Chapter 5

In vitro & *in vivo* anticancer efficacy of green synthesized Copper Oxide nanoparticles

INTRODUCTION:

In the previous chapter, it was observed that the toxicity profile of chemically synthesized CuONPs (S1NPs) was higher than green synthesized CuONPs (S2NPs) in both every *in vitro* and *in vivo* model. So, the S2NPs (green synthesized CuONPs) has been chosen for further studies. The anticancer activity of green synthesized CuONPs was conferred. Ideologically, the study combines the ancient formulae of leaf extract of a medicinal plant with modern metal based nanotechnology. To unearth the mechanism of cancer cell cytotoxicity of CuONPs from *A. indica*, molecular mechanism of anticancer activity was intensively studied and the regulation of pro and anti-apoptotic proteins in *in vivo* and *in vitro* systems were analyzed through the activation of several cytokines.

In cancer therapy, usage of nanoparticles (NPs) as a drug as well as for drug delivery purpose is attributed to their morphology, size, distribution, and surface by volume ratio (Rizvi et al., 2018). Inorganic metal NPs finds extensive use both as a drug and drug carrier in anticancer therapy due to properties like geometries, redox states, reactivity, etc. which are not accessible to organic compounds (Frezza et al., 2010). Among various metal NPs like Ag, Au, Co researchers have preferred Copper (Cu) due to its cost-effectiveness and greater stability (Nasrollahzadeh et al., 2015). Copper can perform surface charge modulation and create oxidative stress inside a cell due to its unique electronic arrangement and its participation as a co-factor for redox cycling of enzymes (Laha et al., 2014).

Among the transition metals, Cu takes part in several biological functions such as electron transfer, structural shaping and catalytic activity, while their cancer cell elimination ability is mainly attributed to the induction of an oxidative stress (Tardito and Marchio, 2009).

NPs can be prepared using both chemical and physical methods. In chemical method, sodium borohydride, hydrazine and microemulsions when used as Cu salt reducing agents, results in skin, nose and eye irritations, pulmonary oedema, affects nervous system, kidney and liver damages (Yugandhar et al., 2017). Meanwhile, CuONPs prepared through microemulsion and evaporation methods are quite expensive (Capek, I. 2004) and also produce several hazardous materials which are environment pollutants (Tavakoli et al., 2007). On the other hand, drawbacks in NPs prepared through physical methods using pulse laser ablation, microwave-assisted and

pulsed/explosion wire discharge methods are health hazards, expensive, high energy consumption and comparatively have lower product efficiency (Kalyanaraman et al., 1998).

To deter these adverse circumstances, researchers have used plant extracts for the synthesis of NPs. Plant derived NPs are considered cost-efficient, safe, better feasibility and adaptability as medicinal, surgical and pharmaceutical drugs. In addition, solvents used in preparing plant derived NPs are considerably safe without any toxicity (Sivaraj et al., 2014).

Although there has been substantive progress on the usage of *A. indica* extracts in anticancer therapy, however, the mechanistic study in its entirety is still unclear. It has been mainly reported that components of *A. indica* suppress NF-κB signalling pathways (Priyadarsini et al., 2010), sensitizes cancer cells during immunotherapy, radiotherapy, exhibited tumor specific anti-proliferative and apoptosis-inducing effects and showed significantly less toxicity on normal cells (Elumalai et al., 2012).

In the present study, a green nano drug using Cu and *A. indica* leaf extracts based on the aforementioned properties of *A. indica* extracts and CuONPs in cancer therapy was designed.

5.1. MATERIALS AND METHODS:

Histopaque 1077, DMEM, penicillin, streptomycin, Doxorubicin (DOX) were procured from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from GIBCO/Invitrogen. MTT and dimethyl sulfoxide (DMSO) were purchased from Himedia, India. Titron X-100, Tris–HCl, Tris buffer, Sodium dodecyl sulphate (SDS), ethidium bromide (EtBr), 2-vinylpyridine and all other chemicals were from Merck Ltd and SRL Pvt. Ltd. Mumbai and of the highest purity grade available.

5.1.1. Cell Culture and maintenance:

Estrogen receptor positive breast cancer cell line MCF-7, cervical cancer cell line HeLa and 4T1 cell line were gifted by Parimal Karmakar, Jadavpur University. These cell lines were maintained in a DMEM complete culture media with 10% FBS, 2 mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin under 5% CO_2 and 95% humidified atmosphere at 37°C in a CO_2 incubator.

5.1.2. p^H and time dependent dissolution study of green synthesized CuONPs:

Green synthesized CuONPs were suspended in a DMEM culture medium (without FBS and antibiotic) and, after incubation with different p^H levels (p^H= 5.4, 7.4 and 9) for varied durations (2, 4, 8, 12 and 24hr) at 37^{o}C temperature. The method was similar with the above mentioned in Chapter 3 Section 3.1.4.8.

5.1.3. Estimation of Cytotoxicity by MTT assay:

In vitro cell viability assay was done through Tetrazolium 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. MCF-7 and HeLa cell lines with 1x10^4 cells per well in 96-well plate were incubated with 1, 5, 10, 25, 50 and 100 µg/ml doses of green synthesized CuONPs for 24hr. 8,12, 24 and 48hr incubation was done with drug at different doses. Among them 24 hr was selected, as the cytotoxicity level was significantly higher at 24hr). DOX was used with a similar concentration of green synthesized CuONPs as a positive control. The method was mentioned in Chapter 4 section 4.2.5.1.

5.1.4. Intracellular uptake of Cu ions in MCF-7 & HeLa cells:

MCF-7(2x10^6 cells/plate) and HeLa cells(2x10^6 cells/plate) were cultured in 35mm cell culture plates and incubated with CuONPs from *A. indica* for 2, 4, 8, 12 and 24hr with 5% CO_2 at 37^{o}C. Subsequently Cu content was measured according to the previously mentioned method in Chapter 4 section 4.2.5.2.

5.1.5. Redox Balance in cancer cells:

Reduced glutathione estimation was performed using cancer cell lysate (2x10^6 cells) with NP40 buffer acting as a lysis buffer. After the centrifugation at 2000×*g* for 15min the protein was precipitated. Before the centrifugation all samples were mixed with TCA. The supernatant of all the samples were diluted to 1ml with sodium phosphate buffer (0.2M; pH 8.0 Ellman's reagent which is DTNB (2ml of 0.6mM) was added and few mins. later, the optical density of the yellow-colored complex was measured at 405nm. The levels of GSH were expressed as µg of GSH mg^{-1} protein (Chakraborty et al., 2011).

The oxidized glutathione level was measured according to Chakraborty et al., 2011. A 0.5ml sample (cancer cell lysate which was lysed with NP40 lysis buffer), 2µl of 2-vinylpyridine was mixed. Deprotenized was done with sulfosalicylic acid after incubation for 1hr at 37°C.

Precipitated proteins were obtained by centrifugation at 1000×*g* for 10min. The supernatant was articulated and GSSG level was assessed with the reaction of DTNB at 412nm in a spectrophotometer. A standard GSSG curve was prepared for calculation. The levels of GSSG were expressed as μg of GSSG mg^{-1} protein.

5.1.6. Estimation of Nitric Oxide (NO) level:

NO level was measured according to the previous chapter 4 section 4.1.5.3.

5.1.7. Intracellular ROS intensity measurement & microscopic observation of cancer cells:

ROS measurement of MCF-7 and HeLa cells were performed using 2,7-dichlorofluorescin diacetate ($DCFH_2$-DA) according to the previously mentioned Chapter 4 section 4.1.5.4.

5.1.8. Observation of nuclear morphology by DAPI staining:

MCF-7 and HeLa cells were implanted in a 6 well plate with $2x10^6$ cells in each well. Green synthesized CuONPs were treated against both cells for 24hr with different doses, washed with PBS and stained with DAPI according to (Lin et al., 2006) with some modification. After the treatment, cells fixation was done with 2.5% glutaraldehyde for 10min. 0.1% Triton X-100 was used for Permeabilization of cancer cells. A working concentration of DAPI staining (1μg/ml) was made by PBS. After the staining, cells were kept in a dark place for 5 min. Then the stain was washed with PBS and cells were observed under the fluorescence microscope (Nikon ECLIPSE LV100POL) at excitation 330-380nm and emission 430-460nm.

5.1.9. Detection of cancer cell apoptosis by flow cytometric analysis:

Cellular apoptosis by flow cytometry was performed according to Looi et al., 2013. Cells were plated at $1x10^5ml^{-1}$ on $25cm^2$ flask overnight and subsequently treated with CuONPs from *A. indica* at various concentrations for 24hr. After the media was removed, cells were trypsinized, centrifuged at 1600rpm and the pallet was incubated with FITC tagged annexin V and propidium iodide (PI) (e Biosciences, India). It was kept in binding buffer for 15min in dark. Stained cells were subjected to flow cytometry analysis and data acquisition and analysis were performed in a Becton-Dickinson FACS verse flow cytometer using Cell-Quest software. For each sample, at least 20,000 events were acquired in a flow cytometry. The FITC-annexin V stained cell indicate apoptotic population lies in X-axis and PI stained cancer cell which indicate necrotic population were lies in Y axis.

