Chapter 7

IN VITRO

CYTOTOXICITY STUDY

7.1 Introduction

Isolated tumor cells which are cultured in *in vitro* conditions to investigate the biological mechanism of tumor formation have been used widely. This technique is also used to access the efficacy of new drug molecules and novel pharmaceutical products. In vitro cell line study results provide an idea about the in vivo fate with regard to the efficacy or toxicity of the developed formulation [1-5].

The developed spherulites formulation separately loaded with Gemcitabine Hydrochloride (GCH) and Vinorelbine tartrate (VLB) was intended to be delivered by intravenous route to animals. However, it was necessary that the formulations should be evaluated for their safety and efficacy by performing in vitro cell line studies. The aim of present study was to evaluate the cell cytotoxicity of the developed formulation by performing MTT assay, cell cycle analysis and apoptosis studies by FACS analysis.

7.1.1 Cytoxicity study by MTT Assay

The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, there is a reduction in cell viability. The MTT Reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation.

The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric method [6].

7.1.2 Cell Cycle analysis and Apoptosis study

The life cycle of an individual cell consists of series of events which leads to its division and replication of DNA to form two daughter cells. Flow cytometry is the tool to analyze the DNA distribution when the cell is in replication state. Normal cell cycle consists of 4 phases; i) G1 phase: Metabolic changes prepare the cell for division. At a certain point - the restriction point - the cell is committed to division. G1 phase will have 1X fluorescence intensity as it contains one set of DNA. ii) S phase. DNA synthesis replicates the genetic material. Each chromosome now consists of two sister chromatids, having fluorescence

intensity between 1X and 2X. iii) G2 phase: Metabolic changes assemble the cytoplasmic materials necessary for mitosis and cytokinesis. iv) M phase: A nuclear division (mitosis) followed by a cell division (cytokinesis). Both G2 and M phase will have 2X fluorescence intensity as DNA gets replicated in two sets. Figure 7.1 is the schematic representation of cell cycle and its Flow cytometry analysis.

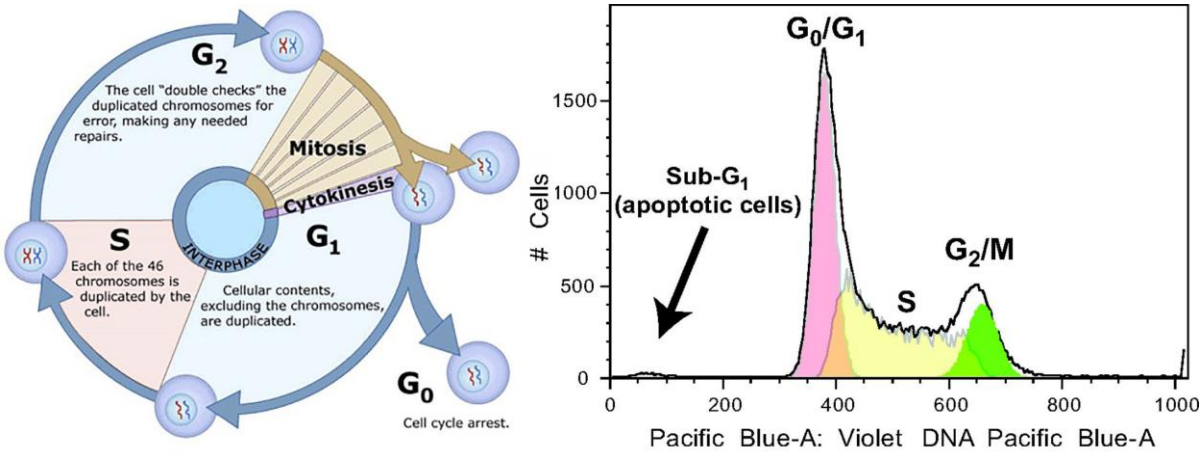


Figure 7.1: Schematic representation of steps involved in cell cycle and its analysis by using DNA intercalating florescence probe in flow cytometry.

Apoptosis is a normal homeostatic process to maintain cell population in tissues, however, this mechanism is altered when a cell mutates and starts multiplying abnormally. Apoptosis study was carried out by using Propidium Iodide (PI) staining procedure. Since PI is economical and stable, it is the first choice for nuclear staining over other stains. Moreover, PI is an excellent cell viability indicator as it has the ability to enter the cell depending on its permeability. Live or early apoptotic cells have intact plasma membrane which restricts the entry of PI. Whereas, late apoptotic and necrotic cells lose the integrity of plasma and nuclear membranes which allows PI to pass, intercalating the nucleic acids and yielding red colored fluorescence [7].

7.2 Materials and Equipment

7.2.1 Cell Line

A549 (Human adenocarcinomic alveolar basal epithelial cells) were obtained from NCCS, Pune, India. Cell culture was maintained at 37 ºC in a humidified atmosphere (95% air and 5% CO_2) and sub cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml) and 10% FBS.

7.2.2 Materials

DMEM, FBS, Antibiotic antimycotic solution and Trypsin-EDTA were purchased from Himedia lab Pvt. Ltd., Mumbai, India. Tissue culture plates (6-well type and 96-well type), tissue culture flask (25 and 75 cm^2), and other sterile material for cell culture were purchased from Thermo scientific, India. MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and Propidium Iodide (PI) were purchased from Sigma Aldrich, Bengaluru, India. All other chemicals were of analytical reagent grade and obtained commercially.

7.2.3 Equipments

- ELISA plate reader (FLUOstar, Germany)
- Jouan IGO150 5% CO2 incubator (Thermo-Fischer, Germany)
- Weiber vertical Laminar Air Flow (Weiber, India)
- Nikon H600L Microscope (Nikon, Japan)
- Fluorescence activated cell sorter (BD FACSCalibur™, BD Biosciences, USA)

7.3 Methods

7.3.1 Cell Cytotoxicity study by MTT Assay

MTT solution: 5mg/ml MTT solution was prepared in phosphate buffer saline pH 7.4 (PBS). This solution was passed through a 0.2 mm membrane filter and stored at 2-8°C.

GCH and Spherulites formulation solution: A stock solution of GCH was prepared. Briefly, 10 mg of the drug was dissolved in 10 ml sterile PBS pH 7.4. Similarly, the spherulites formulation loaded with GCH was also diluted to obtain a final stock solution of 1000 μg/ml. Appropriate aliquots of the stock solution of GCH and its spherulites formulation were supplemented into the wells containing DMEM to get the final concentration 0.001 μg/ml, 0.01 μg/ml, 0.1 μg/ml, 1 μg/ml and 10 μg/ml of GCH.

VLB and Spherulites formulation solution: A stock solution of VLB was prepared. Briefly, 10 mg of the drug was dissolved in 10 ml sterile PBS pH 7.4. Similarly, the spherulites formulation loaded with VLB was also diluted to obtain a final stock solution of 1000 μg/ml. Appropriate aliquots of the stock solution of VLB and its spherulites formulation were supplemented into the wells containing DMEM to get the final concentration 0.001 μg/ml, 0.01 μg/ml, 0.1 μg/ml, 1 μg/ml and 10 μg/ml of VLB.

