Copper (II) Oxide Nanoparticles Induce High Toxicity in Human Neuronal Cell

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Abstract

Copper (II) oxide nanoparticles (CuO-NPs) are widely used in industry, cosmetics and medicine. People have increasingly been exposed to these active materials. Several studies indicate that CuO-NPs could be taken up by different organs and cause toxicities. However, there is still a lack of data on the toxicological effects of CuO-NPs in neuronal system. In the present study, the toxic potentials of CuO-NPs were investigated in human SH-SY5Y neuroblastoma cells. After assessment of their cellular uptake potential, cytotoxicity by MTT and neutral red uptake (NRU) and genotoxicity by comet assay were evaluated. Enzyme-Linked Immune Sorbent Assays (ELISA) determination of malondialdehyde (MDA), 8- hydroxy-deoxyguanosine (8-OHdG), protein carbonyl (PC), and glutathione (GSH) levels for oxidative damage, and Annexin V-FITC with propidium iodide (PI) for apoptosis were used. In conclusion, CuO-NPs were found to accumulate in the cells and induced significant cytotoxic and genotoxic, and oxidative and apoptotic effects. CuO-NPs are hypothesized to dangerously affect human health, especially neuronal system. However, further studies should be done to elucidate their toxic mechanism.

Index terms— copper oxide; nanoparticle; neurotoxicity; cellular uptake; genotoxicity; apoptosis.

1 I. Introduction

uO-NPs are widely used in gas sensors, catalysts, high temperature conductors, solar energy converters and antimicrobial agents owing to their high temperature conductivity, electron correlation effects, antimicrobial activity and special physicochemical properties in various fields (Chang et al., 2012;Huang et al., 2010). Indeed, as it is well known, nanoparticles exist as contaminants in water, air and food products as outputs of natural phenomena or due to the high increase in the anthropogenic activity (Ahamed et al., 2013;Elsaesser et al., 2011;Kim et al., 2010). CuO-NPs caused changes in different organs like lung, kidney, renal tubular, liver, spleen, gastrointestinal tract and stomach tissue (Barceloux, 1999;Cho et al., 2012;Lei et al., 2008;Manna et al., 2012). Acute death, abnormalities in the embryo and gill damage were observed in Zebra fish exposed to CuO-NPs (Griffitt et al., 2007; **??**eo et al., 2009). The toxicity studies of CuO-NPs have been focused more generally on the pulmonary system and to a lesser extent on skin, breast, intestine and liver **??**Ahamed et Perreault et al., 2012). Therefore, it was aimed to evaluate the toxicity and possible mechanism of action of CuO-NPs in neuroblastoma cells following their cellular uptake potential.

2 II. Materials and Methods

Chemicals: Eagle's minimum essential medium (EMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS, 10X), antibiotic solutions and ethylene diamine tetraacetic acid (EDTA) were purchased from Multicell Wisent (Quebec, Canada). Triton X-100 and MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) were purchased from Biomatik (Ontario, Canada). GSH, 8-OHdG, MDA and PC ELISA kits were

purchased from Yehua Biological Technology Co., Ltd. (Shanghai, China). Annexin V-FITC apoptosis detection kit with PI and dye reagents for protein assay were obtained from Exbio (Vestec, Czech Republic) and Biorad (Munich, Germany), respectively. All other chemicals were obtained from Merck (NJ, USA).

CuO-NPs were obtained from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). The CuO-NPs suspensions in milli-Q water and cell culture medium with 10% FBS, were measured by Transmission Electron Microscopy (TEM) (Jem-2100 HR, Jeol, USA) (Abudayyak et al. 2016;2016a). The average diameter was calculated by measuring over 100 particles in random fields of TEM view.

