Chapter 6

Preparation, Characterization and, Toxicity of Chitosan coated green Copper Oxide nanoparticles towards normal cells

INTRODUCTION:

It was observed in the previous chapter that green synthesized CuONPs was less toxic than chemical CuONPs. Green synthesized CuONPS had IC_{50} of 42.46µg/ml on lymphocytes compared to chemical CuONPS that had IC_{50} of 26.48µg/ml on same cells indicating that the green synthesized CuONPS was less toxic at these concentrations. At a dose of 25µg/ml, green CuONPs killed 47% lymphocyte which was also very significant. To reduce the toxicity of green CuONPs and to overcome its limitations for application in cancer therapy Chitosan polymer was used.

Copper oxide NPs with other metal oxide NPs are responsible for severe toxicity in the system (Yang et al., 2010). Generally, the surface of Copper NPs easily oxidizes which is the main reason for ROS generation (Shi et al., 2012; Pettibone et al., 2008). From the previous chapter, a high level of ROS generation due to the internalization of Cu ions inside lymphocytes was also observed. The coating of metallic nanoparticles with chitosan can improve stability, improving size distributions and increasing biocompatibility of the NPs (Dias et al., 2011).

Chitosan is a deacetylated cationic polymer with no cytotoxic effect. Hence in this present chapter, the green CuONPs was coated with Chitosan to reduce its toxicity. Chitosan has an interesting property. It can easily dissolve in an acidic environment, keeping that in mind pH responsive role of chitosan can easily protect the normal lymphocytes and can specifically attack the cancer cells. Chitosan being positively charged can easily bind to negatively charged cell membranes. It helps to degrade the cancer cells by binding with the plasma membrane, redistributes F-actin, fixes with occludin, and depleted the trans-epithelial electrical resistance (TEER) of cells in so doing increasing transcellular and paracellular diffusion of the bound drugs (Artursson et al., 1994; Dodane et al., 1999; Schipper et al., 1996; Schipper et al., 1997; Thanou et al., 2001).

Chitosan can penetrate the tight junction of the epithelial layer and facilitates the paracellular and transcellular drug transport (Mohammed et al., 2017). The toxicity of metal oxides is the major concern for their application. Surface coating with biopolymer increased their bioavailability, prolonged release of Cu ions, easily biodegradable and makes a platform for the conjugation with other effective functional groups that can improve the drug delivery to the targeted site.

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

Therefore in the present study, multi-layered surface coated CuONPs was synthesized to overcome the toxic effect of green CuONPs and the targeting delivery of NPs to the specific site was simultaneously improved.

6.1. MATERIALS AND METHODS:

6.1.1. Chemicals and Reagents:

Chitosan, 3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium bromide (MTT reagent), Rhodamine-B (RhB) were purchased from Sigma Aldrich. TNF-α and IL-10 ELISA Kit, and Caspase 3, 8, p38, pAKT antibodies were obtained from eBiosciences. DMEM, fetal bovine serum (FBS), penicillin, streptomycin, sodium carbonate (Na_2CO_3), sodium chloride (NaCl) and ethylene diamine tetra acetate (EDTA) were obtained from Himedia, India. Tris buffer, KH_2PO_4, K_2HPO_4, alcohol and other chemicals were acquired from Merck Ltd., and SRL Pvt. Ltd., Mumbai, India. Commercially available dimethyl sulfoxides (DMSO) were procured from Himedia. All other chemicals were from Merck Ltd., SRL Pvt. Ltd.

6.2. Methodology:

6.2.1. Cell culture:

Cell culture was performed by the previously mentioned method in Chapter 5 Section 5.1.1.

6.2.2. Preparation of Chitosan Coated green synthesized Copper Oxide Nanoparticles:

Green synthesized CuONPs were prepared according to Dey et al., 2019. A homogeneous 50ml Chitosan (CS) solution was made with the help of 1% acetic acid. The entire mixture was stirred overnight at 60^{o}C. Then 50mg of green CuONPs were mixed and stirred for overnight and then centrifuged. Then conjugated particles (*green CuONPs@CS) were collected by filtration technique. The surfaces of the newly formed particles were washed with acetic acid several times. Desiccator was used to dry the particles (Dey et al., 2020).

6.2.3. Physical Characterizations:

6.2.3.1. FT-IR:

The method of FT-IR was previously mentioned in Chapter 3 Section 3.1.4.1.

6.2.3.2. SEM:

The method of SEM was previously mentioned in Chapter 3 Section 3.1.4.4.

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6.2.3.3. XRD:

The method of XRD was previously mentioned in Chapter 3 Section 3.1.4.3.

6.2.3.4. EDX:

The method of EDX was previously mentioned in Chapter 3 Section 3.1.4.5.

6.2.3.5. DLS and Surface Zeta potential:

The method of DLS and Surface Zeta potential was previously mentioned in Chapter 3 Section 3.1.4.2

6.2.4. Isolation and preparation of lymphocytes:

The isolation and preparation of lymphocytes for lymphocyte toxicity experiments was performed according to Chattopadhyay et al., 2013. The total procedure was mentioned in Chapter 4 section 4.2.2

6.2.5. Preparation of Surface coated NPs as a drug:

Several doses of CuONPs@CS (1-100µg/ml) were used as a drug in all experiments. The CuONPs@CS suspension was prepared by phosphate buffer saline (pH 7.4).

6.2.6. MTT assay against lymphocytes:

The viability of lymphocytes against CuONPs@CS were estimated by 3-(4,5-dimethylthiazol) -2-diphenyltetrazolium bromide (MTT) assay was described in Chapter 4 section 4.1.5.1.

6.2.7. Estimation of Intracellular uptake of Cu ions per cells (lymphocytes):

Estimation of Cu ions uptake by normal cells was described in Chapter 4 Section 4.1.5.2.

6.2.8. ROS intensity measurement and observation of lymphocytes under microscope after stained with $DCFH_2$-DA:

Intracellular ROS generation was measured using 2,7-dichlorofluorescein diacetate ($DCFH_2$-DA) staining assay under microscope was described in Chapter 4 Section 4.1.5.4.

6.2.9. Apoptosis in lymphocytes:

This procedure was performed according to Chapter 4 Section 4.1.5.5.

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6.2.10. Estimation of Cytokines from cell lysate (lymphocytes):

TNF-α and IL-10 cytokines level was estimated by ELISA method in accord with Chapter 4 Section 4.1.5.6.

6.2.11. Measurement of pro apoptotic and anti-apoptotic proteins by ELISA method:

The pro-apoptotic markers Caspase 3 and 8, p38, p53 and anti-apoptotic markers BCL-2, pAKT levels were assessed using an ELISA were described in Chapter 4 Section 4.1.5.7.

6.3. Animals:

Animals were maintained for the study according to the previously mentioned method in Chapter 4 section 4.2.1. Permission for animal ethics is already provided in chapter 4 in 4.2.1 section.

6.3.1. Collection of mice serum and tissue after *in vivo* i.p. treatment:

Following one week of maintenance, the mice were randomly divided into 6 groups with each group containing 6 mice (five experimental groups and one control). Surface coated CuONPs in PBS was injected by intraperitoneal injection with several doses (100, 200, 500, 1000µg/Kg body and 2000µg/Kg Body Weight). There were five groups of different doses and one group was control. After the treatment period animals were sacrificed and serum and tissues were collected.