Sample Coding:

R1: Standard GCH

R2: GCH Spherulites

R3: Standard VLB

R4: VLB Spherulites

Protocol:

A549 cells were maintained in appropriate conditions and were seeded into 96 well plates at cell density of $3x10^3$ cells/well. Cells were allowed to attach and grow for 24 hours. After 24 hours each well bearing cells was treated with different concentration of test samples viz. R1, R2, R3 and R4, separately and the well plate was incubated at 37°C, 5% CO_2 for 8, 16 and 24 hours. Post incubation, treatment media was removed and 20 µL (5 mg/ml) of MTT reagent was added in each well and allowed to incubate for 4 hours. Subsequently, Culture media was replaced with 200 µL DMSO to dissolve the dark formazan product and read at 570 nm on a plate reader (FLUOstar, Ortenberg, Germany). Percentage inhibition, IC50 values and statistical significance were calculated using GraphPad Prism Version 5.00 for Windows (GraphPad Software, La Jolla, CA, USA).

7.3.2 Apoptosis study and Cell cycle analysis by FACS

Protocol:

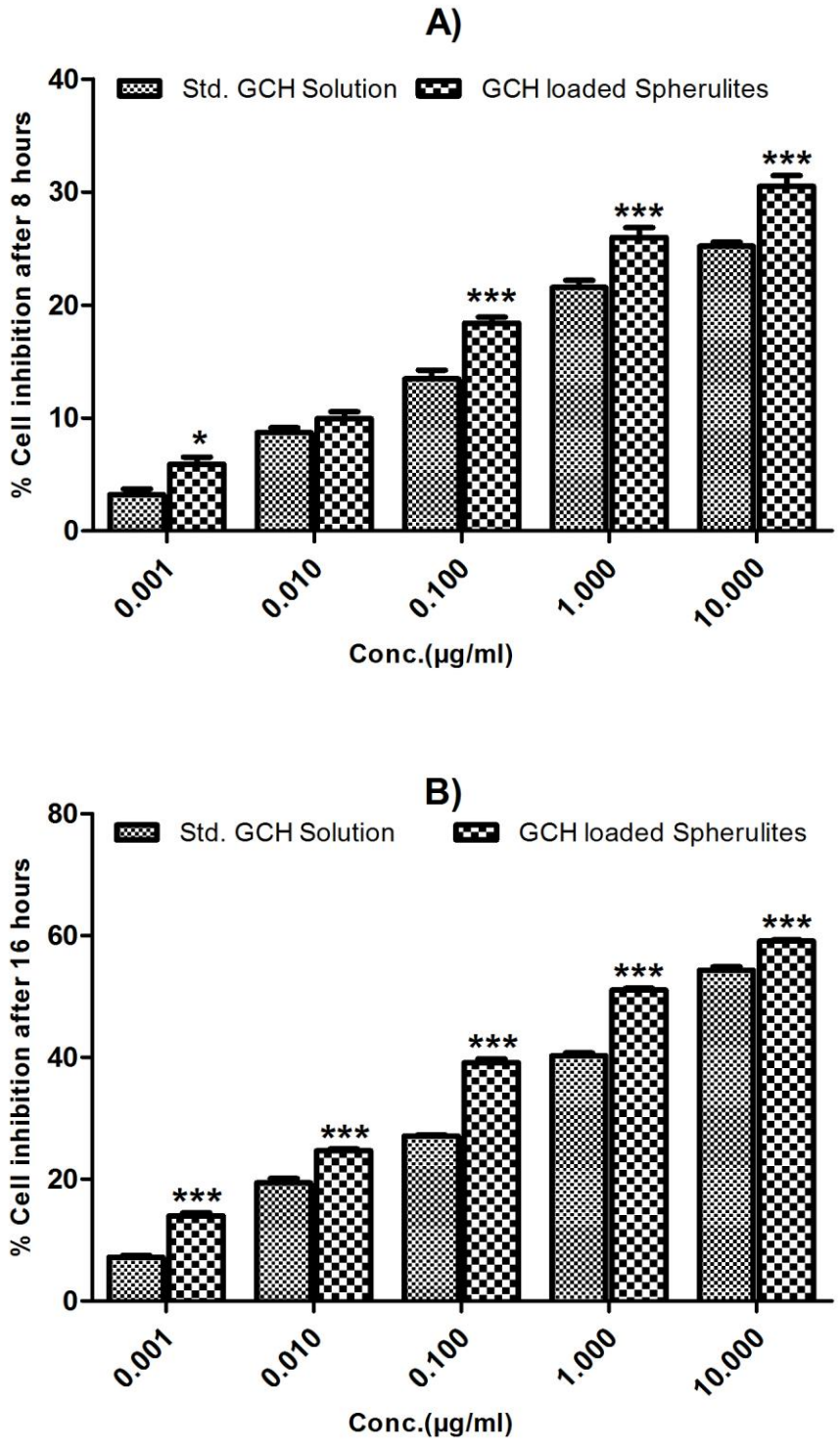
About $1x10^5$ A549 cells were seeded on six well plates and allowed to grow for 24 hours. Cells were treated with 0.1µM of test samples viz. R1, R2, R3 and R4 for 12 hours and 24 hours. Cells were gently trypsinized and washed with PBS pH 7.4 twice. Cells were fixed in methanol and stained with 2 µL of 0.1% propidium iodide (PI) and incubated at 4 ^oC for 10-15 min and the suspension was analyzed for cell cycle and apoptosis on flow cytometer (BD FACSCalibur™; BD Biosciences, Gurgaon, India). Histogram of count vs. intensity was made to calculate ratio of cells under G0/G1 (2n), S (2n+), G2/M phase (4n) and under apoptosis (2n-). Statiscal analysis was performed by applying Two way ANOVA followed by Bonferroni Post-tests using GraphPad Prism Version 5.00 for Windows (GraphPad Software, La Jolla, CA, USA).

7.4 Results and Discussion

7.4.1 Results of MTT Assay

MTT assay is a widely used procedure to evaluate test samples for their ability to cause cell cytotoxicity. MTT assay was performed on A549 cell line and it was found that GCH loaded

spherulites exhibited significantly ($p<0.001$) high degree of cytotoxicity than the standard GCH drug. GCH loaded spherulites required lesser concentration to cause cytotoxicity, whereas, high concentration was required for standard GCH drug. Figure 7.2 depicts the % cell inhibition vs. concentration graph. IC50 values of both the test samples were calculated for each time point. Cell inhibition curves were used to calculate IC50 value.



