Chapter 9

Folic acid-Chitosan conjugation facilitates targeted delivery of nanoparticles in *in vitro* and *in vivo*: A biocompatible anticancer therapy

INTRODUCTION:

In cancer therapy, nanoscale therapeutic materials are considered an effective tool attributing to its nontoxic biodegradable, non-immunogenic, biocompatible, and sustained drug release. However, conventional tumor therapeutic approaches like surgery, radiation, and chemotherapy destroy tumor and as well as normal cells during therapy (Hosseini et al., 2016). Applications of nanoparticles (NPs) are wide ranged for various purposes including drug delivery, regenerative medicine, diagnosis and drug carriers. Generally common anti-cancer drugs having non-specific mode of action, can destroy both tumor and normal cells at the same time. Modifications of different NPs can increase the competence of existing therapeutic approaches for specific targeting (Talekar et al., 2011). Over expression of folate receptor (FR) was observed on the surface of breast, lung, and ovary, kidney and brain cancer cells (Parker et al., 2005). Several studies showed that FR is able to easily bind with Folate and assisted the internalization of folate-targeted NPs via receptor facilitated endocytosis. The cellular uptake of folic acid containing drug in the cancer cells occurs through non-destructive and endosomal pathway (Parker et al., 2005, Murthy, SK. 2007) as because the affinity of FR was high for folic acid (Chen et al., 2013). Generally the FR expression was high on the surface of cancer cells as well as limited expression was observed on normal cells (Parker et al., 2005). So, the main purpose of the folate decorated NPs will be targeting tumor cells in NP-based cancer therapies. Copper being a transitional element showed good anticancer property and especially the green synthesized metal NPs are grabbing the attention of the researcher due to less toxicity (Wang et al., 2015, Sankar et al., 2014). Linear polysaccharide CS is a derivative of alkaline N-deacetylation of chitin. It is consists of glucosamine residues with β-1,4- linkage and casually distributed N-acetyl-glucosamine (Liu et al., 2005). CS can be applied for drug delivery system in an acidic environment as CS is easily soluble in acidic environment (Sadigh-Eteghad et al., 2013, Ye et al., 2014). Chitosan has been considered as a potential nanocarrier system for targeted cancer therapy due to its high biocompatibility and low cytotoxicity. Low transfection efficiency and Incompetent targeting ability is the major problem with drug delivery system. Targeting molecule FA has been conjugated with chitosan coated NPs to improve the therapeutic potential through selective operation of FA. Several studies showed that FR-expressing SMMC-7221 cells were killed by FA conjugated DOX-loaded chitosan NPs (Song et al., 2013). This phenomenon was an example of targeted drug delivery system.

***In this Chapter, from here on, unless otherwise mentioned, CuONPs@CS@FA shall refer to Folic Acid conjugated Chitosan coated green synthesized CuONPs.**

In the present study, an anti-cancer tool of nano sized that can effectively reduce the CuONPs toxicity and selectively kill the cancer cells through FR mediated endocytosis pathway was designed. The *in vitro* and *in vivo* studies of apoptosis in cancer cells were done. Based on cytotoxicity assay, microscopic image, FACS analysis, Cytokines analysis and apoptotic proteins alteration, the anticancer efficacy of the conjugate (CuONPs@CS@FA*) was analyzed.

9.1. MATERIALS AND METHODS:

Rhodamine-B and Doxorubicin were obtained from Sigma (St. Louis, MO, USA). FBS from Gibbco and MTT, DMSO were obtained from Himedia, India. Ethidium bromide (EtBr) and glutaraldehyde were purchased from Merck Ltd. Chitosan, folic acid and Histopaque 1077 obtained from sigma (St. Louis, MO, USA).

9.1.1. Synthesis of CuONPs@CS:

Green synthesized CuONPs from *Azadirachta indica* leaves has been achieved according to previous lab method by Dey et al., 2019. After successful synthesis of green CuONPs, CS was loaded onto its surface by adopting slightly modified procedure as reported previously (Dey et al., 2020).

9.1.2. Synthesis of Folic acid grafted CuONPs@CS:

FA coating has been done according to Chattopadhyay et al., 2013. CS coated CuONPs surface were grafted with folic acid for targeting the cancer cells MCF-7 and HeLa where the folate receptor expression level was high. At first, FA was dissolved in 10ml of DMSO (0.025mM). FA was very low solubility in water. So, DMSO was used to improve the solubility of FA. Then 5mg of dried green CuONPs@CS was suspended in 10ml of MilliQ water. The aqueous suspension of NPs was added to the FA solution. After that the suspension was kept in a shaking incubator at 37^{o}C for 24h (200 rpm). The total suspension was centrifuged at 1500g for 5min to collect the pallet of FA conjugated green CuONPs@CS (CuONPs@CS@FA). The pellet was washed three times using MilliQ water to remove the unbounded FA. Then the conjugate was dried and stored at room temperature.

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9.1.3. Physical Characterization of CuONPs@CS@FA:

9.1.3.1. Fourier Transform Infrared (FTIR) Spectroscopy:

The conjugation of FA with CuONPs@CS was investigated according to the previous method in chapter 3 section 3.1.4.1.

9.1.3.2. Scanning Electron Microscopy:

SEM image of the conjugate was observed according to previously mentioned method in Chapter 3 Section 3.1.4.4.

9.1.3.3. Transmission electron microscopy (TEM):

TEM was performed to know the actual size of the nano composite according to previous method in Chapter 3 Section 3.1.4.6.

9.1.3.4. X-ray Diffraction (XRD) study:

XRD was performed according to the previous method mentioned in Chapter 3 Section 3.1.4.3.

9.1.3.5. Energy-dispersive X-ray (EDX) study:

Elemental analysis was performed according to previous method in Chapter 3 Section 3.1.4.5.

9.1.3.6. Thermogravimetric Analysis (TGA):

The thermal stability was determined by thermogravimetric analysis according to Rehana et al., 2017.

9.1.4. Estimation of Folic acid Binding:

Percentage of folic acid binding was done by spectrophotometrically (Chattopadhyay et al., 2013).

9.1.5. Uptake of Cu ions inside the cell:

Uptake of Cu ions from CuONPs@CS@FA inside the both cancer cells and lymphocytes was estimated by AAS. The total process was performed in accordance with the previous method in Chapter 4 Section 4.1.5.2.

9.1.6. Cell Culture:

Detailed cell (MCF-7, HeLa and 4T1) culture procedure was provided in the Chapter 4 Section 4.1.3.

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9.1.7. Selection of Human subjects and Isolation of Peripheral blood Lymphocytes:

Selection of human subject was done according to Helsinki. The total procedure was already mentioned in Chapter 4 Section 4.1.2.

Peripheral blood lymphocytes collection from the whole blood was done in compliance with Hudson and Hay method.

9.1.8. MTT assay:

The cell (Normal human lymphocytes and MCF-7 and HeLa) viability study was done by 3-(4,5-dimethyl-thiazol)-2-diphenyltetrazolium bromide (MTT) as previously reported (Dey et al., 2019). All the *in vitro* assesments were performed by three times.

9.1.9. Uptake of Rh-B tagged CuONPs@CS@FA visualized by Fluorescence microscopic images:

After the treatment it was observed that fluorescence microscopic images of Rh-B tagged CuONPs @CS@FA inside the cancer cells using NIKON ECLIPSE LV100POL microscope at 400X magnification (Sahu et al., 2010).

9.1.10. ROS generation inside cancer cells:

ROS generation inside the cancer cells was visualized by DCHF2DA staining under the fluorescence microscope previously mentioned in Chapter 4 Section 4.1.5.4.

9.1.11. Mitochondrial membrane potential:

Mitochondrial membrane potential was estimated by spectrofluorometric method (M'Bemba-Meka et al., 2006) with the help of Rh-123.

9.1.12. Apoptosis/ necrotic study by Etbr/AO double staining:

Cellular apoptosis was visualized by EtBr/AO double staining (Liu et al., 2015). To observe the cells, fluorescence microscope (NIKON ECLIPSE LV100POL) at 400X magnification had been used.

9.1.13. Nuclear Changes by DAPI staining:

MCF-7 and HeLa cells ($2x10^5$cells/ml) were treated with CuONPs@CS@FA (50µg/ml) for 24hr. After that cancer cells were trypsinized for isolation from the culture plate. Isolated cells were used for DAPI staining. The process was already mentioned in chapter 5 section 5.1.8.

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9.1.14. Cellular morphology by scanning electron microscopy:

Surface ultra-structure of cancer cells was done by Wahab et al (Wahab et al., 2009) with some modification. MCF-7 and HeLa cells were cultured in a 35mm culture plate. Then CuONPs@CS@FA (50µg/ml) was treated on these cells for 24hr and the cells were kept in an incubator at 37^{o}C temperature. Then the cancer cells were trypsinized from the culture plate and centrifuged at 3000rpm for 5mins. The cell pallet was fixed with 50µL of 4% glutaraldehyde for 4hr in 4°C. Then the cell pallet washed with (1X) PBS for three times for 10mins each. One drop of cell which was previously fixed by glutaraldehyde was taken on a glass plate. After that cells were dehydrated by using ascending grades of acetone (35%, 50%, 75%, and 100%) and dried for 30mins. Finally the cells were affixed to a metal SEM stub and sputter was gold coated. Then scanning electron microscope (SEM, Hitachi S-3000N) has been used for imaging.

9.1.15. Cellular apoptosis by Annexin V-FITC analysis:

HeLa cells and MCF-7 cells were treated with CuONPs@CS@FA with a dose of 50µg/ml. Then MCF-7 and HeLa cells were washed in ice-cold PBS. Annexin binding buffer (100µl) was used for the incubation of cells. Then Annexin V-FITC (5µl) was added to the previously incubated cell suspension and kept in the dark for incubation again at room temperature for 15min. Before the FACS analysis, 5µl of PI was added and subsequently apoptosis was analyzed with FACS instrument (BD Biosciences, FACS Calibur, USA).

9.1.16. Cell cycle analysis:

Cell cycle analysis was done after the treatment of the HeLa cells and MCF-7 cells with the CuONPs@CS@FA. Cells were harvested with a dose of 50µg/ml and fixed with 70% ethanol for 2hr at 4°C. Before the staining process with propidium iodide (PI, Abgenex) at a concentration of 50µg/ml, cancer cells were incubated for 1h with DNAse free RNAse A (SRL, India) at a concentration of 100µg/ml at room temperature. FACS instrument (BD Biosciences, FACS Calibur, USA) was used for the analysis of cell cycle.

9.1.17. Cytokine analysis:

Pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines were estimated through ELISA assay kit (Enzyme linked immunosorbent assay, E-bioscience). TNF-α and IL-10 has sensitivity limit of 4.0pg/ml and 2.0pg/ml respectively.

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9.1.18. Analysis of apoptotic markers:

Apoptotic markers (Caspases 3, 8 and Bcl2) were analyzed by western blot method according to Gao at al., 2009; Pramanik et al., 2016.

