Chapter 8

IN VITRO CELL

CYTOTOXICITY STUDY

8.1 Introduction:

Tumor cell lines have been widely used for research purposes and are, in fact, an excellent model for the study of the biological mechanisms involved in tumor formation [1, 2]. In fact, the use of the appropriate in vitro model in tumor research is crucial for the investigation of genetic, epigenetic and cellular pathways [3], for the study of proliferation deregulation, apoptosis and tumor progression [3], to define potential molecular markers [4] and for the screening and characterization of therapeutics. The results of the research in tumor cell lines are usually extrapolated to in vivo human tumors [4] and its importance as models for drug testing and translational study have been recognized by many biomedical and pharmaceutical companies.

After the preliminary studies of formulation preparation and characterization, the final objective was to deliver the Liposomal formulation by intravaginal route to animals. But before the animal studies, the formulations should be evaluated for their safety and efficacy at cell culture level. The aim of the study was to evaluate prepared Liposomes for cell cytoxicity in order to establish its efficacy for in vivo tumor regression. Hence, cytotoxicity studies by MTT Assay, cell cycle analysis and apoptosis studies by FACS analysis were performed prior to in- vivo studies.

8.1.1 Cytoxicity study by MTT Assay

The antiproliferative activity of the drugs can be evaluated by MTT assay. Cell proliferation and viability assays are of particular importance in cell biology. Tetrazolium salts (e.g., MTT, XTT, WST- 1) are particularly useful for this type of analysis. Tetrazolium salts are cleaved to formazan by the "succinate-tetrazolium reductase" system, which belongs to the respiratory chain of the mitochondria, and is only active in metabolically intact cells. MTT is used for the quantitative determination of cellular proliferation and activation (e.g., in response to growth factors and cytokines such as IL-2 and IL-6). It is also used for the quantification of antiproliferative or cytotoxic effects (e.g., mediated by tumor necrosis factor-alpha or -beta) and for the measurement of interferon action.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] is a water soluble dye. The assay is based on the cleavage of the yellow tetrazolium salt MTT into purple formazan by metabolically active cells. [5] The MTT formazan crystals are insoluble in aqueous solution, but may be solubilized by adding dimethyl sulphoxide (DMSO) solution, then incubating the plates overnight in a humidified atmosphere (e.g., 37°C, 5% CO_2). The solubilized formazan product can be spectrophotometrically quantitated using an ELISA reader. An increase in the number of living cells results in an increase of total metabolic activity, which leads to a stronger color formation. The absorbance of dissolved formazan in the visible region correlates with the number of intact alive cells. Cytotoxic compounds damage and destroy the cells, and thus decrease the reduction of MTT to formazan. For each cell type a linear relationship between cell number and absorbance is established, enabling accurate, straightforward quantification of changes in proliferation.

8.1.2 Cell Cycle analysis and Apoptosis study

The cell cycle is the series of events that take place in a cell leading to its division and duplication of its DNA (DNA replication) to produce two daughter cells. Flow cytometry is used to determine the distribution of DNA in the cell replication state. Quiescent and G1 cells will have one copy of DNA having 1X fluorescence intensity. G2/M phase of the cell cycle will have two copies of DNA having 2X fluorescence intensity. S phase synthesizing DNA will have fluorescence values between the 1X and 2X populations. The schematic representation for the same is given in Figure 8.1

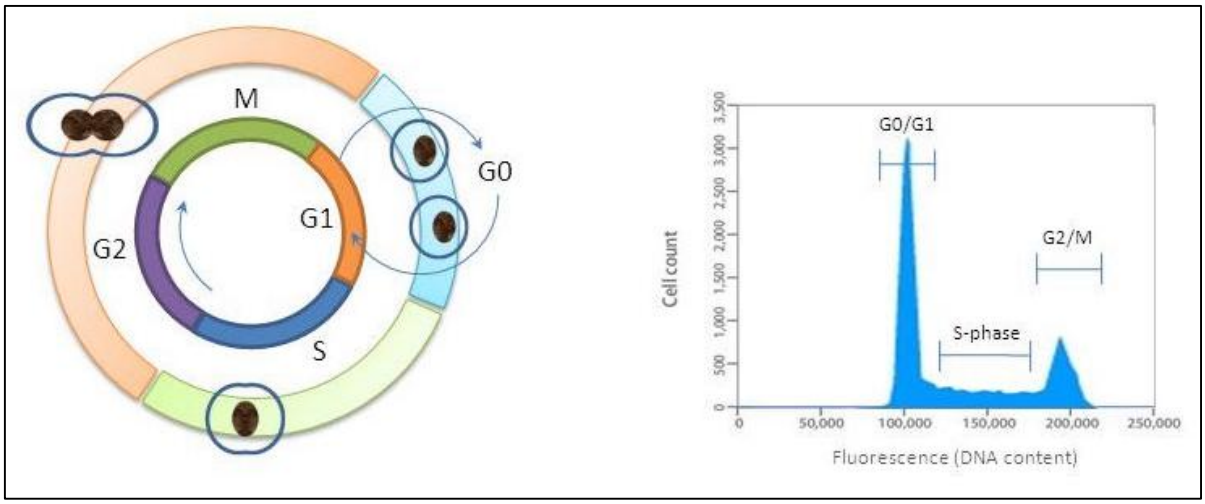


Figure 8.1 Schematic presentation of principle for cell cycle analysis using DNA intercalating florescence probe in flow cytometry

A process of Programmed cell death that may occur in multicellular organisms is called as apoptosis. Apoptosis study was conducted using Propidium Iodide (PI) staining procedure. PI is used more often than other nuclear stains because it is economical, stable and a good indicator of cell viability, based on its capacity to exclude dye in living cells. [6] The ability of PI to enter a cell is dependent upon the permeability of the membrane; PI does not stain live or early apoptotic cells due to the presence of an intact plasma membrane. In late apoptotic and necrotic cells, the integrity of the plasma and nuclear membranes decreases, allowing PI to pass through the membranes, intercalate into nucleic acids, and display red fluorescence. [7]

8.2 Materials and Equipment

***Cell Line*:**

MCF-7 (Human Breast Cancer cell line) was obtained from NCCS, Pune, India. MCF-7 is an epithelial, breast adenocarcinoma cell line that was derived from a metastatic pleural effusion. It over expresses estrogen and progesterone receptors on it, suitable to use for studying the efficacy of drug and/or drug loaded formulation acting on uterine leiomyoma. Cell line was cultured in MEM (Minimum Essential Medium) supplemented with 10 % v/v Fetal Bovine Serum (FBS) and 1% Antibiotic Antimycotic solution

(Penicillin G, Streptomycin and Amphotericin B) and incubated at 37 ºC, 5 % CO_2 in an incubator.