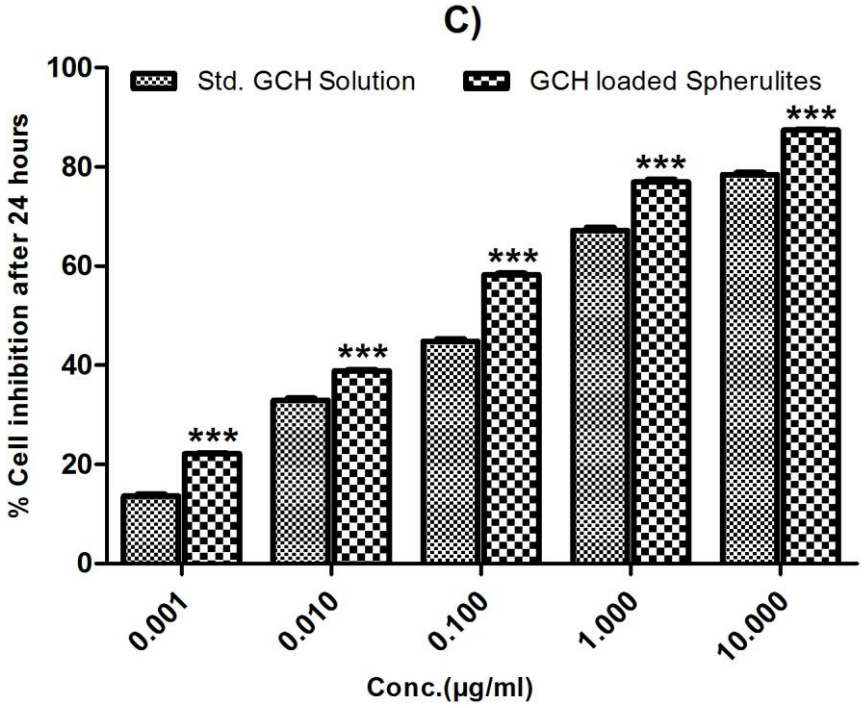


Figure 7.2: Results of cell cytotoxicity by MTT assay A) at 8 hours, B) at 16 hours and C) at 24 hours (where, Standard GCH Solution Vs. GCH loaded Spherulites; *$p<0.05$, *** $p<0.001$). Experiment was performed in triplicate (n=3). Data represents mean±SD.

Figure 7.2 and Table 7.1 shows that increasing incubation time from 8 to 24 hours, the % cell inhibition increased. Cytotoxic effect on A549 cells exerted by GCH loaded spherulites was significantly ($p<0.001$) higher than standard GCH drug at all levels of concentration. Moreover, it was observed that the % cell inhibition was concentration dependent where high concentration of drug showed greater cell inhibition. It is because the cell count in each well remained constant however, the GCH concentration was increased which inhibited cell growth.

Table 7.1: % Cell Inhibition values with respect to time of standard GCH drug and GCH loaded spherulites on A549 cell line.

Concentration (µg/ml)	% Cell Inhibition after 8 hours		% Cell Inhibition after 16 hours		% Cell Inhibition after 24 hours	
	Std GCH	GCH loaded spherulites	Std GCH	GCH loaded spherulites	Std GCH	GCH loaded spherulites
0.001	3.21±0.91	5.89±1.15	7.19±0.52	13.98±0.87	13.64±0.67	22.17±0.34
0.01	8.73±0.72	9.95±1.03	19.41±1.24	24.67±0.54	32.91±0.79	38.87±0.47
0.1	13.47±1.32	18.41±0.87	27.09±0.39	39.15±1.03	44.85±0.73	58.22±0.54
1	21.56±1.09	25.97±1.52	40.28±0.79	51.07±0.46	67.23±0.96	76.98±0.82
10	25.19±0.65	30.51±1.65	54.34±0.95	59.11±0.38	78.42±0.74	87.43±0.27

* Data is represented as Mean± SD (n=3)

IC50 values were calculated for standard GCH drug and GCH loaded spherulites. IC50 values for both the test samples are presented in Table 7.2. The GCH loaded Spherulites had shown better growth-inhibiting activity than the free drug in A549 cell line in terms of dose-dependent and incubation time effects, leading to enhanced antiproliferative activity of the antitumoral compound bearing formulation.

Table 7.2: IC50 values of standard GCH drug and GCH loaded spherulites in A549 cell line.

Sample	IC50 Values (µg/ml)		
	8 hours	16 hours	24 hours
Std GCH	0.2	0.5	0.2
GCH loaded spherulites	0.05	0.04	0.04

Graphical representation of the comparison of IC50 values between standard GCH drug and GCH loaded spherulites is shown in Figure 7.3. Results clearly indicate that the spherulites formulation loaded with GCH have lower IC50 values at all the time points than that of the standard drug. Lower dose ($1/5^{th}$) was required for spherulites formulation for inhibition of 50% total cell population as compared to standard drug. This indicates that GCH-loaded spherulites are more cytotoxic as lesser concentration of formulation is being able to inhibit the 50% cell population than as compared to GCH plain drug. Moreover, colloidal systems are readily internalized by cancer cells via endocytosis. The spherical shape of the vesicles

influence the rate of endocytosis. The spherical shape of spherulites contributes more to its cellular uptake than the plain drug showing higher cytotoxicity [8].

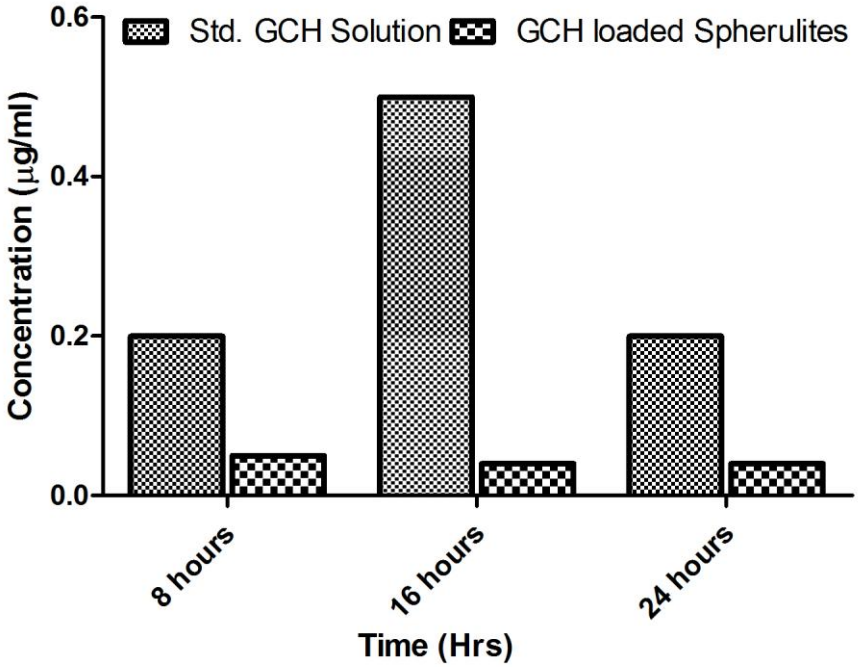
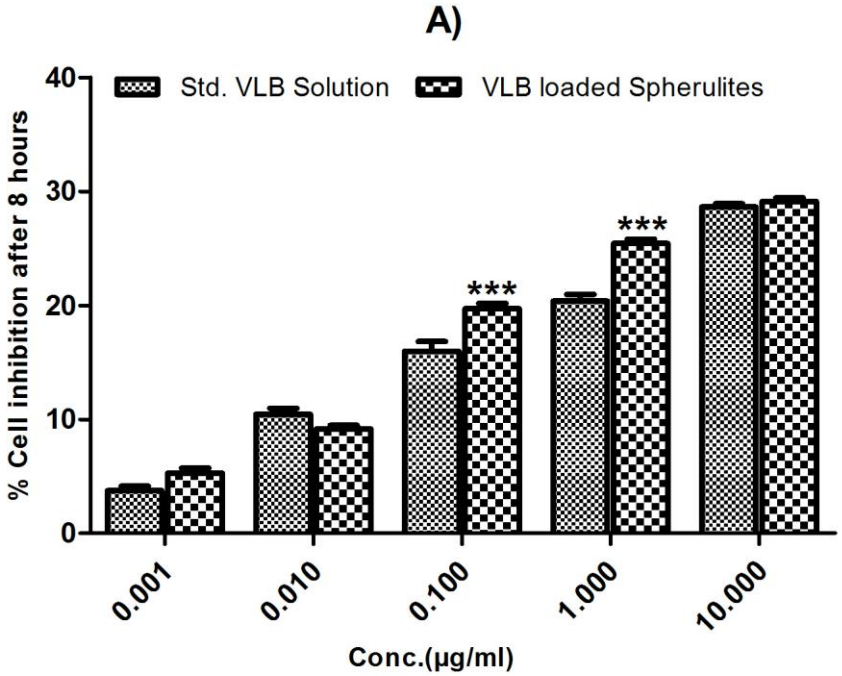
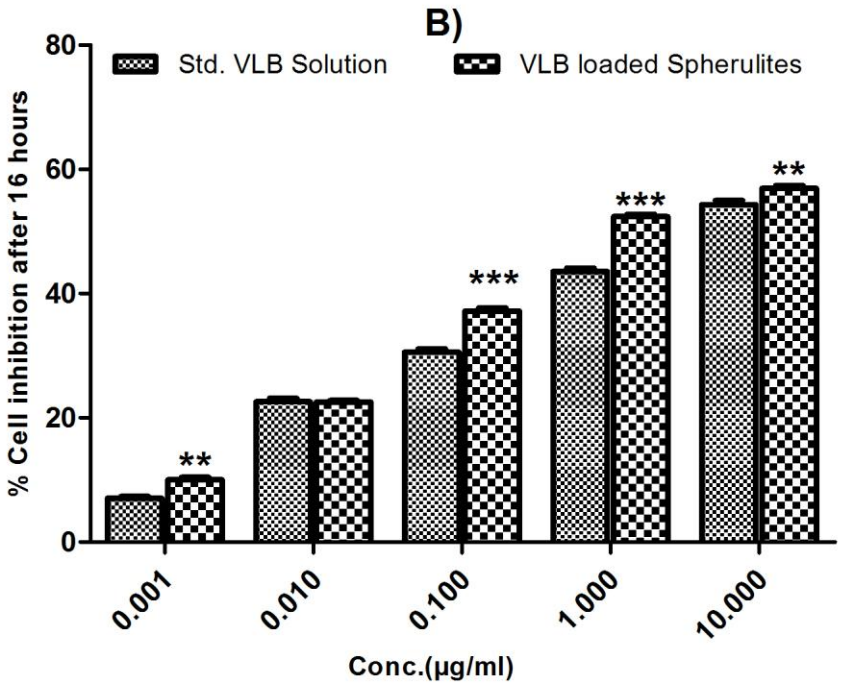


