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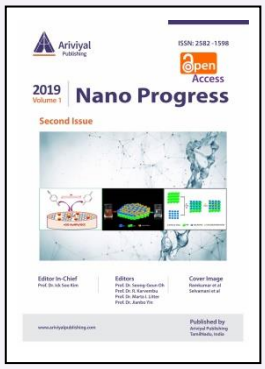

Medicinal Plants Derived Silver Nanoparticles: Cytotoxicity Assay Against Human Monocytic Leukemia (THP-1) Cell Line

Niraj Kumari,*[a] Priti Kumari,[a] Anal K. Jha[b] and Kamal Prasad*[c]

[a]*Aryabhatta Centre for Nanoscience and Nanotechnology, Aryabhatta Knowledge University, Patna, 800001, India.*
[b]*University Department of Chemistry, T.M. Bhagalpur University, Bhagalpur, 812007, India.*
[c]*University Department of Physics, T.M. Bhagalpur University, Bhagalpur, 812007, India.*

***Corresponding author E-mail address**: prasad_k@tmbuniv.ac.in (Kamal Prasad)

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Abstract: The silver nanoparticles have been extensively studied as antimicrobial agents in recent past but very limited information about its antitumor and/or anticancer activity are available in the literature. In this work, a comparative study of the cytotoxic potentials of *Phyllanthus niruri, Achyranthes aspera* and *Azadirachta indica* mediated biosynthesized silver nanoparticles (SNPs) against THP-1 cells, a human leukaemia cell lines have been undertaken. These SNPs showed a dose-dependent cytotoxicity using the WSA-1 assay against THP-1 cells and the activation of reactive oxygen species (ROS) generation by DCFH-DA assay. The results distinctly indicated its probable use as a therapeutic drug for future cancer treatment. The formations of SNPs were ascertained by X-ray diffraction, scanning and transmission electron microscopy, and UV-visible spectroscopy techniques. A biosynthetic mechanism accomplishing nano-transformations has also been proposed where the responsible phytochemicals such as different alkaloids, flavonoids, tannins, saponins and organic acids present in the leaves' parenchyma were considered.

Keywords: Silver; nanoparticles; bionanotechnology; green synthesis; cytotoxicity; THP-1 cells

1. Introduction

With a recent upsurge in the field of bionanotechnology, benign microbes, medicinal plants along with their individual parts and food beneficiaries have attracted the considerable attention of different workers worldwide. By adopting such a green method of synthesis not only ensures a particle size reduction, rather a radial functionalization of the burgeoned nanoparticles of choice that adds tremendously towards their biological (antimicrobial/anticancer) activities. Among these, the syntheses of different nanoparticles using plant extract have an added advantage over the other green bionanotechnology methods which eliminate the need of the intricate process of preserving the cell-cultures, in the sense that in one hand they negotiate the synthetic cues while the unused metabolites add further to their antimicrobial and/or medicinal properties.[1,2] It is seen that the plants like *Phyllanthus niruri, Achyranthes aspera*, and *Azadirachta indica* have the potential medicinal properties and are being used in Ayurveda since the Vedic period. These plants harbor medicinally precious phytochemicals such as alkaloids, flavonoids, terpenes, saponins and many others in their leaves.[3–6] Also, these plants were reported as antimicrobial, antiviral and/or anticancer agents to treat cancer and other types of infections. It is observed that cancer is a group of diseases identified from excessive cell growth, number of hallmarks, reprogramming of energy metabolism and suppression of immune response against tumor cells. Also, the cell lines have played important roles in the studies of cancer for the development of new drugs and other molecular mechanisms. Among them, a human monocyte leukemia (THP-1) cell line which is derived from the perimetric blood of acute monocytic leukemia patient which display single spherical cell morphology. It expresses Fc as well as C3b receptors without surface or cytoplasmic immunoglobulins.[7–10] Furthermore, silver nanoparticles (SNPs) are considered to have very good antimicrobial, antiviral as well as the anticancer properties[1,11–14] and the therapeutic potentiality of SNPs has been a matter of contention since long. Also, it is known that the anti-proliferative properties of SNPs bolstered with its ROS production ability have contrived it as a potent therapeutic agent for cancer therapy. An extensive literature survey indicated the no work has been undertaken yet to observe the cytotoxic potential of *P. niruri*, *A. aspera* and *A. indica* leaves negotiated SNPs against human monocytic leukemia (THP 1) cell lines.

