

# Characterization and Anti-Cancerous Effect of *Putranjiva roxburghii* Seed Extract Mediated Silver Nanoparticles on Human Colon (HCT-116), Pancreatic (PANC-1) and Breast (MDA-MB 231) Cancer Cell Lines: A Comparative Study

This article was published in the following Dove Press journal:  
International Journal of Nanomedicine

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**Introduction:** A comparative study of *Putranjiva roxburghii* Wall. seed extract and developed silver nanoparticles (PJSNPs) for improving bioavailability that enhance their anti-cancer activity against HCT-116 (colon carcinoma), PANC-1 (pancreatic carcinoma), MDA-MB 231 (breast carcinoma) cell lines was performed.

**Materials and Methods:** The green synthesis of PJSNPs (*Putranjiva* silver nanoparticles) was performed using PJ (*Putranjiva*) extract, and characterization of synthesized nanoparticles was accomplished through UV-Vis spectrum, X-ray diffraction (XRD), transmission electron microscopy, energy-dispersive X-ray spectroscopy (TEM-EDAX), scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), atomic force microscopy (AFM), and Raman spectroscopy.

**Results:** The results revealed that PJSNPs are homogeneous, spherical in shape,  $\sim 8 \pm 2$  nm in size, and negatively charged with a zeta potential of about  $-26.71$  mV. The cytotoxicity pattern observed was  $\text{AgNO}_3 > \text{PJSNPs} > \text{PJ extract}$ . The morphological changes of the cells were observed by flow cytometry and also by the DNA ladder pattern on gel electrophoresis, which indicated that the process of cell death occurred via the apoptosis mechanism and PJSNPs were exerting late-stage apoptosis in all the tested cell lines. The small size and negative value of zeta potential could be the factors responsible for greater bioavailability and thus increased uptake by the tumor cells.

**Conclusion:** The MTT assay and morphological changes observed by various methods indicate that the novel PJSNPs are a better anticancer agent than PJ extract. All the above properties make biologically synthesized PJSNPs an important target in the field of anti-cancer drug discovery.

**Keywords:** green synthesis, *Putranjiva*, silver nanoparticles, anti-cancer, apoptosis

## Background

Nanotechnology has gained tremendous popularity in recent years and has become a vital part of the drug discovery and development particularly, the drug delivery system. Due to their distinctive properties, metal nanoparticles have proved their worth in electronics, photonics, as well as biomedicine.<sup>1-3</sup> Amongst all the metals used, silver is the most desirable one as pure silver has the maximum electrical and

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thermal conductivity and minimum contact resistance.<sup>4</sup> Silver nanoparticles are being used in industries<sup>5</sup> and also have shown to improve antimicrobial and anti-cancer activity besides having a significant role in the drug delivery system.<sup>6,7</sup>

Various chemical<sup>8–10</sup> and physical<sup>11–13</sup> methods have been used for the synthesis of nanoparticles. However, these methods are losing reputation due to low yield, high energy supplies and generation of toxic by-products.<sup>14</sup> The concept of green synthesis has evolved to overcome the above limitations for the synthesis of nanoparticles as it is cost-effective, bio-compatible and eco-friendly in nature. Various green methods such as using the microwave, electrochemical reduction, sonochemical preparation, or supercritical technology<sup>15–17</sup> have been deployed for the synthesis of SNPs. Other than that, the use of micro- or marine organisms and plant extracts for the synthesis of SNPs are well accepted due to their biocompatibility or biomimetic approach.<sup>18,19</sup>

Moreover, the plant extracts not only act as reducing agents but also stabilize the preparation of nanoparticles.<sup>20,21</sup> They are rich in biomolecules that act as bio-reductants for the formation of metallic nanoparticles in the solution resulting in color change of the solution due to the surface plasmon resonance phenomenon.<sup>22</sup>

In the present study, a facile green method for the synthesis of silver nanoparticles (SNPs) using silver nitrate solution and *Putranjiva roxburghii* Wall. extract as the reducing agent has been reported.

*Putranjiva roxburghii* Wall. (Family Putranjivaceae) is an evergreen tree found growing in wild as well as cultivated in the Indian subcontinent. It is regarded as one of the best herbs for rejuvenation and restoration of the female reproductive system in the Indian traditional system of medicine. Apart from having analgesic, antipyretic, and anti-inflammatory activities<sup>23,24</sup> it is also used for treating azoospermia,<sup>25</sup> elephantiasis, eye infection, habitual abortion, sterility, constipation, cough, cold and fever.<sup>26,27</sup> In spite of tremendous ethnobiological importance, its anti-cancer properties have not been fully explored yet and also there is no report on green synthesis of AgNPs (Silver nanoparticles) from *Putranjiva roxburghii* Wall. seed extract till now; hence, in the present article, we have prepared a novel nano-composite named 'Putranjiva roxburghii Wall. seed extract mediated SNPs' (abbreviated as PJSNPs) to target tumor cells for the development of new anti-cancer agents, and characterized them using UV-Vis absorption spectrum, X-ray Diffraction

(XRD), Transmission Electron Microscopy Energy-Dispersive X-ray Spectroscopy (TEM-EDAX), Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), Atomic Force Microscopy (AFM), Zeta potential and Raman spectroscopy. Additionally, the cytotoxic properties of PJSNPs and their anti-cancerous effects were investigated against HCT-116 (colon carcinoma), PANC-1 (pancreatic carcinoma), MDA-MB 231 (breast carcinoma) cell lines.

## Materials and Methods

### Materials

The seeds of *Putranjiva roxburghii* Wall. were a generous gift by Patanjali Ayurveda Ltd., Haridwar, India. The specimen was deposited at PHD, Patanjali Yogpeeth, Haridwar (Voucher no. PRIA/06/05/2017/002). The solvents, chemicals and kits were purchased from Merck, Sigma-Aldrich, and Invitrogen. The cell lines HCT-116 (colon carcinoma), PANC-1 (pancreatic carcinoma), MDA-MB 231 (breast carcinoma) were purchased from NCCS, Pune, India. The microorganism for antimicrobial study were procured from NCL, Pune, India. The blinded blood sample of a healthy volunteer was taken after obtaining his written consent by a registered clinician at Pathology lab, Patanjali Ayurvedic Hospital, Haridwar, India and was generously gifted to us. The study was approved by the Bioethical Committee for Scientific Research at the Patanjali Ayurvedic Hospital, Haridwar, India.