Figure 7.3: IC50 values with respect to time of standard GCH drug and GCH loaded spherulites in A549 cell line.

Table 7.3 shows that VLB loaded Spherulites displayed more antiproliferative activity than standard VLB drug at all the time points. VLB loaded spherulites required lesser concentration to cause cytotoxicity, whereas, high concentration was required for standard VLB drug. Figure 7.4 depicts the % cell inhibition vs. concentration graph. IC50 values of both the test samples were calculated for each time point. Cell inhibition curves were used to calculate IC50 value.


A)
Std. VLB Solution
VLB loaded Spherulites
% Cell inhibition after 8 hours
40
30
20
10
0
***
***
0.001
0.010
0.100
1.000
10.000
Conc.(µg/ml)


B)
Std. VLB Solution
VLB loaded Spherulites
% Cell inhibition after 16 hours
80
60
40
20
0
**
***
***
**
0.001
0.010
0.100
1.000
10.000
Conc.(µg/ml)

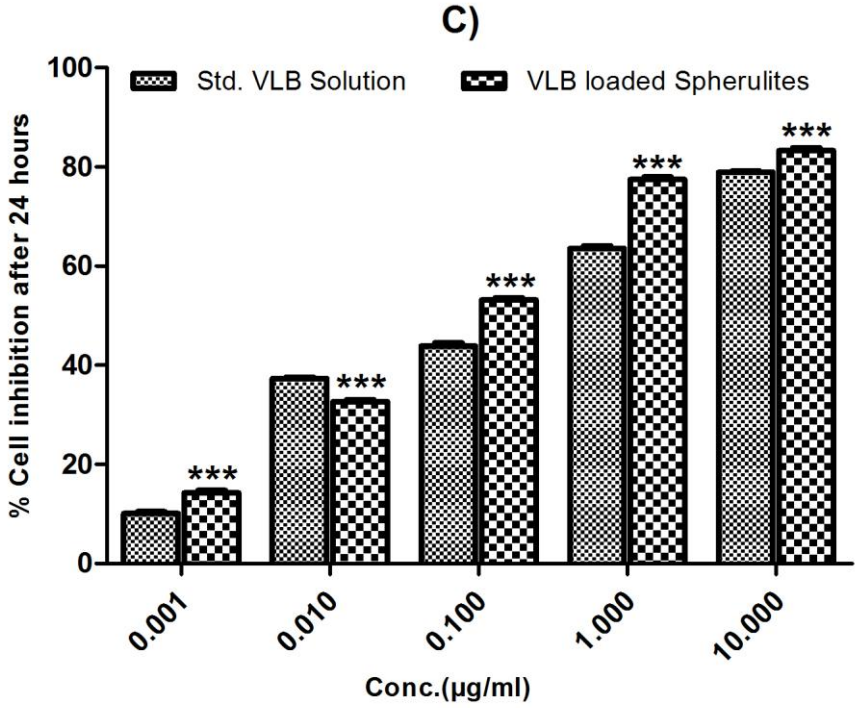


Figure 7.4: Results of cell cytotoxicity by MTT assay A) at 8 hours, B) at 16 hours and C) at 24 hours (where, Standard VLB Solution Vs. VLB loaded Spherulites; **p<0.01, *** p<0.001). Experiment was performed in triplicate (n=3). Data represents mean±SD.

Table 7.3: % Cell Inhibition values with respect to time of standard VLB drug and VLB loaded spherulites on A549 cell line.

Concentration (µg/ml)	% Cell Inhibition after 8 hours		% Cell Inhibition after 16 hours		% Cell Inhibition after 24 hours	
	Std VLB	VLB loaded spherulites	Std VLB	VLB loaded spherulites	Std VLB	VLB loaded spherulites
0.001	3.75±0.67	5.29±0.79	7.04±0.57	10.02±0.69	10.08±0.68	14.27±0.91
0.01	10.44±0.93	9.15±0.54	22.57±1.03	22.56±0.39	37.32±0.51	32.66±0.67
0.1	15.96±1.51	19.72±0.82	30.61±0.84	37.19±0.93	43.90±1.09	53.18±0.72
1	20.37±1.03	25.43±0.68	43.58±0.97	52.39±0.74	63.57±0.82	77.48±0.83
10	28.63±0.49	29.11±0.59	54.34±1.15	56.95±0.76	78.94±0.46	83.27±1.02

* Data is represented as Mean± SD (n=3)

Figure 7.4 and Table 7.3 shows that increasing incubation time from 8 to 24 hours, the % cell inhibition increased. Cytotoxic effect on A549 cells exerted by VLB loaded spherulites was significantly ($p<0.001$) higher than standard VLB drug at all levels of concentration.