5.1.10. Release of Cytokines from cancer cells:

After the 24hr treatment with green synthesized CuONPs with different doses, cytokine levels were analysed from MCF-7 and HeLa cells. The cytokine levels were measured from the supernatant of the cell pellet using eBioscience kit ELISA method for IL-10 (eBioscience; cat# 88-7104-22) and TNF-α (eBioscience; cat# 88-7342-29). The method was previously mentioned in chapter 4 section 4.1.5.6.

5.1.11. Apoptotic protein measurement by ELISA method:

Pro and anti-apoptotic markers level was measured according to previous Chapter 4 Section 4.1.5.7.

5.2. Animals:

Animals were maintained according to previously mentioned method in Chapter 4 section 4.2.1. Each experimental group contained 6 mice.

5.2.1. Tumor development in Balb/c mice model by 4T1 cells:

6-8 weeks old female Balb/c mice were injected subcutaneously in the abdominal mammary pad with $1x10^5$ 4T1 cells suspended in PBS on day 0 of the study. Mice in the untreated group were similarly given doses of 0.1ml of PBS. 4T1 cells rapidly multiplied resulting in highly metastatic tumors. The abnormal growth of the mice abdominal and increased weight within 10-14 days confirmed the development of tumors. Mice were examined every other day for conditions including rough coat appearance, discoloration of skin and swollen abdomen.

5.2.2. Determination of mean survival time and tumor growth restriction assay:

Within 14 days of inoculating the Balb/c mice with $1x10^5$ numbers of 4T1 cells, the tumors developed, which was followed by treatment with green synthesized CuONPs for 15 days at 3 days interval. The mean survival time was calculated by using this following formula

Increase in life span = (T-C) x100

T = Number of days the treated animals survived

C= Number of days the control animal survived

5.2.3. Euthanasia of experimental animals:

Euthanasia was done according to the previous Chapter 4 section 4.2.3. After the dissection of mice, the primary tumor was weighed and homogenized for the further experiments.

5.2.4. Release of Cytokines from tumor homogenate:

After completion of the total treatment schedule, tumor tissues from the mice were taken, weighed, minced and homogenized. Subsequently, the homogenized sample was centrifuged (7000gx10min) and the supernatant was used for TNF-α and IL-10 cytokines measurement according to the above mentioned method in Section 5.1.11.

5.2.5. Estimations of Apoptotic markers by ELISA:

Apoptotic protein markers were also estimated from primary tumor homogenate of Balb/c mice, according to the above-mentioned method of Section 5.1.12.

5.2.6. Protein Estimation by Lowry method:

Protein estimation was done according to previous Chapter 4 Section 4.2.7.

5.2.7. 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.

5.3. RESULTS:

5.3.1. pH and time dependent dissolution study of green synthesized CuONPs:

In the present study, the releasing amount of Cu ions from green synthesized CuONPs were different at different pH levels (Fig.5.1A). At different time duration, the release of Cu ions in acidic environment was higher compared to the basic and physiological environment. After 24hr the release of Cu ion at acidic pH was 0.509 PPM.

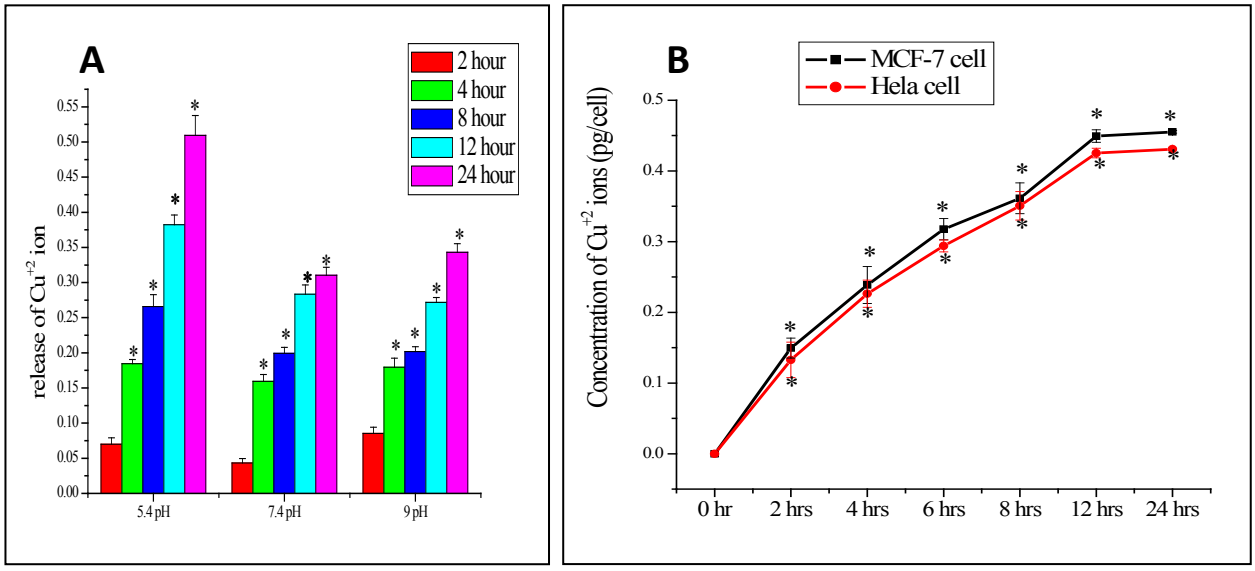


Figure 5.1: (A) Release of Cu^{+2} ions from green synthesized CuONPs at different pH for different time durations. **(B)** Cu^{+2} ions concentration in MCF-7 and HeLa cells after treatment with CuONPs at different time durations, measured by AAS

5.3.2. Intracellular uptake of Cu ions in MCF-7 & HeLa cells:

At 100µg/ml dose of green synthesized CuONPs against MCF-7 and HeLa cells, the internalization of Cu ion in the MCF-7 and HeLa cells were measured by AAS. After 24 hrs, 0.455 and 0.436pg Cu ions/cell were released from MCF-7 and HeLa cells significantly (Fig.5.1B). The Fig 5.1C showed the concentration of Cu ions in cancer cells without any treatment.

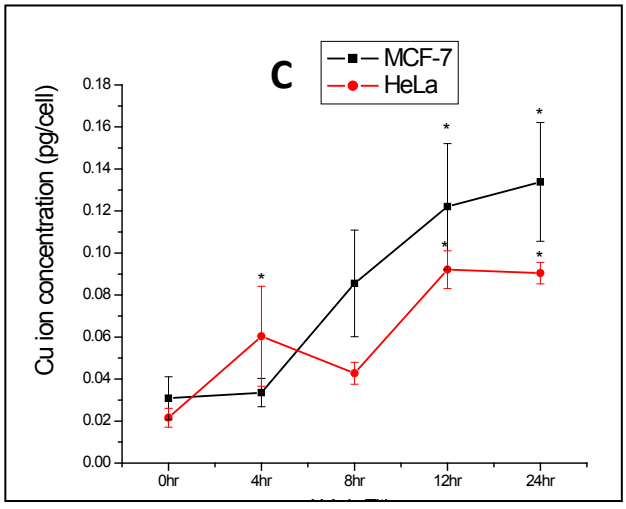


Figure 5.1: (C) Estimation of free Cu ions in cancer cells measured by AAS without treament of nanoparticles.

5.3.3. Estimation of cytotoxicity by MTT assay against cancer cells:

Green synthesized CuONPs showed significant toxicity on MCF-7 and HeLa cells at doses of 1, 5, 10, 25, 50 and 100µg/ml compared to the control group. MCF-7 and HeLa cells were killed significantly (P<0.05) by 13.76%, 29.45%, 57.37%, 72.92%, 78.41% , 87.41% and by 12.98%, 28.94%, 54.17%, 70.22%, 76%, 84.28% respectively compared to the control group (Fig.5.2). The IC_{50} values were 21.56 and 24.74 µg/ml in case of MCF-7 and HeLa cells.

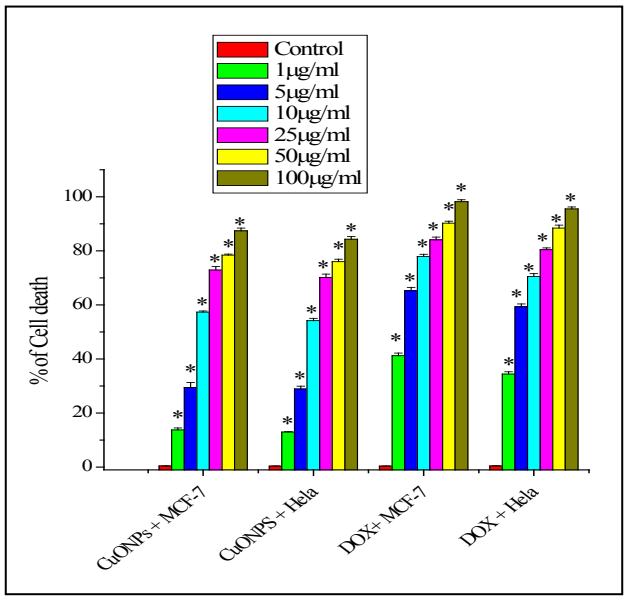


Fig. 5.2: Dose dependent cell death assay of both green synthesized CuONPs and DOX on MCF-7 and HeLa cells. Values were expressed as mean ± SEM. Superscripts indicated a significant difference as (P<0.05) compared with control