### Preparation of Extract

About 10 g of seeds were milled into a fine powder and boiled for 2 hrs in 100 mL of deionized water. The extract was filtered to remove the particulate matter to get the clear solutions which were then refrigerated (4°C) for further experiments. At each and every step of the experiment, sterility conditions were maintained for the accuracy of the results.

### Synthesis of PJSNPs

During the initial optimization procedure, different concentrations of silver nitrate solutions (1–10 mM) were prepared and treated with different amount of the seed extract (1–5 mL). The mixture was heated at 80°C for 1 hr. The best results were obtained with 1 mM AgNO<sub>3</sub> and 5 mL extract. So we proceeded with the best conditions for further experiments.<sup>28</sup>

On reduction of  $\text{Ag}^+$  to  $\text{Ag}^0$  in  $\text{AgNO}_3$  solution, through the *Putranjiva* seed extract, the color of the solution changes. The formation of SNPs (Silver Nano Particles) was furthermore confirmed by spectrophotometric investigation (Absorbance scan 200–800 nm). The precipitate was collected by filtration, washed with deionized water several times, and finally dried in air at  $60^\circ\text{C}$  for 6 hrs.

## Characterization of Nanoparticles

The prepared silver nanoparticles were analyzed and characterized using UV-Vis spectrophotometer (Shimadzu UV-1800), crystalline metallic silver was examined by X-ray diffractometer (Bruker D8), AFM (Shimadzu SPM9500J2), TEM (TEM-TECNAI-20-G2), FTIR (Cary 630), Raman spectrometer (Nuspec 2.0), Zeta-Sizer (Anton Paar MCP 300), and EDAX (JEOL, JSM-2100F).

## Cell Lines & Culture Conditions

The human cancer cell lines, namely, HCT-116 (colon carcinoma), PANC-1 (pancreatic carcinoma) and MDA-MB 231 (breast carcinoma) were cultured in DMEM medium supplemented with 10% FBS (Invitrogen) and 1% antibiotic (Invitrogen) and grown overnight at  $37^\circ\text{C}$  in a humidified atmosphere with 5%  $\text{CO}_2$ .

## Peripheral Blood Mononuclear Cell (PBMC) Preparation and Cell Culture

The PBMCs were isolated from 5 mL of whole blood consisting of anti-coagulant EDTA (Sigma, MO, USA) from a healthy adult donor on a Ficoll-Hypaque (Hornby, Ontario, Canada) density gradient according to the method described earlier.<sup>29</sup> The cells were cultured within T25 culture flask (Corning Incorporated, NY, USA) for overnight in the supplemented RPMI (Sigma, MO, USA) with fetal bovine serum (FBS), 100 U/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin (Sigma, MO, USA) and 2 mM L-glutamine (Gibco, NY, USA) at  $37^\circ\text{C}$  for 24 h before any treatments. Before performing the experiment, the medium was discarded and the separated cells were washed and counted.

## Cytotoxicity Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a colorimetric assay for determining cell viability. The NAD(P)H oxidoreductase enzymes may possibly reveal the number of viable cells as these enzymes can reduce the tetrazolium dye MTT to

purple insoluble formazan. Cytotoxicity evaluation of  $\text{AgNO}_3$ , PJ & PJSNPs was performed in cancer cells (HCT-116, PANC-1 and MDA-MB 231) using MTT assay as described by Mosmann.<sup>30</sup> The cytotoxic effect of  $\text{AgNO}_3$  & PJSNPs was also evaluated in PBMC cells. The cells were harvested and seeded at a density of  $1 \times 10^5$  cells/mL in a 96-well plate and were incubated for 24 h in an incubator ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ). A series of dilution (10 to 0.0097 mg/mL) of nanoparticles in the medium was added to the cells. After 24 h of incubation, 12 mM MTT stock solution was added to each well and was further incubated at  $37^\circ\text{C}$  for 4 hrs. Formazan crystals formed after 4 hrs in each well were dissolved in 100  $\mu\text{L}$  of SDS-HCl solution and kept for incubation in a humidified chamber at  $37^\circ\text{C}$  for 4 hrs. The plate was read on a multi-mode plate reader (EnVision, Perkin Elmer) at 570 nm.

$$\% \text{ Cell viability} = \frac{A_{570} \text{ of treated cells}}{A_{570} \text{ of control cells}} \times 100$$

## Cell Apoptosis Assay

The number of apoptotic cells induced by PJSNPs with different concentrations was measured by flow cytometry using Annexin V-FITC-PI kit (Sigma-Aldrich, Germany). The Annexin V-PI assay evaluates phosphatidylserine translocation from the inner to the outer layer of the plasma membrane which is an event typically associated with apoptosis. HCT-116, PANC-1 and MDA-MB 231 cells ( $2 \times 10^5$  per well) were seeded into 6-well plates and treated with  $\text{IC}_{50}$  concentrations of PJSNPs. Both control and treated cells were incubated for 24 hrs in an incubator ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ). Following the incubation, the cells were washed twice with PBS. Later, the cells ( $1 \times 10^6$  cells/mL) were resuspended in 1X binding buffer. Subsequently, 5  $\mu\text{L}$  each of AnnexinV-FITC and propidium iodide were added to control and treated cell lines. The tubes were incubated at room temperature for exactly 15 min and protected from light. Finally, the cells were analyzed by flow cytometry (FC500, Beckman-Coulter, Hialeah, FL, USA). The controls used to set up compensation and quadrants were unstained cells, cell stained with FITC and Annexin V and cell stained with PI. Flow cytometric analysis was performed using Partec FloMax software.

## DNA Fragmentation Assay

The suspension of HCT-116, MDA-MB 231, PANC-1 ( $10^6$  cells/mL) were seeded in 6-well microplates and

treated with  $IC_{50}$  concentration of PJSNPs. The DNA extraction was done using a salting-out method. Extracted DNA was run on a 1% agarose gel for 20 min by applying 100 V, which was then stained with ethidium bromide, and the bands were detected using an ultraviolet transilluminator.

## Statistical Analysis

The results are presented as mean  $\pm$  standard deviation of three experiments. Analysis of the dose–response curve was done using the Software GraphPad Prism 7.  $IC_{50}$  values were determined by plotting triplicate data points over a concentration range and calculating values using regression analysis of Prism software using a 95% confidence level.

