7.1 Introduction

Cell lines have been seen as an exceptional element for the determination of the various cellular pathways, cell-cell interaction, and characterization of a specific gene involved in cancer development. Nevertheless, cell lines provide comprehensive fundamentals of carriers' system before its actual application to animal models (1). Characterization of cell lines provides better insight into a polygenetic etiology of the cancer cells along with the biological reflexes involved in the reinforcement of a disease. Cell characterization has played a significant role in the development of novel carriers, understanding of different uptake mechanisms along with alterations involved in current therapy resistivity, and development of targeted carriers with available chemotherapeutic agents (2). In fact, the appropriate in-vitro model can help in determination of the cellular pathway, genetic as well epigenetic pathway, to study the downregulation of proliferation, apoptotic pathways, progression of the disease, to define the endocytic route for uptake, quantification of cellular uptake and characterization of the novel cancer therapeutics. The results of the cell line study have been extrapolated to the in-vivo studies on animals and humans. To assess the biological effects of the developed PLHNCs and MSNs, the in-vitro characterization was performed on MCF – 7 and MDA MB 231 cell lines.

PART -A Fulvestrant and Exemestane Loaded Polymer Lipid Hybrid Nanocarriers (PLHNCs)

7.2 General Methods and Preparations

The MDA MB 231 cell line (Human Breast adenocarcinoma cells) was from the cell repository facility, National Centre of Cell Science (NCCS), Pune, India. The cell line was maintained at 37°C in humidified 5% CO2 atmosphere (Stericycle i160, Thermofischer Scientific, Germany). The cultures were maintained in Dulbecco's Minimum Essential Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% antibiotic solution (3).

7.2.1 Media Preparation

Complete media was prepared by mixing DMEM with antibiotic solution (1% v/v) and 10% v/v heat inactivated fetal bovine serum (FBS). This complete media was stored in a sterile screw capped bottle in refrigerated condition (NMT 25° C). The bottle was then sealed with parafilm and wrapped with aluminium foil. The process was carried out in a vertical laminar air flow cabinet (4).

7.2.2 Preparation of Phosphate Buffer Saline pH 7.4

Sodium chloride (8 gm), potassium chloride (200 mg), di-sodium hydrogen phosphate (1.44 gm), potassium dihydrogen phosphate (240 mg) was added in 1 litre of double distilled water and pH was checked. Finally, the buffer was autoclaved.

7.2.3 Preparation of FACS buffer

Bovine serum albumin (0.5% W/v) and fetal bovine serum (0.5% v/v) were added in sterilized phosphate buffer pH 7.4 along with 2 mM EDTA and 2 mM NaN_3.

7.2.4 Subculturing of cell line

The cells were maintained as monolayer culture in T-25 cell culture flasks and subcultured twice every week. The Subcultured cell lines were then placed at 37°C in a humidified atmosphere at 95% air and 5% CO2 (Stericycle i160, Thermofischer Scientific, Germany) in complete media. Fresh complete medium was replaced every 3 days (5).

Detailed Procedure:

- ✓ The complete medium was prepared on pre-warmed water bath at 37°C.
- ✓ The cells were taken from incubator and observed for cell growth under the microscope (made sure cells have grown ~80% confluence).
- ✓ The medium was removed from flask by aspiration. The cells attached to the flask were washed twice with culture medium without serum.
- ✓ 1 mL of trypsin EDTA solution was added and then allowed to stand for 5 min occasionally swirling to dislodge the cells.
- ✓ Then 5 mL of complete medium was added to stop the trypsin activity.
- ✓ After gently pipetting the cells up and down to disrupt cell clumps, the cells were counted and reseeded in fresh flask at desired seeding density.
- ✓ The flasks were then incubated properly.

7.2.5 Cell counting using Hemacytometer (6)

1. Preparing hemacytometer

- ✓ The hemacytometer was cleaned using 70% ethanol.
- ✓ The coverslip was firmly fixed using gentle pressure.

2. Preparing cell suspension

- ✓ After trypsinization of adherent cells, cell suspension was prepared. The trypsin action was deactivated by adding FBS. After centrifugation at 1500 rpm, the pellet was resuspended in a smaller volume of complete media.

3. Counting

- ✓ After gentle vortex, 20 µL of the cell suspension was transferred to the edge of a coverslip.
- ✓ After removing any surplus fluid carefully, cells in 4 corner squares of the grid were calculated.
- ✓ The average number of cells in 4 squares was calculated as:

$$Average\ cell\ count\ per\ square = \frac{Total\ number\ of\ cells\ in\ 4\ squares}{4}$$

- ✓ Total cell count was calculated as follows:

$$Cell\ count\ \left(\frac{cells}{ml}\right) = Average\ cell\ count \times 10^4 \times Dilution\ factor$$

7.3 In Vitro cell line studies

7.3.1 *In-vitro* cytotoxicity studies

MTT assay is a colorimetric assay for determining the viable cell count depending on the mitochondrial dehydrogenase activity measurement. The MTT (3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole dye) is taken up and reduced inside the living cells which have mitochondrial dehydrogenase (reductase) enzyme activity intact. This reduction produces a purple-coloured formazan compound that gives a direct estimate of number of viable cells when measured spectrophotometrically (As shown in Figure 7-1).

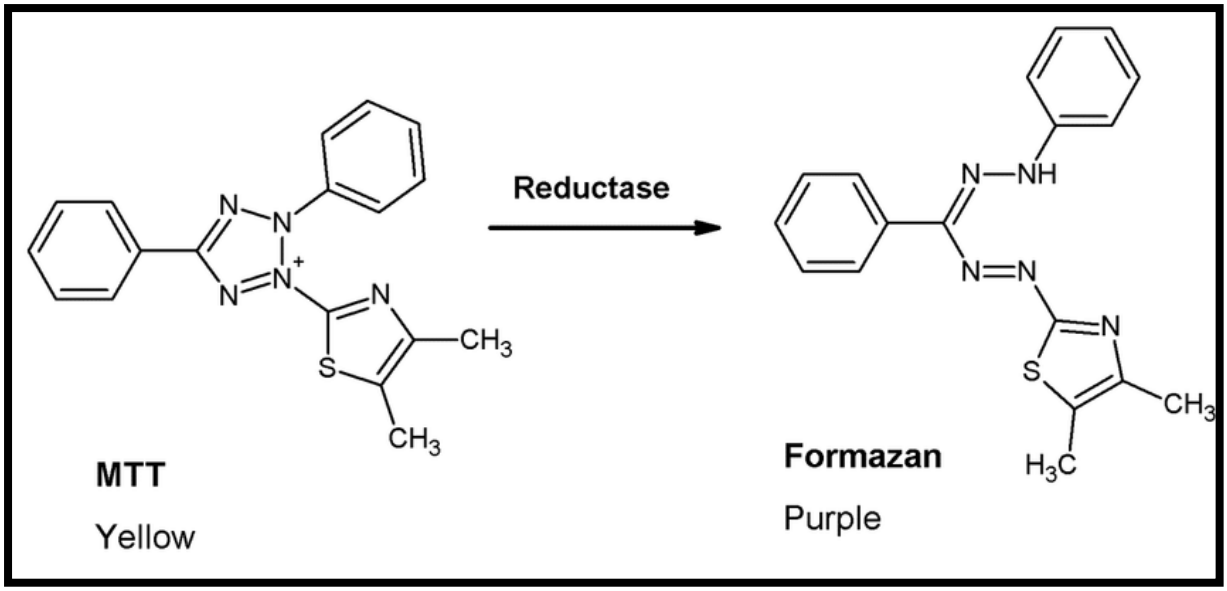

Figure 7.1 MTT Dye reduction by mitochondrial reductase enzyme of viable cells

Formazan formed is water insoluble, so solubilization of formazan precipitates is done by using chemicals like dimethyl sulfoxide (DMSO), an acidified ethanol solution or a surfactant (Sodium lauryl sulphate) solution in diluted hydrochloric acid. Formazan derivative absorbs UV radiation which can be used for estimation of quantity of formazan formed. This assay can be of use to determine the viable cell count of any culture. This in turn can be useful in determining the cytotoxicity of any chemical of medical interest or any delivery system developed for delivery of drug or therapeutic genes (7, 8).

In-vitro cytotoxicity of the PLHNCs was estimated with the help of MTT assay. MDA MB 231 cells were seeded in 96 well-plates with a cell density of 5000 cell per well. After the growing phase of 24 h, cells were exposed to various ranges of formulation (Fulvestrant and Exemestane equivalent to 0.025, 0.05, 0.1, 0.2, 0.5, 1.0 µM and 0.1, 0.25, 0.5, 1.0, 2.0, 3.0 µM)) in complete media. The cells were incubated for 24 h, 48 h and 72 h. After incubation, 100µl of MTT solution (1 mg/mL) was added to each well and incubated for 4 h. Once the MTT incubation was conducted, the culture medium was removed and 200 µL of DMSO was added to each well. Live cells were able to reduce the MTT dye (yellow coloured) to formazan crystals (purple coloured) with mitochondrial enzymes. The absorbance of the plate was recorded at 570 nm with a reference band at 655 nm on the ELISA microplate reader, Multiskan, Thermo Scientific. Micro well with 0.5% Triton X100 was taken as positive control in 8 wells for each experiment, whereas for negative control, wells of untreated cells were considered. Cell

viability was calculated and plotted based on the concentration of Fulvestrant and Exemestane on the X-axis against % viability on the Y-axis.

7.3.2 Cell migration assay

Migration of the cancer cells was assessed using the scratch assay. Briefly, the confluent monolayer of the cells was grown in 6 well-plates at a concentration of 5000 cells/well. The cells were allowed to grow for a period of 24 h. Consequently, scratches were made on the centre of wells using a 200 µL sterile pipette tip (9). Markings of the scratch were highlighted from the bottom with the help of a marker. The later treatment was delivered to all the wells with the F-PLHNCs, FA-F-PLHNCs, Fulvestrant suspension and for Exemestane, E-PLHNCs, FA-E-PLHNCs, Exemestane suspension. at the dose of IC_{50} as found in MTT assay and incubated for 24 h. Images of the scratch assay were captured after the incubation period with Nikon microscope. Captured images were quantified using ImageJ software to assess the inhibitory effect on the scratch width concerning formulation and % scratch closure was then computed.

7.3.3 Cellular uptake studies

Cell uptake studies were performed using FITC labelled FA-F-PLHNCs and FA-E-PLHNCs. The blank nanoparticles were also used for further investigations. To measure the quantitative cell uptake, confocal laser microscopy was performed, while to analyse the quantitative uptake, flowcytometric study was employed (10).

7.3.3.1 Qualitative cellular uptake by confocal microscopy

MDA MB 231 and MCF -7 Cells were seeded into six well-plates with initial cell density of 10,000 cell/well on the covered surface of a sterilized coverslip. After the span of 24 h, the cells were transfected with different formulation and free drug at concentration of IC_{50} values. Subsequently, cells were incubated for 6 h and then, washed with cold phosphate buffer saline (PBS). Finally, washing was followed by live imaging of the cells, which was subjected to confocal laser scanning microscopy for the ultimate visualization (11, 12).

7.3.3.2 Quantitative uptake studies

MDA MB 231 cells were seeded with an initial cell density of 5000 cells/well and proliferated for 24 h. The quantitative analysis was carried out using HPLC analysis of Fulvestrant and Exemestane. The growth media was replaced with FLV suspension, FLV PLHNCs, FA FLV

PLHNCs, EXE suspension, EXE PLHNCs, and FA EXE PLHNCs with different concentrations of the drug and incubated for 1, 3 and 6 hours, separately to observe the effect of concentration and time on cellular uptake. The washing of the cells (3 times) was carried out by PBS and lysed with 0.1% Triton X-100 after incubation. The internalised FLV and EXE were extracted using ACN and cell lysate was centrifuged for 20 minutes at 18,000 rpm. The supernant was analysed by HPLC with an isocratic mobile phase (Methanol:Water:ACN; 65:20:15) at 280nm and 243nm respectively (13, 14).

7.3.4 Apoptosis Studies

7.3.4.1 Caspase -3 Induction assay

Apoptosis plays a major role in the assessment of chemotherapeutic agents since it is a major pathway for cell death (15). The role of caspase-3 in apoptosis is well determined. Hence, we measured the activity of caspase executioner in response to Fulvestrant formulation and Exemestane formulation. The colorimetric assay is based on Ac-DEVDpNa (acetyl Asp-Glu-Val-Asp p-nitroanilide) hydrolysis by the caspases-3 enzyme. The resulting hydrolysis would release p-nitroaniline which could show absorbance at 405 nm. The absorbance of treated cells compared to untreated control cells showed the fold increase in the activity of caspase-3. Briefly, MDA MB 231 and MCF – 7 cells were seeded at an initial cell density of 10,000 cells/well in a 96 well-plate. Once the 80% confluency was achieved, cells were treated with various formulations (Fulvestrant suspension, Blank PLHNCs, F-PLHNCs, FA-F-PLHNCs at the dose of IC_{50} found in MTT assay) and (Exemestane suspension, Blank PLHNCs, E-PLHNCs, FA-E-PLHNCs at the dose of IC_{50} found in MTT assay) and incubated for 6 h and 12 h with individual plates (16). After the treatment cells were washed thrice with PBS and caspase assay buffer containing 50 mM HEPES, 1 mM EDTA, 100 mM NaCl, 10% glycerol, 10 mM DTT, and 0.1% CHAPS with final pH of 7.4. After that, cell lysis was carried out with cell lysis buffer (1X) and cells were pelletized with centrifugation at 2000 rpm for 10 min. Lysate of cells were then transferred to another well plate and incubated with the caspase-3 colorimetric substrate (CASP-3-C, Sigma-Aldrich) at 37 °C for at least 2 h. After incubation, absorbance was measured using an ELISA plate reader at 405 nm (17).

7.3.4.2 Cell apoptosis detection

The detection of cell apoptosis was accomplished using a flow cytometer. MDA MB 231 and MCF – 7 cells were seeded at an initial cell density of 2000 cells per well in 6-well plates. Cells were incubated for 24 h. Cells were treated with Fulvestrant suspension, Fulvestrant PLHNCs,

FA-F-PLHNCs, Exemestane suspension, Exemestane PLHNCs and FA-E-PLHNCs (at dose of IC_{50} analysed by MTT assay) and incubated at 37°C for 24 h. Untreated cells were selected as control. Once the incubation period was over, cells were trypsinized using 0.0025% trypsin solution and harvested in 200 mL of binding buffer (0.2 µ sterile filtered 0.1M HEPES (pH 7.4), 1.4M NaCl, and 25 mM CaCl2 solution) in the form of a cell suspension. Immediately, propidium iodide (8 mL) and annexin V-FITC (5 mL) were added to the cell suspension and gently vortexed for uniform mixing and kept aside for 2.5 hrs for permeation of dyes into the cells (16). The proportion of the apoptotic cells and stained cells were measured using BD FACS ARIA microscope (18, 19).

PART – B Fulvestrant and Exemestane Loaded Mesoporous Silica Nanoparticles (MSNs)

7.4 Cytotoxicity studies of Fulvestrant, Exemestane and Quercetin co loaded Mesoporous Silica Nanoparticles (MSNs).

MTT assay:

In-vitro cytotoxicity of the MSNs was estimated with the help of MTT assay. MCF – 7 cells were seeded in 96 well-plates with a cell density of 5000 cell per well. After the growing phase of 24 h, cells were exposed to various ranges of formulation (Fulvestrant and Exemestane equivalent to 0.025, 0.05, 0.1, 0.2, 0.5, 1.0 µM and 0.1, 0.25, 0.5, 1.0, 2.0, 3.0 µM) in complete media. The cells were incubated for 24 h, 48 h and 72 h. After incubation, 100µl of MTT solution (1 mg/mL) was added to each well and incubated for 4 h. Once the MTT incubation was conducted, the culture medium was removed and 200 µL of DMSO was added to each well. Live cells were able to reduce the MTT dye (yellow coloured) to formazan crystals (purple coloured) with mitochondrial enzymes(20, 21). The absorbance of the plate was recorded at 570 nm with a reference band at 655 nm on the ELISA microplate reader, Multiskan, Thermo Scientific. Micro well with 0.5% Triton X100 was taken as positive control in 8 wells for each experiment, whereas for negative control, wells of untreated cells were considered. Cell viability was calculated and plotted based on the concentration of Fulvestrant and Exemestane on the X-axis against % viability on the Y-axis.

$$\%\, Viability = \frac{Mean\ absorbance\ of\ sample - Mean\ absorbance\ of\ blank}{Mean\ absorbance\ of\ control - Mean\ absorbance\ of\ blank} \times 100$$

where, absorbance of sample and control cells represent the amount of formazan determined for cells treated with the different formulations and for control cells (non-treated), respectively while the absorbance of blank refers to absorbance of empty well (without cells).

