Chapter III
3.1 Introduction

Chitin is a renewable, non-toxic, non-immunogenic, biodegradable and biocompatible co-polymer of N-acetyl glucosamine and glucosamine [212-214]. Chitin and its derivatives can be modified into hydrogels, membranes, nanofibers, beads, micro/nanoparticles, scaffold and sponges and holding excellent biological and scientific properties [215-221]. Chitin and its derivatives are used for various types of biomedical applications such as drug and gene delivery, wound healing, anticancer, antimicrobial and tissue engineering [222]. The anticancer activity of chitooligosaccharides, a low molecular weight chitin derivative, with varied deacetylation (90%, 75% and 50%) and molecular weight (below 1 kDa, 1-5 kDa and 5-10 kDa), was reported to have cytotoxic effect against human myeloid leukemia HL-60 cells [79]. The distinct biological effects of chitosan against three human melanoma cell lines (A375, SKMEL28, and RPMI7951) were studied and reported that, the antitumor effect of chitosan was specific towards cell line [82]. Kaya et al. [223] studied the cytotoxic and antiproliferative effects of three-dimensional chitosan ring produced from the body segments of a diplopod in fibroblast L929 and HeLa cells. Subhapradha and Shanmugam [224] proved the anticancer potential of β-chitosan nanoparticles against human hepatoma cells. Anticancer activity of chemically prepared low molecular weight chitin (2480 kDa) from shrimp shell was also evaluated with the human monocyte leukemia cell line, THP-1 [225]. Therefore, the direct anticancer study of chitin nanoparticles (CNP) against human breast cancer cells (MCF-7) could contribute to the development of new anticancer agents.
Polymer/metal nanocomposite is a class of hybrid materials composed of an organic polymer matrix with dispersed metallic nanoparticles [226]. In recent years, the application of nanoparticles and nanocomposites have significantly increased in medicinal field which includes molecular imaging, drug delivery, diagnosis/treatment of cardiovascular diseases, wound healing, development of materials and medical devices with antimicrobial properties, cell labeling, pharmaceutical application, cancer therapy, biosensing and material chemistry [227-233]. The metal nanoparticles exhibited novel physico-chemical properties which were not observed in individual molecules or bulk metals [234]. Due to the special characteristics, (small-size effect, surface effect and quantum-size effect) the metal nanoparticles have potential applications in the fields of catalysis, magnetic recording, sensing, medical diagnosis and treatment [235]. Some metal nanoparticles, such as Pt, Pd, Ag, Au, Cu, Co, Fe and Ni have very good catalytic activities and used in various applications such as biosensing, imaging and treatment of diseases [236-239,113]. Among the noble metal nanoparticles, silver and copper are widely used due to their inexpensive and attractive biological properties such as antibacterial, antifungal, larvicidal, antiparasitic and anticancer properties [241-242]. Recently, silver nanoparticles (AgNPs) have attracted much research interest due to its applications in biomedical, drug delivery, food industries, agriculture, textile industries and water treatment [243]. Likewise, copper is an essential element for humans and most aerobic organisms and also important for the function of several enzymes and proteins involved in energy metabolism, respiration and DNA synthesis [244-246]. Most of the copper nanoparticles (CuNP) and Cu-based materials have been investigated and reported that copper ions are less toxic for normal cells, than to cancer cells [247]. The synthesis of chitin nanoparticles was already reported for the enhancement of the surface to volume ratio, physical, thermal and mechanical properties [248]. Also, the synthesis of
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Chitin/metal nanocomposites has recently generated extensive research interest for their novel optical, electronic, mechanical and catalytic properties. The chitin/silver nanocomposite (CNP/AgNP) has showed enhanced antimicrobial and mosquito larvicidal activity. Similarly, chitin/copper nanocomposite (CNP/CuNP) has exhibited enhanced non-enzymatic glucose sensing and antimicrobial property [249,250]. With above considerations, the present study was aimed to evaluate the anticancer activity of CNP/AgNP and CNP/CuNP against the MCF-7 cells, in order to develop a potent chemotherapeutic agent. The compatibility of CNP/AgNP and CNP/CuNP was also studied using a non-cancerous human embryonic kidney (HEK 293T) cell line.

