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Invitro cytotoxic effect of green synthesized Copper Nanoparticles on Mcf-7 cells

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Abstract

Copper nanoparticles were synthesized from green tea leaves extract. This method was proved to be an efficient method for the preparation of copper nanoparticles. The extract was prepared in deioised water. The extract was added to 10mMol of copper sulphate solution as 1:1 ratio and 0.5 m L- ascorbic acid was added as capping agent. The colour change of the solution indicates the formation of copper nanoparticles. The biosynthesized copper nanoparticles were characterized with the help of visual inspection, SEM (Scanning electron microscopy), EDX (Energy dispersive x ray spectroscopy), XRD (X ray spectroscopy).The size of the particle was found to be in the range of 60-80 nm. The synthesized copper nanoparticle showed cytotoxic effect against MCF-7 cell line. This method can be used for eco-friendly biosynthesis of copper nanoparticles.

Key words: copper nanoparticles, Green synthesis, cytotoxic effect.

Introduction

Nanotechnology is a broad-based science involving manipulation of atoms, electrons, protons and neutrons in a variety of ways to generate new understanding of how materials can be developed to solve many problems in medicine, engineering, agriculture, biology, chemistry, surface science, space exploration, ocean and marine science, geography and geology. In some cases the research will develop new machines or ways to deliver new products (Kawazoe and Meeche, 2005).

Nanoparticles (NPs), abbreviate form of nano scale particles, are atomic or molecular aggregates with at least one dimension comprised between 1 and 100 nm. NPs have attracted interest because of their small size, exhibition of emergent properties like quantum dots, non-linear optical, thermal, electrical and chemical properties. A number of studies showed the importance of NPs to improve human health, electronic, magnetic and optoelectronic, biomedical, pharmaceutical, cosmetic, energy, environmental, catalytic and material applications (Youtie *et al*., 2008; Porter and Youtie, 2010).

Engineered NPs are produced by a number of chemical and physical approaches. Traditional technologies use a top-down approach when constructing materials. Most objects are created starting from a bulk materials and then breaking it into smaller pieces using mechanical, chemical or other forms of energy until they precisely form the desired construction (e.g. integrated circuits in microelectronics) (Kawazoe and Meeche, 2005). Alternatively, the bottom-up approach recognizes that the building blocks of life (enzymes, and other components of each living cell), already act as machines at the nanoscale. Nanoscale materials are synthesized from atomic or molecular species via chemical reactions, allowing for the precursor particles to grow in size (Prathna *et al*., 2010).

However, such methods are harmful as the chemicals used are often toxic, flammable, not easily disposable due to environmental issues, have low production rate and are expensive (Kowshik *et al*., 2003). Striving for alternative and cheaper pathways for nanoparticle synthesis, scientists contributed to the development of a relatively new and largely unexplored area of research based on the biosynthesis of nanomaterials (Mohanpuria *et al.,* 2008). A great deal of effort has been put into the search for methods utilizing biological systems in order to produce metal nanoparticles at ambient temperature and pressure without requiring hazardous agents and generating poisonous by-products.

Biosynthesis of NPs is a type of bottom-up approach where the main reaction occurring is reduction/oxidation. The use of plants for nanoparticle synthesis is a comparatively new and under-researched technique. Synthesis of metal NPs using plant extracts is very cost effective, so can be used as an economic and valid alternative for the large-scale production of metal nanoparticles (Huang *et al*., 2007).

Cancer is the second most frequent cause of death in the world. The discovery of antitumor activity of cisplatin began a search for other metal complexes with cytotoxic properties

against cancer cells. One of the transition metal, whose complexes are extensively tested for antitumor application is copper. Copper is a trace element essential for human life. It is a building element of several important enzymes (e.g.superoxide dismutase, cytochrome oxidase, tyrosinase) and it regulates the intracellular redox potential, while its complexes possess antibacterial, antifungal, antiviral, anti-inflammatory and anticancer properties.