Copper release into cell medium: Copper release from CuO-NPs into the cell culture medium was determined using the Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Thermo Elemental X series 2, USA) method (Abudayyak et al. 2016;2016a). The released amount of copper was analyzed by ICP-MS. Cu content of the cell culture medium was also measured. Cell culture conditions: Human neuronal cell line (SH-SY5Y) was obtained from the American Type Culture Collection (CRL-2266?, ATCC, VA, USA). The cells were incubated in EMEM medium supplemented with FBS 10% and antibiotics at 5% CO 2 , 90% humidity and 37°C for 24 h (60-80% confluence). Cell densities were in the range from 1× 10 5 to 1×10 7 cells/mL for all assays (Abudayyak et al. 2016;2016a). Exposure occurred for 24 h. Cellular uptake and morphology examinations: It was evaluated by ICP-MS and TEM (Abudayyak et al. 2016;2016a). The cells were washed several times with equal volumes of PBS and cell culture medium with 10% FBS and counted via Luna cell counter (Virginia, USA) following exposure to two different concentrations of the particle suspension (2.5 and 25 µg/mL). Ultra-thin sections (50-60 nm) were cut by an ultra-microtome (Reichert UM 3, Austria). Sections were analyzed and photographed using a TEM (Jeol-1011, Tokyo, Japan) with attached digital camera (Olympus-Veleta TEM Camera, Tokyo, Japan). Cytotoxicity assays: Cytotoxic activities of CuO-NPs on SH-SY5Y cells were determined by MTT and NRU assays based on different cellular mechanisms (Abudayyak et al. 2016;2016a; **??**epetto et al., 2008; **??**an Meerloo et al., 2011). Optical density (OD) values were read at 590 and 540 nm for MTT and NRU, respectively, using a microplate spectrophotometer system (Epoch, Germany). In every assay, unexposed cells were served as a negative control. The inhibition of enzyme activity was calculated as compared to a negative control. The half-maximal inhibitory concentration (IC 50) was then expressed as the concentration of the sample causing a 50% inhibition of enzyme activity in cells. The CuO-NP concentrations were 2.5-60 µg/mL in the cytotoxicity assays. Genotoxicity assay: Genotoxic activities of CuO-NPs were determined by comet assay (Abudayyak et al. 2016;2016a;Collins et al., 2004; **??**peit et al., 1999). Hydrogen peroxide (H 2 O 2) (100 µM) and PBS were used as positive and negative controls, respectively. The number of DNA breaks was scored under a fluorescent microscope (Olympus BX53, Olympus, Tokyo, Japan) at 400X magnification using an automated image analysis system (Comet Assay IV, Perceptive Instruments, Suffolk, UK). DNA damage to individual cells was expressed as a percentage of DNA in the comet tail (tail intensity %). The CuO-NP concentrations were 5-50 µg/mL in the comet assay. Oxidative damage assays: The oxidative damage potentials of CuO-NPs were measured by human GSH, MDA, 8-OHdG, or PC ELISA kits with different endpoints according to the manufacturer's instructions. The OD value was read at 450 nm using a microplate spectrophotometer system. In every assay, the unexposed cells served as a negative control. The protein amount in 10 6 cells was measured according to Bradford (1976). Results were expressed as µmol, µmol, µg, and µg per g protein for GSH, MDA, 8-OHdG, and PC, respectively, using a standard calibration curve. The CuO-NP concentrations were 5-25 µg/mL in the oxidative damage assays. Apoptosis assay: The cellular apoptosis or necrosis was determined by Annexin V-FITC apoptosis detection kit with PI (Abudayyak et al. 2016;2016a). In every assay, the untreated cells served as a negative control. The results were expressed as a percentage of the total cell amount. The CuO-NP concentrations were 10-80 µg/mL in the apoptosis assay. Statistical analysis: The assays were done in triplicate and repeated four times. Data were expressed as mean±standard deviation (SD). Significant differences between untreated and treated cells were calculated by one-way ANOVA Dunnett t-test using SPSS version 17.0 for Windows. p values of less than 0.05 were considered significant.

