Chapter 7

Anticancer efficacy of Chitosan coated green Copper Oxide nanoparticles: An *in vitro* & *in vivo* study

INTRODUCTION:

It was observed from the previous study that Chitosan coated green CuONPs effectively was able to reduce the toxicity against normal lymphocytes in *in vitro* mice model up to 50µg/ml and 1000µg/ml dose for *in vivo* model due to less amount of release of Cu ions inside the cells and tissue. Less amount of release of Cu ions influenced little amount of ROS generation which helped to reduce TNF-α level and ultimately leads to less apoptosis of normal lymphocytes upto certain specific dose.

In this present study it was intended to evaluate the anti-cancer activity of CS coated green CuONPs against MCF-7 and HeLa cells and as well as *in vivo* Balb/c mice model. The main obstacle in cancer therapy is to differentiate between normal and malignant cells. To overcome this issue, active (Byrne et al., 2008) and passive targeting (Maeda, H. 2001; Maeda, H. 2012) increases the concentration of NPs in the target specific site. Blood vessels in tumors are leaky and abnormal compared to the normal blood vessels (Maeda et al., 2006). This abnormal blood vessel leads to enhance EPR effect which is a marker of solid tumor vasculature. In this context surface modification of NPs played a major role to minimize the toxicity and uptake by macrophage phagocytic system and increased circulation time (Nie, S. 2010). p^H dependent solubility of CS helped to specifically target cancer cells and enhances the accumulation of NPs inside the cancer cells. So, surface modification will be an important strategy for developing anticancer therapy with NPs.

Therefore in this chapter, the detailed mechanism of the anticancer activity of CS coated green CuONPs in both *in vitro* and *in vivo* models will be studied.

7. MATERIALS:

7.1. List of Chemicals and Reagents:

Ethylene diamine (EDTA), sodium carbonate (Na2CO3), dimethyl sulfoxides (DMSO) and sucrose were purchased from Himedia, India. Rhodamine-B, TNF-α, IL-10 kit, Caspases-3,8, pAKT, Bcl2 were procured from Sigma (St. Louis, MO, USA) and eBiosciences.Alcohol, Tris-buffer, formaldehyde were purchased from Merck Ltd., SRL Pvt. Ltd., Mumbai, India.

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7.2. METHODS:

7.2.1. Cell culture:

The process of MCF-7 and HeLa cell culture was previously mentioned in Chapter 5 Section 5.2.1.

7.2.2. Preparation of conjugate as a drug:

Different doses (1-100µg/ml) of *green CuONPs@CS were prepared in phosphate buffer saline (p^H 7.4). All the doses prepared were then used for the treatment of cancer cells in *in vitro* system.

7.2.3. *In vitro* Cytotoxicity of cancer cells:

Detailed description was provided in Chapter 4 Section 4.1.5.1.

7.2.4. Intracellular uptake of Cu ions:

Intracellular uptake of Cu ion was done through AAS which was described in the previous chapter 4 section 4.1.5.2.

7.2.5. Microscopic observation of intracellular uptake:

Cu ions uptake by cancer cells (MCF-7 and HeLa) from CuONPs@CS was studied by fluorescence microscopy (Dey et al., 2020).

7.2.6. ROS Measurement by microscopic assay and O.D. measurement:

Intracellular ROS generation was estimated using 2,7-dichlorofluorescein diacetate ($DCFH_2$-DA) describe in chapter 4 section 4.2.9.

7.2.7. Pretreatment with ROS scavenging agent (N-acetyl-L-cysteine):

NAC pretreatment was done to measure ROS attribution for cancer cell death according to (Hanley et al., 2009).

7.2.8. Microscopic observation of Apoptosis by AO/EtBr double staining:

The apoptosis or necrosis event was analyzed using AO/EtBr double staining according to method describe in Chapter 4 Section 4.1.5.5.

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7.2.9. Analysis of Cytokines:

CuONPs@CS influenced cytokine production was determined by ELISA describe in Chapter 4 Section 4.1.5.6.

7.2.10. Pro apoptotic and anti-apoptotic markers analysis by ELISA:

Pro-apoptotic (Caspases 3, 8 and p38) and anti-apoptotic markers (pAKT, Bcl2) levels were determined by previously mentioned Chapter 4 Section 4.1.5.7.

7.2.11. Cancer cells immunofluorescent staining by antibodies:

Cancer cells treatment was performed with the CuONPs@CS conjugate at a dose of 50µg/ml for 24hr. Then the cells were washed several times with PBS. Washed cells were put in a paraformaldehyde solution for 15min at 4^{o}C for fixation. Again cells were washed with blocking buffer for three times. The component of blocking buffer is 5% bovine serum albumin in PBS. Subsequently cells were permeabilized in PBS using 0.5% Triton X-100 at 25^{0}C for 20 min. Thereafter cells were incubated in an incubator for 1hr at 25^{0}C with mouse antibodies specific for Caspase-8, cleaved Caspase-3, Phosphorylated-p38.

Dilutions of antibodies were done in blocking buffer at a ratio of 1:100. Cells were then washed with the blocking buffer. Thereafter cells were probed with Rhodamine-conjugated secondary antibodies for 1h at 25^{o}C and kept in the dark. Glass slides mounted with coverslip were viewed using a fluorescence microscope (Carl Ziess microscope; Model Axio scope A1).

7.2.12. Animals:

Maintenance of Balb/c mice was described in previous Chapter 4 Section 4.2.1.

7.2.13. *In vivo* tumor development:

Tumor development was done according to previously mentioned Chapter 5 Section 5.2.1.

7.2.14. Estimation of mean survival time and measurement of increase in life span:

Mean survival time and percentage increase in lifespan was estimated according to method describe in Chapter 5 Section 5.2.2.

7.2.15. Euthanasia of experimental animals:

After the treatment all the mice were euthanized by cervical dislocation which was mentioned previously in chapter 5 section 5.2.3.

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7.2.17. Estimations of Pro and anti-inflammatory cytokines:

Cytokines were estimated by according to the above mentioned method section 7.2.9.

7.2.18. Estimation of Apoptotic markers:

Pro and anti-apoptotic markers were estimated according to above mentioned method section 7.2.10

7.2.19. Protein Estimation:

Protein estimation was performed in accordance with Lowry et al. (1951).

7.2.20. Statistical analysis by Origin 6.1:

It was done by one-way ANOVA (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) which was detail in chapter 4 section 4.2.8.