3.2 Materials and methods

3.2.1 Materials

CNP, AgNP, CuNP, CNP/AgNP and CNP/CuNP (synthesized and characterized in Chapter I & II) were used without further modifications. Penicillin G, streptomycin, Trypsin–EDTA, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dual fluorescent staining solution (AO/EB), Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) were obtained from Sigma Chemicals. 2'-dichlorodihydrofluorescein diacetate (DCFH-DA) was procured from Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA. In vitro toxicology assay kit TOX7 was purchased from Sigma-Aldrich Co. OxiSelect superoxide dismutase activity assay kit was purchased from Cell Biolabs, Inc.

3.2.2 Cell culture

Human breast cancer cells (MCF-7 - ATCC HTB-22) and non-cancerous cells (HEK-293T - ATCC CRL-3216) were cultured in DMEM supplemented with 10% of FCS, 1% of penicillin/streptomycin and 10 μg/mL of insulin at 37°C in 5% CO₂ water-saturated atmosphere. Cells passaged at pre-confluent densities were used for further studies.
3.2.3 Cell viability assay

The cell viability was determined by standard MTT dye uptake method [251]. Briefly, MCF-7 and HEK 293T cells were trypsinized and plated into 96-well plates at a density of $3.0 \times 10^3$ cells/well. The cells were treated with various concentrations (20-100 µg) of CNP, AgNP, CuNP, CNP/AgNP and CNP/CuNP in triplicates. After 48 h incubation, 10 µL of MTT solution (2.5 mg/mL) was added and further incubated at 37°C and 5% CO$_2$ for 4 h. The amount of colored formazan derivative was determined by measuring optical density (OD) using TECAN microplate reader (InfiniteM200 PRO, Switzerland AG) at 570 nm. OD values were subjected to calculate the percentage of viability by using the following formula [251]

$$\text{Percentage of cell viability} = \frac{\text{OD of sample}}{\text{OD of control}} \times 100 \ldots \ldots \ldots \ldots (3.1)$$

The IC$_{50}$ values were calculated using StatPlus software (AnalystSoft Inc., USA).

3.2.4 Morphological analysis

The MCF-7 cells ($0.5 \times 10^6$) cultured in 6-well plates were treated with CNP (100 µg/mL), AgNP, CuNP, CNP/AgNP and CNP/CuNP (72, 85, 31 and 48 µg respectively) for 24 h. Similarly, the HEK 293T cells ($0.5 \times 10^6$) were also treated with CNP (100 µg/mL), AgNP, CuNP, CNP/AgNP and CNP/CuNP (76, 88, 108 and 124 µg respectively) for 24 h. After 24 h, the media was removed from the plates and washed with sterile phosphate-buffered saline (PBS) twice. The cells were observed under a computer aided inverted microscope (Olympus, PA, USA) and images were captured at 20× magnification. A minimum of 100 numbers of total cells were counted by ImageJ software (ImageJ, NIH, USA) and the numbers of morphologically varied cells were recorded for the quantification of cells with morphological changes after treatment. The experiment was conducted in triplicates. The percentage of morphologically varied cells were determined according to the following formula [252]
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Percentage of morphologically varied cells =
Number of morphologically varied cells / cells counted × 100 …………………. (3.2)

Further assays were performed using MCF-7 cells only.