6.3.2. Body weight and Organ weight estimation of Balb/c mice:

CuONPs@CS was injected by three days interval for 15 days and 30 days in Balb/c mice. After the treatment for 15 days there was no significant toxicity in body weight. After 30 days 2000µg/Kg body weight dose showed significant decreased in body weight and as well as organ toxicity. But 1000µg/Kg body showed no significant toxicity for 30 days. So, 1000µg/Kg Body Weight dose has been selected for *in vivo* experiment for 30 days at an interval of 3 days.

6.3.3. Biochemical toxicity markers level from mice serum:

Serum LDH, SGOT and creatinine levels were measured by kit method (Span diagnostic, Mumbai, India) after the isolation of serum from the whole blood.

6.3.4. Cell viability assay against lymphocytes:

The cell viability was estimated by according to the above mentioned method in Chapter 4 Section 4.1.5.1.

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6.3.5. Cytokines Release estimation:

Cytokines were estimated by according to the above mentioned method in Chapter 4 Section. 4.1.5.6.

6.3.6. Estimation Apoptotic markers by ELISA method from mice serum:

Apoptotic markers were assessed by according to previous mentioned method in Chapter 4 Section 4.1.5.7.

6.3.7. Estimation of protein by Lowry method:

Total protein was estimated according to Lowry et al., 1951.

6.3.8. Statistical analysis by Origin 6.1:

Entire data were represented as mean±SEM. Means of two groups were analogized using two-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) with several comparison t-tests. $p<0.05$ was taken as a limit of significance.

6.4. RESULTS:

6.4.1. FT-IR study of the new conjugate (CuONPs@CS):

Generally the peak near about 1601cm^{-1} assign to the NH2 group and the peak near about 1150.2cm^{-1} assign to the peak of C-N stretching of chitosan molecule. These characteristics peak were observed in this conjugate (CuONPs@CS) which was not observed in only CuONPs. So, the conjugation was confirmed.

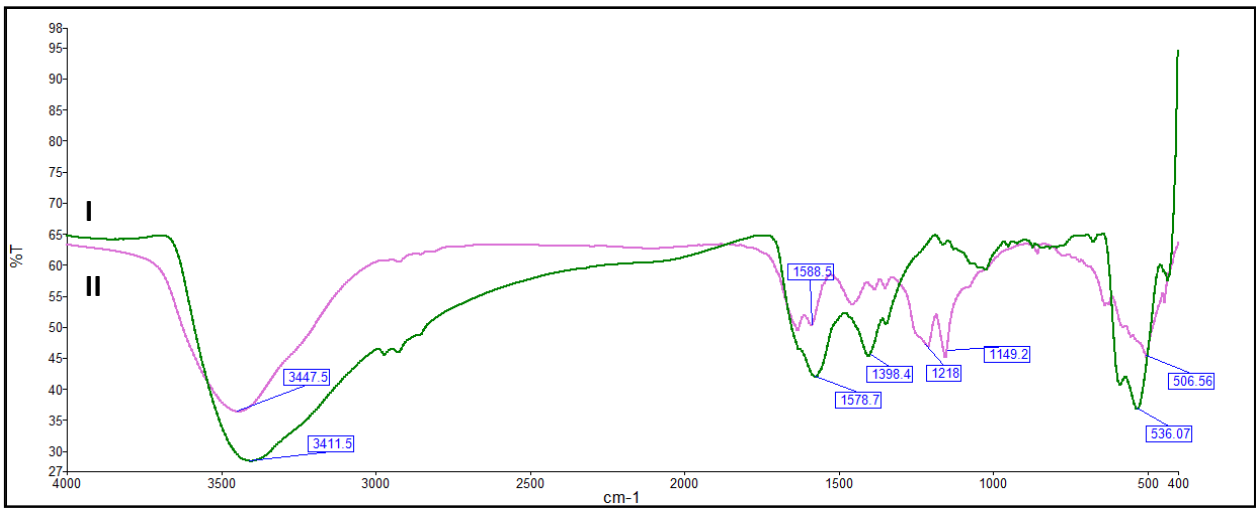

Figure 6.1: FT-IR spectroscopy of **I)** CuONPs@CS (Pink line) and **II)** green synthesized CuONPs (green line)

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6.4.2. XRD study of Conjugate:

The spectrum showed the peak in the range of 21.8°, 25.9°, 32.6°, 45.0°,52.6°, 56.7°, 58.1°, 65.8° and 70.5° which corresponds to the (021), (021), (110), (111), (020), (202), (113), (202), (111) planes respectively. The XRD pattern of CS coated green CuONPs presented the cubic crystalline structure. The corresponding planes obtained were in good agreement with the JCPDS File number (JCPDS NO: 05-0061).

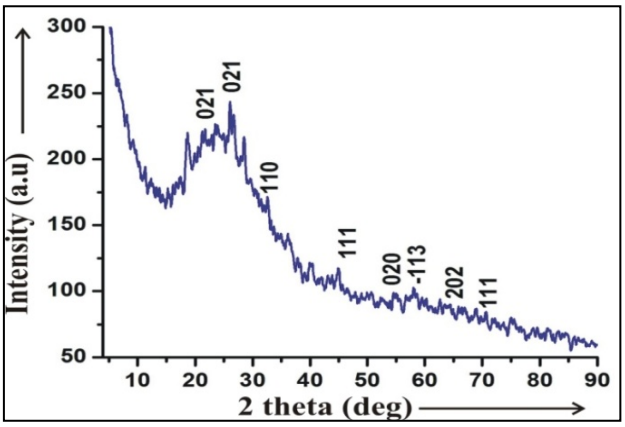

Figure 6.2: XRD pattern of CuONPs@CS.

6.4.3. DLS and Zeta study:

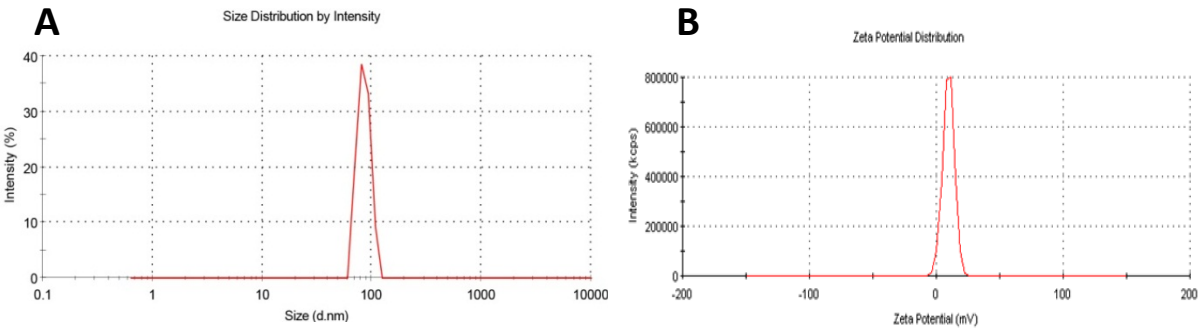

Figure 6.3: A) Hydrodynamic size measurement of CuONPs@CS by DLS **B)** Surface zeta potential of CuONPs@CS

From the DLS study it was observed that the average hydrodynamic size of the conjugate was 81.2nm and the positive surface zeta potential (9.58mV) value of CuONPs@CS indicated the successful coating of CS.