5.3.4. Cellular redox balance:

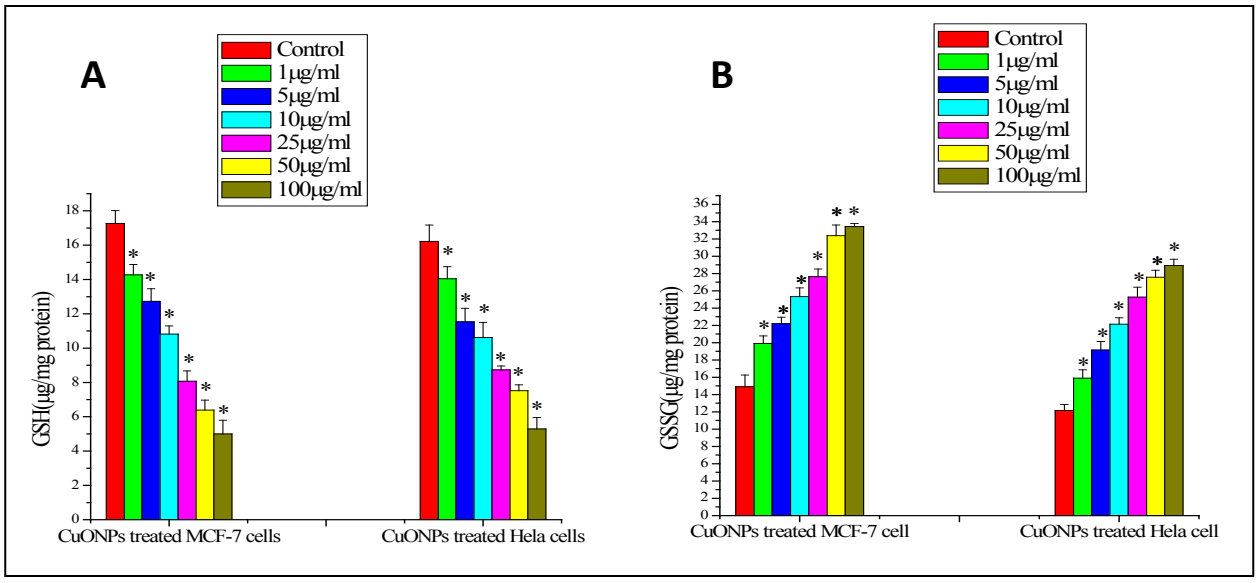


Fig. 5.3: Cellular redox balance was measured by GSH and GSSG level. **(A)** Estimation of GSH level from MCF-7 and HeLa cells. **(B)** Estimation of GSSG level from MCF-7 and HeLa cells. Values were expressed as mean ± SEM. Superscripts indicated a significant difference as (P<0.05) compared with control.

In case of MCF-7 and HeLa cells the GSH level decreased significantly (P<0.05) by 17.37%, 26.285, 37.40%, 53.27%, 63.05%, 71.04% and by 13.38%, 28.87%, 34.54%, 46.14%, 3.60%, 67.36% respectively compared to the control group (Fig.5.3A) in a dose-dependent manner.

The oxidized GSSG level elevated significantly (P<0.05) by 25.16%, 32.85%, 41.17%, 46.07%, 53.96%, 55.41% and by 23.29%, 36.48%, 45.07%, 51.90%, 55.93%, 58% in case of HeLa and MCF-7 cells respectively compared to the control group (Fig.5.3B).

5.3.5. Nitric oxide release assay from cancer cells:

NO release level increased (Fig.5.4) in case of both the cell lines significantly (P<0.05) compared to the control group. The MCF-7 and HeLa cells increased the NO level by 15.63%, 26.84%, 54.24%, 61.03%, 64.77%, 67.12% and by 17.57%, 27.74%, 41.99%, 50.79%, 56.23%, 63.84%, respectively in a dose-dependent manner.

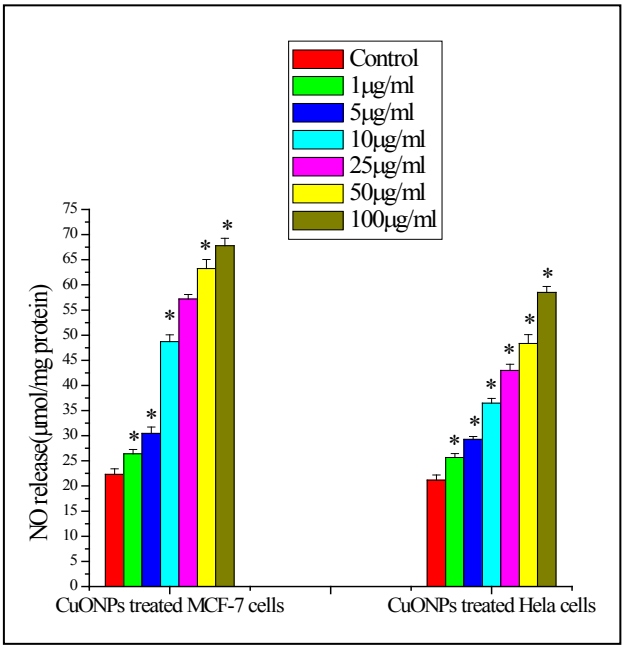
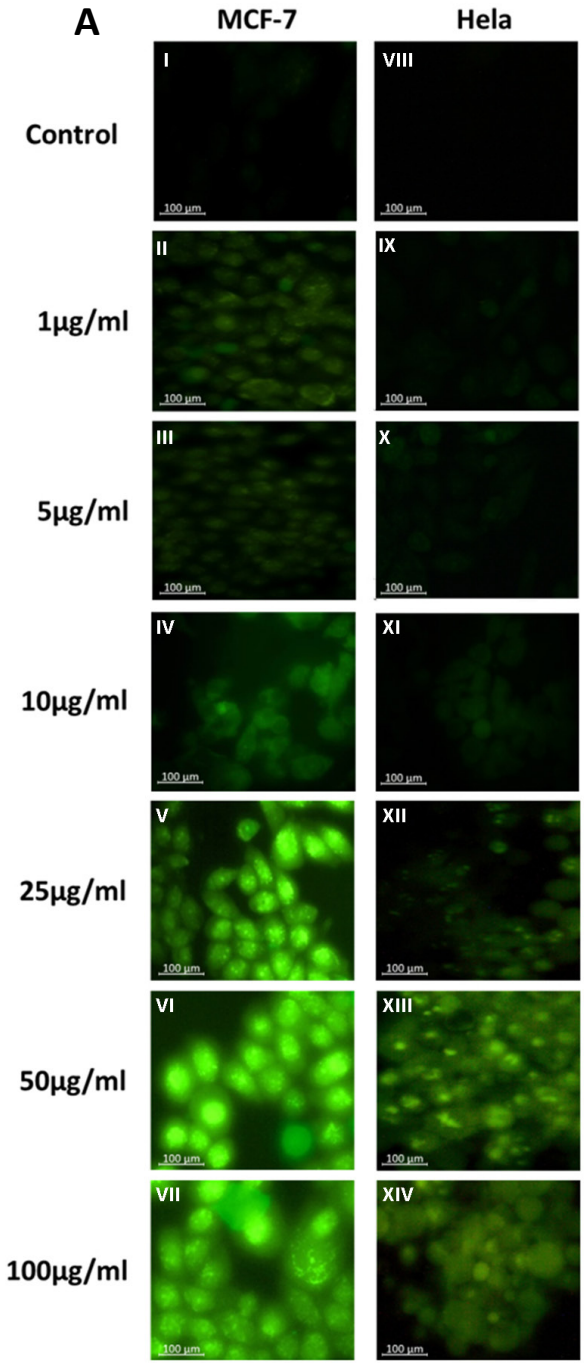


Fig. 5.4: Estimation of NO release level of green synthesized CuONPs treated MCF-7 and HeLa cells. NO level was expressed as micro mole/mg protein. The levels of NO were expressed as percentage of untreated cells. Values are expressed as mean ± SEM of three experiments;

5.3.6. Intracellular ROS intensity measurement & microscopic observation of cancer cells:



The CuONP treated HeLa and MCF-7 cells showed significant ROS generation. After 24 hr treatment, the ROS generation was visualized under the microscope (Fig.5.5A). In case of highest dose of 100 µg/ml, MCF-7 and HeLa cells produces 8.22 and 5.96 folds more ROS inside the cell compared to control as measured through image J software (Fig.5.5B).

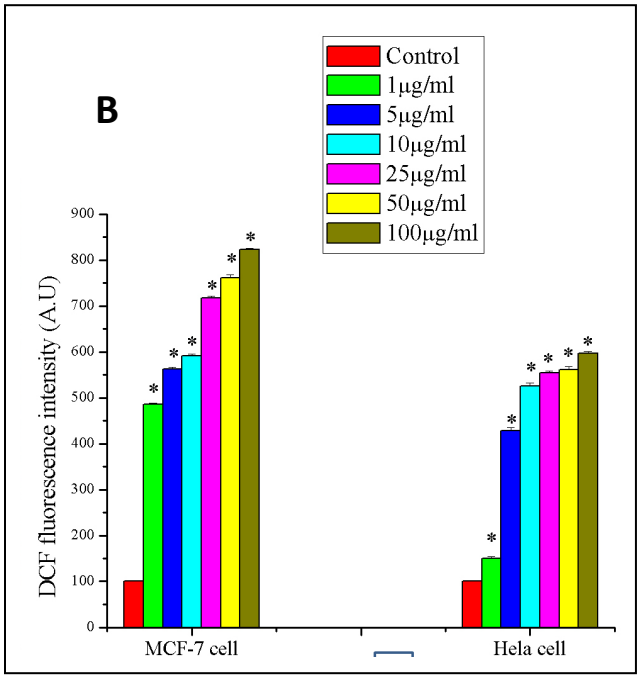


Fig. 5.5: **(A)** Outcome of green synthesized CuONPs on MCF-7 and HeLa cells were visualized under fluorescence microscope by DCHF2DA at a magnification of 40X **(B)** Control cells intensity was adjusted to 100 units. Data is represented as fold of change of the ROS level with the control group. Intensity was measured by image J software. Values were expressed as mean ± SEM of three experiments; superscripts indicated significant differences ($P<0.05$) compared with the control group.