3.2.5 Apoptosis assay

The MCF-7 cells (0.5 × 10^6) were treated with CNP (100 µg/mL), AgNP, CuNP, CNP/AgNP and CNP/CuNP (IC_{50} concentrations - 72, 85, 31 and 48 µg respectively) and incubated for 24 h in CO_{2} incubator at 37°C. The cells were removed by trypsinization and collected by centrifugation at 5000 rpm for 5 min. The pellet was resuspended in medium and 25 µL of the cell suspension was transferred to glass slides. Dual fluorescent staining solution (1 µL), containing 100 µg/mL of acridine orange (AO) and 100 µg/mL of ethidium bromide (EB), was added to each suspension and then covered with a coverslip. The morphology of apoptotic cells was examined and the cells were counted using a fluorescent microscope. A minimum of 100 numbers of total cells were counted and the numbers of apoptotic cells were recorded for the quantification apoptotic cells after treatment. The experiment was conducted in triplicates. The percentages of apoptotic cells were determined according to the following formula [251, 252]
Per chance of morphologically varied cells =
Number of apoptotic cells / cells counted × 100 ……………………… (3.3)

3.2.6 Reactive oxygen species (ROS) determination

Intracellular ROS was measured based on the intracellular peroxide-dependent oxidation of DCFH-DA to form the fluorescent compound 27′ -dichlorofluorescein, as previously described [253]. The cells were seeded in 24-well plates at a density of 0.5 × 10^6 cells/well and cultured for 24 h. After washing twice with phosphate-buffered saline (PBS), fresh media containing 100 µg/mL of CNP and respective IC_{50} concentrations of AgNP, CuNP, CNP/AgNP and CNP/CuNP, were added and incubated for 24 h. The cells
were then supplemented with 20 μM DCFH-DA, and the incubation continued for 30 min at 37°C. The cells were rinsed with PBS and the fluorescence intensity was determined using a multi-model plate reader with an excitation wavelength of 485 nm and emission of 495 nm.

3.2.7 Membrane integrity assay

The membrane integrity of the MCF-7 cells was evaluated by determining the activity of lactate dehydrogenase (LDH) leaking out of the cells according to the manufacturer’s instructions using in vitro toxicology assay kit. Briefly, the cells (0.5 × 10^6) were exposed to 100 μg/mL of CNP and the respective IC₅₀ concentrations of AgNP, CuNP, CNP/AgNP and CNP/CuNP for 24 h. Then 100 μl of cell-free supernatant was transferred into the wells of a 96-well plate; subsequently, 100 μl of the LDH assay reaction mixture was added to each well. After 3 h of incubation at 37°C, the optical density of the colour generated was determined at a wavelength of 490 nm using a reader TECAN microplate reader (InfiniteM200 PRO, Switzerland AG) [253].

3.2.8 Superoxide dismutase (SOD) activity assay

The SOD activity was measured using a commercial OxiSelect superoxide dismutase activity assay kit. Briefly, MCF-7 cells (0.5 × 10^6) were treated with 100 μg/mL of CNP and respective IC₅₀ concentrations of AgNP, CuNP, CNP/AgNP and CNP/CuNP for 24 h at 37°C. After treatment, the cells were washed with ice-cold PBS and then incubated under ice cold condition with 1× lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.5% Triton-100) for 10 min. The cells were centrifuged at 12000 rpm for 10 min and the supernatant (10 μl) was collected and transferred to a 96-well plate. Then 10 μl of 1× SOD assay buffer and 70 μL of deionized water were added to the supernatant. Finally, 10 μl of 3 xanthine oxidase solution was added and immediately
the absorbance was read at 490 nm. The results are expressed as a percentage of SOD activity and calculated as follows [253]

\[
\text{SOD activity} = \left( \frac{A_0}{A_1} \right) \times 100
\]

Where \( A_0 \) is the absorbance of the negative control, and \( A_1 \) is the absorbance of the cells exposed to the nanoparticles and nanocomposites.

**3.2.9 Statistical analysis**

All the experiments and assays were conducted in triplicate and repeated at least three times. The results are presented as mean ± standard deviation. All the experimental data were compared using the Student’s \( t \)-test. \( P<0.05 \) was considered statistically significant (SPSS 24.0, IBM Analytics, USA).