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6.4.4. SEM study of the conjugate (CuONPs@CS):

SEM image revealed the surface morphology and average size of the surface coated NPs. From the image it was observed that after the CS coating the surface of the NPs became very smooth and properly spherical shape in nature. The average size of the double layered NPs was 62±3nm.

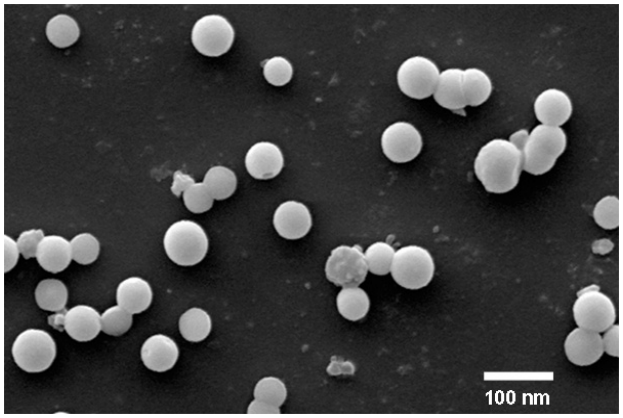

Figure 6.4: Scanning electron Microscopic image of CuONPs@CS.

6.4.5. TGA study of CuONPs@CS:

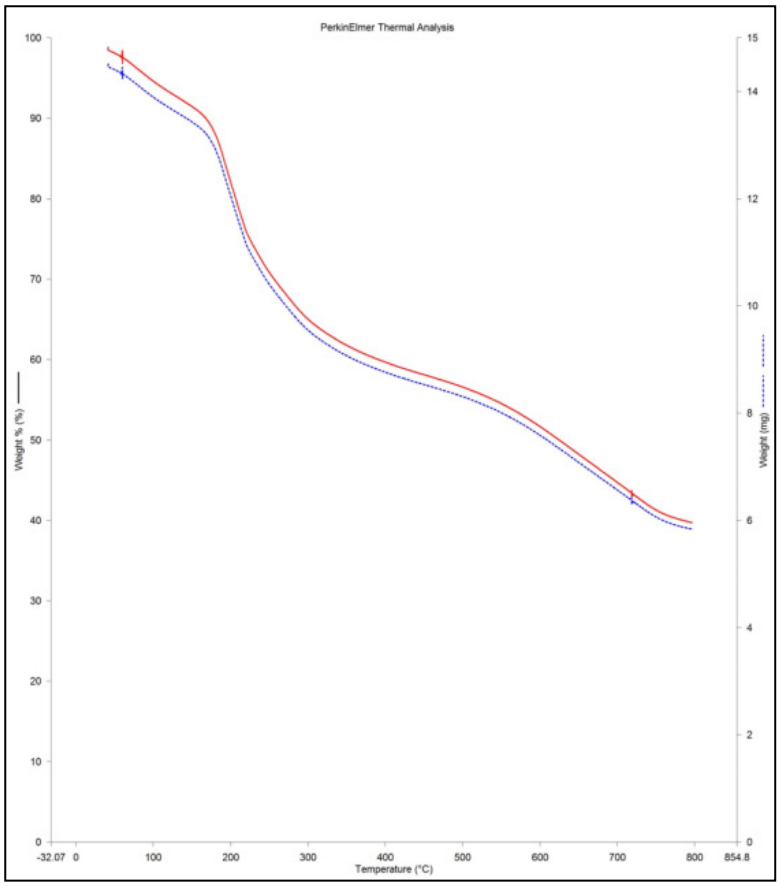

Figure 6.5: Thermogravimetric study of CuONPs@CS

From the thermogravimetric experimental analysis a weight loss was observed occurring near 400^{o}C temperature indicate the decomposition of organic compounds. These organic compounds were present due to surface coating of green CuONPs.

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6.4.6. TEM study of chitosan coated green synthesized CuONPs:

Actual mean diameter of the particle was 34.5±5nm.

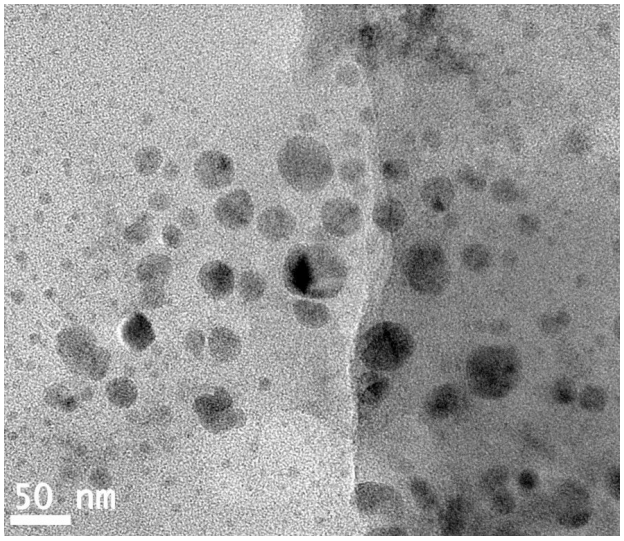

Figure 6.6: Size measurement by TEM analysis

6.4.7. Cytotoxicity assay by MTT against lymphocytes:

Cytotoxicity assay revealed the % of lymphocytes death after the surface coating with CS and green component of *A. indica* leaves on to the surface of CuONPs. It was observed that the cell death increased gradually in a dose dependent manner but significantly increased at a dose of