7.3. RESULTS:

7.3.1. *In vitro* Cytotoxicity of cancer cells:

After the CS coating on green CuONPs, the % of cell death in case of MCF-7 was 9.55, 17.34, 28.11, 48.28, 68.45 and 73.26 and in case of HeLa cell % of cell death was 9.05, 14.08, 25.8, 46.7, 65.33 and 70.19% at concentrations of 1, 5, 10, 25, 50 and 100µg/ml doses respectively. Now the % of cancer cell death was decreased than the previous green CuONPs at same doses but the dose become less toxic which as observed in the previous chapter. So, the higher dose

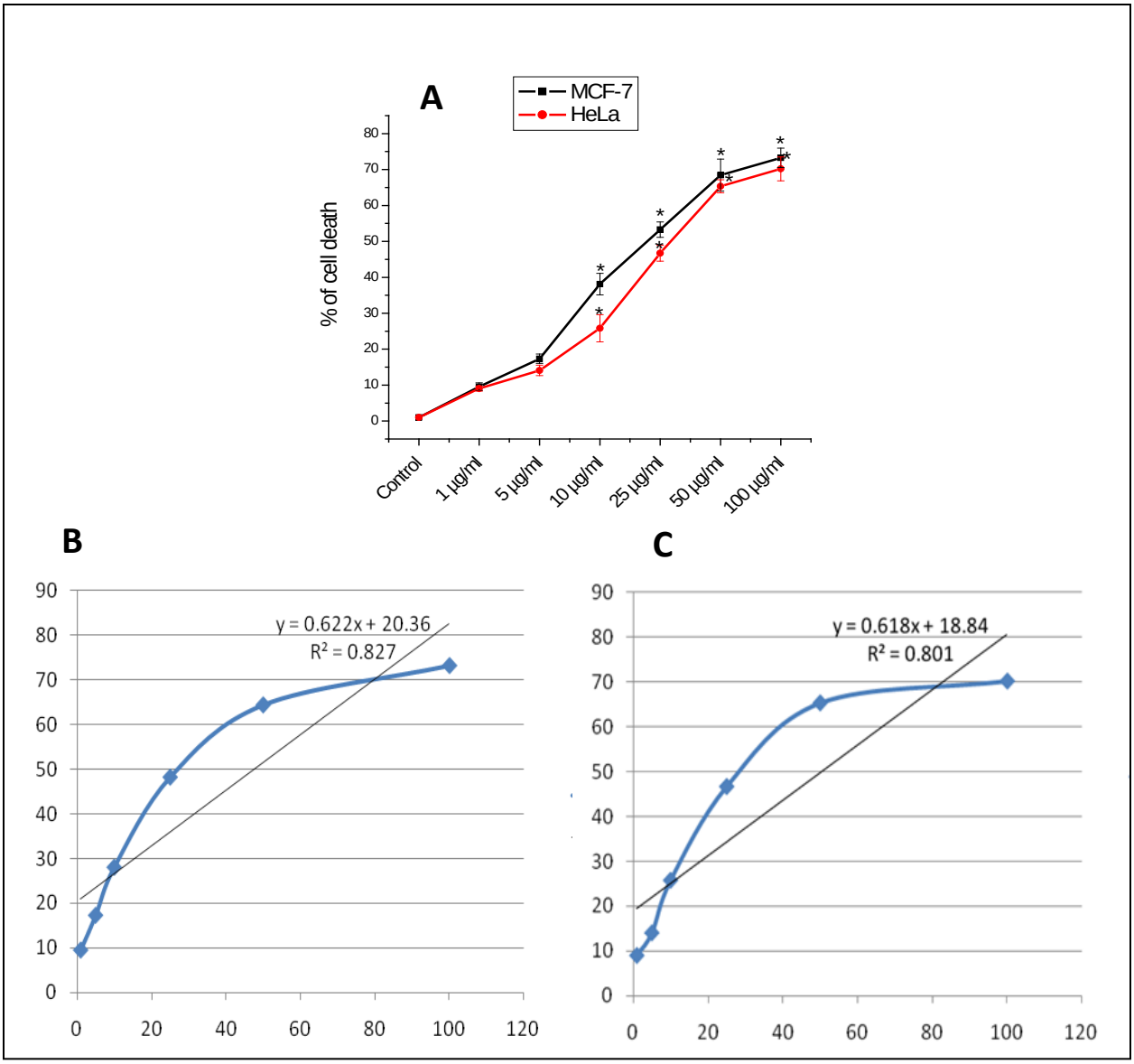

Figure 7.1: **(A)** Cell death of MCF-7 and HeLa cells **B)** IC_{50} of MCF-7 C) IC_{50} of HeLa cells. n=3. Values were expressed as mean±SEM. *Superscripts indicated a significant difference as ($p<0.05$) compared with control.

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(50µg/ml) can be used. Previously the IC_{50} value against MCF-7 and HeLa cell was 24.53 and 25.07µg/ml dose. But now the IC_{50} values became 47.65µg/ml in case of MCF-7 and 50.42µg/ml in case of HeLa cells. IC_{50} value against lymphocytes was 75.10µg/ml dose which indicate that 50µg/ml dose has become totally non-toxic. This selected dose can be used for further study.

7.3.2. Intracellular uptake of Cu ions in MCF-7 and HeLa cells:

Intracellular uptake inside the both cancer cells is the major cause of cancer cell death. After 24hr inside the MCF-7, 0.423pg ion/cells were internalized and in case of HeLa cells, 0.399pg ion/cells were internalized.