5.3.7. Nuclear fragmentation by DAPI staining:

In case of untreated HeLa and MCF-7 cells, the nuclei remained intact, whereas in case of green synthesized CuONPs treated cells showed fragmented nuclei. As the green synthesized CuONPs dose increased gradually, the nuclear fragmentation increased in case of both the cells (Fig.5.6).

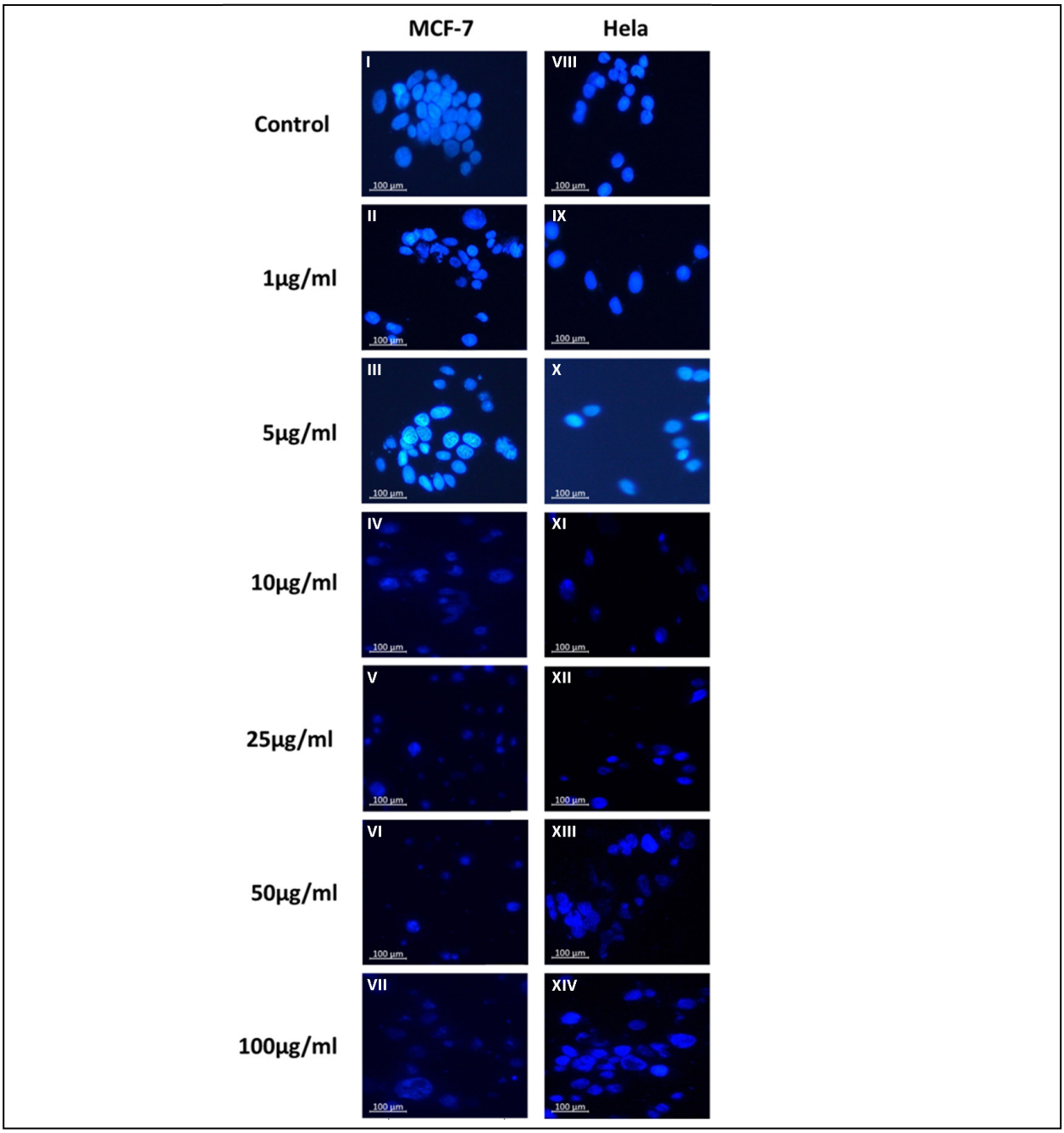


Figure 5.6: Visualization of nuclear morphology by DAPI staining using fluorescence microscopy at a magnification of 40X were visualized at excitation 330-380 nm and emission 430-460 nm.

5.3.8. Cellular apoptosis analysis of cancer cells by flow cytometry:

To know the percentage of apoptosis and necrosis, MCF-7 and HeLa cells were stained with annexin V and PI. Flow cytometry analysis of stained cells distinguished them into four groups. Early apoptosis (Annexin V+ve and PI-ve), late apoptosis and early necrosis will be in Annexin V+ve and PI+ve quadrant (Fig.5.7)

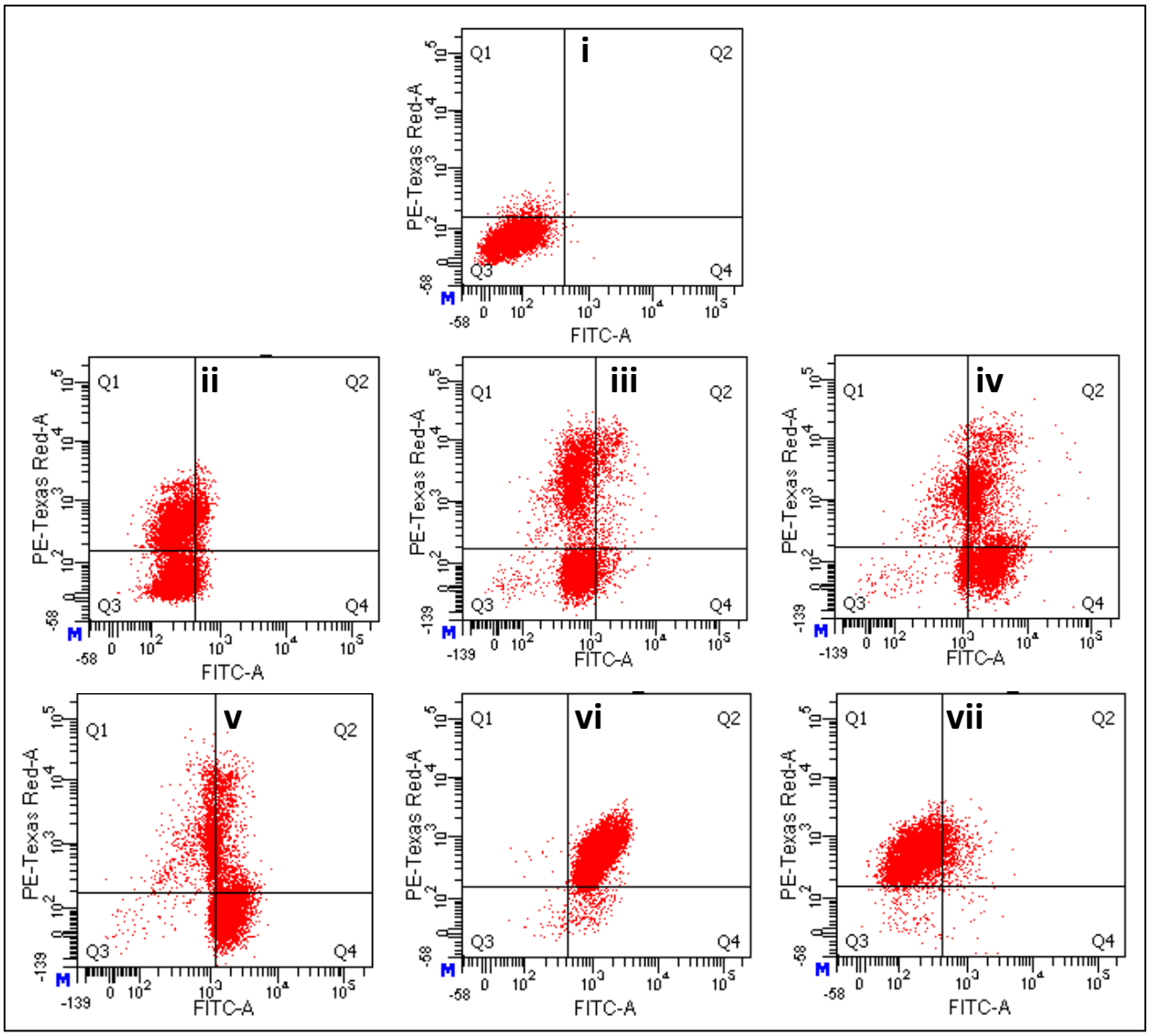


Fig. 5.7A: Estimation of apoptotic cell population by flow cytometry using Annexin-V/PI staining. The percentage of cell population of CuONPs treated MCF-7 cells at Lower Left (LL: viable cells), Lower Right (LR: Annexin V-FITC positive early apoptotic cells), Upper Right (UR: Annexin V-FITC and PI dual positive) and Upper Left (UL: Only PI positive necrotic cells) was estimated before and after treatment. Population of **(A)** MCF-7 cells after treatment with Control (i), 1 µg/ml (ii), 5 µg/ml (iii), 10 µg/ml (iv), 25 µg/ml (v), 50 µg/ml (vi) and 100 µg/ml (vii) of green synthesized CuONPs.

