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Cytotoxicity Analysis from *Punica granatum* Peel Silver Nanoparticles (PGP-AgNPs) Treated HGT-1 Cells

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Abstract: Metal nanoparticles have numerous applications in various fields of science and technology such as catalysis, optoelectronics and biomedicine. In the present study we synthesized and characterized silver nanoparticles (AgNPs) from *Punica granatum* peel (PGP) extract and evaluated its cytotoxic activities. The formation of nanoparticles was confirmed by visual assessment. The PGP-AgNPs was characterized by UV spectroscopy, SEM, FTIR and XRD. UV–Visible spectroscopy are important techniques to determine the formation and stability of silver nanoparticles in an aqueous solution. XRD peaks indicated that the PGP-AgNPs are crystalline in nature. FTIR spectrum of synthesized PGP-AgNPs showed the six absorption bands which indicated the presence of active functional groups in the region of 600-3500 cm^{-1}. The reduction of cell viability observed in this study is suggestive of anticancer effects of AgNPs synthesised from *Punica granatum* peel extract. Results showed reduced cell viability of cancer cell lines with increasing concentrations (10–200 µg/ml) of AgNPs. In this study, silver nanoparticle showed the minimum cell viability (32.59%) and maximum inhibitions (67.41%) in 200 µg/ml concentrations against HGT-1 cell line. The present study concluded that the PGP-AgNPs exhibited strong inhibitory activities against the HGT-1 cell line.

Keywords: *Punica granatum*, XRD, PGP-AgNPs, HGT-1, Cell viability, Nanoparticles

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Introduction

Nanotechnology provides a good platform to modify and develop the important properties of metal in the form of nanoparticles having promising applications in diagnostics, biomarkers, cell labeling, contrast agents for biological imaging, antimicrobial agents, drug delivery systems and Nano drugs for treatment of various diseases. Large number of physical, chemical, biological, and hybrid methods are available to synthesize different types of nanoparticles. Although physical and chemical methods are more popular in the synthesis of nanoparticles, the use

of toxic chemicals greatly limits their biomedical applications, in particular in clinical fields. Hence, development of reliable, nontoxic, and eco-friendly methods for synthesis of nanoparticles is of utmost importance to expand their biomedical applications (Colvin *et al.,* 1994).

The application of nanotechnology in cancer, which includes liposomes, nanoparticles, polymeric micelles, dendrimers, nanocantilever, carbon nanotubes and quantum dots have significantly changed the cancer therapy. The nanoparticles interacted with receptors and expressed on the surface of cancer cells. Thus, nanotechnology gave the positive result in the field of cancer treatment. Application of nanotechnology decreases the side effects due to normal cell damage, which produced better results and leads to lengthening of patient survival (Atul *et al.*, 2010).

Nowadays, silver nanoparticles (AgNPs) have a wide spread of applications and the highest level of commercialization among the nanomaterials. For instance, among different nanoparticles investigated, AgNPs are the most promising particles that are used in the field of nanomedicine for their antimicrobial activity against different microbes. However, there is not sufficient information concerning the biological effects of AgNPs on human cells. In addition, there are limited studies on the potential of biogenic AgNPs as anticancer agents. Previous studies reported that AgNPs induced genotoxicity and cytotoxicity in both cancer and normal cell lines, altered cell morphology, reduced cell viability, caused oxidative stress in lung fibroblast and glioblastoma cells, human and rat liver cells HeLa cells, and THP- 1 monocytes (Lee *et al.*, 2008). The importance of nanoparticles belongs to their goals, cancer cell Nano science and virus HIV (Elechiguerra *et al*., 2005), thus; used in producing drugs in Nano medicine. Recently silver nanoparticles used in anticancer therapy for several types of cancer are Hep2 cell line (Rosarin *et al*., 2013), HT-29 cell lines (Devi *et al.*, 2012), Vero cell line (Renugadevi *et al*., 2012) and breast cancer line MCF-7 (Thangaraju *et al.*, 2012).

