7. CELL LINE STUDIES

Table 7.1: List of Chemicals.

Sr. No.	Chemicals	Source and Place
1	Doxorubicin Hydrochloride	Gift sample from Sun Pharmaceuticals, India
2	Dulbecco's Modified Eagle Medium (DMEM)	Himedia, India
3	Fetal Bovine Serum (FBS)	Himedia, India
4	Trypsin-EDTA solution	Himedia, India
5	Antibiotic / Antimycotic solution (Penicillin G, Streptomycin and Amphotericin B)	Himedia, India
6	Trypan blue dye	Himedia, India
7	Isopropyl alcohol	Rankem, India
8	Dimethyl Sulfoxide (DMSO)	Himedia, India
9	Methanol	Rankem, India
10	MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)	Himedia, India
11	Dichloro-dihydro-fluorescein diacetate (DCFHDA)	Sigma Aldrich, India
12	Poly-l-lysine	Sigma Aldrich, India

13	Rhodamine 123	Sigma Aldrich, India
14	Annexine V-Fluoresceine isothiocyanate (Annexin-FITC) and propidium iodide	Invitrogen, India

Table 7.2: List of Instruments:

Sr. no.	**Equipment**	**Company Name and Place**
1	Digital weighing machine AX 120	Shimadzu, Japan
2	Bath Sonicator	Fast clean ultra-cleaner, India
3	Incubator	JGUAN quality system, India
4	Laminar Air Flow	Weiber, India
5	Inverted Microscope	Nikon, USA
6	Centrifuge	Remi, India
7	Micro plate reader	Bio-Rad,
8	Refrigerator	Whirlpool, India
9	BD FACSAria II	BD Biosciences, USA
10	Autoclave	Hitech Labs, India.

7.1 Cytotoxicity studies:

The prospective anticancer drug undergoes a series of qualifying studies before it is subjected to human testing.[1] Large scale drug screening using human tumor cell line was initiated in 1990.[2] This in vitro human tumor cell line screen shifted to a disease-orientated screening strategy from being a compound orientated.[3] NCI60 human tumor cell line anticancer drug screening protocol is used for the primary in vitro screening of the potential anticancer agents in order to evaluate the ability of a potential candidate to inhibit the growth of tumor cells in culture. This modern pharmaceutical in vitro screening protocol comprises a panel of 60 human tumor cell lines and it was developed by the National Cancer Institute (NCI, USA). At pesent, the NCI60 is the generally used system for the preliminary screening of potential anticancer drugs.[4] The rationale for this in vitro prescreening is to remove inactive compounds from unnecessary and costly full-scale evaluation.[3] Furthermore, in vitro cell line studies possess several advantages such as homogeneity, highly controlled conditions throughout the experiment, possible identification of molecular mechanisms, and very high reproducibility.[5]

7.1.1 Cytotoxicity studies of blank mesoporous silica nanoparticles (MSNs):

- **Cell line storage and maintenance:**

  The human breast cancer cell line (MCF-7), purchased from the National Centre for Cell Sciences (NCCS, Pune, India) was used for the cytotoxicity study. It was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-glutamine (2 mM), 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (100 µg/ml streptomycin, 100 units/ml penicillin and 50 µg/ml amphotericin). The culture was maintained at 37 ºC under a humidified atmosphere with 5% CO_2. The culture medium was changed at regular time intervals.[6]

- **MTT assay:**

  The in vitro cytotoxicity of the nanocarriers was assessed on MCF-7 breast cancer cells by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay.

Briefly, the cells were counted using hemocytometer and seeded in 96 well plate with 200 µl medium/well (10,000 cells/well). After 24 hr the media was removed and the cells were washed with PBS (pH 7.4) and blank MCM-41 type MSNs synthesized as per procedure described in chapter 4 were added to the wells in triplicate in different concentrations (1-100 µg/mL). After 24 hours, the cells were washed with PBS pH 7.4 and 100 µl of MTT solution (1mg/ml) was added to each well and incubated for 4 hours at 37ºC. Thereafter, the MTT solution was removed and 100µl of DMSO was added for solubilisation of formazan crystals. Absorbance was taken at 570 nm with a reference filter of 630 nm using ELISA microplate reader (Bio-Rad, USA). Cell viability was computed using following formula,

$$\% \text{ Viability} = \frac{\text{Mean Absorbance of sample} - \text{Mean Absorbance of blank}}{\text{Mean Absorbance of Control} - \text{Mean Absorbance of blank}} * 100 \qquad (7.1)$$

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]

7.1.2 Cytotoxicity studies of metal oxide loaded mesoporous silica nanoparticles (MO-MSNs):

MTT assay was performed to determine the cytotoxic effect of MO (CuO or ZnO) loaded MSNs. MCF-7 cells were seeded in 96 well plate with 200 µl medium/well (10,000 cells/well) and after 24 hr the media was removed and the cells were washed with PBS (pH 7.4). Then MO-MSNs synthesized as per procedure described in chapter 4 were added to the wells in triplicate in different concentrations (1-100 µg/mL). The remaining procedure is same as described in 7.1.1.

7.1.3 Cytotoxicity studies of drug loaded MSNs and CuO-MSNs:

- **Cell line storage and maintenance:**

  Two different cell lines MCF-7 and MDA-MB-231 (both human breast cancer cell lines), purchased from the National Centre for Cell Sciences (NCCS, Pune, India) were

used for the study. Both the cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-glutamine (2 mM), 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (100 µg/ml streptomycin, 100 units/ml penicillin and 50 µg/ml amphotericin). The cultures were maintained at 37 ºC under a humidified atmosphere with 5% CO_2. The culture medium was changed at regular time intervals.[6]

- **MTT assay:**

MTT assay was performed to determine the cytotoxic effect of free DOX and drug loaded nanoparticles DOX-MSN and DOX-CuO-MSN as well as surface functionalized MSNs and CuO-MSNs (DOX-MSN-CH-FA, DOX-MSN-SS-CH-FA, DOX-CuO-MSN-CH-FA, and DOX-CuO-MSN-SS-CH-FA) against two different human breast cancer cell line MCF-7 and MDA-MB-231. The cells were seeded in 96 well plate with 200 µl medium/well (10,000 cells/well) and after 24 hr the media was removed and the cells were washed with PBS (pH 7.4). Then respective samples were added to the wells in triplicate in different concentrations (equivalent to 0.01-20 µg/mL of DOX) and incubated up to 72 hrs (24 hr, 48 hr and 72 hr). The remaining procedure is same as described in 7.1.1.

7.2 Estimation of reactive oxygen species (ROS) generation:

ROS generated upon addition of the sample were measured by using Dichloro-dihydro-fluorescein diacetate (DCFHDA) dye.

- **Coating of the cover slips:**

The cover slips were placed in each well of 12 well plates and coated with poly-l-lysine prior to addition of MCF-7 cells. The cover lips were treated with 50 µg/ml poly-l-lysine for 2 h at 37 °C in an incubator without rotation or shaking. Unbound poly-l-lysine was removed and then cover slips were blocked with 2 mg/ml bovine serum albumin at at 37 °C for 1 h. The cover slips were washed once with PBS and refilled with 1-2 ml of media before plating the cells.[8]

In a typical procedure, $1*10^5$ MCF-7 cells/well were seeded on the cover slip in 12 well plates and incubated for 24 hours. The media was removed and the cells were washed with PBS (pH 7.4) and various samples such as DOX, DOX-MSN, DOX-MSN-SS-CH-FA, DOX-CuO-MSN, and DOX-CuO-MSN-SS-CH-FA were added to wells at a concentration equivalent to 1 μg/ml of DOX and 1 μg/ml CuO-MSNs was also added in one well. Then the plates were again incubated and upon completion of 24 hours incubation period, the wells were emptied and the cells were washed with the PBS 7.4 to remove the traces of the original medium. Immediately prior to use, a fresh stock solution (1mg/ml) of DCFHDA was prepared in sterile DMSO. DCFHDA at a final concentration of 10 μM in regular culture medium (DMEM) with reduced serum (2%) was added to every well and the plates were again incubated for 30 minutes in the dark. DCFHDA containing medium was discarded and the cells were washed twice with PBS. Everything was performed in the dark and the cells were protected from light. The stained cover slips were examined using 40× objective fluorescence microscope (Nikon, USA) and the cell images were captured. DCFHDA loaded untreated cells and unstained untreated cells were used as a control in the present study.[9]

7.3 Estimation of change in the mitochondrial membrane potential (MMP):

The fluorescent dye rhodamine 123 (Rh-123) staining method was utilized to determine the alterations in MMP ($\Delta\psi m$) of MCF-7 cells using fluorescence microscopy.