Accordingly, in this study synthesis of SNPs using the ethanolic leaf extracts of *P. niruri, A. aspera* and *A. indica* and their cytotoxicity assay against THP-1 cells have been carried out. The as-prepared SNPs were characterized from the standard techniques like X-ray diffraction, scanning and transmission electron microscopy, and UV visible spectroscopy. Besides, a biosynthetic mechanism

Table 1. (a) X-ray diffraction patterns and (b) suitably magnified X-ray diffraction (111) peaks of SNPs at room temperature synthesized using *P. niruri*, *A. aspera* and *A. indica* leaves.

Parameters	SNPs from *A. indica*	SNPs from *P. niruri*	SNPs from *A. aspera*
Crystal system	*fcc*	*fcc*	*fcc*
Crystal system	4.071Å	4.076 Å	4.078 Å
Apparent particle size (X-ray)	11 nm	23 nm	28 nm
Lattice strain	0.0023	0.0018	0.0016
Particle size (TEM)	2–7 nm	2–12 nm	2–18 nm

accomplishing nanotransformations has been proposed where the responsible phytochemicals such as different alkaloids, flavonoids, tannins, saponins and organic acids present in the leaves' parenchyma were considered.

2. Experimental Section

2.1. Biosynthesis of SNPs using medicinal plants

Prior to conducting the experiment, freshly collected leaves of *P. niruri, A. aspera*, and *A. indica* were rinsed thoroughly using de ionized water and were dried and cut separately into fine pieces. The leaf extracts were prepared by taking 15 g of each plant leaf and were poured in 100 ml of 50% ethanol in three 250 ml conical flasks and each mixture was boiled for 20 min. before decanting till the colour changes from clear transparent to deep green. Thereafter the leaf masses were pressed separately by wrapping in serene cloth and 50 ml of each plant leaf extract was collected under laminar flow. They were doubled in volume by adding 50 ml sterile distilled water and were treated as source extracts. Analytical reagent grade silver nitrate ($AgNO_3$) was taken into use for preparing a solution of 0.025M strength at room temperature in sterile distilled water. Now, 15 ml of aq. $AgNO_3$ solution was added to the 100 ml of *P. niruri, A. aspera* and *A. indica*, leaf extracts in three 250 ml flasks and were getting heated keeping temperature 50-60°C until the light green colored solution turned in to black color. This was perceived as the initiation of nanotransformation. The as-synthesized nanoparticles were centrifuged for 15 min at 5000 rpm and the solid masses (SNPs) as well as their colloids were kept separately for further studies.

2.2. Characterization of biosynthesized SNPs

The formations of SNPs were ascertained by X-ray diffraction (XRD) technique. The XRD spectra were obtained with X-ray diffractometer (Bruker D8 Advance) using CuKα radiation λ = 1.5406 Å over a range of Bragg angles 20° to 80° at room temperature. The scanning electron micrograph (SEM) image of SNPs was taken using a computer controlled scanning electron microscope (EVO 18 of Carl Zeiss Microscopy Ltd., UK). A high-resolution Bruker transmission electron microscope, operated at an accelerated voltage of 200 keV, was used to procure TEM images of all the SNPs. The absorption spectra of all the samples were obtained from a computer-controlled UV-visible-NIR spectrophotometer (PerkinElmer, UK).

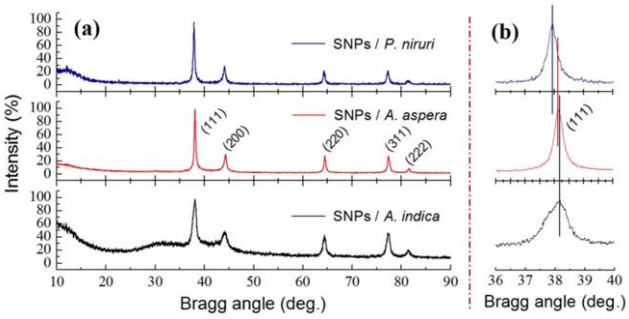

Fig. 1. (a) X-ray diffraction patterns and (b) suitably magnified X-ray diffraction (111) peaks of SNPs at room temperature synthesized using *P. niruri*, *A. aspera* and *A. indica* leaves.