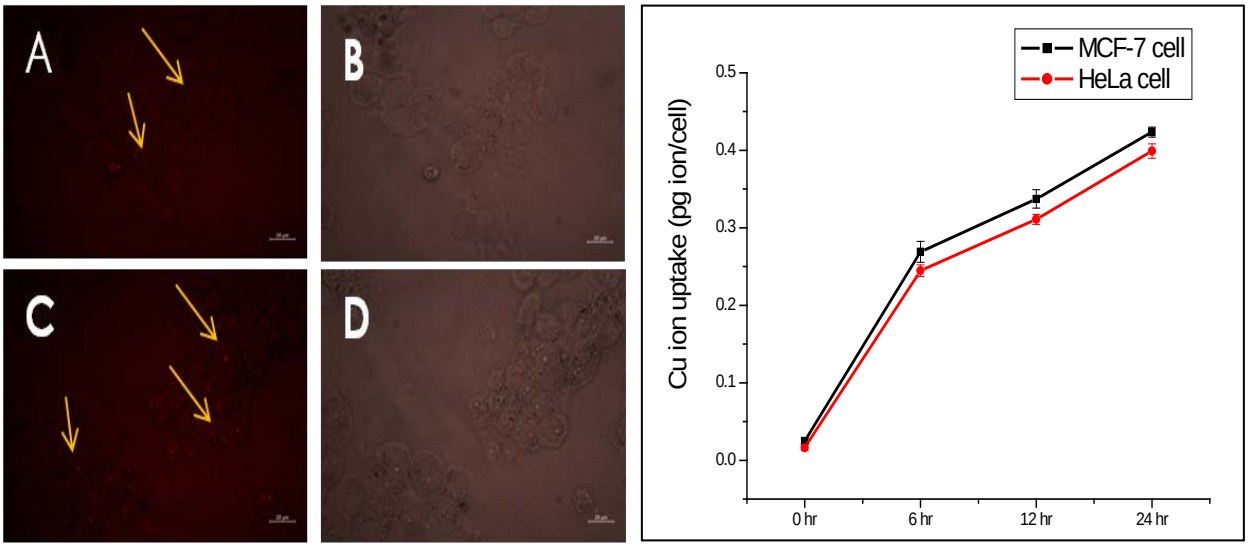

Figure 7.2: Intracellular uptake of Rh-B tagged CuONPs@CS at a dose of 50µg/ml **A)** fluorescence image of uptake of CuONPs@CS inside the MCF-7 cells. **B)** Grey scale image of uptake of CuONPs@CS inside MCF-7 cell. **C)** Fluorescence image of uptake of CuONPs@CS inside the HeLa cells. **D)** Grey scale image of uptake of CuONPs@CS inside HeLa cells. **(E)** Estimation of Cu ions concentration by AAS inside the cancer cell

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7.3.3. Microscopic observation of Apoptosis by AO/EtBr double staining:

Figure 7.3 clearly demonstrates that after the treatment with green CuONPs@CS the cancer cells showed condensed and fragmented chromatin which indicated early and late apoptotic cells. Apoptotic bodies' formation was another characteristic of apoptosis.

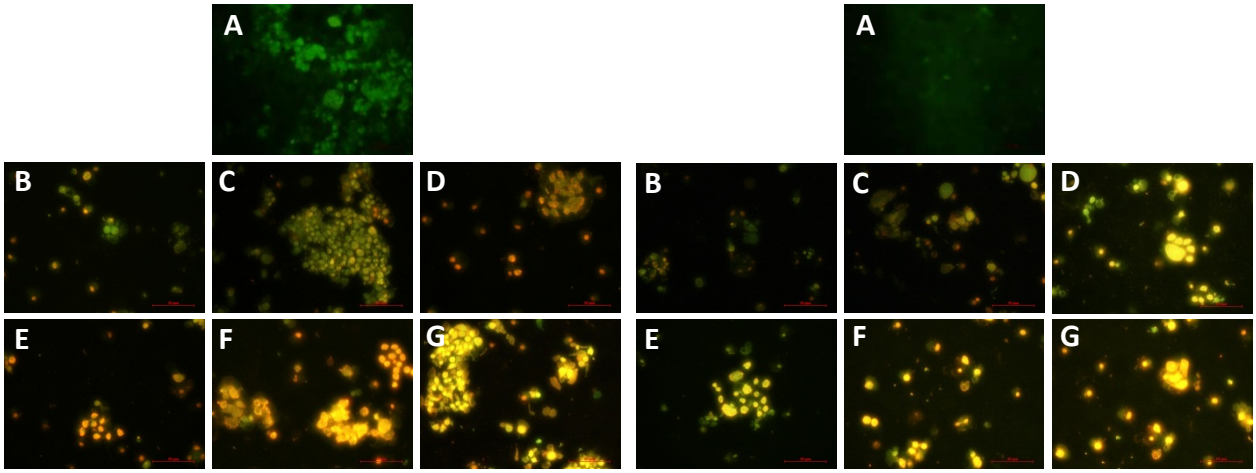

Figure 7.3: Represents the apoptotic phenomenon of cancer cells by Etbr/AO double staining. (Panel I) **A** represents control MCF-7 cells, **B**=1µg/ml dose of CuONPs@CS+ MCF-7, **C**=5µg/ml dose of CuONPs@CS+MCF-7, **D**=10µg/ml dose of CuONPs@CS+MCF-7,**E**=25µg/ml dose of CuONPs@CS+MCF-7,**F**=50 µg/ml dose of CuONPs@CS+MCF-7,**G**=100µg/ml dose of CuONPs@CS+MCF-7. (Panel II) **A** represents Control HeLa cells, **B**=1µg/ml dose of CuONPs@CS+ MCF-7, **C**=5µg/ml dose of CuONPs@CS+MCF-7, **D**=10 µg/ml dose of CuONPs@CS +MCF-7, **E**=25µg/ml dose of CuONPs@CS +MCF-7, **F**=50 µg/ml dose of CuONPs@CS +MCF-7, **G**=100µg/ml dose of CuONPs@CS +MCF-7

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7.3.4. ROS Measurement by microscopic assay and O.D. measurement:

From the image it is observed that a significant amount of ROS generations inside the cancer cells produced. In case of MCF-7 the ROS level was increased significantly compared to the control. In case of HeLa cells the ROS intensity was also increased significantly compared to the control group.