Human breast cancer (MCF-7) cells were seeded in 12-well plates at a seeding density of 1×10^5 cells/well and incubated for 24 hours. At the end of 24 hours, the media was removed and the cells were washed with PBS (pH 7.4) and treated with various samples such as DOX, DOX-MSN, DOX-MSN-SS-CH-FA, DOX-CuO-MSN, and DOX-CuO-MSN-SS-CH-FA at a concentration equivalent to 1 μg/ml of DOX and 1 μg/ml of CuO-MSN for 24 hours. Upon completion of 24 hours incubation period, the wells were emptied and the cells were washed with the PBS 7.4 to remove the traces of the original medium. Later on, trypsinization was carried to detach the cells and resulting cell pellets were given two times PBS washing. 2 ml fresh medium containing Rh123 (Rhodamine 123) (1.0 μM) was then added to the cell pellets and were incubated at 37 °C for 20 min with gentle shaking. The cells were centrifuged and

resulting cell pellets were washed again with PBS two times, then subjected to flow cytometry analysis.[10]

7.4 Clonogenic assay:

In order to determine the effect of various formulations on the colony formation ability of MCF-7 cells, clonogenic assay was performed. MCF-7 cells were seeded at 2.5×10^3 cells/ml/well in 6-well plates. The culture medium was changed after 24 h, and fresh medium was added. Treatment was given to the cells with specified samples such as DOX, DOX-MSN, DOX-MSN-SS-CH-FA, CuO-MSN, DOX-CuO-MSN, and DOX-CuO-MSN-SS-CH-FA at a concentration equivalent to 1 μg/ml of DOX and incubated for another 7 days at 37 °C with 5% CO_2. Thereafter, 70% chilled methanol was used to fix the obtained colonies followed by staining with crystal violet solution (0.5%). Subsequently, colonies were observed in the plates and photographed.[11]

7.5 Scratch 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 p200 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 DOX, DOX-MSN, DOX-MSN-SS-CH-FA, CuO-MSN, DOX-CuO-MSN, and DOX-CuO-MSN-SS-CH-FA at a concentration equivalent to 1 μg/ml of DOX. In control 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.[8]

7.6 Detection of apoptosis:

The apoptosis-inducing effect of different samples was investigated by analyzing the percentage of early and late apoptotic cells by Annexin V-FITC and propidium iodide (PI) dual staining.

Human breast cancer (MCF-7) cells were seeded in 6-well plates at a seeding density of 2×10^5 cells/well and incubated for 24 hours. At the end of 24 hours, the media was removed and the cells were washed with PBS (pH 7.4) and the cells were treated with various samples such as DOX, DOX-MSN, DOX-MSN-SS-CH-FA, CuO-MSN, DOX-CuO-MSN, and DOX-CuO-MSN-SS-CH-FA at a concentration equivalent to 1 μg/ml of DOX for another 48 hours. Control wells were not treated with any samples. Upon completion of 48 hours incubation period, the wells were emptied and the cells were washed with the PBS 7.4 to remove the traces of the original medium. The attached cells were trypsinized using trypsin/ethylenediaminetetraacetate and 10^5 cells were incubated for 10 minutes, at 4°C with fluorescein isothiocyanate (FITC)-conjugated annexin V and 12.5 mg propidium iodide. Then, a diluting binding buffer was added and the cells were analyzed using flow cytometer.[12]

7.7 Results and discussion:

7.7.1 MTT Assay:

MTT assay, developed by Mosmann in 1983, is the earliest of all the methods used for the cytotoxicity studies. This assay is based on the conversion of colourless tetrazolium salt to coloured insoluble formazan in proportion to viable cells. MTT assay is one of the most simple, rapid and convenient method of determination of cytotoxicity.[13]

Figure 7.1 represents the cytotoxicity of blank MSNs. The viability of untreated cells was assumed to be 100% and when compared with the control, the cell viability of treated cells was also approximately 100% even when the cells were incubated with MSNs at concentrations as high as 100 μg/mL for 24 h. It proved that the blank MSNs did not show any inhibition of cell growth or cause cell death.

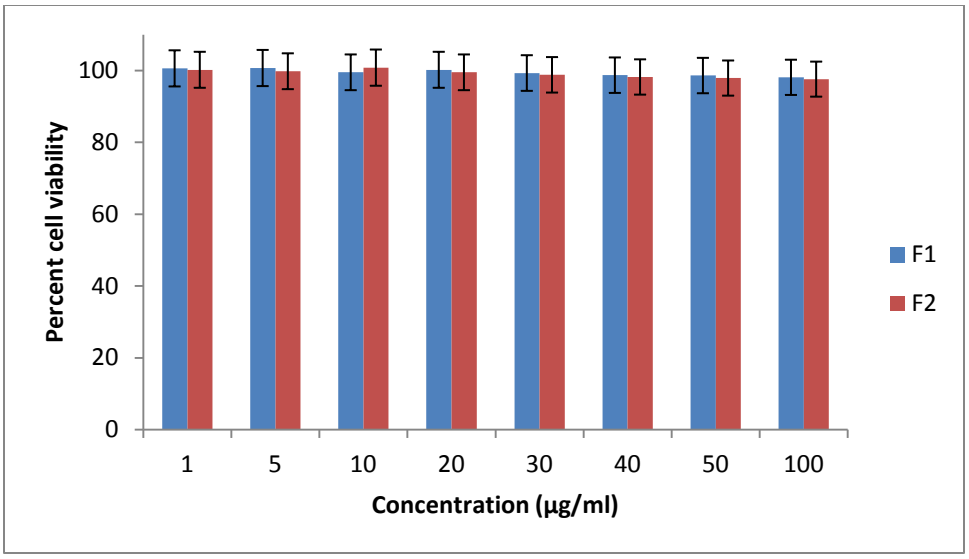


Figure 7.1: Cytotoxicity of blank MSNs against MCF-7 cells after 24 h incubation.

Metal oxides such as copper oxide (CuO) and zinc oxide (ZnO) are known to demonstrate cytotoxic activity. A significant reduction in the viability of MCF-7 cells was recorded when treated with CuO loaded MSNs (Figure 7.2). This decrease in the viability was concentration dependant and decreased with increase in concentration. The IC_{50} values for two different batches of CuO loaded MSNs (MO1 and MO2) were found to be 35.4 µg/ml and 61.6 µg/ml, respectively. Batch MO2 showed greater cytotoxic potency which might be attributed to the higher CuO content in it.

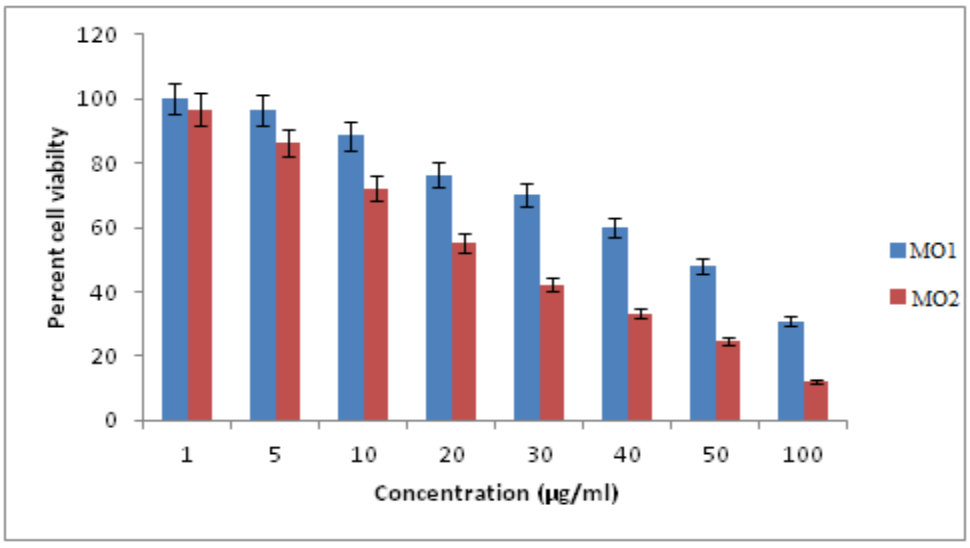


Figure 7.2: Cytotoxicity of CuO-MSNs against MCF-7 cells after 24 h incubation.