Moreover, it was observed that the % cell inhibition was concentration dependent where high concentration of drug showed greater cell inhibition. It is because the cell count in each well remained constant however, the VLB concentration was increased which inhibited cell growth.

IC50 values were calculated for standard VLB drug and VLB loaded spherulites. IC50 values for both the test samples are presented in Table 7.4. The VLB loaded Spherulites had shown better growth-inhibiting activity than the free drug in A549 cell line in terms of dose-dependent and incubation time effects, leading to enhanced antiproliferative activity of the antitumoral compound bearing formulation.

Table 7.4: IC50 values of standard VLB drug and VLB loaded spherulites in A549 cell line.

Sample	**IC50 Values (µg/ml)**		
	8 hours	**16 hours**	**24 hours**
Std VLB	0.2	0.2	0.3
VLB loaded spherulites	0.04	0.04	0.04

Graphical representation of the comparison of IC50 values between standard VLB drug and VLB loaded spherulites is shown in Figure 7.5. Results clearly indicate that the spherulites formulation loaded with VLB have lower IC50 values at all the time points than that of the standard drug. Lower dose ($1/5^{th}$) was required for spherulites formulation for inhibition of 50% total cell population as compared to standard drug. Spherulites significantly exhibited lower IC50 value which indicated that VLB loaded spherulites require lesser concentration to inhibit 50% cells as compared to VLB plain solution. Vesicles having spherical shape have a major effect on cellular uptake. Cell uptake is dependent on morphology of the vesicle, as, spherical shaped particle shows greater uptake than irregularly shaped particles. Due to this reason VLB loaded spherulites showed more cell cytotoxicity than plain drug solution. [9].

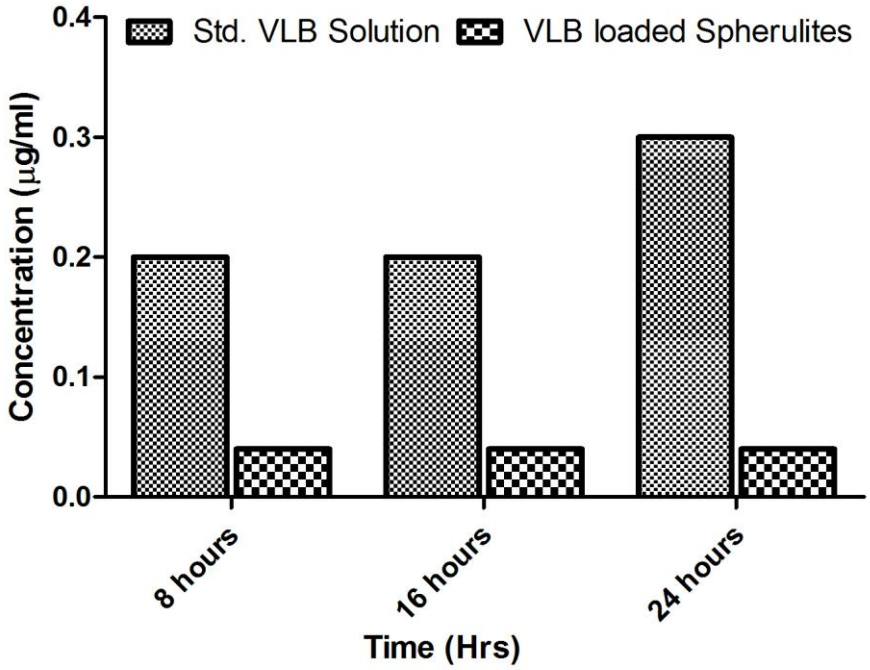


Figure 7.5: IC50 values with respect to time of standard VLB drug and VLB loaded spherulites in A549 cell line.

7.4.2 Results of Apoptosis study and Cell Cycle Analysis

Apoptosis study was performed by staining A549 cells using PI to access the degree of apoptosis or necrosis caused by anticancer drug loaded spherulites. PI have the property to penetrate through the membrane of dead or damaged cell membrane, which is measured upon the intensity of fluorescence and identification of cell population showing apoptosis or necrosis. Histogram shown in Figure 7.7 and 7.9 depicts that more cells were found in apoptotic phase treated with spherulites formulation loaded with both the drugs separately than cells treated with standard drug. Formulation exhibited higher degree of apoptosis which can be explained by higher uptake of spherulites within the cells as compared to the standard drug. The exact mechanism of cellular uptake of spherulites is unknown, however, numerous mechanisms possible for it includes; endocytosis, micro pinocytosis, diffusion of particles through cell membrane, channel facilitated entrance or further by adhesive interactions as for instance electrostatic forces, Van der Waals or steric interactions [10-12]. Moreover, exposure time of test samples contributed significantly in the apoptosis of cells. Separately loaded formulations with GCH and VLB yielded the results which shows significant increase in apoptosis than standard drug.

Cultured population of cells are heterogeneous and asynchronous, in other words, cells subpopulations are composed of different phases of cell cycle [13]. GCH is a S-phase cell cycle specific drug i.e. it caused cell cycle arrest in S-phase. Results shown in Table 7.5 and Figure 7.6 indicate that GCH loaded spherulites exhibited significantly ($p<0.001$) higher apoptosis than standard drug. Higher cellular uptake of formulation was responsible for apoptosis as cell counts in the cell cycle G0-G1, S and G2-M phases were significantly reduced ($p<0.001$). GCH arrests cell cycle in S-phase by inhibition of cellular DNA synthesis. In cells, drug is converted to its triphosphate form, which gets incorporated into DNA and terminates DNA strand elongation. After incorporation of gemcitabine nucleotide into the DNA strand, one more deoxynucleotide is incorporated, and thereafter the DNA polymerases are unable to proceed ("masked chain termination"). It also inhibits DNA synthesis indirectly via inhibition of ribonucleotide reductase [14]. Histogram shown in Figure 7.7 also depicts the higher apoptosis in formulation followed by plain drug solution.

Table 7.5: Cell Cycle Analysis of A549 cell line after treatment with untreated cells as control, Standard GCH (Std GCH) and GCH loaded spherulites, by PI staining using FACS technique.