From the initial doses to the higher doses of green synthesized CuONPs showed apoptotic and necrotic population in MCF-7 and HeLa cells significantly compared to the control group. MCF-7 cells showed 93.9% of late apoptosis. In case of HeLa cells at a dose of 50µg/ml showed 96.9% of necrotic cells and 10.9% of late apoptosis and 100µg/ml dose showed 97.5% of necrotic cells.

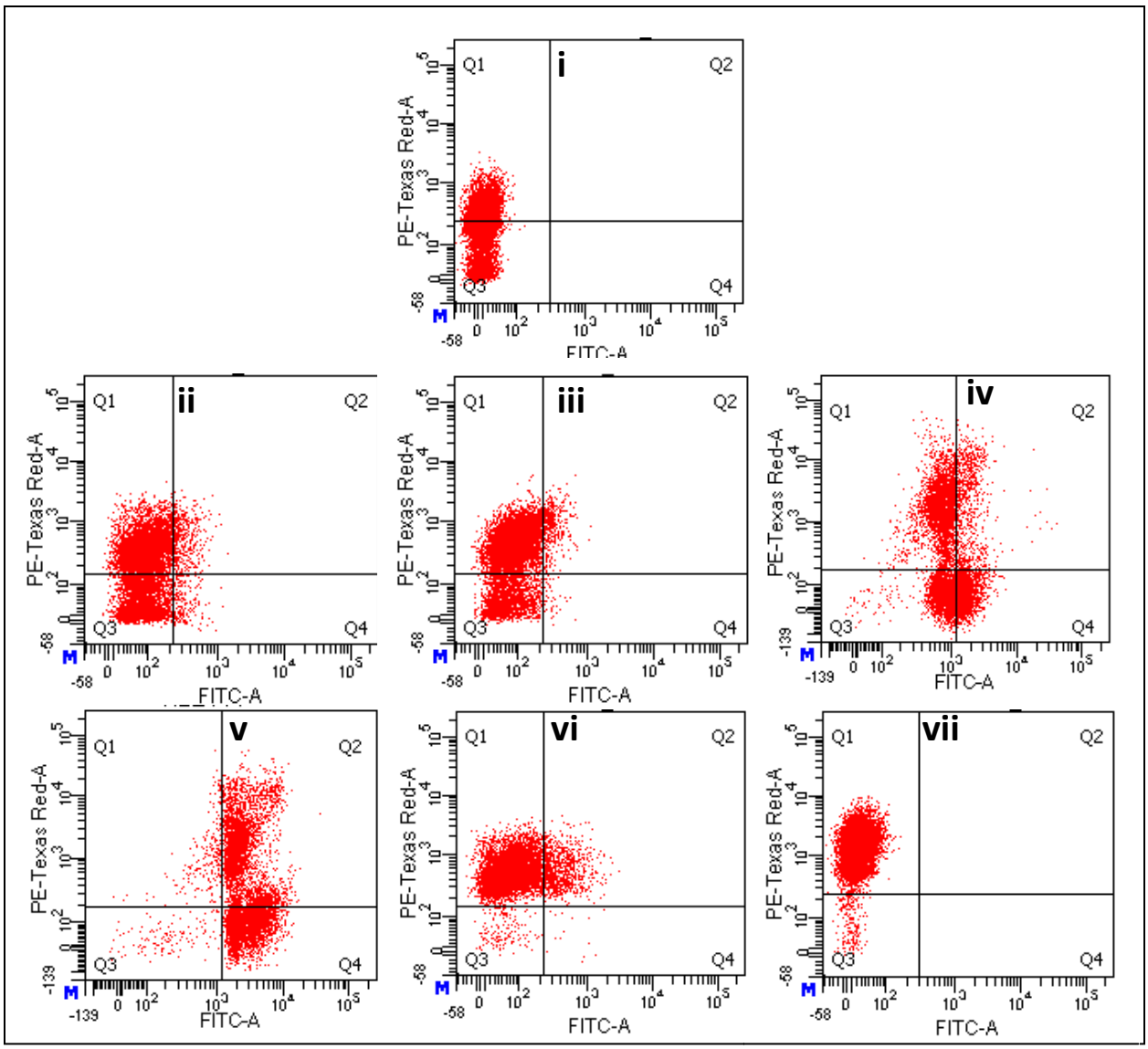


Fig. 5.7B: Estimation of apoptotic cell population by flow cytometry using Annexin-V/PI staining. The percentage of cell population of CuONPs treated HeLa cells at Lower Left (LL: viable cells), Lower Right (LR: Annexin V-FITC positive early apoptotic cells), Upper Right (UR: Annexin V-FITC and PI dual positive) and Upper Left (UL: Only PI positive necrotic cells) was estimated before and after treatment.**(B)** Population of HeLa cells after treatment with Control (i), 1 µg/ml (ii), 5 µg/ml (iii), 10 µg/ml (iv), 25 µg/ml (v), 50 µg/ml (vi) and 100 µg/ml (vii) of green synthesized CuONPs.

5.3.9. Pro and Anti-inflammatory cytokines analysis by ELISA:

Green synthesized CuONPs induced the pro-inflammatory cytokine (TNF-α) level in MCF-7 and HeLa cells significantly ($P<0.05$) by 1.34, 1.83, 2.44, 2.99, 3.83, 4.56 folds and by 1.24, 1.86, 2.50, 2.85, 3.89, 4.37 folds respectively, as compared to the control.

The anti-inflammatory cytokine (IL-10) level in case of MCF-7 and HeLa cells, decreased significantly ($P<0.05$) by 1.17, 1.32, 1.63, 1.98, 3.09, 3.90 folds and by 1.12, 1.23, 1.56, 1.92, 2.61, 3.50 folds compared to the control when treated with green synthesized CuONPs, at doses of 1µg/ml to 100µg/ml (Fig.5.8).

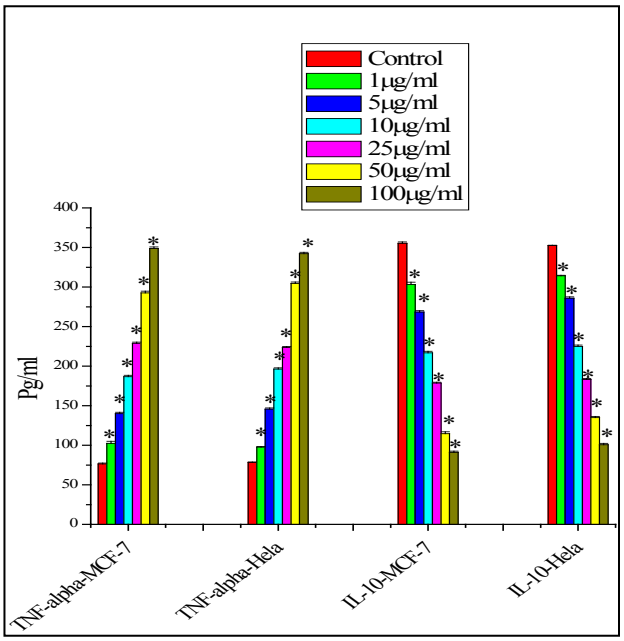


Fig. 5.8: ELISA data of Pro (TNF-α) and anti-inflammatory (IL-10) cytokine level on MCF-7 and HeLa cells after 24hr of treatment of green synthesized CuONPs. Values are expressed as mean±SEM of three experiments; superscripts indicate significant differences ($p < 0.05$) compared with the control group.

5.3.10. Apoptotic protein markers by ELISA:

After 24hr incubation of the cells with green synthesized CuONPs, the cell lysate was used for the pro and anti-apoptotic protein analysis by ELISA. From Fig.5.9 it was observed that the level of caspase 8, caspase 3 and P^{38} increased gradually as the dose increased.

The P^{38} expression level continuously increased by 21.81%, 36.29%, 48.80%, 57.02%, 64.16% and 68.79% for MCF-7 cells whereas in case of HeLa cells, the expression level increased by 27.34%, 11.17%, 41.87%, 51.30%, 60.59% and 65.03% significantly (Fig.5.9A&B). Caspase 9 increased by 18.46%, 36.26%, 50.27%, 55.88%, 63.56% and 67.85% and cytochrome C level increased by 19.92%, 30.11%, 47.65%, 56.38%, 60.95% and 65.05% compared to the control group in case of MCF-7 cells (Fig.5.10A). The expression of Bax protein was also elevated after the implication of green CuONPs at different doses. The Bax protein increased significantly

(P<0.05) by 27.27%, 41.17%, 47.10%, 61.09%, 66.83% and 73.98% compared to the control group.