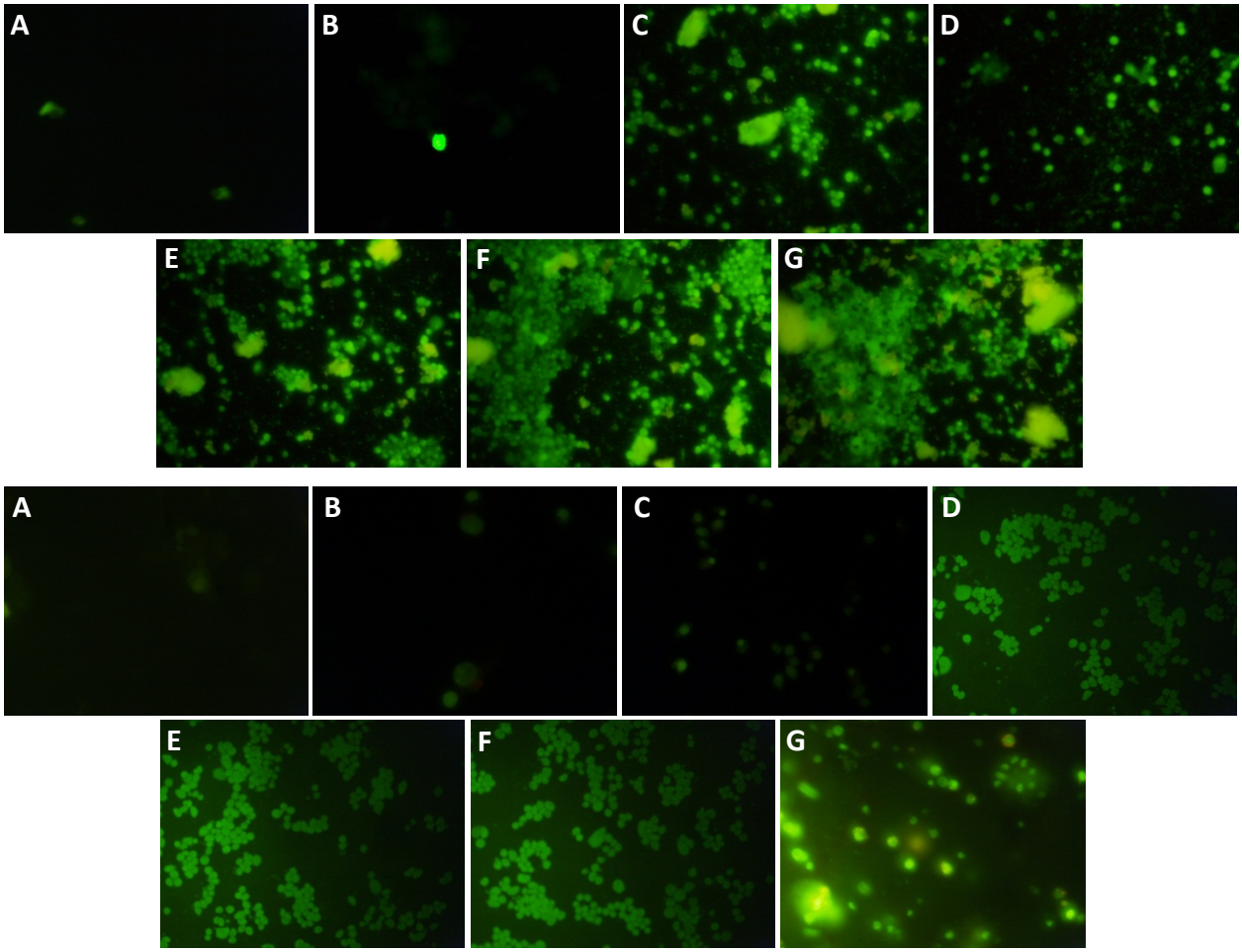

Figure7.4: Cancer cells were visualized under fluorescence microscope by DCHF2DA staining with different doses at 400 X magnification.

7.3.5. Pretreatment with ROS scavenging agent (N-acetyl-L-cysteine):

NAC is a precursor of cysteine that replenishes the intracellular level of GSH. 10mM NAC was used to ensure the effect of CuONPs@CS against cancer cell ROS generation. It was observed that nearly 100% viability was retained which indicate conjugate play the major role in ROS generation inside the cancer cells.

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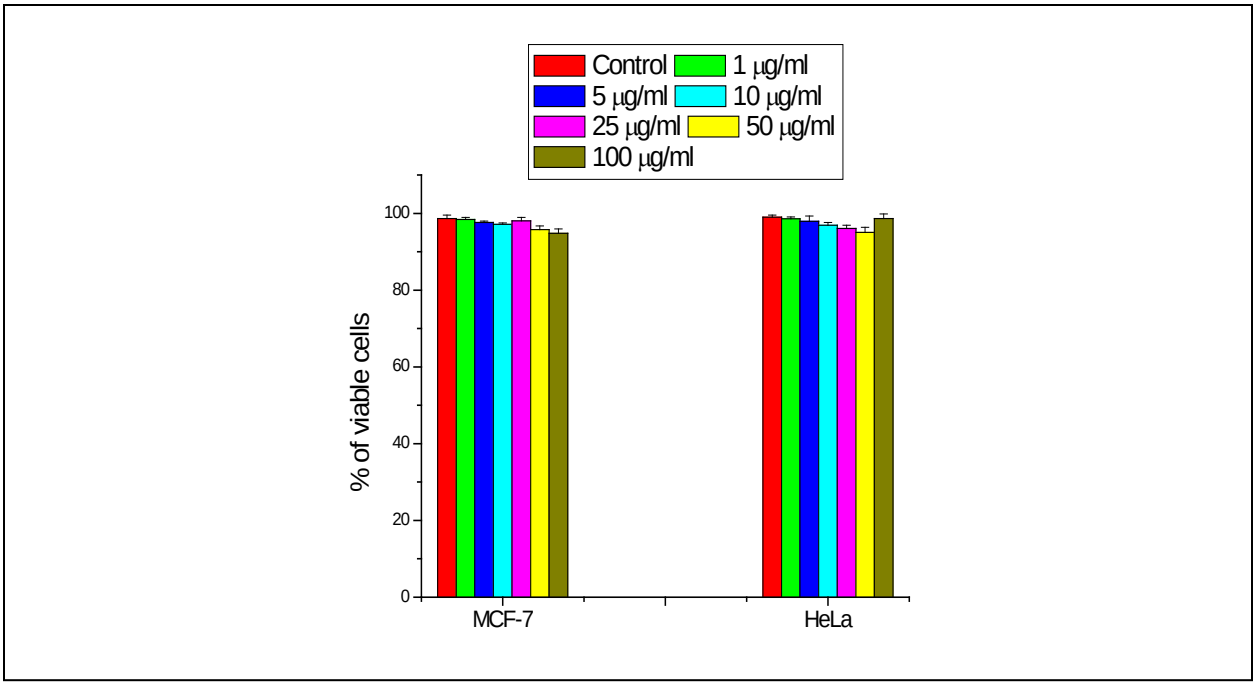

Figure 7.5: NAC (10mM) pretreatment with cancer cell MCF-7 and HeLa. Cell viability of both cancer cells after ROS scavenging agent (NAC) pre-treatment. Results represent the means of three separate experiments, and error bars represent the standard error of the mean.

7.3.6. Analysis of Cytokines:

Pro-inflammatory cytokine level was increased in case of both cell lines. In case of MCF-7 cell line the TNF-α level was increased by 1.43,1.61, 1.63, 1.97, 2.08 and 2.19 fold compared to the