Various researchers have reported ZnO as an anticancer substance but in our study we did not observe any significant reduction in the cell viability when MCF-7 cells were treated with ZnO-MSNs (Figure 7.3). MTT assay was performed for two different batches of ZnO loaded MSNs (MO3 and MO4) and both the samples showed more than 90% cell viability at the end of the study. The reason for observing high viability of MCF-7 cells after treatment with ZnO-MSNs, might be related with very low ZnO content present in the synthesized MSNs and at such low concentration it might not be capable enough to cause cancer cell death significantly.

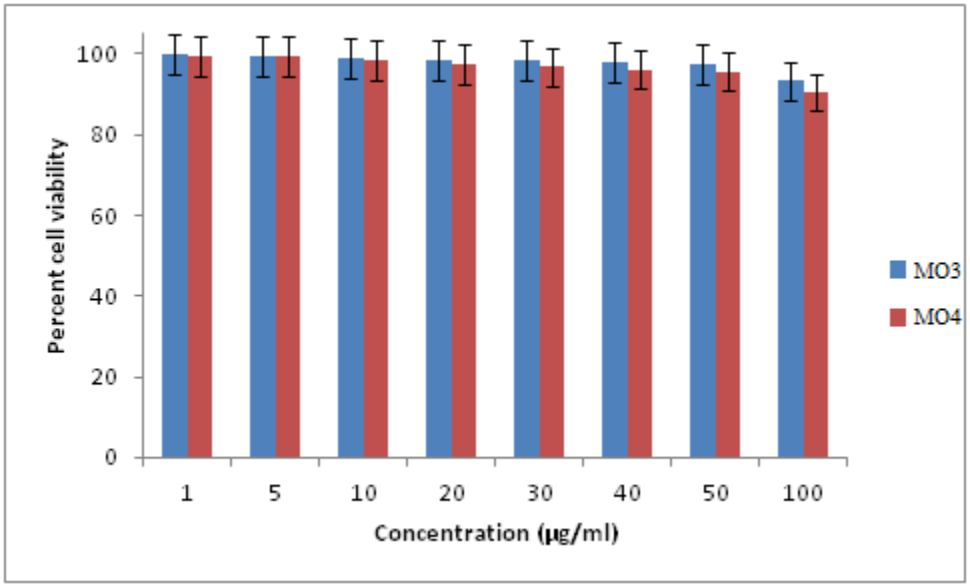
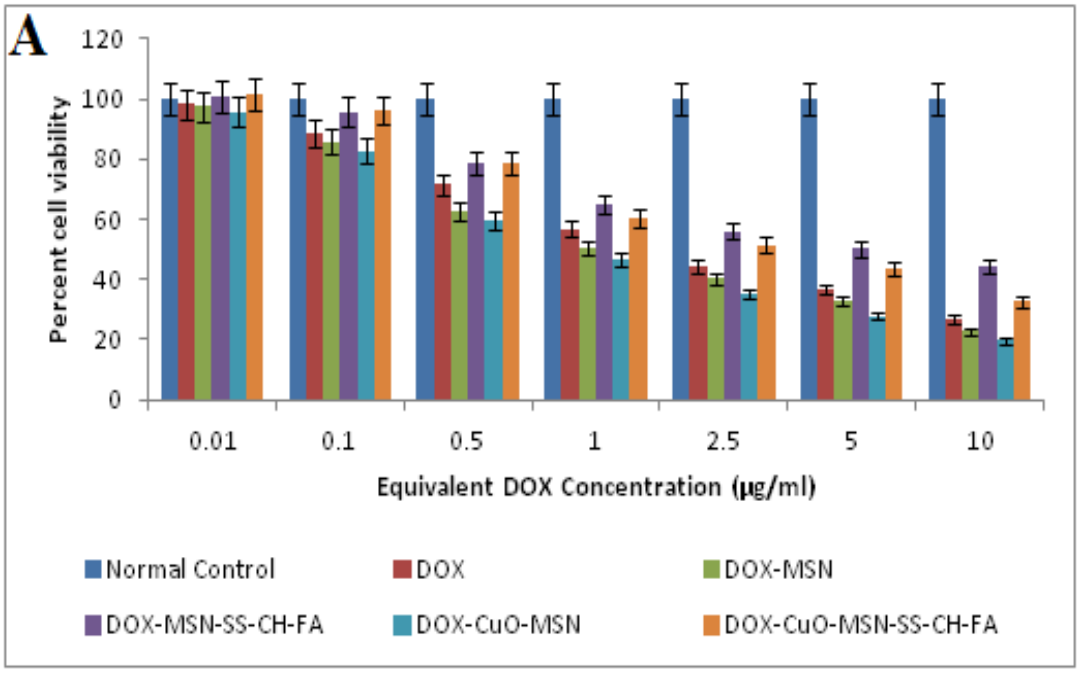
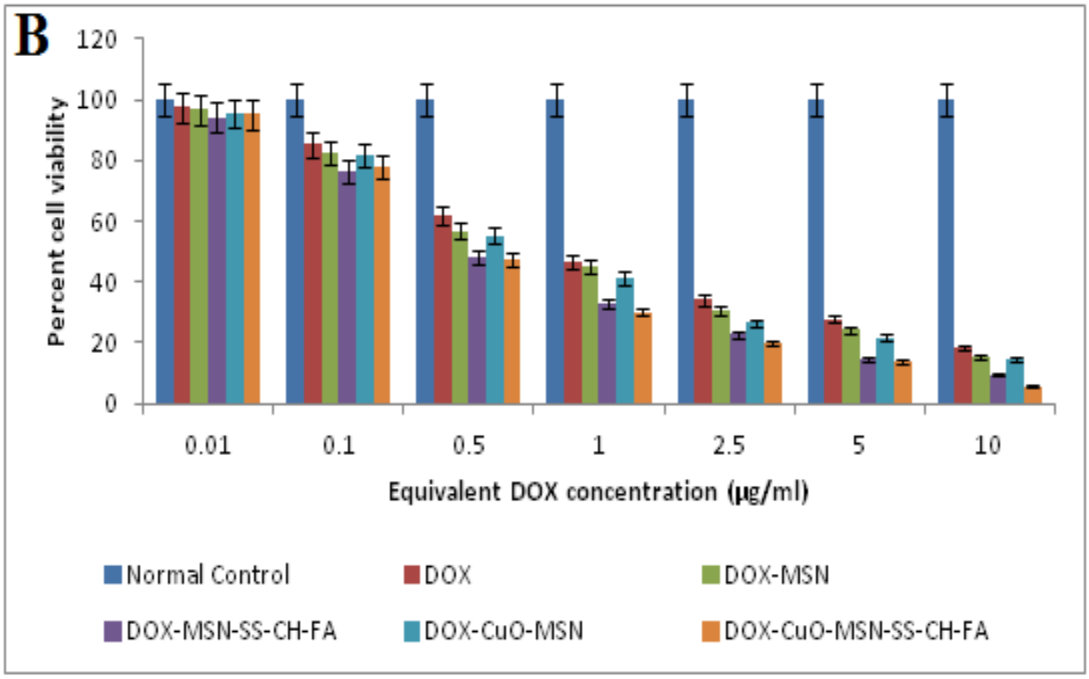


Figure 7.3: Cytotoxicity of ZnO-MSNs against MCF-7 cells after 24 h incubation.

Figure 7.4 represent the effect of different formulations treatment on percent cell viability as a function of concentration. As the concentration increased, a decrease in the cell viability was observed. As seen in figure 7.4 A, the cytotoxic effect shown by CH-FA capped MSNs as well as CuO-MSNs was little lesser than that of DOX alone or DOX loaded uncapped nanoparticles (about 0.6 and 0.83 times as compared to free DOX at highest concentration). This might be due to less release of DOX from CH-FA capped nanoparticle within 24 hours. As compared to results of 24 hours treatment, 48 and 72 hours treatments showed enhanced cytotoxic effect demonstrated by DOX-MSN-SS-CH-FA and DOX-CuO-MSN-SS-CH-FA (about 1.9 and 3.2 times as compared to free DOX at highest concentration). Apart from this, at lower

concentrations, CuO loaded nanoparticles showed similar or little higher cell viability as compared to DOX-MSNs. It may happen only if CuO increased the proliferation of MCF-7 cells at very low concentrations. Very less difference was observed in the results of 48 hours and 72 hours treatment. This might be indicative of the release of most of the drug from CH-FA capped nanoparticles within 48 hours.