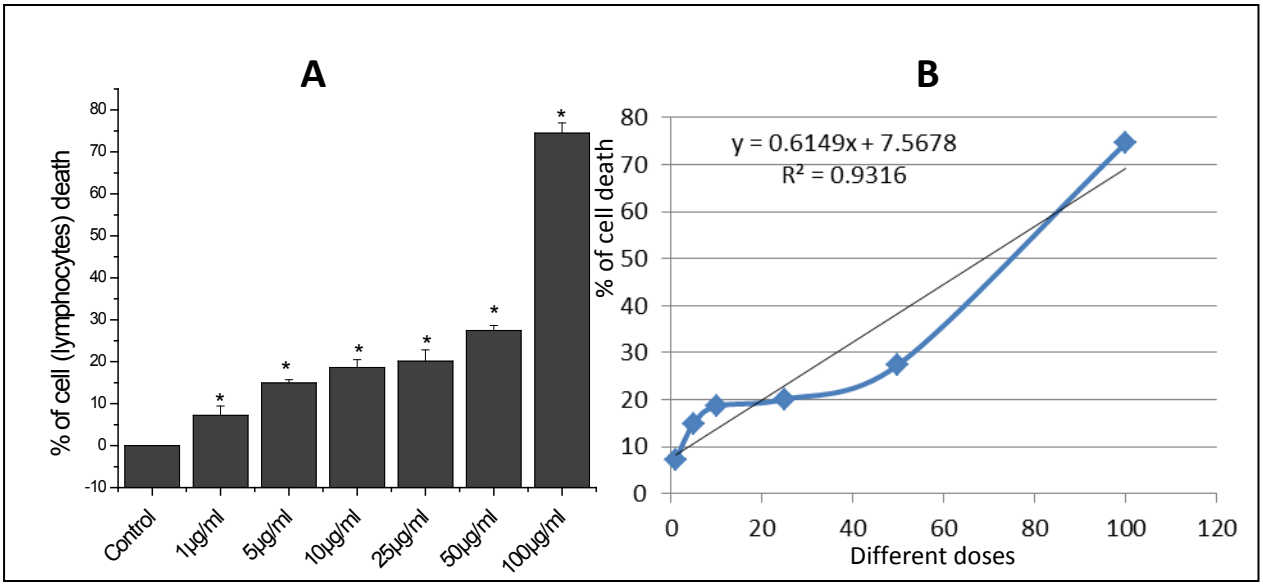

Figure 6.7: (A) Dose and duration dependent percentage of Lymphocytes death by CuONPs@CS was estimated by MTT assay **(B)** IC_{50} of CuONPs@CS against lymphocytes. Values were expressed as mean±SEM. *Superscripts indicated a significant difference as ($P<0.05$) compared with control. n=3.

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100µg/ml dose. From the dose 1 to 100µg/ml dose CuONPs@CS killed the lymphocytes by 7.25%, 14.92%, 18.65%, 20.14%, 27.45% and 74.49%. Before the surface coating CuONPs killed 47% lymphocytes at a dose of 25µg/ml which was the IC_{50} value against cancer cells. So, this dose cannot be used for anti-cancer therapy. At this dose the lymphocytes killing activity was significantly ($p<0.05$) high compared to the control. But after the surface coating the IC_{50} value became 69.12µg/ml dose (Fig. 6.8B). The 25µg/ml and 50µg/ml dose become nontoxic.

6.4.8. Estimation Intracellular of Cu ions uptake per cells (lymphocytes):

After 24hr Cu ion inside the lymphocytes were measured by AAS. 0.203pg ion/cell was estimated by AAS. This amount of Cu ion at a dose of 50µg/ml can be considered as a safe for further use.

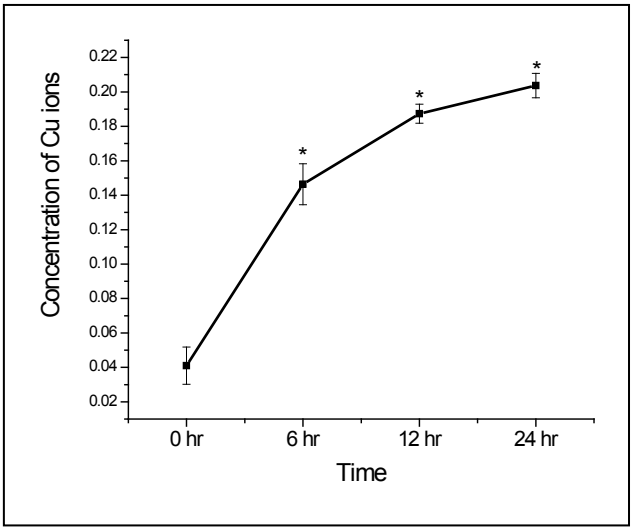

Figure 6.8: Estimation of Cu ions concentration from CuONPs@CS

6.4.9. ROS intensity measurement and observation of lymphocytes under microscope after stained with $DCFH_2$-DA:

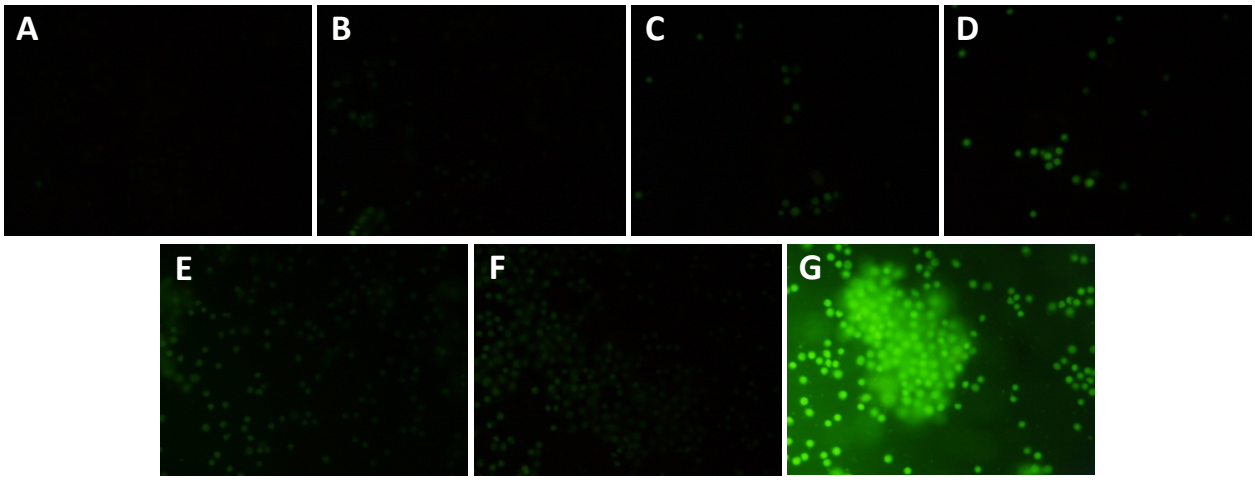

Figure 6.9: (A-G) Effects of CuONPs@CS on lymphocytes were visualized under fluorescence microscope by DCHF2DA staining with different doses.

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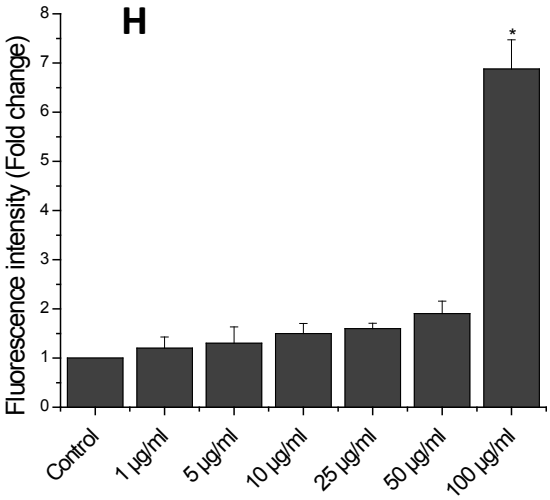
ROS generations inside the lymphocytes were measured by fluoroscensence dye DCHF2DA staining. After the treatment with CuONPs@CS for 24hr no such significant amount of ROS generation was observed up to 50µg/ml dose. But after that 100µg/ml dose CuONPs@CS created severe toxicity which was visualized through fluorescence microscopy and by the spectrophotometer. Intensity of control set as 1 unit.