9.1.19. Folate receptor knockdown:

The FR knockdown in MCF-7 and HeLa cells were done in the lab of Jadavpur University, West Bengal, India. Knockdown was done according to Laha et al (Laha et al., 2015). FR negative HeLa cells and MCF-7 cells were developed using a αFR sh-RNA kit according to the manufacturer's instructions (Santa Cruz Biotechnologies, Inc., USA). The efficiency of the targeting of the CuONPs@CS@FA was studied by MTT assay.

9.1.20. Animals:

Female Balb/c mice of 25-35gm weight were used for the experiments. The animals were fed water *ad libitum*. Polypropylene cage (Terson) was used and 12hr light & dark cycle under room temperature was maintained for mice. Animals were maintained in accordance with the Guidelines of the National Institute of Nutrition, Hyderabad, India. Study approval was given by the institutional ethical committee (IEC) of Vidyasagar University. For the *in vivo* experiment, 5 mice were used in each group.

9.1.20.1. Tumor development *in vivo* model and Euthanasia of experimental animals:

Detailed procedure of tumor development and Euthanasia of experimental animals were provided in Chapter 5 Section 5.2.1 and Chapter 4 Section 4.2.3 respectively.

9.1.20.2. CuONPs@CS@FA induced anti-cancer activities *in vivo*:

9.1.20.2.1. Tumor Weight:

After 30 days treatment (three days interval) with CuONPs@CS@FA at 1000µg/kg body weight dose, tumor weight was measured. From untreated tumor control, tumor was collected and weighted for comparison.

9.1.20.2.2. Increase in survival Days:

The survival days of tumor bearing (untreated mice) mice and CuONPs@CS@FA treated mice has been done after the 30 days treatment.

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9.1.20.2.3. Cytokines analysis:

Cytokines level was estimated by the above mentioned method.

9.1.20.3. Protein Estimation:

Protein estimation was done according to Lowry et al., 1951.

9.1.20.4. Statistical Analysis:

All experiments were repeated five times and standard deviation was calculated from them. ANOVA test (using a statistical package, Origin 6.1, Northamptom, MA 01060, USA) was used for the comparisons of the means of the experiments in all the present study. The limit of significance of the studies were $p<0.05$.

9.2. RESULTS:

9.2.1. Physical Characterization of CuONPs@CS@FA:

FT-IR spectroscopy is a very constructive tool to know the presence of any functional group on the surface of a compound. Fig. 9.1-I represents the spectrum of only green synthesized CuONPs. Subsequently, CS was conjugated with green CuONPs (Fig. 9.1-II). The bioactive components of leaves of *A. indica* were analyzed by GC mass and summarized in Chapter 1. Fig. 9.1-I showed strong peak at 3467.2cm^{-1} which, being a characteristic band of O-H stretching vibration may be due to bioactive components of plant extract.

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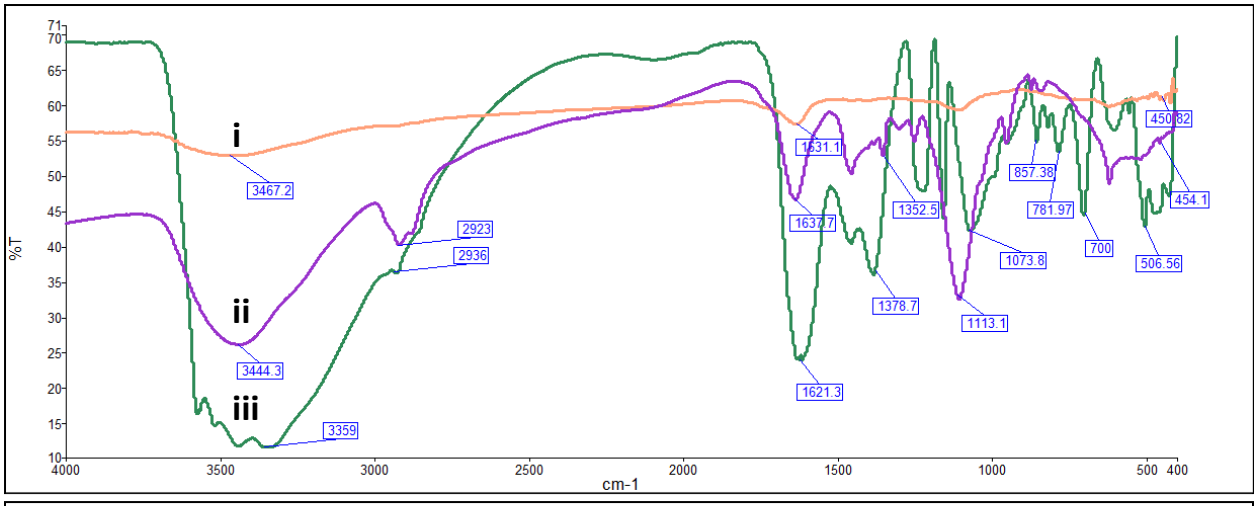

Figure 9.1: FT-IR spectroscopy of **i)** Only green synthesized CuONPs, **ii)** CuONPs@CS, **iii)** CuONPs@CS@FA

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vibration may be due to bioactive components of plant extract.

Bands near about 1631cm^{-1} correspond to amide I. Peak near about 1100 cm^{-1} is the characteristic stretching vibration of C-O and Cu-O vibrational bond corresponds to 450.82 cm^{-1}. From Fig.1A-II, it was observed that the strong bond in the region of 3444.3cm^{-1} which assigns O-H stretching frequency of alcohol or phenols. Peak near about 2923cm^{-1} can be attributed to C-H symmetric stretching which is characteristic peak of chitosan. Peak near about 1637cm^{-1} and 1352cm^{-1} is due to C=O and C-N stretching vibration respectively. Low intensity peaks are due to metal oxygen vibrational frequency (Logpriya et al., 2018). Finally for CuONPs@CS@FA, (Fig.9.1-III) these characteristics peaks of chitosan like C=O (amide I) and C-N all are present with a little shift in the peaks along with low intensity peak 800-400cm^{-1}. The band near about 1500cm^{-1} indicated vibration of N-H (2^{o} amide) of folic acid. From the final conjugate, the presence of CuONPs, CS and FA in the nano composite was confirmed.

9.2.2. XRD study:

Fig 9.2B, X-ray diffraction analysis of CuONPs@CS@FA showed the presence of peak at 2θ = 19.8, 23.1, 25.9, 31.7, 38.60, 45.4, 53.6, 56.2, 66.1 and 76.9^{o} corresponding to the planes of (220), (021), (021), (110), (111), (111), (020), (202), (-113), (111) respectively. 2θ= 31.7, 38.60, 53.6 showed corresponding planes (110), (111) and (020) which observed in the only CuONPs diffractogram pattern. These planes showed better settlement with the diffraction data card

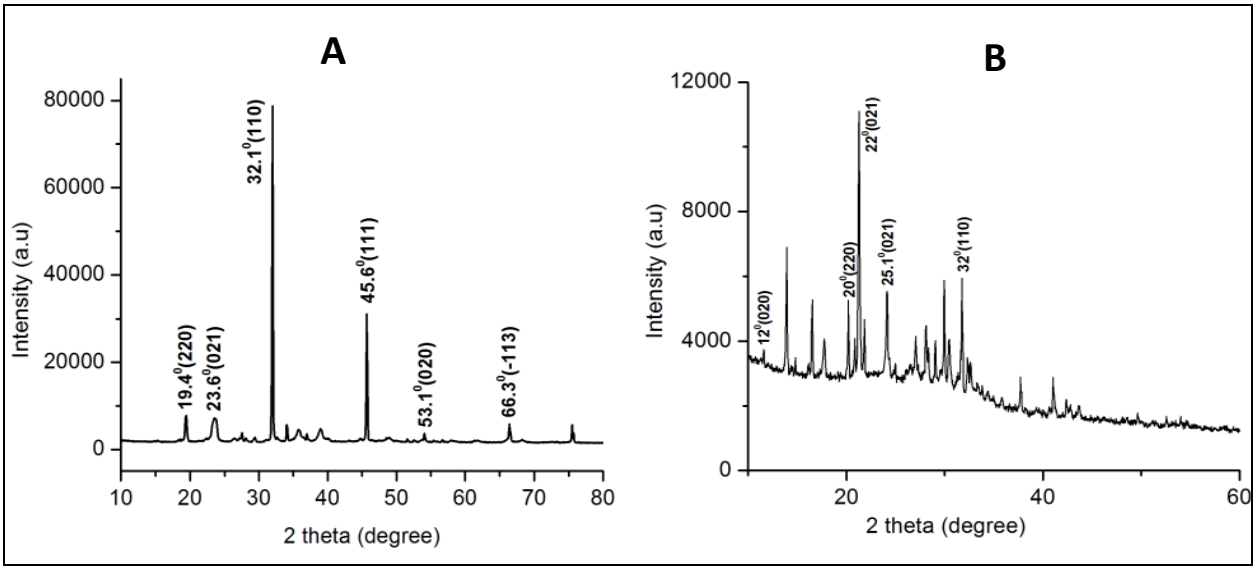

Figure 9.2: XRD study of **(A)** green synthesized CuONPs **(B)** CuONPs@CS@FA

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JCPDS No.05-0661 (Rehana et al., 2017). Other peaks may be due to surface coated materials. So it can be concluded that the CuONPs was successfully coated with CS followed by FA.

9.2.3. EDX:

From the EDX study, the elemental analysis of the conjugate was performed. It was observed the presence of Cu as the only metal element. Presence of other elements (S, Cl, N, O) (Fig.9.3) might be attributed to presence of biopolymer and other organic components (Zhang et al., 2009).