Continuous demand for new anti-cancer drugs has stimulated chemotherapeutic research based on the use of metals since potential drugs developed in this way may be less toxic and more prone to exhibit anti-proliferative activity against tumors (Sorenson 1992; Studer *et. al*., 2010). Transition metal complexes have been extensively studied for their nuclease like activity using the redox properties of the metal and dioxygen to produce reactive oxygen species to promote DNA cleavage by direct strand scission or base nodification (Burrows and Muller 1998). More recent trend in this area has been testing of metal nanoparticles such as gold and platinum nanoparticles of DNA degradation studies. Use of metal nanopartricles can be in particular advantageous in generating singlet oxygen (Lopez *et. al*., 2010; Shen *et. al*., 2009; Lipovsky *et. al.,* 2009). Therefore the present study aims at studying the cytotoxic effect of green synthesized copper nanoparticles.

Materials and Method

Materials

Copper sulphate pentahydrate ($CuSo_4.5H_2o$), L-Ascorbic acid, Ehanol, Acetone and standard antibiotic disc were purchased from Himedia (P) Ltd, Mumbai are used without further chemical treatment and purification. Milli-Q water was used for synthesis of nanoparticles.

Preparation of Extract

Processed green tea leaves were purchased from tea farm, Nilgris. They were washed and cleaned with Millipore water. 10 g of leaves were boiled with 100 ml of distilled water at 70-80- ^oC for 15 – 20 minutes. The extract was filtered using Whatman No. 1 filter paper. The resulted infusion was collected in a clean and dried conical flask by standard sterilized filtration method was stored and used as a reducing agent.

Preparation of Copper Nanoparticles

The particles were prepared in aqueous phase by biological reduction of cupric salt solutions using the green tea extract as reducing agent. The solutions used were purged with nitrogen gas for 20 minutes to remove the dissolved oxygen. For the synthesis of copper nanoparticles

both the precursor (0.01 M) and the reducing agent (extract) were mixed in a clean conical flask in 1:1 proportion and heated at 50^{o}C in magnetic stirrer. The color change was observed from typical copper blue to sea green indicated reduction. To avoid oxidation of copper nanoparticles 0.1 M L-Ascorbic acid were added drop wise to the reaction mixture as capping agent. The heating and mixing continued till the color changed to dark brown. The particles were then extracted by ultracentrifugation at 10,000 rpm for 45 minutes. The particles were washed twice with alcohol and finally with acetone. The small amount of sediment collected were dried at 60^{o}C over night and used for characterization.

Characterization of nanoparticles

Visual observation

The reduction of metal ions was visually observed by the change of color in the reaction solution.

Scanning electron microscopy

Size and morphology of the nanoparticles was examined by SEM (SU1510) operated at 5 kV, magnification x10 k. Thin film of the sample was prepared on a carbon coated copper grid by just dropping the suspension of nanoparticles in water on the grid, extra solution was removed using blotting paper and then the film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min. The sample surface images were taken at different magnifications.

Energy dispersive spectroscopy

EDS was used for the determination of elemental composition and purity of the samples by atom percentage of metal. Elemental analysis on nanoparticles was carried out using EDS instrument (JSM 35 CF JEOL) in a resolution of 60 Å, operated at 15.0 kV with a magnification of about 5 k. Samples were prepared on a carbon coated copper grids and kept under vacuum desiccation for 3 h before loading them onto a specimen holder.

The X-ray diffraction (XRD) Analysis:

Technique used to establish the metallic nature of particles gives information on translational symmetry size and shape of the unit cell from peak positions and information on electron density inside the unit cell, namely where the atoms are located from peak intensities {77}.

XRD patterns were calculated using X'per Rota flex diffraction meter using Cu K radiation and λ=1.5406 A^{o}. crystallite size is calculated using Scherrer equation.

$CS=k\lambda/\beta \cos\theta$

Where CS is the crystallite size

Constant[K]=0.94

β is the full width at half maximum [FWHM]

Full width at half maximum in radius [β] = FWHM x π/180

λ=1.5406 x 10-10, Cosθ =Bragg angle.