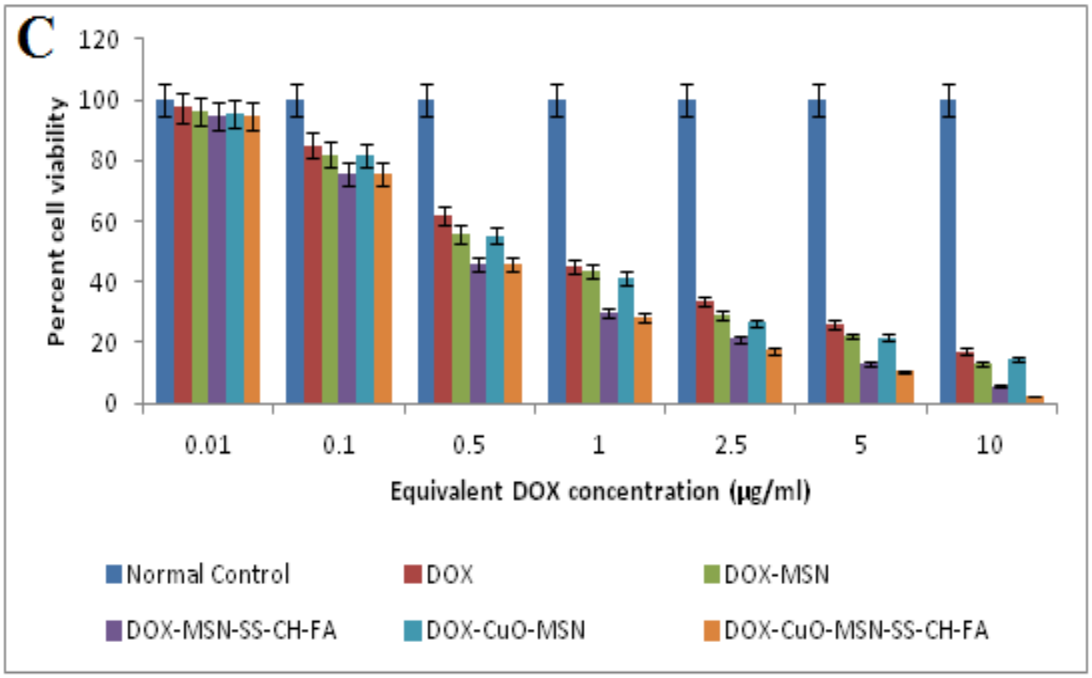
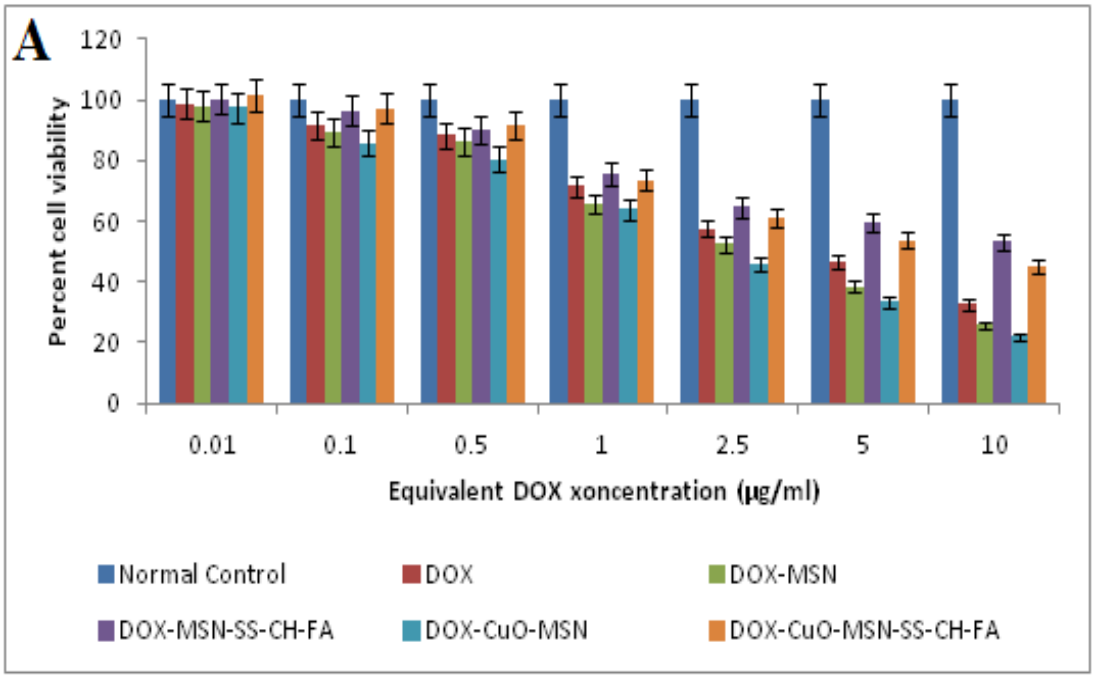
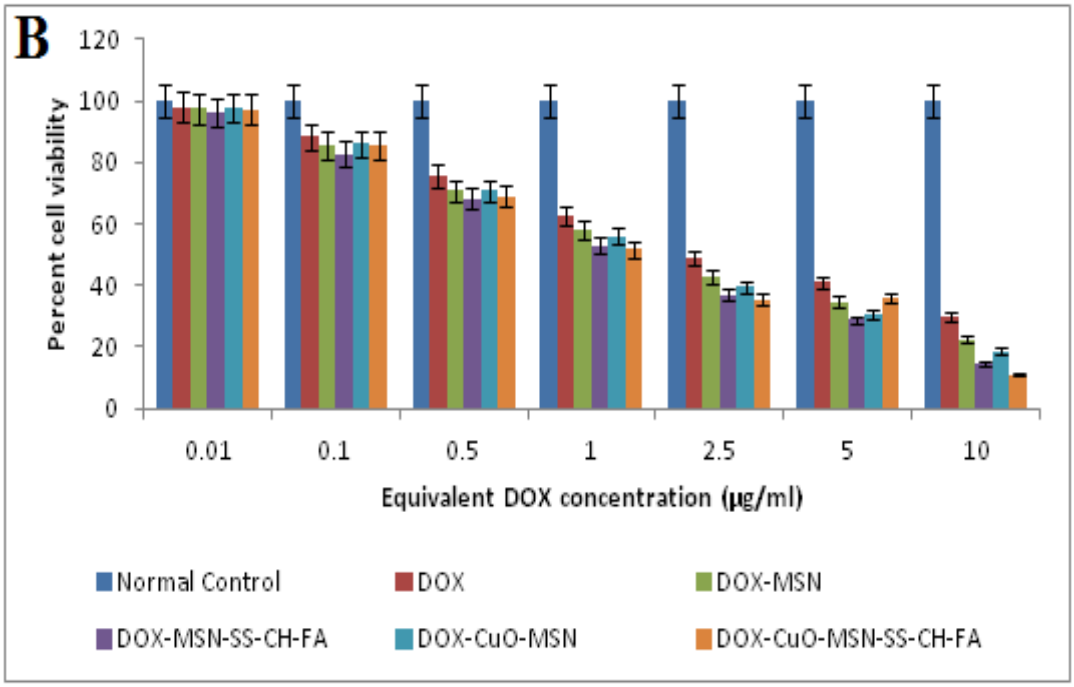


Figure 7.4: Cytotoxicity of free DOX and DOX loaded nanoparticles measured by MTT assay against MCF-7 cells at 24 hours (A), 48 hours (B) and 72 hours (c).