Various extracts of plant parts which are rich in secondary plant metabolites have been used for biosynthesis of AgNPs. *Punica granatum* Linn is a plant of Punicaceae family; the tree may grow up to five meters in height. It has glossy, leathery leaves and bears red flowers at the branch tips (Khalil, 2004). The pomegranate is fruit of this plant, locally known as romane variety safferi. The tree is native in Asian countries; it has been cultivated and naturalized over the whole Mediterranean region since ancient times (Ahangari and Sargolzaei, 2012). Pomegranates have prominent medical history, and possess remarkable medicinal properties (Longtin , 2003) such as anti-inflammation, anti-diabetes, anti-diarrhea, treat dental plaque and aphtae and to combat intestinal infections and malarial parasites. Few studies also revealed the efficacy of the pomegranate fruit against cancer, athero-sclerosis, infectious and coronary heart diseases (Lansky and Newman, 2007; Fischer *et al*., 2011). The pomegranate peels represent 50% of total weight of fruit and are an important source of bioactive compounds such as phenolics, flavonoids, ellagitannins, and proanthocyanidin compounds (Li *et al.*, 2006), minerals, mainly potassium, nitrogen, phosphorus, magnesium, sodium, and calcium, and complex polysaccha-rides. Pomegranate peel extracts have been used for the synthesis of AgNPs. The current study was designed to synthesize and characterize AgNPs from *Punica granatum* peel extract and to evaluate its biological activities.

Materials and Methods

Sample preparation:

Fresh fruits of *Punica granatum* were collected from a local market Thanjavur, Tamil Nadu, India. The fruit was thoroughly washed using distilled water and the seeds were subsequently separated to obtain the peel. The isolated peels were dried under shade at the room temperature for 3-5 days. The dried peels were ground into uniform coarse powder using a blender. Approximately 200 g of

Punica granatum peel powder was extracted with ethanol (4–5 h, 90 °C) using a Soxhlet apparatus which yielded a bright red colour ethanolic *Punica granatum* peel extract (PPE). The extract was concentrated under reduced pressure (45–50 °C) using a rotary evaporator to obtain the viscous mass (42 g; 21.0% w/w). The PPE was stored at 4 °C in the refrigerator for further use in the experiment.

Green synthesis of Silver oxide nanomaterial:

Synthesis of Silver nanoparticles wascarried out using PPE. 0.1 M $AgNO_3$ was prepared in double distilled water. $AgNO_3$ and PPE were mixed together in ratios of 5:5, 6:4, 7:3, 8:2 and 9:1. In this different ratio concentration 5:5 ratio concentration were selected for the bulk preparation because it showed the higher production than other ratios. The reaction mixture was heated below the boiling point and continuously stirred at 800 rpm using magnetic stirrer. The mixture turned into Grey color within 1 h. The obtained suspension was centrifuged at 15,000 rpm for 15 min. The pellet containing Silver nanoparticles was washed 3–4 times with deionized water to remove impurities. The precipitated nanoparticles were lyophilized and stored in a cool, dry, and dark place and their characterization was carried out (Rautela *et al*., 2019).

Characterization of nanoparticles:

The formation of AgNPs was confirmed by measuring the absorbance on a PerkinElmer Lambda-19 UV-Vis Spectrophotometer (Perkin Elmer, Buckinghamshire, UK) in the wavelength range of 300–900 nm and and X-ray diffraction (XRD) spectrum measurement using a XRD powder method (MiniFlex 600, Rigaku) as per the standard experimental procedure. The chemical composition of the synthesized silver nanoparticle was studied by using FT-IR spectrometer (Perkin-Elmer LS-55-Luminescence spectrometer). The solutions were dried at 75°C and the silver nanoparticles were synthesized and characterized in the range 4000–400 cm^{-1} using KBr pellet method. Surface morphology, shape of the nanoparticles, and elemental composition of AgNPs were analyzed through field emission scanning electron microscope (FE-SEM; JOEL JSM-7800F).

In vitro cytotoxicity analysis:

Cell culture:

The cell line such as HGT-1 was obtained from National Centre for Cell Sciences (NCCS), Pune, India. HGT-1 cells were maintained in RPMI 1640 medium containing 2 mM L-glutamine and supplemented with 10% fetal bovine serum (FBS) and 100 100 µg ml^{-1} streptomycin. Cell line was cultured at 37°C in a humidified atmosphere of 5% CO_2.