Figure 6.9: (H) Fluorescence intensity of DCF measured at 485nm excitation and 520nm emission using a spectrofluorometer. Intensity of control cells was set to 1.00. Data is represented as the fold change of the ROS level in the control group. Values are expressed as mean±SEM of three experiments; *superscripts indicate significant difference (P<0.05) compared with the control group.



6.4.10. Estimation of Cytokines from cell lysate (lymphocytes):

Pro-inflammatory cytokines level of lymphocytes indicates the inflammation inside the system. After the treatment with CuONPs@CS the pro-inflammatory cytokines (IL-12, TNF-α) level increased significantly at a dose of 100µg/ml. Before that upto 50µg/ml dose the pro-inflammatory cytokines level was not significantly higher compared to the control group. Similarly the anti-inflammatory cytokines (IL-10, TGF-β) level was significantly depleted at a dose of 100µg/ml which may be due to the toxic effect of CuONPs@CS (Fig. 6.11C, D). This incident indicates the toxic effect of CuONPs@CS at a dose of 100µg/ml but upto 50µg/ml dose it was non-toxic towards the lymphocytes.

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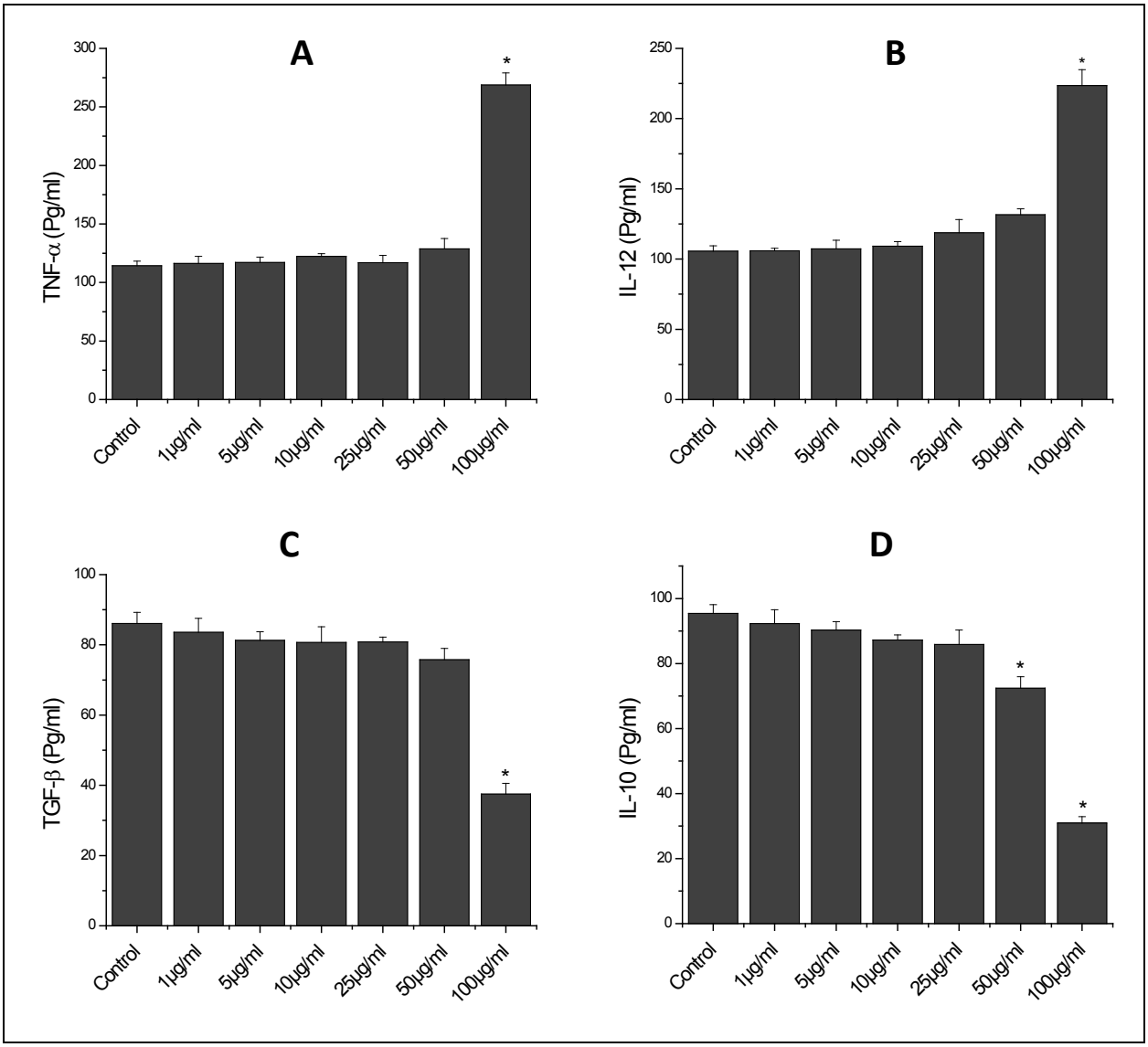

Figure 6.10: Pro and anti-inflammatory level assesment of CuONPs@CS on lymphocytes after 24hr treatment (TNF-α, IL-10). **(A)** TNF-α **(B)** IL-12 **(C)** TGF-β and **(D)** IL-10. Values were expressed as mean ± SEM. *Superscript indicates significant difference compared to the control group. n=3.

6.4.11. Apoptosis in lymphocytes:

Apoptotic phenomenon of lymphocytes is a marker of toxicity. CuONPs@CS treated lymphocytes with several doses from 1-100µg/ml dose were applied on lymphocytes for 24hr. After the treatment schedule the lymphocytes up to 50µg/ml dose showed no significant amount of apoptosis inside lymphocytes but at a dose of 100µg/ml dose it created significant apoptosis against lymphocytes.

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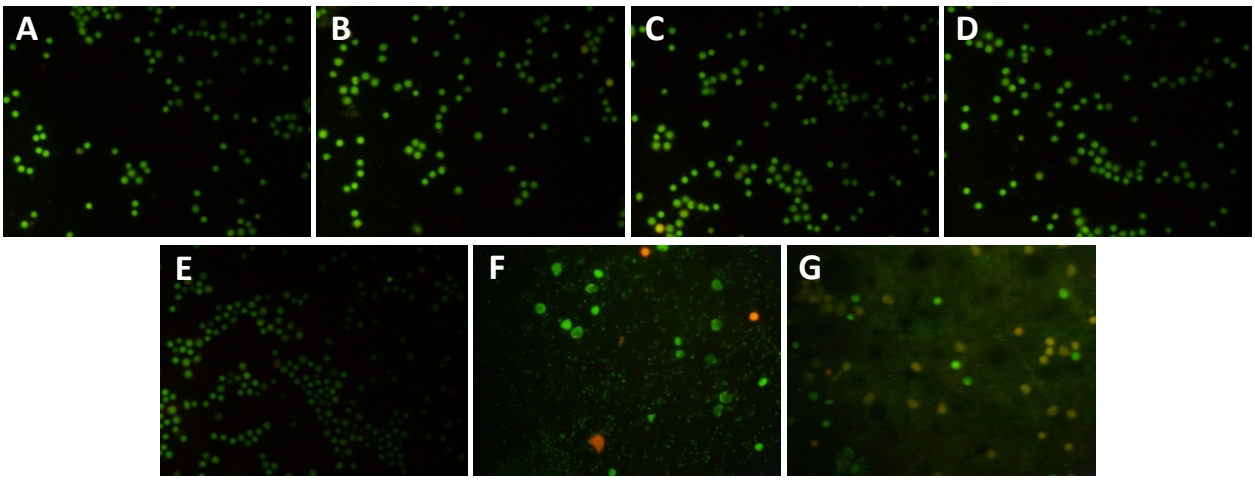