7.5 Cell Migration assay:

Human breast cancer (MCF-7) cells were plated in 6-well plates at a seeding density of 2×10^5 cells/well and incubated to create a confluent monolayer. Once a confluent monolayer was formed, the cell monolayer was scrapped in a straight line to create a ‘‘scratch’’ with a 200 µL pipette tip. The debris were removed, and the edge of the scratch was smoothened by washing the cells once with the growth medium and then replaced with 1 ml of medium specific for the in vitro scratch assay. To obtain the same field during the image acquisition, markings were created to be used as reference points close to the scratch. Then treatment was given to the cells with specified samples such as FLV, FMSN, FQMSN, Q-MSN, EXE, EMSN, EQMSN and blank MSN at a concentration obtained from MTT assay. l group wells, no sample was added. The plates were placed in a tissue culture incubator at 37 °C. The plates were taken out of the incubator daily to be examined and then returned to resume incubation. This process was continued until the scratch in the control group wells was filled completely with cells(23).

7.6 Cellular uptake studies

Cell uptake studies were performed using FITC labelled FA-F-MSNs and FA-E-MSNs. The blank nanoparticles were also used for further investigations. To measure the quantitative cell uptake, confocal laser microscopy was performed, while to analyse the quantitative uptake, flow cytometric study was employed.

7.6.1 Qualitative cellular uptake by confocal microscopy

MDA MB 231 and MCF -7 Cells were seeded into six well-plates with initial cell density of 10,000 cell/well on the covered surface of a sterilized coverslip. After the span of 24 h, the cells were transfected with different formulation and free drug at concentration of IC_{50} values. Subsequently, cells were incubated for 6 h and then, washed with cold phosphate buffer saline (PBS). Finally, washing was followed by live imaging of the cells, which was subjected to confocal laser scanning microscopy.

7.6.2 Quantitative uptake studies

MDA MB 231 cells were seeded with an initial cell density of 5000 cells/well and proliferated for 24 h. The quantitative analysis was carried out using HPLC analysis of Fulvestrant and Exemestane. The growth media was replaced with FLV, FMSN, FQMSN, Q-MSN, EXE, EMSN, EQMSN with different concentrations of the drug and incubated for 1, 3 and 6 hours, separately to observe the effect of concentration and time on cellular uptake. The washing of

the cells (3 times) was carried out by PBS and lysed with 0.1% Triton X-100 after incubation. The internalised FLV and EXE were extracted using ACN and cell lysate was centrifuged for 20 minutes at 18,000 rpm. The supernant was analysed by HPLC with an isocratic mobile phase (Methanol:Water:ACN; 65:20:15) at 280nm and 243nm respectively.

7.7 Apoptosis Studies

7.7.1 Caspase -3 Induction assay

Apoptosis plays a major role in the assessment of chemotherapeutic agents since it is a major pathway for cell death. The role of caspase-3 in apoptosis is well determined. Hence, we measured the activity of caspase executioner in response to Fulvestrant formulation and Exemestane formulation. The colorimetric assay is based on Ac-DEVDpNa (acetyl Asp-Glu-Val-Asp p-nitroanilide) hydrolysis by the caspases-3 enzyme. Briefly, MDA MB 231 and MCF – 7 cells were seeded at an initial cell density of 10,000 cells/well in a 96 well-plate. Once the 80% confluency was achieved, cells were treated with various formulations (FLV, FMSN, FQMSN, Q-MSN, EXE, EMSN, EQMSN and blank MSN at the dose of IC_{50} found in MTT assay) and incubated for 6 h and 12 h with individual plates. After the treatment, cells were washed thrice with PBS and caspase assay buffer containing 50 mM HEPES, 1 mM EDTA, 100 mM NaCl, 10% glycerol, 10 mM DTT, and 0.1% CHAPS with final pH of 7.4. After that, cell lysis was carried out with cell lysis buffer (1X) and cells were pelletized by centrifugation at 2000 rpm for 10 min. Lysate of cells were then transferred to another well plate and incubated with the caspase-3 colorimetric substrate (CASP-3-C, Sigma-Aldrich) at 37 °C for at least 2 h. After incubation, absorbance was measured using an ELISA plate reader at 405 nm. The absorbance of treated cells was compared to untreated control cells to find the fold increase in the activity of caspase-3.

7.7.2 Cell apoptosis detection

The detection of cell apoptosis was accomplished using a flow cytometer. MDA MB 231 and MCF – 7 cells were seeded at an initial cell density of 2000 cells per well in 6-well plates. Cells were incubated for 24 h. Cells were treated with FLV, FMSN, FQMSN, Q-MSN, EXE, EMSN, EQMSN and blank MSN (at dose of IC_{50} analysed by MTT assay) and incubated at 37°C for 24 h. Untreated cells were selected as control. Once the incubation period was over, cells were trypsinized using 0.0025% trypsin solution and harvested in 200 mL of binding buffer (0.2 μm sterile filtered 0.1M HEPES (pH 7.4), 1.4M NaCl, and 25 mM CaCl2 solution) in the form of a cell suspension. Immediately, propidium iodide (8 mL) and annexin V-FITC (5 mL) were

added to the cell suspension and gently vortexed for uniform mixing and kept aside for 2.5 hrs for permeation of dyes into the cells (16). The proportion of the apoptotic cells and stained cells were measured using BD FACS ARIA microscope.

7.8 Results and Discussion

PART A: Fulvestrant and Exemestane Loaded Polymer Lipid Hybrid Nanocarriers (PLHNCs)

7.8.1 Cytotoxicity assay of various PLHNCs formulations on MDA MB 231 and MCF – 7 cells

The results of cytotoxicity studies are shown in Figure 7.2 and IC_{50} of the MDA MB 231 cells with various formulations is presented in Table 7.3. The cytotoxicity of the placebo PLHNCs as seen in Figure 7.2 A, showing more than 95% viability, implying that the carriers and excipients are non-toxic in nature. The fold change in the IC_{50} of F-PLHNCs and FA-F-PLHNCs was found to be 1.39, 1.51 and 2.04 and 1.84, 2.42 and 2.96 as compared to fulvestrant suspension after 24, 48 and 72 h of treatment respectively. For Exemestane, the fold change in the IC_{50} of the E-PLHNCs and FA-E-PLHNCs was found to be 1.19, 1.31 and 1.84 and 2.06, 2.72 and 3.36 after 24, 48 and 72 h of treatment respectively. The IC_{50} values and cell viability data revealed that FA-F PLHNCs and FA-E-PLHNCs had a greater impact on cancer cells than FLV suspension and EXE suspension. Furthermore, as compared to FLV-suspension and EXE-suspension, NPs were more efficiently absorbed by tumor cells by directly trafficking into the tumor cells through endocytosis. The increased cytotoxicity of FA-F-PLHNCs and FA-E-PLHNCs shows the efficiency of folic acid in penetration of NPs via folate transport, which accounts for the increased drug concentration in the cells. Thus, the combinatorial approach of drug encapsulation in lipid-polymer core and attachment of ligand led to a higher cellular accumulation of fulvestrant and exemestane.

The results of cytotoxicity studies of fulvestrant and exemestane formulations are shown in Figure 7.4 and 7.5, and IC_{50} of formulations on MCF – 7 cell lines is presented in Table 7.4.

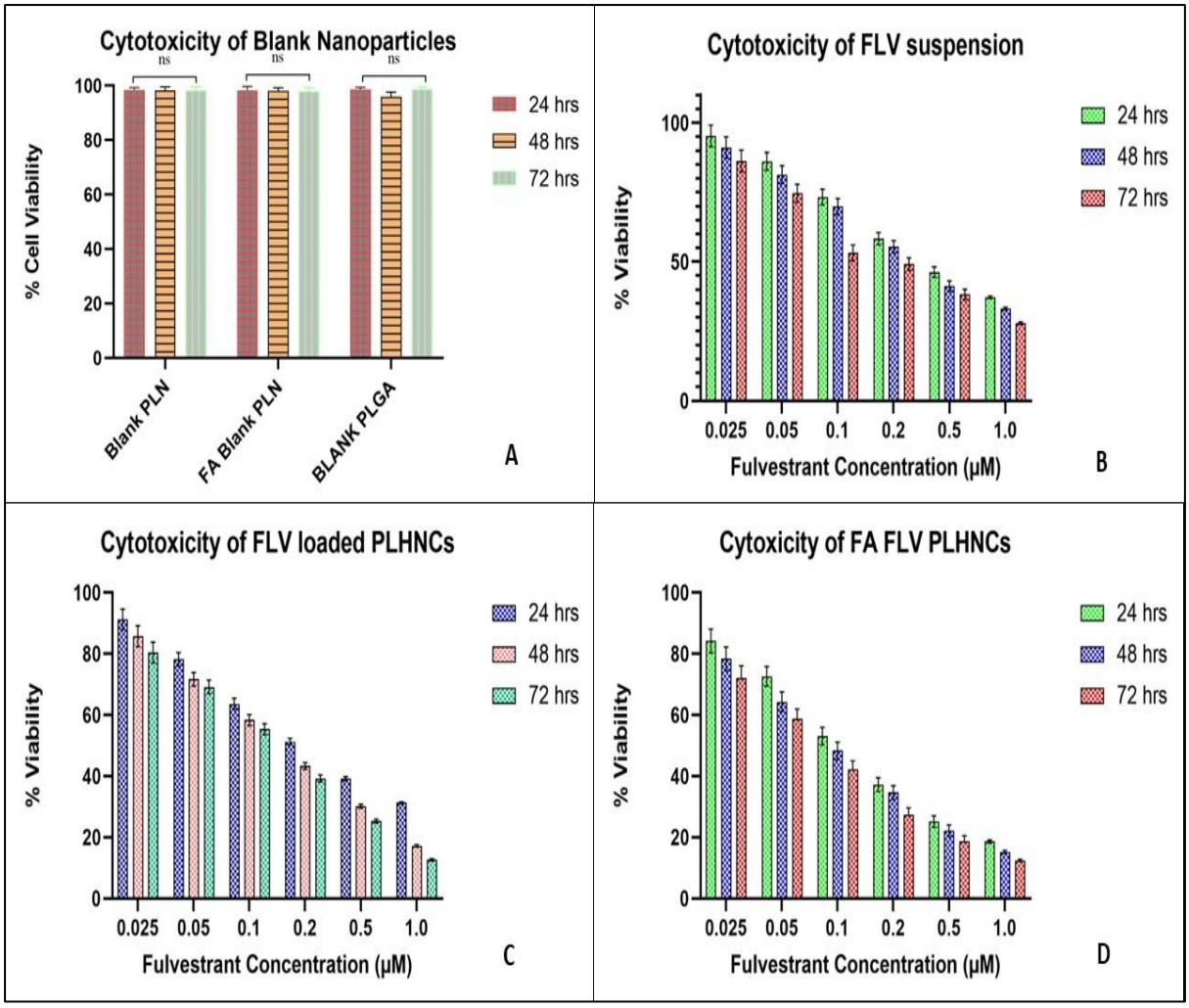

Figure 7.2 Invitro cell cytotoxicity studies of different fulvestrant formulations on MDA MB 231 cells

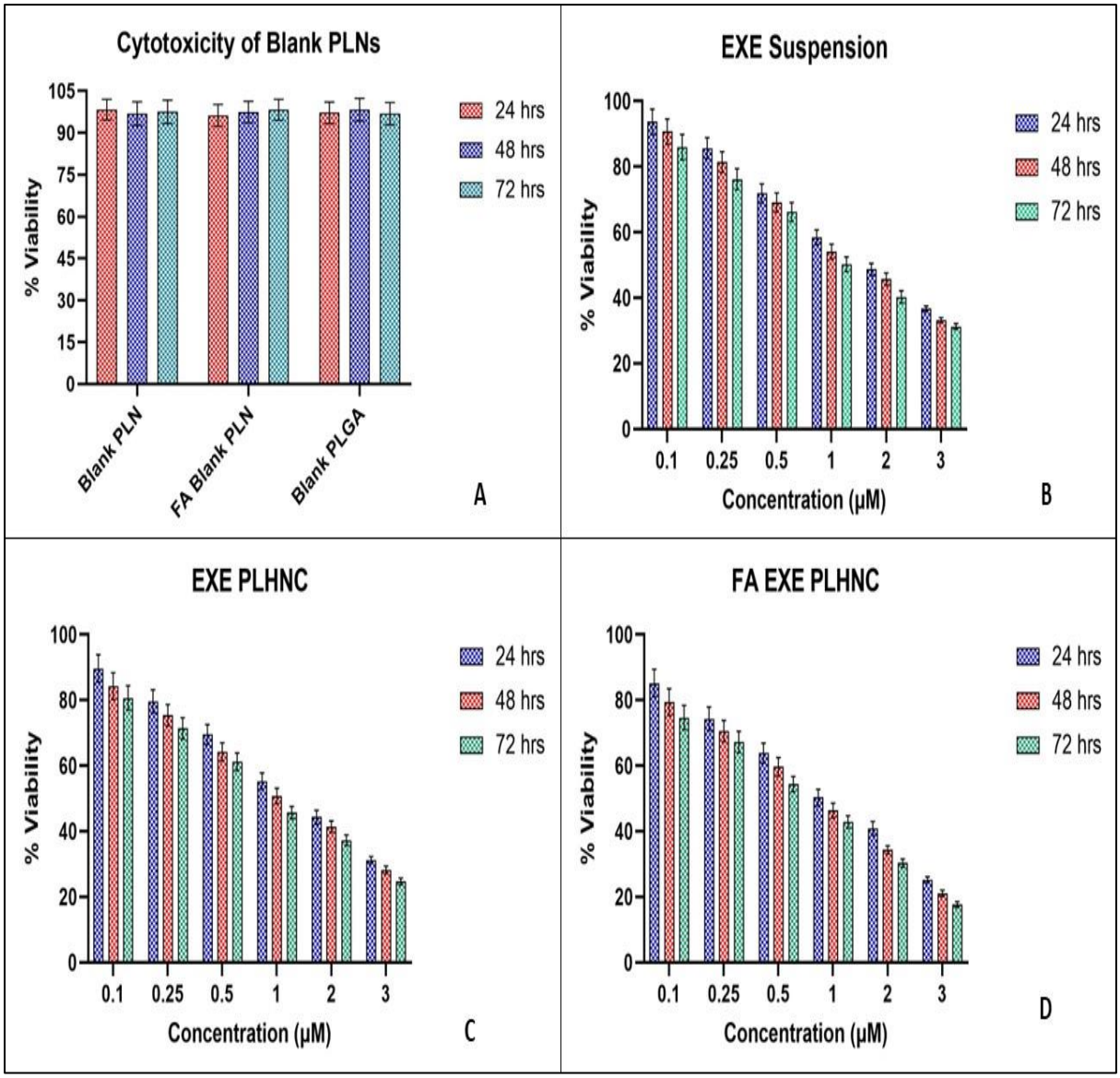

Figure 7.3 Invitro cell cytotoxicity studies of different exemestane formulations on MDA MB 231 cells.

