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In vitro anticancer activity of silver nanoparticle synthesized from *Punica granatum* dried peel against cancer cell lines

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The main aim of the investigation was to screen the silver and gold nanoparticle synthesized from the methanol concentrate of *Punica granatum* for its *in vitro* anticancer activity against MCF 2, PC3, A-549, HeLa, and HepG2 cell lines. Silver and gold nanoparticles were prepared from methanol concentrate of *P. granatum* and nanoparticle synthesized was analyzed by UV and TEM analysis. The impact of nanoparticles synthesized on MCF-2, PC3, A-549, HeLa, and HepG2 disease cell lines was assessed by MTT colourimetric assay and the impact on cell cycle was assessed by flow cytometric method. After assessing the cytotoxicity effect, the impact of apoptosis was also analyzed. The TEM analysis showed the particle size of 25.56 and 22.02 nm for gold and silver nanoparticles respectively. The adequacy of silver nanoparticles synthesized from *P. granatum* against MCF-2, PC3, A-549, HeLa, and HepG2 cell line demonstrated that the hatching of malignancy cells decreased the suitability of PC-3 and A 549 cancer cells lines only with IC_{50} values as 108.7 and 88.42 µg/mL respectively. Gold nanoparticles didn't have any activity against all the cancer cell lines. Cell cycle analysis and apoptosis study showed that Silver nanoparticles are effective in controlling the cell cycle and blocking the apoptosis in all the cell lines used. *P. granatum* can be a very good anticancer drug for various cancer cell lines. All in all, *P. granatum* has a critical cell reinforcement movement and anticancer action.

Keywords: Apoptosis, Cell Lines, Cell cycle, *In vitro* anticancer activity, *Punica granatum*, *Silver nanoparticle*.

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Introduction

Cancer being a deadly disease have a high rate of multiplication because of independent development signals, continued angiogenesis and can attack tissues and metastasize apart from that malignancy, cells show protection from apoptosis. Plants have a huge number of functioning constituents with remedial properties. Various traditional systems of treatment includes cancer preventing agent by mitigating, DNA fix, and acceptance of apoptosis, invulnerable initiation and hindrance of cell cycle movement[1,2]. Hindrance of cell cycle movement, relocation, and attack, together with activating apoptosis can be viewed as techniques for disease treatment.

Punica granatum L. (Family Punicaceae/Lythraceae) commonly known as the pomegranate is a large deciduous shrub or a small tree up to 5-10 m in height, wild and cultivated throughout India up to an altitude of 2000 m in the hills[3]. In the traditional system of medicine, root and stem bark are used as astringent, cooling, anthelmintic, good for tapeworm, strengthening gums and diarrhoea; flowers are used for styptic to gums, ophthalmic pain, hematuria, intrinsic haemorrhage, haemorrhoids, diarrhoea, dysentery, ulcer, pharyngitis, and epistaxis; fruits are sweet, sour, astringent, cooling, tonic, aphrodisiac, laxative, diuretic, anaemia, hyperdipsia, dyspepsia, pharyngitis, ophthalmic pain, pectoral disease, splenic disorder, bronchitis, earache, and diarrhoea; fruit rind is used for dysentery, gastric disorder, bleeding piles, freckles, and gonorrhoea; seeds are used as astringent, stomachic, diuretic, cardiotonic, vomiting, excessive thirst, hepatic and splenic disorder[4-9].

This plant has been reported to have antibacterial, antifungal, hypoglycemic, anti-oxidative, hypolipidemic, analgesic, immunomodulatory, anticonvulsant, anthelmintic, antifertility, antidiabetic, anti-inflammatory, gastroprotective, uterine stimulant cytotoxic, carcinogenesis, angiogenesis, atherosclerosis, hypertension, carotid artery stenosis, myocardial perfusion, dental conditions, ultraviolet radiation, erectile dysfunction, male fertility, neonatal hypoxia

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ischemic brain injury, Alzheimer disease, obesity and anticancer activities[10-16].

The aim of the present investigation was to green synthesis silver and gold nanoparticles and evaluate the *in vitro* anticancer movement against MCF-2, PC3, A-549, HeLa, and HepG2 cell lines, cell cycle impact, and apoptosis.

Materials and Methods

The *P. granatum* fruit was collected from the local market [Sarraki Market, J P Nagar] Bangalore, Karnataka (India), and verified by Department of Pharmacognosy, The Oxford College of Pharmacy. A Voucher specimen (TOCOP/02/2017-18) was deposited in The Oxford College of Pharmacy, Bangalore. The dried peel was air-dried.

Preparation of extract

The dried peel was air-dried and controlled with a mechanical processor, going through a strainer and put away in a sealed shut compartment. At that point, 50 g of air-dried powder was constantly refluxed with 50% methanol at 55 ^{o}C for 30 minutes on a water bath[17]. The solution was filtered and made up to 100 mL.

Drugs and chemicals

Silver nitrate and gold chloride were purchased from SD Fine Chemicals Ltd, Mumbai. Propidium iodide: Cat # P4864 from Sigma and RNase A: Cat # 109169 from Boehring Mannheim GmbH were purchased. All chemicals and reagents used in this study were at least of analytical grade.

Synthesis of nanoparticles

A set of 1, 2, and 3 mM aqueous solution of silver nitrate and gold chloride was prepared for synthesis for silver and gold nanoparticles[17]. Exactly 9 mL of each 1, 2, and 3 mM silver nitrate and gold chloride solution respectively was added to 0.1, 0.2, 0.3, 0.4, and 0.5 mL methanol extract of the dried peel fruit of *P. granatum* to obtain silver and gold nanoparticles. The different concentrations of silver nitrate, gold chloride, and extracts were used to standardize the optimum concentration of silver nitrate, gold chloride, and extract needed for the synthesis of silver and gold nanoparticles. We found 0.3 mL and 1 mM solutions were found to give the best yield. The nanoparticles were synthesized at room temperature and the formation of nanoparticles was confirmed by checking λ_{max} using UV spectrophotometer.

Lyophilisation procedure

After the desired reaction period, the solution containing silver and gold nanoparticles were lyophilized. The reluctant samples were centrifuged 10,000 rpm for 15 minutes. After 15 minutes, discard the supernatant and collect the pellet and freeze-dry. The lyophilized samples were kept in the freezer at 4 °C for further analysis.

Characterization of silver and Gold nanoparticles

Ultra violet visible spectroscopy analyses were carried out by UV–visible spectrophotometer Shimadzu in the range of 200–800 nm, with a scanning speed of 100 mm/min. The morphology examination of dried powder samples was analyzed by TEM analysis which gives information about the particle size.

In vitro anticancer activity

Preparation of test solutions

The 32 mg/mL sample stock was prepared in sterile DMSO. For cytotoxicity studies, serial two-fold dilutions from 320 to 10 µg/mL were prepared which is then used for treatment.

Cell lines and culture medium

All the cell lines were procured from ATCC, stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100 µg/mL) in a humidified atmosphere of 5% CO_2 at 37 ^{o}C until confluent. The cell was dissociated with cell dissociating solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The viability of the cells is checked and centrifuged. Further, 50,000 cells/well were seeded in a 96 well plate and incubated for 24 h at 37 ^{o}C, 5% CO_2 incubator.

The cytotoxic assay screening was performed using MTT assay as per Mosmann[18] method to test the cytotoxicity of *P. granatum* synthesized silver and gold nanoparticles against MCF 2, PC3, A-549, HeLa, and HepG2 cell lines. In brief, the monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/mL using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100 µL of the diluted cell suspension (50,000 cells/well) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once with medium and 100 µL of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37 ^{o}C for 24 h in a 5% CO_2 atmosphere. After incubation, the test solutions in the wells were discarded and 100 µL of MTT (5 mg/10 mL of MTT in PBS) was added to each well. The plates were

incubated for 4 h at 37 ^{o}C in a 5% CO_2 atmosphere. The supernatant was removed and 100 µL of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) values is generated from the dose-response curves for cell lines.

% Inhibition = 100 – (OD of sample/OD of Control) x 100

Cell cycle analysis

Silver nanoparticle-induced changes in cell cycle were measured using the protocol[19]. In brief, MCF 2, PC3, A-549, HeLa, and HepG2 cell lines were exposed for 24 h at 10–50 µg/mL PGDSN. After the treatment, cells were fixed in chilled 70% ethanol for 1 h. Then, cells were washed twice by centrifugation, and cells were stained with propidium iodide for 60 minutes in dark. Stained cells were acquired by flow cytometer.