***Materials*:**

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

Equipment:

- ELISA plate reader (FLUOstar, Germany)
- Jouan IGO150 5% CO_2 incubator (Thermo-Fischer, Germany)
- Weiber vertical Laminar Air Flow (Weiber, India)
- Nikon H600L Microscope (Nikon, Japan)
- Fluorescence activated cell sorter (BDFACSCalibur, BD Biosciences, USA)

8.3 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 filtered through a 0.2 mm filter and stored at 2-8°C.

Leuprolide acetate and liposomal formulation solution: Stock Solution of Leuprolide acetate was prepared by dissolving 10 mg of the drug in 10 ml sterile PBS pH 7.4. The liposomal formulation containing leuprolide acetate was also diluted to give a stock solution of final concentration of 1000 µg/ml.

Suitable aliquots of the stock solution of leuprolide acetate and its liposomal formulation were added into the wells containing MEM medium to give a final concentration 0.001 µg/ml, 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml and 10 µg/ml of leuprolide acetate.

Raloxifene Hydrochloride and liposomal formulation solution:

Stock Solution of Raloxifene was prepared by dissolving 10 mg of the drug in 2ml methanol and volume was made to 10 ml using sterile PBS pH 7.4. The liposomal formulation containing Raloxifene Hydrochloride was also diluted to give a stock solution of final concentration of 1000 μg/ml.

Suitable aliquots of the stock solution of Raloxifene Hydrochloride and its liposomal formulation were added into the wells containing MEM medium to give a final concentration 0.001 μg/ml, 0.01 μg/ml, 0.1 μg/ml, 1 μg/ml and 10 μg/ml of Raloxifene Hydrochloride.

Sample Coding:

A1- Standard Raloxifene HCl (Std RLX)

A2- Liposomal Raloxifene HCl (RLX Liposomes)

A3- Standard Leuprolide Acetate (Std LA)

A4- Liposomal Leuprolide Acetate (LA Liposomes)

A5- Liposomal formulation having both drugs Raloxifene HCl and Leuprolide Acetate (RLX-LA Liposomes)

Protocol:

Cells maintained in appropriate conditions were seeded in 96 well plates ($3x10^3$ cells/well) and allowed to attach and grow. After 24 hours cells were treated with different concentrations of the test samples (A1, A2, A3, A4 and A5) individually and incubated at 37°C, 5% CO_2 for 12, 24, 48 hours. After incubation, treatment media was removed and cells were treated with 20 μL (5 mg/ml) of MTT dye [3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyl tetrazolium bromide] and incubated for 4 hours. The dark blue formazan product formed by the cells was dissolved in 200 μL DMSO under a safety cabinet and read at 570 nm on a plate reader (FLUOstar, Germany). Percentage inhibitions were calculated and plotted with the concentrations and IC_{50} values were calculated.

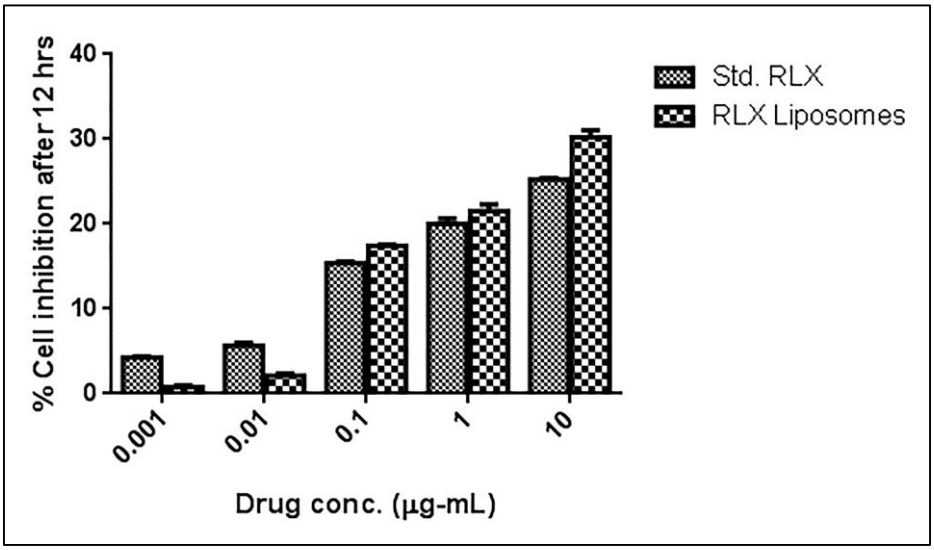
8.4 Apoptosis study and Cell cycle analysis by FACS

Protocol: $1x10^5$ MCF-7 (Human Breast cancer cell line having estrogen receptors) were seeded on 6 well plates and allowed to grow for 24 hours. Cells were treated with 0.1μM of test sample (A1, A2, A3, A4, A5) 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 and incubated at 4 ^oC for 10-15 minutes. After incubation suspension was analyzed for cell cycle and apoptosis on flow cytometer (BD FACSCalibur, BD Biosciences, 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-).

8.5 Results and Discussion

8.5.1 Results of MTT Assay

MTT assay is a widespread method to assess cell cytotoxicity. In vitro cytotoxicity studies were performed on MCF-7 cell line and it is demonstrated that liposomal formulation of Raloxifene was more effective in causing cell cytoxicity than the standard Raloxifene drug as depicted in Figure 8.2, at a higher concentration range. The results are have been shown in Table 8.1. % Cell Inhibition and IC50 values were determined for each time point.