2.3. Generation of Reactive Oxygen Species (ROS) measurements

ROS generation of green synthesized SNPs has been carried out by using 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) dye. The THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) which was grown in 5% CO_2 atmosphere at 37°C. This experiment was performed in 96-well plates. The cells were harvested into the well plates keeping the density of 1×10^6 cells/ml and were washed with the phosphate buffered saline (PBS) having the pH value 7.4. Thereafter, the harvested cells treated with different concentrations (0-25 µg) of *P. niruri, A. aspera*, and *A. indica* mediated SNPs and were incubated for 24 h. After washing twice with PBS, 20 µM DCFH-DA dyes were added to the THP-1 cells and were incubated further in dark place at 37°C for 30 min. An LS 55 spectrofluorimeter (PerkinElmer, UK) was taken into use to determine the fluorescence intensity at excitation 492 nm and emission 530 nm where the net fluorescence intensity is directly proportional to the intracellular ROS of cells. The dye re-suspended in PBS without cells was utilized as the blank control and was subtracted from the values of treated cells.

2.4. Cell viability assays

The cell viability assay was carried out with WST-1 (Cell proliferation reagent, Roche) reagent according to manufacturer's instructions. The THP-1 cells were harvested in 96-well plates and treated with different concentrations (0-50 µg/ml) of *P. niruri, A. aspera* and *A. indica* mediated SNPs. All the cultures were incubated for 24 h at 37°C in an incubator. After 24 h of incubation, 10 µl of WST-1 reagent was added to each culture well and were allowed to incubate further at 37°C for 4 h. The well of blank media was used as background control and the value of which was subtracted from the absorbance values of the treated well culture cells. The absorbance values were determined in dual mode (A450 nm – A655 nm) using an *i*Mark ELISA plate reader (Biorad). The results shown in this work are the mean of three independent experiments. The cell viability was calculated by using the following formula: Cell viability (%) = (Mean OD/Control OD)×100. The IC50 values were also calculated for the concentration of SNPs at which cell viability reduced to half of its original value. IC50 was calculated according to the following equation using straight line (linear regression): IC50= (0.5-b)/a.

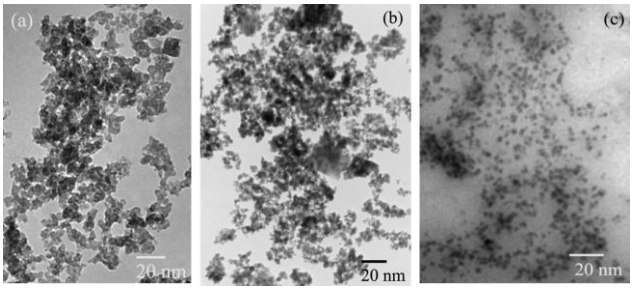

Fig. 2. TEM images of SNPs synthesized using the ethanolic extract of (a) *P. niruri*, (b) *A. aspera* and (c) *A. indica* leaves.

3. Results and Discussions

Fig. 1(a) and (b) respectively show the XRD profiles and magnified (111 peaks) XRD profiles of SNPs synthesized from *P. niruri, A. aspera*, and *A. indica* broths. The XRD patterns show the presence of broad peaks which clearly indicated the formations of small sized particles. The peaks of the XRD profiles were indexed and lattice parameters were obtained with a standard computer program 'POWD' using the least squares regression fitting of the XRD data. The obtained lattice parameters of SNPs are depicted in Table 1. The cubic (fcc) phase of all the SNPs was found to be in agreement with the standard literature (ICDD no. #89-3722). A little difference between the cell parameter of bulk silver (a = 4.085Å) and the prepared SNPs has been observed which could be due to the nanosizing effect. Also, it is observed from Table 1 that the lattice parameter follows the sequence $a_{P.\ niruri} > a_{A.\ aspera} > a_{A.\ indica}$ which is due to the fact that the XRD peaks are shifting toward the higher wavelength side as we change the leaf (Fig. 1b). The reduction in edge length (or cell volume, a^3) is considered due to the presence various phytochemicals in these leaf parenchyma where different biochemical processes occur and able to generate energy inside the nano-transforming medium differently.[15] The apparent particle sizes and lattice strains of SNPs were calculated by analyzing the XRD peaks broadening using the Williamson-Hall approach: $\beta\cos\theta = (K\lambda / D) + 2(\Delta\xi/\xi)\sin\theta$. Here D is the apparent particle size, β is diffraction peak width at the half intensity and $\Delta\xi/\xi$ is the lattice strain and K is the Scherrer constant (~0.89). The term $K\lambda/D$ serves as the Scherrer particle size distribution. The values of apparent particle sizes and average lattice strains were estimated using the linear least-square fitting of $\beta\cos\vartheta$ – $\sin\vartheta$ data (Table 1). It is seen that the values of D follow the same sequence as the a whereas the lattice strains follow the opposite trend. The low values of lattice strain clearly indicated that the procedure adopted in the biosynthesis of SNPs is natural one where seemingly no constraints are imposed.