In brief, 1 x10^6 cells were seeded and cultured for 24 hours in a 6-well plate containing 2 mL of serum-free media. Cells were then treated with desired concentrations of given samples that were prepared in complete media and incubated for another 24 hours. Cells were then harvested and centrifuged at 2000 rpm for 5 minutes at room temperature and the supernatant was discarded carefully retaining the cell pellet. The cell pellet was washed by resuspending in 2 mL of 1XPBS. The washing was repeated another time with the same conditions. The supernatant was discarded retaining the pellet. Cells were fixed by resuspending in 300 µL of Sheath fluid followed by the addition of 1 mL of chilled 70% EtOH drop by drop with continuous gentle shaking and another 1 mL of chilled 70% EtOH added at once. The cells were then stored at 4 °C overnight. Post fixing, the cells were centrifuged at 2000 rpm for 5 minutes. The cell pellet was washed twice with 2 mL of cold 1XPBS. The cell pellet was then resuspended in 500 µL of sheath fluid containing 0.05 mg/mL PI and 0.05 mg/mL RNase A and incubated for 15 minutes in dark. The percentage of cells in various stages of the cell cycle in compounds treated and un-treated populations were determined using FACS Caliber (BD Biosciences, San Jose, CA).

Apoptosis assay

Apoptosis/necrosis induced by silver nanoparticles in cancer cell lines was analyzed using Annexin-V and 7- AAD Kit (Beckman Coulter). The amount of apoptosis/necrosis in the treated MCF 2, PC3, A-549, HeLa, and HepG2 cell lines were analyzed by flow cytometry following the protocol[20].

In brief, the day before induction of apoptosis, plated 1 X 10^6 MCF-2, PC3, A-549, HeLa, and HepG2 cell lines per well for a 6-well plate using DMEM media with 10% FBS and 1% Pen Strep, incubated overnight at 37 ˚C at 5% CO_2. The media was replaced with test solutions of different concentrations in the media containing 10% FBS. The treated cells were incubated for 24 hours at normal culture conditions. The cells were harvested and well contents were completely transferred to the sterile FACS tubes. The cell contents were centrifuged at 2000 rpm for 5 minutes and the supernatant was discarded. Washed the cells twice with cold PBS following the centrifugation and then resuspended the cells in 1 mL 1X Binding Buffer at a concentration of ~1 x 10^6 cells/mL. Transferred 500 µL of the cell suspension (~5 x 105 cells) to a new FACS tube. Exactly 5 µL Annexin V and 10 µL PI were added to the tubes, cells were gently mixed and incubated for 20 minutes at room temperature in the dark. The cells were analyzed by flow cytometry as soon as possible (within 1 hour).

Results and Discussion

The UV spectroscopy of 50% methanol extract of dried peel (0.3 mL) of *P. granatum* at 55 ^{o}C are taken on Shimadazu UV spectrophotometer and graphs were taken from the software which is given in Fig. 1 & 2. The peak at 400 to 450 nm and 570 to 590 nm formation means silver and gold nanoparticles are formed[21]. In TEM analysis, it was very clear that nanoparticles were synthesized and characterized as nanoparticles. The average particle size was 22.02 and 25.56 nm for silver and gold nanoparticle respectively (Fig. 3 & 4).

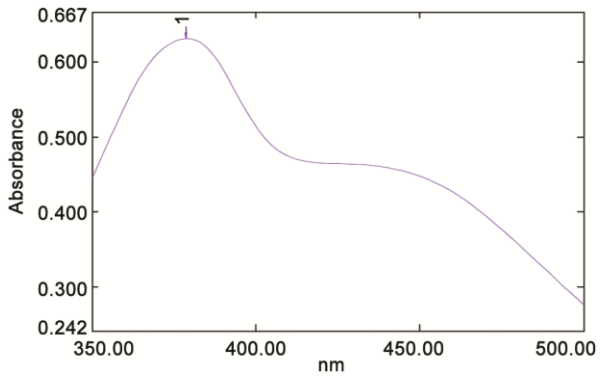


Fig. 1 — UV spectroscopy of silver nanoparticles synthesised from *Punica granatum*

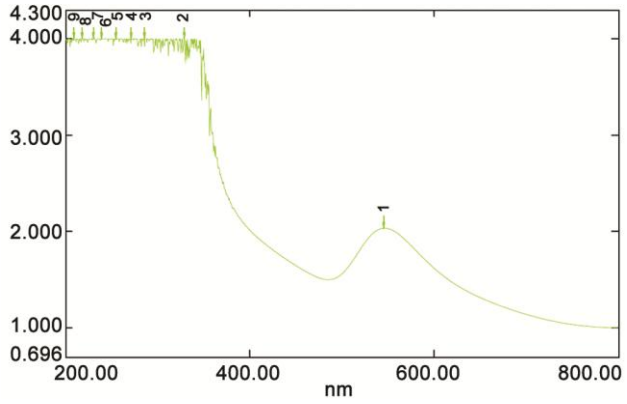


Fig. 2 — UV spectroscopy of gold nanoparticles synthesised from *Punica granatum*

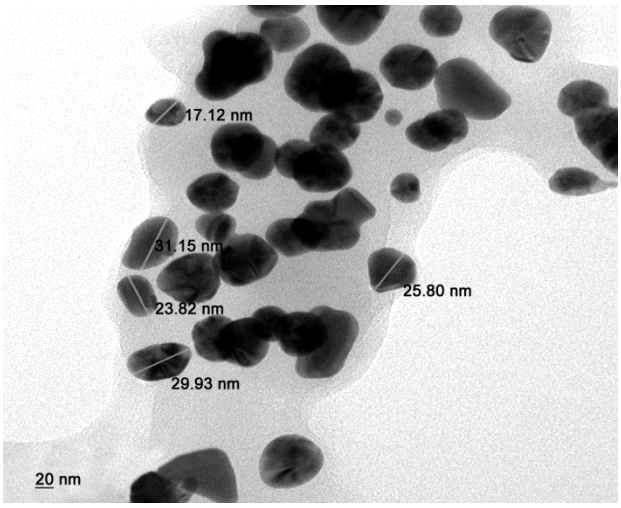


Fig. 3 — TEM analysis of gold nanoparticles synthesised from *Punica granatum*

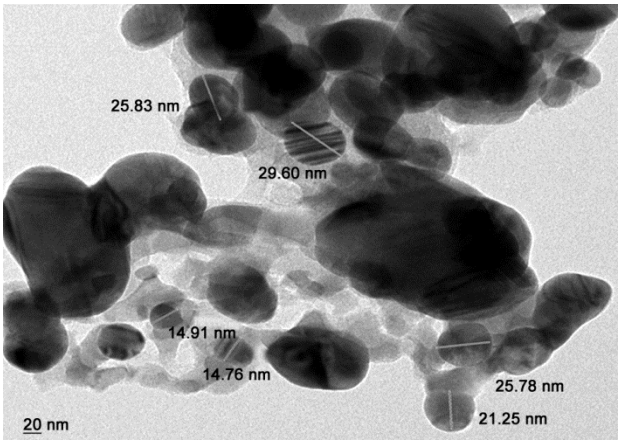


Fig. 4 — TEM analysis of silver nanoparticles synthesised from *Punica granatum*

It is seen that the administration of malignancy and irresistible sicknesses dependably require the quest for new medications. Albeit various medications are at present being used for malignant growth chemotherapy, they display cell poisonous quality, actuates genotoxic, cancer-causing, and teratogenic impacts in non-tumour cells. These reactions limit the utilization of chemotherapeutic specialists regardless of their high viability in treating target threatening cells. Along these lines, the quest for novel medications that are both viable and non-harmful bioactive plant items have been expanded. As of late, ethnobotanical and customary employments of normal mixes, particularly of plant inception got much consideration as they are very much tried for their viability and for most of it, accepted to be all right for human use.

Traditionally, the *in vitro* determinations of toxic effects of unknown compounds have been performed by counting viable cells after staining with a vital dye. Alternative methods used are the measurement of radioisotope incorporation as a measure of DNA synthesis, counting by automated counters, and others that rely on dyes and cellular activity. The MTT system is a means of measuring the activity of living cells via mitochondrial dehydrogenases. The MTT method is simple, accurate, and yields reproducible results. The key component is (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide), or MTT, which is a water-soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells. This water-insoluble formazan can be solubilized using DMSO, acidified isopropanol, or other solvents (Pure propanol or ethanol).

The resulting purple solution is spectro-photometrically measured. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of effects caused by the test material[18].

The effect of silver nanoparticle synthesized from *P. granatum* against MCF-2, PC3, A-549, HeLa, and HepG2 cell line demonstrated that the hatching of malignancy cells decreased the suitability of PC-3 and A 549 cancer cells lines only with IC_{50} values as 108.7 and 88.42 µg/mL respectively (Table 1, Fig. 5). It is also seen that gold nanoparticles didn't have any activity against any MCF 2, PC3, A-549, HeLa, and HepG2 cell lines. Similar results were observed by other authors for HeLa and colon cancer on silver nanoparticles synthesized from other parts of *P. granatum*[22,23].