Figure 6.11: CuONPs@CS on lymphocytes was visualized under fluorescence microscope by Etbr/AO double staining with different doses with original magnification of 400X**.**

6.4.12. Measurement of proapoptotic and antiapoptotic proteins by ELISA method:

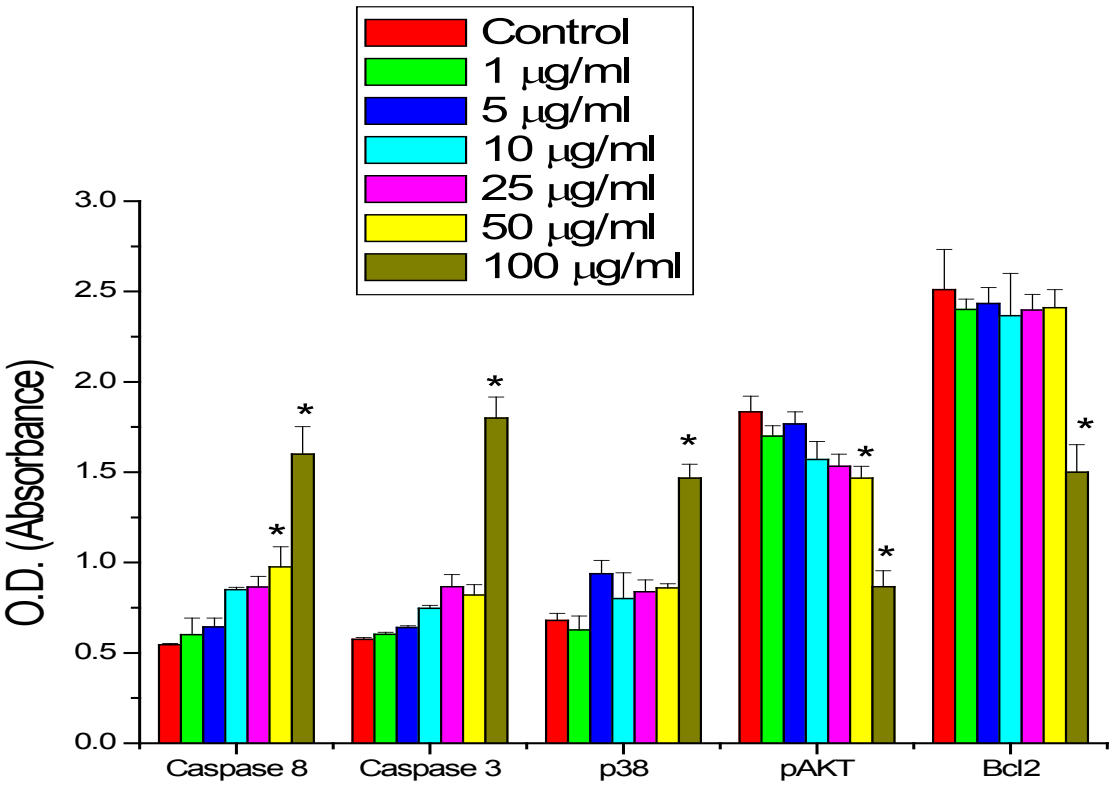

Figure 6.12: Data of pro-apoptotic (Caspase-8, Caspase-3, p38) and anti-apoptotic (pAKT, Bcl2) protein level after treatment of CuONPs@CS. Values are expressed as mean±SEM of three experiments; *superscripts indicate significant differences ($P<0.05$) compared with the control group.

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As the doses increases the pro-apoptotic marker level increased and anti-apoptotic level decreased compared to the control group. At a dose of 100µg/ml caspase 3, 8 and p38 level increased by 3.15, 2.63 and 2.14 fold respectively compared to the control group (Fig. 6.13). Among the all pro-inflammatory apoptotic markers the caspase 3 increment level was highest compared to caspase 8 and p38. The anti-apoptotic markers pAKT and Bcl2 level decreased by 2.09 and 1.67 fold respectively compared to the control group. But at low doses no remarkable change was observed.

6.4.13. *In vivo* study:

6.4.13.1. Estimation of Body Weight and organ weight in Balb/c mice after the treatment with CuONPs@CS:

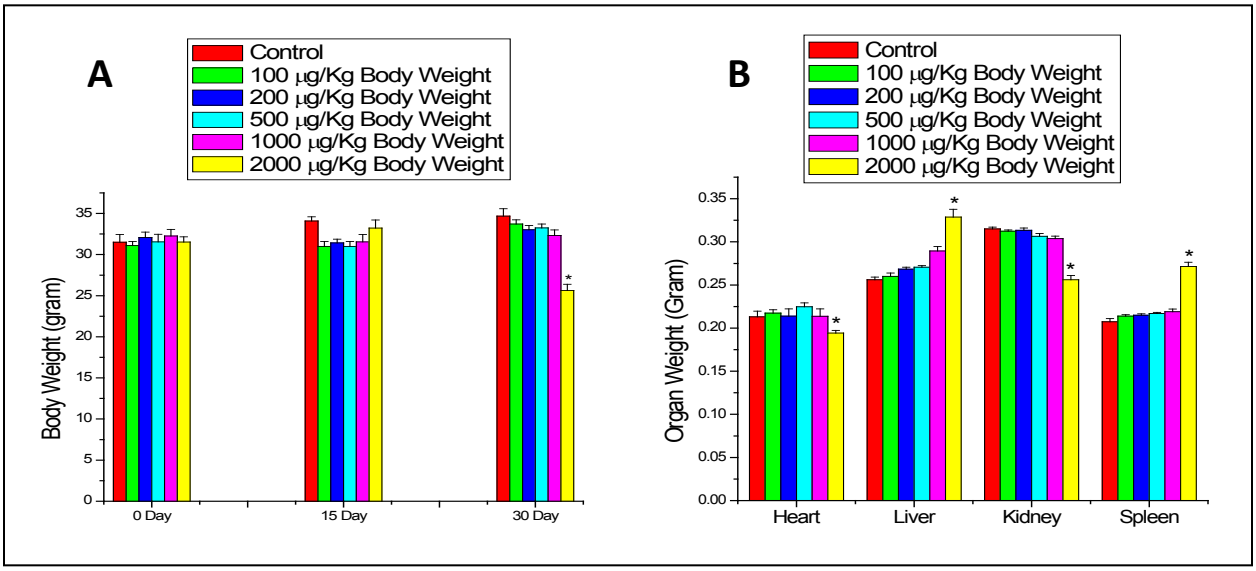

Figure 6.13: (A) Assesment of body weight of CuONPs@CS treated groups for 15 days and 30 days. **(B)** Assesment of organ weight of CuONPs@CS treated groups for 30 days .Values are expressed as mean±SEM; *superscripts indicate significant differences ($P<0.05$) compared with the control group. n=6.