**3.3. Results and discussion**

**3.3.1 Cell viability assay**

The *in vitro* cytotoxicity of CNP, AgNP, CuNP, CNP/AgNP and CNP/CuNP on MCF-7 breast cancer cells was determined using the MTT assay (Fig. 3.1). CNP showed 92.77% viability at the highest concentration (100 \( \mu \)g/mL) and confirmed that nontoxic against MCF-7 cells (Fig. 3.1a). The nontoxic, week bacteriostatic and fungistatic nature of chitin was already reported in earlier studies [249,250]. However, dose dependent inhibition of cell viability was observed in AgNP, CuNP, CNP/AgNP and CNP/CuNP in the range of 20-100 \( \mu \)g/mL concentration (Fig. 3.1b-e). At the concentration 100 \( \mu \)g/mL AgNP, CuNP, CNP/AgNP and CNP/CuNP showed 23.50%, 38.17%, 11.27% and 18.67% of cell viability respectively. From the assay, the determined IC\(_{50}\) values for AgNP, CuNP, CNP/AgNP and CNP/CuNP were 72 \( \mu \)g, 85 \( \mu \)g, 31 \( \mu \)g and 48 \( \mu \)g respectively (Table 3.1).

The composite materials (CNP/AgNP and CNP/CuNP) showed a higher degree of inhibition (31 \( \mu \)g and 48 \( \mu \)g) than free nanoparticles (CNP, AgNP and CuNP). The increase in the inhibitory activity of the composite material could be explained by the
stable interaction between the silver and copper nanoparticles with the CNP matrix [254]. Furthermore, the enhanced surface area and inhibition of agglomeration of AgNP and CuNP in the composite material are important factors for the enhancement of cytotoxicity [255]. Altogether, CNP/AgNP has showed pronounced inhibitory effect in cell viability than CNP, AgNP, CuNP and CNP/CuNP. The phenomenon may attribute to the combined effect of the supporting material and the metallic nanoparticle [256].

In the case of HEK-293T cells, similar to MCF-7 cells, CNP exposure showed 92.50% viability at the highest concentration (100 \( \mu g/mL \)) and confirmed that nontoxic against HEK-293T cells (Fig. 3.2a). However, dose dependent inhibition of cell viability was observed in AgNP, CuNP, CNP/AgNP and CNP/CuNP in the range of 20-100 \( \mu g/mL \) concentration (Fig. 3.2b-e). At the concentration of 100 \( \mu g/mL \) AgNP, CuNP, CNP/AgNP and CNP/CuNP showed 24.16%, 58.16%, 55.60% and 65.66% of cell viability respectively. From the assay, the determined IC\(_{50}\) values for AgNP, CuNP, CNP/AgNP and CNP/CuNP were 76 \( \mu g \), 88 \( \mu g \), 108 \( \mu g \) and 124 \( \mu g \) respectively (Table 3.1). Compared to MCF-7 cells, the toxicity was reduced in CNP/AgNP and CNP/CuNP against HEK-293T whereas the AgNP and CuNP retained their toxicity in HEK-293T cells also. The results confirmed that, after incorporation with CNP, the activity of the composite material was specific towards cell lines. The specificity of chitin derivative (chitosan) towards cell line was already reported by Gibot et al. [82]. Relating to the present study, CNP acted as a supporting material that decreased the agglomeration of AgNP and showed enhanced anticancer activity against MCF-7 cells and decreased toxicity against HEK-293T.
Figure 3.1: Cytotoxic effects and percentage of viability of MCF-7 cells treated with (a) CNP, (b) AgNP, (c) CuNP, (d) CNP/AgNP and (e) CNP/CuNP. Error bar represent SD from the mean (n = 3).
Figure 3.2: Cytotoxic effects and percentage of viability of HEK-293T cells treated with (a) CNP, (b) AgNP, (c) CuNP, (d) CNP/AgNP and (e) CNP/CuNP. Error bar represent SD from the mean (n = 3).
**Table 3.1:** Cytotoxic activity (IC$_{50}$ values (µg/mL)) of synthesized nanoparticles and nanocomposites in MCF-7 and HEK-293T cells.