MTT Assay

Cell viability, after treatment with different concentrations of AgNPs, was assessed using MTT assay (Mosmann, 1983). This colorimetric test, which is based on the reduction of yellow tetrazolium salt ([3-(4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide; MTT; Sigma Aldrich, Darmstadt, Germany) to blue formazan product by mitochondrial dehydrogenase in the metabolically active cells, reflects the metabolic rate of treated cells. Cells (1×10^5/well) were plated in 96-well plates and incubated in 37ºC with 5% CO_2. After the cell reaches the confluence, the various concentrations of AgNPs was added and incubated for 24 h. After incubation, the samples were removed from the well and washed with phosphate-buffered saline (pH 7.4) or MEM without serum. Then 100 µl of 0.5% of MTT (3- 4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl - tetrazolium bromide) was added to each well and incubated for 4 h. After incubation, 1 ml of DMSO was added in all the wells. The absorbance at 570 nm was measured with UV-Spectrophotometer using DMSO as the blank. The % cell viability was calculated using the following formula:

Cell viability (%) = A570 of treated cells/A570 of control cells X 100

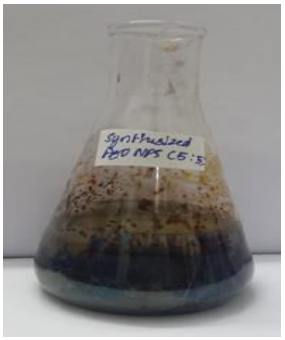

Fig.1: Bulk production using $AgNO_3$ + (PG) Plant extract.

All the data were from three independent experiments with six wells for each experiment. The half inhibitory concentrations (IC_{50}) of AgNPs were determined using GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, United States).

Results and Discussion

Green synthesis of Silver oxide nanoparticles:

Punica granatum peel extract was used for the synthesis of silver nanoparticles. Reduction of $AgNO_3$ was visually observed from the change in reaction mixture colour from transparent to brownish yellow after 30 min of reaction. The formation of nanoparticles was confirmed by visual assessment. The intensity of brown colour gradually intensified in direct proportion to the reaction period (Fig. 1). The reaction mixture turning to grey colour from brownish-green indicated the synthesis of Ag-NPs and the excitation of surface plasmon resonance effect and reduction of silver nitrate. In the case of the negative control, no change in colour was observed. Different ratio of $AgNO_3$ and PPE were mixed. But ratio concentration 5:5 was selected for the bulk preparation.

Green synthesis of nanoparticles used in this experiment is found to be eco-friendly, non-toxic and less usage of chemicals compared to physical and chemical method. In the previous study reported that the intensity of brown colour gradually intensified in direct proportion to the reaction period. Also, the colour of the solution gradually intensified on heating, which indicates the formation of Ag nanoparticles (Vinothkumar *et al*., 2015). In the case of the negative control (silver nitrate solution only), no change in colour was observed. Nabikhan *et al.* (2010) have reported that the colour of the sea weed *S. Fusiformis* extract becomes turbid after the addition of aqueous $AgNO_3$ solution signifying the initiation of the reaction.

Characterization of nanoparticles:

UV-Visible spectroscopy:

UV–Visible spectroscopy is an important technique to determine the formation and stability of silver nanoparticles in an aqueous solution. The formation of AgNP was evident from the change in solution colour from light yellow to grey colour as well as from the presence of the typical Plasmon peak in the range of 300-900 nm with a peak maximum in the range of 480 nm in the UV spectrum (Fig. 2).

UV-Visible absorption spectroscopy is one of the main techniques to examine the size and shape of the nanoparticles in aqueous suspensions (Kasthuri *et al.*, 2009). For instance, previous research has shown that spherical AgNP contribute to the absorption band at around 400–480 nm in the UV–Vis spectrum. Based on the high intensity, we confirmed that the surface Plasmon resonance was eminent. Similar study reported that the SPR band was located at 450 nm for gold nanoparticle synthesized using *Gonoderma lucidium* (Vinotha and Rajakumar, 2020).

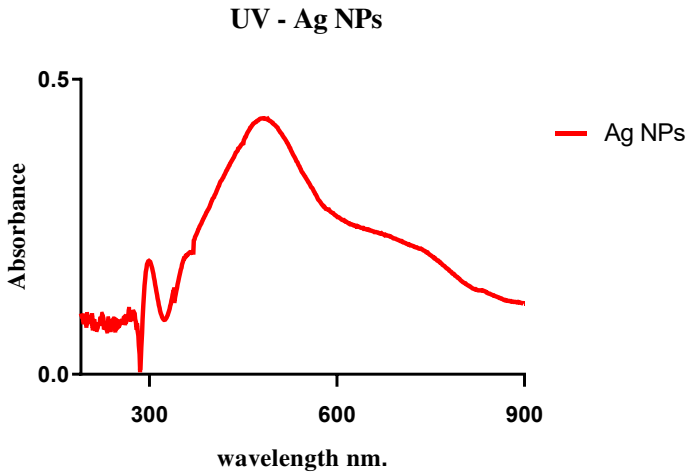

Fig. 2: UV spectrum of AgNPs from *Punica granatum* peel extract.