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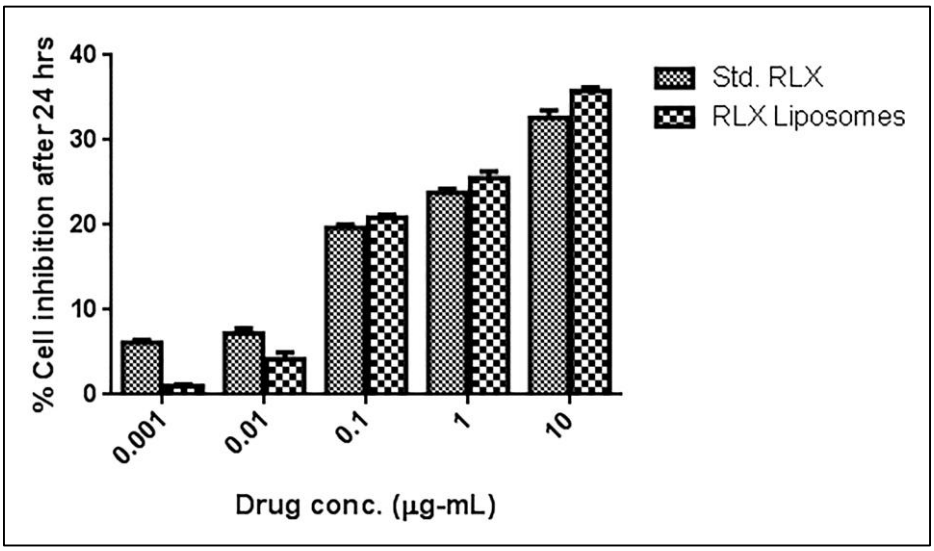

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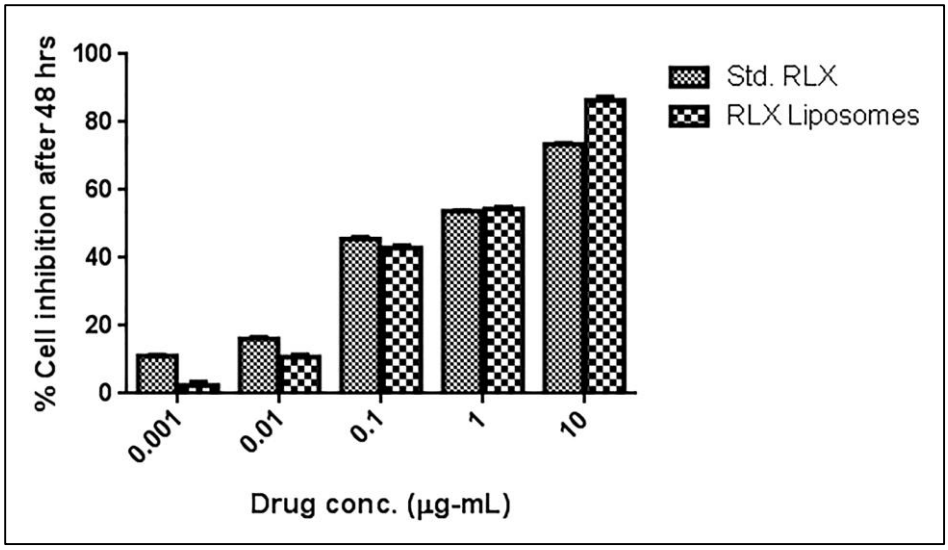

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Figure 8.2: Cytotoxicity of different concentrations of Std RLX and RLX Liposomes at A) 12 hours B) 24 hours C) 48 hours

As seen in the graphs, as the time of incubation increased from 12 to 48 hours, the cell cytotoxicity increased. The values of % Cell Inhibition for Std. RLX at lower concentration range (0.001 and 0.01 µg/ml) was significantly higher than RLX liposomes indicating that Std. RLX was more cytotoxic than RLX Liposomes due to its direct action

on estrogen receptor present on MCF7 cells. However, as the concentration was gradually increased (0.1, 1 10 µg/ml) liposomal formulation showed better results than Std. RLX possibly due to higher uptake of Liposomal formulation within the cells.

Table 8.1: % Cell Inhibition values of Std RLX and RLX Liposomes on MCF7 cell line

Concentration (µg/ml)	**% Cell Inhibition after 12 hours**		**% Cell Inhibition after 24 hours**		**% Cell Inhibition after 48 hours**	
	Std RLX	RLX Liposomes	Std RLX	RLX Liposomes	Std RLX	RLX Liposomes
0.001	4.27±0.11	0.78±0.21	6.13±0.32	1.03±0.19	11.2±0.11	2.47±0.95
0.01	5.66±0.35	2.12±0.28	7.21±0.62	4.2±0.75	16.18±0.42	10.8±0.63
0.1	15.35±0.24	17.42±0.17	19.66±0.42	20.89±0.32	45.58±0.53	42.87±0.82
1	20.01±0.66	21.53±0.77	23.76±0.53	25.51±0.80	53.75±0.12	54.37±0.68
10	25.28±0.13	30.21±0.87	32.62±0.89	35.74±0.47	73.44±0.35	86.41±0.97

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

IC50 values were determined for std. RLX and RLX-Liposomes in MCF-7 breast cancer cell lines and data is presented in Table 8.2. Enhanced cytotoxic activity of RLX liposomes compared to std RLX clearly demonstrates the high affinity/uptake of liposomes in MCF-7 cells.

Table 8.2 IC50 values of Std. RLX and RLX-Liposomes in MCF-7 cell line.

Sample	**IC50 Values (µg/ml)**		
	12 hours	**24 hours**	**48 hours**
Std. RLX	25.51	18.20	0.96
RLX-Liposomes	18.65	15.22	0.83

Figure 8.3 represents graphically the comparison of IC50 values between Std. RLX and RLX-Liposomes. It is clearly seen that liposomal formulation is showing lower values of IC50 at all the time points than the standard drug which can be interpreted in a way that lower dose of liposomal formulation is required to inhibit cell growth than the standard

drug. IC50 values were found to decrease with increase in exposure time of drug and/or drug-loaded formulations.