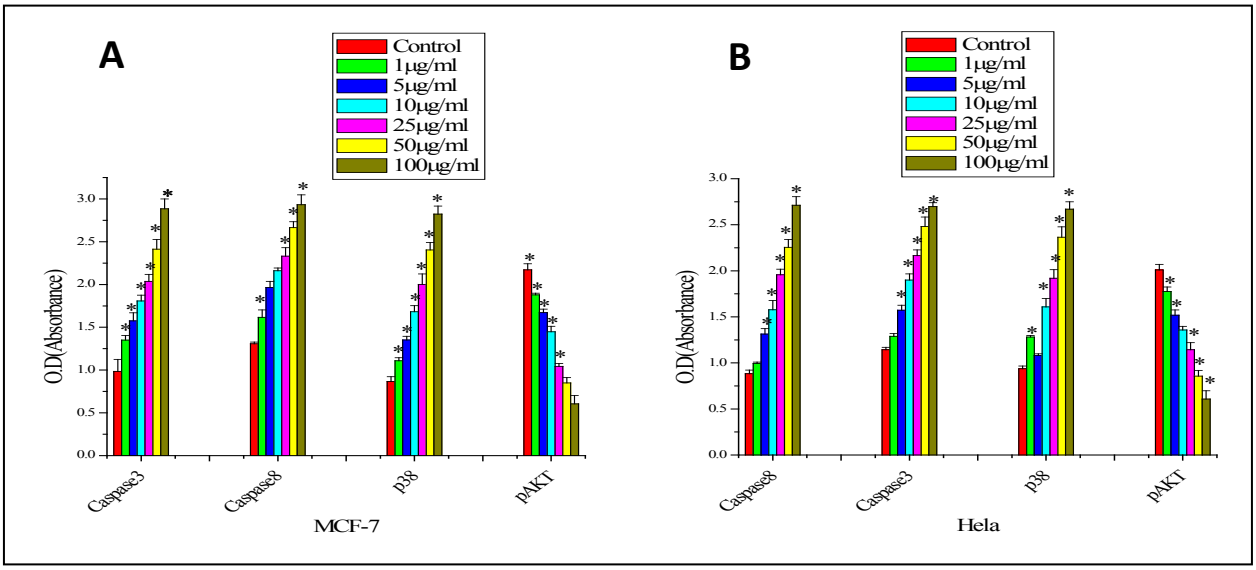


Fig. 5.9: Changes of pro-apoptotic (Caspase 8, Caspase 3, p38) and anti-apoptotic (pAKT) protein response of green CuONPs on MCF-7 and HeLa cells. **(A)** Pro and anti-apoptotic protein expression in MCF-7 cells, **(B)** Pro and anti-apoptotic protein expression in HeLa cells. Values are expressed as mean ± SEM of three experiments; superscripts indicate significant differences (P<0.05) compared with the control group.

In case of HeLa cells, Caspase 9 level increased by 14.05%, 29.43%, 41.92%, 50.32%, 58.40% and 61.18% and the cytochrome C expression level increased by 26.66%, 36.78%, 38.20%, 51.75%, 59.25% and 65.51% significantly compared to the control group. Bax protein also elevated by 21.11%, 32.80%, 45.95%, 51.52%, 57.38% and 63.81% (Fig.5.10B).

But the pAKT level dropped down compared to the control group. pAKT is an anti-apoptotic protein. This phenomenon was observed in case of both the cell lines. In case of HeLa cells the pAKT level decreased by 11.89%, 24,83%,32.45%, 43.25%, 57.39% and 70.13% (Fig.5.10B) and tumorigenic triple positive breast cancer cells also decreased pAKT level by 13.82%, 23.04%, 33.64%, 52.07%, 61.29% and 72.35% significantly (P<0.05) compared to the control group (Fig.5.9A).

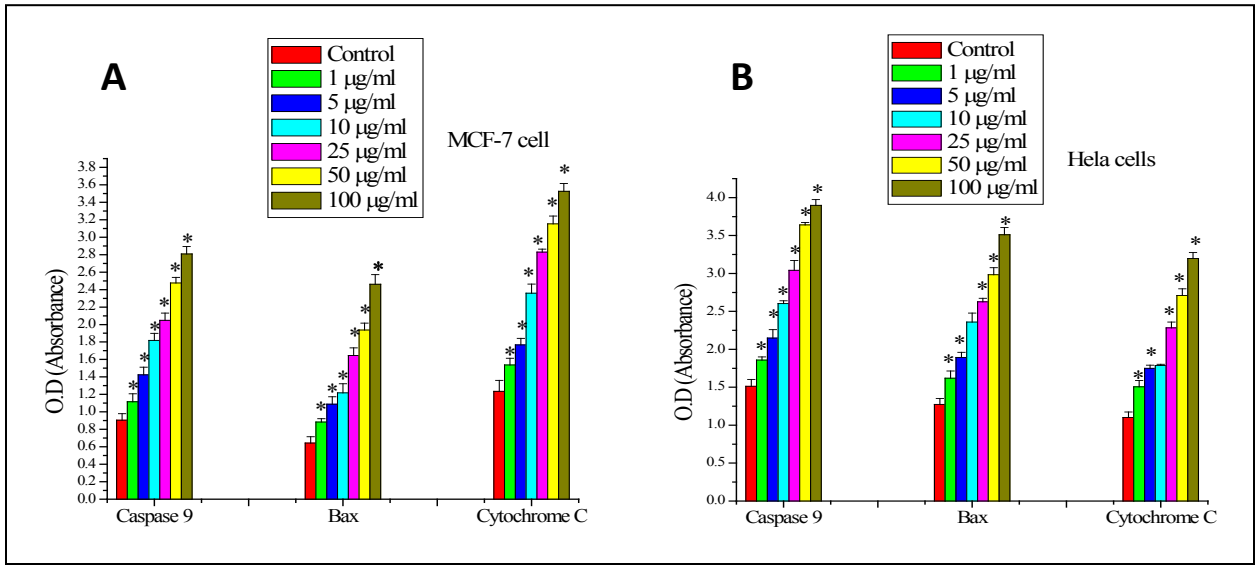


Fig. 5.10: Changes of pro-apoptotic (caspase 9, cytochrome C and Bax) protein level after treatment with green CuONPs on MCF-7 and HeLa cells. **(A)** MCF-7 and **(B)** Pro-apoptotic protein expression in HeLa cells. Values are expressed as mean ± SEM of three experiments; superscripts indicate significant differences (P<0.05) compared with the control group.

5.4. *In vivo* effect:

A solid tumor bearing Balb/c mice generally survived for 18±2 days but, after the administration

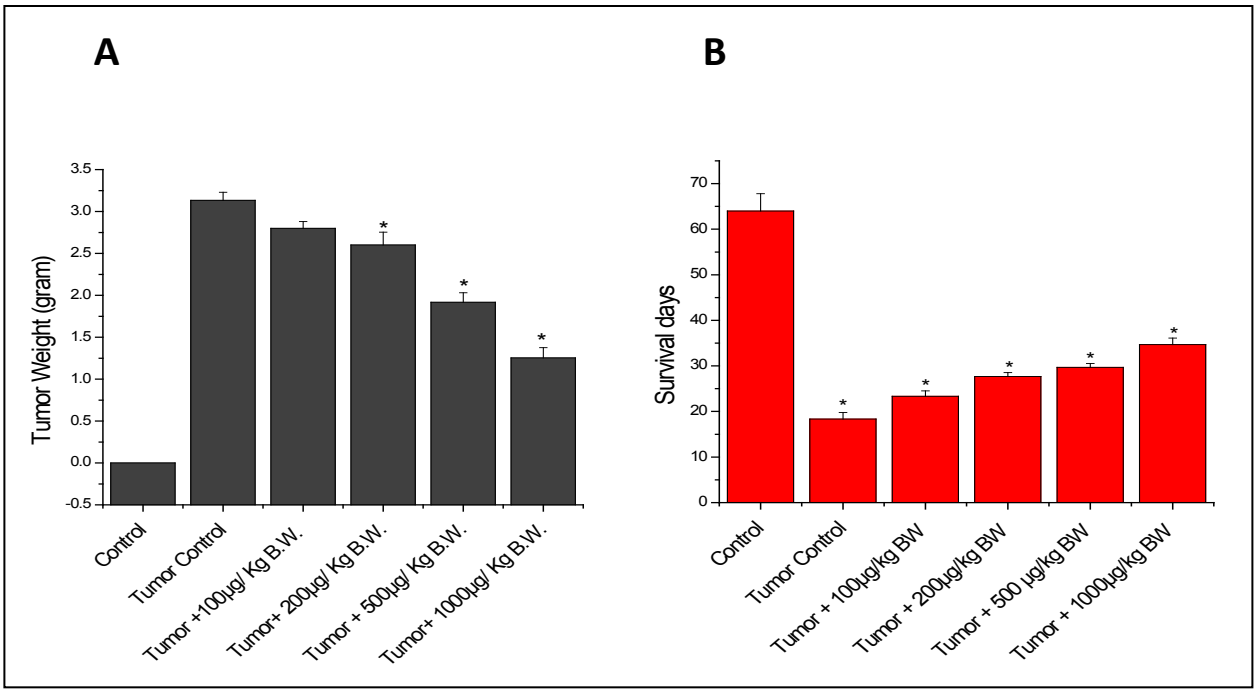


Fig. 5.11: (A) Tumor weight of green synthesized CuONPs treated 4T1 infected Balb/c mice. **(B)** Survival days of green synthesized CuONPs treated 4T1 infected mice. Values are expressed as mean ± SEM, n=6; *superscripts indicate significant differences (P<0.05) compared with the both control group.

of green synthesized CuONPs the tumor growth restricted (Fig.5.11A) and the survival days increased up to 34±2 days (Fig.5.11B). *In vivo* tumor weight reduced significantly (P<0.05) by 60.06% at 1000µg/kg body weight compared to the control breast tumor bearing mice.

