

34. Sreejayan N , Roa MNA. 1997 Nitric oxide scavenging by curcuminoids. J Pharm Pharmacol 49:105–107.

35. Roy A, Ganguly A, Dasgupta S, et al. 2008 Mitochondria-dependent reactive oxygen species-mediated programmed cell death induced by 33- diindolylmethane through inhibition of F0F1-ATP synthase in unicellular protozoan parasite Leishmania donovani. Mol Pharmacol 74: 1292–1307.

36. Dash SK, Chattopadhyay S, Ghosh T, et al. 2013 Antileukemic efficacy of monomeric manganese based metal complex on KG-1A and K562 cell lines. ISRN Oncol 69: 70-92.

37. Mollick MMR, Bhowmick B, Mondal D, et al. 2014 Anticancer (*in vitro*) and antimicrobial effect of gold nanoparticles synthesized using Abelmoschusesculentus (L) pulp extract via a green route. RSC Adv4: 37838–37848.

38. Singh NP, McCoy MT, Tice RR, et al. 1988 A simple technique for quantification of low levels of DNA damage in individual cells. Exp Cell Res 175: 184- 191.

39. Paul S, Bhattacharyya SS, Samaddar A, et al. 2011 Anticancer potentials of root extract of Polygala senega against benzo-pyrene-induced lung cancer in mice. J Chin Integr Med 9: 320–327.

40. AshaRani PV, Low Kah Mun G, Hande MP, et al. 2009 Cytotoxicity , genotoxicity of silver nanoparticles in human cells.ACS Nano 3: 279-290.

41. Guo D, Zhao Y, Zhang Y, et al. 2014 The cellular uptake and cytotoxic effect of silver nanoparticles on chronic myeloid leukemia cells. J Biomed Nanotechnol 10(4): 669–678.

42. Akbay P, Basaran AA, Undeger U, et al. 2003 *In vitro* immunomodulatory activity of flavonoid glycosides from Urtica dioica L. Phytother Res 17: 34-37.

43. Mukhopadhyay M, Shaw M, Nath D 2017 Chemopreventive potential of major flavonoid compound of methanolic bark extract of *Saraca asoca* (Roxb) in benzene-induced toxicity of acute myeloid leukemia mice. Pharmacogn Mag 13:216-223.

44. Netchareonsirisuk P, Puthong S, Dubas S et al. 2016 Effect of capping agents on the cytotoxicity of silver nanoparticles in human normal and cancer skin cell lines. J Nanoparticle Res.18: p322.

45. Lim HK, Asharani PV , Hande MP 2012 Enhanced genotoxicity of silver nanoparticles in DNA repair deficient Mammalian cells. Front Genet 3: 104.

46. El-Sherief HAM, Youssif BGM, Bukhari SNA et al. 2018 Novel 1,2,4-triazole derivatives as potential anticancer agents: Design, synthesis, molecular docking and mechanistic studies. Bioorg Chem 76:314-325.

47. Trachootham D, Alexandre J , Huang P 2009 Targeting cancer cells by ROS-mediated mechanisms: A radical therapeutic approach? Nat Rev Drug Discov 8: 579–591.

48. Khorrami S, Zarrabi A, Khaleghi M et al. 2018 Selective cytotoxicity of green synthesized silver nanoparticles against the MCF-7 tumor cell line and their enhanced antioxidant and antimicrobial properties. Int J Nanomedicine 13: 8013-8024.

49. Stefaan S, Demeester J , De Smedt, S et al. 2013 Turning a frown upside down: exploiting nanoparticle toxicity for anticancer therapy. Nano Today 8. p.121- 125.

50. Arvizo RR, Saha S, Wang E, et al. 2013 Inhibition of tumor growth and metastasis by a self-therapeutic nanoparticle. Proc Natl Acad Sci USA 110:6700-6705.