## Results and Discussion

### Characterization of Silver Nanoparticles

#### UV-Vis Spectra Analysis

The synthesis of the SNPs in aqueous solution was monitored by recording the absorption spectra at a wavelength range of 200–800 nm (Figure 1A). As the plant extract was mixed with  $AgNO_3$ , the color of the reaction mixture changed from brown to reddish brown (Figure 1A inset) due to excitation of surface plasmon resonance (SPR) vibration of silver nanoparticles,<sup>22</sup> while no color change was observed in the absence of plant extract in  $AgNO_3$ . The complete color change took after about 30 mins; thereafter, no further color of the reaction mixture changed, indicating that the silver salt present in the reaction mixture has been reduced completely. Nanoparticles absorb light at a different wavelength and get excited due to charge density at the interface between conductor and insulator, the mechanism known as surface plasmon resonance. The absorbance peak between 400 and 450 nm by UV-Vis analysis is the characteristic of SNPs<sup>31</sup> and the results obtained are in complete correlation with the earlier studies.<sup>21,32</sup> The change in color and  $\lambda_{max}$  with a prominent peak around 420 nm correspond to SNPs formation. SPR peak located between 410 and 450 nm has been observed for SNPs and might be attributed to spherical nanoparticles.<sup>31,33,34</sup>

#### Raman Spectroscopic Analysis

Raman spectroscopy (Deitanu Rock Hound Nuspec 2.0) can be used to study chemical identification, characterization of molecular structures, effects of bonding, environment and the stress of the compounds. The *Putranjiva roxburghii* Wall. capped silver nanoparticles are fine black powder,

highly photosensitive towards a wide range of light wavelength similar to the majority of silver compounds.

The most representative  $Ag_2O$  spectrum shows a very intense band with a peak at  $146\text{ cm}^{-1}$  attributing to Ag lattice vibrational modes, ie, phonons (Figure 1B). The range  $200\text{--}580\text{ cm}^{-1}$  is characterized by a broadband in which it is possible to define Raman shifts at 288 and  $537\text{ cm}^{-1}$ . The bands at  $537\text{ cm}^{-1}$  can be ascribed to the n(Ag–O) vibrations for sub-surface species and n(O–O) mode for adsorbed molecular oxygen. The peak observed at  $1030\text{ cm}^{-1}$  is probably due to chemisorbed atomic/molecular oxygen species.<sup>35</sup> Although sub-surface species might promote adsorption of molecular oxygen, it could also be a probable consequence of surface restructuring in silver nanoparticles.<sup>36</sup>

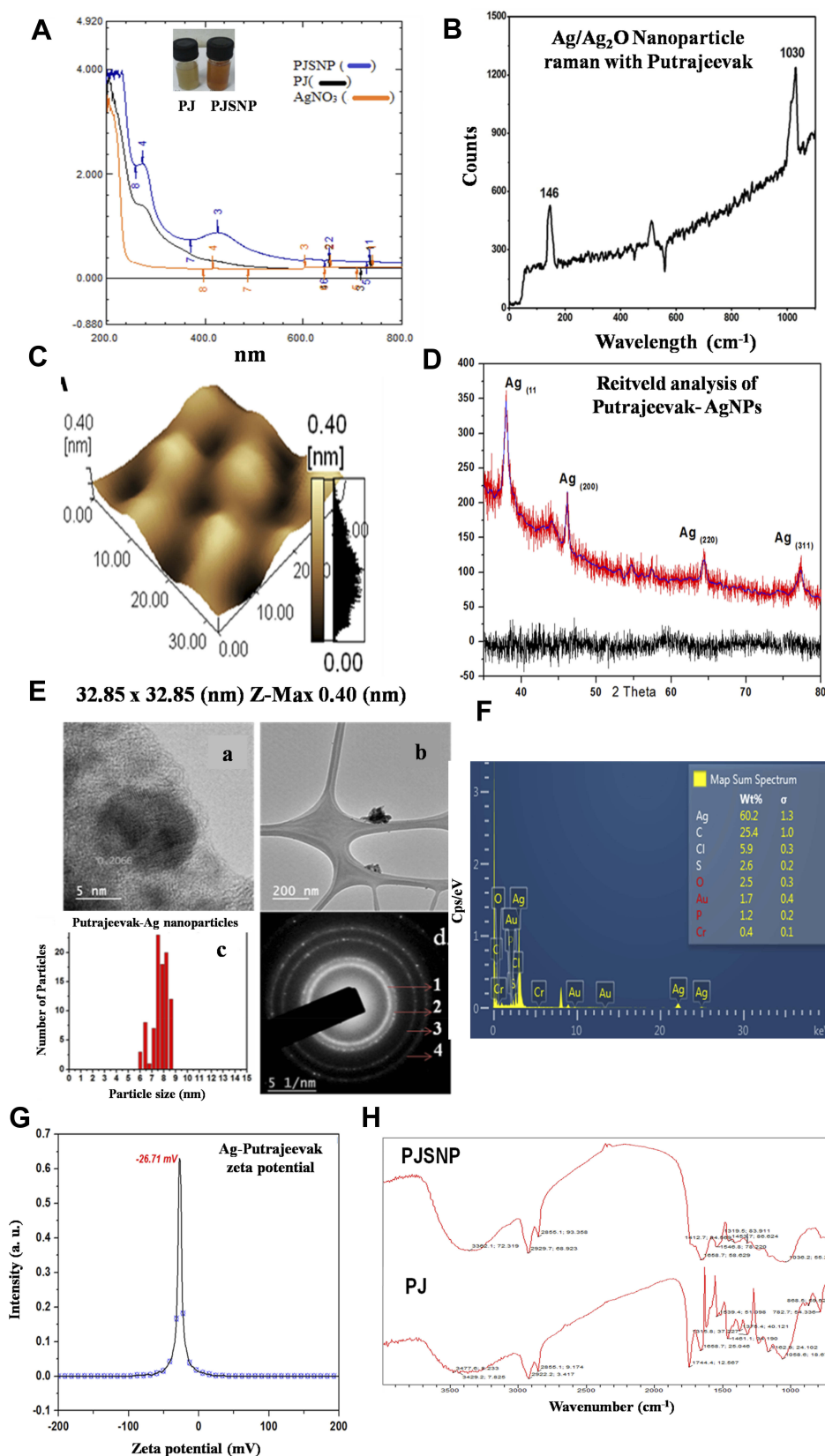
#### Atomic Force Microscopy

The surface topology of the PJSNPs was studied by atomic force microscopy analysis (Figure 1C). This method is used as a primary method to monitor SNPs dissolution and agglomeration pattern. The biologically synthesized silver nanoparticle size was measured using line profile determination of individual particles in the range of  $32.85 \times 32.85\text{ nm}$ .<sup>37</sup> The silver nanoparticles were imaged by AFM to understand the accurate configuration and to confirm that the silver nanoparticles were more or less homogeneous in size and shape. The topography of AFM micrograph clearly indicates that PJSNPs possess spherical shape with  $\sim 8 \pm 2\text{ nm}$  size measured using line profile determination of individual spherical-shaped particles.