Table 1 — Effect of cytotoxicity of different concentration of silver nanoparticles against cancers cell lines

Nanoparticles cell lines	10µg/mL	20µg/mL	40µg/mL	80µg/mL	160µg/mL	320µg/mL
PC3 PGDGN	6	11	18	26	32	40
PC3 PGDSN	9	19	24	40	54	67
A 549 PGDGN	8	14	22	27	35	43
A 549 PGDSN	11	23	32	40	59	69
MCF2 PGDGN	5	11	17	24	30	37
MCF 2 PGDSN	3	9	20	25	29	35
HEPG2 PGDGN	2	7	15	23	31	37
HEPG2 PGDSN	4	12	20	26	30	39
HeLa PGDGN	5	12	19	29	34	38
HeLa PGDSN	3	10	23	31	38	42

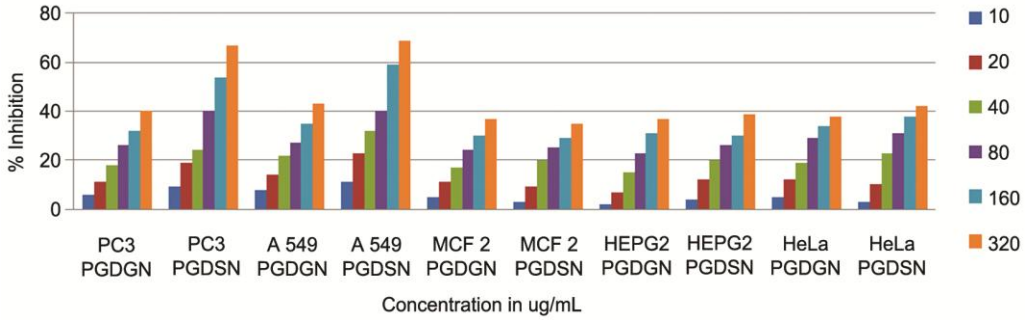


Fig. 5 — Effect of cytotoxicity of different concentration of silver nanoparticles against cancers cell lines

The cycle of increase in components (growth) and division followed by growth and division of these daughter cells, etc., is called the cell cycle. The two most obvious features of the cell cycle are the synthesis and duplication of nuclear DNA before division, and the process of cellular division itself - mitosis. These two components of the cell cycle are usually indicated in shorthand as the "S phase" and "mitosis" or "M". When the S phase and M phase of the cell cycle were originally described, it was observed that there was a temporal delay or gap between mitosis and the onset of DNA synthesis, and another gap between the completion of DNA synthesis and the onset of mitosis. These gaps were termed G1 and G2, respectively. One of the earliest applications of flow cytometry was the measurement of DNA content in cells. This analysis is based on the ability to stain the cellular DNA in a stoichiometric manner. A variety of dyes are available to serve this function, all of which have high binding affinities for DNA. The location to which these dyes bind on the DNA molecule varies with the type of dye used. The most common DNA binding dye in use today is the blue-excited dye Propidium Iodide (PI). PI is an intercalating dye which binds to DNA and double-stranded RNA (and is thus almost always used in conjunction with RNase A to remove RNA). When diploid cells which have been stained with a dye that stoichiometrically binds to DNA are analyzed by flow cytometry, a "narrow" distribution of fluorescent intensities are obtained. The study suggests that the cells treated with the samples have shown a marked increase in % of cells in the S phase & G2M phase compared to control; as the checkpoints at these phases play a major role in DNA replication & cell division[20,24-26]

The result of silver nanoparticle PGDSN showed that G0/G1, S and G2M phase are 79.28, 7.44, 13.04; 80.66, 8.12, 10.74; 73.14, 13.48, 12.74; 72.94, 12.42, 14.14 and 81.78, 3.28, 14.7 for MCF 2, PC3, A 549, HEPG2, HeLa cells when compared to control as 84.04, 3.10, 9.96; 73.18, 12.8, 13.88; 79.63, 4.93, 10.19; 68.92, 10.99,18.16 and 82.14, 5.8, 11.56, respectively (Table 2, Fig 6 & 7). From the cell cycle analysis, it is clear that MCF2, A 549, and HEPG2 cause an increase in S and G2M stage and HeLa causes an increase only in the G2M stage suggesting that PGDSN has a control on the cell replication and cell division.

Apoptosis is a cell death process characterized by morphological and biochemical features occurring at different stages. Once triggered apoptosis proceeds with different kinetics depending on cell types and culminates with cell disruption and formation of

apoptotic bodies. A critical stage of apoptosis involves the acquisition of surface changes by dying cells that eventually results in the recognition and uptake of these cells by phagocytes. Different changes on the surface of apoptotic cells such as the expression of thrombospondin binding sites, loss of sialic acid residues, and exposure of a phospholipid like phosphatidylserine (PS) were described.

Phospholipids are asymmetrically distributed between inner and outer leaflets of the plasma membrane with phosphatidylcholine and sphingomyelin exposed on the external leaflet of the lipid bilayer, and phosphatidylserine predominantly observed on the inner surface facing the cytosol. Exposure of PS on the external surface of the cell membrane has been reported for activated platelets and senescent erythrocytes. Recently, it was shown that cells undergoing apoptosis break up the phospholipid asymmetry of their plasma membrane and expose PS which is translocated to the outer layer of the membrane. This occurs in the early phases of apoptotic cell death during which the cell membrane remains intact. This PS exposure may represent a hallmark (early and widespread) in detecting dying cells. Annexin V, belonging to a recently discovered family of proteins, the annexin, with anticoagulant properties has proven to be a useful tool in detecting apoptotic cells since it preferentially binds to negatively charged phospholipids like PS in the presence of Ca^{2+} and shows minimal binding to phosphatidylcholine and sphingomyelin.

Changes in PS asymmetry, which is analyzed by measuring Annexin V binding to the cell membrane, were detected before morphological changes associated with apoptosis have occurred and before membrane integrity has been lost. By conjugating FITC to Annexin V it is possible to identify and quantitate apoptotic cells on a single cell basis by flow cytometry. Staining cells simultaneously with FITC-Annexin V (green fluorescence) and the propidium iodide (red fluorescence) allows the discrimination of intact cells, early apoptotic and late apoptotic or necrotic cells[19]. A low concentration of AgNPs was also reported to be able to induce apoptosis[27].

The result of silver nanoparticle PGDSN showed that viable cell, early apoptotic cell, late apoptotic cell and necrotic cell are 65, 3.42, 21.76, 9.82; 80.32, 2.24, 4.02, 13.42; 70.97, 4.84, 20.52, 3.67; 84.66, 4.46, 6.68, 4.2 and 80.94, 3.02, 4.44, 11.62 for MCF 2, PC3, A 549, HEPG2, HeLa cells when compared to control as 95.42, 0.38, 0.40, 3.80; 92.42, 2.84, 3, 1.74, 92.76, 0.19, 6.09, 0.96; 96.86, 1.28, 0.46, 1.4 and 96.6, 0.14, 1.68, 1.58 respectively (Table 3, Fig. 8 & 9).

Table 2 — Effect of silver nanoparticles against cancers cell lines on cell cycle

	Sub G0	G0/G1	S	G2M
MCF 2 control	3.10	84.04	03.10	09.96
MCF 2 PGDSN	0.52	79.28	07.44	13.04
PC3 control	0.68	73.18	12.80	13.88
PC3 PGDSN	0.76	80.66	08.12	10.74
A 549 control	5.86	79.63	04.93	10.19
A 549 PGDSN	1.32	73.14	13.48	12.74
HEPG2 control	2.50	68.92	10.99	18.16
HEPG2 PGDSN	0.84	72.94	12.42	14.14
HeLa control	0.82	82.14	05.80	11.56
HeLa PGDSN	0.38	81.78	03.28	14.70