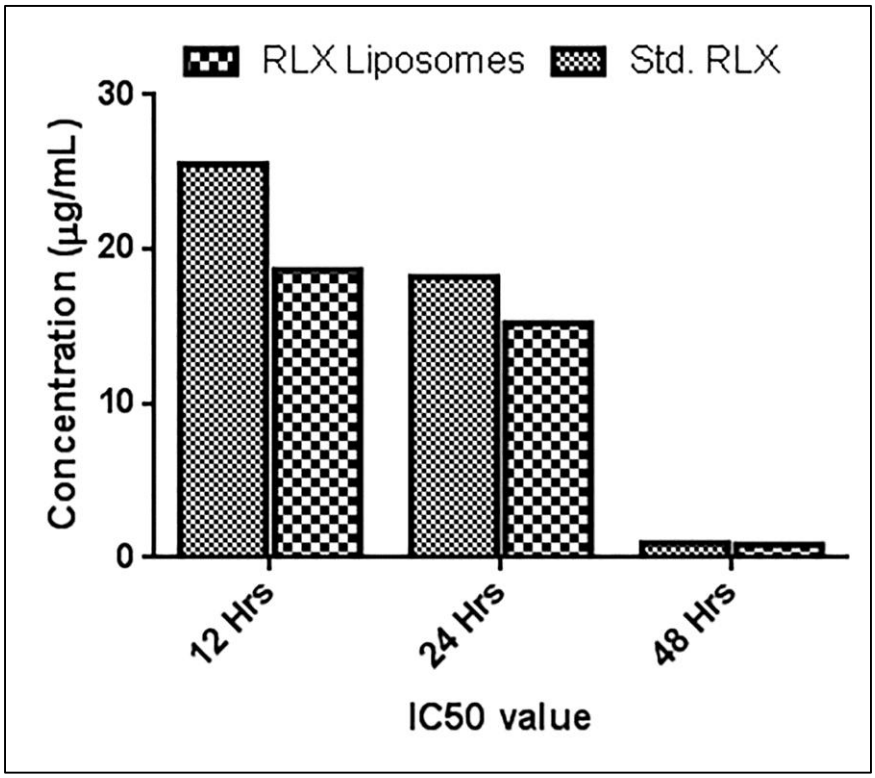


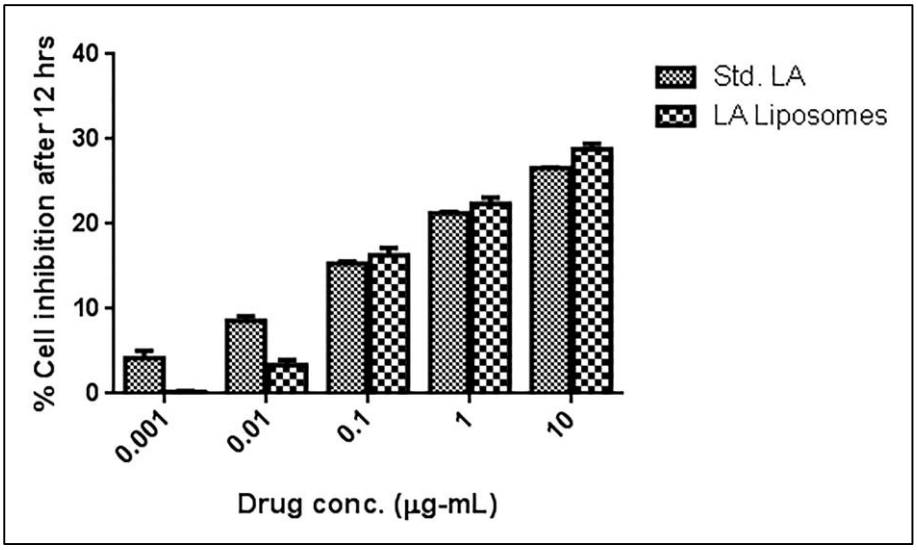
Figure 8.3 IC50 values of Std. RLX and RLX-Liposomes in MCF-7 cell line

As seen in Table 8.3, standard Leuprolide drug solution caused more cell death than the liposomal formulation at lower concentration range (0.001 and 0.01 µg/ml) at all time points. While at 0.1 µg/ml, there was no significant difference between the std. drug and liposomes at 12 hours and 24 hours of incubation. Whereas, after 24 hours at same concentration, LA liposomes were found to exhibit more antiproliferative action than the standard drug. Similar observation could be made for the concentration range 1 and 10 µg/ml, which showed no significant difference between the cytotoxic effect of std. drug and liposomes at 12 and 24 hours. While at 24 hours, LA liposomes were more cytotoxic than std. drug. The reason may be due to higher uptake of liposomes by endocytosis through cells on long exposure. Figure 8.4 depicts the data in graphical form.

Table 8.3: % Cell Inhibition values of Std LA and LA Liposomes on MCF7 cell line

Concentration (µg/ml)	**% Cell Inhibition after 12 hours**		**% Cell Inhibition after 24 hours**		**% Cell Inhibition after 48 hours**	
	Std LA	LA Liposomes	Std LA	LA Liposomes	Std LA	LA Liposomes
0.001	4.19±0.89	0.21±0.11	7.10±0.21	0.85±0.42	14.28±0.76	2.47±0.74
0.01	8.56±0.57	3.36±0.63	13.27±0.31	7.21±0.22	29.14±0.89	11.14±0.34
0.1	15.31±0.26	16.33±0.82	21.35±0.78	20.15±0.40	48.27±0.65	38.27±0.59
1	21.26±0.15	22.35±0.78	27.05±0.36	29.78±0.24	56.35±0.57	60.24±0.47
10	26.57±0.13	28.82±0.67	32.13±0.33	35.42±0.56	74.19±0.78	88.24±0.34

*Experiment was performed in triplicate (n=3)



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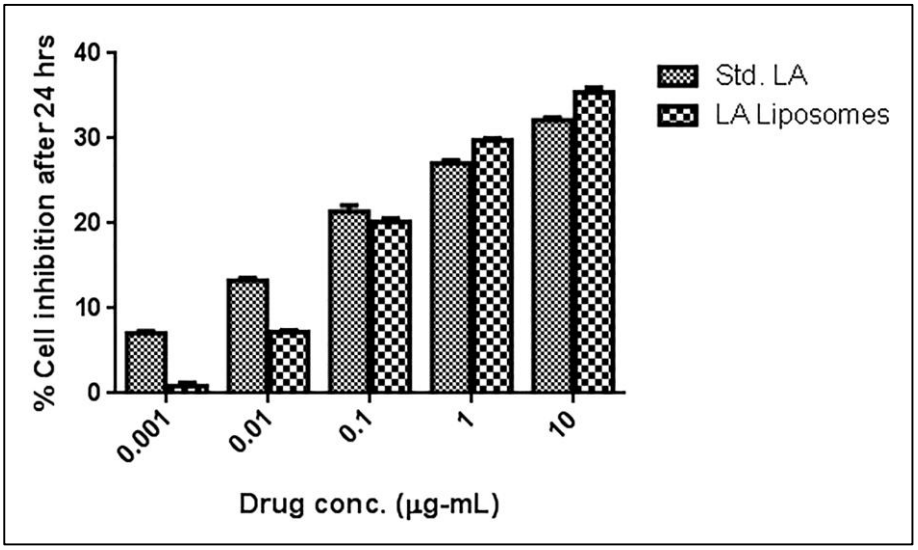