Fig. 2 shows the TEM images of SNPs being formed using (a) *P. niruri*, (b) *A. aspera* and (c) *A. indica* broths. The measurement of sizes was performed along the largest diameter of the particles using the *ImageJ* software. The particles are found to be almost spherical in shape having the sizes ranged between 2 nm and 19 nm. Also, it is seen that *A. indica* negotiated SNPs are having a lower distribution of particles compared to other two leaf broths (Table 1).

The UV–vis. spectroscopy is an important technique to confirm the formation and stability of nanoparticles in an aqueous medium. Fig. 3 shows the UV-vis. spectrum recorded for SNPs synthesized

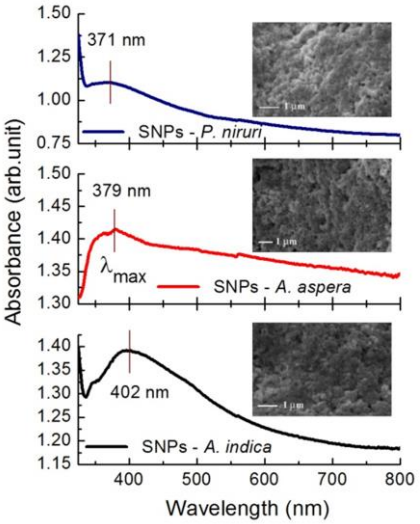

Fig. 3. UV-vis spectra of SNPs synthesized using the ethanolic extract of (a) *P. niruri*, (b) *A. aspera* and (c) *A. indica* leaves Inset: Respective SEM image.

using *P. niruri*, *A. aspera*, and *A. indica* leaf extracts. The surface plasmon resonance (λmax) was observed at 371 nm, 379 nm, and 402 nm, respectively for *P. niruri*, *A. aspera*, and *A. indica* leaves synthesized SNPs. It is observed that the value of λmax shift towards higher wavelength side with the SNPs prepared from *P. niruri* to *A. aspera* to *A. indica*. Also, the broadened plasmon bands, asymmetric in nature, with absorption tails in the longer wavelengths, could be seen, which might be due to the size distribution of particles.

Besides, the biosynthesized SNPs were kept at room temperature in the laboratory and inspected after three months. The absorbance values appeared to be almost the same. This simply suggested that the SNPs synthesized by all the three leaves extract bears excellent stability which might be due to the encapsulation by the different phytochemicals present in the respective leaf parenchyma. Inset of Fig. 3 shows the SEM micrograph of SNPs synthesized using *P. niruri*, *A. aspera*, and *A. indica* broths. These micrographs also support the formation of very small sized particles in little nm range, the TEM results (Fig. 2).

The phytochemical analysis of the ethanolic extracts of *P. niruri*, *A. aspera*, and *A. indica* leaves showed that these plants contain both primary as well as secondary metabolites. They have been screened, isolated, characterized, and studied for different biological activities.[16–21] These plants are rich in alkaloids, benzenoids, coumarins, flavonoids, lignans, lipid, sterol, tannins, triterpene, saponins, glycosides, and these compounds are known to exhibit the physiological activities and medicinal properties. The UV-vis., IR and mass spectroscopic studies of *P. niruri* leaves extract showed that it contains gallic acid, ellagic acid, ricinoleic acid, quercetin, quercitrin, norsecurinine, *etc.*[16,17] The phytochemical analysis of *A. aspera* suggested that it is rich in reducing sugar, saponin protein, glycosides, alkaloids, flavonoids phenolic compounds, while the UV-vis., IR, NMR and Mass spectroscopic studies of *A. indica* leaves extract showed that it is rich in nimbin, nimbiol, nimban-diol, nimbanene, nimbolide, ascorbic acid, n-hexacosanol and amino acid, 6-desacetylnimbinene, 7-desacetyl-7-benzoylgedunin, 7-desacetyl-7-benzoylazadiradione, 17-hydroxyazadiradione, *etc.*[18–21] These