Similarly, the cytotoxic effect of free DOX and DOX loaded MSNs as well as CuO-MSNs was measured against MDA-MB-231 cells and the results demonstrated dose dependent cytotoxicity. As seen in figure 7.5 A, The cytotoxic effect shown by CH-FA capped MSNs as well as CuO-MSNs was little lesser than that of DOX alone or DOX loaded uncapped nanoparticles (about 0.61 and 0.72 times as compared to free DOX at highest concentration). This might be due to less release of DOX from CH-FA capped nanoparticle within 24 hours. As compared to results of 24 hours treatment, 48 and 72 hours treatments showed enhanced cytotoxic effect demonstrated by DOX-MSN-SS-CH-FA and DOX-CuO-MSN-SS-CH-FA (about 2 and 2.7 times as compared to free DOX at highest concentration). Very less difference was observed in the results of 48 hours and 72 hours treatment. Furthermore, CuO and DOX loaded nanoparticles showed better cytotoxicity as compared to DOX loaded MSNs alone. So, it can be said that the incorporation of CuO into the MSN frameworks positively improved the cytotoxicity of the formulation.


A
120
100
80
60
40
20
0
Percent cell viability
0.01
0.1
0.5
1
2.5
5
10
Equivalent DOX xoncentration (µg/ml)
Normal Control
DOX
DOX-MSN
DOX-MSN-SS-CH-FA
DOX-CuO-MSN
DOX-CuO-MSN-SS-CH-FA


B
120
100
80
60
40
20
0
Percent cell viability
0.01
0.1
0.5
1
2.5
5
10
Equivalent DOX concentration (µg/ml)
Normal Control
DOX
DOX-MSN
DOX-MSN-SS-CH-FA
DOX-CuO-MSN
DOX-CuO-MSN-SS-CH-FA

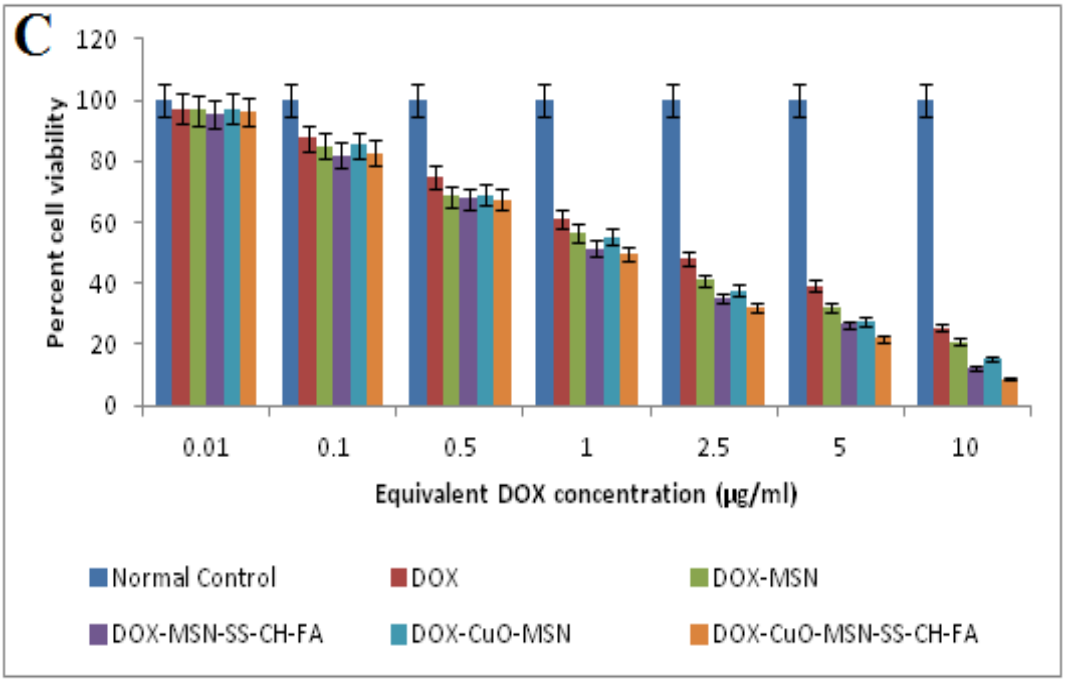


Figure 7.5: Cytotoxicity of free DOX and DOX loaded nanoparticles measured by MTT assay against MDA-MB-231 cells at 24 hours (A), 48 hours (B) and 72 hours (c).

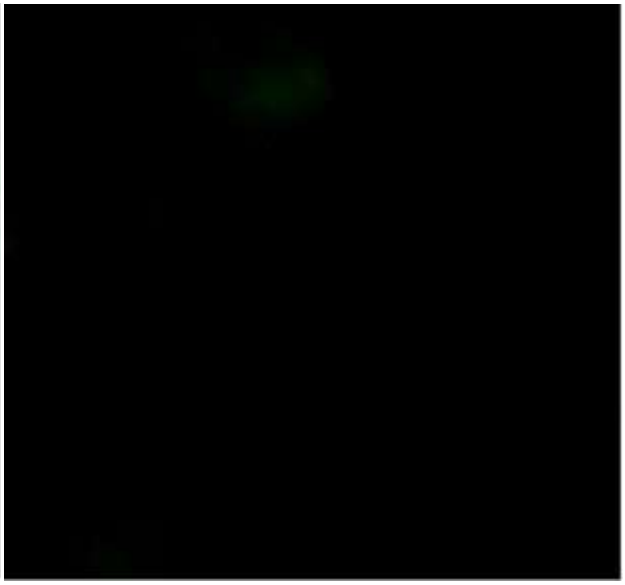
7.7.2 Estimation of ROS:

Reactive oxygen species comprise a number of molecules containing an oxygen and include H_2O_2 (hydrogen peroxide), NO (nitric oxide), O_2^- (oxide anion), peroxynitrite ($ONOO^-$), hydrochlorous acid (HOCl), and hydroxyl radical (OH^-) that damage DNA and RNA and oxidize proteins and lipids. Oxidative species are known to produced under various pathological situations such as cancers, ischemic/reperfusion, neurologic and cardiovascular pathologies, infectious diseases, inflammatory diseases, autoimmune diseases, etc and ROS can be detrimental (it is then referred to as "oxidative and nitrosative stress") when produced in high amounts in the intracellular compartments.[14]

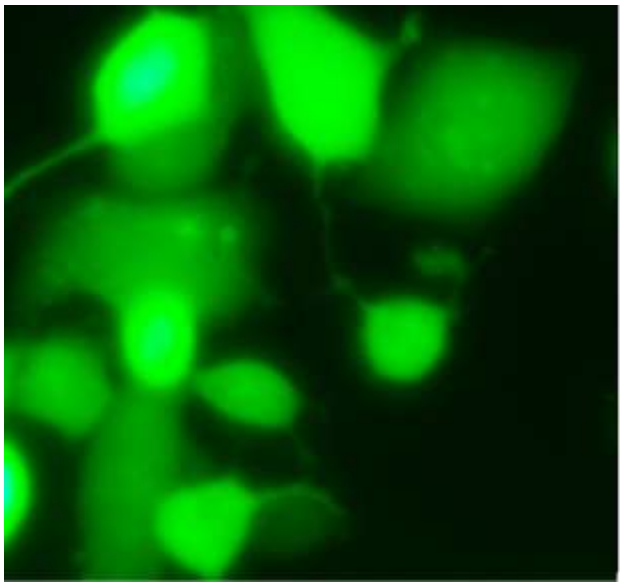
Figure 7.5 stands for microscopic images of formulation treated MCF-7 cells after treatment with DCFHDA, a non-fluorescent dye that produces green fluorescent upon oxidation in the presence of ROS. As seen in the images, control didn't show any fluorescence which clearly meant that the control cells didn't produce any ROS. As compared to control, all the samples produced significant ROS revealed by the strong green

fluorescent. DOX -CuO-MSN and DOX -CuO-MSN-SS-CH-FA were found to show very high fluorescent which might be attributed to the ROS producing capability of additional CuO incorporated within the MSNs framework.