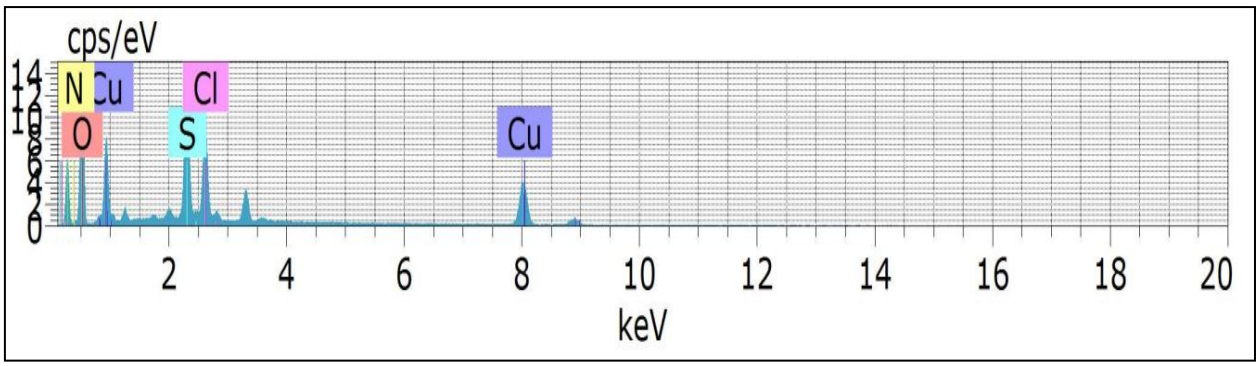

Figure 9.3: EDX study of CuONPs@CS@FA

9.2.4 SEM and TEM:

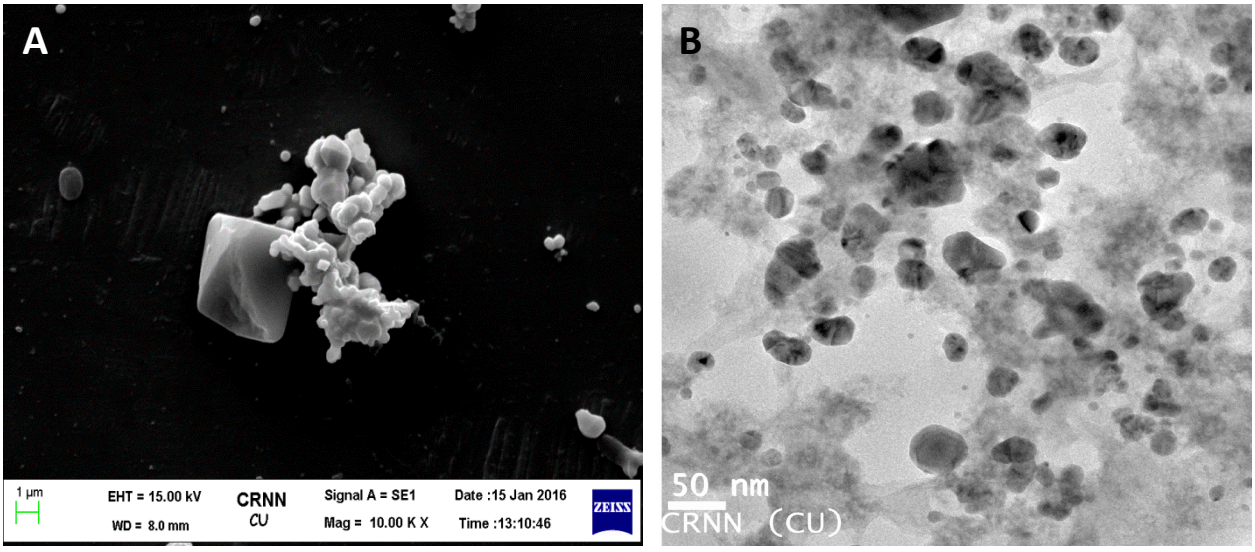

Figure 9.4: A) SEM and **B)** TEM images of CuONPs@CS@FA

The average size of the nano composite (CuONPs@CS@FA) was 52±4nm (Fig.9.4B) and SEM image revealed the attachment of CS and folic acid with CuONPs.

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9.2.5. TGA analysis:

From the TGA data (Fig. 9.5), the thermal stability of the total conjugate upto 600^{o}C temperature was observed. Near about 200^{o}C temperature the conjugate became unstable and a sudden decreased in weight was observed. Again instability was observed near about 400^{o}C temperature.

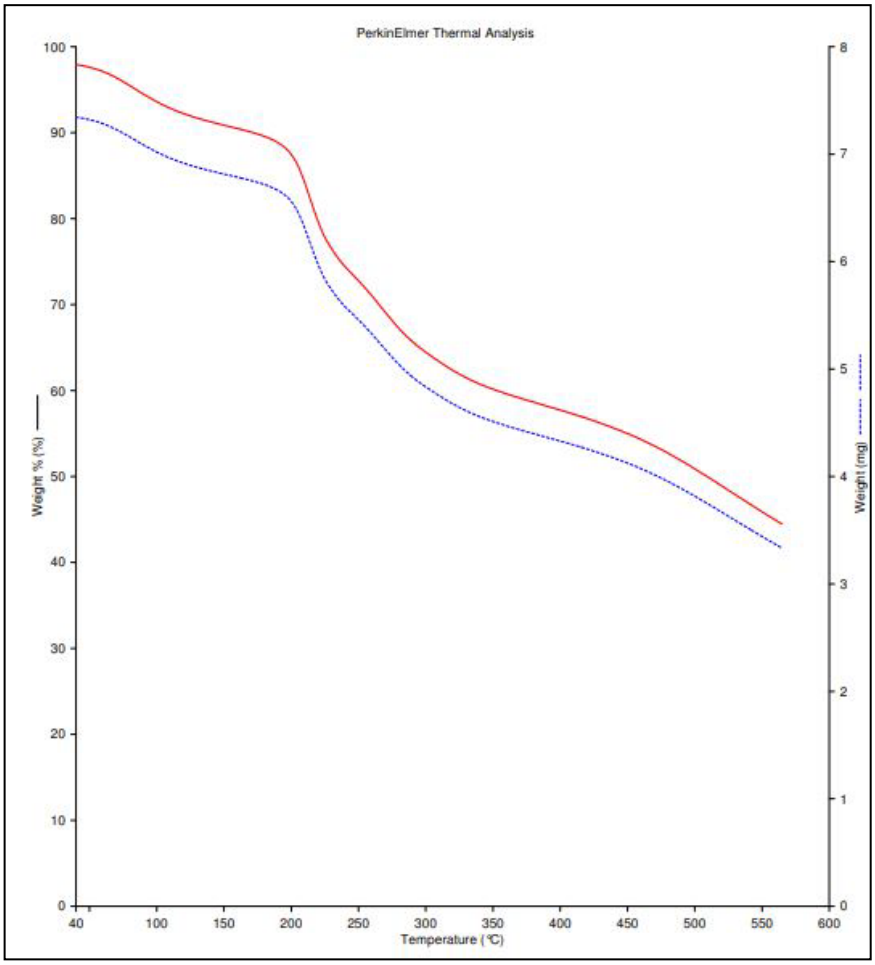

Figure 9.5: TGA analysis of CuONPs@CS@FA

9.2.6. Folic acid binding:

The % of folic acid binding with the CuONPs@CS was estimated by spectrophotometrically (Fig.9.6A). In a suspension of 5mg/ml CuONPs@CS equal concentration of FA solution was mixed. At first the O.D. of Folic acid solution was measured. As the time increased, the O.D. of the sup where the excess folic acid will remain was measured and the remaining folic acid was conjugated with the CuONPs@CS. After 24h the O.D of folic acid will be minus from the initial

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FA O.D. From the O.D. value the % of FA binding was calculated. After 24h, 88.63% of FA was bonded with CuONPs@CS which was very significant (P<0.05).

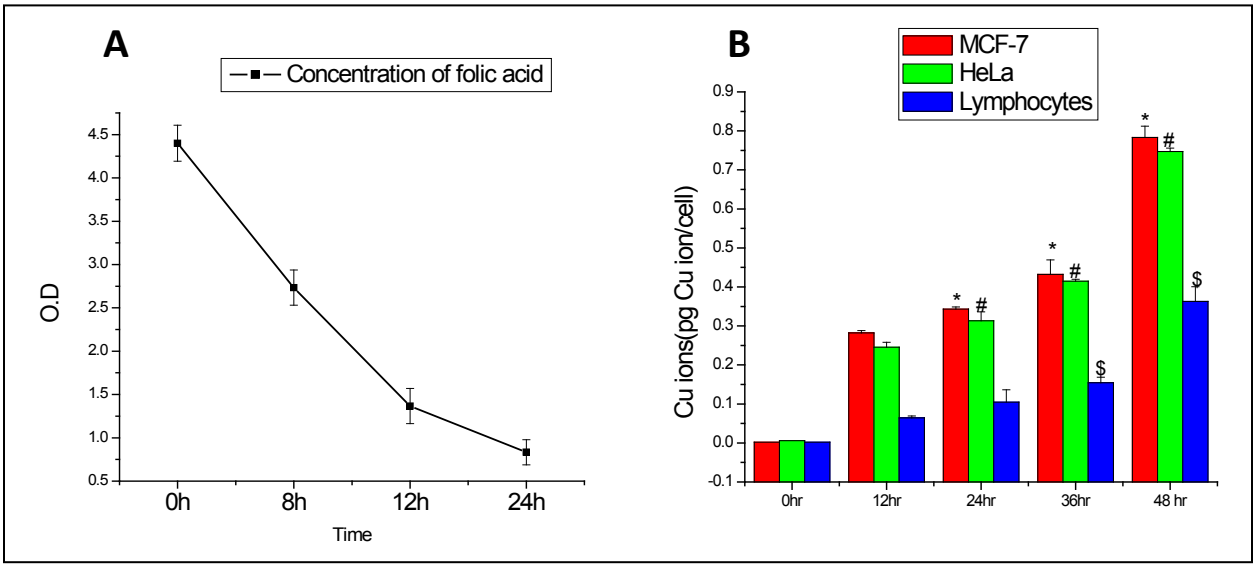

Figure 9.6: (A) Estimation of Folic acid binding with CuONPs@CS by spectrophotometrically after different time duration. **(B)** Intracellular uptake of Cu ion inside the MCF-7, HeLa and Lymphocytes. Values were expressed as mean ± SEM. n=5; *Superscripts indicated a significant difference as (P<0.05) compared with control MCF-7. #Superscripts indicated a significant difference as (P<0.05) compared with HeLa control group and $ Superscripts indicated a significant difference as (P<0.05) compared with Lymphocytes control group

9.2.7. Intracellular Uptake of CuONPs@CS@FA:

After 24h Cu ion internalization inside the MCF-7 and HeLa cells was 0.432 and 0.415pg Cu ion/cell and 0.15pg Cu ion was internalized inside the lymphocytes at normal p^H (Fig.9.6B). But after 48h the Cu ion internalization inside the cancer cell increased significantly but lymphocytes internalization also increased which may create severe toxicity. Hence 24h treatment was chosen for further experiments.

9.2.8. Cytotoxicity assay:

CuONPs@CS@FA was applied on MCF-7, HeLa and Lymphocytes in a dose dependent manner. MCF-7 cells were killed by 9.05, 18.22, 38.66, 63.29, 71.20 and 83.27% and HeLa cells were killed by 12.82, 24.30, 35.20, 52.11, 69.87 and 74.78% significantly (P<0.05) Fig. 9.7A. The IC_{50} values of CuONPs@CS@FA against MCF-7 and HeLa cells were 35.84µg/ml and 40.64µg/ml respectively. Subsequently DOX has been used to evaluate the efficacy of the conjugate. The IC_{50} values of DOX against MCF-7 and HeLa were 1.24µg/ml and 3.54µg/ml

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respectively. But the cytotoxicity assay against lymphocytes killed the cells by 7.07, 13.78, 16.80, 20.38, 23.89 and 60.33% (Fig. 9.7B).