Table 7.3 IC50 (µM) of various formulations for MDA MB 231 cell lines

Formulation Treatment	IC_{50} (µM)		
	24H	48H	72H
Fulvestrant Suspension	4.13 ± 0.28	3.38 ± 0.16	2.07 ± 0.08
FLV PLHNCs	3.68 ± 0.21	2.24 ± 0.12	1.06 ± 0.04
FA FLV PLHNCs	3.12 ± 0.18	1.86 ± 0.11	0.74 ± 0.09

Exemestane Suspension	8.63 ± 0.38	6.84 ± 0.31	5.68 ± 0.26
EXE PLHNCs	6.62 ± 0.31	5.09 ± 0.23	4.68 ± 0.19
FA EXE PLHNCs	5.56 ± 0.26	4.18 ± 0.17	3.26 ± 0.12

Table 7.4 IC50 (µM) of various formulations for MCF – 7 cell lines

Formulation Treatment	**IC_{50} (µM)**		
	24H	**48H**	**72H**
Fulvestrant Suspension	3.12 ± 0.28	2.21 ± 0.16	1.23 ± 0.08
FLV PLHNCs	2.56 ± 0.21	1.38 ± 0.12	0.76 ± 0.04
FA FLV PLHNCs	2.13 ± 0.18	0.96 ± 0.11	0.55 ± 0.09
Exemestane Suspension	7.89 ± 0.31	5.79 ± 0.27	4.76 ± 0.16
EXE PLHNCs	6.38 ± 0.27	4.62 ± 0.21	3.51 ± 0.14
FA EXE PLHNCs	5.63 ± 0.22	4.13 ± 0.18	3.24 ± 0.12

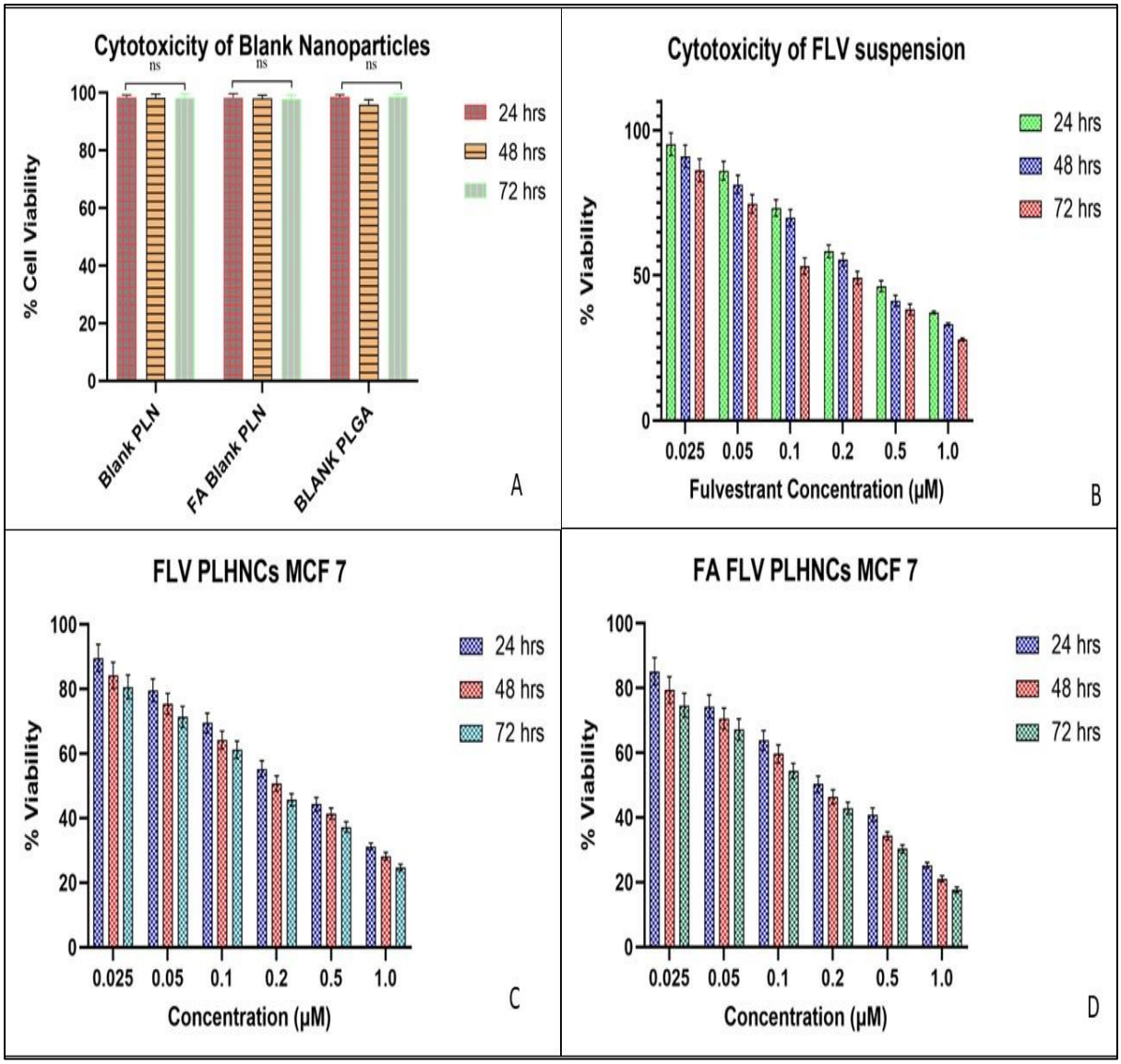

Figure 7.4 Invitro cell cytotoxicity studies of different fulvestrant formulations on MCF – 7 cells

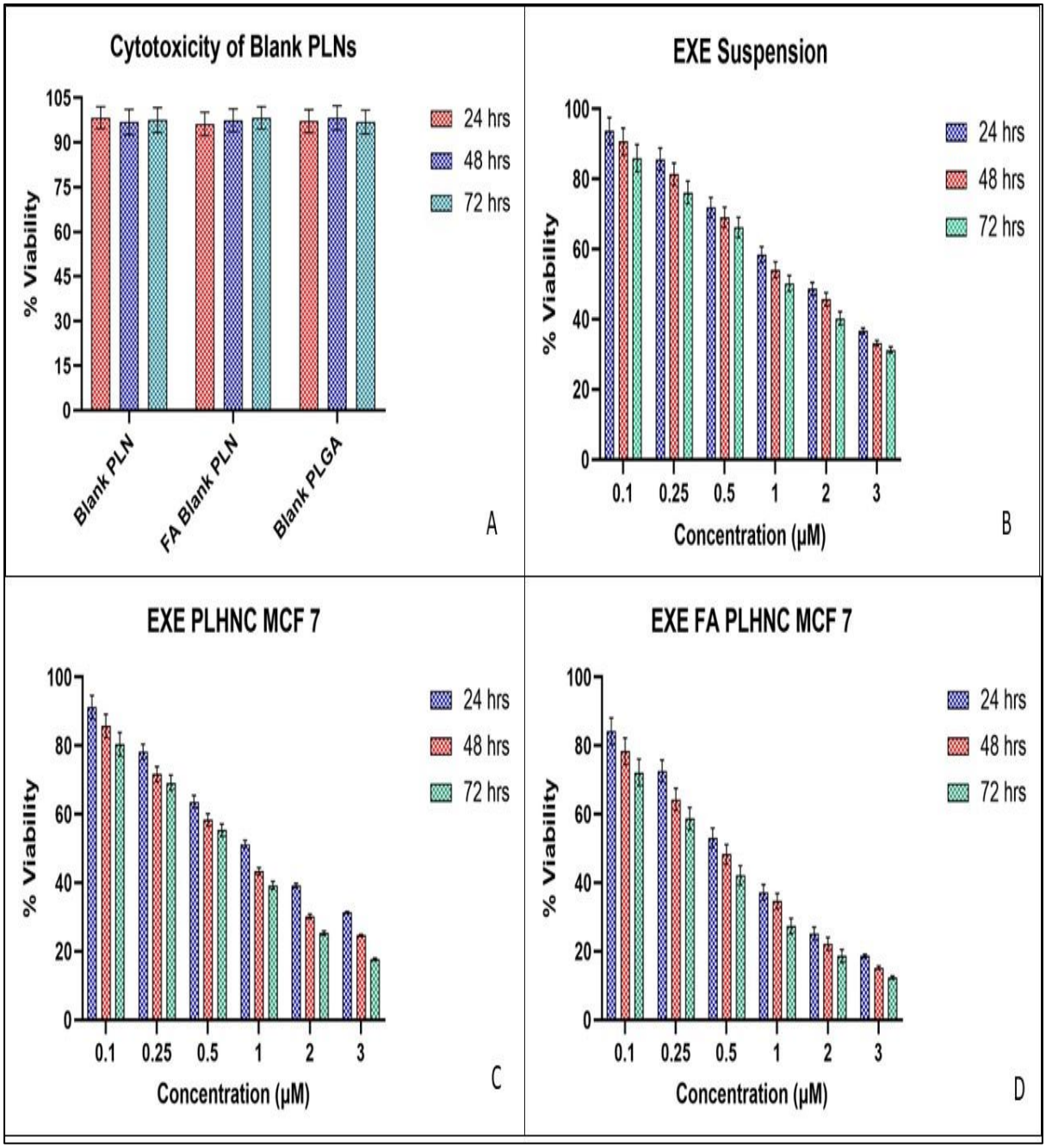

Figure 7.5 Invitro cell cytotoxicity studies of different exemestane formulations on MCF – 7 cells

7.8.2 Cell migration (Scratch assay) for MDA MB 231 and MCF – 7 cells

From Figure 7.6, it can be observed that there was significant reduction in the scratch area after 24 h in the control cells and the cells had fully regrown and multiplied after 48 h (no scratch was visible). A significant decrease was noticed in the FLV PLHNCs, EXE PLHNCs, FA FLV PLHNCs, and FA EXE PLHNCs formulations as the scratch area remained almost the same

even after 48 h. Again, standard Fulvestrant and Exemestane suspension showed some cell-cell interaction and migration after 24 h and 48 h which showed that cells had effluxed the drug, making plain drug treatment ineffective over time.

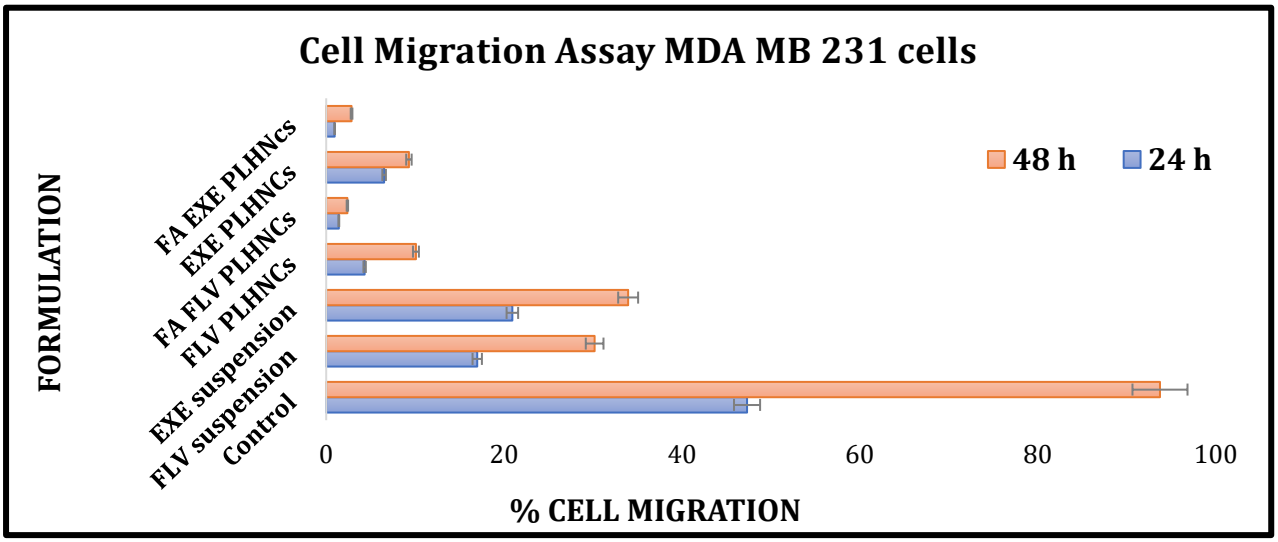

Figure 7.6 Cell migration assay for PLHNC formulations for MDA MB 231 cells

For MCF – 7 cells, from Figure 7.7 it can be observed that there was significant reduction in the scratch area after 24 h in the control cells and the cells had fully regrown and multiplied after 48 h (no scratch was visible). A significant decrease was noticed in the FLV PLHNCs, EXE PLHNCs, FA FLV PLHNCs, and FA EXE PLHNCs formulations as the scratch area remained almost the same even after 48 h. Again, standard Fulvestrant and Exemestane suspension showed some cell-cell interaction and migration after 24 h and 48 h which showed that cells had effluxed the drug, making treatment ineffective over time.

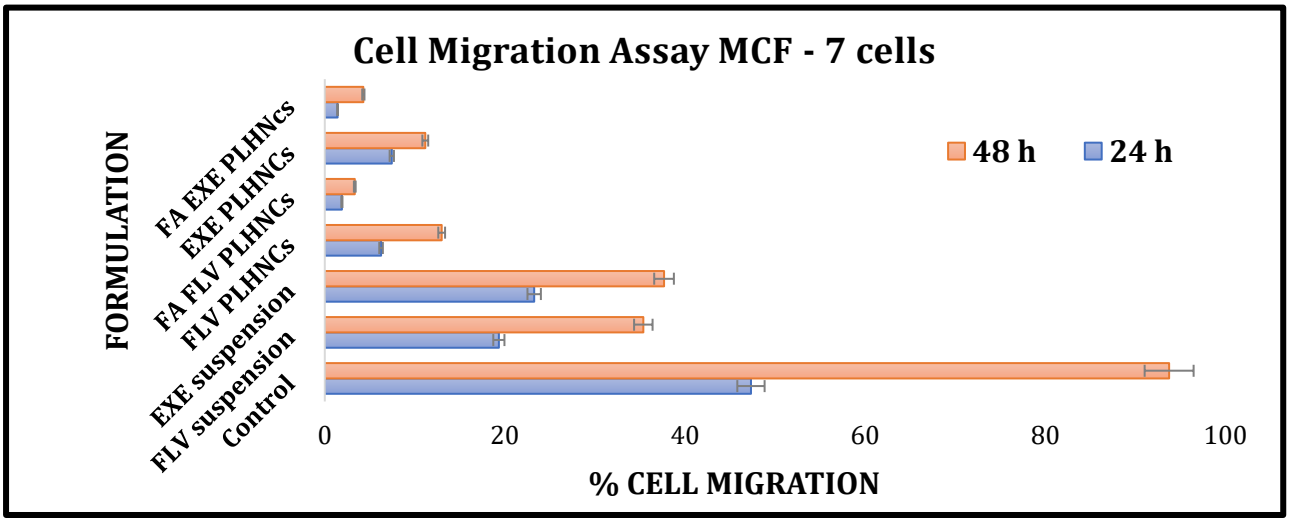

Figure 7.7 Cell migration assay for PLHNC formulations on MCF – 7 cells

7.8.3 Qualitative uptake studies using confocal microscopy

Confocal images shown in Figure 7.8 suggests the effect of targeting on the cellular internalization and the role of formulation development in targeting tumor cells. From Figure 7.8, it can be observed that the cellular uptake of folate targeted formulation drastically increased over time compared to non-targeted PLHNCs. An increase in fluorescence with time suggested the internalization process of PLHNCs. At any time-point, non-targeted PLHNCs have shown less fluorescence compared to the targeted ones. PLHNCs formulation, both targeted and non-targeted, could penetrate cellular bilayer very easily and with high efficiency. Folate targeted PLHNCs had more uptake (i.e., more fluorescence inside the cell) due to an enhanced engulfing of folate receptors on the cell surface. The fusogenic properties of the cationic lipids may have facilitated cellular uptake via micropinocytosis transfer and resulted in more fluorescence in both targeted and non-targeted PLHNCs compared to plain drug suspension.