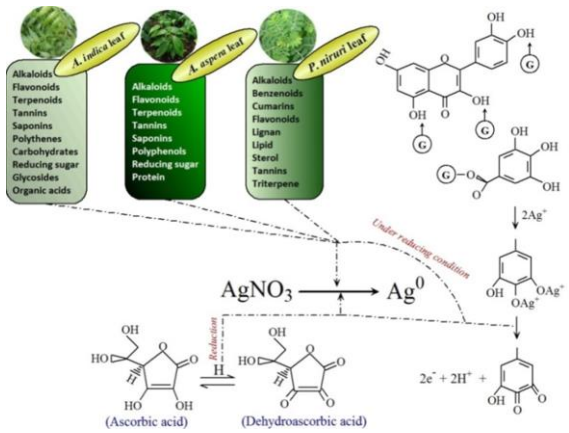

Fig. 4. Probable biosynthetic mechanism for the preparation of SNPs using *P. niruri*, *A. aspera* and *A. indica* leaves.

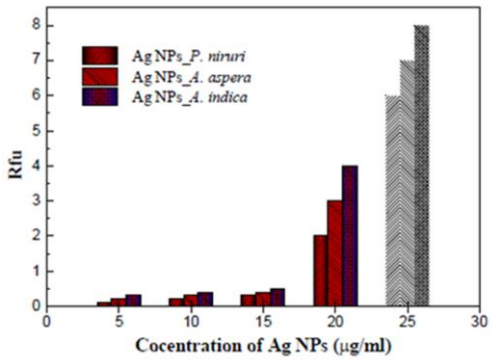

Fig. 5(a). Dose dependent ROS generation in THP-1 cells after treatment with *P. niruri*, *A. aspera* and *A. indica* mediated SNPs (Rfu - relative fluorescence unit).

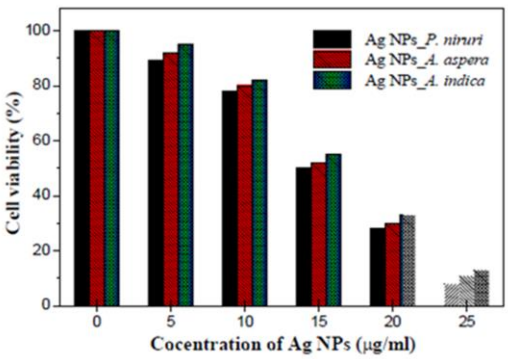

Fig. 5(b). Dose dependent cytotoxicity assay in THP-1 cells after treatment with *P. niruri, A. aspera*, and *A. indica* mediated SNPs.

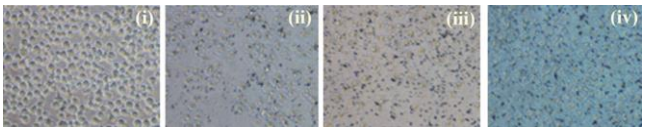

Fig. 5(c). In vitro cytotoxicity effect of SNPs on THP-1 cell lines showing (i) untreated cells with (ii) *P. niruri*, (iii) *A. aspera* and (iv) *A. indica* mediated SNPs at a concentration 25 µg/ml.

phytochemicals have been used as traditional medicine which inhibits proliferation, induce apoptosis and cell death, and reduce cellular oxidative stress. Flavonoids, as one of the most diverse and widespread groups of natural compounds are probably the most important natural phenolic holding prodigal promises. These compounds are found to have a wide range of chemical as well as biological activities along with radical scavenging properties. Besides, phenol is a quite versatile molecule due to the presence of a functional group (-OH) and its basic properties involve hydrophilicity through H-bond, dipole-dipole interaction, hydrogen atom transfer, metal chelation, single electron transfer, π-stacking and van der Waals interactions. Further, the ethanolic extract of these plants contains ascorbic acid which is an oxido-reductive labile molecule. It is known that the ascorbic acid and dehydroascorbic acid are readily inter-convertible requiring and/or releasing an appreciable amount of free energy in an aqueous medium which is sufficient enough to realize the nano-transformation.[22] Therefore, taken together with the gallic and other organic acids, coumarin, tannins, along with other metabolites like flavonoids are oxido-reductively agile and may effectively contribute towards nanotransformation of SNPs.[1,15,23,24] The schematics for the biological synthesis of SNPs using *P. niruri*, *A. aspera*, and *A. indica* are illustrated in Fig. 4.