The IC_{50} value of CuONPs@CS@FA against lymphocytes was 86.52µg/ml. So, the 50µg/ml dose can be totally nontoxic and can be used as a safe dose for further experiments. DOX has been used as a positive control. DOX killed lymphocytes by 25.38, 55.70, 67.04, 72.67, 85.74 and 92.70% (Fig. 9.7B). The IC_{50} value of green CuONPs@CS was 66.25µg/ml whereas; the IC_{50} value of only green CuONPs was 30.72µg/ml against lymphocytes.

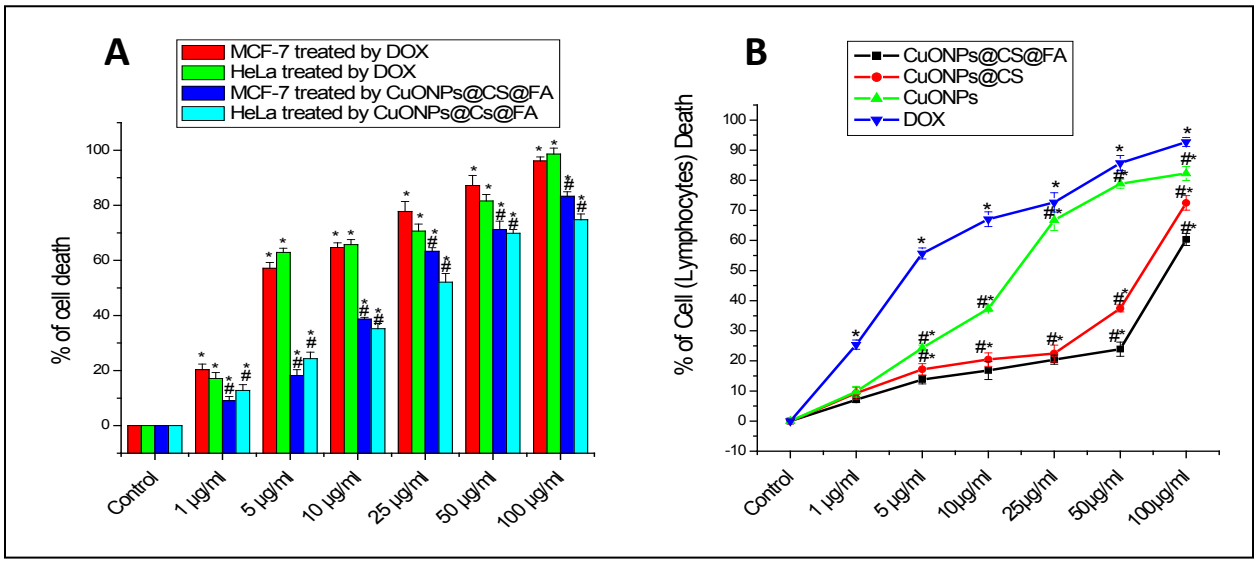

Figure 9.7: (A) Dose and duration dependent percentage of MCF-7 & HeLa cell **(B)** Lymphocytes death by CuONPs@CS@FA, DOX, green CuONPs@CS and only green synthesized CuONPs was estimated by MTT assay. Values were expressed as mean ± SEM, n=5. *Superscripts indicated a significant difference as ($p<0.05$) compared with control. #Superscripts indicated a significant difference as ($p<0.05$) compared with DOX treated group

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9.2.9. Fluorescence microscopic images of Rh-B tagged CuONPs@CS@FA inside cancer cells:

The total conjugate was tagged with Rh-B. After the successful tagging of Rh-B with the dose of 50µg/ml CuONPs@CS@FA, was applied on MCF-7 and HeLa cells. Successful internalization inside the cancer cells was confirmed by microscopic imaging (Fig. 9.8 A-F).

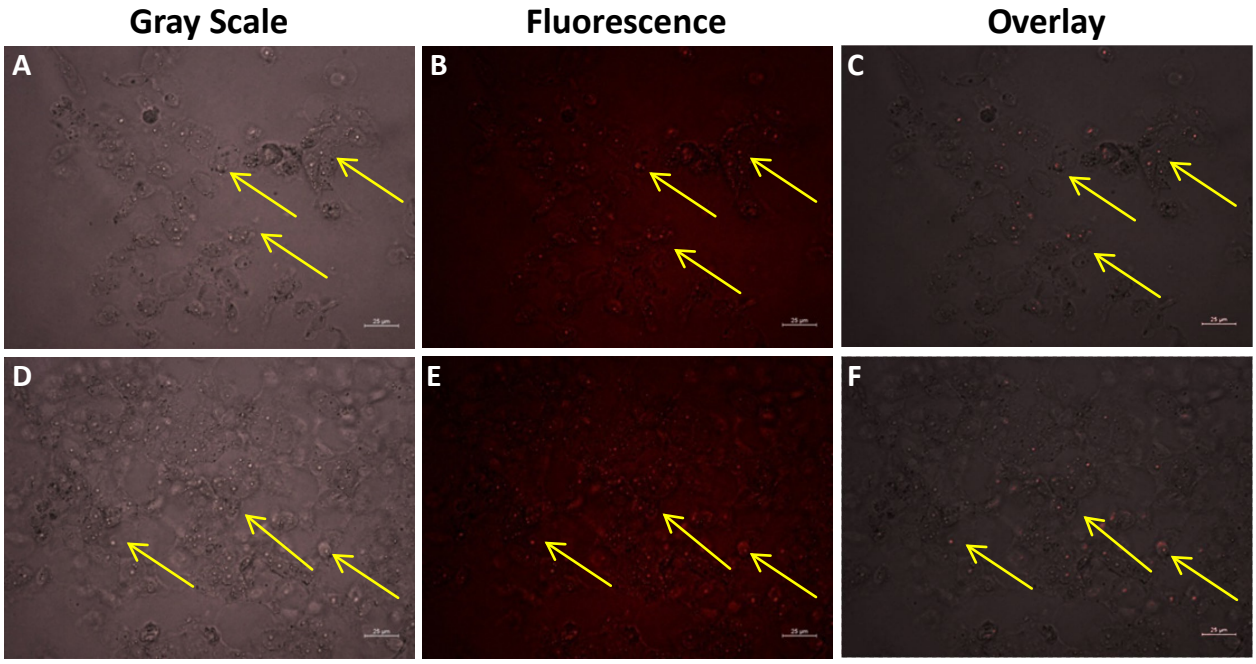

Figure 9.8: Grey scale, fluorescence and merged images of Rh-B tagged CuONPs@CS@FA treated **(A-C)** MCF-7 cell and **(D-F)** HeLa cells revealed the localization of conjugate NPs.

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9.2.10. ROS generation inside cancer cells:

Significant amount of ROS generation inside the both MCF-7 and HeLa cells were observed compared to the control group after the treatment with CuONPs@CS@FA (Fig 9.9A-F). ROS intensity was measured by spectrophotometer Fig. 9.9 G.

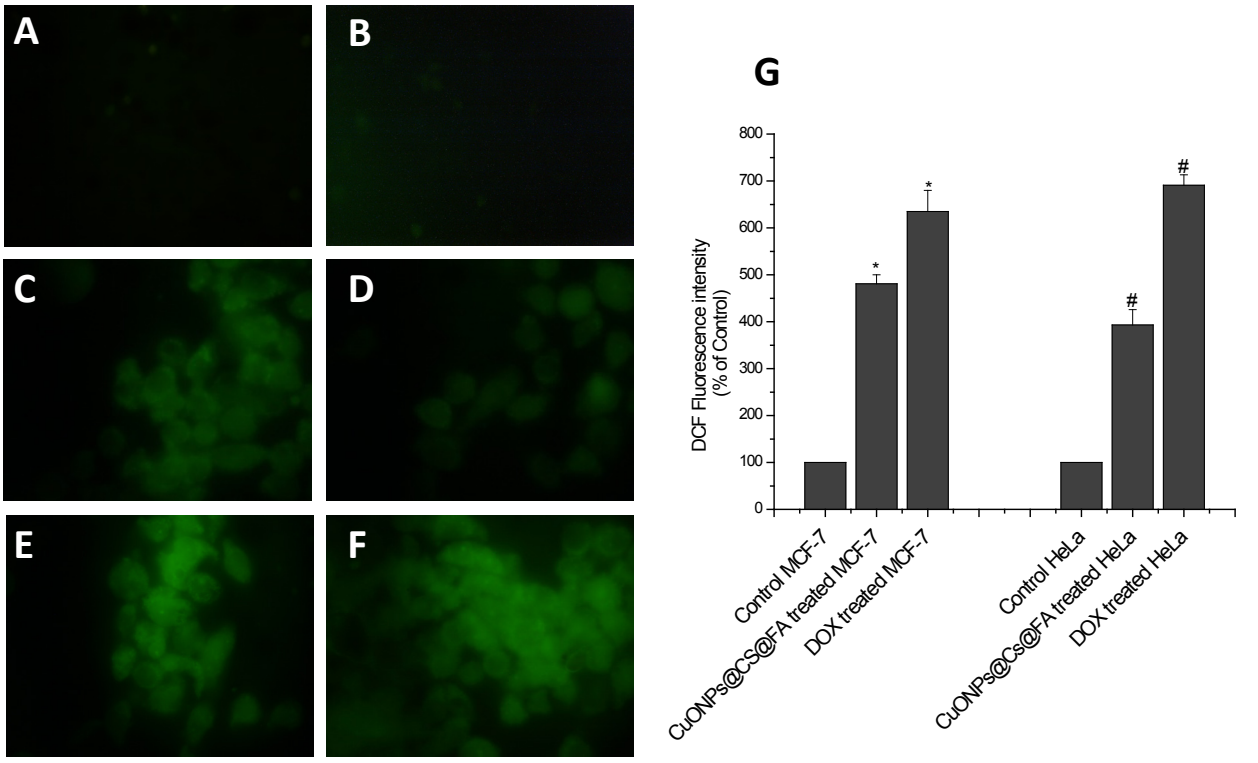

Figure 9.9: Visualization of ROS production by DCHF2DA staining inside MCF-7 and HeLa cells via fluorescence microscope after the treatment with CuONPs@CS@FA and DOX. **(A)** Control MCF-7 and **(B)** Control HeLa cell **(C)** treated MCF-7 cells with a dose of 50µg/ml. **(D)** CuONPs@CS@FA treated HeLa cells with a dose of 50µg/ml. **(E)** DOX (50µg/ml) treated MCF-7 **(F)** DOX (50µg/ml) treated HeLa cell **(G)** DCF fluorescence intensity was expressed in term of ROS production. The data of ROS in control group was set at 100 units. Treated groups have been presented as a fold change compared to the control group. Values were expressed as mean ± SEM, n=5. *Superscripts indicated a significant difference as ($P<0.05$) compared with control MCF-7. #Superscripts indicated a significant difference as ($P<0.05$) compared with control HeLa group**.**

9.2.11. Mitochondrial membrane potential:

The reduction in MMP level was observed through the measurement of intensity of Rh-123. Fig. 9.10 indicates the reduction of MMP by CuONPs@CS@FA was 61.56% (MCF-7) and 55.54% (HeLa) compared to the control group.