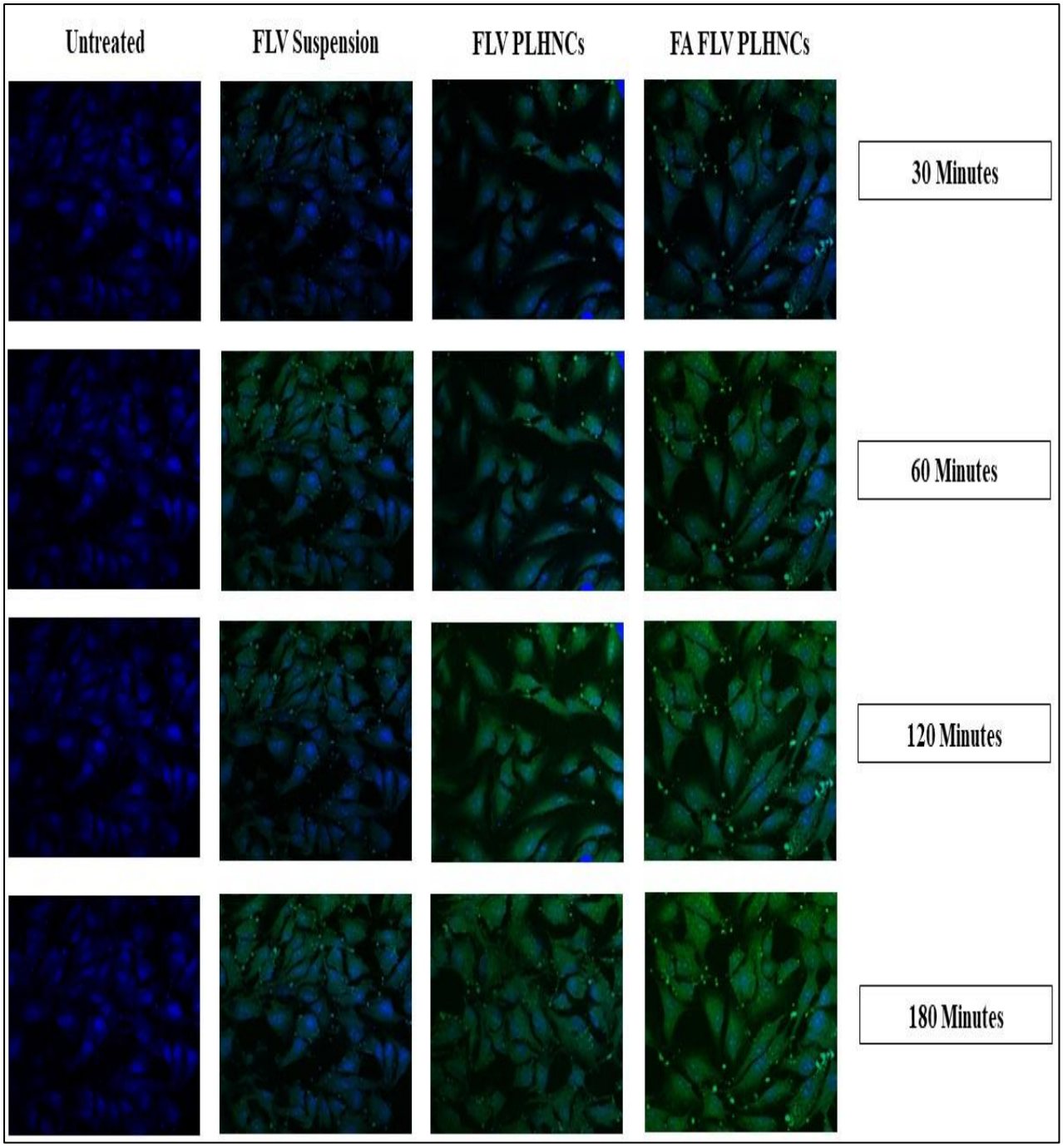

Figure 7.8 Confocal microscopy of cellular uptake at different time intervals for fulvestrant formulations

7.8.4 Quantitative uptake studies

Quantitative studies were carried out using HPLC method. The results of the quantitative uptake of Fulvestrant and Exemestane by MDA MB 231 and MCF – 7 cells are shown in Figure 7.9 and 7.10 respectively. The increased cell uptake of Folate conjugated PLHNCs was accredited to the cytosolic drug delivery of nanoparticles. Quantitative results obtained at

various concentrations were supported by qualitative observations of the confocal microscopy. The results suggested that the internalization of all formulations was in concentration dependent manner. The results also showed that the uptake increased in time dependent manner as the polymer sustains the release of drug from its matrix. Also, the efficiency of cellular uptake for both the drugs was found to be maximum in folate conjugated PLHNCs compared to drug loaded PLHNCs, whereas the least cellular uptake was found with the drug suspension.

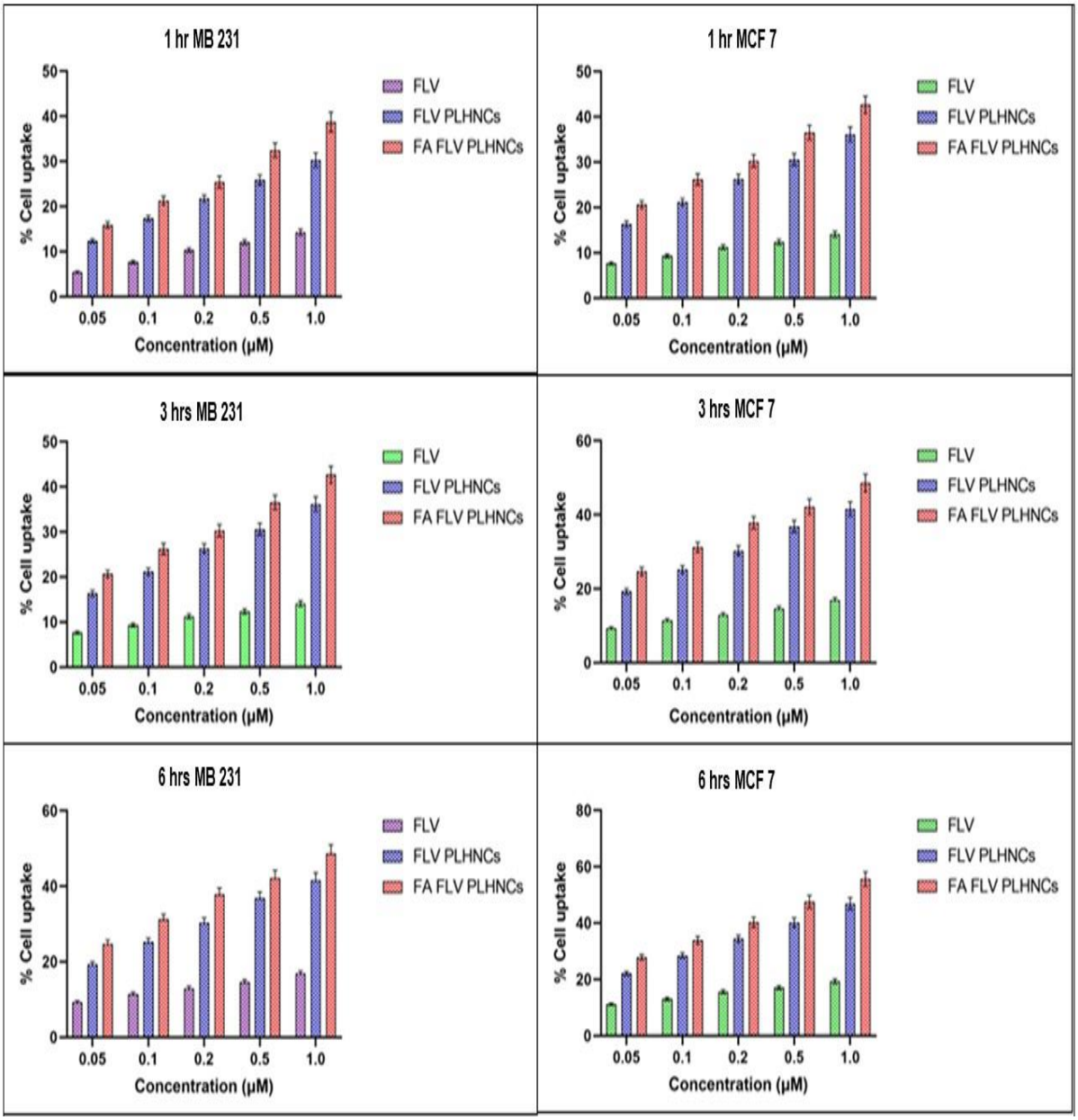

Figure 7.9 Quantitative cellular uptake of FLV suspension, FLV PLHNCs and FA FLV PLHNCs at different time intervals and different concentration

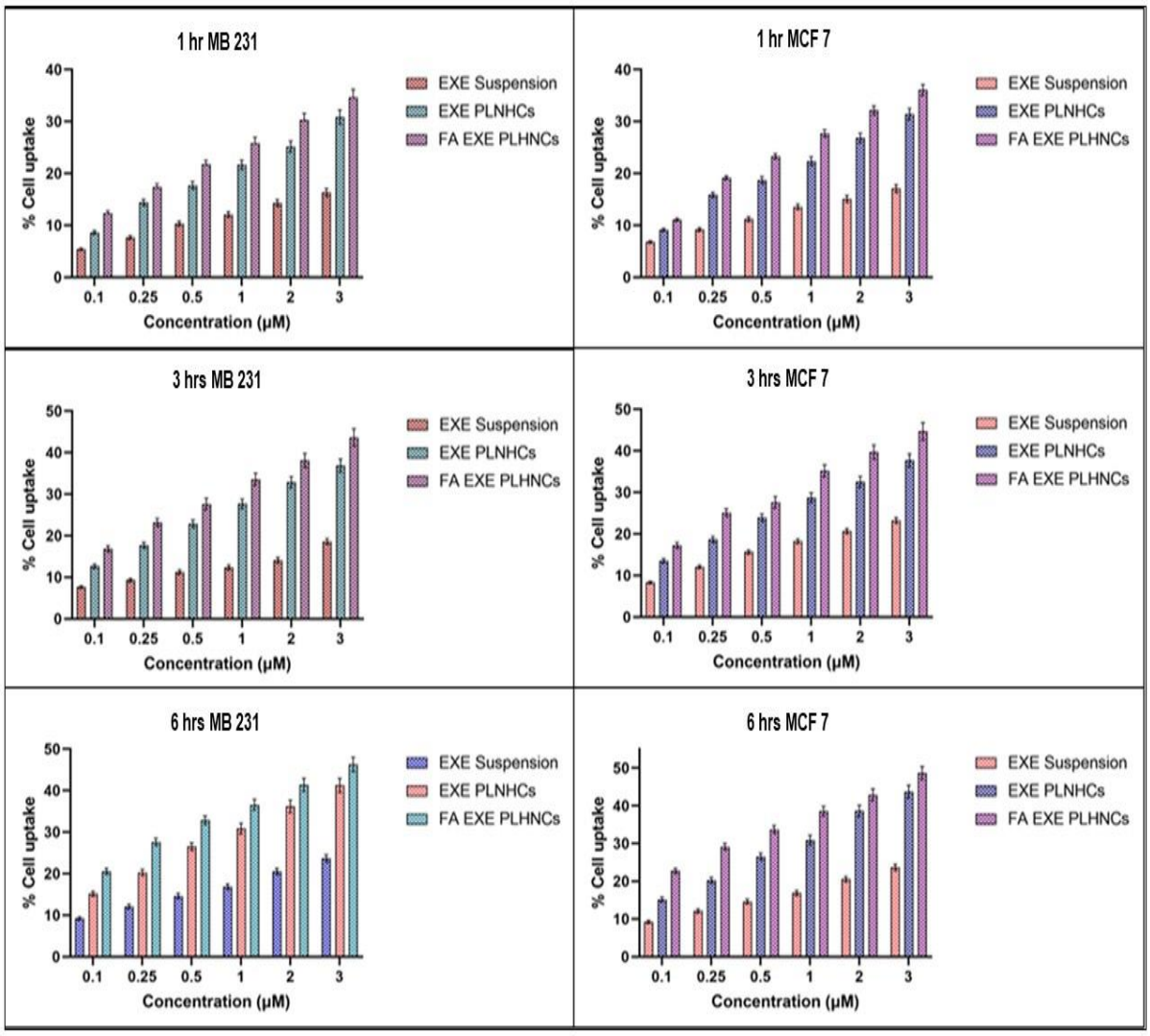

Figure 7.10 Quantitative cellular uptake of EXE suspension, EXE PLHNCs and FA EXE PLHNCs at different time intervals and different concentration

7.8.5 Caspase -3 Induction assay

Caspase-3 acts as a mediator in nuclear apoptosis which includes chromatin condensation, DNA fragmentation along with cell blebbing (24). From figure 7.11, it can be concluded that control cells with no treatment showed almost negligible cell apoptosis, while cells treated with fulvestrant and exemestane suspension possessed caspase-3 activity. A significant increase was observed when the cells were treated with FLV PLHNCs and EXE PLHNCs which was almost twice more than that of suspension, which postulates higher cellular internalization and higher apoptotic efficiency (25). Though there was significant increase in cells treated with folate conjugated nanoparticles compared to drug suspension, but not significant than that of the drug

loaded PLHNCs, as it is established that folate conjugation plays an important role in targeting but does not play a significant role in increasing apoptotic activity (26).

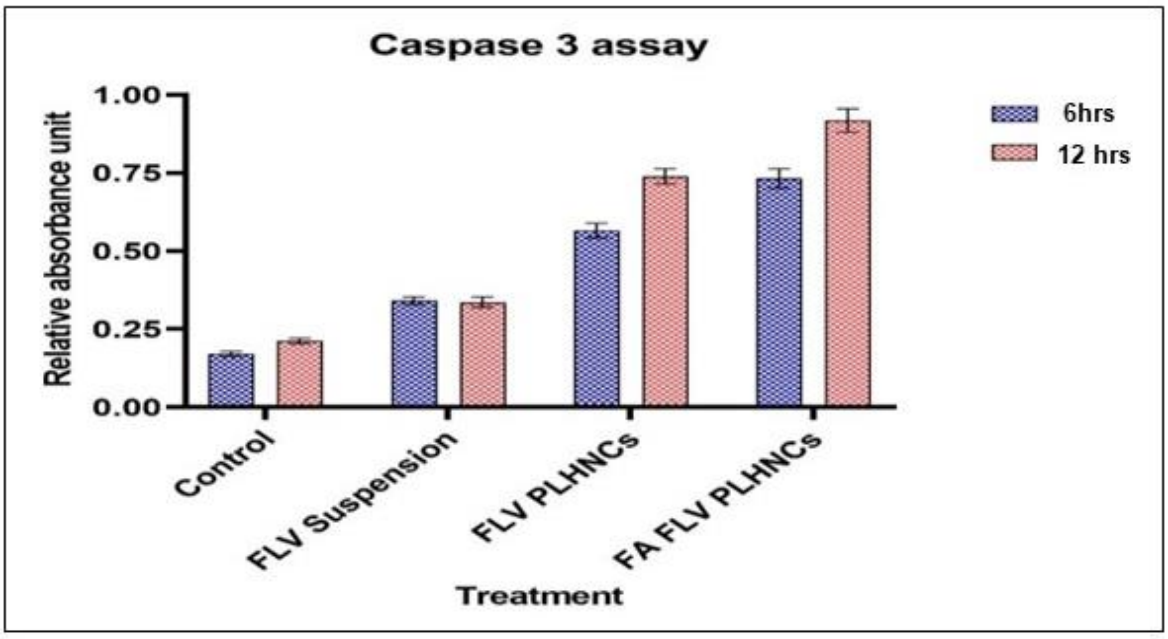

Figure 7.11 (A) Detection of caspase-3 level in MDA MB 231 cell line using CASP-3-C kit, treated with different fulvestrant formulations and incubated for 6 and 12 hrs.