Sample	**Apoptosis**		**G0-G1**		**S-Phase**		**G2-M Phase**	
	12 hrs	**24 hrs**	**12 hrs**	**24 hrs**	**12 hrs**	**24 hrs**	**12 hrs**	**24 hrs**
UT*	7.39± 1.37	9.44± 1.03	58.67± 2.61	54.38± 1.21	15.90± 1.89	17.2± 0.63	16.43± 2.03	14.54± 1.32
Std GCH	34.5± 1.97	73.71± 1.15	43.04± 2.03	17.79± 1.87	13.19± 1.65	8.44± 0.94	9.79± 1.57	2.65± 0.86
GCH Spherulites	52.7± 2.19	93.48± 1.61	32.43± 0.83	2.98± 0.97	8.73± 0.47	0.7± 0.39	4.67± 0.64	0.65± 0.43

*UT stands for Untreated sample/Control; data represents the cell counts after 12 hours and 24 hours of treatment. Experiment was performed in triplicate (mean±SD)

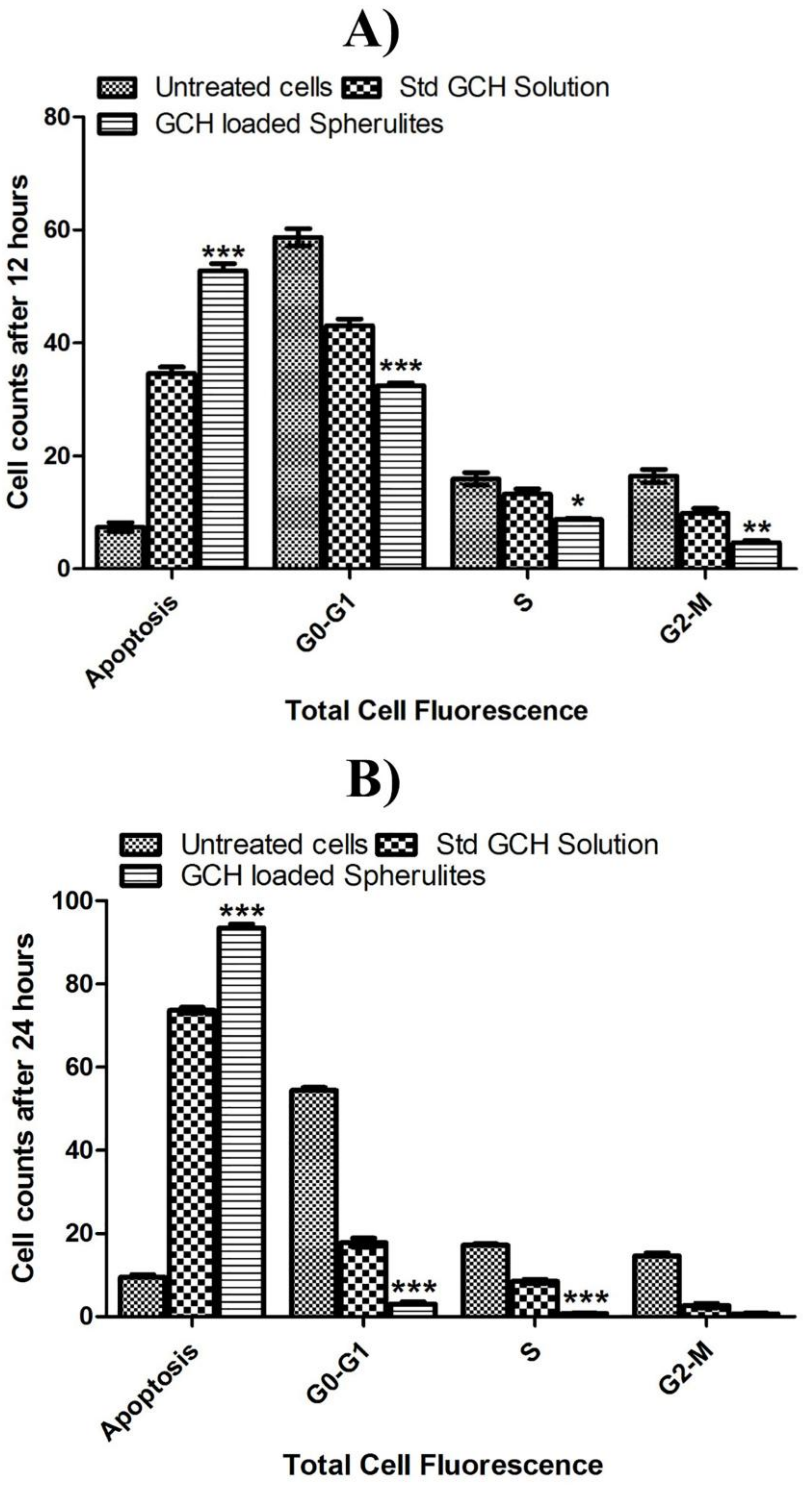


Figure 7.6: Graphical representation of Cell Cycle Analysis of A549 cell line with respect to time A) After 12 hours and B) After 24 hours by PI staining using FACS technique (where, Standard GCH Solution Vs. GCH loaded Spherulites; *p<0.05, **p<0.01, ***p<0.001) Experiment was performed in triplicate (n=3). Data represents mean±SD.

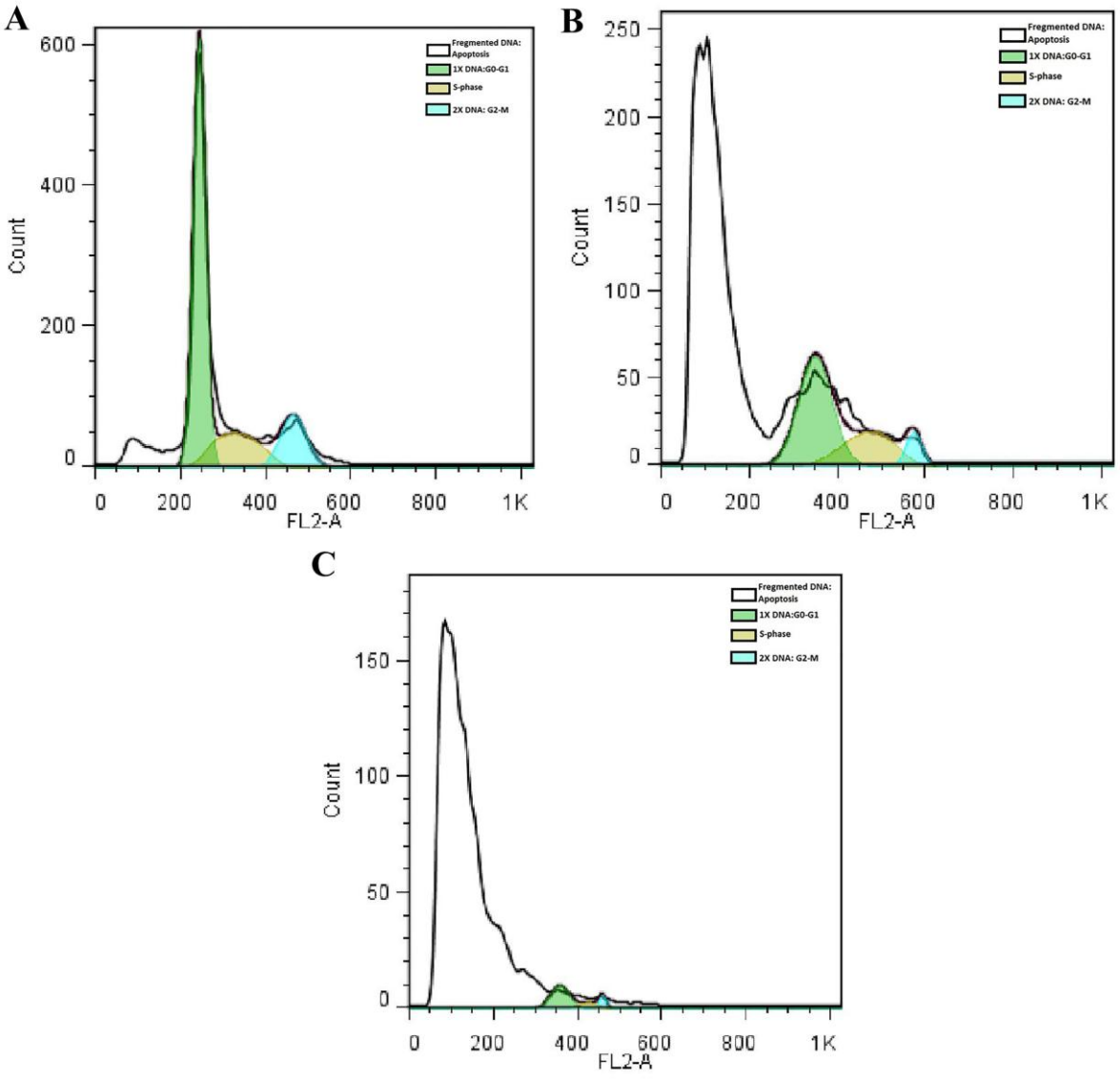


Figure 7.7: Cell cycle analysis of A549 cells; where A) Untreated cells, B) Cells treated with Std GCH, C) Cells treated with GCH loaded spherulites.