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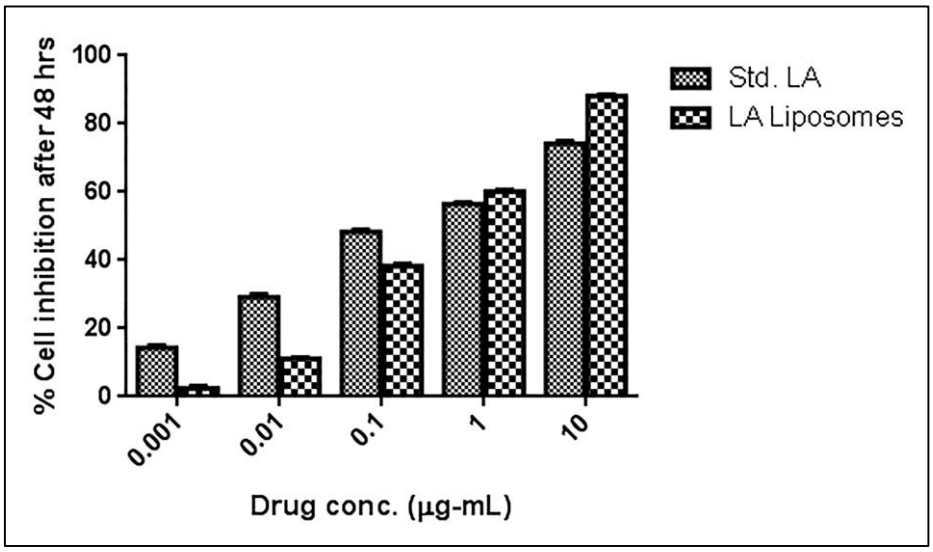


C)

Figure 8.4: Cytotoxicity of different concentrations of Std LA and LA Liposomes at A) 12 hours B) 24 hours C) 48 hours

Results of IC50 values of Std. LA and LA-Liposomes are given in Table 8.4. The data has been represented graphically in Figure 8.5. The results reveal that LA-Liposomes are more cytotoxic than std. LA, showing lesser IC50 value than the plain drug at all the time points. IC50 values were found to decrease with increasing incubation time.

Table 8.4 IC50 values of Std. LA and LA-Liposomes in MCF-7 cell line.

Sample	IC50 Values (µg/ml)		
	12 hours	24 hours	48 hours
Std. LA	24.38	20.22	0.79
LA-Liposomes	19.92	15.61	0.56

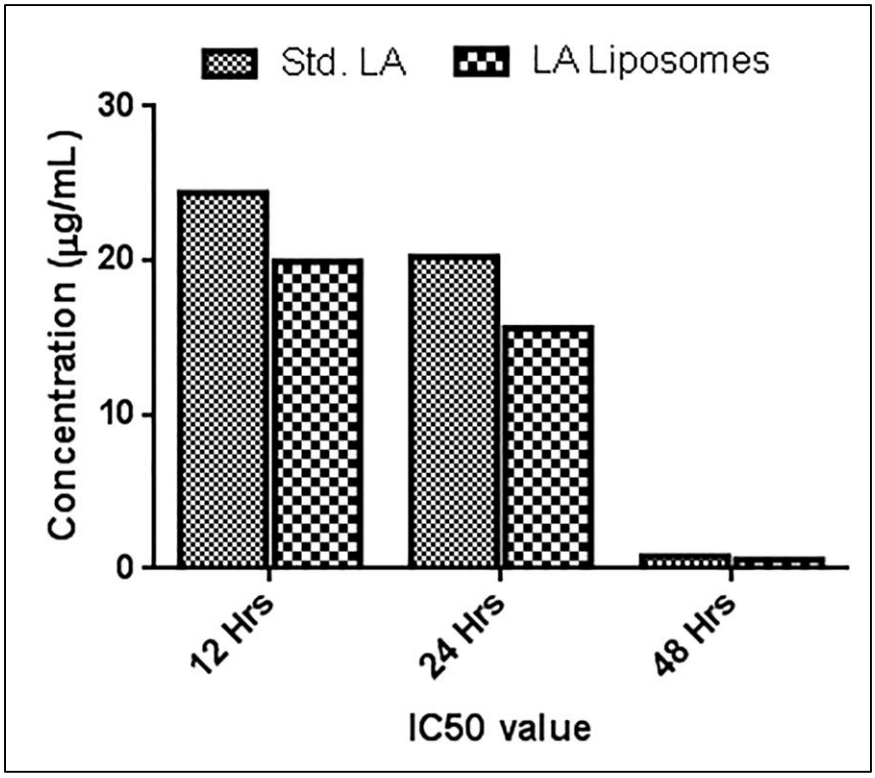


Figure 8.5 IC50 values of Std. LA and LA-Liposomes in MCF-7 cell line

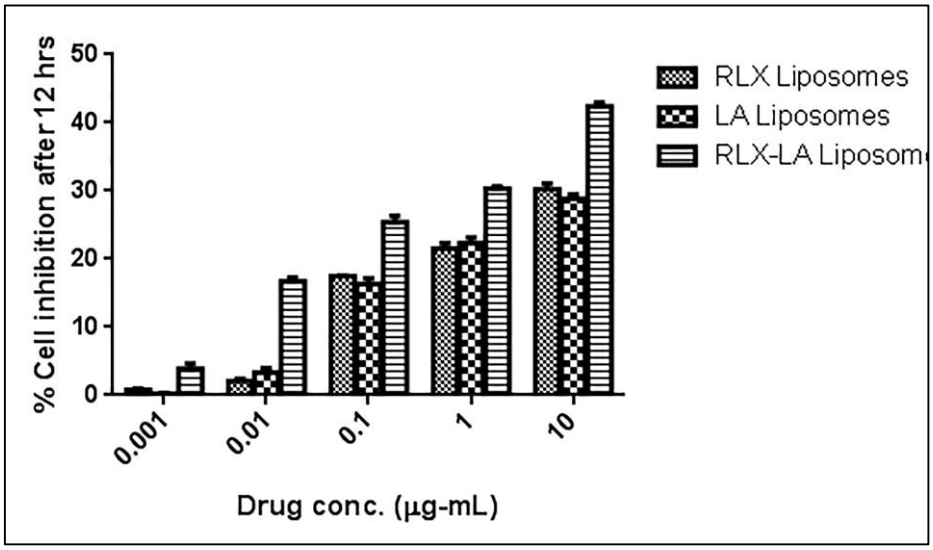
When comparison is made between the two standard drugs cytotoxicity of Leuprolide was found to be more than Raloxifene but the difference was not significant. Similar was the case for Liposomal formulation of Leuprolide, which is showing greater efficacy than the liposomal formulation of Raloxifene, but the difference is not statistically significant. The results are given in Table 8.5. When both the drugs were entrapped within the same formulation, the cytotoxicity was significantly more than the individual formulations as seen in Figure 8.6. Liposomal formulation having both the drugs is showing high anti-proliferative action at all concentrations demonstrating the synergistic effect of the drugs. The results are in accordance with the study carried out by Youn-Jee Chung and co

workers, who demonstrated that Raloxifene when administered with Leuprolide shows more antiproliferative effect on myoma cells. [8] As seen in the Figure 8.6, as the exposure time increases, cell death also increases showing significant difference between % cell inhibition at 12, 24 and 48 hours. This anticipates the dose reduction of both the drugs when given in combined form than as compared to individual administration.