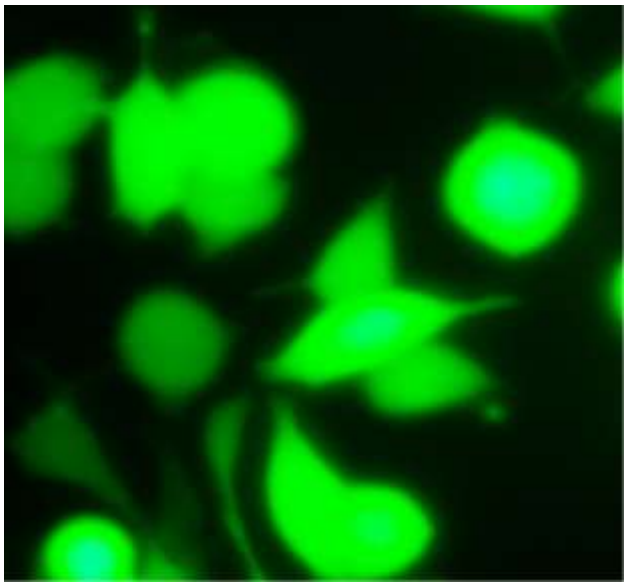
Unstained untreated



Stained untreated



DOX



DOX-MSN

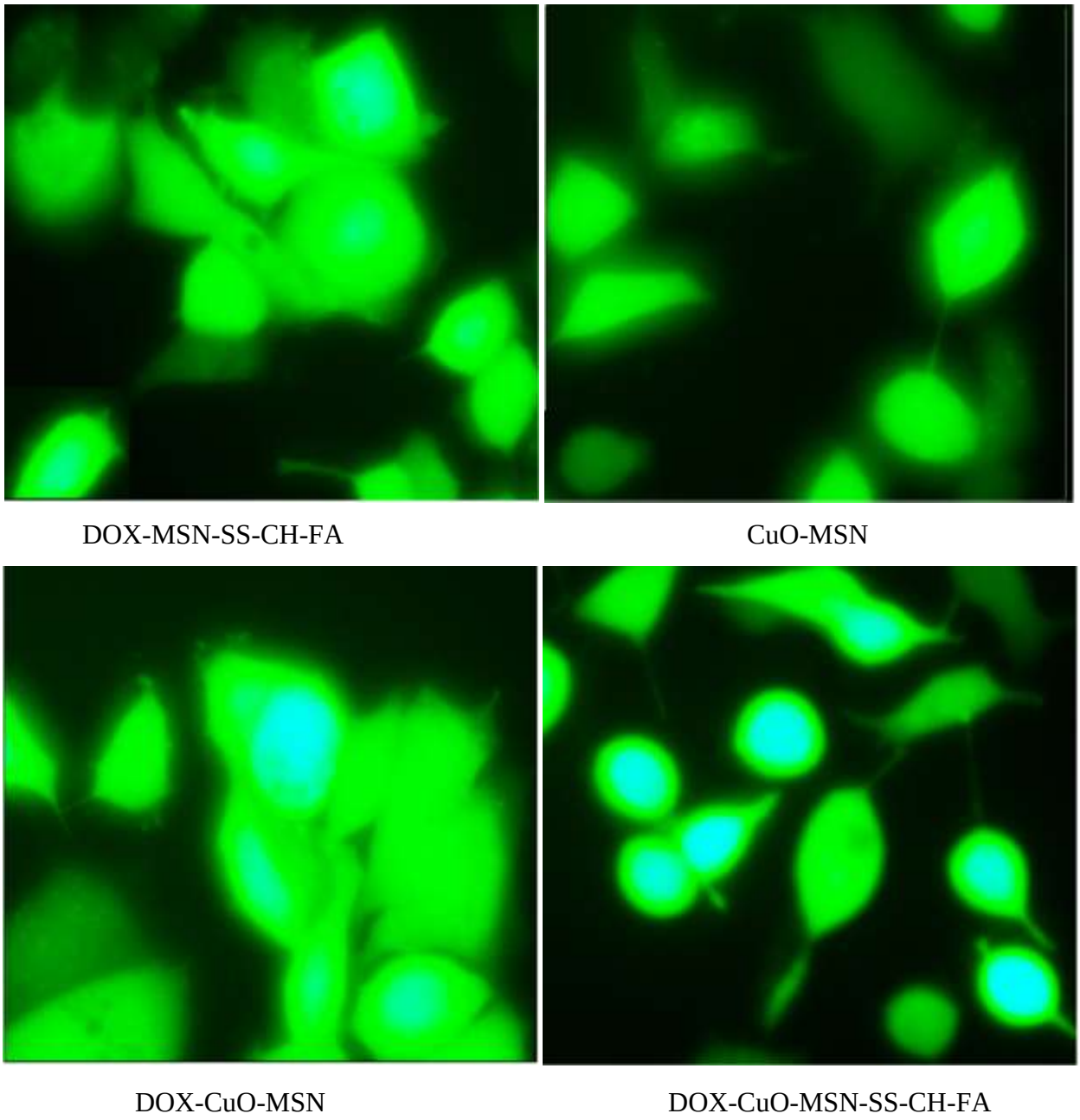


Figure 7.6: Estimation of intracellular ROS by fluorescence microscopy.

7.7.3 Estimation of MMP:

It is believed that the increased ROS levels within cancer cells leads to free radical attack over membrane phospholipids resulting in a loss of Δψm. As mitochondria are the key source of ROS generation in the cell, overproduction ROS and the accumulation of ROS may causes damage to the lipids, proteins, and alteration in mitochondrial functions which ultimately leads to mitochondrial membrane permeabilization that further instigates apoptosis.

As seen in figure 7.7, all the formulations cause significant loss in the mitochondrial membrane potential. The drug loaded nanoparticles showed increased loss in the Δψm as compared to free drug which was further improved by the capping of CH-FA over the surface of nanoparticles. DOX-CuO-MSN-SS-CH-FA showed highest loss in Δψm signifying its ability to cause apoptosis.

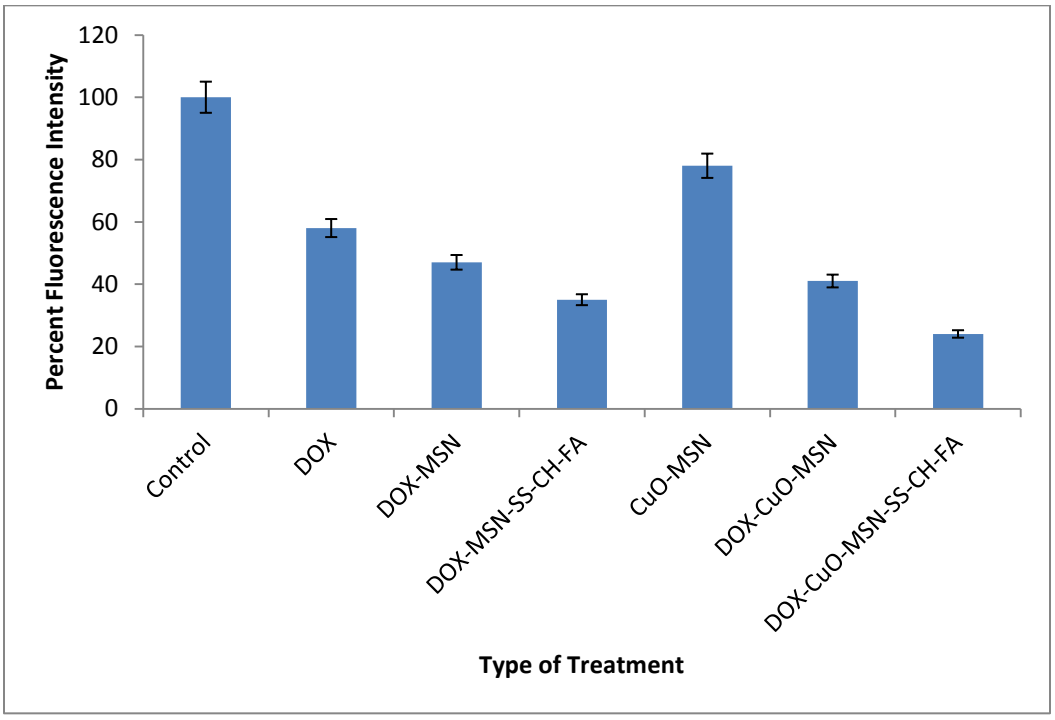


Figure 7.7: The mitochondrial membrane potential (Δψm) of the cells after treatment with various formulations, measured by flow cytometry.

7.7.4 Clonogenic assay:

As the tumor is composed of heterogeneous cell types, some subpopulation may contain long term clonal renewal ability which can be the reason for tumor refractoriness to chemotherapy. Clonogenic potential is the renewal capacity of cells or tumorigenic potential of cancer cells once the chemotherapy or radiation therapy is withdrawn.