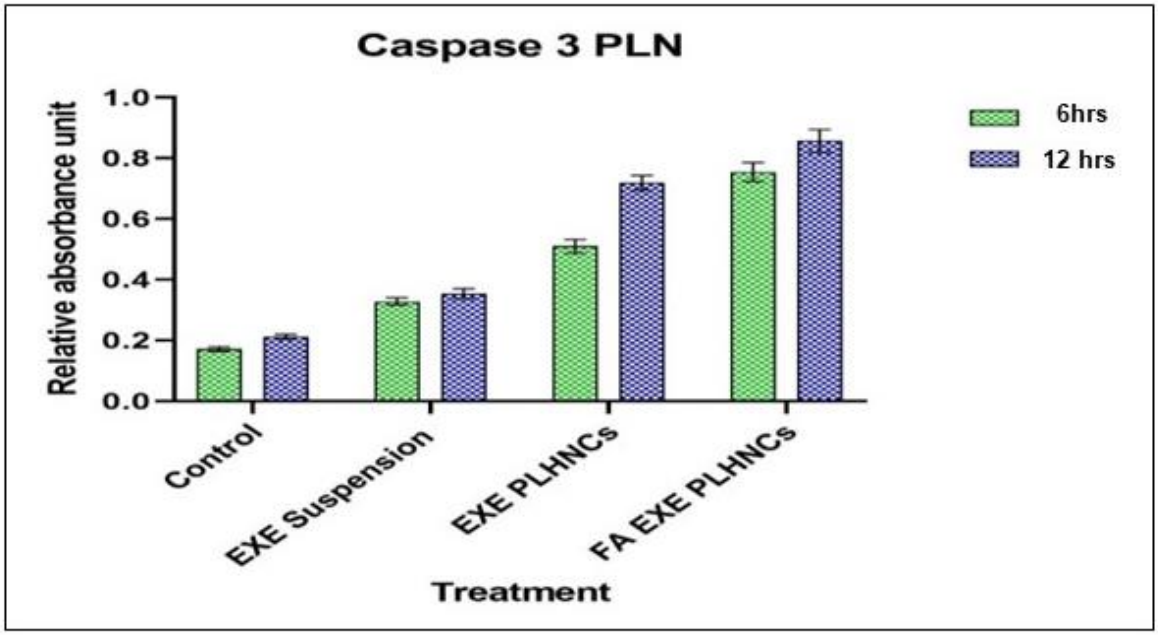

Figure 7.11 (B) Detection of caspase-3 level in MDA MB 231 cell line using CASP-3-C kit, treated with different exemestane formulations and incubated for 6 and 12 hrs.

7.8.6 Cell Apoptosis detection

FITC- Annexin V- Propidium Iodide (PI) was used to differentiate live cells, early apoptotic cells, and late apoptotic cells (11). The intact membranes of viable cells do not permit PI interaction, while dead cells permit the PI interaction. Hence, FITC-Annexin V/ PI staining would help to differentiate early apoptosis from late apoptosis in cell populations. As seen in Figure 7.12, after the incubation with MDA MB 231 cells for 24 h, fulvestrant loaded hybrid nanocarriers treated cells shows a shift towards the upper right quadrant in the cell chamber that indicates initiation of early as well late apoptosis (10). Untreated cells show most cells in the lower left quadrant of the cell chamber which suggested a high number of live cells with negligible DNA fragmentation. An extreme upward right shift of the folate targeted formulation suggested a predominant number of cells in the early as well as late stages of apoptosis, which proves the high DNA fragmentation and condensation of chromatin due to fulvestrant inside the cells.

The apoptosis assay of exemestane formulations shows a slightly different apoptosis approach compared to fulvestrant formulations, there are 68% cells, that shows signs of late apoptosis that indicates the regeneration of cells due the different mechanism of action of exemestane in breast cancer treatment (27). EXE hinders the aromatase enzyme that is responsible for estrogen synthesis, but it doesn’t have any effect on already synthesized estrogen which serves as media for growth of tumor cells (28). Hence, EXE formulations showed lesser apoptotic effect compared to fulvestrant formulations.

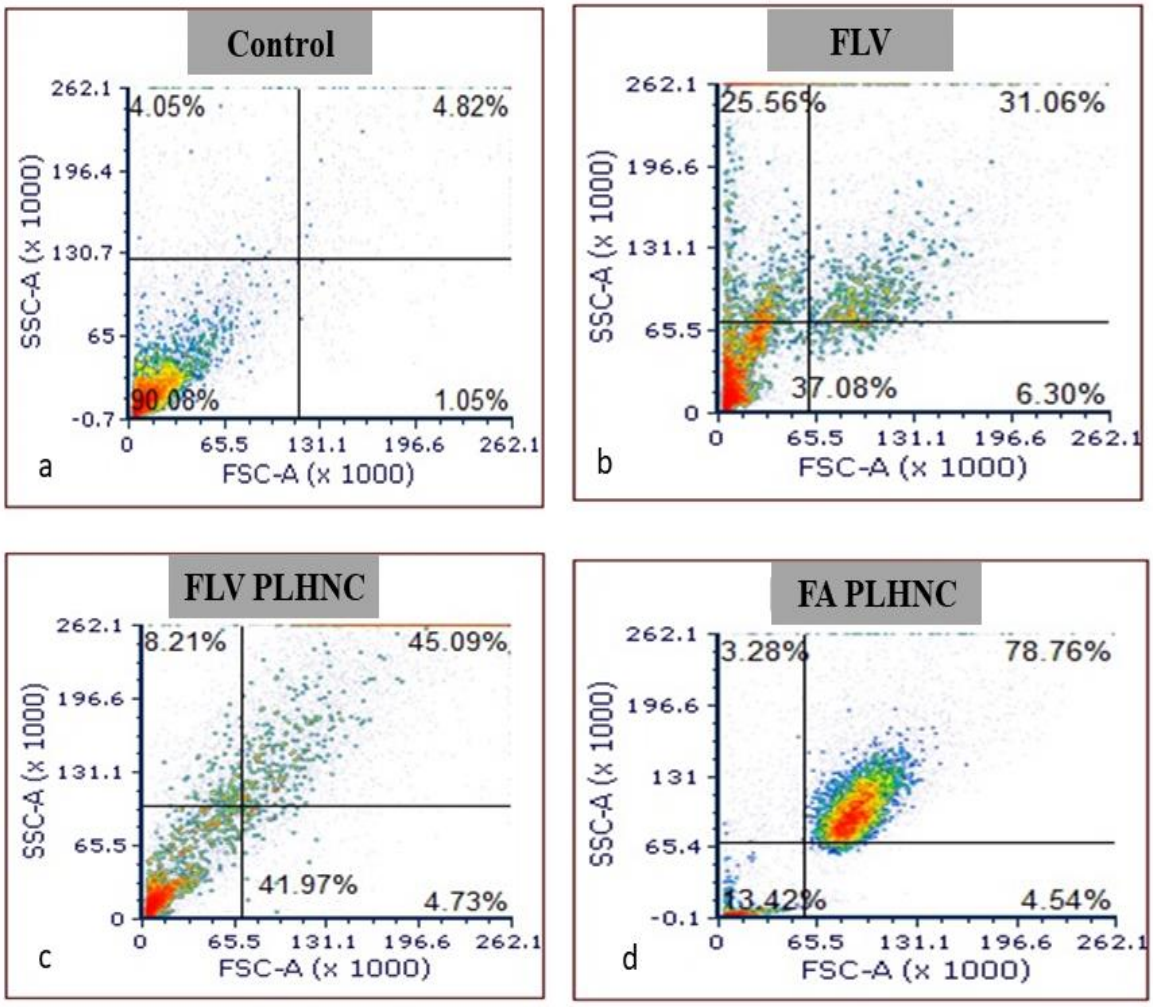

Figure 7.12 Apoptosis cell analysis of MDA MB 231 cell line for a) Control cells, b) FLV suspension, c) FLV PLHNCs, d) FA FLV PLHNCs

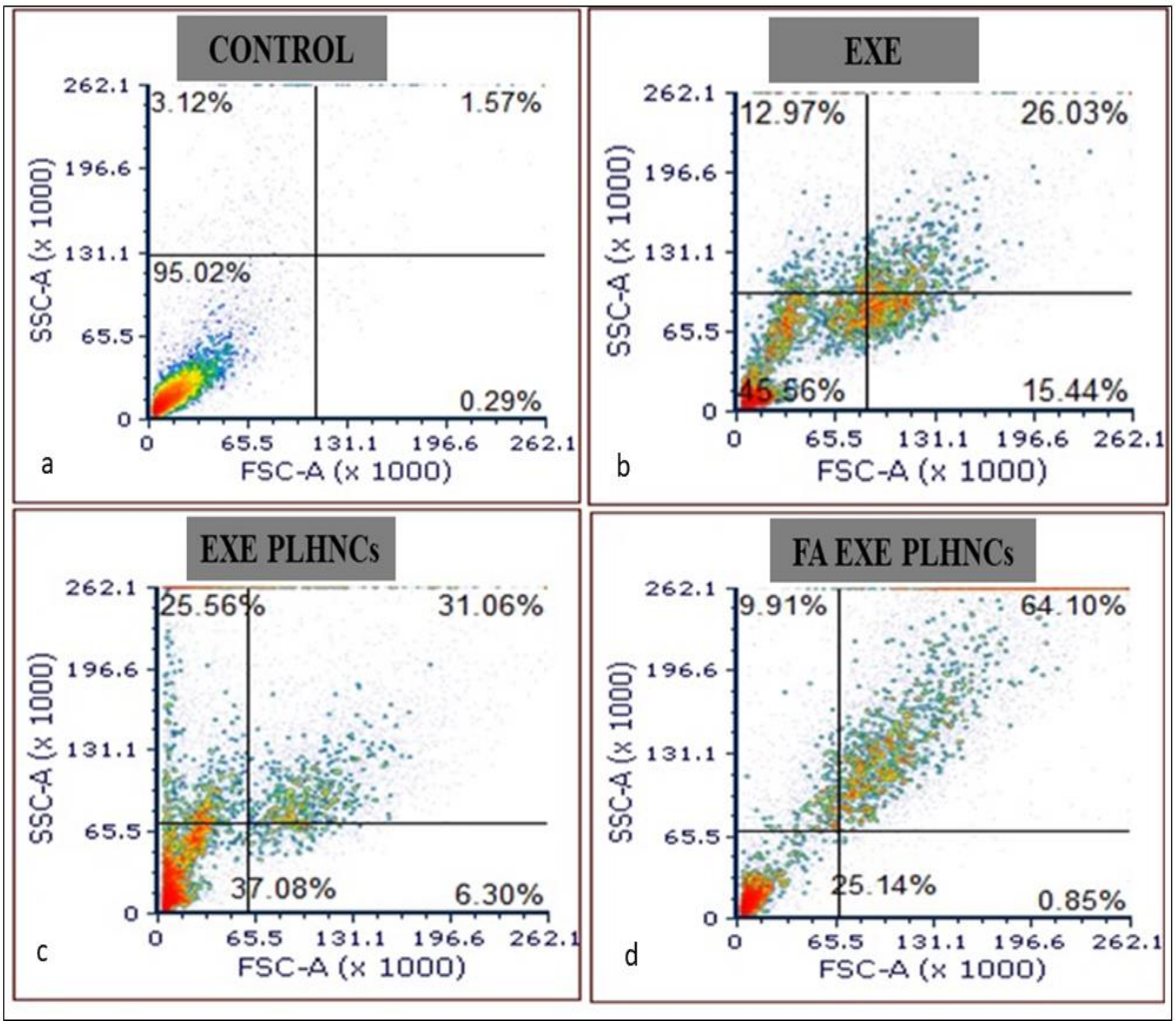

Figure 7.13 Apoptosis cell analysis of MDA MB 231 for a) Control cells, b) EXE suspension, c) EXE PLHNCs, d) FA EXE PLHNCs

7.9 Results and Discussion

PART B: Fulvestrant and Exemestane Loaded Mesoporous Silica Nanoparticles (MSNs)

7.9.1 Cytotoxicity assay of various MSN formulations on MDA MB 231 and MCF – 7 cells

The results of cytotoxicity studies are shown in Figure 7.14 and IC50 of the MDA MB 231 cell lines with various formulations is presented in Table 7.5. The IC_{50} values and cell viability data revealed that FLV MSN, FQMSN and EXE MSN, EQMSN had a greater permeation in cancer cells than FLV suspension and EXE suspension. Furthermore, as compared to FLV-suspension and EXE-suspension, NPs were more efficiently absorbed by tumor cells by passive diffusion into the tumor cells. The increased cytotoxicity of FQMSN and EQMSN shows that

nanoparticulate system improves the cellular internalization of fulvestrant and exemestane in the cellular atmosphere. However, addition of quercetin to the formulation improves the sensitivity of the drugs to the cells, also reverses the estrogen resistance in the MDA MB 231 cells and also provides synergistic anticancer activity against the breast cancer cells (29). The results of cytotoxicity studies on MCF – 7 cells are shown in Figure 7.16 and IC50 of the MCF – 7 cell lines with various formulations is presented in Table 7.6.

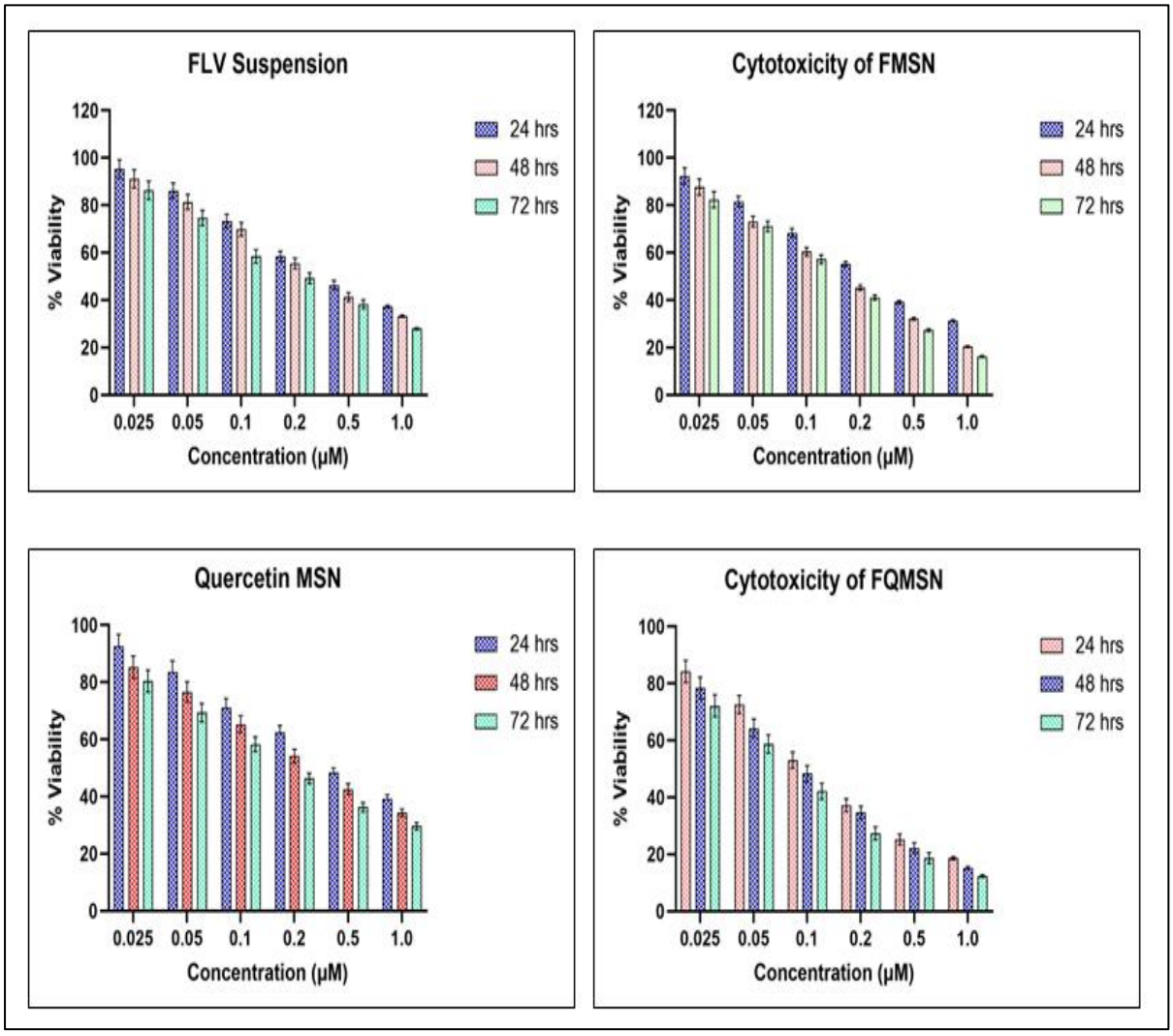

Figure 7.14 Invitro cell cytotoxicity studies of different fulvestrant formulations on MDA MB 231 cells.