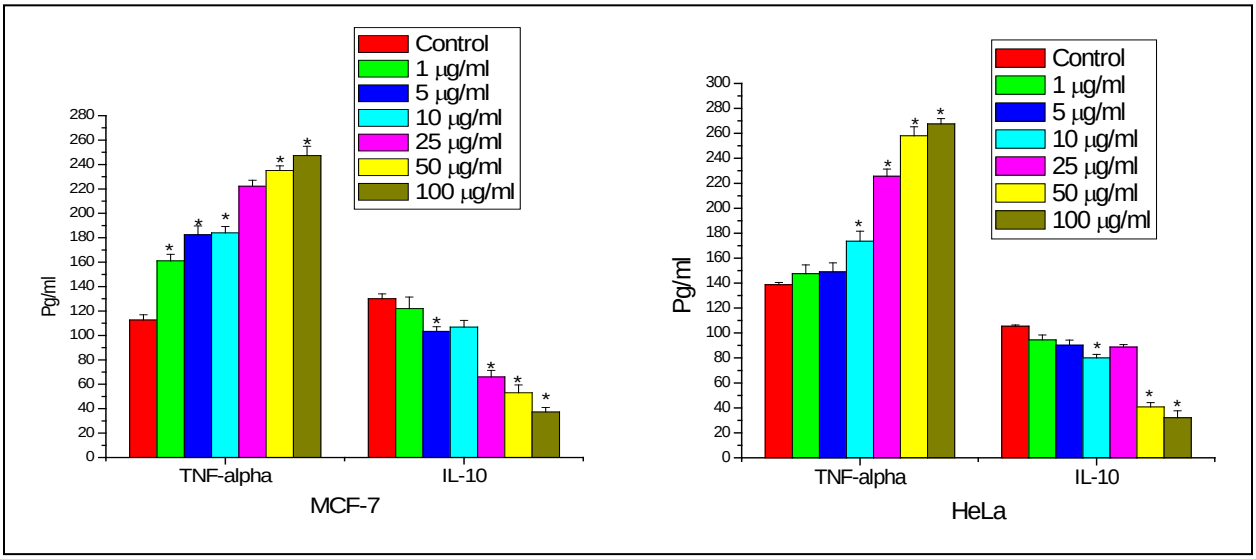

Figure 7.6: (A) Changes of cytokine level in MCF-7 and **B)** HeLa cells. Results represent the means of three separate experiments, and error bars represent the standard error of the mean. *Superscripts indicate significant difference ($P < 0.05$) compared with the control group.

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control group but the anti-inflammatory cytokine IL-10 level decreased by 1.06, 1.25, 1.21, 1.97, 2.45 and 3.48 fold significantly ($P<0.05$) compared to the control group.

In case of HeLa cells the TNF-α level increased by 1.06, 1.07, 1.25, 1.62 and 1.86 fold as well as IL-10 level deceased by 1.11, 1.16, 1.31, 1.18, 2.58 and 3.27 fold. Both the values are significant ($P<0.05$) compared to the control group.

7.3.7. Cytotoxicity using TNF-α blocker:

Pentoxifylline has the ability to suppress monocyte- macrophage derived TNF-α production. This study showed that the pentoxifylline in culture medium was able to increase the MCF-7 cell viability up to 97.50%, 93% and 87.24 % in the presence of CuONPs@CS at 25, 50 and 100µg/mL, respectively. In case of HeLa cells, the viability increased up to 98.81%, 96.05%, 92.43% and 91.78% at (10,25, 50, 100µg/ml) different doses. The viability of cancer cells were increased in the presences of TNF-α inhibitor.

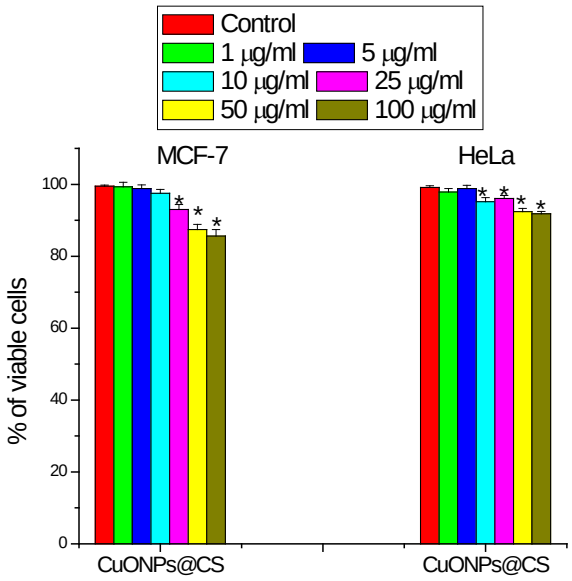

Figure 7.7: Cytotoxicity of CuONPs@CS against the cancer cells after pretreatment with TNF-α inhibitor Pentoxifyline. Values are expressed as mean±SEM of three experiments; *superscripts indicate significant differences ($P<0.05$) compared with the control group.

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7.3.8. Cancer cells Immunofluorescent staining by antibodies:

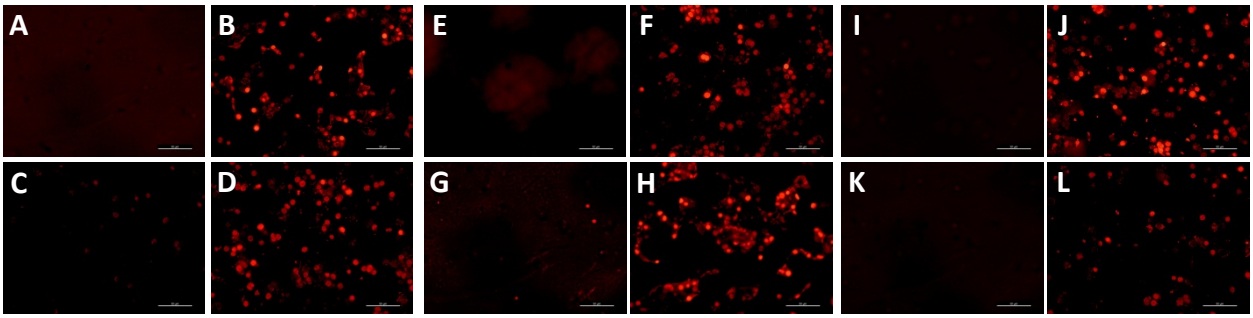

Figure 7.8: Cancer cells treatment with CuONPs@CS for 24hr at a dose of 50µg/ml induced apoptosis that was estimated by expression of Caspase 8, cleaved Caspase 3 and P-p38 proteins by immunofluroscent staining **A)** Control MCF-7, **B)** Treated MCF-7, **C)** Control HeLa, **D)** Treated HeLa immunofluroscent staining of Caspase 8, **E)** & **G)** are Control MCF-7 and HeLa cells, **F) & H)** are Cleaved Caspase 3 expression, **K) & L)** are p-p38 protein and **I) & J)** are control MCF-7 and HeLa cells. Fluorescence intensity was measured by the help of image J software (NIH)

The immune-fluorescence study (Fig. 7.8) indicated the involvement of Caspases 3, 8 and Phosphorylated-p38 (p-p38) in apoptosis of the cancer cells. The fluorescence staining was performed at a dose of 50µg/ml dose. All the protein activation was confirmed compared to the

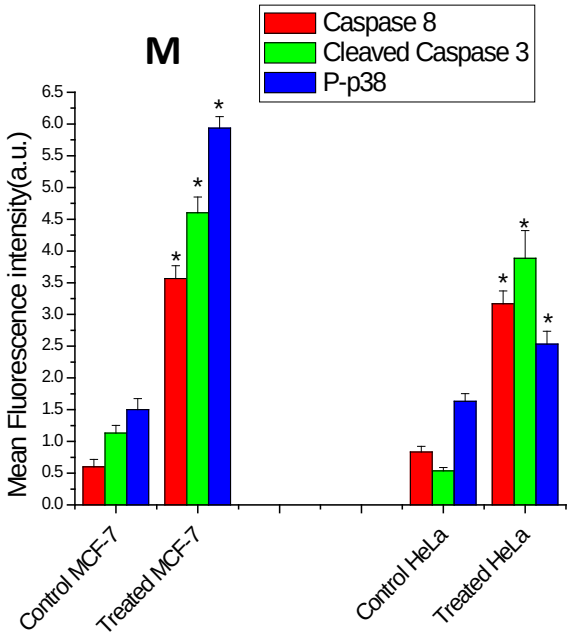

Figure 7.8: (M) Graphical representation of mean fluorescence intensity of pro-apoptotic proteins at a dose of 50 µg/ml of figure 7.8 **(A-L)**. *Superscripts indicate significant differences (P<0.05) compared with the control group.