All the formulations illustrated marked inhibition MCF-7 colony formation and growth as compared to untreated control group (Figure 7.8). As seen in the image, DOX-CuO-MSN-SS-CH-FA inhibited the colony formation in MCF-7 completely and no colonies were observed after treatment while very few colonies were observed in cells treated with DOX-MSN-SS-CH-FA and DOX-CuO-MSN. DOX-MSN also showed increased inhibition of colonies when compared to DOX alone. Hence, it can be said that the formulations showed better inhibition of MCF-7 colony formation as compared to plain drug, DOX-CuO-MSN-SS-CH-FA being the best inhibitor as no colonies were observed at the end of treatment.

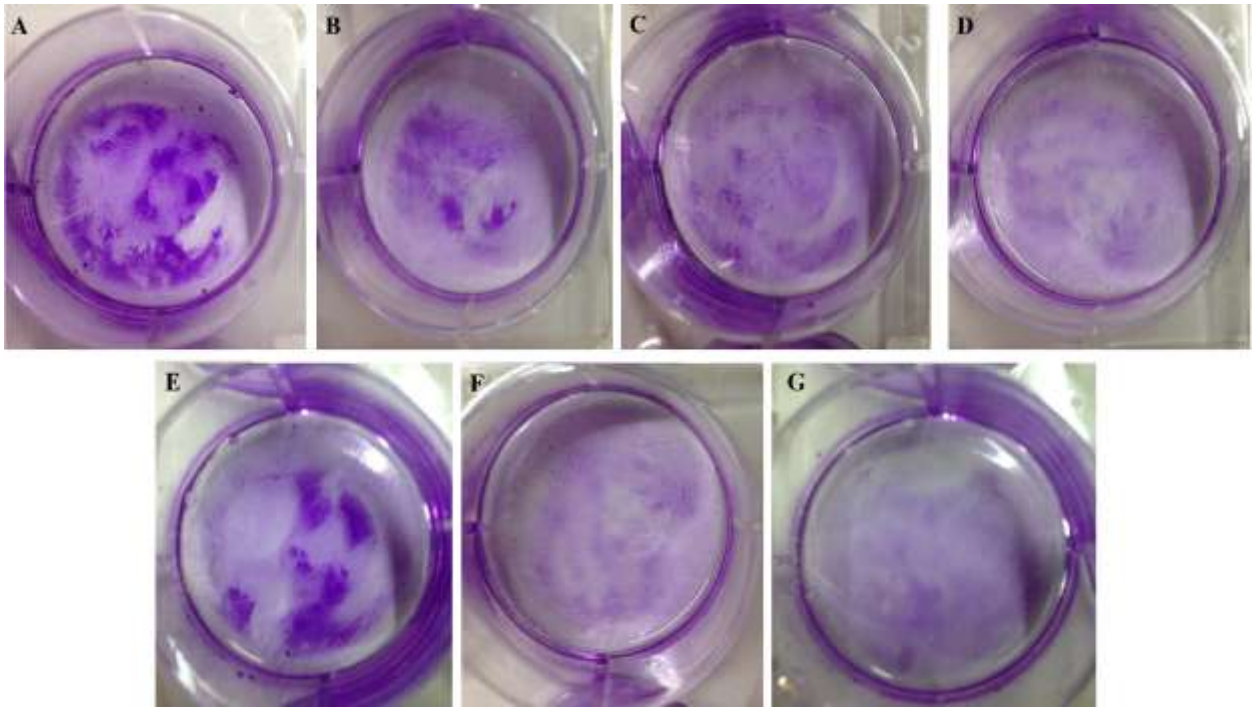
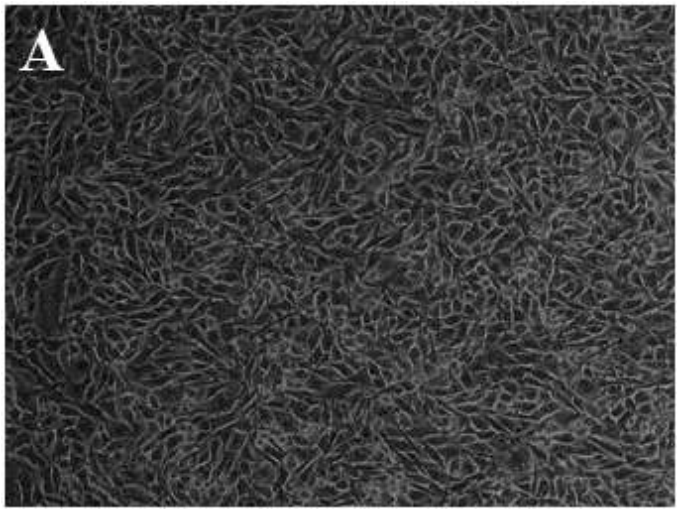


Figure 7.8: Estimation of MCF-7 tumor colony inhibition mediated by different formulations: Control (A), DOX (B), DOX-MSN (C), DOX-MSN-SS-CH-FA (D), CuO-MSN (E), DOX-CuO-MSN (F), DOX-CuO-MSN-SS-CH-FA (G).

7.7.5 Scratch assay:

The in vitro scratch assay is an easy, low-cost and well-developed method to measure cell migration in vitro. This method is based on the observation that, upon creation of a new artificial gap, so called ‘‘scratch’’, on a confluent cell monolayer, the cells on the edge of the newly created gap will move toward the opening to close the ‘‘scratch’’ until new cell–cell contacts are established again.

Figure 7.9 represent the migration of MCF-7 cells after being treated with different formulations. CuO-MSN alone was not able cause restrict the migration of cells to significant extent. As compared to DOX alone, DOX-MSN and DOX-CuO-MSN showed better inhibition of cell migration. CH-FA capping further improved their efficacy and remarkable reduce the migration of MCF-7 cells. Hence, it can be said that the DOX-MSN-SS-CH-FA and DOX-CuO-MSN-SS-CH-FA can considerable restrict the metastasis of the MCF-7 calls.