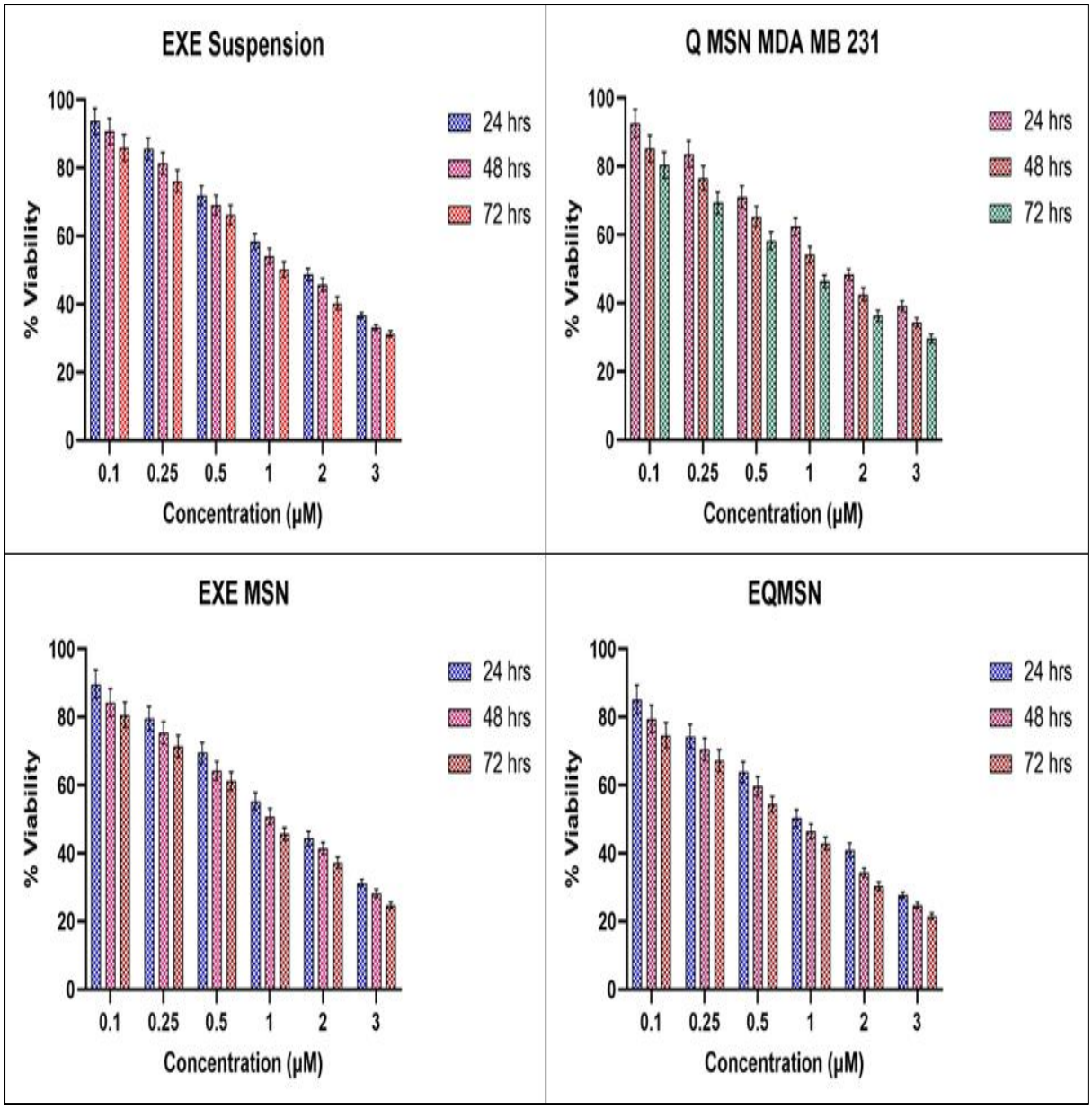

Figure 7.15 Invitro cell cytotoxicity studies of different exemestane formulations on MDA MB 231 cells.

Table 7.5 IC50 (μM) of various formulation for MDA MB 231 cell lines

Formulation Treatment	IC_{50} (μM)		
	24H	48H	72H
Fulvestrant Suspension	4.13 ± 0.28	3.38 ± 0.16	2.07 ± 0.08
Exemestane Suspension	8.63 ± 0.38	6.84 ± 0.31	5.68 ± 0.26

Quercetin MSN	8.19 ± 0.28	6.89 ± 0.23	5.92 ± 0.19
FLV MSN	2.87 ± 0.18	1.68 ± 0.12	1.06 ± 0.08
FQMSN	2.18 ± 0.12	1.19 ± 0.06	0.59 ± 0.04
EXE MSN	6.24 ± 0.36	3.78 ± 0.19	2.76 ± 0.18
EQMSN	3.64 ± 0.21	2.83 ± 0.16	2.08 ± 0.09

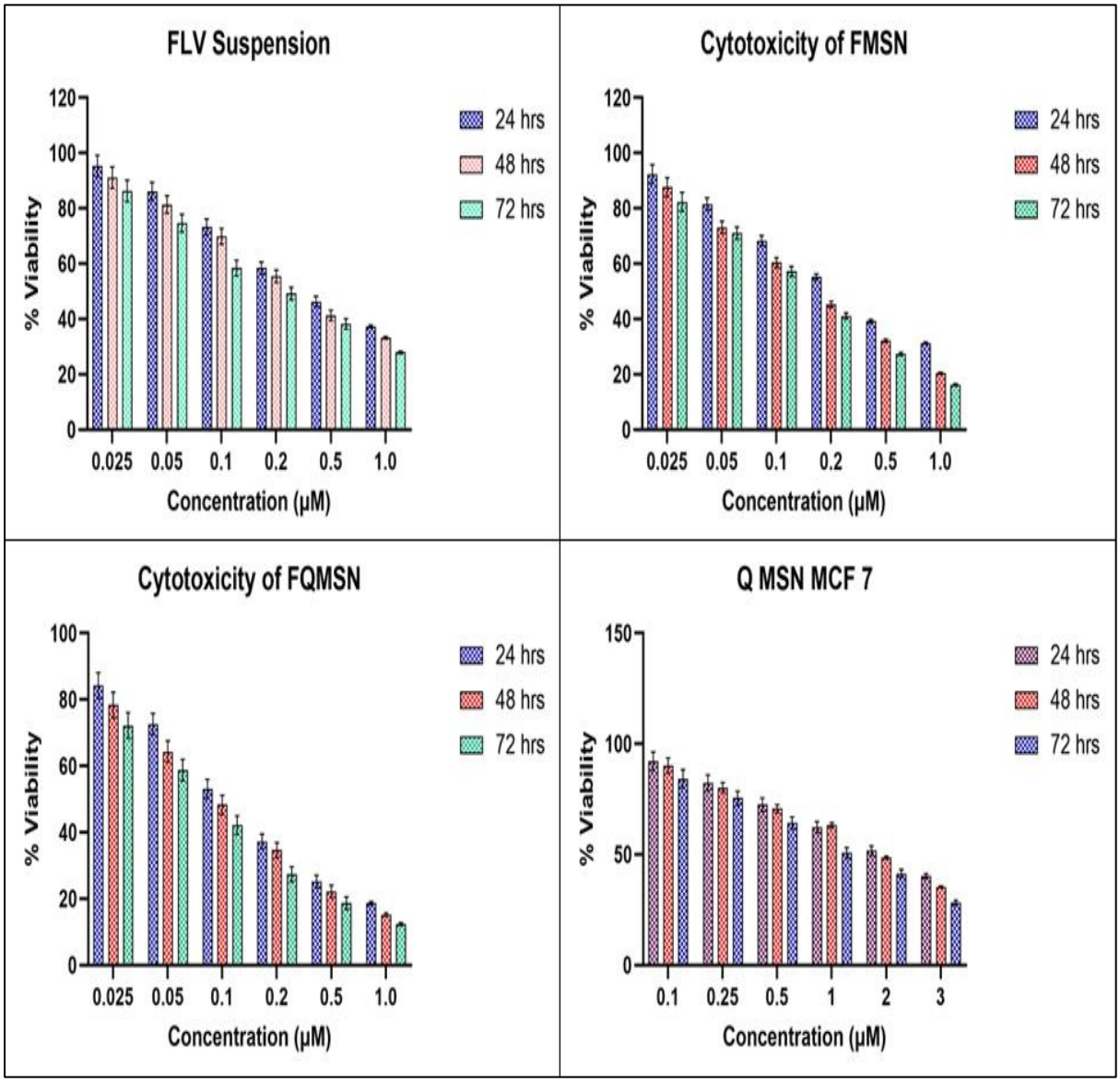

Figure 7.16 Invitro cell cytotoxicity studies of different fulvestrant formulations on MCF – 7 cells.

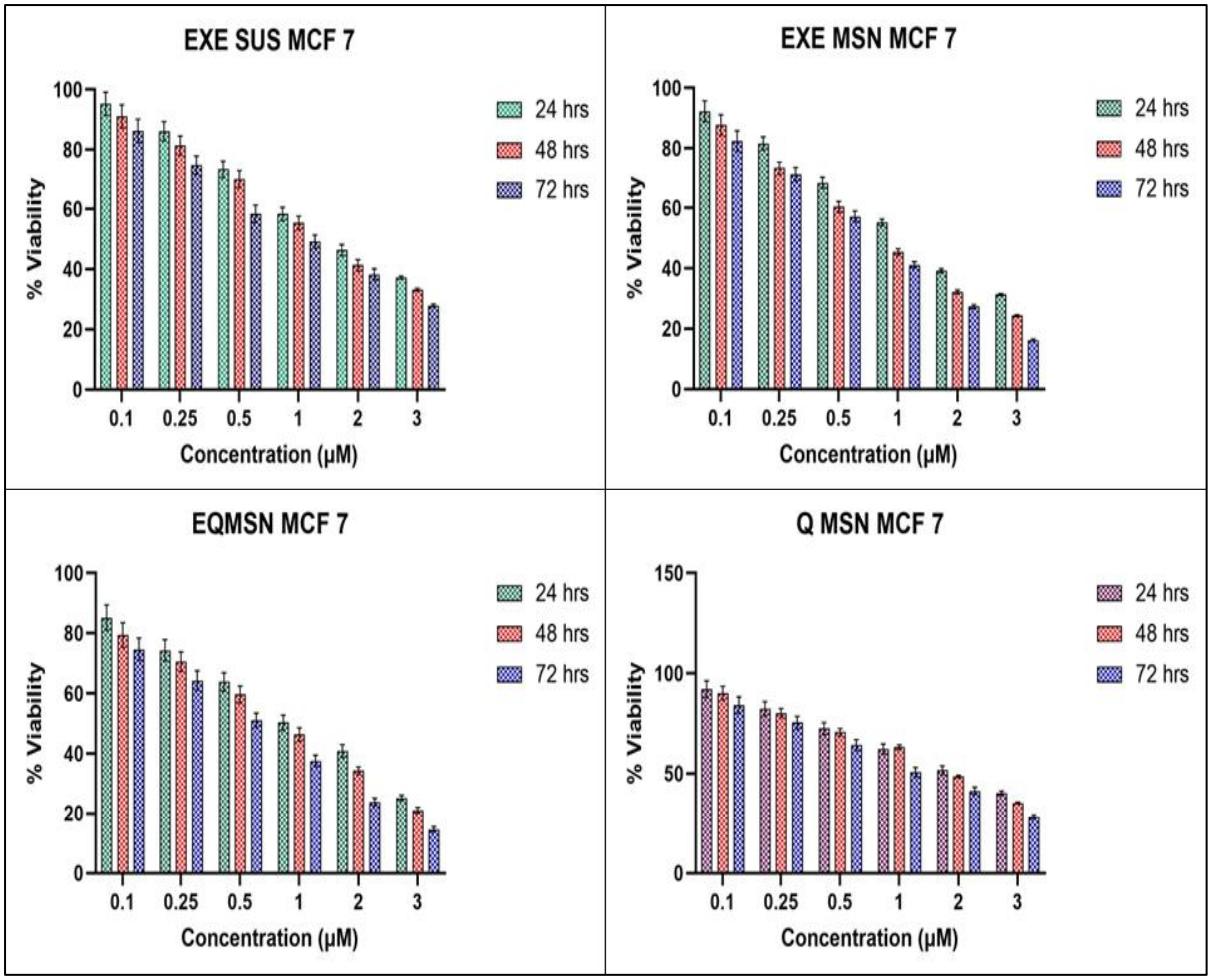

Figure 7.17 Invitro cell cytotoxicity studies of different exemestane formulations on MCF – 7 cells.

Table 7.6 IC50 (µM) of various formulation for MCF – 7 cells

Formulation Treatment	IC_{50} (µM)		
	24H	48H	72H
Fulvestrant Suspension	3.12 ± 0.28	2.21 ± 0.16	1.23 ± 0.08
Exemestane Suspension	7.89 ± 0.31	5.79 ± 0.27	4.76 ± 0.16
Quercetin MSN	7.19 ± 0.29	5.18 ± 0.24	4.21 ± 0.24
FLV MSN	2.37 ± 0.18	1.36 ± 0.12	0.96 ± 0.08
FQMSN	1.58 ± 0.12	0.89 ± 0.06	0.48 ± 0.04
EXE MSN	5.14 ± 0.36	2.94 ± 0.19	2.26 ± 0.18
EQMSN	2.87 ± 0.21	2.13 ± 0.16	1.58 ± 0.09

7.9.2 Cell migration (Scratch assay) for MDA MB 231 and MCF – 7 cells.

From Figure 7.18 & 7.19, it can be observed that there was significant reduction in the scratch area after 24 h in the control cells and the cell population had increased after span of 48 h (no scratch was visible). A significant decrease was noticed in the FLV MSN, FQMSN and EXE MSN, EQMSN formulations as the scratch area remained almost the same after 48 h ($p < 0.0074$). Again, standard Fulvestrant and Exemestane suspension showed some inhibition of cellular growth and apoptosis as well as migration after 24 h but after 48h, the scratch area had contracted and the cells had regrown as they had developed resistance to the drug, making the treatment ineffective over time.