#### X-Ray Diffraction Analysis

The XRD spectrum was recorded to confirm the crystalline structure of synthesized AgNP. The crystallographic structure of biologically synthesized silver nanoparticles (Table 1; Figure 1D) depicted four distinct peaks at  $37.96^\circ$ ,  $45.95^\circ$ ,  $64.36^\circ$  and  $77.10^\circ$  which were the corresponding values of (1 1 1), (2 0 0), (2 2 0) and (311) lattice planes of the face-centered cubic (FCC) silver, closely matching with the reported reference values.<sup>38</sup> All diffraction peaks could be well indexed with the cubic structure of silver (Ag) [JCPDS No. 087–0720]. The absence of any other chemical phase indicated the purity and crystallinity of the synthesized PJSNPs. A systematic increase in the broadening of the diffraction peaks with increasing  $2\theta$  indicated a concomitant reduction in particle size. The obtained diffraction spectrum strongly suggested the presence of silver nanoparticles in accordance with AFM and Raman spectrum analysis. The  $2\theta$  positions of lattice planes were





**Figure 1** Characterization of silver nanoparticles synthesized using *P. roxburghii* seed extract. (A) UV-Vis spectra of PJ and PJSNPs (B) Raman spectra of PJSNPs (C) Atomic force microscopic image of PJSNPs (D) Calculated (Blue line) Rietveld refinement and Observed (Red line) plot of the powder XRD patterns for the Putrajeevak-Ag NPs (PJSNPs). The difference plot is at the bottom of the figure (blue line) and tick marks represent allowed reflections for each sample (E) Transmission electron microscopy of PJSNPs. HTEM micrograph (a and b) particle size distribution histogram (c) selected area diffraction pattern (d) (F) EDAX of PJSNPs (G) Zeta potential of PJSNPs (H) Fourier transformation infrared spectra PJSNPs and PJ extract.

**Table I** A Summary of Observed d-Spacings, hkl and Lattice Constant for the Biologically Synthesized PJSNPs

Sample Name	2θ (Degree)	FWHM (Degree)	Lattice Constant (Å)	(hkl)	Standard d-Value	Observed d-Value
PJSNP	37.96	0.63	4.092	(111)	2.36	2.36
	45.95	1.16		(200)	2.04	2.01
	64.36	0.77		(220)	1.44	1.44
	77.10	0.67		(311)	1.23	1.23

slightly shifted towards the lower angle because of strain generation in silver nano-crystals due to the presence of *Putranjiva roxburghii* Wall. The resulting PJSNPs are highly uniform in phase & composition. The crystalline phase purity was confirmed by Rietveld refinement of XRD and the uniformity in composition was deduced from the Vegard's law because the occupancy refinement of PJSNPs cannot give a satisfactory measurement of the stoichiometry. The obtained results were consistent with the sizes of SNPs obtained from TEM analysis.

Apart from the characteristic peaks FCC Ag, the diffractogram also showed small hump-like peaks at 44.05° and 54.61° which may be due to the crystallization of the bio-organic phase on the surface of the SNPs. In the Rietveld analysis, the d-value at 44.05° and 54.61° were also under consideration limit but not matched with the SNPs lattice plane. Some other studies reported similar results with various additional peaks<sup>39,40</sup> and bio-organic compound/protein (s) might be responsible for such pattern. The authors have suggested that magnesium ions present in the chlorophyll might act as strong X-ray scattering centers in the bio-organic crystalline phase.<sup>41</sup>

### Transmission Electron Microscopy and EDAX

The Transmission electron microscopy (TEM) provided further insight into the morphology and size details of the synthesized SNPs. Transmission electron micrograph reveals that PJSNPs are spherical, as can be observed in Figure 1E, HTEM micrograph (Figure 1E-a and b) and particle size distribution histogram (Figure 1E-c) correspond to silver nanoparticles formed after 24 h of reaction. PJSNPs sizes are  $\sim 8 \pm 2$  nm. A typical selected area diffraction pattern is shown in the inset of (Figure 1E-d). Main Diffraction rings can be indexed as (111), (200), (220) and (311) reflections (indicated by numbers 1, 2, 3 and 4, respectively), corresponding to a face-centered cubic (FCC) crystalline structure of PJSNPs. In Figure (1E-a), few particles appeared with contrast difference, while the inorganic core Ag nanoparticles can be seen as darker contrast and polymer shell can be seen as lighter contrast.<sup>42</sup> The results are being in good

agreement with XRD data. The SAED pattern of nanoparticles sample has also been shown in Figure (1E-d), which revealed a characteristic circular diffraction pattern corresponding to (111), (200), (220) and (311) planes of the face-centered cubic silver nanoparticles.

EDAX analysis shows the presence of eight elements (Ag, C, Cl, S, O, Au, P and Cr) in the PJSNPs powder (Figure 1F), where the silver (60.2 weight%) is present as major element followed by carbon (25.4 weight%), chlorine (5.9 weight%), sulfur (2.6 weight%), oxygen (2.5 weight%) and gold (1.7 weight%). However, some other unidentified peaks were also seen (contributing 1.7 weight%). Thus, this study indicates that pure crystalline nature is solely composed of silver. Similar studies exhibiting weak signals from Cl in synthesized SNPs colloid have also been reported which may be due to the presence of impurities in bacterial supernatant.<sup>43</sup>

As TEM coupled Oxford EDAX, microprobe analysis is a quantitative analysis of several spots was carried out, and finally, an average value of elements is considered. TEM and EDAX studies revealed the spherical nature of particles synthesized from silver metal.