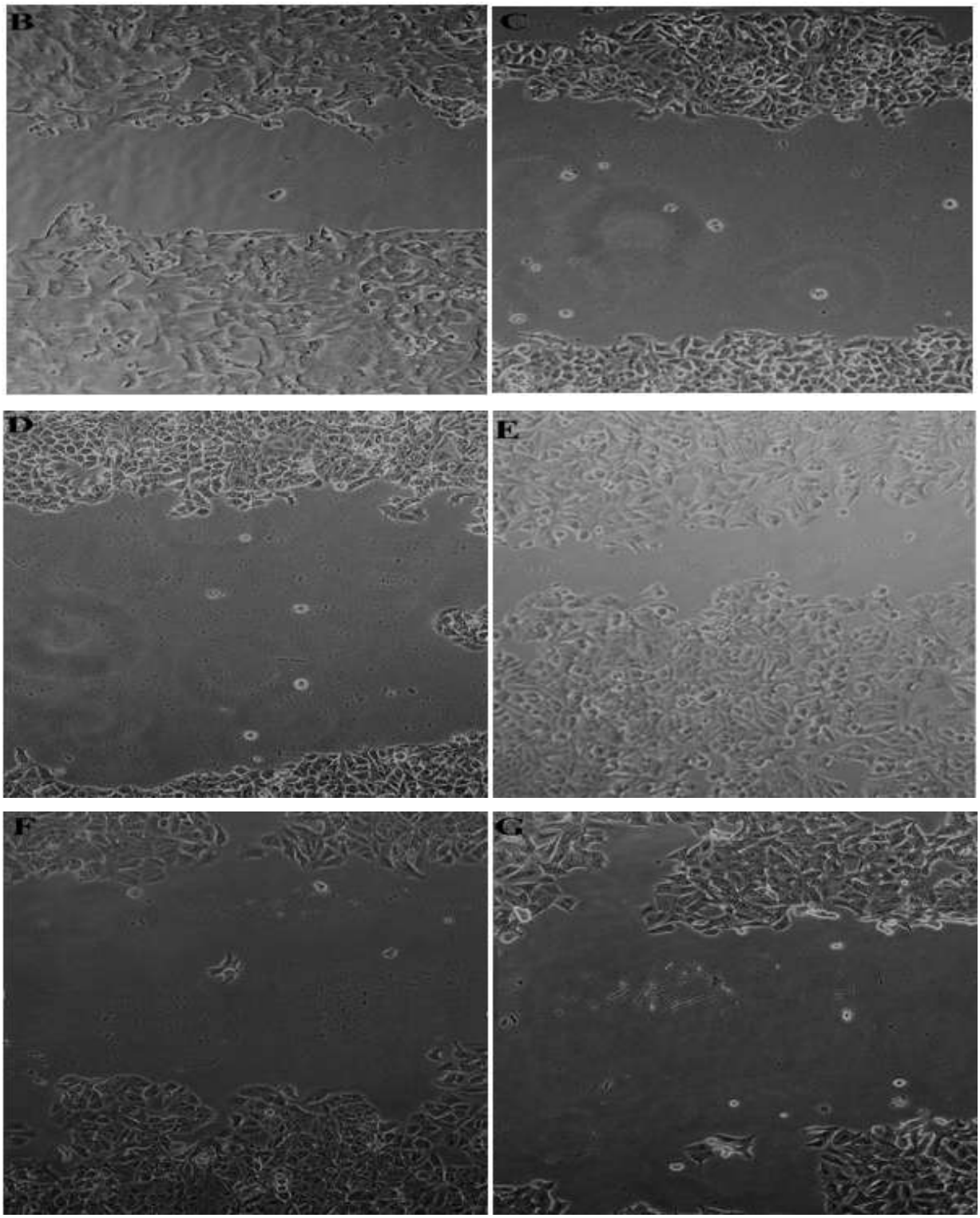
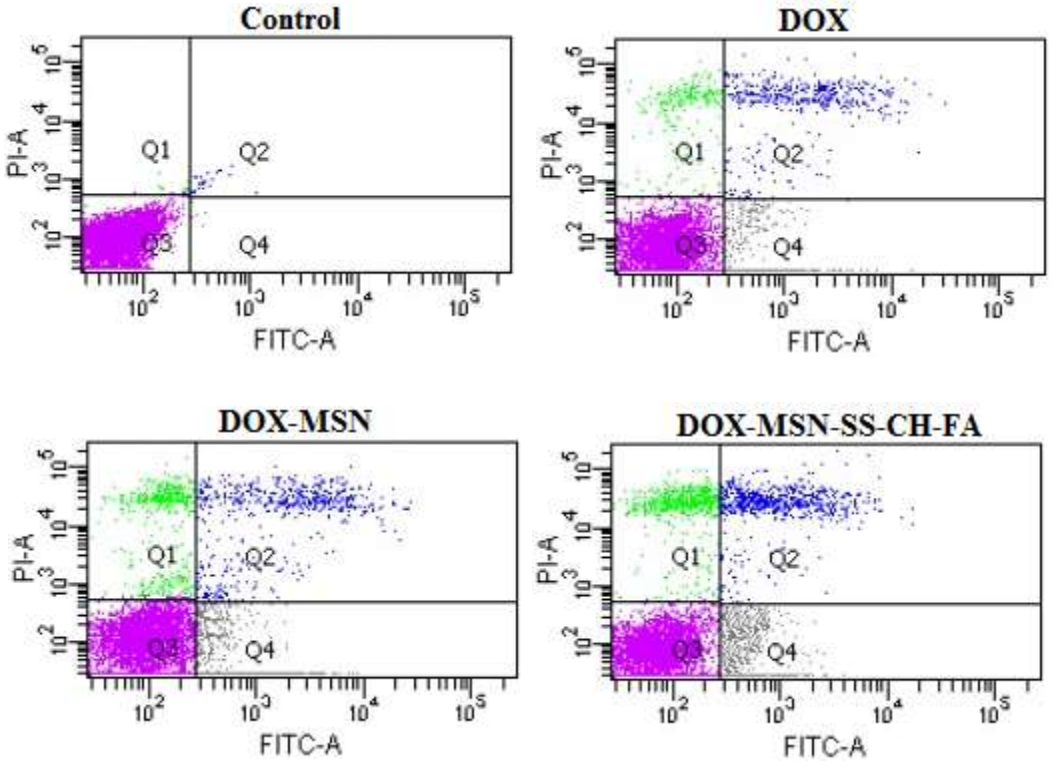


Figure 7.9: In vitro scratch assay to determine effect of different formulations: Control (A), DOX (B), DOX-MSN (C), DOX-MSN-SS-CH-FA (D), CuO-MSN (E), DOX-CuO-MSN (F), DOX-CuO-MSN-SS-CH-FA (G) on cell migration.

7.7.6 Apoptosis study:

As the synthesized nanoparticles were found to generate ROS and disrupt mitochondrial membrane potential, apoptosis and necrosis induced by these nanoparticles were studied due to the involvement of mitochondria in the regulation of apoptosis. The loss of the $\Delta\psi m$ can trigger the release of apoptogenic factors from mitochondria to the cytosol that ultimately leads to the sequential death of the cell.

Figure 7.10 represent the apoptosis mechanism of MCF-7 cells upon treatment with different formulations. As seen in the images, almost all the cells were viable in the control group. As compared to control, all the treatment groups showed marked increase in the apoptotic and necrotic cells. The proportion of the apoptotic and necrotic cells was higher in DOX-MSN and DOX-CuO-MSN as compared to DOX alone. DOX-MSN-SS-CH-FA and DOX-CuO-MSN-SS-CH-FA showed very high proportion of necrotic cells as compared to others. This clearly indicate that these two formulations were highly toxic to the MCF-7 cells causing death of the cancer cells.



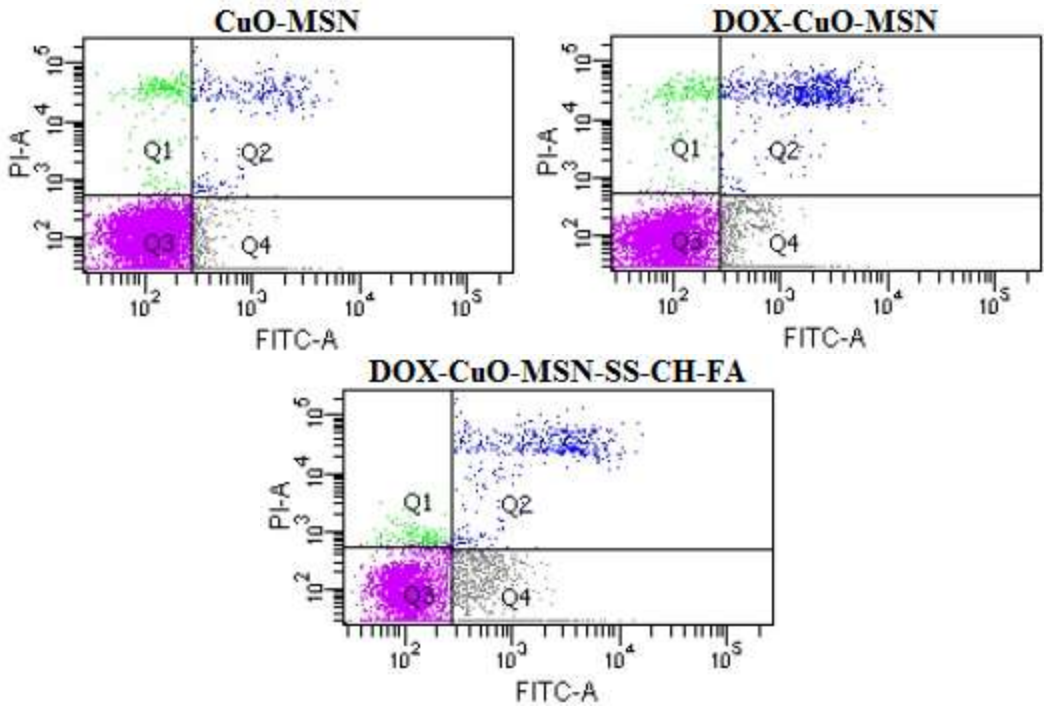


Figure 7.10: Evaluation of the apoptosis mechanism of MCF-7 breast cancer cells upon treatment with different formulations. Q1 means early apoptotic cells, Q2 means apoptotic or dead cells, Q3 means non apoptotic viable cells and Q4 means necrotic cells.

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