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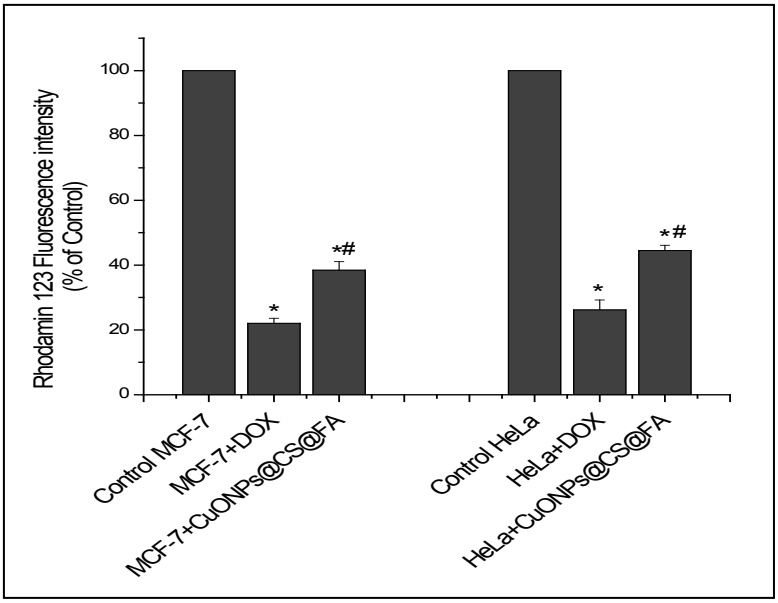

Figure 9.10: MMP was determined by measuring Rhodamin-123 fluorescence intensity using fluorescence spectrophotometer. Values are expressed as mean±SEM of three experiments; *superscripts indicate significant difference ($P<0.05$) compared with the control group and $^{\#}$superscripts indicate significant differences of CuONPs@CS@FA treated group compared to DOX treated group. n=5.

9.2.12. Apoptosis/necrotic study of cancer cells by Etbr/AO double staining:

Several morphological changes occur during apoptosis. In the Fig. 9.11A-F indicated the apoptotic body formation inside the cancer cells which is a sensitive indicator of apoptosis and the orange color inside the

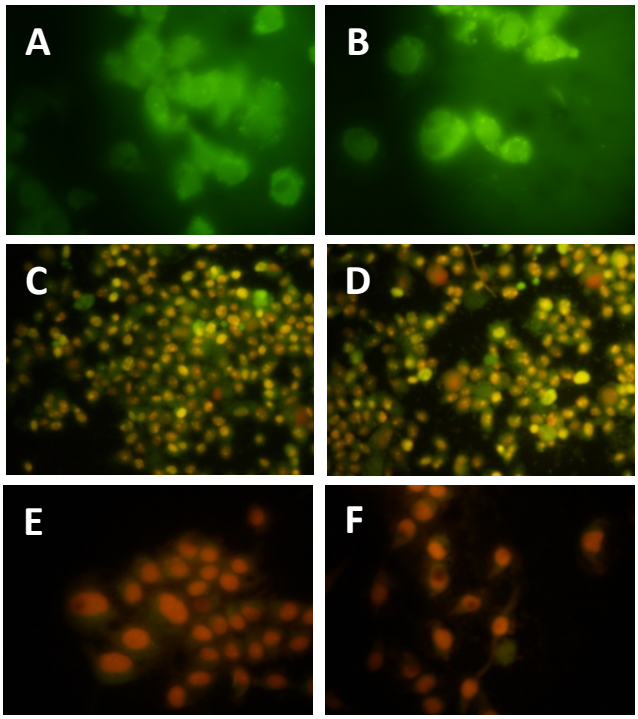

Figure 9.11: Displayed membrane integrity and apoptosis by EtBr/AO double staining. **(A)** Control MCF-7 **(B)** MCF-7 cells treated with green CuONPs@CS@FA (50µg/ml dose). **(C)** Control HeLa cells **(D)** HeLa cells treated with green CuONPs@CS@FA (50µg/ml dose). **(E)** DOX (50µg/ml) treated MCF-7. **(F)** DOX (50µg/ml) treated HeLa cells.

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cancer cells indicated the late apoptosis after the treatment with CuONPs@CS@FA.

9.2.13. Nuclear Changes by DAPI staining:

Over production of ROS inside the cancer cells may induce nuclear fragmentation which was observed by DAPI staining. The Fig.9.12A-F indicate the significant amount of fragmentation inside the cancer cells nucleus compared to the control group.

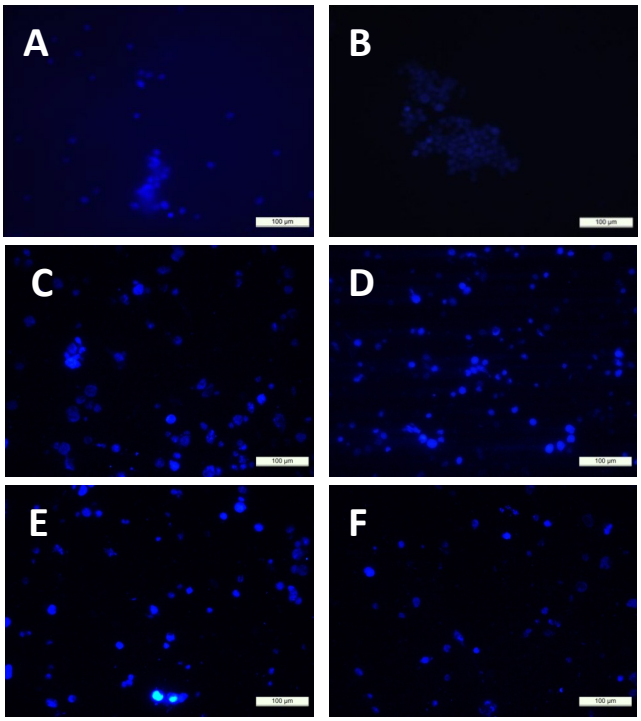

Figure 9.12: Displayed morphological changes of nuclei by DAPI staining **(A)** Control MCF-7 **(B)** MCF-7 treated with CuONPs@CS@FA at a dose of 50µg/ml **(C)** DOX treated MCF-7 **(D)** Control HeLa cells **(E)** HeLa cells treated with CuONPs@CS@FA at a dose of 50µg/ml. **(F)** DOX treated HeLa cells.

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9.2.14. Cancer cell images by Scanning Electron Microscopy:

After the treatment with CuONPs@CS@FA (50µg/ml) for 24h, cancer cells ultra-structure imaging were performed with the help of scanning electron microscopy (Fig.9.13A-D). After the treatment, apoptotic body and membrane blebbing was observed inside the MCF-7 cell and HeLa cell which corresponds to the surface morphology of apoptosis.

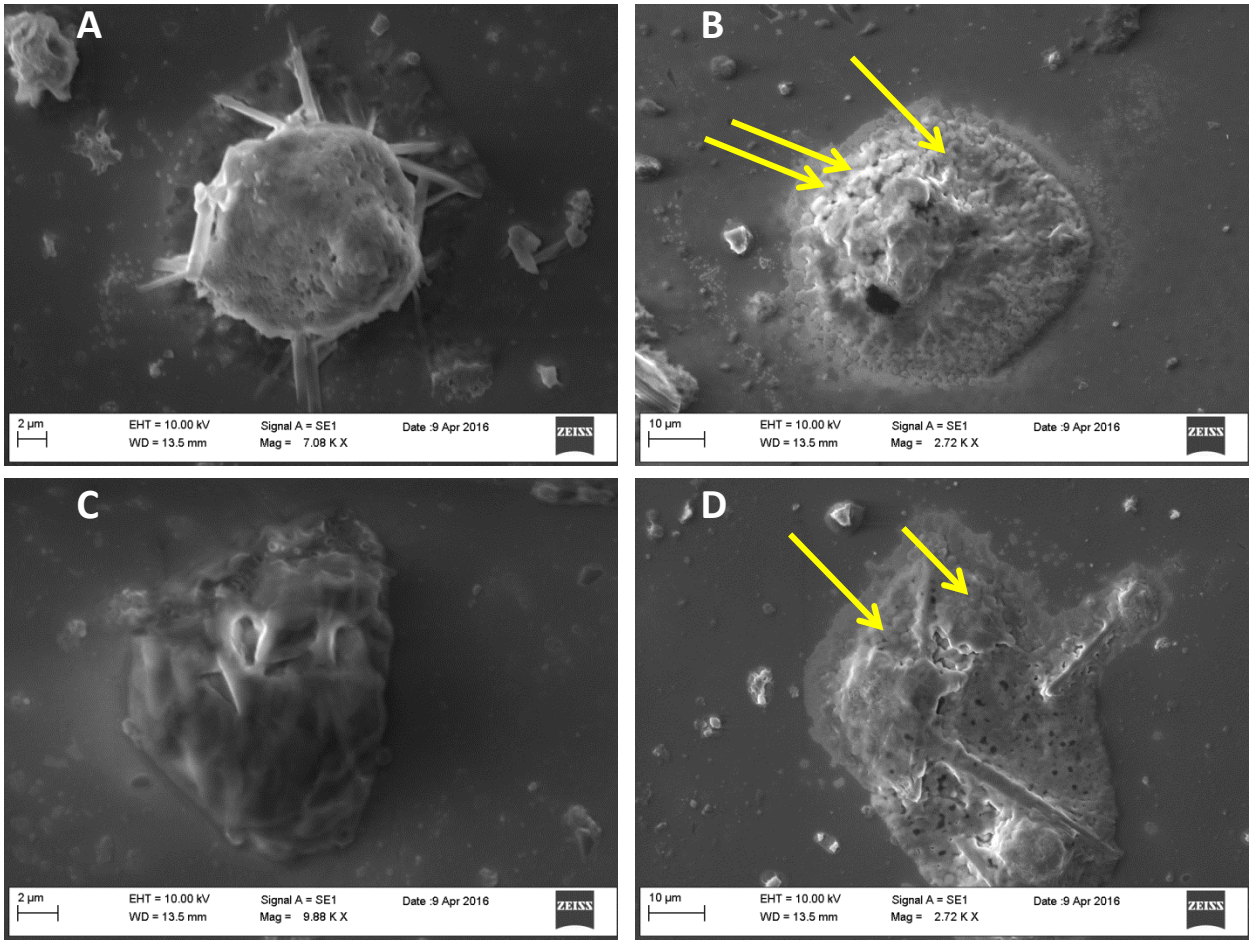

Figure 9.13: Displayed ultra-structure of MCF-7 and HeLa cells by scanning electron microscopy **(A)** Control MCF-7 **(B)** CuONPs@CS@FA treated MCF-7 cells **(C)** Control HeLa cells **(D)** CuONPs@CS@FA treated HeLa cells. Arrows in the picture indicate the observed changes.