### Zeta Potential

Zeta potential experiments were carried out to investigate the electrostatic stability of the synthesized PJSNPs. The magnitude of the zeta potential is a measure of electrostatic repulsion between adjoining and similarly charged particles in a dispersion. Molecules or colloids with high zeta potential values are electrostatically stable in comparison to colloids with low zeta potential as in the later case attractive forces are greater than the repulsive forces resulting in coagulation.<sup>44</sup> In the present study, PJSNPs owned a zeta potential of  $-26.71$  mV (Figure 1G) which suggests that the surface of the nanoparticles is negatively charged and dispersed in the medium. The negative value confirms the repulsion between the particles and proves that they are very stable. Also, the negatively charged particles are less cytotoxic compared to positively charged and the later ones are rapidly cleared from the bloodstream.<sup>45</sup>

## Fourier Transform Infrared Spectroscopy

The FTIR measurement was studied to identify the possible biomolecules responsible as capping and reducing agent for the SNPs synthesized by the extract (Figure 1H). The intense broadbands at 3600 and 3200  $\text{cm}^{-1}$  are probably due to the O–H and C–H stretching modes, respectively. The PJSNPs did not show any band at 1744  $\text{cm}^{-1}$ , whereas the prominent band of the same frequency was observed in the PJ extract, indicating that the SNPs are stabilized through the C=O bond. Carbonyl groups of the amino acid residues and peptides of proteins have a strong affinity for metal binding, signifying the importance of protein as an encapsulating agent.<sup>46</sup> Similarly, the absence of a peak at 1461  $\text{cm}^{-1}$  (C=C group) in PJSNPs is probably due to the reduction of  $\text{AgNO}_3$  to Ag.<sup>47</sup> Comparison of the FTIR spectra of PJSNPs with PJ extract discloses the occurrence of functional groups such as amines, carboxylic acids, aldehydes and alcohols which are most likely to be accountable for reducing and capping the silver ions.<sup>48</sup>

## Anticancer Potential of PJ Extract and PJSNPs

### Brine Shrimp Lethality Assay

It is an important tool for the preliminary cytotoxicity screening of plant extracts based on the ability to kill laboratory-cultured brine shrimp. Criteria of toxicity followed were  $\text{LC}_{50}$  values >1000  $\mu\text{g/mL}$  (non-toxic), 500 to 1000  $\mu\text{g/mL}$  (weak toxicity) and <500  $\mu\text{g/mL}$  (toxic).<sup>49,50</sup>

In the present study, four different concentrations (2, 20, 200 and 2000  $\mu\text{g/mL}$ ) of PJ extract, as well as PJSNPs, were used to access their cytotoxicity using the brine shrimp lethality assay.  $\text{LC}_{50}$  values for PJ (2000  $\mu\text{g/mL}$ ) were three times higher than the PJSNPs (632.45  $\mu\text{g/mL}$ ). As already reported  $\text{LC}_{50}$  values > 1000  $\mu\text{g/mL}$  are non-toxic; consequently, PJ is non-cytotoxic in nature; however, PJSNPs exhibited weak toxicity in the brine shrimp lethality assay. The toxicity of silver nanoparticles against brine shrimp has also been reported in the literature.<sup>51,52</sup> Collectively, the cytotoxic effects of PJSNPs to brine shrimp can be associated with anti-cancer properties and could be developed further as an unconventional source of anti-cancer drugs. Therefore, we further studied the cytotoxic effect of the PJ extract and PJSNPs in the different cell lines using the MTT assay.

### MTT Assay

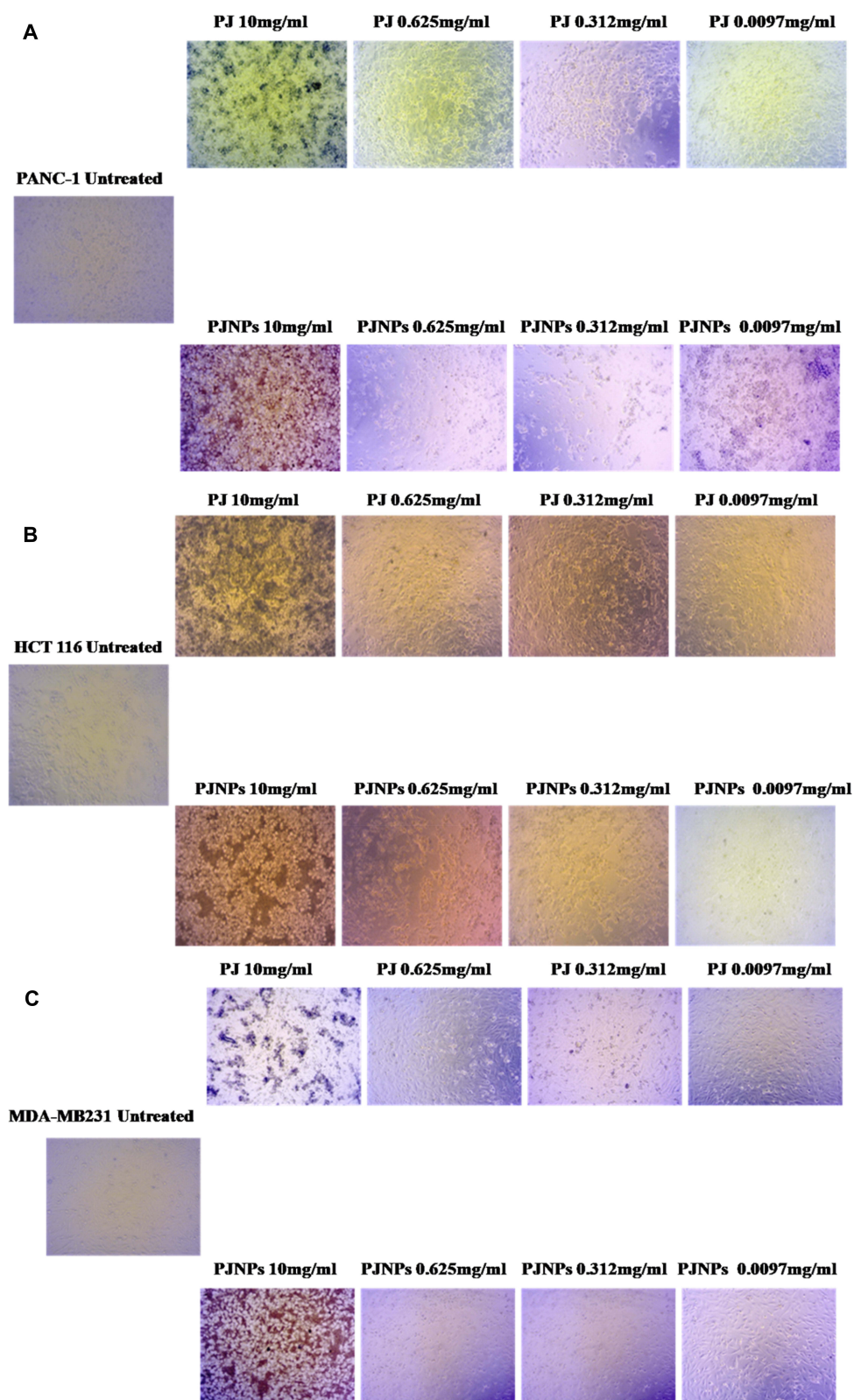
The MTT reduction assay is used for the determination of the cytotoxic effect of a substance in the liver cells. The  $\text{AgNO}_3$ ,