<table>
<thead>
<tr>
<th>Samples</th>
<th>MCF-7</th>
<th>HEK-293T</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNP</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>AgNP</td>
<td>72</td>
<td>76</td>
</tr>
<tr>
<td>CuNP</td>
<td>85</td>
<td>88</td>
</tr>
<tr>
<td>CNP/AgNP</td>
<td>31</td>
<td>108</td>
</tr>
<tr>
<td>CNP/CuNP</td>
<td>48</td>
<td>124</td>
</tr>
</tbody>
</table>

### 3.3.2. Morphological analysis

To confirm the cell viability assay, the effect of CNP, AgNP, CuNP, CNP/AgNP and CNP/CuNP on MCF-7 cell’s morphology was evaluated. Figure 3.3a-f shows the microscopic images of the MCF-7 cells in the presence and absence (control) of respective nanoparticles and nanocomposites. Control cells (Fig. 3.3a) retained their normal polygonal shape with intact monolayer appearance. Similarly, cells treated with CNP (Fig. 3.3b) also showed polygonal shape with intact monolayer appearance, whereas, the reduced cell number and significant difference in cell morphology were observed in the cells treated with AgNP, CuNP, CNP/AgNP and CNP/CuNP (Fig. 3.3c-f). The morphological variations like cellular shrinkage, membrane blebbing, apoptotic bodies were observed (Table 3.2) and which were leads to growth retardation, reduction of the cell monolayer area and poor adherence. Morphological analysis of the treated cells suggested that AgNP, CuNP, CNP/AgNP and CNP/CuNP induce apoptosis in MCF-7 cells. Number of apoptotic cells and morphologically varied cells was observed more during CNP/AgNP and CNP/CuNP treatment in MCF-7 cells. Pronounced effect on cell morphology was observed in the CNP/AgNP treated cells than on AgNP, CuNP, and CNP/CuNP treated cells. The results are in concordance with the previous report of Gurunathan et al. [257], suggested that nanocomposite with silver nanoparticle embedded.
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in reduced graphene oxide showed potential morphological alterations in cancer cells than free graphene oxide and silver nanoparticle. Menon et al. [258] also reported the nano-encapsulation of polyoxometalate by chitosan with enhanced toxicity towards different cancer cell lines like KB, MCF-7, PC-3 and A549.

The effect of CNP, AgNP, CuNP, CNP/AgNP and CNP/CuNP on HEK-293T cell’s morphology was also evaluated. Figure 3.4a-f shows the microscopic images of the HEK-293T cells in the presence and absence (control) of respective nanoparticles and nanocomposites. Control cells and the cells treated with CNP retained their normal polygonal shape with intact monolayer appearance (Fig. 3.4a,b). By contrast to MCF-7 cells, the reduced cell number and difference in cell morphology were observed higher (Table 3.2) in cells treated with AgNP and CuNP than CNP/AgNP and CNP/CuNP (Fig. 3.4c-f). The morphological analysis also confirmed the cell line specific activity of the CNP/AgNP and CNP/CuNP. This study confirmed that the composite materials (CNP/AgNP and CNP/CuNP) showed higher level of toxicity against cancerous cells compared to non-cancerous cells. Silva et al. [259] also reported that the composite material (ZnO-Fe₃O₄) showed highly selective activity towards cancerous cells than the normal cells.

**Table 3.2:** Percentage of morphologically varied cells after treated with synthesized nanoparticles and nanocomposites in MCF-7 and HEK-293T cells.