Measurement of body weight of Balb/c mice helps to understand the overall toxic effect of CuONPs@CS. But interestingly after 15 days treatment no significant decreased in body weight was observed. At a dose of 2000µg/kg body weight, significant decreases in body weight were observed after 30 days (Fig. 6.13A). So, 15 days treatment becomes totally nontoxic after double layered coating. No mortality was observed. From the experiment it was observed that the liver weight increased at highest dose but the kidney weight decreased after the intraperitoneal administration of CuONPs@CS at a dose of 2000µg/Kg body Weight for 30 days treatment (Fig.

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6.13B). Spleen weight was also increased may be due to toxic effect of CuONPs@CS at a dose of 2000µg/kg Body Weight.

6.4.13.2. Biochemical toxicity markers level from mice serum:

LDH level from the mice serum is a marker of *in vivo* toxicity. LDH level increased significantly ($P<0.05$) by 2.02 folds at 2000µg/Kg Body Weight dose compared to the control group. Serum creatinine level increased by 1.77 folds significantly after the treatment with CuONPs@CS for

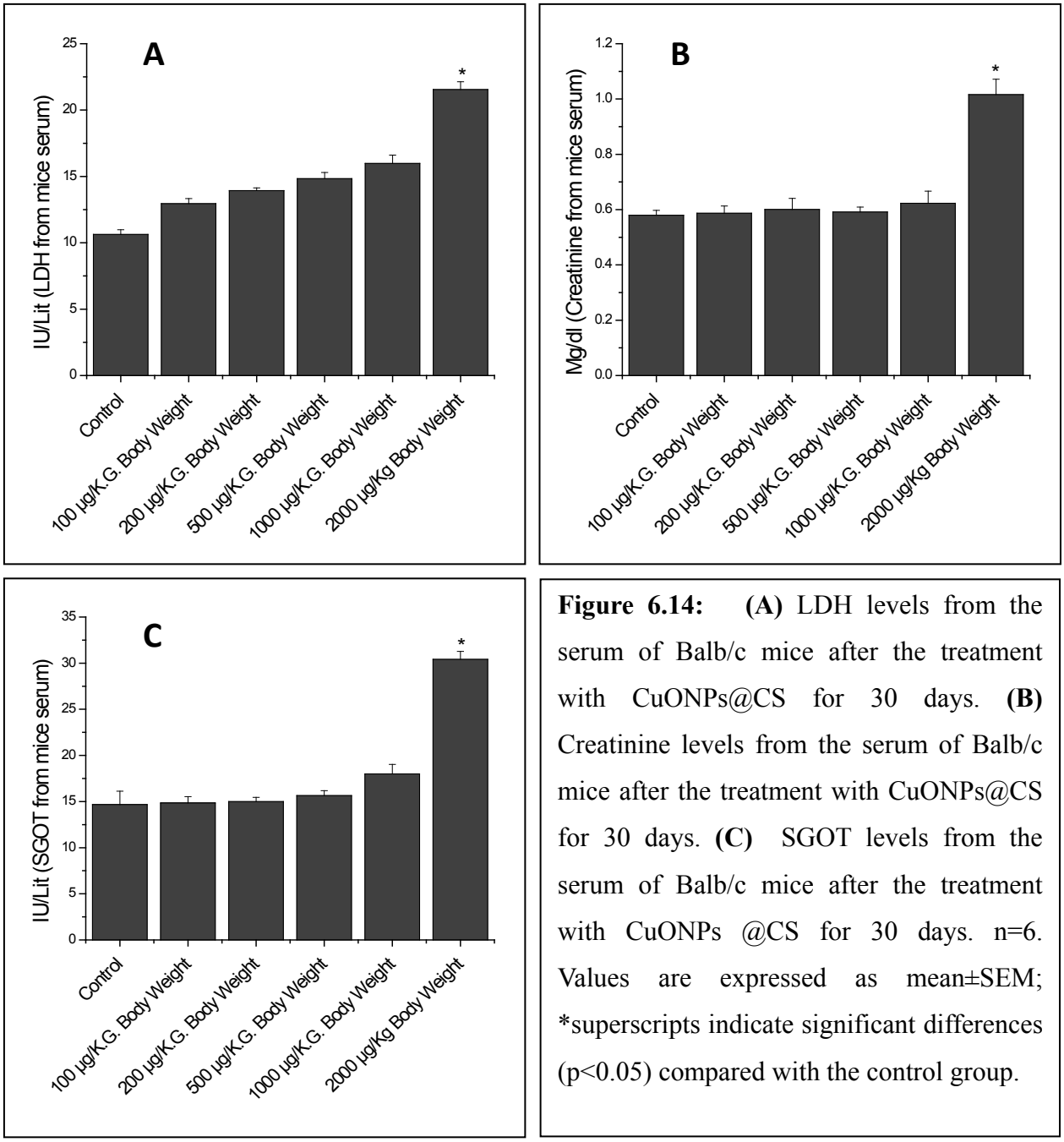

Figure 6.14: **(A)** LDH levels from the serum of Balb/c mice after the treatment with CuONPs@CS for 30 days. **(B)** Creatinine levels from the serum of Balb/c mice after the treatment with CuONPs@CS for 30 days. **(C)** SGOT levels from the serum of Balb/c mice after the treatment with CuONPs @CS for 30 days. n=6. Values are expressed as mean±SEM; *superscripts indicate significant differences ($p<0.05$) compared with the control group.

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30 days compared to the control group (Fig. 6.14B). Serum SGOT level increased by 2.07 fold significantly ($p<0.05$) compared to the control group (Fig. 6.14C).

6.4.13.3. Estimation of release of Cytokine level and Apoptotic markers from mice serum:

TNF-α level increased by 1.45 folds compared to the control dose at a dose of 2000µg/Kg Body Weight but 100-1000µg/Kg Body Weight dose did not showed any significant toxicity after the treatment with CuONPs@CS (Fig.6.15A). Significant increment of pro-apoptotic markers level was observed and at the same time anti-apoptotic markers level depleted at a dose of 2000µg/Kg body weight compared to the control group.