Table 8.5: % Cell Inhibition values of RLX-LA Liposomes on MCF7 cell line

Concentration (µg/ml)	**% Cell Inhibition after 12 hours**	**% Cell Inhibition after 24 hours**	**% Cell Inhibition after 48 hours**
0.001	3.89±0.74	6.27±0.10	17.11±0.59
0.01	16.74±0.55	19.55±0.82	41.78±0.67
0.1	25.41±0.89	31.29±0.15	65.22±0.78
1	30.34±0.29	36.47±0.41	77.43±0.45
10	42.40±0.56	50.56±0.55	90.47±0.82

* Experiment was performed in triplicate (n=3)



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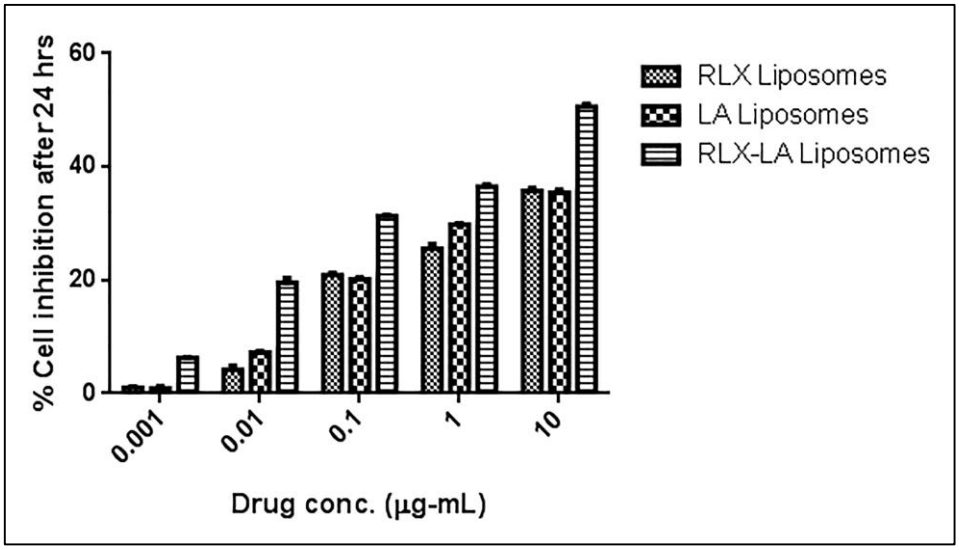

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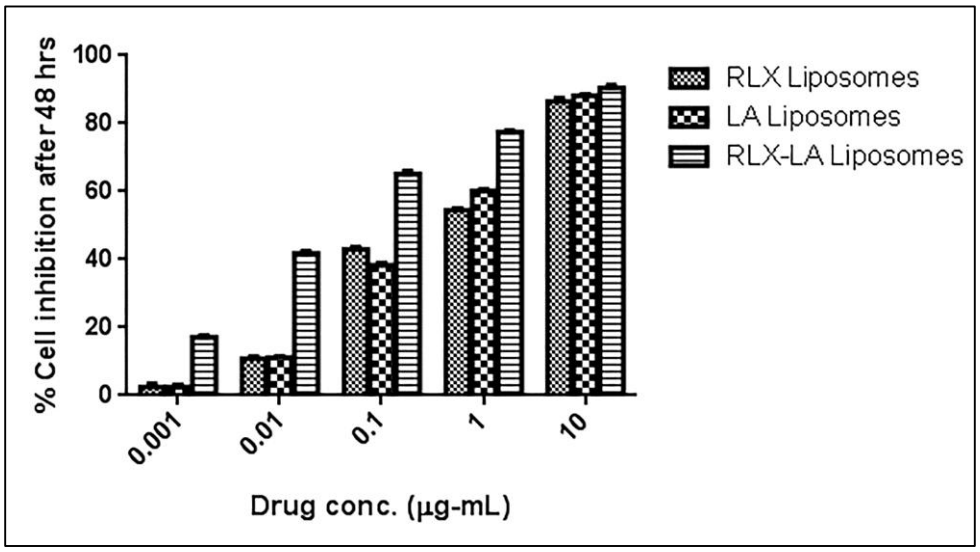

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Figure 8.6: Cytotoxicity comparison of RLX-LA Liposomes, RLX Liposomes and LA Liposomes at A) 12 hours B) 24 hours C) 48 hours

IC50 values for dual drug entrapped liposomes have been given in Table 8.6, and the graphical form of the same in Figure 8.7. As seen from the values, IC50 of combined formulation (RLX-LA Liposomes) is significantly lower than the individual liposomal formulation. This indicates the synergism between the two drugs to exhibit the cytotoxic effect. IC50 value after 48 hours of incubation in the case of dual drug entrapped liposomes lowest than the individual formulations. IC50 values of LA liposomes was 28

fold higher while that of RLX liposomes was 41 fold higher than dual drug entrapped liposomes indicating the highest efficacy of the dual drug entrapped liposomal formulation (RLX-LA Liposomes) requiring lesser dose to exhibit cytotoxicity.

Table 8.6 IC50 values of RLX-LA-Liposomes in MCF-7 cell line.