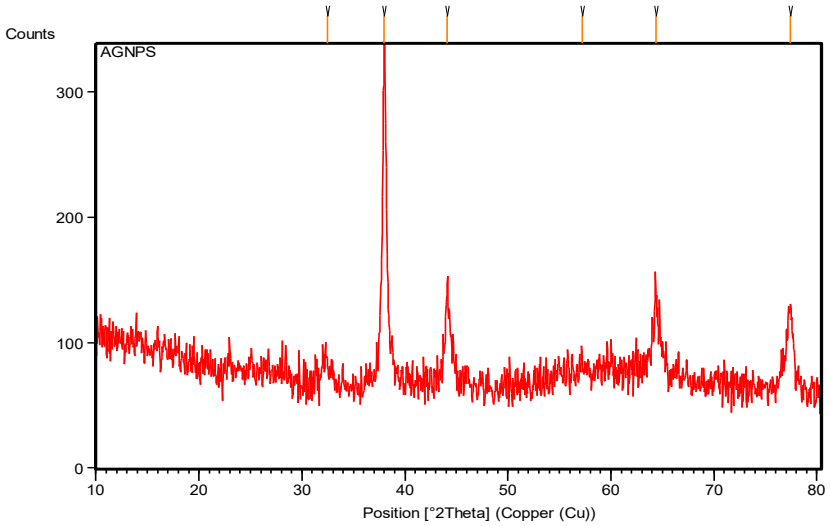

Fig. 3: XRD analysis of AgNPs.

XRD

XRD is wildly used technique to elucidate the structure, crystalline nature and to estimate the purity of nanoparticles synthesized. X-ray diffractogram of the biosynthesized Nano silver exhibits Bragg reflections, corresponding to face-centered cubic (FCC) crystal lattice. XRD peaks indicate that the AgNPs are crystalline in nature. The sharp diffraction pattern of the XRD spectra obtained by the annealing at 25°C indicates a pure crystalline structure. It showed strong diffraction peaks position (2ɵ) at 32.4450°, 38.0032°, 44.1196°, 57.2429°, 64.3652° and 77.3859° attributed to (6.28%), (100%), (30.39%), (3.25%), (25.12%) and (23.21%) relative intensity of the face-centered hexagonal silver structure (Fig. 3).

XRD is widely used technique to elucidate the structure, crystalline nature and to estimate the purity of nanoparticles synthesized. In this study, The sharp diffraction pattern of the XRD spectra obtained by the annealing at 25°C indicates a pure crystalline structure of AgNPs. Comparable XRD

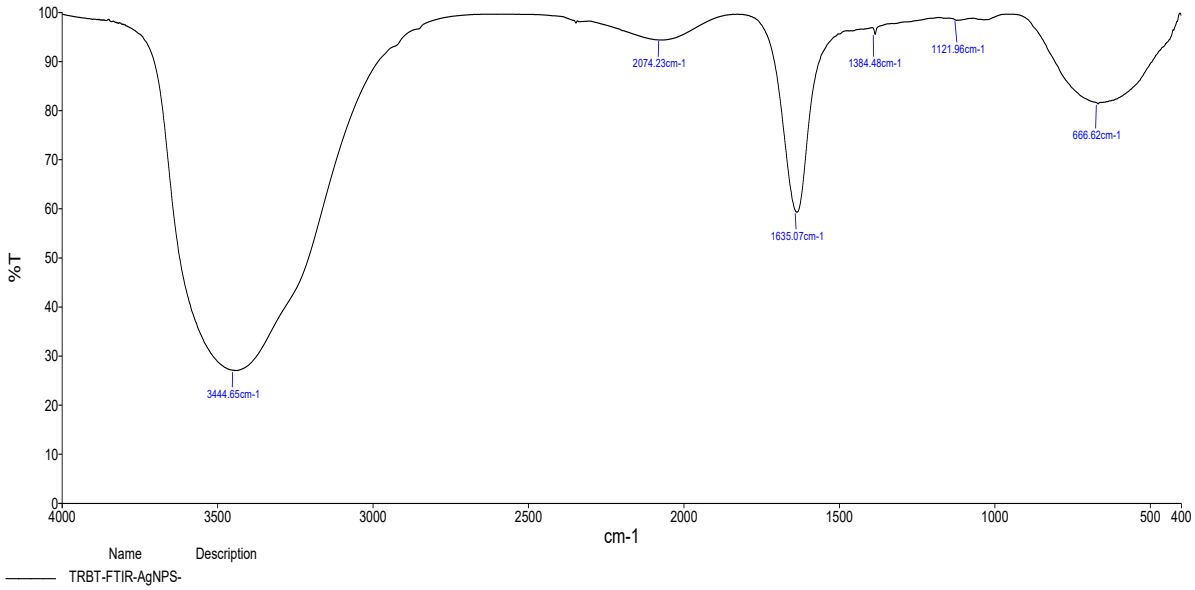

Fig. 4: FTIR spectrum of AgNPs.