ROS generation is a marker of oxidative stress and it is considered as an important tool to study cell toxicity. Also, the oxidative damage of cellular components causes cell death. The interaction of SNPs with mammalian cells can promote oxidative stress by inducing the cellular ROS generation which beat the cellular antioxidant capacity. Further, oxidative stress plays a crucial role in a number of normal and abnormal biochemical functions of the cellular system which results in pathological processes. Production of excess ROS is known to stimulate apoptosis in the response of a variety of signals into the different cellular system. ROS have been measured on the basis of intracellular peroxide-dependent oxidation of 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) into 2', 7'-dichlorofluorescein (DCF) compound to form the fluorescent.[25–27] ROS generation levels have been studied and compared with untreated cells. As expected, much higher ROS levels have been observed in human monocytic leukemia cell line i.e. peripheral blood cells of acute monocytic leukemia patient denoted as THP-1 cells treated with *P. niruri*, *A. aspera*, and *A. indica* mediated SNPs compared with untreated THP-1 cells. ROS levels have been found to increases with the increase in dose concentration which authenticated excellently well with dose-dependent cytotoxicity and growth retardation of THP-1 cells. Accordingly, the values of Rfu increases significantly with the increment in dose concentration (Fig. 5a).

The cell viability assay is the most important methods for analysis of cytotoxicity which determine cellular response to different nanoparticles and provide cell information *i.e*. cell survival, death and metabolic activities.[28-29] In this experiment, the THP-1 cells have been treated with various concentrations (0–25 µg/ml) of *P. niruri*, *A. aspera* and *A. indica* mediated SNPs, which able to reduce the viability of human monocytic leukemia cell line *i.e*. THP-1 cells in a dosedependent manner. After 24 h of treatment, SNPs have been found cytotoxic to THP-1 cells at different concentrations and the significant cytotoxic effect (92%, 89% and 87%) was observed at 25 µg/ml concentration of *A. indica*, *A. aspera*, and *P. niruri* mediated SNPs, whereas, at 10.836 ± 0.2265 µg/ml, 11.071 ± 0.2265 µg/ml and 11.289 ± 0.2265 µg/ml 50% death (50% viability) was observed in bar

diagram (Fig. 5b). The half maximal inhibitory concentration (IC50) has been calculated as the concentration of SNPs required to inhibit the growth of cells in culture by 50% compared to the untreated cells. The cellular morphologies of treated cell lines (THP-1 cells) showed cytotoxicity effect which undergo significant cell damage and the cell death was observed more at the concentration of 25 µg/ml of *P. niruri*, *A. aspera*, and *A. indica* mediated SNPs which shown in (Fig. 5c) with compared to untreated cell. Seemingly, the action of SNPs depends on the shape, size, dose, time, type of cells and condition of media. Therefore, the SNPs biosynthesized and stabilized from the metabolite rich sources (*P. niruri*, *A. aspera*, and *A. indica*) are encircled with many active, therapeutically significant phytochemicals, adding to their biocompatibility and nontoxicity.

4. Conclusions

The present experiments were performed with the aim to fabricate biocompatible SNPs from three medicinal plants *P. niruri, A. aspera*, and *A. indica* and to assess their anticancer potential at minimal doses on the human monocytic leukaemia (THP-1) cell line. Among the biosynthesized SNPs especially *A. indica* mediated SNPs owing to their small dimensions, shape and surface groups exhibited cytotoxic potential against THP-1 cells at a minimal dose of 25 µg/ml. The assay was carried out for a short span of ~28 h with a low dose of SNPs. Also, the present biosynthetic method is prudishly green, fast, high yield and cost-effective. It is therefore suggested that functionalized SNPs especially the *A. indica* mediated one could effectively be used as a future therapeutic drug for cancer (human leukaemia THP-1) treatment. The further research is on-going and there are many avenues to be unfolded.

Acknowledgements

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Conflicts of Interest

The authors declare no conflict of interest.

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