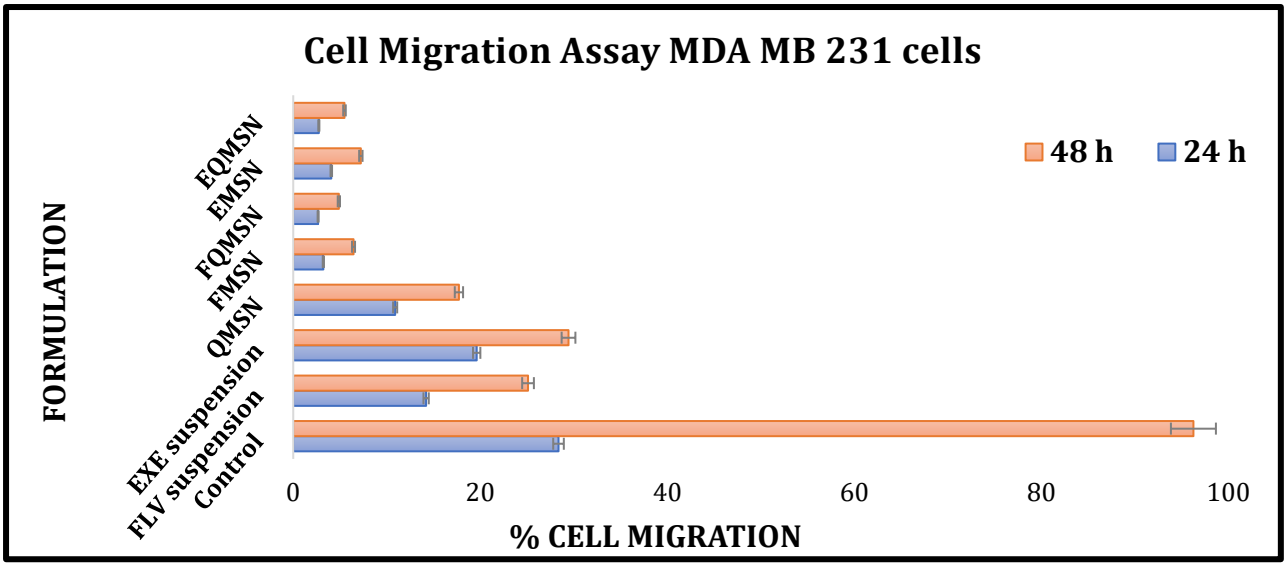

Figure 7.18 Cell migration assay for MSN formulation on MDA MB 231 cells

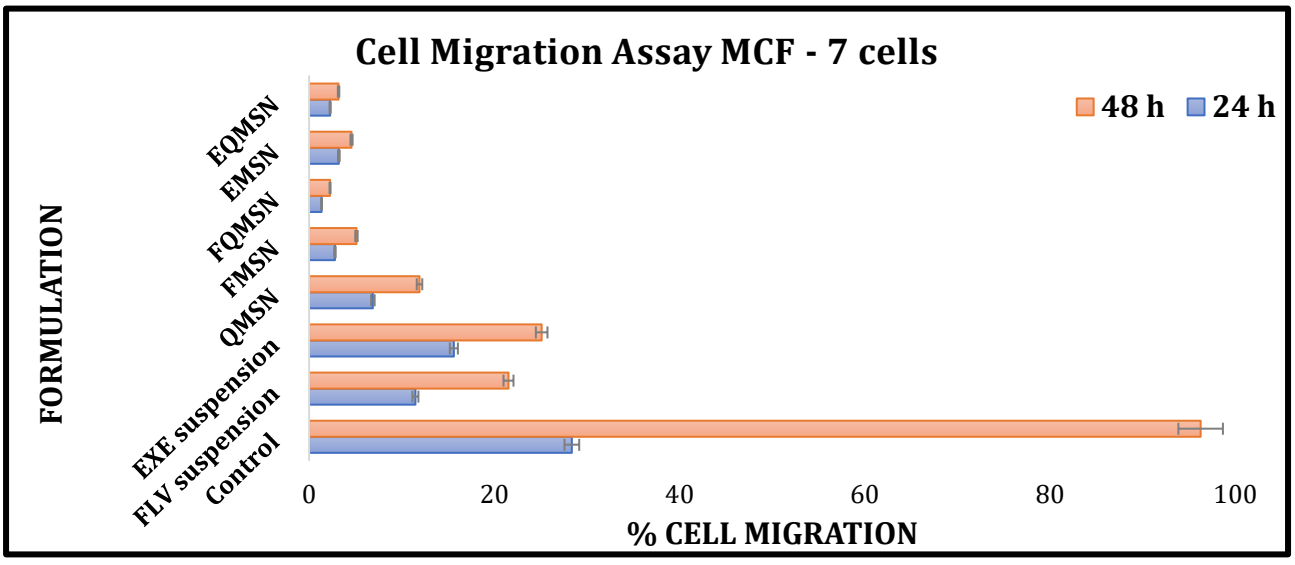

Figure 7.19 Cell migration assay for MSN formulation on MCF – 7 cells

7.9.3 Qualitative uptake studies using confocal microscopy

Confocal images shown in Figure 7.20 suggests the effect of targeting on the cellular internalization and the role of formulation development in targeting tumor cells. It can be observed that the cellular uptake studies of FQMSN and EQMSN postulated more cellular internalization compared to non-targeted and pure drug suspension. The higher uptake can be attributed to the overexpressed folate receptors in MDA MB 231 cells and the nanoparticulate size of particles and enhanced permeability and retention. An increase in fluorescence with time has suggested the internalization process of MSNs. At any time-point, non-targeted MSNs have shown less fluorescence compared to the targeted ones. MSNs formulation, both targeted and non-targeted, could penetrate cellular bilayer very easily and with high efficiency. Folate targeted MSNs had more uptake (i.e., more fluorescence inside the cell) due to abundant presence of folate receptors on the cell surface that form folic acid folate conjugate and release the drug in cellular matrix.

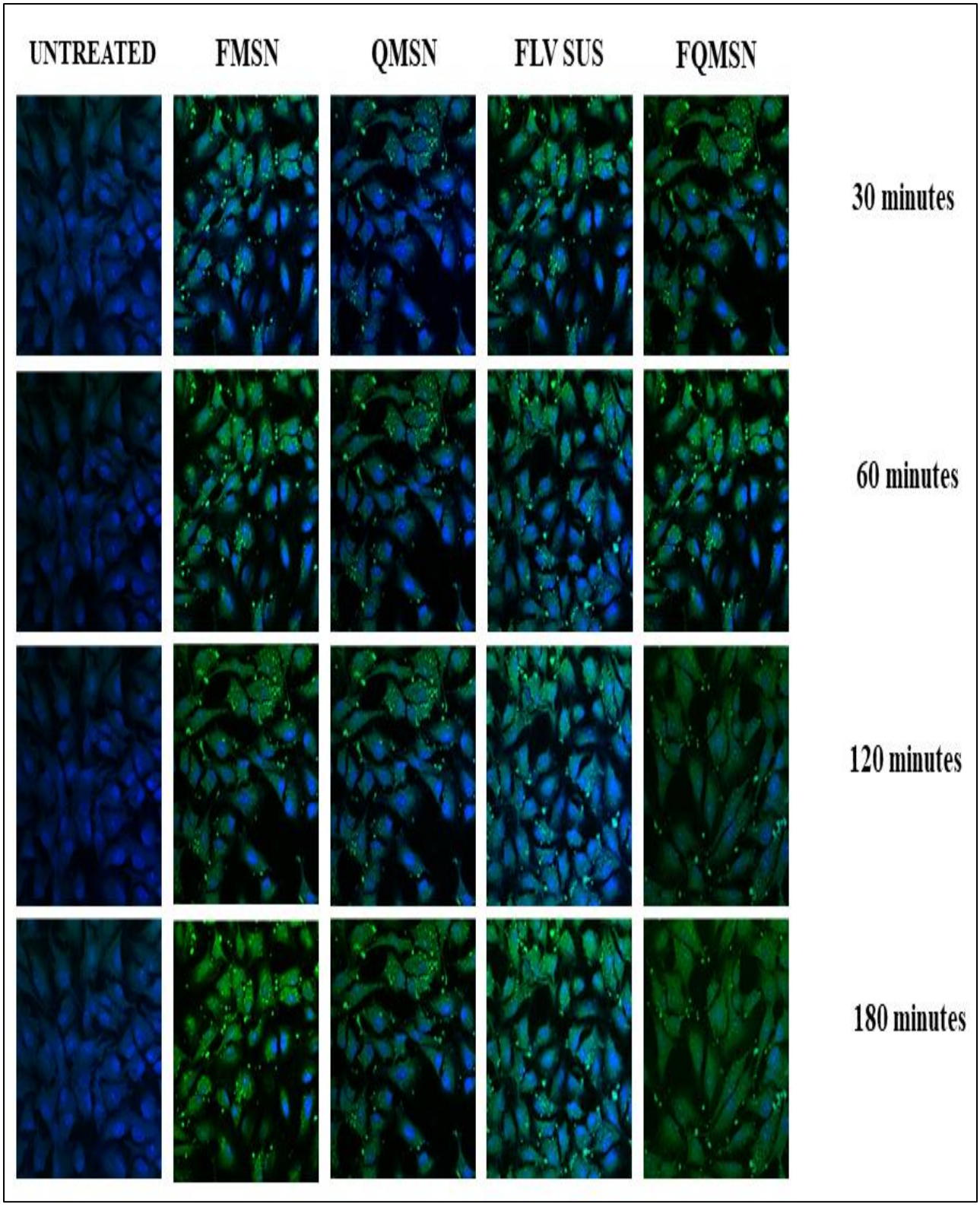

Figure 7.20 Confocal microscopy of cellular uptake at different time intervals for Untreated, FLV suspension, FLV MSN, QMSN and FQMSN.

7.9.4 Quantitative uptake studies

The results are shown in Figure 7.21 and 7.22 for fulvestrant and exemestane respectively. The higher cellular uptake of MSN-FA can be primarily attributed to the receptor mediated uptake mechanism via folate receptor. Quantitative results obtained at various concentrations were supported by qualitative observations of the confocal microscopy. The results suggested that the internalization of FLV MSN, EXE MSN, FQMSN and EQMSN was in time and concentration dependent manner. Also, the efficiency of cellular uptake for both the drugs was found to be maximum in folate conjugated MSNs compared to drug loaded MSNs, whereas the least cellular uptake was found with the drug suspension.

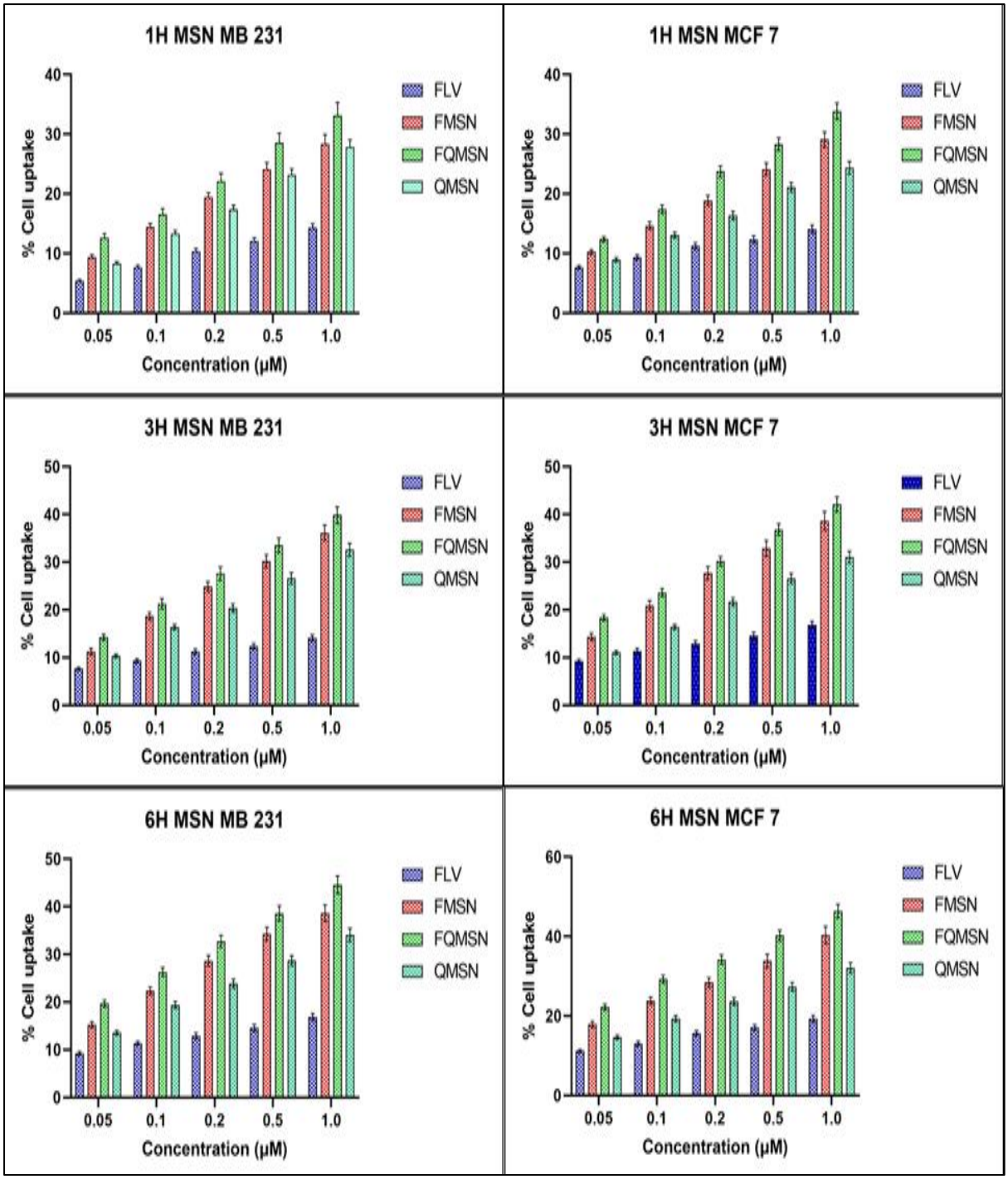

Figure 7.21 Quantitative cellular uptake of FLV suspension, FLV MSN, QMSN and FQMSN at different time intervals and different concentration

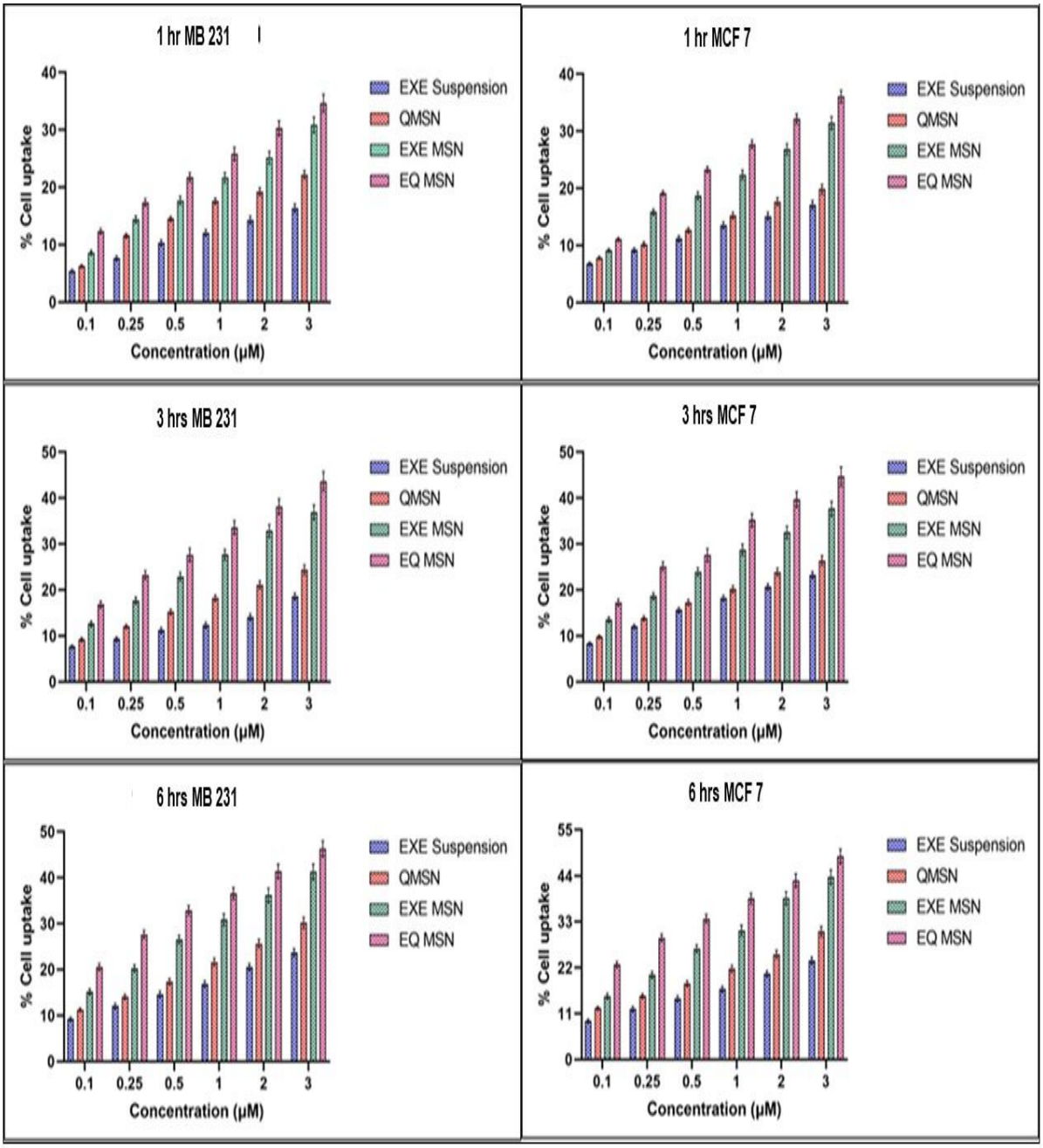

Figure 7.22 Quantitative cellular uptake of EXE suspension, EXE MSN, QMSN and EQMSN at different time intervals and different concentration

7.9.5 Caspase -3 Induction assay

From figure 7.23, it can be concluded that untreated cells showed no cell apoptosis, but cells treated with drug suspension showed increase in caspase – 3 concentrations indicating the start of apoptosis but was non-significant. The cellular caspase – 3 levels for nanoparticulate formulations were significantly high as compared to the drug suspension. The quercetin loaded MSN showed higher caspase activity compared to drug but lower compared to drug

nanoparticulate formulation as the anticancer activity of quercetin is lower compared to FLV and EXE (30).