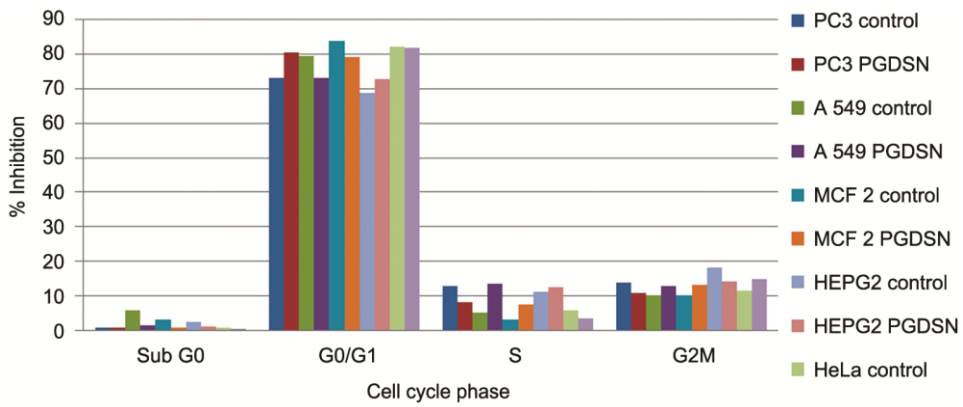
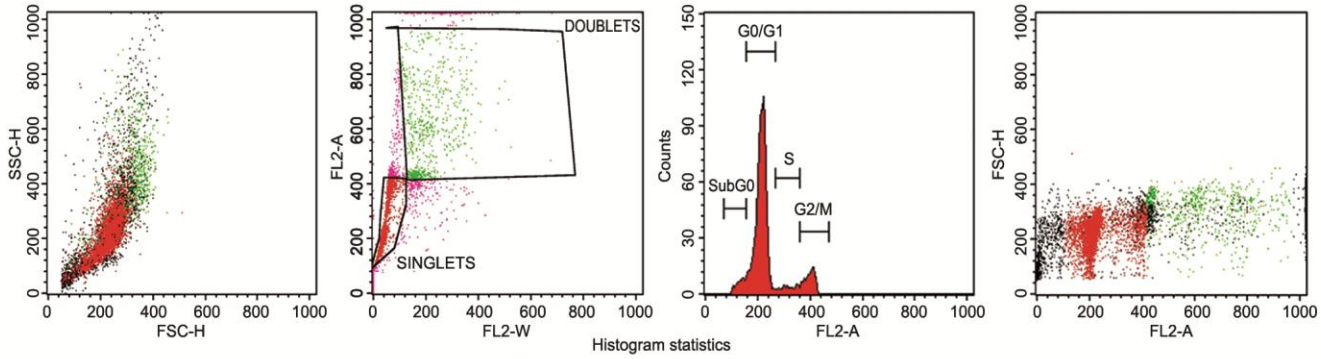


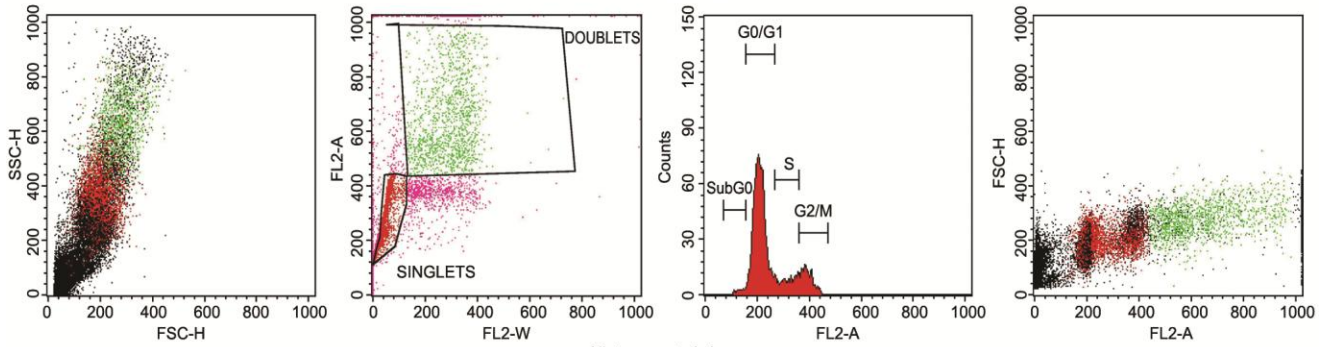
Fig. 6 — Effect of silver nanoparticles against cancers cell lines on cell cycle



Histogram statistics

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	5052	100.00	69.53	231.72	28.28
Sub G0	72, 158	296	5.86	4.07	135.63	11.35
G0/G1	156, 268	4023	79.63	55.37	212.01	8.75
S	268, 362	249	4.93	3.43	319.43	8.41
G2/M	362, 474	515	10.19	7.09	396.06	4.21

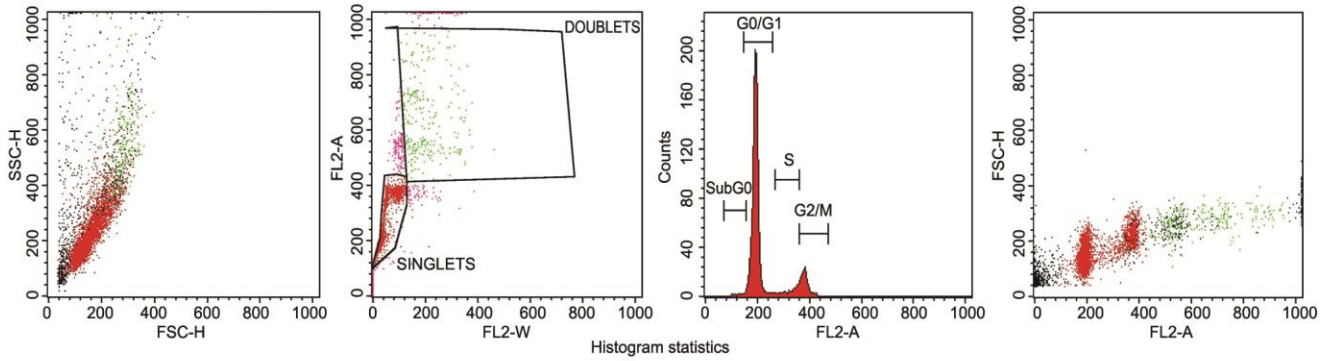
A-549 untreated cell



Histogram statistics

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	5000	100.00	18.33	246.16	28.77
Sub G0	72, 158	66	1.32	0.24	138.68	10.43
G0/G1	156, 268	3657	73.14	13.40	209.45	10.09
S	268, 362	674	13.48	2.47	318.90	9.06
G2/M	362, 474	637	12.74	2.33	392.26	5.02

A-549 cells treated with 320 µg/mL of PGDSN

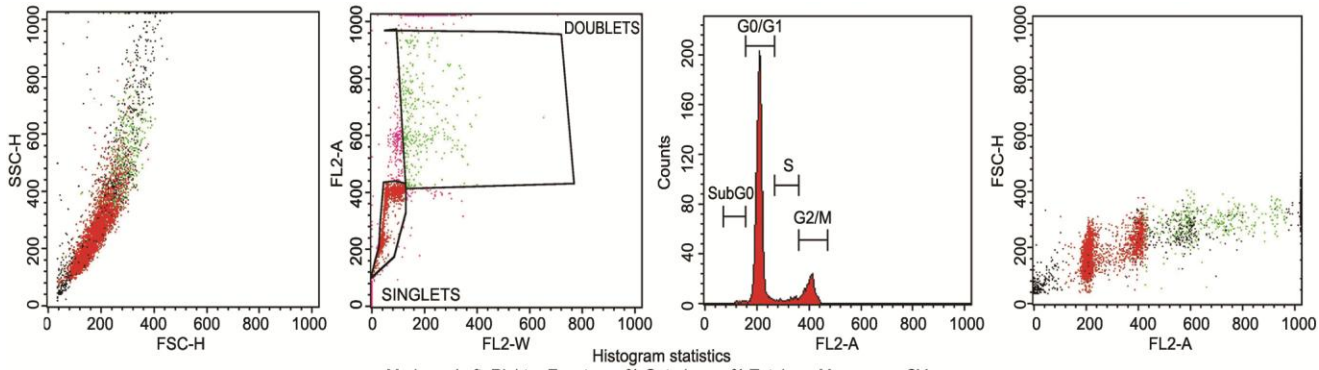


Histogram statistics

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	5000	100.00	82.41	221.91	30.21
Sub G0	72, 158	41	0.82	0.68	137.17	13.69
G0/G1	147, 259	4107	82.14	67.69	192.61	6.21
S	268, 362	290	5.80	4.78	332.77	7.96
G2/M	362, 474	578	11.56	9.53	380.24	3.28

HeLa untreated cell

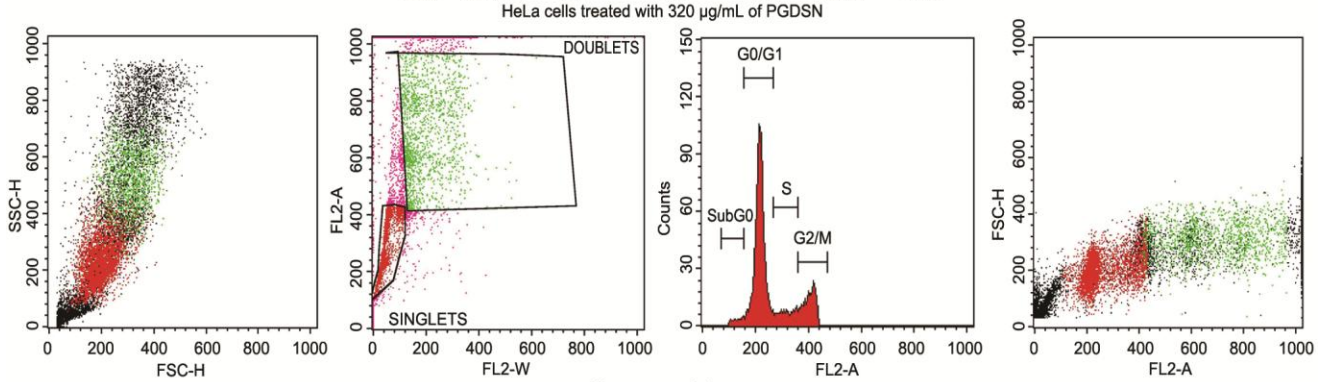
(*Contd*.)