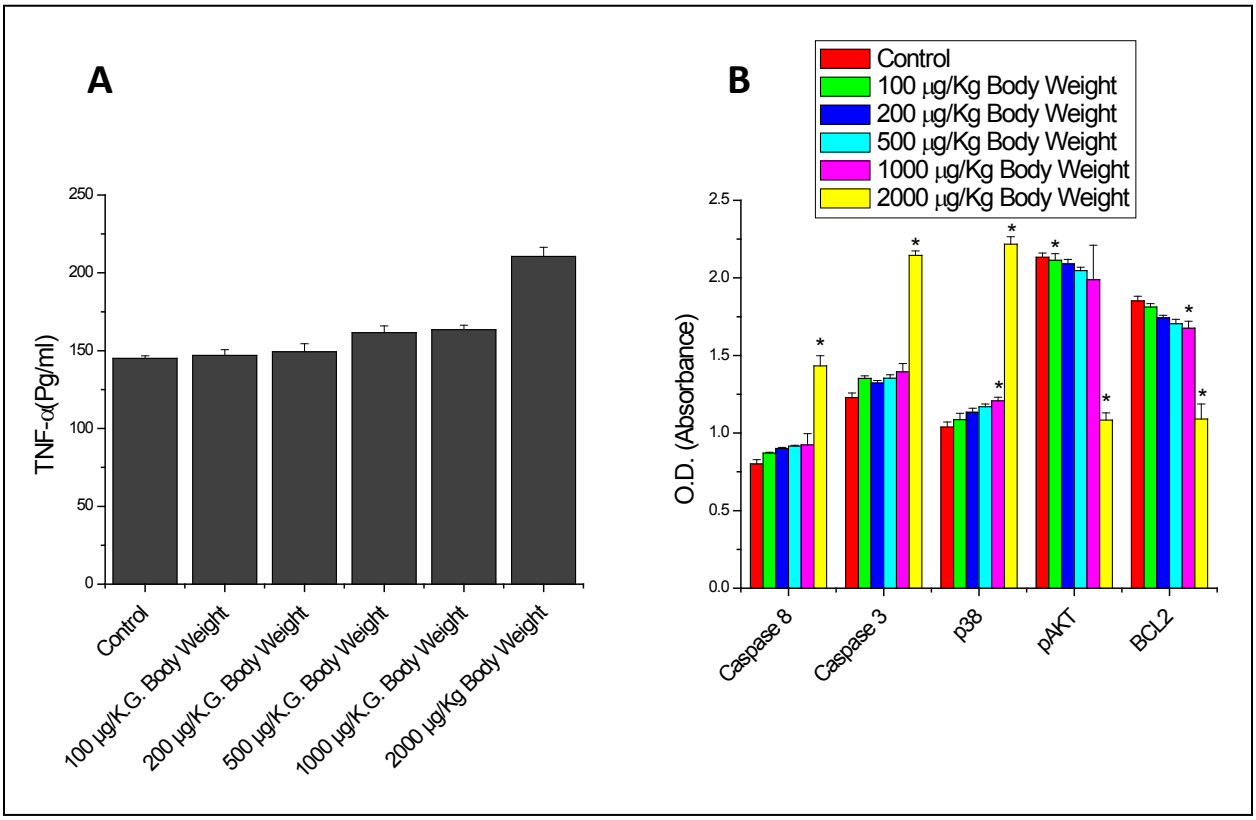

Figure 6.15: (A) Pro and anti-inflammatory cytokine changes of CuONPs@CS treated mice serum after 30 days (TNF-α, IL-10). **(B)** Alteration of pro-apoptotic (Caspase-8, Caspase-3, p38) and anti-apoptotic (pAKT, Bcl2) response of CuONPs@CS. n=6. Values are expressed as mean±SEM; *superscripts indicate significant differences ($P<0.05$) compared to the control group

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6.5. DISCUSSION:

In this study, double layered CS coated CuONPs were successfully synthesized. With the help of several physical techniques, the shape and size of the CuONPs@CS were estimated. It was spherical in shape with an average diameter of 34.5±5nm. The XRD pattern revealed the conjugation of CuONPs with CS (Fig.6.2). A similar result was observed by Logpriya et al., 2018. When the metal oxide nanoparticles are suspended in solution, it exerts highly toxic ions (Ivask et al., 2015). After coating with the green components of *A. indica,* the release of Cu ions measured was 0.34pg ions/cell which indicates toxicity towards normal cells. Multi layered coating decreased the release of Cu ions inside the lymphocytes. The release of Cu ions inside lymphocytes reduced to 0.20pg ions/cell. CS has an interesting property which enables it to readily dissolve in acidic environment and release the Cu ions which helps to target specific cancer cells. But the pH of lymphocytes being neutral was unable to dissolve CS and hence release of Cu ions from the conjugate was minimal. Hence the CuONPs@CS became nontoxic to the lymphocytes up to 50µg/ml dose. From the MTT assay, it was observed that up to 50µg/ml dose, CuONPs@CS killed only 27% lymphocytes but at 100µg/ml dose it killed near about 61% lymphocytes, which was significantly higher compared to the control group. Certain metal oxide NPs produces spontaneous ROS generation pertaining to the surface material composition and characteristics of the oxides, while nanomaterials generate ROS in presence of specific cell environment (Abdal Dayem et al., 2017; Manke et al., 2013). From Fig.6.10, it was observed that up to 50µg/ml dose, ROS generation was minimal but at a dose of 100µg/ml significant ($P<0.05$) amount of ROS generation was observed. Cu ions released from the CuONPs@CS induced apoptosis in lymphocytes at high doses. Fig.6.11 provided strong evidence of apoptosis as observed by Etbr/AO double staining at a dose of 100µg/ml. Apoptosis in lymphocytes is a major marker of toxicity (Al-Assaf et al., 2013). Pro-inflammatory cytokine TNF-α levels increased significantly only at 100µg/ml dose after 24hr treatment. From the *in vitro* study, it can be concluded that CuONPs@CS at 50µg/ml dose shows no significant toxicity.

During the *in vivo* study, organ weight and body weight markers (Fig. 6.13) were the major indicators of toxicity. Also no mortality was observed. From the body weight and organ weight distribution assay, it was found that no significant toxicity was observed up to 1000µg/Kg Body Weight. However at 2000µg/Kg Body Weight dose, significant toxicity was observed as the body weight decreased significantly. The weight of spleen and liver increased and the weight of

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kidney decreased which also indicated toxicity at a dose of 2000µg/Kg Body Weight. Similar result was observed by Hilaly et al., 2004 and Kluwe, WM. 1981. Several serum biochemical markers also support the toxicity outcome at a dose of 2000µg/Kg body weight. Serum LDH, SGOT and creatinine levels are the indicators of the proper functioning of liver and kidney. Significant elevation of these markers at a dose of 2000µg/kg Body Weight compared to the control group indicated dysfunctioning of these major organs.

Pro-inflammatory cytokines level was amplified after the treatment with CuONPs@CS in Balb/c mice serum at a dose of 2000µg/Kg Body Weight dose. Also the pro-apoptotic markers Caspase 3, 8 and p38 level increased significantly ($P<0.05$) compared to the control group at the same dose (2000 µg/kg Body Weight) (Fig. 6.15). The anti-apoptotic markers level decreased significantly only at a higher dose.

From the total study, it can be concluded that multi-layered surface coating effectively reduces the toxicity of CuONPs by pH responsive sustained released of Cu ions in *in vitro* and *in vivo* model. Sustained released of Cu ions up to 50µg/ml dose in *in vitro* and 1000µg/Kg body weight in *in vivo* model does not create any severe toxicity, however after 50µg/ml dose and 1000µg/Kg body weight produced significant toxicity in both models. So, higher doses CuONPs@CS induced apoptosis by TNF-α, p38 and MAPK activated pathway in lymphocytes.

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