5.4.1 Cytokines analysis:

Green synthesized CuONPs increased the TNF-α level by 16.54%, 23.29%, 42.12% and 53.70% after the treatment with 100, 200, 500, 1000µg/kg body weight of Balb/c mice respectively. But at these above mentioned same doses, the anti-inflammatory cytokine IL-10 level decreased significantly (P<0.05) by 7.20%, 22.64%, 53.02% and 62.69% as compared to the control group (Fig.5.12).

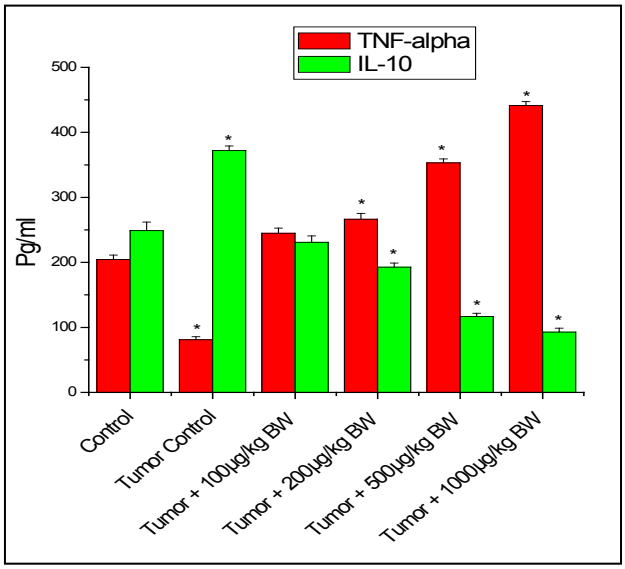


Fig. 5.12: ELISA data of serum Pro (TNF-α) and anti-inflammatory (IL-10) cytokine of green synthesized CuONPs on 4T1 cells *in vivo* model after 24hr treatment. Values are expressed as mean ± SEM, n=6; *superscripts indicate significant differences (P<0.05) compared with the both control group.

5.4.2. Apoptotic protein markers analysis by ELISA:

In case of caspases 8 and 3, the expression of protein level was increased significantly (P<0.05) by 33.85%, 46.49%, 50.58%, 59.42% and 27.14%, 8.10%, 43.01%, 49.50% respectively in comparison with the normal control group. p38 protein expression level was increased by 35.33%, 45.30%, 49.74% and 50.99% after the treatment with 100, 200, 500 and 1000 µg/kg body weight (Fig.5.13A). Caspase 9 protein, cytochrome C and Bax protein expression increased after the implication of green synthesized CuONPs which was observed from (Fig.5.13). The caspase 9 level increased by 21.61%, 29.67%, 46.44%, 52.94% and Bax level increased by 32.98%, 55.17%, 68.86% and 74.40% significantly (P<0.05) compared to the control group (Fig.5.13B). The cytochrome c expression level was elevated by 18.27%, 40.85%, 50.32% and 58.46% compared to the control tumor group. pAKT level decreased significantly (P<0.05) by 15.68%, 29.41%, 42.15%, 54.50% compared to the control group (Fig.5.13A)

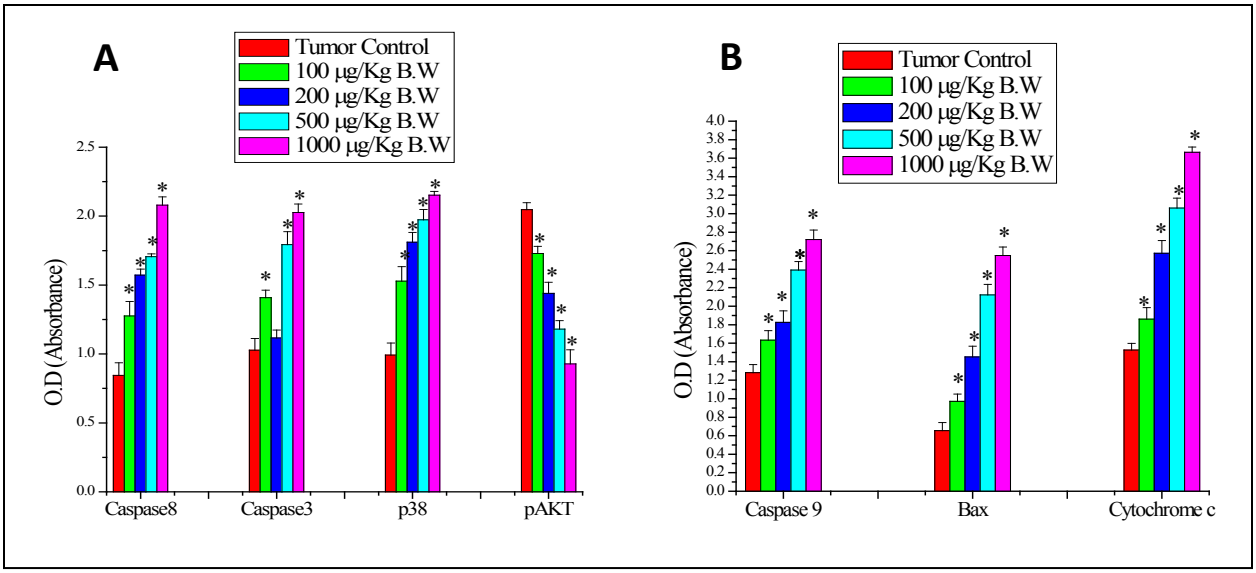


Fig. 5.13: Changes in the level of of pro-apoptotic (caspase 8, caspase 9, caspase 3, p38, cytochrome C and Bax) and anti-apoptotic (pAKT) proteins after the treatment with green synthesized CuONPs on 4T1 cells. **(A)** Caspases 3 and 8, p38 and pAKT were estimated from the 4T1 cells of Balb/c mice. **(B)** Caspase 9, Bax and cytochrome C were estimated from 4T1 cells of Balb/C mice. Values are expressed as mean ± SEM, n=6; *superscripts indicate significant differences (P<0.05) compared with the both control group.

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5.5. DISCUSSION:

In the present study, the green synthesized CuONPs, the MTT assay was performed against HeLa and MCF-7 cell lines. At 100 µg/ml, green synthesized CuONPs killed MCF-7 and HeLa cells by 87.41% and 84.28%. Mitochondrial respiration occurs outside the mitochondrial inner membrane and involves NADH and NADPH dependent mechanism that are insensitive to respiratory chain inhibitors (Berridge and Tan, 1993). Mitochondrial toxicity assay (MTT assay) being dependent on mitochondrial respiration, is involved in the successful killing activity for dosages from 1µg/ml to 100µg/ml in both cell lines. Green synthesized CuONPs showing prominent anticancer activity according to Nagajyothia et al., 2017 has been entirely supported by this present study.

At a particular dose of 100µg/ml, Cu ion internalization in the MCF-7 and HeLa cells were measured by AAS. Metal ions internalize through endocytosis depending upon the shape and size of the particles (Gyenge et al., 2011). The pH of cancer cells being different from the normal

cellular environment also influences more particle internalization in the cancer cells compared to the normal cells (Wongrakpanich et al., 2016).

Reduced Glutathione maintains a crucial role in maintaining the cellular redox balance and execution of apoptosis. Recently in case of cancer therapy, altered redox balance is the promising targeted approach for several drugs (Choi et al., 1998, Clarke, MJ. 2003). In this metal based nanotherapy, the cellular redox homeostasis was disturbed by their redox properties. When the reduced glutathione level oxidizes it converts into oxidized glutathione, which reacts with several proteins using thiol group and accumulates inside the cells (Valko et al., 2006).

In this study, elevated levels of NO generation in case of both the cancer cells (Fig.5.4) were found. The excess production of NO indicates massive oxidative stress (Gow et al., 1996) which provokes cell death and cell damage. Oxidative stress is induced mainly due to the generation of NO and ROS, produced through the mitochondrial respiratory chain (Dröse and Brandt, 2012), leading to DNA fragmentation (Fig.5.6) (Asare et al., 2012), as also verified in this study.

After confirming successful nuclear fragmentation and significant ROS generation, the apoptotic event of MCF-7 and HeLa cells were analyzed at different doses of NPs through flow cytometry study. The FACS analysis using PI and Annexin V staining showed a significant amount of early and late apoptosis of cancer cells, whereas very little amount of necrotic event also observed at high doses. This apoptotic event indicates that the cytotoxic drug green CuONPs induced apoptosis againt cancer cells (Siddiqui et al., 2013).

Most of the transition metal NPs showed toxicity by ion leaching (Pachón and Rothenberg, 2008). The NPs were stable at normal pH 7.4 as already observed in this present study. But when these NPs enter into the cancer cell microenvironment at acidic pH, the stability of NPs disrupted and these NPs released maximum Cu ions by ion leaching which was observed from pH dependent ion dissolution study (Fig.5.1B). So, the intracellular Cu ions concentration increased in acidic pH as the dose increases, which influenced the generation of ROS in cancer cells. Similar result was obtained by Chattopadhyay et al., 2015. In this study, the solid tumor weight of Balb/c mice was reduced by 60.06% compared to control tumor (Fig.5.11A) after the treatment with *A. indica* derived green synthesized CuONPs and its mean survival days increased from 18±2 to 34±2 days (Fig.5.11B).