Histogram statistics

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	5000	100.00	81.98	240.51	28.49
Sub G0	72, 158	19	0.38	0.31	135.26	11.11
G0/G1	156, 268	4089	81.78	67.04	208.89	5.32
S	268, 362	164	3.28	2.69	325.27	8.41
G2/M	362, 474	735	14.70	12.05	400.97	4. 08

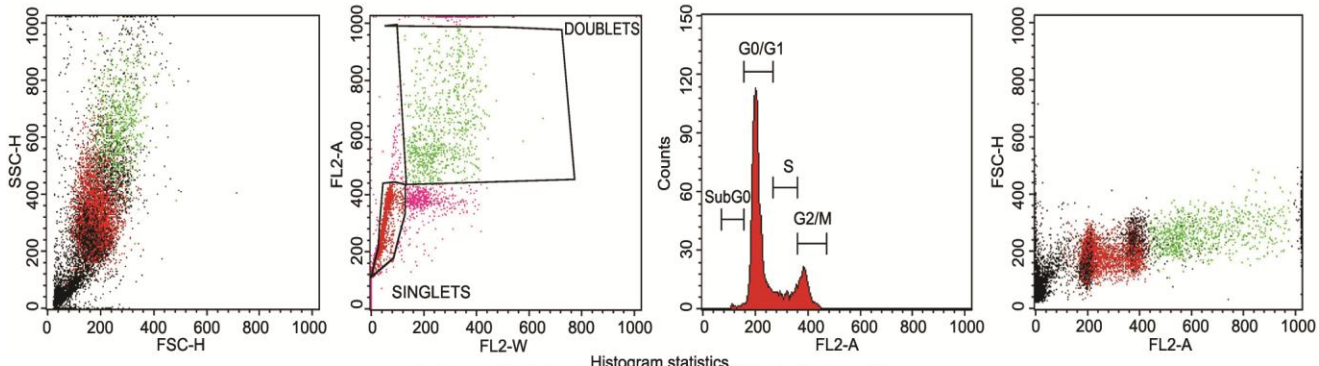
HeLa cells treated with 320 µg/mL of PGDSN



Histogram statistics

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	5006	100.00	43.27	259.13	30.10
SubG0	72, 158	125	2.50	1.08	133.90	12.15
G0/G1	156, 268	3450	68.92	29.82	216.36	8.46
S	268, 362	550	10.99	4.75	318.07	8.59
G2/M	362, 474	909	18.16	7.86	402.05	4.92

HEP-G2 untreated cell

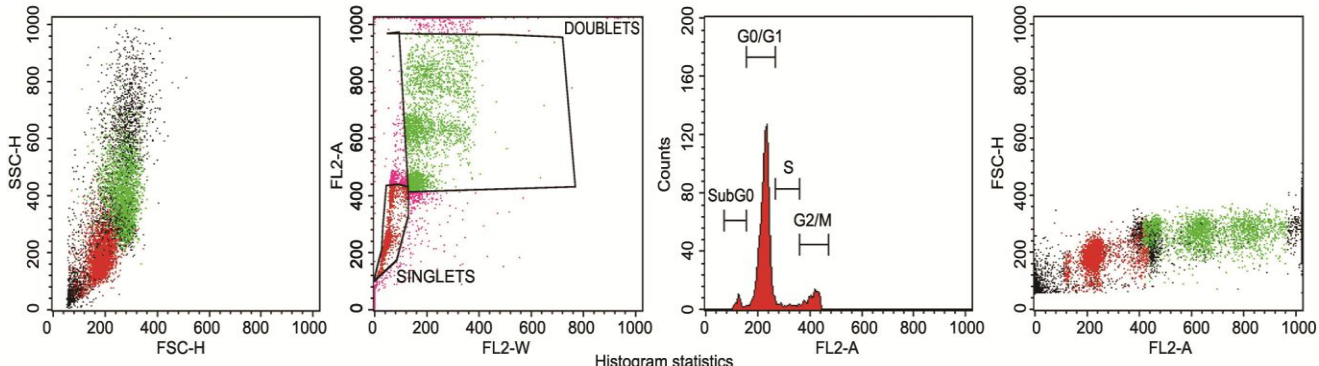


Histogram statistics

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	5000	100.00	40.70	245.34	28.92
SubG0	72, 158	42	0.84	0.34	137.90	10.91
G0/G1	156, 268	3647	72.94	29.69	206.99	8.86
S	268, 362	621	12.42	5.05	317.17	9.12
G2/M	362, 474	707	14.14	5.75	387.74	4.31

HEP-G2 cells treated with 320 µg/mL of PGDSN

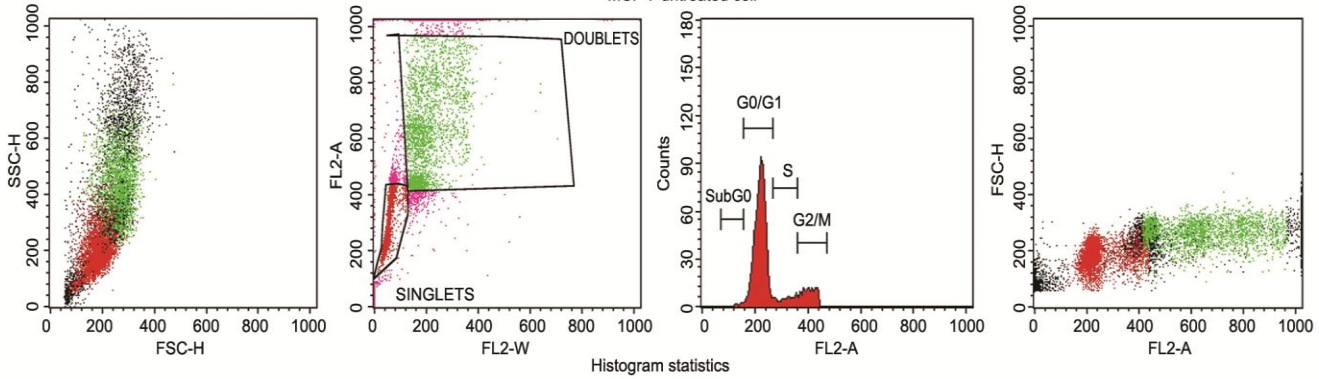
(*Contd*.)



Histogram statistics

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	5000	100.00	47.23	243.39	25.62
SubG0	72, 158	155	3.10	1.46	127.57	8.52
G0/G1	156, 268	4202	84.04	39.69	225.42	7.48
S	268, 362	155	3.10	1.46	312.07	9.47
G2/M	362, 474	498	9.96	4.70	409.90	4.87

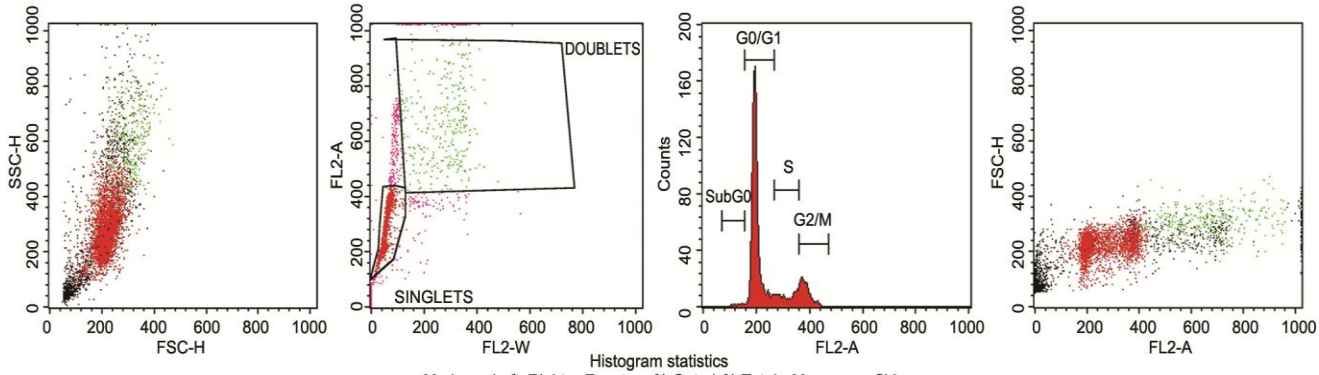
MCF-7 untreated cell



Histogram statistics

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	5000	100.00	50.46	249.83	27.26
SubG0	72, 158	26	0.52	0.26	144.31	9.69
G0/G1	156, 268	3964	79.28	40.00	218.73	8.68
S	268, 362	372	7.44	3.75	319.52	9.01
G2/M	362, 474	652	13.04	6.58	403.15	5.28