<table>
<thead>
<tr>
<th></th>
<th>CNP</th>
<th>AgNP</th>
<th>CuNP</th>
<th>CNP/AgNP</th>
<th>CNP/CuNP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MCF-7</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>1.67±0.58</td>
<td>9.67±1.53</td>
<td>9.67±0.58</td>
<td>15.33±0.58</td>
<td>13.33±1.15</td>
</tr>
<tr>
<td>MB</td>
<td>1.33±0.58</td>
<td>18.00±2.00</td>
<td>10.33±1.53</td>
<td>15.67±1.53</td>
<td>13.00±1.00</td>
</tr>
<tr>
<td>AB</td>
<td>0</td>
<td>21.67±1.15</td>
<td>18.33±0.58</td>
<td>37.33±3.21</td>
<td>24.67±2.51</td>
</tr>
<tr>
<td><strong>HEK-293T</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>0.33±0.53</td>
<td>8.66±0.58</td>
<td>12.33±1.53</td>
<td>12.00±1.73</td>
<td>10.33±2.08</td>
</tr>
<tr>
<td>MB</td>
<td>0.67±0.58</td>
<td>19.67±1.15</td>
<td>10.67±0.58</td>
<td>14.67±1.15</td>
<td>10.33±3.51</td>
</tr>
<tr>
<td>AB</td>
<td>0</td>
<td>19.00±1.73</td>
<td>16.67±0.58</td>
<td>14.33±2.08</td>
<td>12.67±2.08</td>
</tr>
</tbody>
</table>

CS-Cellular shrinkage; MB- Membrane blebbing; AB- Apoptotic bodies

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Figure 3.3: Morphology of MCF-7 cells: (a) control cells (b) treated with CNP, (c) AgNP, (d) CuNP, (e) CNP/AgNP and (f) CNP/CuNP. Arrows show (1) Normal cell, (2) cellular shrinkage, (3) membrane blebbing and (4) apoptotic bodies. The numbers of morphological varied cells are higher in CNP/AgNP and CNP/CuNP treatment.
Figure 3.4: Morphology of HEK-293T cells: (a) control cells (b) treated with CNP, (c) AgNP, (d) CuNP, (e) CNP/AgNP and (f) CNP/CuNP. Arrows show (1) Normal cell, (2) cellular shrinkage, (3) membrane blebbing and (4) apoptotic bodies. The numbers of morphological varied cells are higher in CNP/AgNP and CNP/CuNP treatment.
3.3.3 Apoptosis assay

Apoptosis is defined by a characteristic set of changes in cell morphology during cell death [260]. Following the observation of cell morphology, differential uptake of fluorescent DNA binding dyes such as acridine orange/ethidium bromide staining (AO/EB) is a suitable method for the evaluation of cellular viability and apoptosis. In the present study, AO/EB fluorescence staining was used to confirm the nanoparticle and nanocomposite induced apoptosis in MCF-7 cells. Control cells and CNP treated cells (Fig. 3.5a, b) were observed as uniform green cells, which confirm the presence of viable cells. Since ethidium bromide stain was impermeable to viable cells; the live cells have round nuclei which were stained green [261]. On the contrary, the cells treated with AgNP, CuNP, CNP/AgNP and CNP/CuNP (Fig. 3.5c-f) showed the mixture of apoptotic cells and viable cells. Apoptotic cells in the early-stage were marked by granular yellow-green and apoptotic cells in the late-stage were marked by concentrated and asymmetrically localized orange nuclei [262]. Necrotic cells were marked by round nuclei which were stained orange. From the AO/EB staining, it was confirmed that AgNP, CuNP, CNP/AgNP and CNP/CuNP were inducing apoptosis in MCF-7 cells (Table 3.3). The number of apoptotic cells (both early and late-stage apoptotic cells) was higher in CNP/AgNP than AgNP, CuNP, and CNP/CuNP. The results were in concordance with MTT assay and cell morphology of the present study.