3 III. Results and Discussion

Particle size and distribution: According to the X-ray diffraction results supplied by the manufacturer (Sigma Chemical Co. Ltd., USA), the surface area of CuO-NPs was 29 m 2 /g (Figure 1). The average size was observed to be 34.9 nm with a narrow size distribution (ranging from 16.7-64.2 nm) after suspending in water. When suspending in the culture medium, the size of the particles was found to be slightly agglomerated and/or aggregated with 38.8 nm (ranging from 18.8-73.8 nm) (Figure 2). The copper ion release of CuO-NPs was evaluated in the cell culture medium. Although the concentration was 3.1 ± 0.322 µg/mL, which represented 15.5% of the nanoparticles, in the CuO-NPs cell culture suspension, there was no observed copper ions in the cell culture medium. Based on that, the observed toxicological endpoints and morphological changes were mainly due to CuO-NPs.

Cellular uptake: ICP-MS revealed that the particles were taken up by SH-SY5Y cells in the range of 0.390-0.917 µg/10 5 cells in concentration dependent manner following exposure to CuO-NPs at 5-25 µg/mL concentrations (Table **??**). Some researchers reported iron oxide and two different types of titanium dioxide nanoparticles to enter SH-SY5Y cells in concentration dependent manner (Kilic et al., 2016; **??**aldiglesias et al., 2013).

Cellular morphology by TEM: The particles were observed in the cytoplasmic vacuoles. Mitochondria were visible in few of the cells exposed to both 2.5 and 10 µg/mL CuO-NPs. Some cells exposed to 2.5 µg/mL CuO-

NPs revealed nuclear fragmentation. The electronlucent cytoplasmic vacuoles lead to complete disruption of the cytoplasm in few of the cells (Figure 3). respectively. The reduction in cell viability was concentration-dependent (Figure 4). The CuO-NPs were found to cause cytotoxic effects to HaCaT keratinocytes, BALB3T3 embryonic fibroblasts **??** (Akhtar et al., 2016). Also, it could be via disruption of cell membrane integrity (Cronholm et al., 2011). However, there was no study about genotoxicity on SH-SY5Y cells.

Oxidative damage: The oxidative damage potential of CuO-NPs was evaluated by measuring cellular levels of GSH, MDA, 8-OHdG, and PC (Table **??**). CuO-NPs induced oxidative damage resulting in significant decrease in the GSH levels (?46.1%). Although an increase on the levels of MDA (?1.33 fold) was observed it was not significant. On the other hand, the levels of PC and 8-OHdG protein and DNA oxidative damage biomarkers did not change. In previous studies, it was observed that CuO-NPs induced oxidative damage in HaCaT keratinocytes (Alarifi et al., 2013) (Piret et al., 2012; **??**iddiqui et al., 2013). The reduction in cell viability observed could be due to an increase in oxidative stress after CuO-NPs exposure.

Apoptosis: Death in SH-SY5Y cells was significantly induced by CuO-NPs, with a maximum percentage of 73.4 and 40.0% for apoptosis and necrosis, respectively. According to our results, apoptosis was seen to be the main pathway for cell death in the SH-SY5Y cell line. At the highest exposure concentration (40 µg/mL), the apoptosis percentage was 79.2% of the dead cells (Figure 6). The previous studies showed CuO-NPs could induce apoptosis in the following cells: MCF7 breast cancer (Laha et al., 2014) **??**013) observed CuO-NPs induced apoptosis via a decrease in mitochondrial membrane potential with a concomitant increase in the gene expression ratio of Bax/Bcl2, up-regulation of p53 tumour suppressor and caspase-3 apoptotic genes. Also, the researchers showed apoptosis could be induced by reduction of BAD phosphorylation and an increase in cleaved caspase-3 products (Laha et al., 2014). An et al. (2012) indicated that the apoptosis and cognitive impairment could be via increased cleaved caspase-3 levels on hippocampal CA1 neuron in rats.

4 IV. Conclusion

Generally, the studies about Cu based nanoparticles and CuO-NPs were focused on the pulmonary system. However, very few researchers were concerned about the possible toxicity over other systems. In the present study, it was observed that CuO-NPs taken up by the neuronal cells could produce cytotoxic, genotoxic, and apoptotic effects, as well as oxidative damage in the neuronal cells in vitro. Their commercial and industrial applications should be carefully evaluated because of their potential hazardous effects on human health. Further in vivo studies are needed to fully understand the toxicity mechanisms of CuO-NPs.