**Table 2**  $\text{IC}_{50}$  Values of PJ, PJSNPs and  $\text{AgNO}_3$  Against PANC-1, MDA-MB 231 and HCT-116

Compound	Cell Line	Cell Type	$\text{IC}_{50}$ (mg/mL)
PJ	PANC-1	Pancreatic cancer cell line	8.6
PJ	MDA-MB 231	Breast cancer cell line	7.7
PJ	HCT-116	Colon cancer cell line	6.0
PJSNP	PANC-1	Pancreatic cancer cell line	0.36
PJSNP	MDA-MB 231	Breast cancer cell line	0.26
PJSNP	HCT-116	Colon cancer cell line	0.54
$\text{AgNO}_3$	PANC-1	Pancreatic cancer cell line	0.00025
$\text{AgNO}_3$	MDA-MB 231	Breast cancer cell line	0.00029
$\text{AgNO}_3$	HCT-116	Colon cancer cell line	0.00025

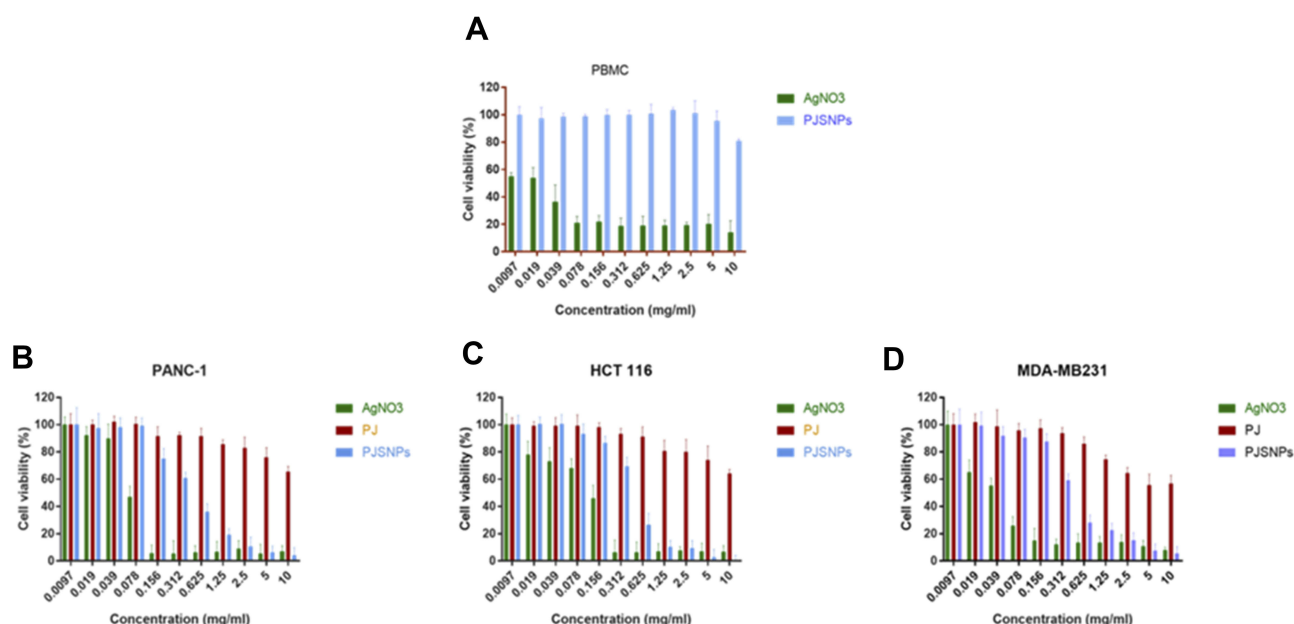
PJ extract and PJSNPs were tested against PANC-1, MDA-MB 231 and HCT-116 cell lines. The results (Table 2; Figure 2) show the cytotoxicity at different concentrations (10 to 0.0097 mg/mL) in a dose-dependent manner which is supported by a previous study in which silver nanoparticles have affected the cell viability in a dose-dependent manner in a variety of mammalian cell lines.<sup>53</sup>

As compared to control, the percentage of cell growth inhibition was found to be very high with different concentrations of  $\text{AgNO}_3$  followed by PJSNPs and PJ. The cytotoxic effect of  $\text{AgNO}_3$  and PJSNPs were also tested in isolated PBMC cells where  $\text{AgNO}_3$  caused cytotoxicity (55% cell viability) even at the lowest concentration (0.0097 mg/mL) whereas PJSNPs showed no toxic effect of the same concentration (Figure 3). The  $\text{IC}_{50}$  values of PJ extract (8.6, 6.0 and 7.7 mg/mL) were higher compared to PJSNPs (0.36, 0.54 and 0.26 mg/mL) and  $\text{AgNO}_3$  (0.00025, 0.0025 and 0.0029 mg/mL) against PANC-1, HCT-116 and MDA-MB 231 cell lines after 24 hrs of incubation (Table 2). From the results, the cytotoxicity of  $\text{AgNO}_3$  > PJSNPs > PJ which signifies the importance of nano-ization of  $\text{AgNO}_3$  to PJSNP as the former is showing toxicity in normal cells also at the lowest tested concentration. The reason for higher toxicity of PJSNPs may be due to the stimulation of reactive oxygen species (ROS) activity leading to various cellular events like cytotoxicity, unregulated cell signaling, DNA damage, apoptosis, and cancer.<sup>54,55</sup> In the previous studies, silver nanoparticles are reported to interfere with the electron-transport chain by activation of NADPH-related enzymes and depolarizing the mitochondrial membrane resulting in increased cellular levels of ROS.<sup>56–58</sup> In another study when human glioblastoma and fibroblast cells were treated with silver



**Figure 2** Effects of PJ extract and PJSNPs on cell viability against human cell lines based on MTT assay after 24 h of incubation. Cell lines treated with various concentrations of PJ and PJSNPs ranging from 0.0097 mg/mL to 10 mg/mL. Phase-contrast microscopy showing cytotoxic effect on (A) Pancreatic Carcinoma PANC-1; (B) Colon carcinoma HCT-116 and (C) Breast cancer MDA-MB 231 cells.