results were found in the biosynthesis of AgNP using the extract of *Eclipta prostrate* leaves and *Jatropha curcas* L. latex (Chauhan *et al.,* 2016).

FTIR:

FTIR spectroscopy was analysed to identify the functional groups in the *Punica granatum* peel extract responsible for the newly synthesized AgNPs. This spectrum shows the six absorption bands indicate the presence of active functional groups in the synthesized nanoparticles. FTIR spectrum of synthesized AgNPs was determined in the region of 600-3500 cm^{-1} (Fig. 4). FTIR spectra of AgNPs showed intense peak at 3444.65, 2074.23, 1635.07, 1384.48, 1121.96 and 666.62 cm^{-1}. The absorption band at 3444.65 cm^{-1} relates to O-H stretching of strong alcohol group. The absorption peak at 2074.23 cm^{-1} indicate the N=C=s stretching strong isothiocyanate. The peaks at 1635.07 and 1384.48 cm^{-1} relates to strong C=C stretching of alkene and C-H bending of alkynes, respectively. The absorption band at 1121.96 cm^{-1} was assigned to be strong C-O stretching of secondary alcohol. The peak at 666.62 cm^{-1} relate to strong C-Br stretching of halo compounds.

In the present study, FTIR spectrum of synthesized AgNPs was determined in the region of 600-3500 cm^{-1}. The absorption band at 3444.65 cm^{-1} relates to O-H stretching of strong alcohol group. In addition, feature result reported that the peak close to 3479 cm^{-1} may be linked to O–H bond stretching of phenolic compounds. Peaks close to 1640 cm^{-1} may also indicate the presence of polysaccharides, due to C═O bond stretching (Rajathi and Sridhar, 2013).

FE-SEM:

The biosynthesized silver nanostructure from *Punica granatum* peel extract was further confirmed by the SEM micrographs. The SEM micrographs showed the high density Ag-NPs synthesis. The SEM analysis of the sample after reduction showed the presence of the $AgNO_3$ in the sample and are spherical shaped, well distributed without aggregation in the solution with the average size of about 9.5 mm. Synthesis of $AgNO_3$ is quite fast and Nanoparticles formed within few hours. Mostly spherical and near spherical shape is obtained, also obtained more nanoparticles by increasing the interaction time. Sometime non spherical polyhedral particle was

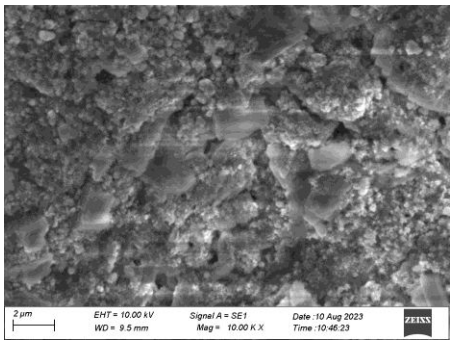

Fig. 5: SEM analysis of AgNPs.

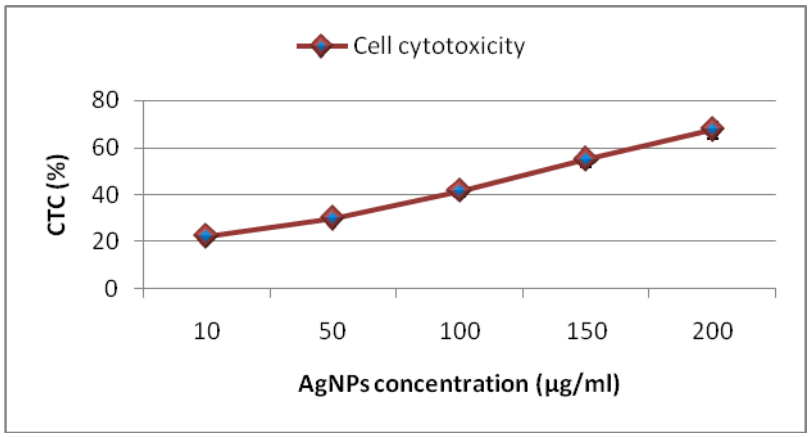

Fig. 6: Cytotoxicity activity of PGP-AgNPs against HGT-1 cell line.

also found, increase with the interaction time the aggregation and anisotropy shape is also increased. The result showed that the silver ions formed have Skew spherical in shape and they have the range of 2 µm (Fig. 5).