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control group.

7.3.9. Estimation of Apoptotic markers of MCF-7 cells and HeLa cells:

In case of MCF-7 cells Caspase-8 level increased by 1.06, 1.10, 1.16, 1.48, 1.84, and 2.25 folds. p38 level increased by 1.04, 1.06, 1.37, 1.61, 1.64 and 2.14 folds. Caspase-3 levels increased by 1.04, 1.06, 1.37, 1.61, 1.64 and 2.14 folds. All the pro-apoptotic markers level increased significantly ($P<0.05$) compared to the control group. pAKT level and BCl2 level decreased by 1.14, 1.29, 1.44, 1.63, 2.09, 2.85 and 1.07, 1.38, 1.47,1.58,2.04 , 2.46 respectively.

In case of HeLa cells caspase 8,3 and p38 level increased by 1.42, 1.68, 2.01, 2.24, 2.79, 2.96; 1.22, 1.60, 1.48, 2.05, 2.70, 2.77 and 1.15, 1.38, 1.45, 2.10, 2.61, 2.76 fold respectively compared to the control group. On the other hand pAKT level and BCl2 level decreased by 1.04, 1.15, 1.38, 1.74, 1.97, 2.12 folds and 1.08, 1.17, 1.98, 1.50, 2.18, 4 folds respectively compared to the control. Both the pro and anti-apoptotic values were significant ($P<0.05$) compared to the control group.

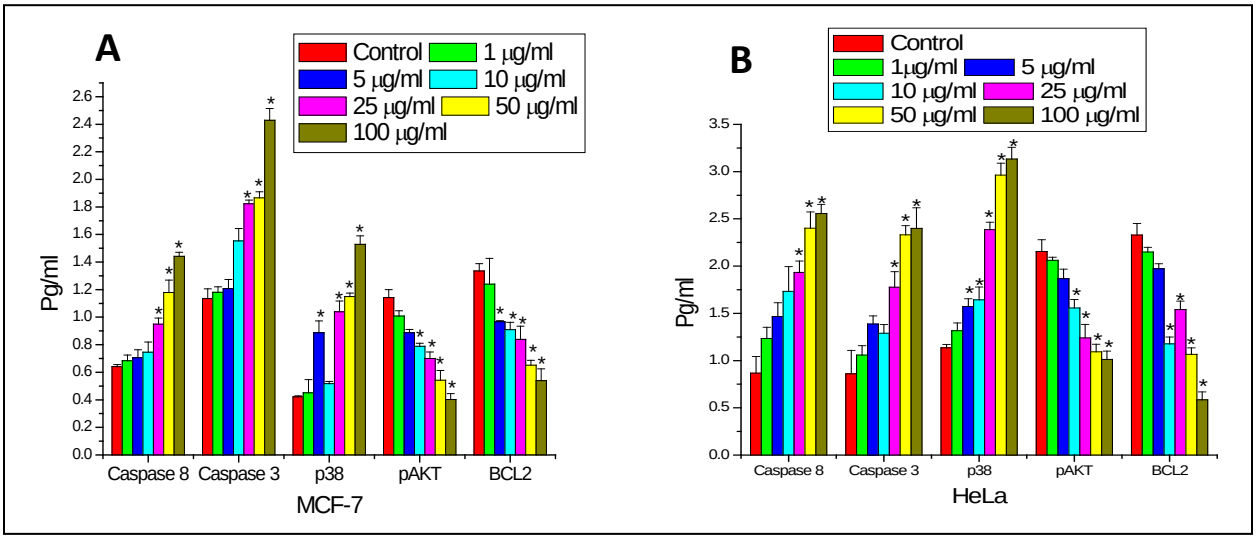

Figure 7.9: Absorbance value of pro and anti-apoptotic proteins markers. Values are expressed as mean±SEM of three experiments; *superscripts indicate significant differences ($P<0.05$) compared with the control group. **A)** Represents the apoptotic proteins expression from MCF-7 cells and **B)** Represents the apoptotic protein expression from HeLa cells.

7.3.10. *In vivo* study:

7.3.10.1. Tumor Weight estimation:

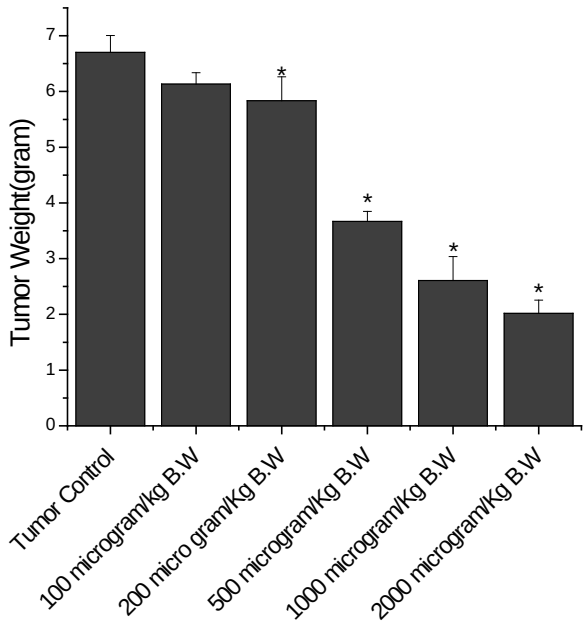

Figure 7.10: Tumor weight of the CuONPs@CS treated group with different doses. n=6; Values are expressed as mean±SEM; *superscripts indicate significant differences (P<0.05) compared with the tumor control group.

The tumor weight decreased from 6.7gm to 3.11gm significantly ($p<0.05$) at a dose of 1000µg/Kg Body Weight. But treatment with 2000µg/Kg body weight dose reduced the tumor weight 6.7gm to 2.02gm which phenomenon carries significant result. Other doses also reduced the tumor weight in a dose dependent manner. The total time period of the treatment with different doses was 30 days at an interval of 3 days.

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7.3.10.2. Survival days of Balb/c mice:

The survival time of the treated Balb/c mice increased up to 39 days from 18 days which was the average survival days of Balb/c tumor bearing mice after the treatment with 1000µg/kg body weight. The conjugate CuONPs@CS helped to increase the life span by reducing the tumor burden. At a dose of 2000µg/kg body weight, Balb/c mice survived for 47 days but were not healthy at all and among the 6 mice, 4 died. Body weight decreased abruptly. Dizziness was observed in the mice and simultaneously hair fall was observed. That mean this dose became toxic most of the mice cant survived.