IC50 Values (µg/ml)		
12 hours	**24 hours**	**48 hours**
12.59	10	0.02

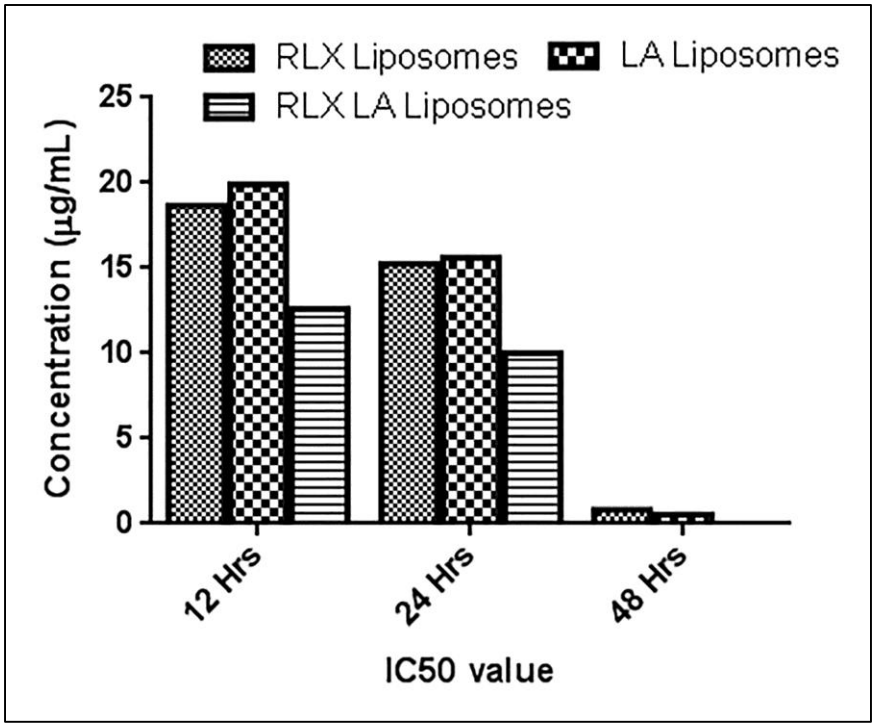
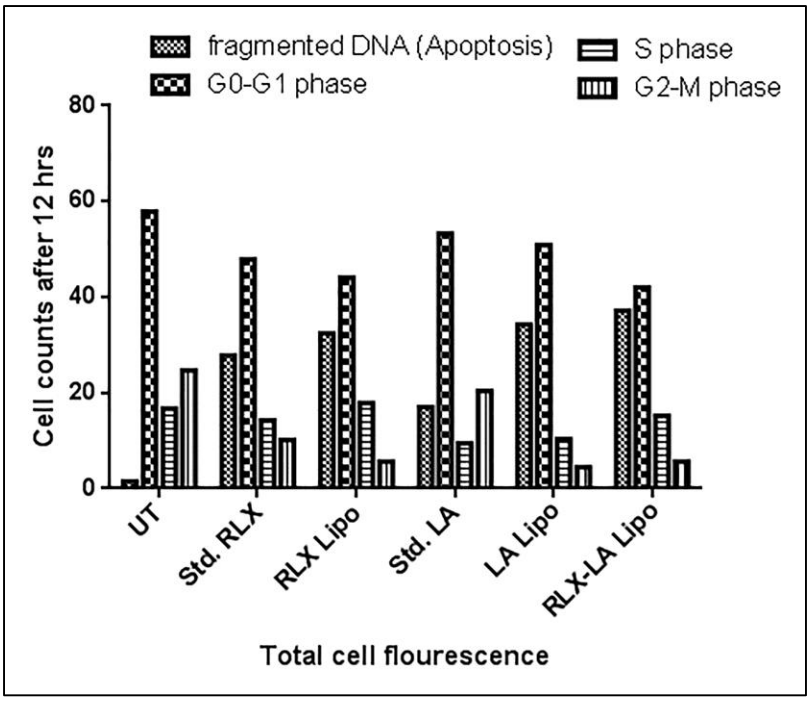


Figure 8.7 IC50 values of RLX-LA-Liposomes in MCF-7 cell line

8.5.2 *Results of Apoptosis study and Cell Cycle Analysis*

Apoptosis studies were carried out using PI staining procedure to determine whether drug loaded Liposomes caused apoptosis or necrosis of MCF-7 cells. The membranes of dead and damaged cells are permeable to Propidium Iodide, identifying the apoptotic cells and necrotic populations. As seen in Figure 8.8, liposomal formulations showed more counts of cells in apoptotic phase as compared to the standard drugs. The reason behind this may

be attributed to higher uptake of the liposomes within the cells than as compared to the drugs due to endocytosis. Also as the time of exposure increased, fragmented DNA count also increased for all the test samples. There was not much difference between the apoptotic cell counts of RLX Liposomes and LA Liposomes both showing the fragmented DNA count almost same at both the time points. However, looking at the standard drugs, RLX was found to be causing more apoptosis than LA. The reason behind this may be due high affinity of Raloxifene towards estrogen receptors over expressed on MCF-7 cells than Leuprolide, which exert its antiproliferative action on GnRH receptors expressed over the MCF-7 cells. [9, 10] Highest apoptotic count could be registered by RLX-LA dual drug entrapped liposomes at both the time points. The augmented apoptotic activity of combined drug formulation in comparison to individual liposomal formulations can be due to the synergism of both the drugs as suggested by Youn-Jee Chung and Co-workers. [8] Also in combination form, RLX and LA may exert their individual action on both the receptor types (depending on their affinities) estrogen-R as well as GnRH-R causing more cell death than individual drug therapy.



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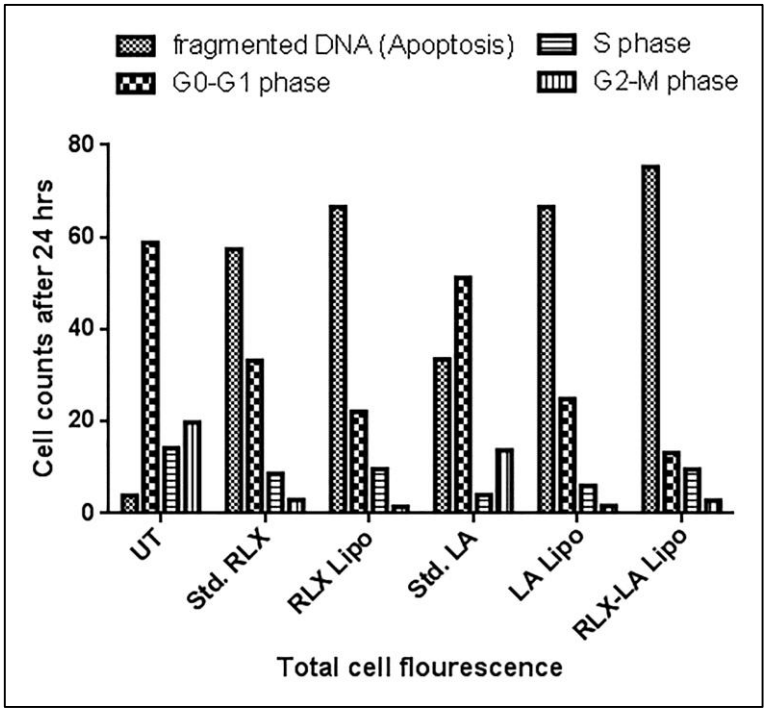

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Figure 8.8: Apoptosis estimation in MCF7 cell line after exposure of standard drugs and liposomal formulations at A) 12 hours and B) 24 hours. *UT stands for Untreated cells (Control)

Drugs are known to act by different pharmacological actions, which include their effect on genes controlling the cell cycle and induction of pro- and anti-apoptotic genes [11]. Raloxifene is known to arrest the cells in G1 phase of cell cycle [12] while Leuprolide is known to inhibit cell growth and proliferation primarily through arresting G2/M phase. [13] Hence, we investigated whether the pharmacological action of Raloxifene and Leuprolide would affect the proliferation of tumor cells by studying the distribution of DNA in cell cycle. The DNA distribution in cell cycle was studied to investigate the effect of prepared formulations on growth of MCF7 cells known to over express the estrogen receptors as well as GnRH receptors. Results of cell cycle analysis as seen in Table 8.7 demonstrates that Standard RLX and LA shows stronger arrest at G0/G1 phase compared to their respective liposomal formulations. Where LA-Liposomes shows more cell arrest in S-phase preventing its progression to M phase. It is also demonstrated that Std LA treated cells showed stronger arrest at G2/M phase as compared to Std. RLX.