Table 3.3: Percentage of morphologically varied cells after treated with synthesized nanoparticles and nanocomposites in MCF-7 cells.

<table>
<thead>
<tr>
<th>MCF-7</th>
<th>Percentage of apoptotic and necrotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CNP</td>
</tr>
<tr>
<td>Early apoptotic cells</td>
<td>0</td>
</tr>
<tr>
<td>Late apoptotic cells</td>
<td>0</td>
</tr>
<tr>
<td>Necrotic cells</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 3.5: (a) AO/EB staining of MCF-7 cells, (b) after treated with CNP, (c) AgNP, (d) CuNP, (e) CNP/AgNP and (f) CNP/CuNP. Cells stained in green are viable cells whereas cells stained in granular yellow-green are early-stage apoptotic cells and the cells stained in orange are late-stage apoptotic cells. Arrows show (1) Normal cell, (2) early apoptotic cells, (3) late apoptotic cells and (4) necrotic cells.
3.3.4. Reactive oxygen species (ROS) determination

Oxidative stress (generation of ROS) is one of the major proposed cytotoxicity mechanisms of various nanomaterials [261]. The effect of CNP, AgNP, CuNP, CNP/AgNP and CNP/CuNP on ROS generation and the fluorescence intensity of the generated ROS was studied (Fig. 3.6). The control MCF-7 cells showed the fluorescence intensity of 0.339 and the cells treated with CNP showed the fluorescence intensity of 0.352, whereas, the cells treated with AgNP, CuNP, CNP/AgNP and CNP/CuNP have generated significantly higher ROS compared to control. The fluorescence intensity observed from the cells treated with AgNP, CuNP, CNP/AgNP and CNP/CuNP were 0.899, 0.826, 1.070 and 0.927 respectively. The data confirmed that the ROS generation from the cells treated with the composite material was significantly higher than the free nanoparticles. CNP/AgNP showed highest ROS generation among the tested nanoforms. The increased generation of ROS in CNP/AgNP treated cell was due to the higher cellular adhesion and uptake of the CNP/AgNP [257]. The enhanced ROS generation was concordance with the results of Menon et al. [258], who reported the enhanced intracellular ROS release by chitosan/polyoxometalate nano-complex compared to the negative control in keratin-forming tumor cell line (KB), which is an indicative of an additional stress contributed by the nano-complex. In the present study, the AgNP, CuNP, CNP/AgNP and CNP/CuNP induce the ROS generation in MCF-7 cells which signify that generating ROS in the cells can be a possible mechanism of cancer cell death. Similar results were observed in previous studies performed in various cell lines. Castro-Aceituno et al. [251] reported the enhanced ROS generation from AgNP synthesized from *Panax ginseng* fresh leaves and the relation between ROS generation and apoptosis. Avalos et al. [252] also reported that increased intracellular ROS generation in the hepatoma and leukemia cells treated with silver nanoparticles. Acilan et al. [247] reported
that copper complexes have the ability to induce the generation of ROS in human cervical cancer (HeLa) and colon adenocarcinoma (HCT-116) cells. The increased ROS generation was responsible for the reduced cell viability in MCF-7 cells treated with composite material. The results were highly correlated with the results of MTT assay and apoptosis study.

**Figure 3.6:** Intracellular ROS generation in MCF-7 cells treated with CNP, AgNP, CuNP, CNP/AgNP and CNP/CuNP. The results are expressed as the mean ± SD of three independent experiments. The AgNP, CuNP, CNP/AgNP and CNP/CuNP treated cells showed statistically significant differences from the control group (* P < 0.05).