5 V. Acknowledgement

This work was supported by the Research Fund of Istanbul University (Project No: 52253). Dr. M. Abudayyak carried out cell culture and exposure conditions, the toxicological assays and the particle characterisation. Prof. Dr. G. Özhan participate the toxicological assays and carried out the evaluation of the results. Dr. E. Guzel carried out the uptake and morphological changes in the cells. All authors wrote, read and approved the manuscript. Also, the authors declare there is no conflict of interest. All experiments were done in triplicate and each assay was repeated four times.

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The results were expressed as the mean cell death (%) compared to negative control (unexposed cell). [1] [2]

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[2] © 2016 Global Journals Inc. (US)Copper (II) Oxide Nanoparticles Induce High Toxicity in Human Neuronal Cell

Figure 1:

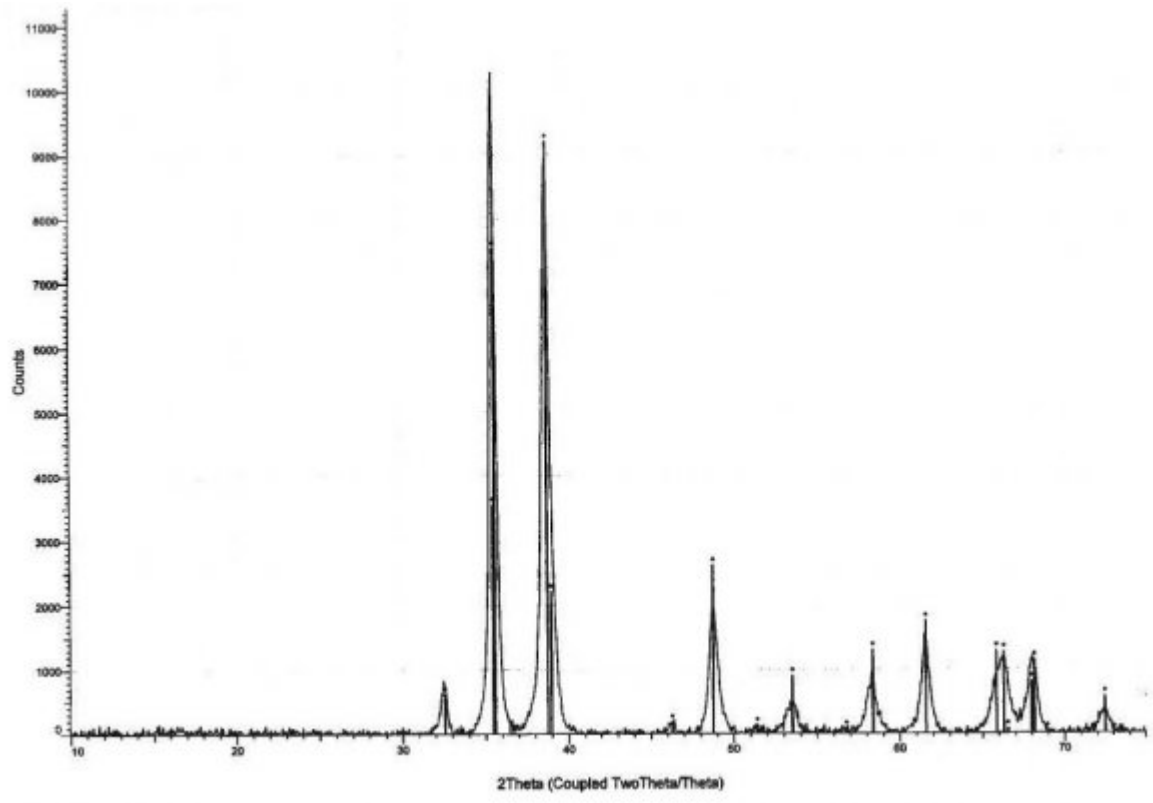

Figure 2:

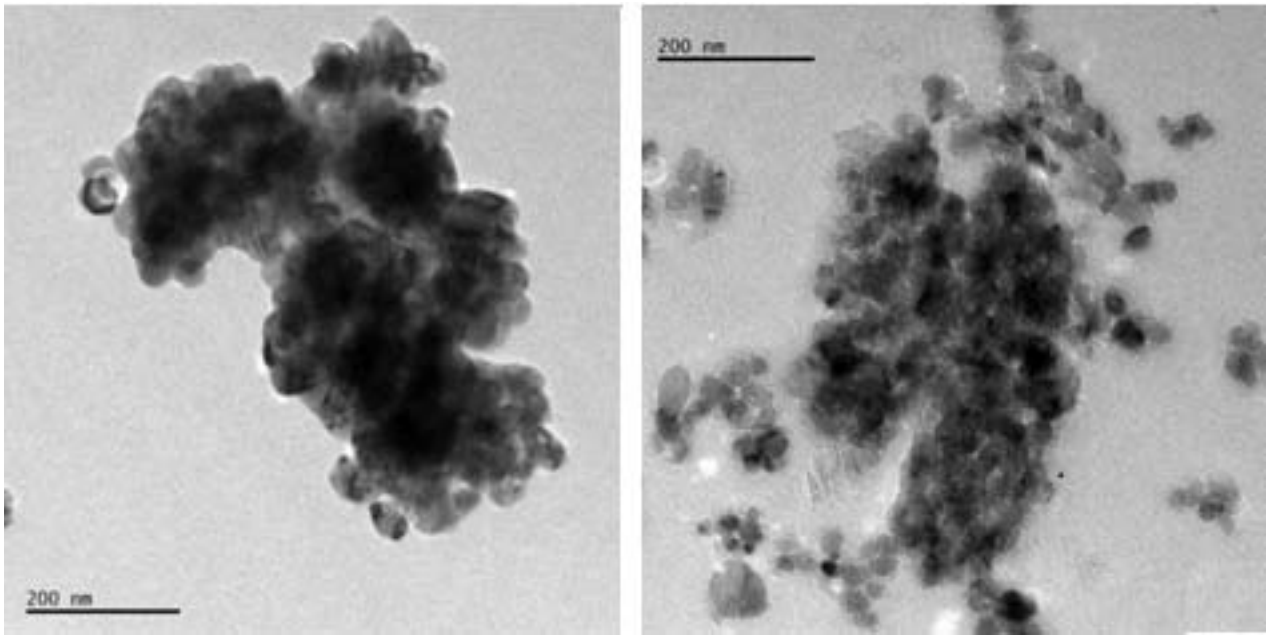

Figure 3:

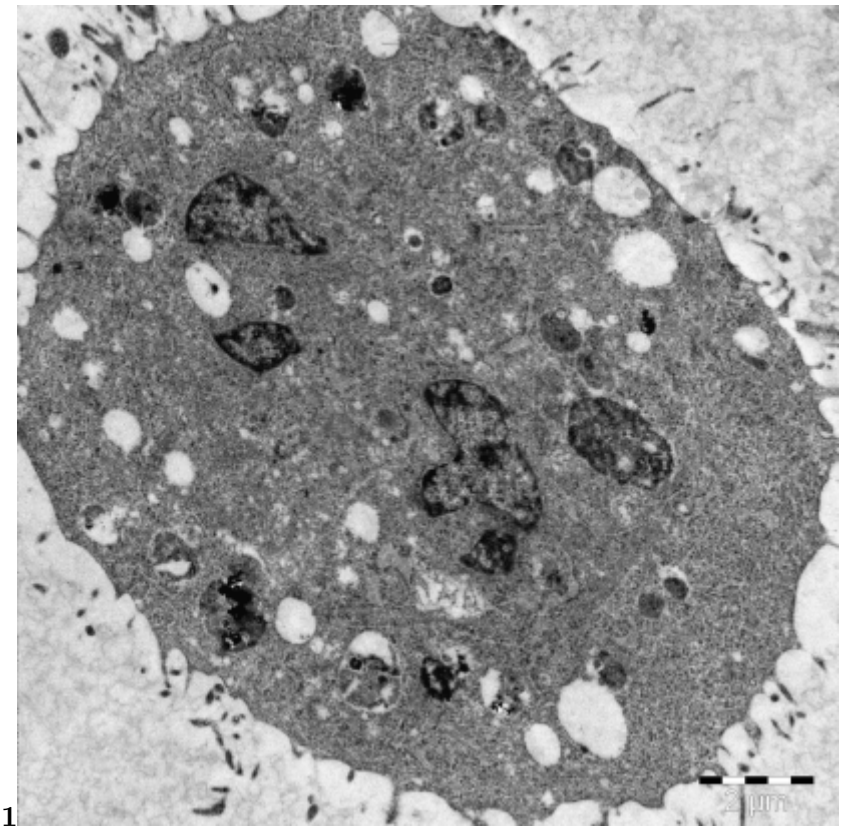

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Figure 4: Figure 1 :

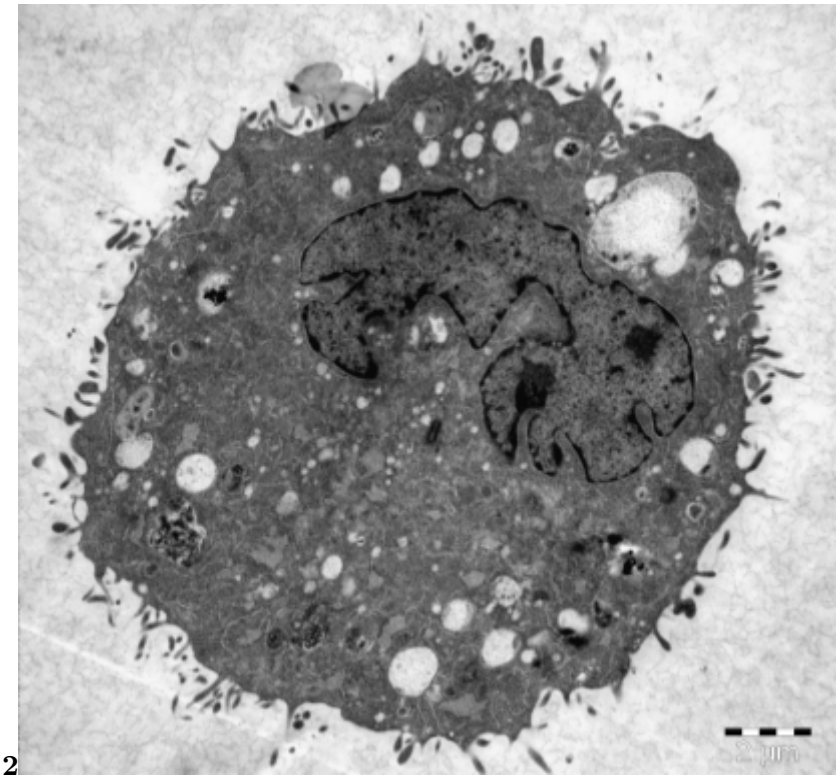

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Figure 5: Figure 2 :