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9.2.15. Cellular apoptosis by Annexin V-PI analysis:

The cancer cell death assay was done by FACS using Annexin-V and PI staining (Fig. 9.14 A-D). Next, at the dose of 50µg/ml, MCF-7 cells showed 56.7% of late apoptosis and 35.6% necrosis. In case of HeLa cells at a dose of 50µg/ml 14.5% of early apoptotic cells were observed. 50µg/ml dose showed 36.9% late apoptotic cells and 25.3% of necrotic cells.

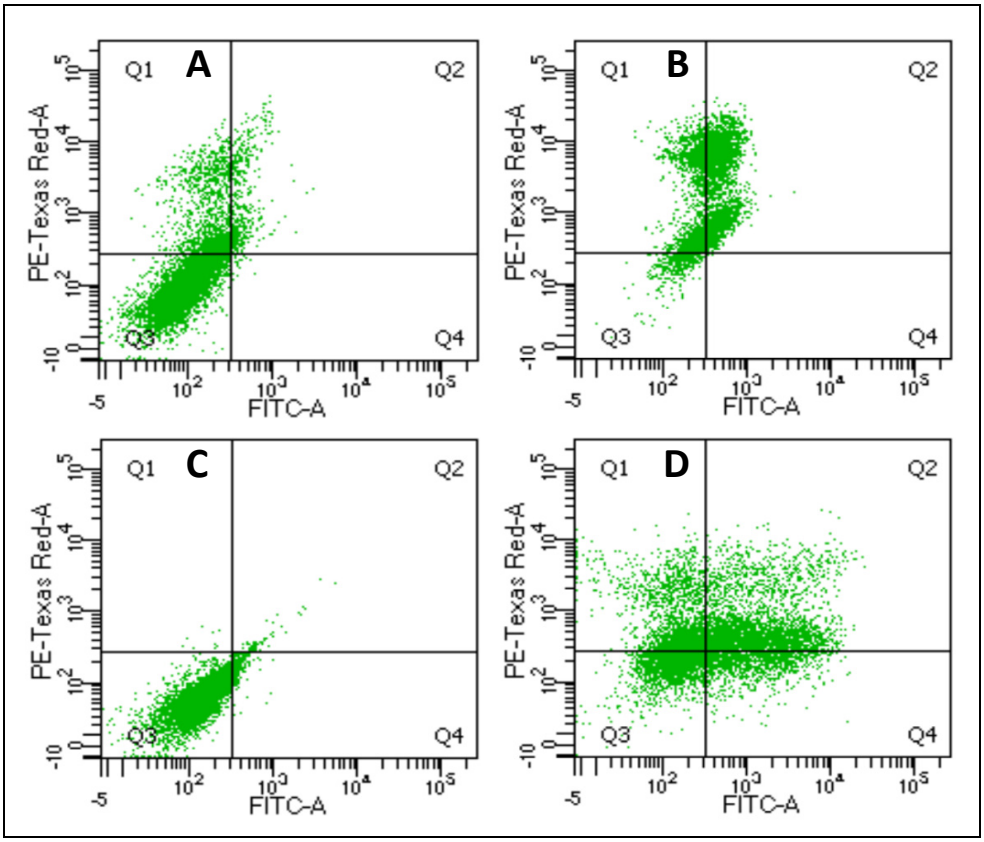

Figure 9.14: Estimation of different cell population by flow cytometry using Annexin-V/PI staining. The percentage of cell population of CuONPs@CS@FA treated MCF-7 and HeLa cells at Lower Left (LL: viable cells), Lower Right (LR: Annexin V-FITC positive early apoptotic cells), Upper Right (UR: Annexin V-FITC and PI dual positive) and Upper Left (UL: Only PI positive necrotic cells) was estimated before and after treatment. Population of **(A)** Control MCF-7 cells and **(B)** Treated (CuONPs@CS@FA 50 µg/ml) MCF-7 cells **(C)** Control HeLa cells **(D)** Treated (CuONPs@CS@FA 50 µg/ml) HeLa cells

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9.2.16. Cell cycle analysis:

As shown in Fig.9.15A-D, the cells exposed to CuONPs@CS@FA (50µg/ml) showed increased cell population at the G2/M phase ($p<0.05$). This may be due to that CuONPs@CS@FA treated MCF-7 and HeLa cells became arrested at G2/M phase. CuONPs@CS@FA helped to inhibit the G2/M checkpoint proteins.

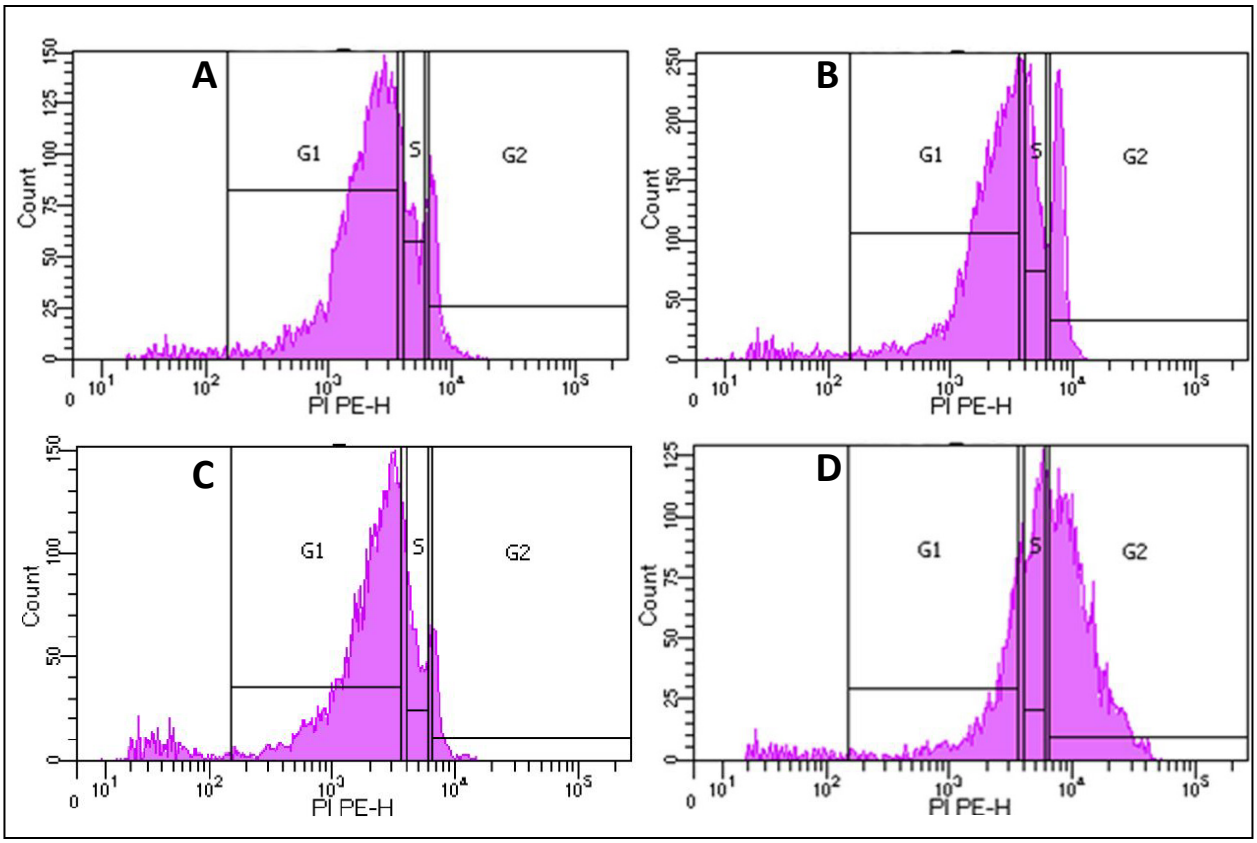

Figure 9.15: Determination of cell cycle arrest by flow cytometry **(A)** untreated MCF-7 cells **(B)** MCF-7 cells treated with CuONPs@CS@FA (50µg /ml) **(C)** Untreated HeLa cells **(D)** HeLa cells treated with CuONPs@CS@FA (50µg /ml).

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9.2.17. Assessment of Cytokine release from cancer cells:

In case of MCF-7 cells the TNF-α level was elevated by 71.94% significantly ($p<0.05$) compared to the control after the treatment with CuONPs@CS@FA. But the anti-inflammatory cytokine IL-10 level depleted by 55.98% significantly ($p<0.05$) compared to the control (Fig 9.16A). Pro-inflammatory cytokine TNF-α level was increased by 65.45% and IL-10 cytokine level reduced by 54.56% significantly ($p<0.05$) compared to the control group in case of HeLa cells (Fig. 9.16B).

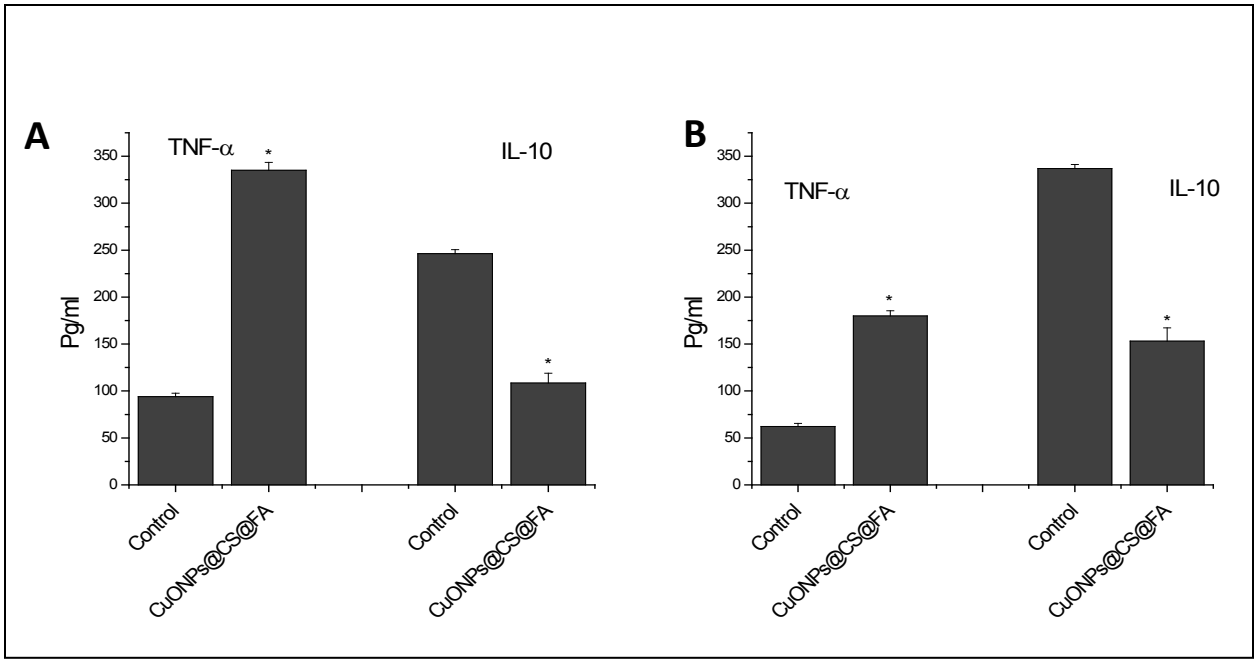

Figure 9.16: Cytokines analysis in vitro by ELISA method. Pro and anti-inflammatory (TNF-α, IL-10) response of CuONPs@CS@FA of **(A)** MCF-7 and **(B)** HeLa cells. Values are expressed as mean±SEM, n=5; *superscripts indicate significant difference ($P<0.05$) compared with the control group.