The synthesized nanoparticles morphology were characterized by scanning electron microscope, The SEM image exposed that the formed nanoparticle was spherical in shape formed with the size range of 40-70 nm (Natarajan *et al.*, 2010). In this study, comparison of experimental results showed that the diameter of prepared nanoparticles in the solution was about 9.5 mm. This is in agreement with recent results reported for green-synthesized AgNP (Nasiriboroumand *et al.,* 2018). In the present result, The SEM micrographs showed the high density Ag-NPs synthesis.

In vitro cytotoxic activity of AgNPs:

The reduction of cell viability observed in this study is suggestive of anticancer effects of AgNPs synthesised from *Punica granatum* peel extract. Results showed that the reduced cell viability of cancer cell lines with increasing concentrations (10–200 µg/ml) of PGP-AgNPs. The AgNPs were able to reduce viability of the HGT-1 ells in a dose dependent manner. The silver nanoparticle showed the minimum cell viability (32.59%) and maximum inhibitions (67.41%) were noted in 200 µg/ml concentrations against HGT-1 cell line. In this cell line, 50 % cytotoxicity concentration (CTC_{50}) was found to be 127.62 µg/ml in PGP-AgNPs which was required to inhibit the cell growth by 50%. The cytomorphological changes of AgNPs on HGT-1 cell lines at different concentrations involve intracellular suicide program possessing morphological changes like cell shrinkage, oxidative stress, coiling and biochemical response leading to apoptosis. It is

quite obvious from the results that the apoptosis rate of HGT-1 cell lines increases with increase in concentration of PGP-AgNPs (Fig. 6).

The *in vitro* anticancer activity detects the reduction of MTT by mitochondrial dehydrogenase to blue formazan product, which reflects the normal function of mitochondria and cell viability. Gopinath *et al.* (2008) proposed an apoptotic effect on cancer and normal cells *in vitro* induced by AgNPs used at low concentrations. AgNPs induce cell responses often specific to cell types, resulting in varying degrees of toxicity. Depending on nanoparticle size, concentration, and exposure time, AgNPs induced different degrees of toxicity *in vitro* in fibroblasts, epithelial cells, melanoma cells, HaCaT cells, and HeLa cells. AgNPs at a concentration of 11–36 µg/ml caused a reduction in mitochondrial function (Zanette *et al.*, 2011). Similar results have been reported in human (0.5–3 µg/ml) and rodent (10–50 µg/ml) liver cells, alveolar macrophages (10–75 µg/ ml), mouse dermal fibroblast cells and liver cells (30 µg/ml), and mouse germ line stem cells (10 µg/ml) (Liu *et al.*, 2010). In previous study, the *in vitro* analysis showed that the AgNPs demonstrated dose-dependent cytotoxicity against RAW 264.7 macrophages and MCF-7 breast cancer cells but higher against the latter than the former (Wypij *et al.,* 2021). Cell viability decrease was found to be 42.2–14.2 and 38.0–15.5% while LDH leakage 14.6–42.7% and 19.0–45.0%, respectively (Magdalena *et al.,* 2021). The use of AgNPs for biomedical purposes, especially *in vivo* applications, requires determination of their cytotoxic effect. Although many studies on the toxicity of biogenic AgNPs in various cell lines have been reported (Hamed *et al.*, 2020). The mechanisms underlying the toxicity in eukaryotic cells are still unclear.

Conclusion

Biosynthesis of AgNPs is regarded as a green, eco-friendly, low-priced process that provides small and biocompatible nanostructures with antimicrobial and anticancer activities and potential application in medicine. In the present study AgNPs were synthesised from *Punica granatum* peel extract and it was characterized by UV spectroscopy, SEM, FTIR and XRD. The formation of nanoparticles was confirmed by visual assessment. The present study concluded that the *Punica granatum* peel extract exhibited strong inhibitory activities against the HGT-1 cell line. In feature PGP-AgNPs may be used in the various biomedical applications.

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