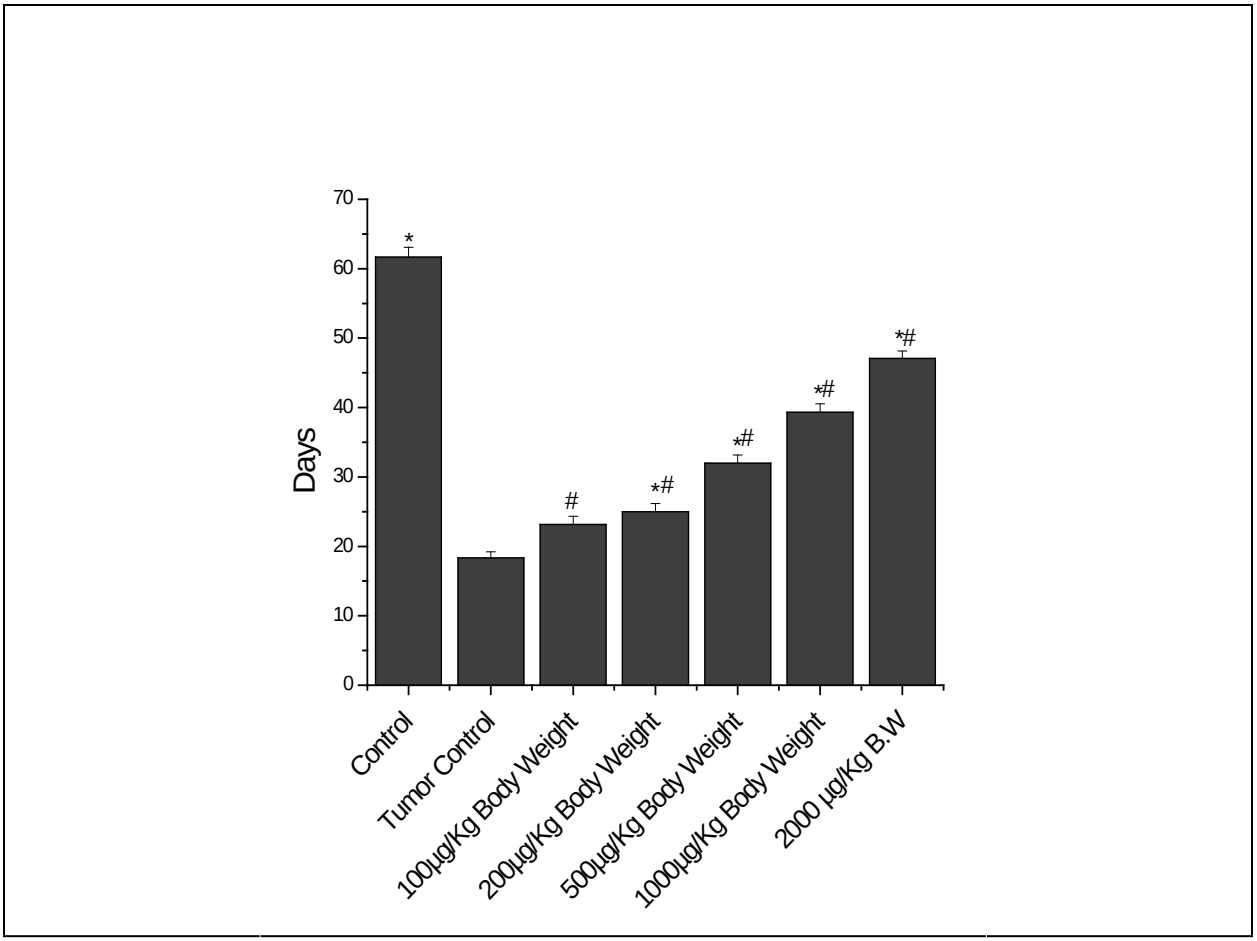

Figure 7.11: Survival days of Balb/c mice. n=6; Values are expressed as mean±SEM; *superscripts indicate significant differences ($p<0.05$) compared with the tumor control group. #superscript indicate significant differences ($p<0.05$) compared with the control group.

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7.3.10.3. Estimation of Cytokine from Tumor homogenate:

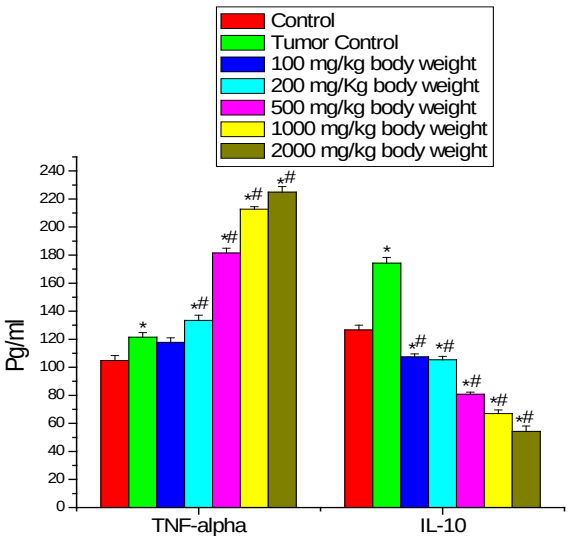

Figure 7.12: Changes of cytokines from Balb/c mice tumor homogenate. Values are expressed as mean±SEMs; *superscripts indicate significant differences (P<0.05) compared with the control group. # Superscripts indicate significant difference compared to the tumor control group. n=6.

As the doses of the conjugate increased as the TNF-α level also gradually increased. TNF-α level increment was observed by 1.12, 1.27, 1.73, 2.02 and 2.14 fold and IL-10 level depleted by 1.17, 1.20, 1.56, 1.88 and 2.32 significantly (P<0.05) compared to the control group. In case of tumor control mice the IL-10 expression level was significantly higher compared to the control. At the same time decrease in IL-10 level was observed compared to the tumor control group.

7.3.10.4. Estimation of apoptotic markers:

After the treatment with CuONPs@CS for 30 days at an interval of 3 days the Caspase 8 level increased by 1.03, 1.16, 1.17, 2.05, 2.22 folds and Caspase 3 level increased by 1.10, 1.69, 1.88, 2.16 and 2.65 folds respectively. The pro-apoptotic protein p38 level increased by 1.03, 1.11, 1.58, 1.98 and 2.07 folds significantly ($P<0.05$) compared to the control group. Anti-apoptotic protein pAKT level depleted by 1.07, 1.08, 2.11, 2.66 and 2.87 simultaneously Bcl2 level decreased by 1.05, 1.41, 1.18, 1.92 and 3.03 fold significantly ($P<0.05$) compared to the control group.