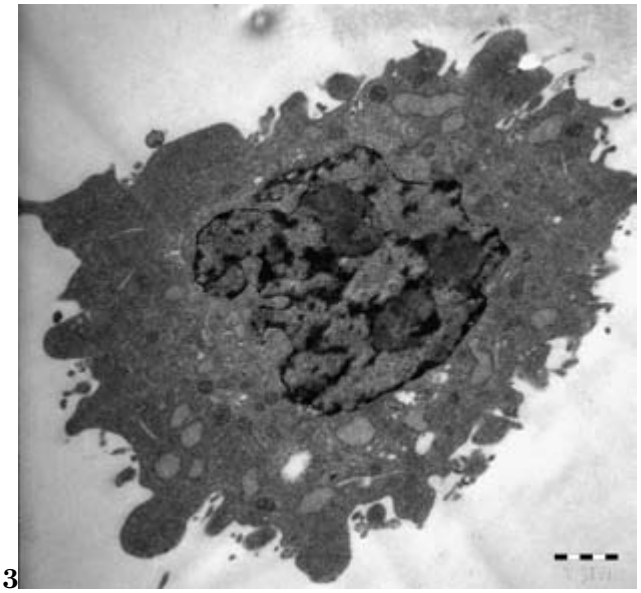

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Figure 6: Figure 3 :

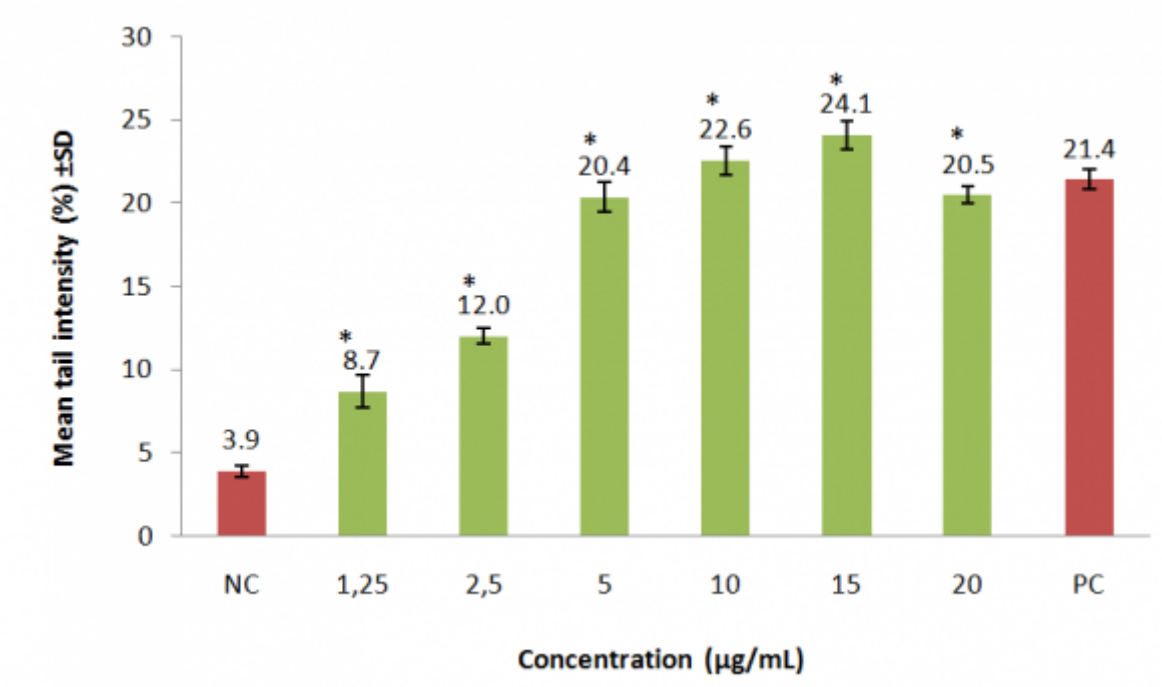

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Figure 7: Figure 4 :

Figure 8:

Figure 9:

All experiments were done in triplicate and each assay was repeated four times.
The results were presented as percentages of the total cell amount.

.1 Necrosis Apoptosis

Cu content of the negative control (unexposed cell) was also measured. Every assay was repeated four times. The results were expressed as mean ± SD. The protein amount calculated for 4x10 4 cells in every assay according to Bradford (1976). The results were expressed as µmol, µmol, µg and µg per g protein for GSH, MDA, 8-OHdG and PC, respectively, using standard calibration curve. *p ?0.05 were selected as the levels of significance.

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