### 3.3.5. Membrane integrity assay

The membrane integrity of the MCF-7 cells was evaluated by measuring the activity of lactate dehydrogenase (LDH) leaking from the cell membrane. The level of LDH in the culture media indicates the extent of damage caused by the toxic agent. The LDH level was expressed as OD value obtained from the experiment (Fig. 3.7). The OD values for non-treated (control) and CNP cells were 0.270 and 0.282 respectively. The results evidenced that CNP was not significantly causing membrane damage and release of LDH.
However, AgNP, CuNP, CNP/AgNP and CNP/CuNP caused significant damage in the membrane integrity, hence the release of LDH was very high. The observed OD value for AgNP, CuNP, CNP/AgNP and CNP/CuNP treated MCF-7 cells were 0.965, 0.777, 1.240 and 1.048 respectively. The level of intracellular LDH released into the culture medium was directly proportional to the cell membrane damage caused by toxic materials [263]. Dose and time-dependent induction of damage in membrane integrity and leakage of LDH from silver and copper nanoparticle treated cells were also reported in earlier studies [264,265]. In the present study, amount of LDH released from the composite treated cells was higher than the cells treated with nanoparticles. The results confirmed that, combined action of CNP and the metallic nanoparticles (AgNP and CuNP) caused damage to the membrane integrity of MCF-7 cells. Although AgNP, CuNP and CNP/CuNP induce LDH leakage, CNP/AgNP has a remarkable response and significant impact on the membrane integrity of MCF-7 cells. Similarly, Gurunathan et al. [257] also reported reduced graphene oxide-silver (rGO–Ag) nanocomposite showed elevated LDH leakage than rGO and AgNP alone. The present study showed that the LDH leakage strongly depend on the supporting material (CNP) as well as physical and chemical properties of the metallic nanoparticles.
Figure 3.7: Leakage of LDH to culture supernatant in MCF-7 cells treated with CNP, AgNP, CuNP, CNP/AgNP and CNP/CuNP. The results are expressed as the mean ± SD of three independent experiments. The AgNP, CuNP, CNP/AgNP and CNP/CuNP treated cells showed statistically significant differences from the control group (* P < 0.05).

3.3.6. Superoxide dismutase (SOD) activity

To investigate the potential role of oxidative stress in cytotoxicity of the nanomaterials, the effects of CNP, AgNP, CuNP, CNP/AgNP and CNP/CuNP on SOD level was measured. SOD is an antioxidant enzyme which regulates the intracellular ROS [266]. In control MCF-7 cells, the level of SOD was considered as 100%, whereas the significant changes were observed in the activity of SOD in the cells treated with CNP/AgNP and CNP/CuNP (58.91% and 65.86% respectively) (Fig. 3.8). The CNP/AgNP treated cells showed very low SOD activity (58.91%) compared to other nanoparticles and composite material. In the present study, the treated cells showed elevated ROS level and low SOD activity which leads to enhanced oxidative stress. The results were in good agreement with the previous study which reported that an increased ROS generation and decreased level of antioxidant enzyme could lead to oxidative stress [257].
Figure 3.8: Effect of CNP, AgNP, CuNP, CNP/AgNP and CNP/CuNP in the SOD level in MCF-7 cells. The results are expressed as the mean ± SD of three independent experiments. The AgNP, CuNP, CNP/AgNP and CNP/CuNP treated cells showed statistically significant differences from the control group (* P<0.05).

3.4 Conclusion

In the present study, the anticancer activity of CNP, AgNP, CuNP, CNP/AgNP and CNP/CuNP was evaluated against MCF-7 cells. The CNP incorporated metal nanocomposites showed enhanced cytotoxicity than AgNP and CuNP alone in MCF-7 cells. The combined activity of chitin and metallic nanoparticle has enhanced the anticancer effect of the nanocomposites. The CNP/AgNP showed significant cytotoxicity and induction of apoptosis in MCF-7 cells. In addition, after incorporating with CNP the toxicity of AgNP and CuNP against non-cancerous HEK-293T cells was reduced, and indicated that the composite material was highly active towards cancerous cells. The application of chitin, in combination with metallic nanoparticles, could be used for the development of novel anticancer therapeutics and may provide potential platform for cancer management.