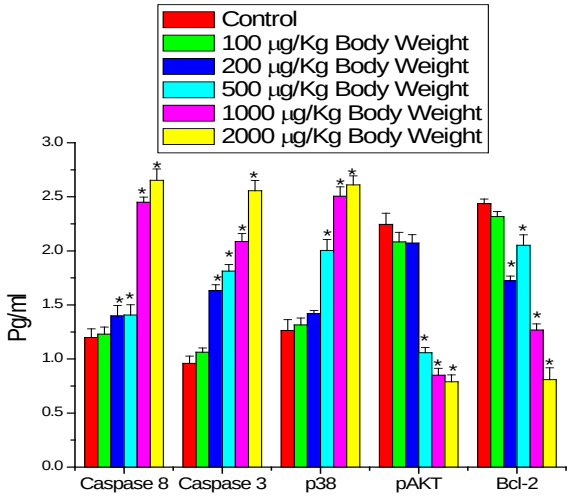

Figure 7.13: Absorbance of pro-apoptotic (Caspase-8, Caspase-3, p38) and anti-apoptotic (pAKT, Bcl2) markers from tumor homogenate after the treatment of solid tumor bearing mice with CuONPs@CS. Values are expressed as mean± SEM; *superscripts indicate significant differences ($P<0.05$) compared with the control group. n=6.

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7.4. DISCUSSION:

In the current study, the physio-chemical properties of surface coated green CuONPs were found to facilitate in modulating the behavior of the NPs in tumor microenvironment across the biological barrier. CS has several advantages such as it is biocompatible, biodegradable and nontoxic by nature. CS having anti-oxidant property easily scavenges the free radical from the cancer cells. CS coated green CuONPs inhibited cancer cell growth in a dose dependent manner. The cancer cell growth was inhibited gradually and significantly ($p<0.05$) as the doses increased. Anti-tumor activity of metal-CS complex interacts with the DNA which causes inhibition of tumor cell growth (Zheng et al., 2006). The previous study also supports the current fact. Intracellular uptake of CuONPs@CS was confirmed by the Rh-B tagged CS@CuONPs internalization as visualized in Fig. 7.2 using fluorescence microscopy. The concentration of Cu ions inside the MCF-7 and HeLa cells were 0.423 and 0.399 pg Cu ion/cell respectively which indicated that leached metal ions from the nano conjugate is responsible for ROS generation inside the cancer cells. The release of free ions from the conjugate influenced the surface oxidation of NPs and provoked several bio-active components, thereby increasing toxicity in the cellular system (Sukhanova et al., 2018). Generally cancer cells membrane consists of a high amount of phospholipid which influences the fixation of Cu ions with the membrane. In this membrane mediated pathway, Cu ions internalizes inside the cancer cells. Cancer cell membranes are leaky in nature which gives an extra advantage for the internalization purpose. Another mechanism of Cu ions internalization is a positive negative interaction between cancer cells and Cu ions. Mostly the cancer cells membrane carries negative charge but the Cu ions from the conjugate are positive in nature. Hence the positive negative interaction helps the Cu ions to invade inside the membrane (Frohlich, E. 2012). Due to double layered coating onto the surface of CuONPs, Cu ions release was reduced significantly. As a result, the half inhibitory concentration of CuONPs@CS against MCF-7 and HeLa cells were 47.65µg/ml and 50.42µg/ml respectively. The pH of cancer cells generally lying between 5-6, influences the release of Cu ions from CuONPs@CS. ROS generation inside the cancer cells was measured using spectrofluorometric analysis (Fig. 7.4). When NPs accumulated inside the cancer cells, it altered the redox status and disrupted the mitochondrial functions which finally led to apoptosis by arresting cell cycle (Nicco and Batteux, 2018). To understand the involvement of nanoconjugate in ROS generation, NAC which is a precursor of GSH molecule was used to treat the cells prior

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to the CuONPs@CS treatment. Almost 100% viability in cancer cells were observed, which indicated that the NAC pretreated cancer cells could decrease cell viability and cell invasion (Schmitt et al., 2015). The strong evidence of apoptosis of both cancer cells were presented in Fig. 7.3. ROS imbalance strategy of cancer cells determines the survival or death of cancer cells. Both receptor and mitochondrial mediated apoptosis of cancer cells depend on ROS (Ozben, T. 2007). TNF-α blocker, pentoxifylline was used to confirm the function of key regulatory protein TNF-α. After the blocking, in case of MCF-7 cells, viability was 85.65% and in the case of HeLa cells, viability was 91.49%. This phenomenon indicated that in cancer cell apoptosis, TNF-α plays a major role (Pastor et al., 2010). Pro and anti-inflammatory cytokines levels estimated, were presented in Fig.7.6. The pro-inflammatory cytokines level increased significantly in a dose dependent manner and simultaneously the anti-inflammatory cytokines level decreased significantly ($P<0.05$) compared to the control group. The increment of Caspase 3, Caspase 8 and p38 proteins observed from Fig. 7.8 indicated the culminate Caspase activation and influenced mitochondrial mediated apoptosis. A similar result was found by Wang et al., 2008. When CuONPs passed through the membrane, they released Cu ions which helped to produce H_2O_2 on the cell membrane (VanWinkle et al., 2009).

In case of *in vivo* study, the cytotoxicity against solid tumor in Balb/c mice model was observed for 30 days treatment at a 3 days interval. Tumor weight reduced up to 2000µg/Kg body weight significantly ($P<0.05$). As the tumor weight reduced, the survivability days also increased. At a dose of 1000µg/Kg body weight, no significant toxicity was experienced, but at a dose of 2000µg/Kg body weight dizziness and significant hair loss was observed. From the cytokines analysis, a significantly increased level of TNF-α and decreased level of IL-10 compared to the control group were observed.

In conclusion, it can be said that CuONPs@CS can be used as a smart nano sized tool which neutralizes the cancer cells from the *in vitro* and *in vivo* system with minimal toxicity. Hence it can be used as a smart and effective drug in cancer therapy. CuONPs@CS influenced the ROS generation, which ultimately triggered the apoptosis in cancer cells. An interesting property of CS enables it to release the Cu ions from the nano conjugate in acidic environment of MCF-7 and HeLa cancer cells. In *in vivo* study, CuONPs@CS reduced the solid tumor weight in a dose dependent manner up to 2000µg/Kg Body Weight. However in the previous chapter, it was

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confirmed that 1000µg/Kg Body Weight dose is safe for *in vivo* model. Hence 1000µg/Kg Body Weight was selected as a safe dose and significant anticancer activity was observed at a 1000µg/Kg Body Weight dose. Further study has been done with 50µg/ml dose for *in vitro* study and 1000µg/Kg Body Weight dose for *in vivo* study.

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