**Figure 3** Comparative cytotoxicity of AgNO<sub>3</sub>/PJSNPs on (A) PBMC (Peripheral Blood Mononuclear Cell as normal cell line); and AgNO<sub>3</sub>/PJ/PJSNPs on (B) Pancreatic Carcinoma PANC-1; (C) Colon carcinoma HCT-116 and (D) Breast cancer MDA-MB 231 cell lines.

nanoparticles, high levels of ROS-mediated cytotoxicity were observed due to disruption of the electron-transfer chain.<sup>59</sup> However, in one of the studies, researchers reported a different mechanism which involves deactivation of enzymes by the formation of stable S-Ag bond with the thiol group of enzymes in the cell membrane; or denaturation of the DNA by breaking hydrogen bonds between nitrogen.<sup>22</sup> Some of the studies claim that the smaller the size of silver nanoparticles, the stronger is the cytotoxicity,<sup>60,61</sup> because the size has an effect on its uptake by the cells. We have also observed similar results in the present study that the small size ( $\sim 8 \pm 2$  nm) of PJSNPs may be the reason for better cellular activity of PJSNPs compared to PJ extract.

The cytotoxicity displayed by PJSNPs at lower concentrations might be linked to the phytoconstituents present in the plant extracts which are involved in AgNP formation.<sup>62</sup> Moreover, the cytotoxic effects of biosynthesized AgNPs against breast cancer MCF-7 cell line,<sup>63</sup> Hep-2 cancer cell line<sup>64</sup> and HeLa cell lines<sup>65</sup> also support our outcomes.

### Apoptosis Induction

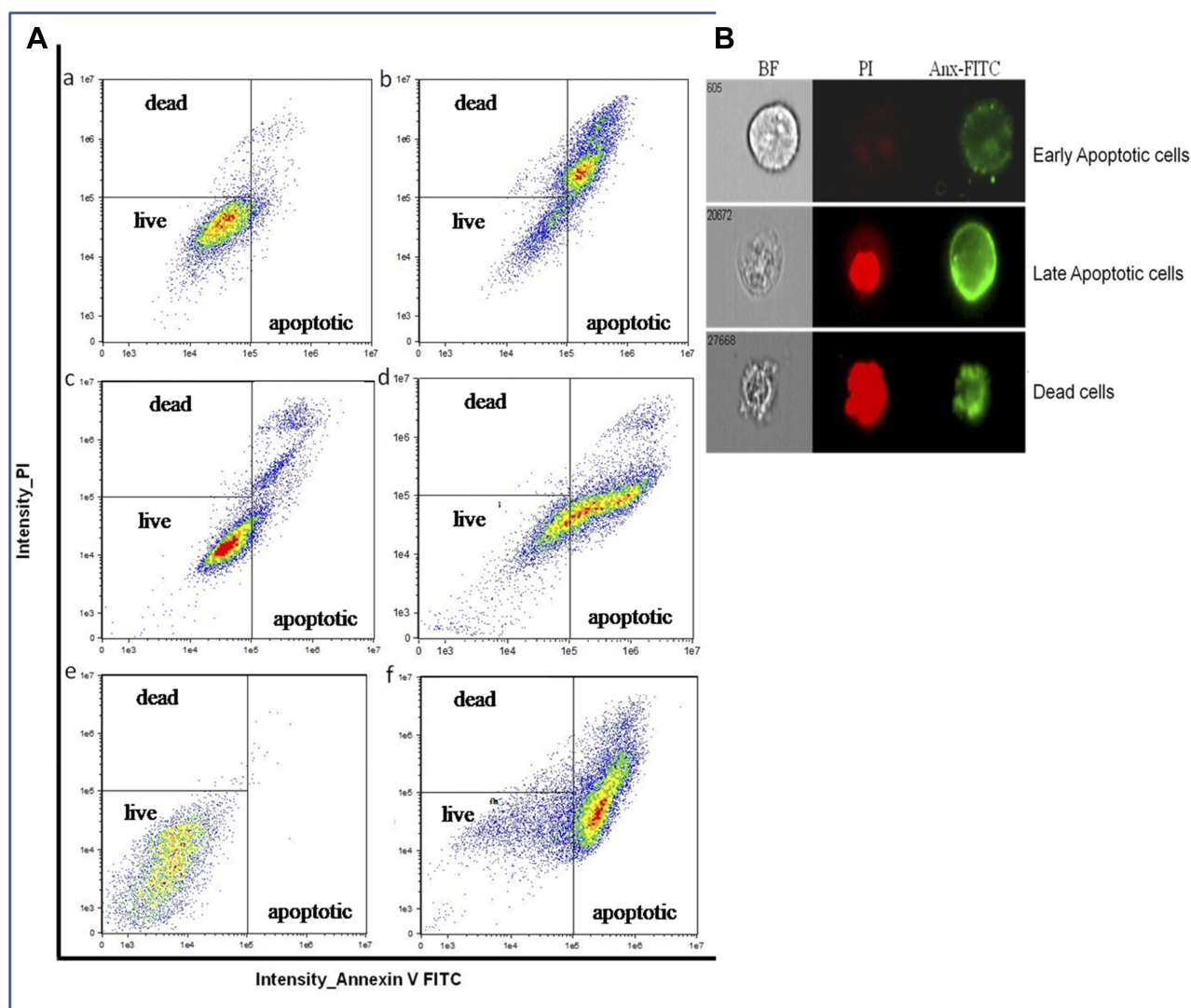
To find out if the mechanism of cell death is by apoptosis, the cancer cell lines were treated at IC<sub>50</sub> concentration of PJSNPs. The results were evaluated by Annexin V-FITC/PI assay and DNA fragmentation assay.