VLB induces cytotoxicity by inhibiting the polymerisation of tubulin dimers into microtubules, which in turn disrupts mitotic spindle formation and prevent cell division, which promotes apoptosis in cancer cells. VLB arrests cell cycle in M phase [15]. Results shown in Table 7.6 and Figure 7.8 indicate the VLB loaded spherulites exhibited significantly ($p<0.001$) higher apoptosis than standard drug solution. Histogram shown in Figure 7.9 also depicts the higher apoptosis in formulation followed by plain drug solution.

Table 7.6: Cell Cycle Analysis of A549 cell line after treatment with Untreated cells as control, Standard VLB (Std VLB) and VLB loaded spherulites, by PI staining using FACS technique.

Sample	Apoptosis		G0-G1		S-Phase		G2-M Phase	
	12 hrs	**24 hrs**	**12 hrs**	**24 hrs**	**12 hrs**	**24 hrs**	**12 hrs**	**24 hrs**
UT*	7.39± 1.37	9.44± 1.03	58.67± 2.61	54.38± 1.21	15.90± 1.89	17.2± 0.63	16.43± 2.03	14.54± 1.32
Std VLB	9.67± 0.83	22.21± 0.97	57.40± 1.37	49.68± 1.20	20.78± 0.79	13.5± 0.39	12.54± 0.82	15.99± 1.03
VLB Spherulites	19.94± 0.42	37.13± 0.68	54.89± 1.13	46.29± 0.97	11.29± 0.63	7.57± 0.37	14.65± 1.01	7.84± 0.92

*UT stands for Untreated sample/Control; data represents the cell counts after 12 hours and 24 hours of treatment. Experiment was performed in triplicate (mean±SD)

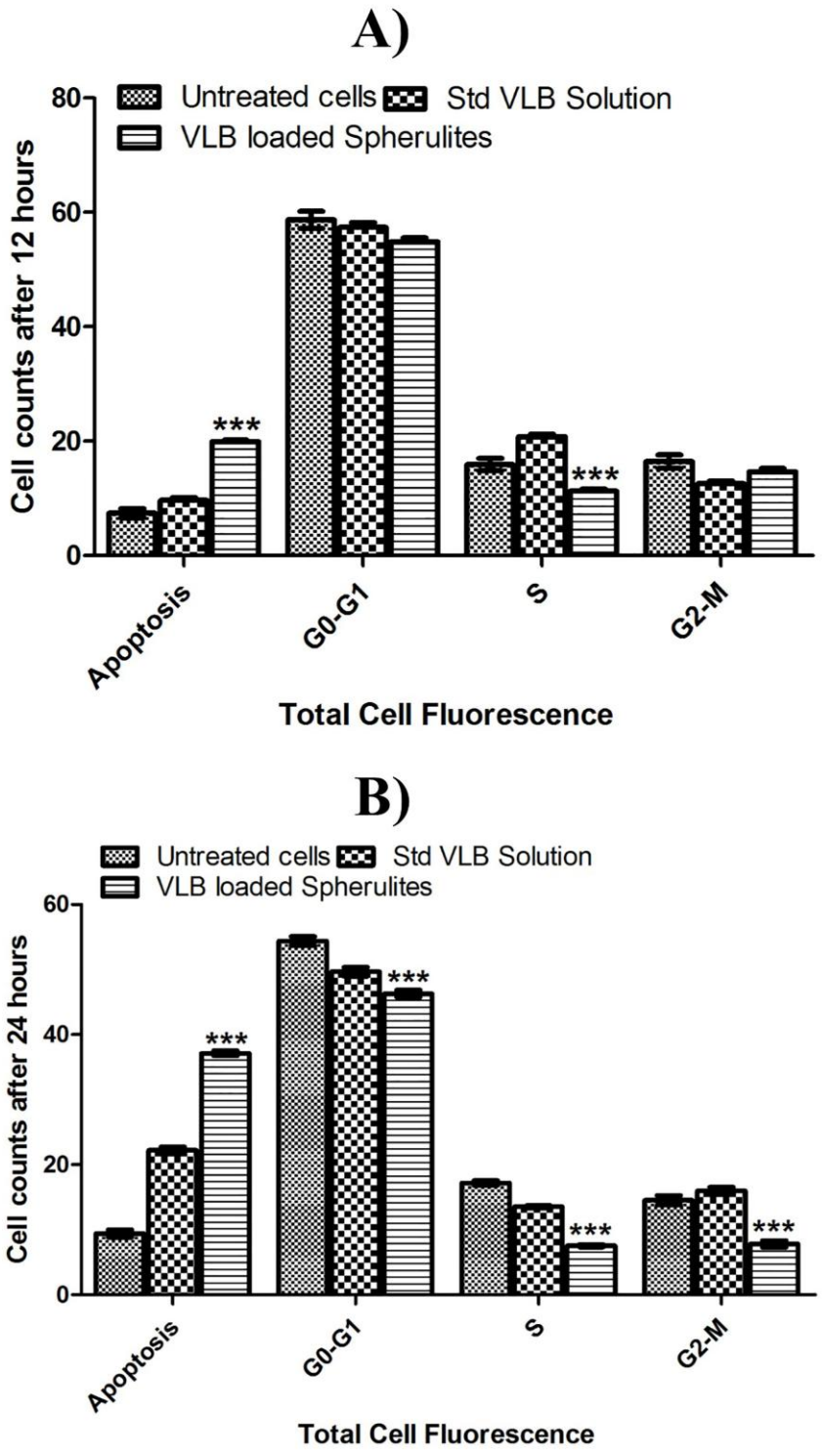


Figure 7.8: Graphical representation of Cell Cycle Analysis of A549 cell line with respect to time A) After 12 hours and B) After 24 hours by PI staining using FACS technique (where, Standard VLB Solution Vs. VLB loaded Spherulites; *** $p<0.001$). Experiment was performed in triplicate (n=3). Data represents mean±SD.