RLX-LA liposomes showed more cell accumulation in G2/M phase than as compared to individual liposomal formulations i.e. RLX-Liposomes and LA-Liposomes. This is suggestive of the synergistic effect for causing the cell inhibition and tumor regression, which can be very well correlated with the results of In-Vivo Pharmacodynamic studies which showed maximum tumor regression after administration of dual drug entrapped than as compared to the individual RLX liposomes and LA liposomes. The results are shown graphically in Figure 8.9.

Table 8.7: Cell Cycle Analysis in MCF7 cell line after treatment with (a) Control/UT (PBS), (b) Standard Raloxifene (Std RLX) (c) RLX Liposomes, (d) Std Leuprolide (Std LA), (d) LA Liposomes e) RLX-LA Liposomes (dual drug) by PI staining using FACS technique.

Sample	**Fragmented DNA (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*	1.45	3.81	57.89	58.77	16.7	14.2	24.67	19.76
Std RLX	27.8	57.4	47.85	33.15	14.23	8.64	10.12	2.94
RLX Liposomes	32.41	66.54	44.12	22.1	17.87	9.63	5.6	1.48
Std LA	16.98	33.44	53.25	51.09	9.45	4.03	20.32	13.72
LA Liposomes	34.26	66.54	50.9	24.88	10.34	6.02	4.5	1.6
RLX-LA Liposomes	37.12	75.22	42.07	13.12	15.14	9.56	5.67	2.74

*UT stands for Untreated sample/Control; data represents the cell counts after 12 hours and 24 hours of treatment

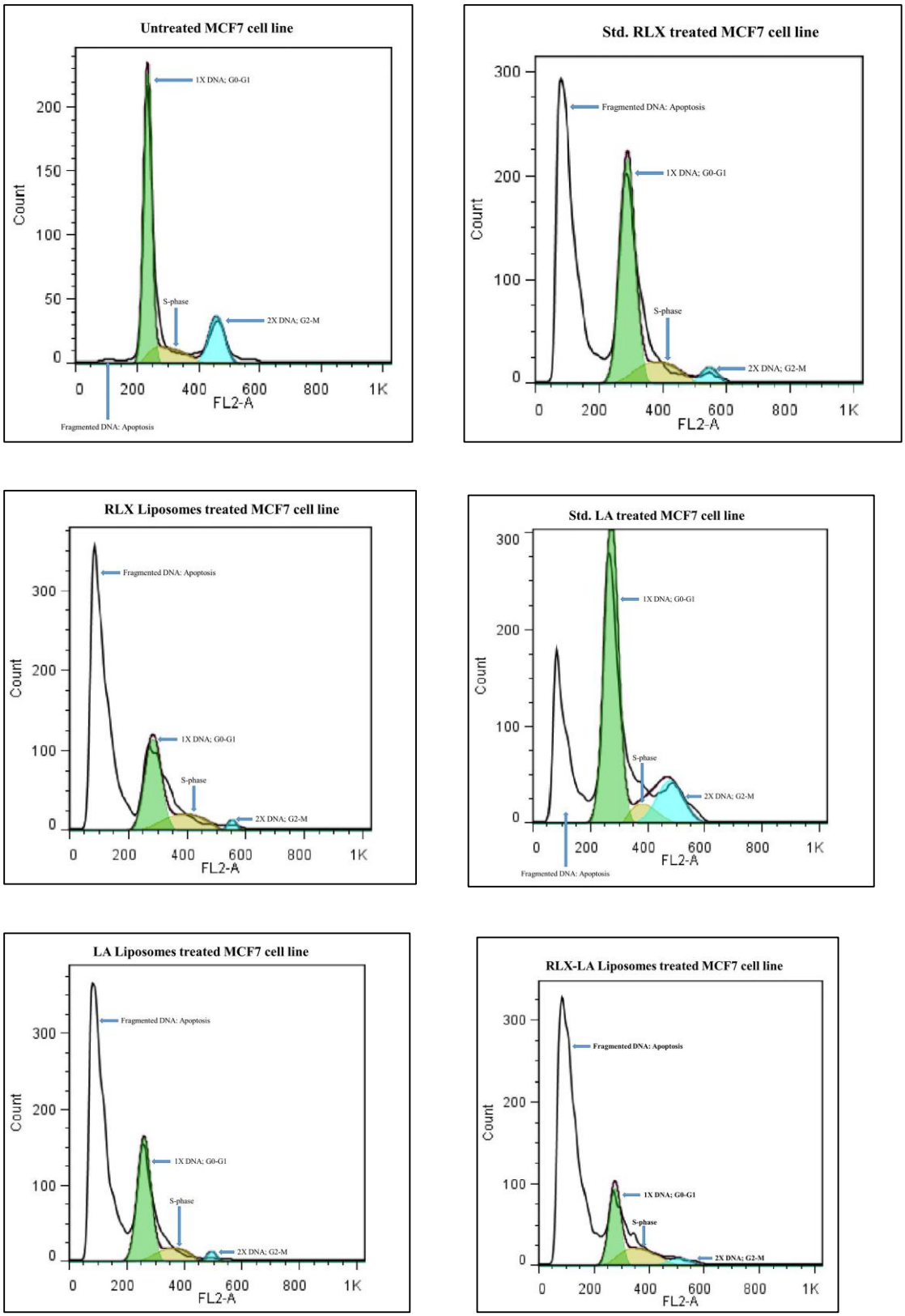

Figure 8.9: Effect of UT (Control), Std. RLX, RLX-Liposomes, Std LA, LA-Liposomes,

RLX-LA Liposomes exposure on cell cycle distribution in MCF7 cells using FACS as an estimation technique.

8.6 References

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