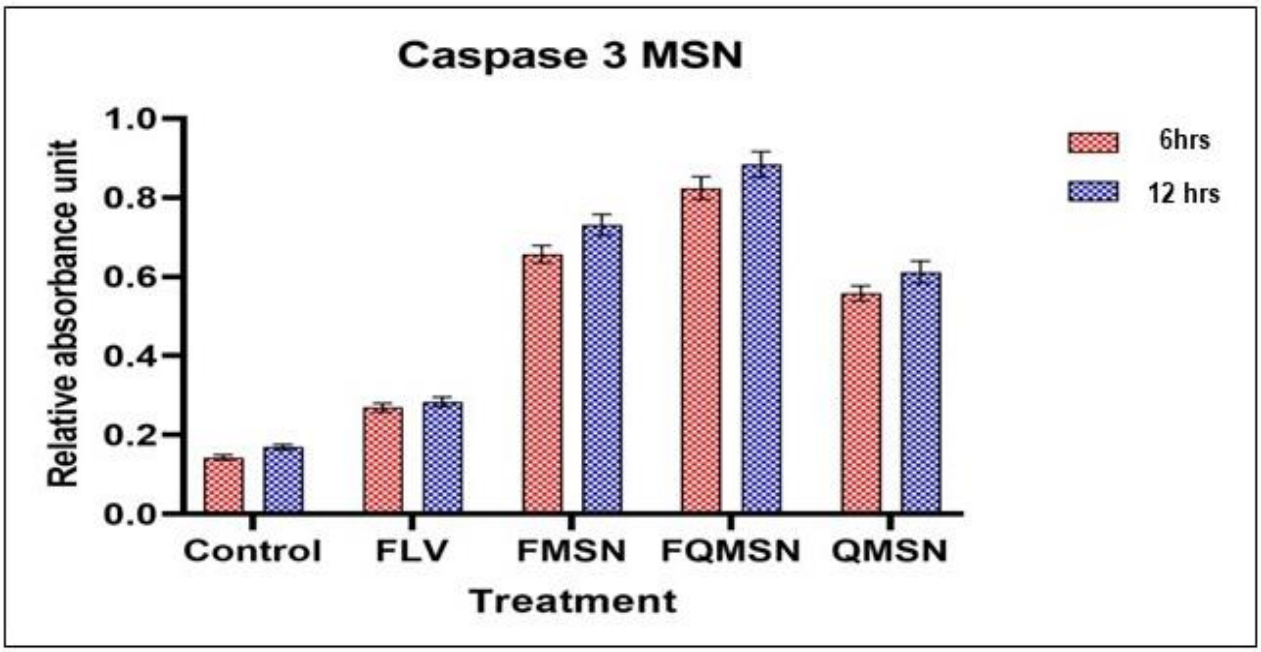

Figure 7.23 (A) Detection of caspase-3 level in MDA MB 231 cell line using CASP-3-C kit, treated with different fulvestrant and respective silica nanoparticles and incubated for 6 and 12 hrs.

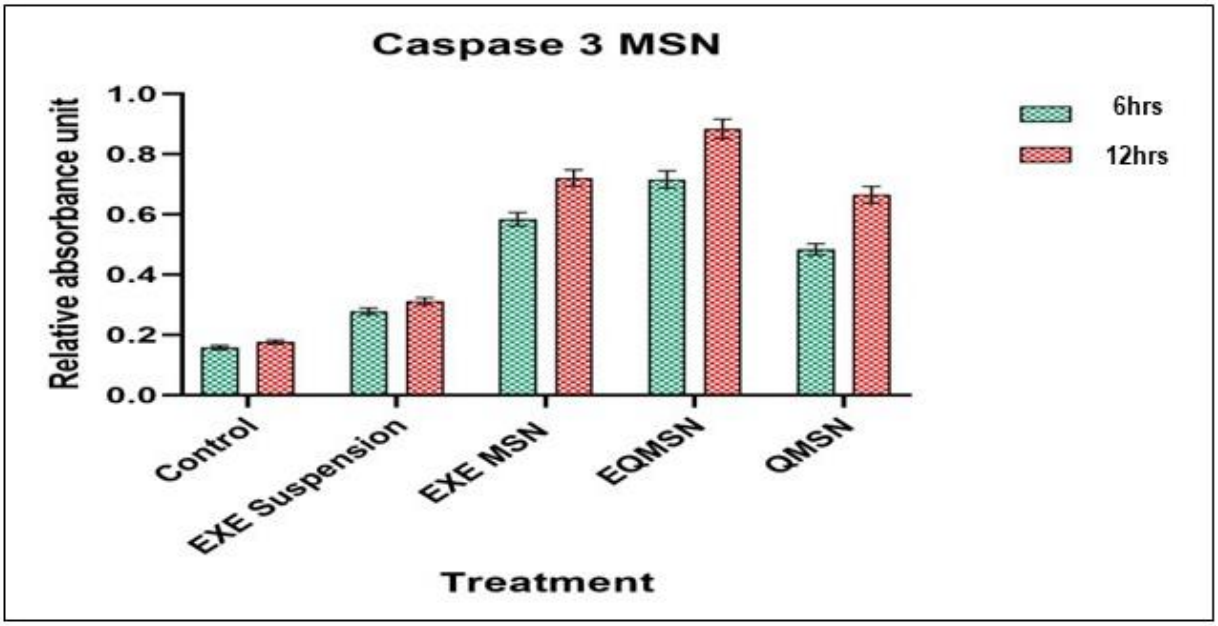

Figure 7.23 (B) Detection of caspase-3 level in MCF – 7 cell line using CASP-3-C kit treated with different exemestane and respective silica nanoparticles and incubated for 6 and 12 hrs.

7.9.6 Cell Apoptosis detection

As seen in Figure 7.24, after the incubation with MDA MB 231 cells for 24 h, fulvestrant and quercetin co-loaded mesoporous silica nanoparticles treated cells have most of the cells towards the upper right quadrant that indicates initiation of early as well late apoptosis (31). Untreated cells show most cells in the Q1 which suggested a high number of live cells with negligible DNA fragmentation. An extreme upward right shift of the folate targeted formulation suggested a predominant number of cells in the early as well as late stages of apoptosis, which proves the high DNA fragmentation and condensation of chromatin due to fulvestrant inside the cells.

The EQMSN have lower number of cells in the late apoptotic cells, as the pharmacological activity of exemestane is to prevent the synthesis of estrogen, but it doesn't affect the circulatory estrogen levels of the body, so it is taken up by the tumor cells, to continue their growth and hence, the rate of apoptosis will be same as the rate of regeneration, so there was lower rate of apoptosis.

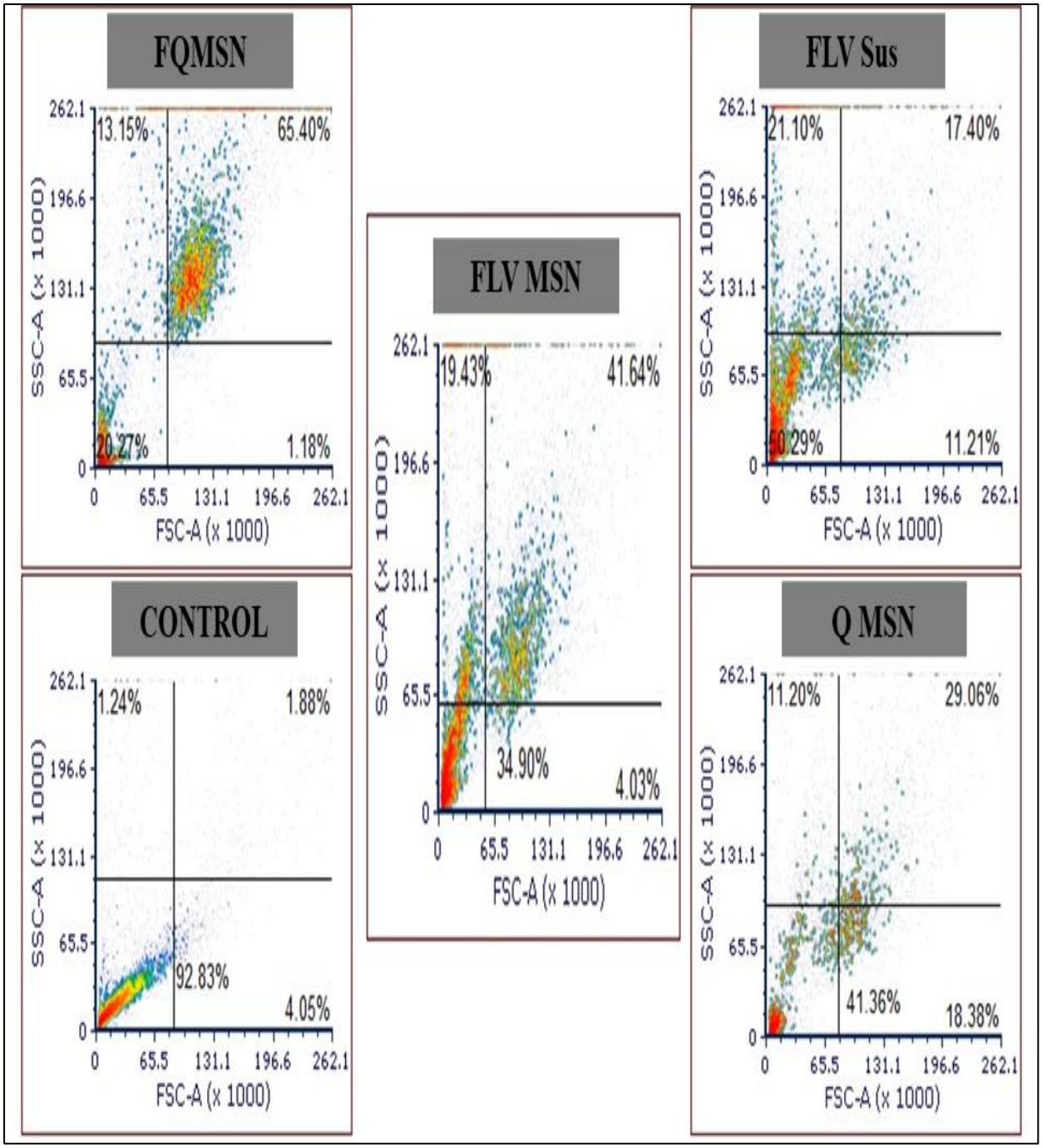

Figure 7.24 Apoptosis cell analysis of MDA MB 231 cell line after different fulvestrant formulation treatment upon 24 h incubation

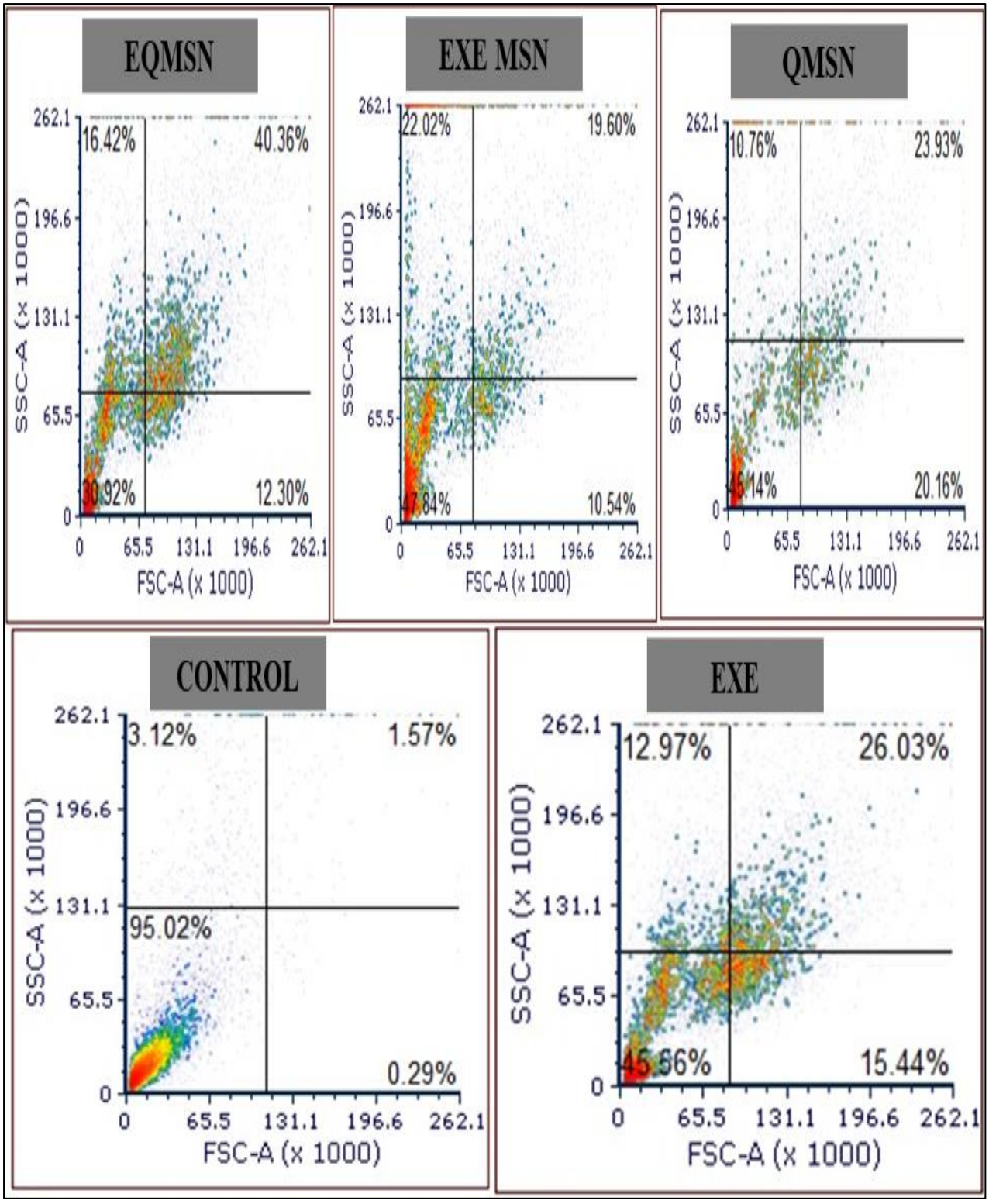

Figure 7.25 Apoptosis cell analysis of MDA MB 231 cell line after different exemestane formulation treatment upon 24 h incubation

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