MCF-7 cells treated with 320 µg/mL of PGDSN



Histogram statistics

Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	5000	100.00	77.54	239.71	30.24
SubG0	72, 158	34	0.68	0.53	136.65	10.63
G0/G1	156, 268	3659	73.18	56.75	199.88	8.67
S	268, 362	640	12.80	9.93	319.14	9.40
G2/M	362, 474	694	13.88	10.76	384.09	4.36

PC-3 untreated cell

(*Contd*.)

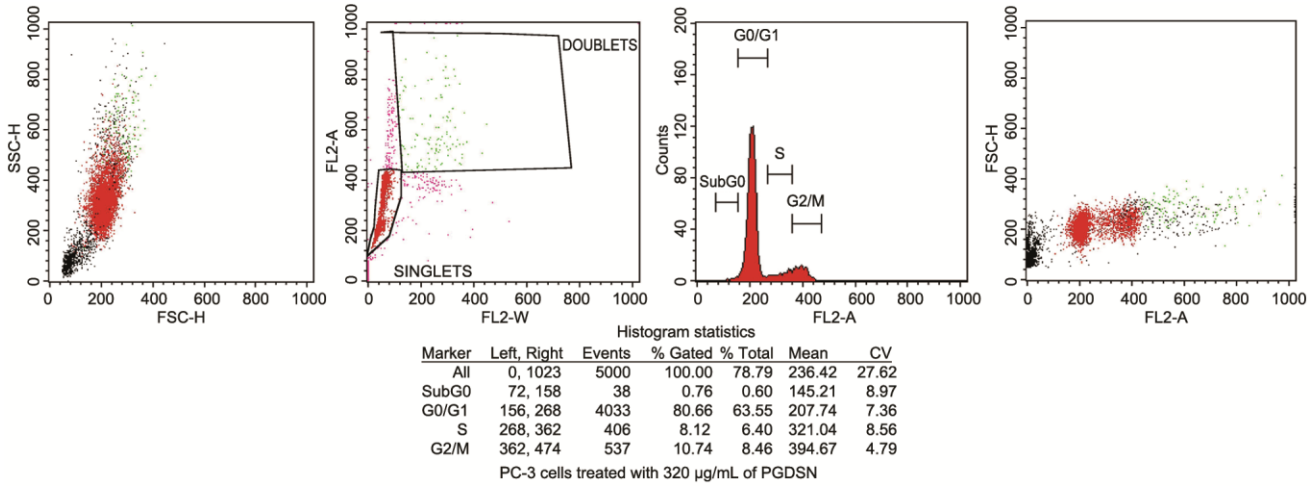


Fig. 7 — Flow cytometry analysis of cell cycle in A 549 cells

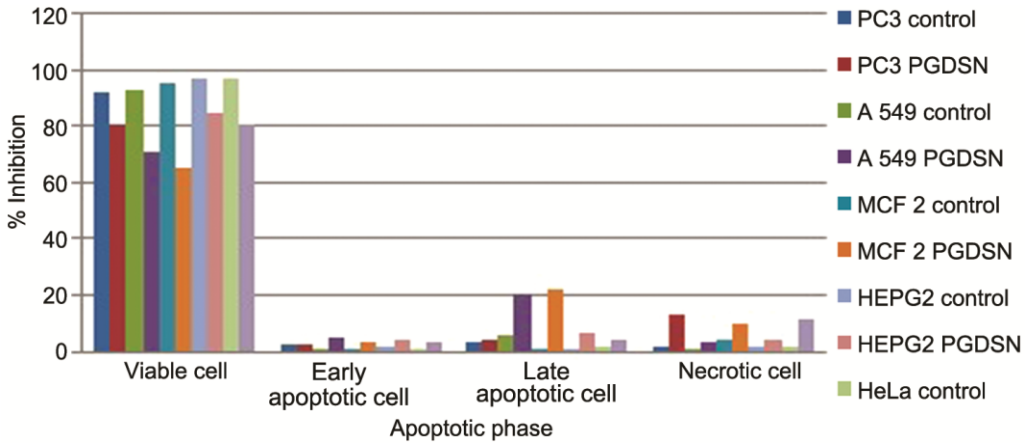


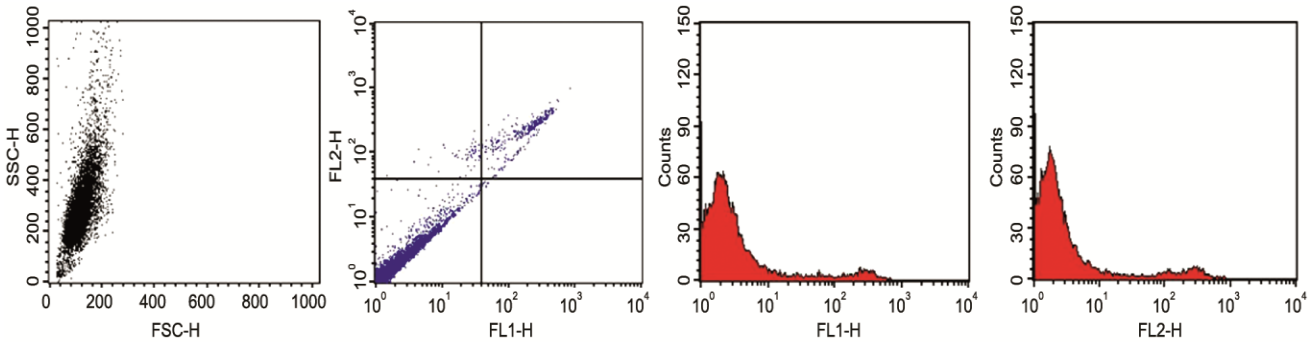
Fig. 8 — Effect of silver nanoparticles against cancers cell lines on apoptosis

Table 3 — Effect of silver nanoparticles against cancers cell lines on apoptosis

	Viable cell	Early apoptotic cell	Late apoptotic	Necrotic cell
MCF 2 control	95.42	0.38	00.40	03.80
MCF 2 PGDSN	65.00	3.42	21.76	09.82
PC3 control	92.42	2.84	03.00	01.74
PC3 PGDSN	80.32	2.24	04.02	13.42
A 549 control	92.76	0.19	06.09	00.96
A 549 PGDSN	70.97	4.84	20.52	03.67
HEPG2 control	96.86	1.28	00.46	01.40
HEPG2 PGDSN	84.66	4.46	06.68	04.20
HeLa control	96.60	0.14	01.68	01.58
HeLa PGDSN	80.94	3.02	04.44	11.62

PGDSN caused decrease in viable cancer cells, increase in the number of late apoptotic and necrotic cells suggesting to possess apoptotic activity.

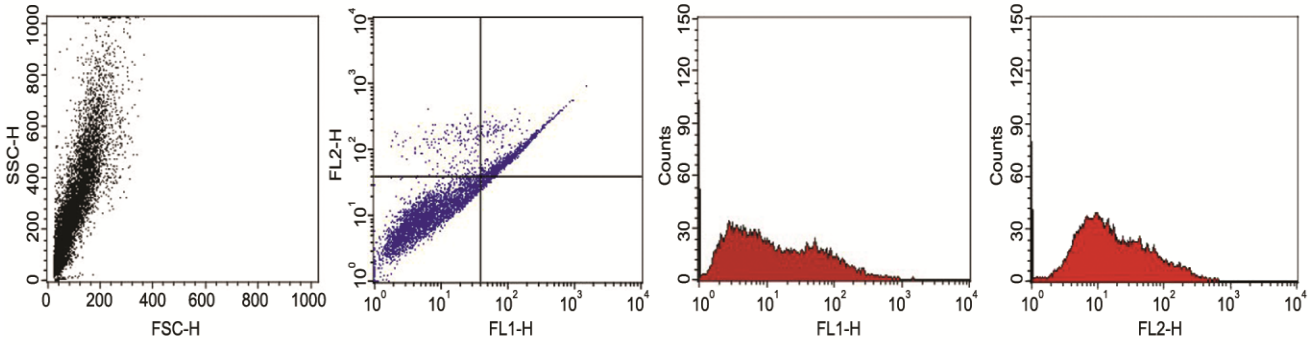
Pomegranate extracts and their constituents are known to exert their activity by diverse mechanisms and are known to inhibit angiogenesis, proliferation, invasiveness, growth, and also to induce apoptosis[28]. These extracts (juice, seed oil, peel) individually and upon combinatorial treatment are reported to inhibit tumour growth. Interestingly, the combinatorial treatment was found to be more effective than treatment with a single extract[29]. Several *in vivo* studies have also elucidated the potential anticancer mechanism of the extracts[30,31].