NPs interact with the components of the immune system and can modulate them to serve specific functions (Dobrovolskaia and McNeil, 2007) like inducing pro-inflammatory cytokines which disrupt the TH1/TH2 cytokines balance (Dwivedi et al., 2011). After their application, the NPs induced immunosuppression or immunostimulation (Dobrovolskaia and McNeil, 2007). The production of pro-inflammatory cytokines like TNF-α and IL-10 (Roy et al., 2014) were also influenced by the shape of the NPs. Nitric oxide plays a crucial role in inflammation and excess production is observed in abnormal physiological states (Sharma et al., 2008).

The increase of NO by NPs may induce a pro-inflammatory response and related diseases (Park and Park, 2009). Metal oxide and carbon black NPs exhibited pro-inflammatory responses as previously reported (Niwa et al., 2008, Monteiller et al., 2007, Renwick et al., 2004). In this study also, the pro inflammatory cytokine levels (Fig.5.12) were higher compared to the control, whereas the anti-inflammatory cytokine levels were lower compared to the control, thereby indicating TH1/TH2 cytokines imbalance in cancer cells and *in vivo* tumor model.

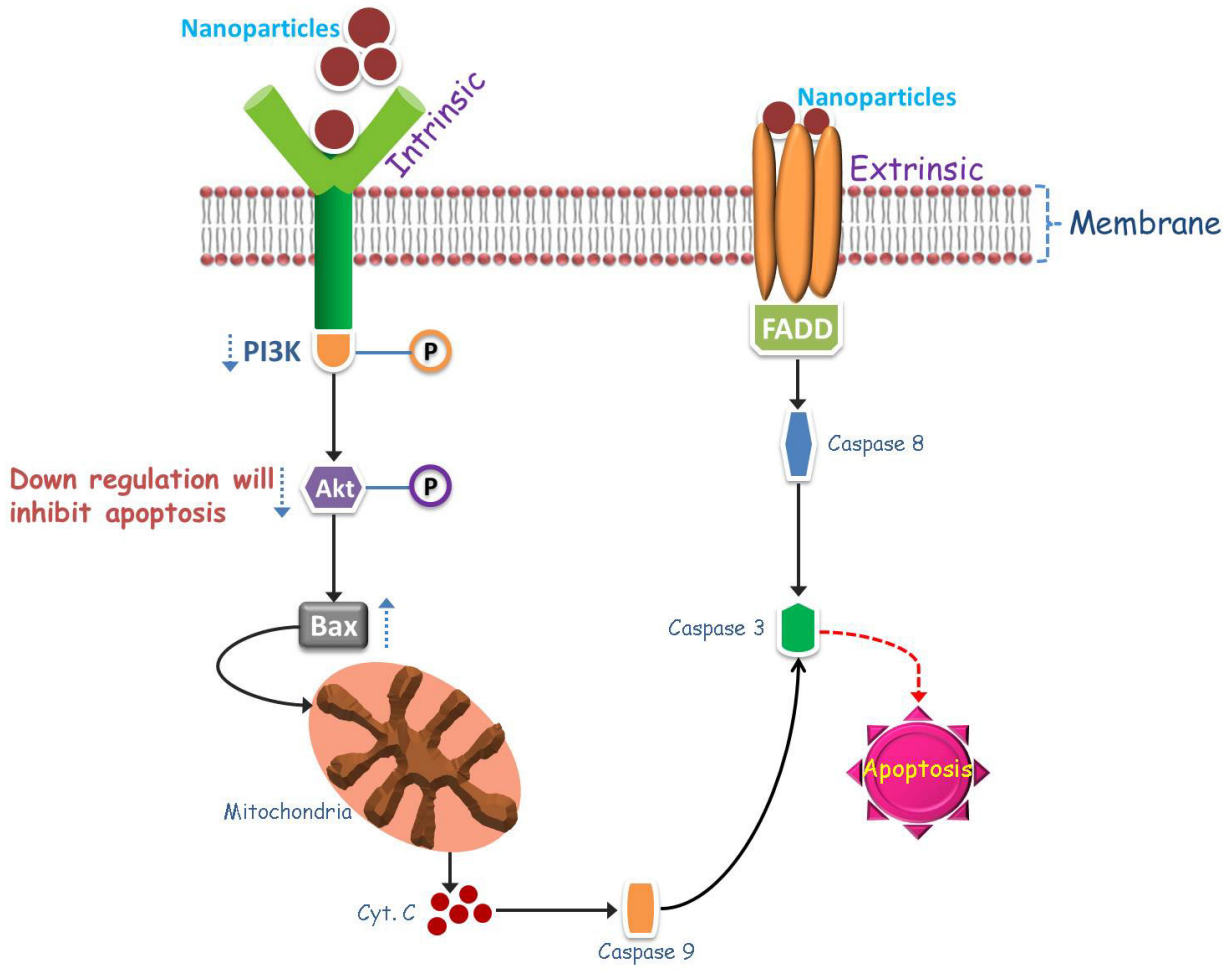
The process of apoptosis is mainly triggered and executed by key regulatory caspase proteins (Ola et al.,2011) through intrinsic and extrinsic pathways (Mo, H. 2000). The intrinsic pathway is activated by caspase 9 and subsequently by caspase 3, while the extrinsic pathway is activated through death receptor-mediated caspase 8 and then by activation of caspase 3. Most of the cytotoxic drugs triggered apoptosis through the mitochondrial pathway by both caspase 9 (Debatin, KM. 2004) and caspase 8 (Lacour et al., 2011). Caspase 8, being an initiator caspase, activates apoptosis when death receptors are stimulated and is also required by other apoptotic stimuli (Sakamaki et al., 2014). In this *in vitro* and *in vivo* study, the cytotoxic agent green synthesized CuONPs also showed a higher level of expression of caspases 3, 8 and 9. This phenomenon analysed the involvement of caspase proteins in the apoptotic event by the green synthesized CuONPs. The apoptosis of HeLa and MCF-7 cells occured due to overexpression of caspase 9 (Druškovič et al., 2006). NPs are able to activate P^{38} mitogen-activated protein kinase through nuclear factor-E2 related factor-2 and nuclear factor Kappa-B signalling pathways leading to DNA damage followed by apoptosis (Eom and Choi, 2010). Also the P^{38} expression level increased after the application of green CuONPs as clarified from the previous result (Mo, H. 2000).

Cytochrome C up-regulation was noted from (Fig.5.10A&B) *in vitro* model and in case of *in vivo* model (Fig.5.13B). The increase in the level of cytochrome C in mitochondria and cytosolic part of the cancer cells influenced transcriptional activation of the intrinsic apoptotic pathway. The release of cytochrome C in the cytosol triggered the activation of caspases in a cascade manner (Baliga and Kumar, 2003). In the present study, as the drug concentration increased the level of cytochrome C also increased. This phenomenon indicated the simultaneous activation of caspases. Caspases are mainly associated with apoptosis of cancer cells. Bax is another pro-apoptotic protein, which provokes apoptosis (Adams and Cory, 2007). Results indicated that up-regulation of Bax was observed in MCF-7 and HeLa cells by green synthesized CuONPs (Fig.5.10). So, toxic effects were observed in case of both the cancer cells. In Balb/c mice similar kind of observation was noted.

The activation of AKT does not inhibit cell death, but instead renders cells more sensitive to metabolic stress. pAKT hyperactivation occurring in many cancer cell types, played an important role in cancer cell survival and contributes to tumor cell resistance to cytotoxic therapies. The anti-apoptotic ability of AKT is anticipated to be coupled with glucose metabolism. Glucose deprivation causes AKT hyperactivation and accelerates cell death by inducing ROS overload (Los et al., 2009). The metabolic activity in the mitochondria rose due to AKT hyperactivation and inhibits FoxO transcriptional activity. At the same time, NPs treated cancer cell also generate higher level of ROS inside the cell. Both these phenomena promote cancer cell apoptosis incuded by oxidative stimuli (Dolado and Nebreda, 2008). Finally, hyper activation of ROS induces cell death, causing decrease in all the essential biomolecules needs for cells to survive.

Green synthesized CuONPs showed anticancer activity against MCF-7 and HeLa cells. Green synthesized CuONPs released higher amount of Cu ions and was successfully internalized into the cancer cells. The uptake of Cu ions in the cells initiates imbalance of GSH:GSSG ratio, elevated level of NO generation and ROS generation leading to DNA fragmentation of cancer cells. From the flow cytometry study, the percentage of apoptotic cells was validated through the increased expression of pro-apoptotic proteins expression. Elevated ROS levels also provoked the pro-inflammatory cytokines level of cancer cells. From *in vitro* and *in vivo* study, the cytotoxicity of cancer cells were significantly observed due to implication of CuONPs and the mean survival time increased in case of Balb/c mice.

The study provided the valuable information to understand the cytotoxic etiology and apoptosis pathway using green synthesized CuONPs against MCF-7 and HeLa cells as well as in Balb/c mice model.



Probable mechanism of anticancer activity by green synthesized CuONPs.