(i) Annexin V-FITC/PI assay. Cell death induced by PJSNPs was investigated for apoptotic activity by monitoring Phosphatidylserine (PS) translocation using the Annexin V-FITC/PI assay. Early apoptosis is characterized by the translocation of PS from the inner layer of the plasma membrane to the outer surface.<sup>66</sup> Apoptotic cells are reflected by the quantification of Annexin V-FITC binding to externalized PS. In flow cytometer analysis, Annexin V/Propidium iodide (AnnV/PI) staining is based on the ability of the protein Annexin V to bind to Phosphatidylserine (PS), which is externalized in the outer cell membrane leaflet upon induction of apoptosis. In viable cells, PS is located in the inner-membrane leaflet, but upon induction of apoptosis, it is translocated to the outer-membrane leaflet and becomes available for Annexin V binding. The addition of PI enabled viable (AnnV<sup>-</sup>/PI<sup>-</sup>), early apoptotic (AnnV<sup>+</sup>/PI<sup>-</sup>), late apoptotic (AnnV<sup>+</sup>/PI<sup>+</sup>), and necrotic (AnnV<sup>-</sup>/PI<sup>+</sup>) cells to be distinguished.<sup>67</sup> The flow cytometry analysis of HCT-116, MDA-MB 231 and PANC-1 and cells showed that the cell population tended to shift from viable to apoptotic, on treatment with PJSNPs. After treatment with IC<sub>50</sub> concentration of PJSNPs, the apoptotic cells were found higher in HCT-116 (71.5%), MDA-MB 231 (69.0%) and PANC-1 (76.8%) cells as compared to the respective control.

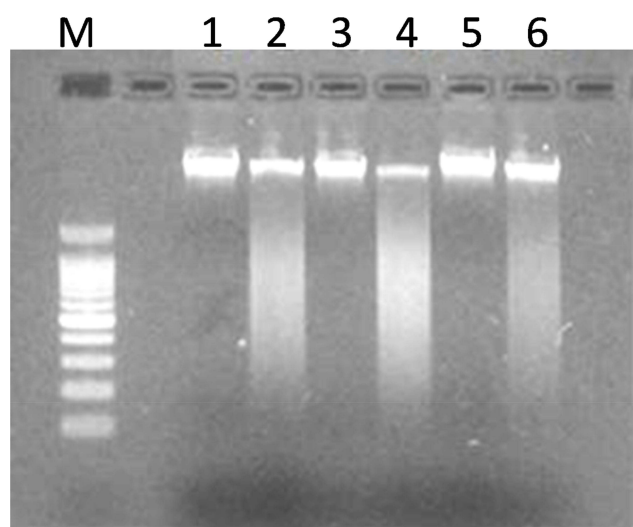
In contrast, the more number of live cells was found in untreated HCT-116 (87.2%), MDA-MB 231 (73.3%) and PANC-1 (99.1%) cells (Figure 4). In addition, the percent of dead (necrotic) cells observed in treated cells were 8.38, 1.18 and 3.78 in HCT-116, MDA-MB 231 and PANC-1, respectively (Figure 4). These results demonstrate the ability of PJSNPs to exert apoptosis in all the tested cancer cell lines.

- (ii) DNA fragmentation assay. The DNA of HCT-116, PANC-1 and MDA-MB 231 cells treated with PJSNPs at  $IC_{50}$  concentration were extracted and loaded on the agarose gel. The results of DNA “laddering” pattern extracted from cells treated

with PJSNPs are shown in Figure 5, (Supplementary Figure 1). The fragmentation pattern we observed was quite similar to that reported for cancer cell lines treated with silver nanoparticle.<sup>68</sup> It appears that during DNA fragmentation the silver particles accumulated inside the nucleus may possibly influence the DNA and cell division<sup>69</sup> by stimulating dose-dependent DNA damage, chromosomal aberrations, errors in chromosome segregation, sister chromatic exchanges and formation of micronuclei.<sup>59,70</sup> Consequently, it can be speculated that the similar pathways are pursued by PJSNPs to induce DNA fragmentation.



**Figure 4** The PJSNPs induced apoptosis in three human cancer cell lines (HCT-116, MDA-MB 231 and PANC-1) after 24 h of incubation. Flow cytometer was used to collect 8000 cell count. Viable cells do not take any color (Annexin V<sup>-</sup>/PI<sup>-</sup>), early apoptotic cells (Annexin V<sup>+</sup>/PI<sup>-</sup>) are green, late apoptotic cells (Annexin V<sup>+</sup>/PI<sup>+</sup>) are green and red, and necrotic cells (Annexin V<sup>-</sup>/PI<sup>+</sup>) are red. (A) In the figure (a-b) represent HCT-116, (c-d) represent MDA-MB 231 cells and, (e-f) represent PANC-1 cells. (B) Figures represent fluorescence images obtained from the flow cytometer.



**Figure 5** DNA ladder on agarose gel electrophoresis. Lane M: 100 bp ladder, L1: MDA-MB 231 control, L2: MDA-MB 231 treated, L3: HCT-116 control, L4: HCT-116 treated, L5: PANC-1 control, L6: PANC-1 treated.

## Conclusion

*Putranjiva roxburghii* Wall. mediated silver nanoparticles (PJSNPs) were synthesized and characterized using various techniques. The silver nanoparticles were  $\sim 8 \pm 2$  nm spherical shaped, face-centered cubic having a negative charge. The small size and negative zeta potential could be the reasons that they are specifically taken up by the tumor cells. The biological studies indicated that aqueous extract of *Putranjiva roxburghii* Wall. seed was non-cytotoxic while the silver nanoparticles displayed weak cytotoxicity in the brine shrimp lethality assay. In MTT assay, PJSNPs displayed better cytotoxicity than PJ extract. However,  $\text{AgNO}_3$  is toxic to normal cell also at the lowest tested concentration whereas PJSNPs showed no toxic effect at the same concentration. This reflects the importance of nanonization of  $\text{AgNO}_3$  to PJSNP as the former is displaying toxic effect in normal cells also. In addition, the flow cytometric studies confirmed that the PJSNPs induced cell death via the apoptosis mechanism.

Thus, our findings propose the anticancer prospective of biosynthesized PJSNPs against human cancer cells and might play a significant role in the development of new and effective therapeutic agent for cancer treatment.

## Acknowledgments

We are thankful to Patanjali Yogpeeth, Haridwar (India) for providing *Putranjiva roxburghii* Wall. seeds and to the Patanjali Ayurvedic Hospital, Haridwar (India) for providing blinded whole blood samples from healthy donors for

the study. We also gratefully acknowledge UGC-DAE Consortium for Scientific Research, Indore (India) for providing the facilities for AFM, XRD and Raman, and the Indian Institute of Petroleum, Dehradun (India) for EDAX facility. The authors sincerely acknowledge Dr. L. N. Misra, Chief Research Advisor, Patanjali Research Foundation, Haridwar (India) for his suggestions and expert guidance in improving the quality of the paper.

## Disclosure

The authors declare that they have no competing interests.

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