9.2.18. Expression of Caspases:

Caspases 3 and 8 expressions were observed in Fig. 9.17A-H after immunofluroscent microscopic study of cancer cells. HeLa and MCF-7 cells were stained with Caspase 3 and 8 immunofluroscent antibody. The cells were treated with CuONPs@CS@FA conjugate at a dose of 50µg/ml dose for 24hr. After the treatment cells were compared with the control cells (without any treatment). The intense red color of treated cells observed, indicated the Caspases 3 and 8 expressions of cancer cells.

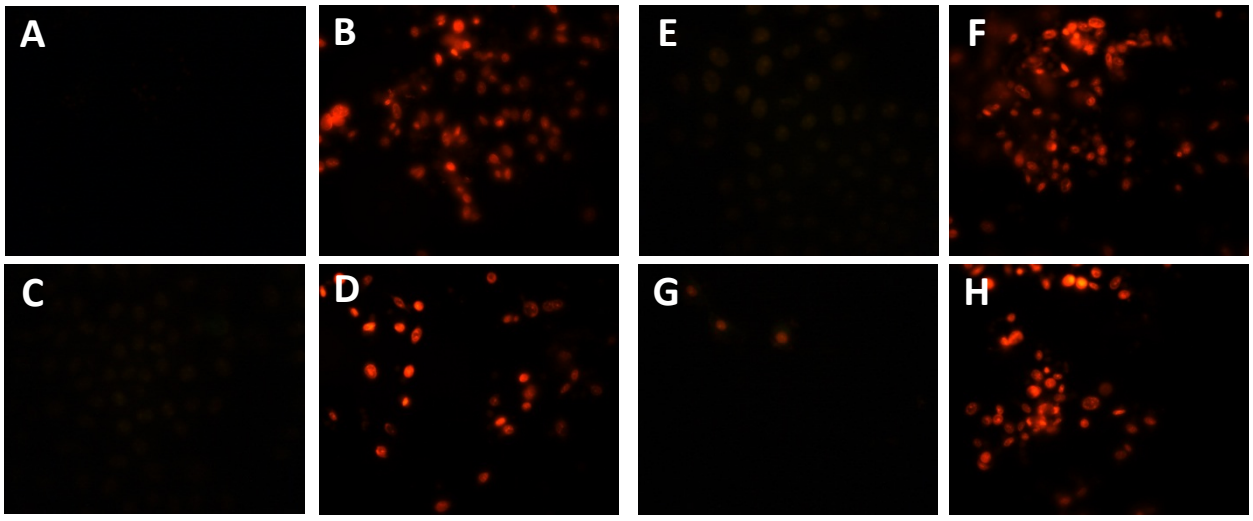

Figure 9.17: Caspases activity of MCF-7 and HeLa cells were expressed by immunofluroscent staining process. **A)** Cleaved Caspase 3 expression of Control MCF-7 cell **B)** Cleaved Caspase 3 expression of Treated MCF-7 cells **C)** Cleaved caspase 3expression of Control HeLa **D)** Cleaved Caspase 3 expression of Treated HeLa cells **E)** Caspase 8 expression of Control MCF-7 **F)** Caspase 8 expression of Treated MCF-7 cells **G)** caspase 8 expression of Control HeLa **H)** Caspase 8 expression of Treated HeLa cells.

9.2.19. Expression of apoptotic markers from cancer cells:

To confirm the apoptotic proteins involvement in the apoptotic phenomenon after the CuONPs@CS@FA treatment both MCF-7 and HeLa cells, the apoptotic marker analysis assay by western blot method was performed. After the treatment significant up regulation was observed in case of Caspase 3 protein but significant down regulation was also observed in case of AKT protein compared to the control group ($p<0.05$) Fig. 9.18.

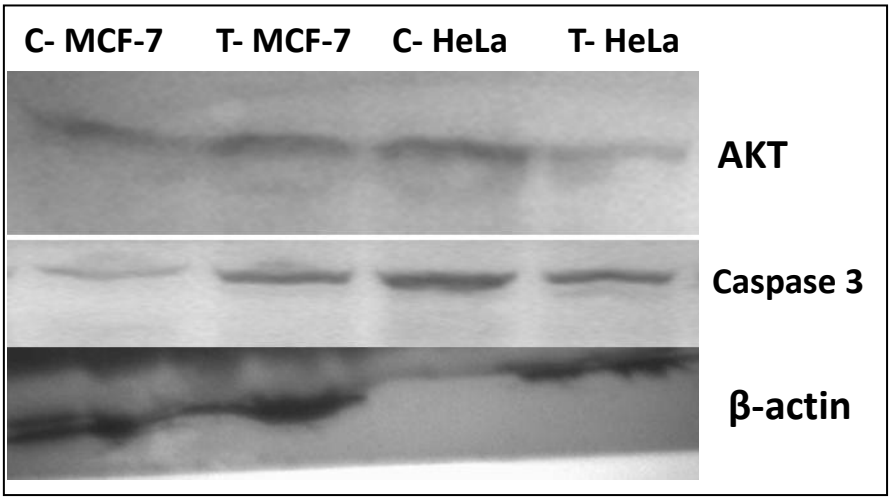

Fig. 9.18: Western blot analysis of Caspase 3 and pAKT proteins of MCF-7 and HeLa cells.

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9.2.20. Folate receptor knockdown:

Then FR negative cells were treated with CuONPs@CS@FA for 24hr. The % of cell death was significantly ($P<0.05$) lowered compared to the FR positive MCF-7 and HeLa cells. At a highest dose of 100µg/ml CuONPs@CS@FA, MCF-7 was killed by 28.33% and HeLa killed by 24.66% of cells compared to the control cells (Fig.9.19). This result indicates the internalization of the CuONPs@CS@FA through FR of cancer cells which ensured the targeted delivery of the NPs.

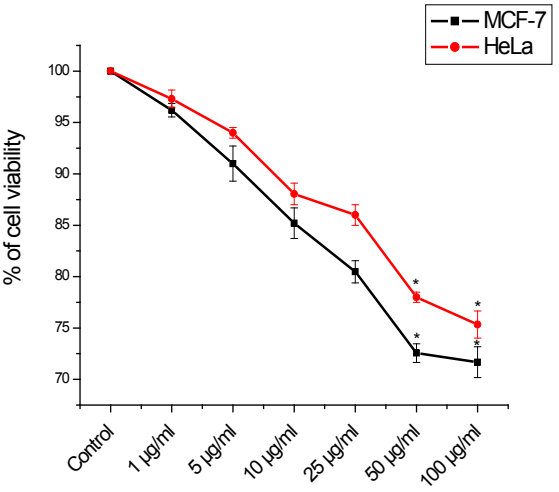

Figure 9.19: Dose and duration dependent MTT assay of MCF-7 and HeLa cells after the blocking of Folate receptor of both these cells. Values are expressed as mean±SEM; *superscripts indicate significant differences ($p<0.05$) compared with the control group. n=5.

9.2.21. *In vivo* study:

9.2.21.1. CuONPs@CS@FA induced anti-cancer activities *in vivo*:

Solid tumor was implanted by 4T1 cell in Balb/c mice model. After the 14 days the mature tumor increased body weight. Next all the tumor bearing mice were treated with CuONPs@CS@FA intraperitoneally for 30 days at three days interval. 1000µg/Kg Body Weight dose has been chosen as a biological safe dose for *in vivo* study.

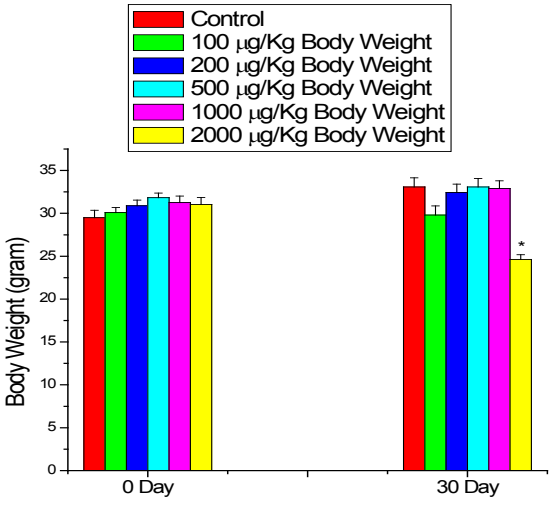

Figure 9.20: Selection of biologically safe dose in vivo treatment by measurement of body weight of mice for 30 days after the treatment with the CuONPs@CS@FA at an interval of 3 days. Values are expressed as mean±SEM; n=5, *superscripts indicate significant differences ($p<0.05$) compared with the control group.

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9.2.21.2. Analysis of tumor weight:

After the above mentioned treatment schedule the tumor weight was reduced compared to the control tumor bearing mice. CuONPs@CS@FA treatment significantly ($p<0.05$) reduced weight by 74.25%(Fig. 9.21A).

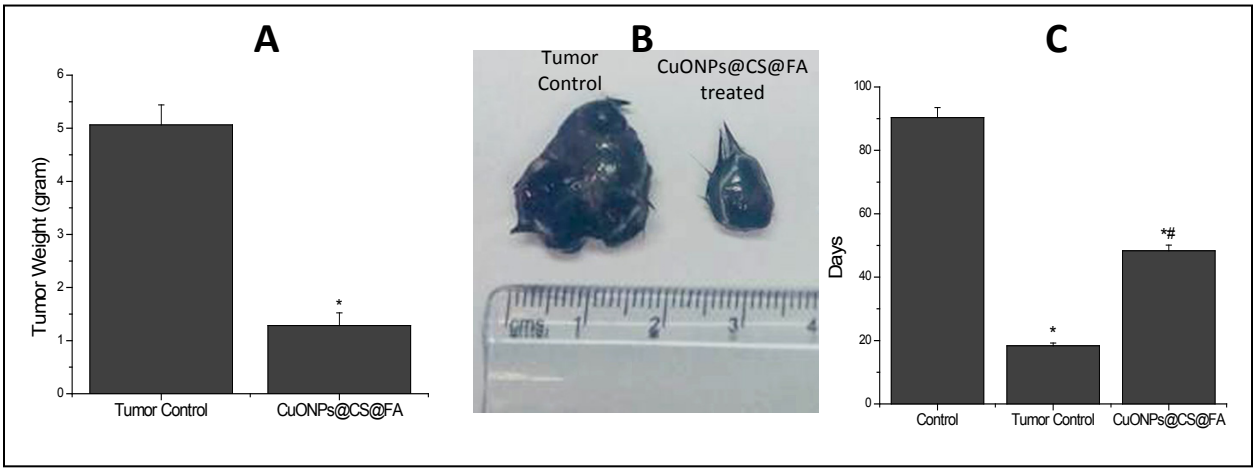

Figure 9.21: (A) Mean weight and **(B)** images of tumor after the treatment of CuONPs@CS@FA for 30 days at a dose of 1000µg/Kg body weight. **(C)** Survival days of Balb/c mice. Values are expressed as mean±SEM; *superscripts indicate significant differences ($p<0.05$) compared with the control group. #Superscripts indicated a significant difference as ($p<0.05$) compared with tumor control group. n=5.