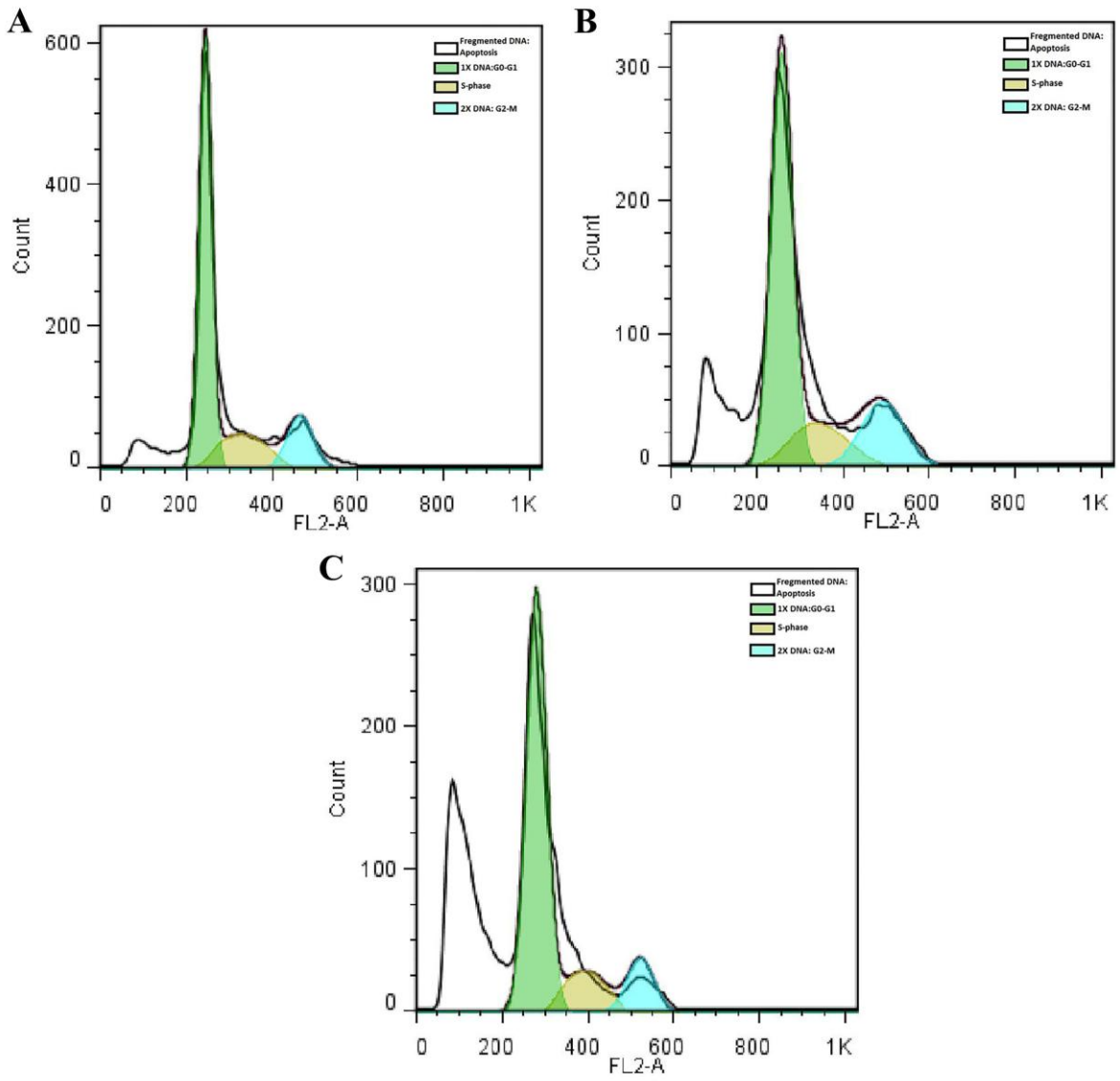


Figure 7.9: Cell cycle analysis of A549 cells; where A) Untreated cells, B) Cells treated with Standard VLB, C) Cells treated with VLB loaded spherulites.

7.5 References

1. Louzada, S., Adega, F., & Chaves, R. (2012). Defining the sister rat mammary tumor cell lines HH-16 cl. 2/1 and HH-16. cl. 4 as an in vitro cell model for Erbb2. PloS one, 7(1), e29923.
2. Vargo-Gogola, T., & Rosen, J. M. (2007). Modelling breast cancer: one size does not fit all. Nature reviews. Cancer, 7(9), 659.
3. Nakatsu, N., Yoshida, Y., Yamazaki, K., Nakamura, T., Dan, S., Fukui, Y., & Yamori, T. (2005). Chemosensitivity profile of cancer cell lines and identification of genes

determining chemosensitivity by an integrated bioinformatical approach using cDNA arrays. Molecular cancer therapeutics, 4(3), 399-412.

4. Kao, J., Salari, K., Bocanegra, M., Choi, Y. L., Girard, L., Gandhi, J., ... & Minna, J. D. (2009). Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. PloS one, 4(7), e6146.
5. Van Staveren, W. C. G., Solís, D. W., Hebrant, A., Detours, V., Dumont, J. E., & Maenhaut, C. (2009). Human cancer cell lines: Experimental models for cancer cells in situ? For cancer stem cells?. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 1795(2), 92-103.
6. Available online: https:// www.atcc.org/~/ media/ DA5 285A1F52C414E8 64C966FD78 C9A79.ashx. (Accessed on October 10, 2017).
7. Crowley, L. C., Scott, A. P., Marfell, B. J., Boughaba, J. A., Chojnowski, G., & Waterhouse, N. J. (2016). Measuring cell death by propidium iodide uptake and flow cytometry. Cold Spring Harbor Protocols, 2016(7), pdb-prot087163.
8. Dhande, R., Tyagi, A., Sharma, R. K., & Thakkar, H. (2017). Biodistribution study of ^{99m}Tc-gemcitabine-loaded spherulites in Sprague–Dawley rats by gamma scintigraphy to investigate its lung targeting potential. Journal of microencapsulation, 34(7), 623-634.
9. Dhande, R., Tyagi, A., Sharma, R. K., & Thakkar, H. (2018). ^{99m}Tc-vinorelbine tartrate loaded spherulites: Lung disposition study in Sprague-Dawley rats by gamma scintigraphy. Pulmonary Pharmacology & Therapeutics (10.1016/j.pupt.2018.01.002).
10. Geiser, M., Rothen-Rutishauser, B., Kapp, N., Schürch, S., Kreyling, W., Schulz, H., ... & Gehr, P. (2005). Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. Environmental health perspectives, 113(11), 1555.
11. Rimai, D. S., Quesnel, D. J., & Busnaina, A. A. (2000). The adhesion of dry particles in the nanometer to micrometer-size range. Colloids and surfaces A: Physicochemical and engineering aspects, 165(1), 3-10.
12. Rothen-Rutishauser, B., Schurch, S., & Gehr, P. (2007). Interaction of particles with membranes.
13. Miao, X., Koch, G., Ait-Oudhia, S., Straubinger, R. M., & Jusko, W. J. (2016). Pharmacodynamic modeling of cell cycle effects for gemcitabine and trabectedin combinations in pancreatic cancer cells. Frontiers in pharmacology, 7.

14. Huang, P., & Plunkett, W. (1995, August). Induction of apoptosis by gemcitabine. In Seminars in oncology (Vol. 22, No. 4 Suppl 11, pp. 19-25).
15. Faller, B. A., & Pandit, T. N. (2011). Safety and efficacy of vinorelbine in the treatment of non-small cell lung cancer. Clinical Medicine Insights. Oncology, 5, 131.