Quadrant statistics

Quad	% Gated	% Total	X Mean	Y Mean
UL	0.96	0.96	23.33	92.26
UR	6.09	6.09	218.58	246.19
LL	92.76	92.76	2.90	2.63
LR	0.19	0.19	44.68	32.60

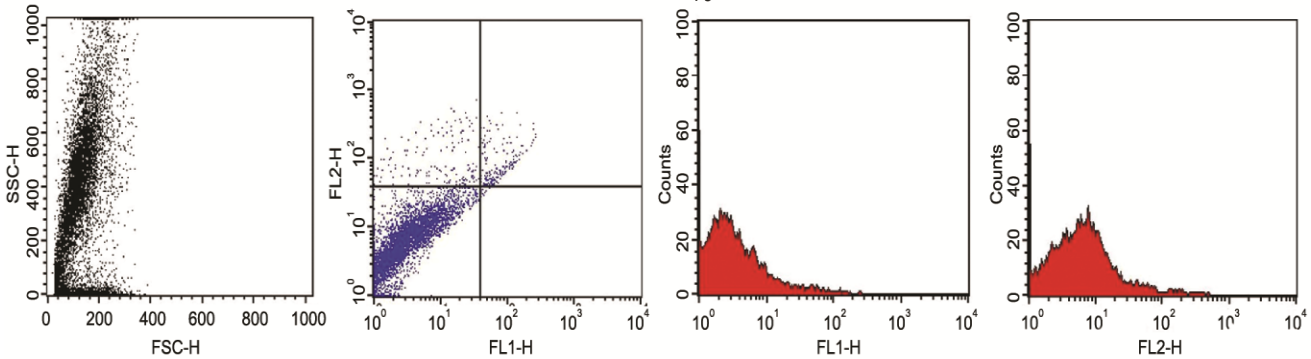
A-549 untreated cell



Quadrant statistics

Quad	% Gated	% Total	X Mean	Y Mean
UL	3.67	3.67	19.06	122.35
UR	20.52	20.52	132.21	101.48
LL	70.97	70.97	9.12	11.41
LR	4.84	4.84	46.83	33.08

A-549 cells treated with 320 µg/mL of PGDSN

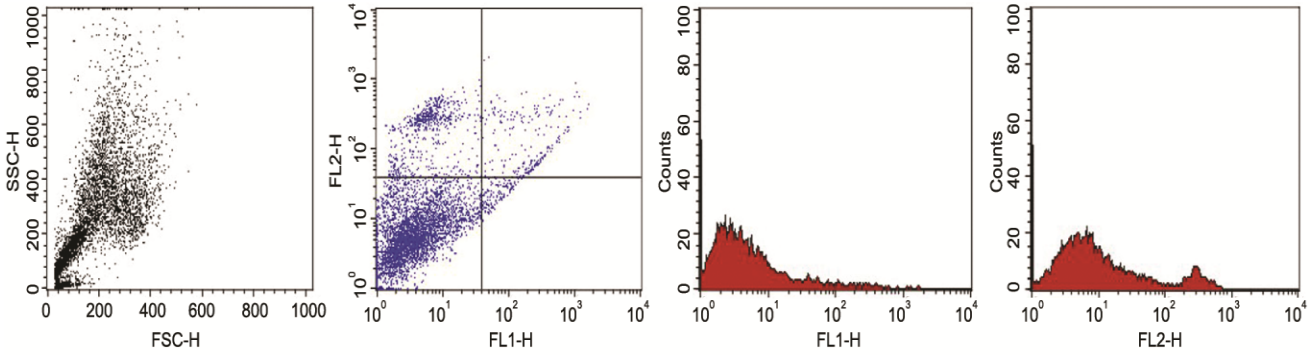


Quadrant statistics

Quad	% Gated	% Total	X Mean	Y Mean
UL	1.58	1.58	14.35	125.04
UR	1.68	1.68	84.91	108.76
LL	96.60	96.60	2.75	4.85
LR	0.14	0.14	45.27	33.95

HeLa untreated cell

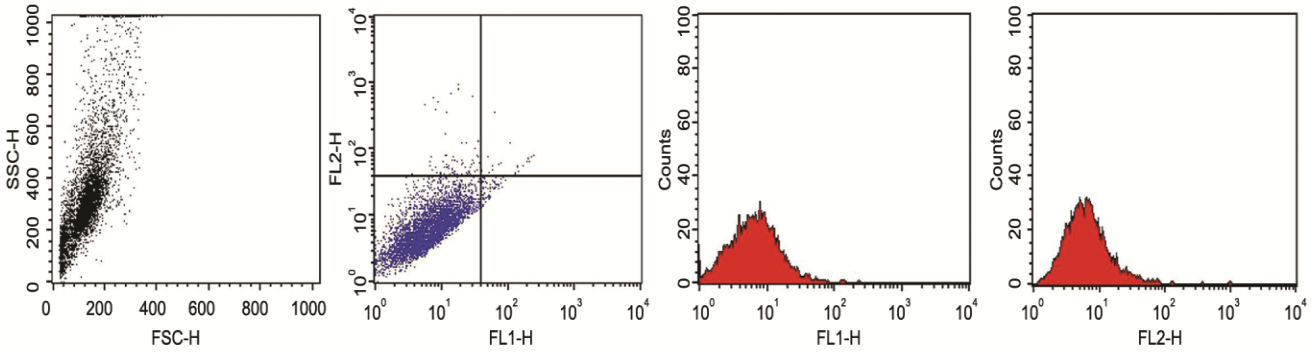
(*Contd*.)



Quadrant statistics

Quad	% Gated	% Total	X Mean	Y Mean
UL	11.60	11.60	8.00	237.45
UR	4.44	4.44	275.82	202.85
LL	80.94	80.94	4.96	7.57
LR	3.02	3.02	71.51	23.37

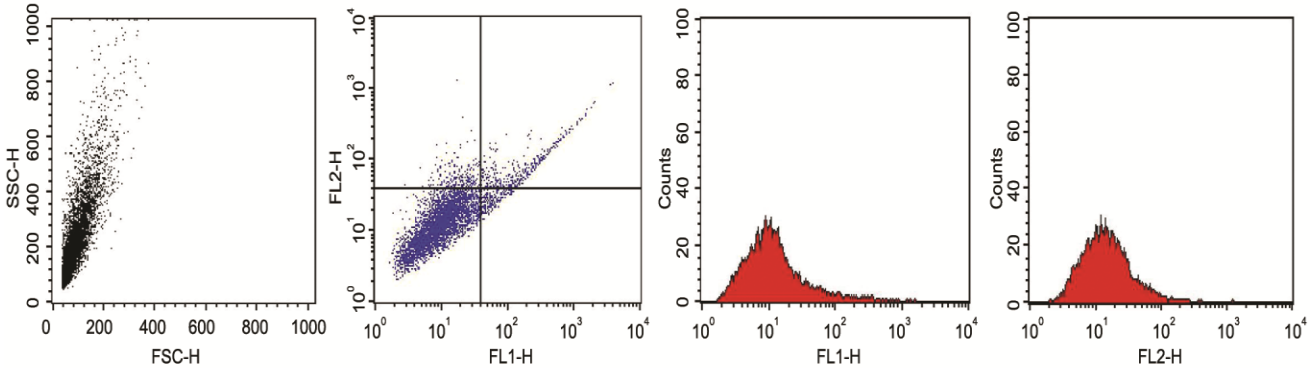
HeLa cells treated with 320 µg/mL of PGDSN



Quadrant statistics

Quad	% Gated	% Total	X Mean	Y Mean
UL	1.40	1.40	17.60	141.83
UR	0.46	0.46	121.15	73.24
LL	96.86	96.86	7.90	7.46
LR	1.28	1.28	53.34	25.63