9.2.21.3. Survivability curve:

The average survival time of tumor bearing mice was 18 days. But after the treatment with CuONPs@CS@FA the average time of survivability increased up to 48 days, which was considered a significant result (Fig. 9.21C).

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9.2.21.4. Cytokines analysis:

TNF-α level increased by 51.79% compared to the tumor control group after the treatment with CuONPs@CS@FA. IL-10 level was decreased by 58.62% compared to the tumor control mice significantly (P<0.05) (Fig. 9.22).

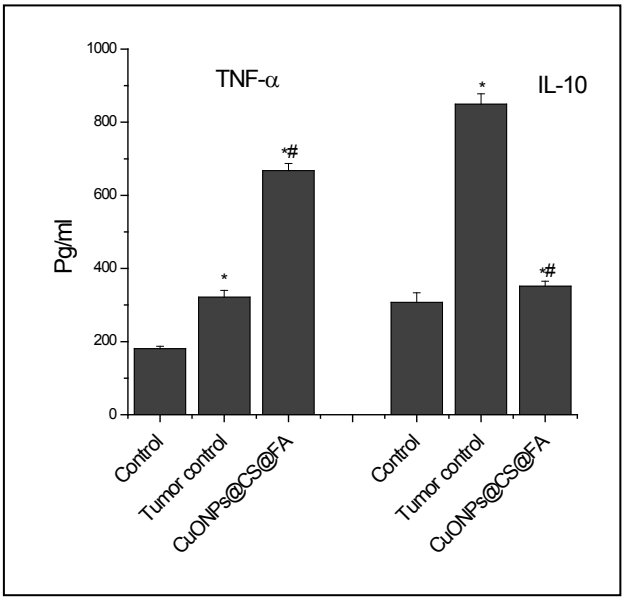

Figure 9.22: Pro and anti-inflammatory (TNF-α, IL-10) response of tumor homogenate after the treatment of CuONPs@CS@FA for 30 days at an interval of 3 days. *superscripts indicate significant differences (p<0.05) compared with the control group and $^{\#}$superscripts indicate significant differences of CuONPs@CS@FA compared to tumor control group. n=5.

9.3. DISCUSSION:

A smart nano sized tool with double layer coating was successfully synthesized to reduce the toxicity. The first layer was composed of components of *A. indica* leaves and the second layer was CS which was attached by physical bonding (Song et al., 2013). After the coating the conjugate (green CuONPs@CS) was grafted with FA to target the cancer cells specifically. The successful conjugation of CS and FA with green CuONPs was confirmed by FT-IR, SEM, XRD, EDX and TGA. FR mediated delivery of the surface coated NPs created severe toxicity in both cancer cells. But the toxicity of CuONPs@CS@FA at 50µg/ml dose in lymphocytes was only 23.89% and 100µg/ml dose killed lymphocytes by 60.33% significantly (p<0.05) which was quite toxic for normal cells. To confirm the folate mediated NPs delivery, the FR knockdown of the MCF-7 and HeLa cells (Fig. 9.19) was performed. After knockdown very low toxicity against cancer cells was observed. This phenomenon proved that the NPs were internalized to the

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cancer cells through FR mediated endocytosis pathway. Generally FRs is linked to the lipid region of the cancer cells. FR and its contents after the migration to the inner membrane gets dissociated from the folate-conjugated NPs rendered by the acidic microenvironment of interior side following which folate-conjugated NPs can be released into the cytosol of the tumor cells (Mansoori et al., 2010). Subsequently CuONPs@CS@FA can enter the mitochondria and produced ROS. Similar kind of result was also observed by Sharma et al (Sharma et al., 2012). After internalization the conjugate will release more NPs from the coating of CS in acidic condition of cancer cells and as a result produced huge amount of ROS. ROS induced oxidative stress can regulate the cell signaling cascade and damage the cancer cells (Benhar et al., 2002). In this study it was also observed the cancer cell damage by CuONPs@CS@FA through nuclear fragmentation and chromatin condensation (Fig.9.12A-F). Apoptosis was proven when blebbing of membrane, DNA fragmentation, cell shrinkage, nuclear condensation and phosphatidylserine (PS) exposure were visualized (Nagata and Golstein, 1995; Vaux and Korsmeyer, 1999). Those incidents were observed in the study from Etbr/AO double staining and cellular SEM image. Late apoptotic phase was the main population stage of cancer cells after the treatment that was confirmed by FACS analysis. The conjugate arrested the cancer cells at G2/M phase (Fig.9.15A-D). Alteration in these check points may provoke cell cycle arrest finally leading to apoptosis (Xue et al., 2012; Majumdar et al., 2001). Excess production of ROS can kill the cancer cells by the elevation of TNF-α level (Yang et al., 2007; Manke et al., 2013). TNF-α shows pivotal role in apoptosis at cellular level and simultaneously in modulation of cellular immunity (Mehta et al., 2018; Müller-Hermelink et al., 2008). The pro (TNF-α) and anti (IL-10) inflammatory cytokines level was considerably higher and lower respectively compared to the control cancer cells.

Activated protein molecules (Caspases 3, 8 & 7) are responsible for apoptotic cell death, destruction of cell component proteins and fragmentation of DNA. Intrinsic apoptosis pathway is achieved by caspase-8 which is activated by BCL-2 interacting domain (Bid), a BH3-only Bcl2 family member. Apoptosis pathway was induced due to cleavage of the key NF-κB mediator RIP which inhibits the cell survival signal. Caspase-8 activation promotes the cleavage of NF-κB (Lin et al., 1999). Caspase 3 which is an executor of mitochondrial apoptotic pathway (Kuang et al., 2016; Xu et al., 2012). Caspase 8 acts as an initiator and activator of tumor necrosis family receptors and Cytochrome C protein. Both these proteins are convoluted in apoptosis pathway. In

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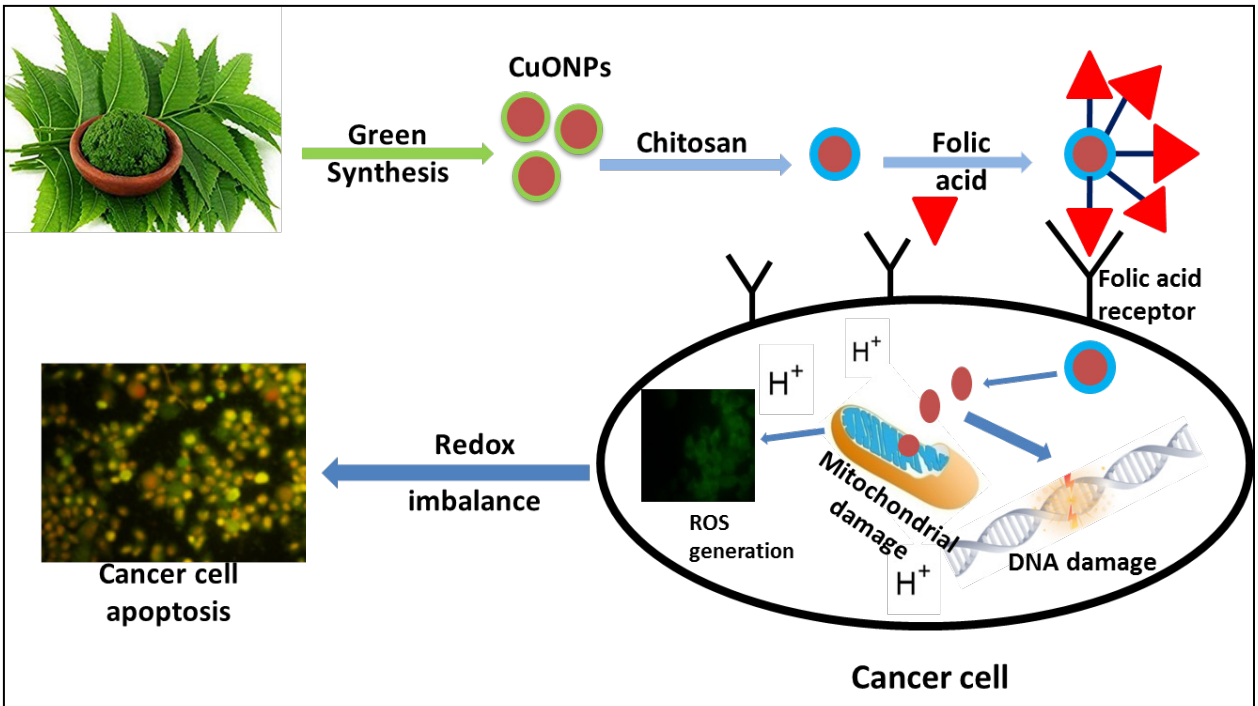
the present study, the (Fig. 9.18) involvement of Caspases-3, 8 and Bcl2 proteins was observed which ensure the intrinsic apoptosis pathway.

In vivo study in Balb/c mice model was done to evaluate the anti-tumor efficacy of the conjugate CuONPs@CS@FA. After the 30 days the tumor weight was reduced significantly ($P<0.05$) by 67.82%. At the same time the survival days also increased up to 48 days compared to the tumor control mice. Similar kind of result was also notified in previous study (Gao et al., 2011; Scarano et al., 2013; Guo et al., 2013). Cytokines level form tumor homogenate also indicate the high level of TNF-α which is the key operator of apoptosis (Horssen et al., 2006).

In conclusion, the present study indicates that a folate decorated conjugate that delivers the NPs directly to the folate receptor containing MCF-7, HeLa cells and 4T1 cells *in vivo* model with very low toxicity was successfully designed. The CuONPs@CS@FA conjugate showed appreciable anti-cancer activity with underlying mechanism. The CuONPs@CS@FA conjugate modulating the pro-inflammatory cytokine level shall be a new cancer cell targeting tool for immunotherapy. However further study is required before its application in clinical basis study for safety.



Probable mechanism of folate receptor mediated apoptosis of cancer cells after the treatment with FA-CS-CuONPs

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