HEP-G2 untreated cell

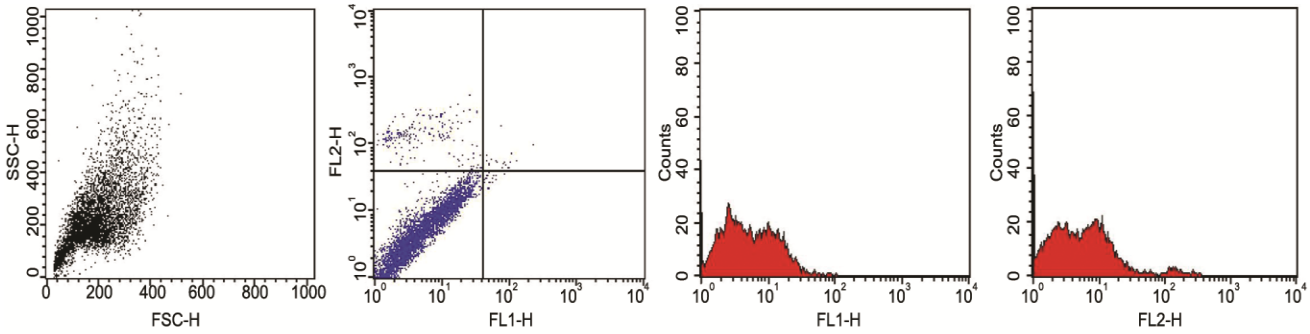


Quadrant statistics

Quad	% Gated	% Total	X Mean	Y Mean
UL	4.20	4.20	19.67	69.21
UR	6.68	6.68	272.79	105.93
LL	84.66	84.66	10.80	13.36
LR	4.46	4.46	60.58	27.04

HEP-G2 cells treated with 320 µg/mL of PGDSN

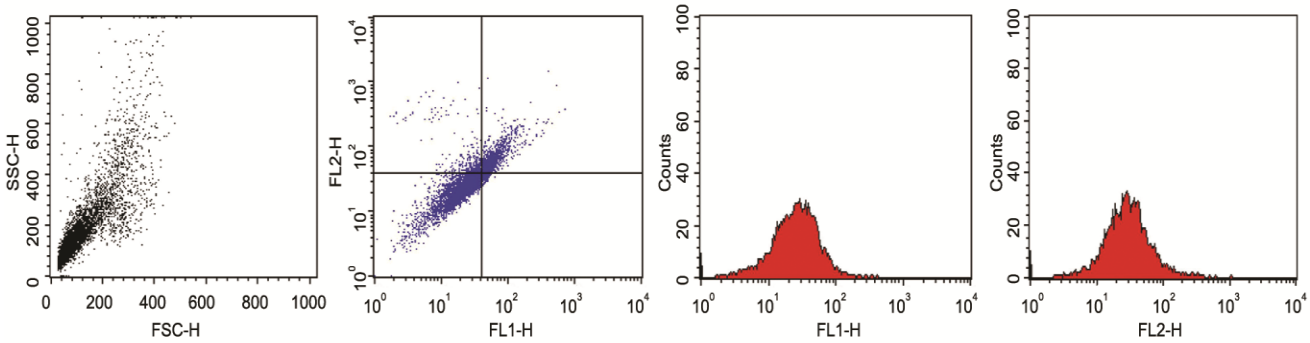
(*Contd*.)



Quadrant statistics

Quad	% Gated	% Total	X Mean	Y Mean
UL	3.80	3.80	8.79	149.02
UR	0.40	0.40	81.39	60.78
LL	95.42	95.42	7.22	6.81
LR	0.38	0.38	53.66	30.07

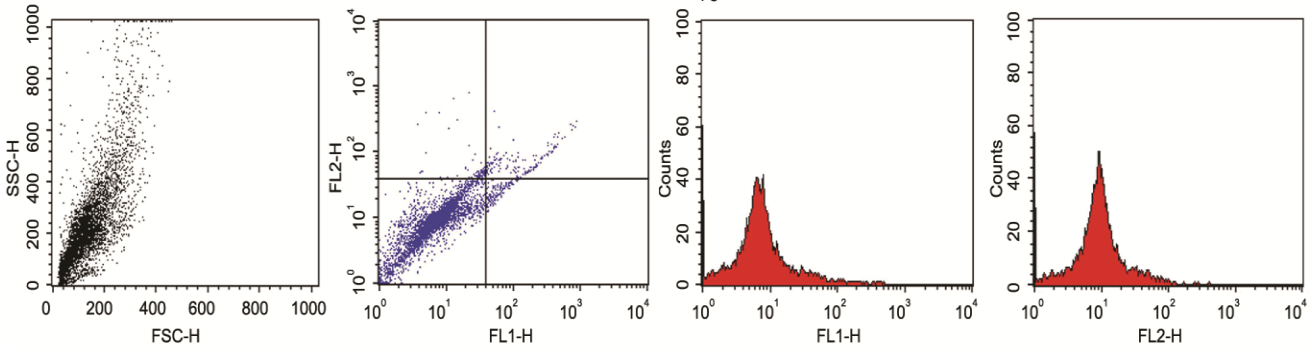
MCF-7 untreated cell



Quadrant statistics

Quad	% Gated	% Total	X Mean	Y Mean
UL	9.82	9.82	29.26	84.41
UR	21.76	21.76	69.86	79.09
LL	65.00	65.00	20.01	21.35
LR	3.42	3.42	45.69	34.25

MCF-7 cells treated with 320 µg/mL of PGDSN



Quadrant statistics

Quad	% Gated	% Total	X Mean	Y Mean
UL	1.74	1.74	27.30	81.70
UR	3.00	3.00	183.23	84.04
LL	92.42	92.42	7.81	9.46
LR	2.84	2.84	60.40	24.70

PC-3 untreated cell

(*Contd*.)

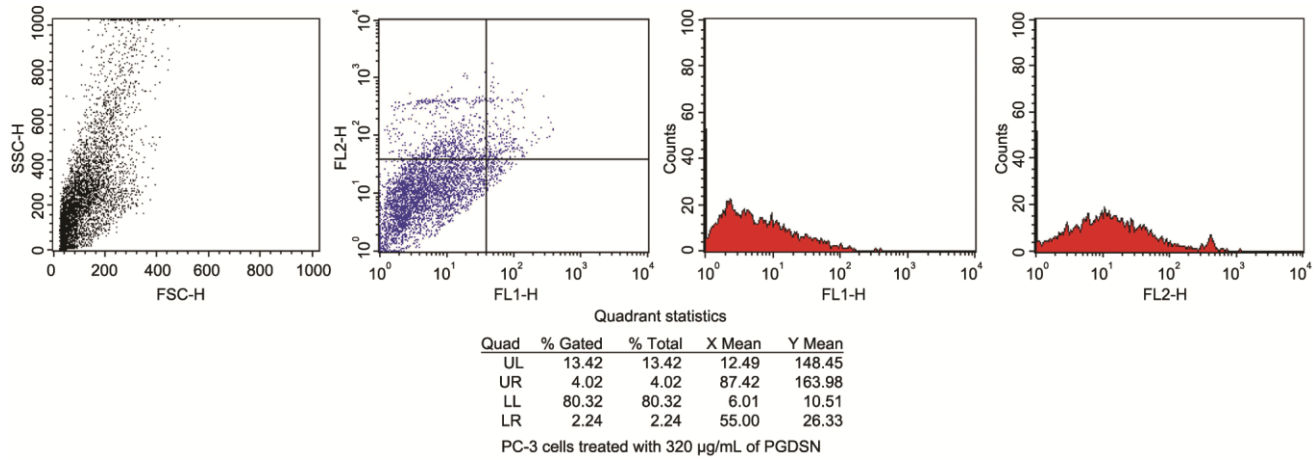


Fig. 9 — Flow cytometry analysis of apoptosis in A 549 cells

As suggested from the results and previous studies on *P. granatum* it has been shown that silver nanoparticle has very good activity against cell lines. It is well known that a high amount of ROS generation could lead to apoptotic and necrotic cell death[32]. Excessive ROS generation has been linked with substantial DNA damage and apoptosis/necrosis[31]. The present results are well in accordance with the recent reports that have shown apoptosis cell death due to the exposure of nanoparticles including the exposure of plant-synthesized silver nanoparticles[33].

Conclusion

The cytotoxicity study, inhibition of cell division, and an increase in the number of late apoptotic cell and necrotic cells suggest the role of silver nanoparticles in the prevention of proliferation of cancer cells. In the future, we have planned to target the silver nanoparticle at the site of the cancer cell itself. Thus, silver nanoparticles synthesized using the dried peel of *P. granatum* PGDSN is a promising agent for nano chemoprevention of various cancer cells used for the study.

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Ethical issues

There is none to be applied.

Conflict of interest

None to be declared.

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