In vitro cytotoxicity assay

Methodology

The human breast adenocarcinoma cell lines (MCF-7) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). The cells were maintained at 37^0 C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted by tryphan blue exclusion assay using a hemocytometer. The cell suspension was diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. one hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37^0C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dispersed in phosphate buffered saline (PBS) and diluted to twice the desired final maximum test concentration with serum free medium. Additional four, 2 fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulted the required final sample concentrations. Following drug addition the plates were incubated for an additional 48 h at 37^0 C, 5% CO_2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

MTT assay

3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

After 48h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

% Cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between % Cell inhibition and Log concentration and IC50 was determined using GraphPad Prism software (Mosmann 1983; Monks et.al., 1991).

Results and Discussion

Characterization of nanoparticles

Visual inspection

The appearance of dark brown colloidal solution for Cu in the reaction mixture indicated the formation of copper nanoparticles . The formation of color in the reaction solution arises from excitation of surface Plasmon vibration in the metel nanoparticles.

Scanning electron microscopy

Scanning electron micrograph of the synthesized nanoparticles is presented in Figure 1.

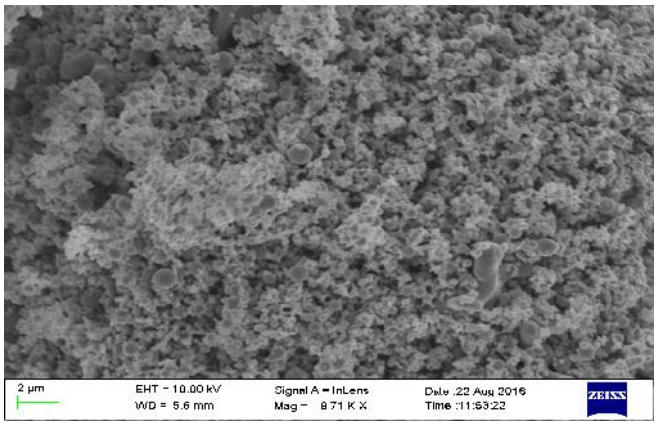


Figure 1 : Scanning electron micrograh of copper nanoparticles

Micrograph shows that the appearance of the particles is spherical in shape. Synthesized particles do not appear as discrete one but form much larger particles. The observarions of such larger nanoparticles are composed of van der Waals clusters of smaller entities and magnetic interactions among the particles.

Energy dispersive spectroscopy

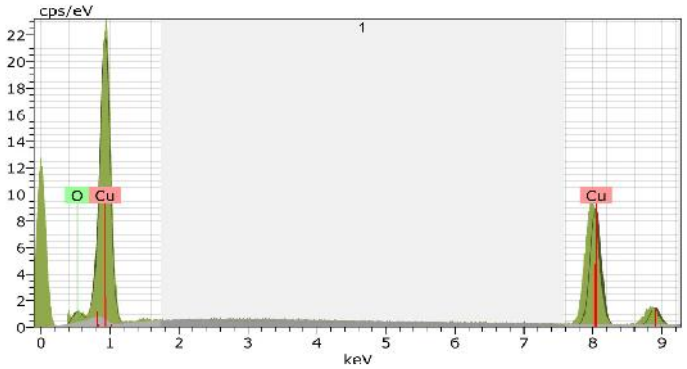


Figure 2 : Energy dispersive spectrum of copper nanoparticles

EDS micrograph explains the surface atomic distribution and chemical composition of nanoparticles. Quantitative measuring results obtained from EDS analysis reflect that 93% and 7% atom particles were of copper and oxygen respectively which confirms the purity of copper metal.

X-ray diffractogram

The XRD spectra depicted in Figure shows an intense peak at 43.24, with plan (111), the crystal plane of Cu, which matched to the FCC of bulk Copper. The low intensity peaks at 50.37 and 74.02 respectively match well with the plane (200), (220) for the sample. These are very close to those in the JCPDS. This indicates that the prepared Cu material is highly pure, crystalline, and well arranged in specific orientation, having no peaks for any impurities. The size of copper crystallites was estimated from the Deby-Scherrer equation. The crystal size of product as calculated by the Scherrer formula is 20-60 nm. Size distribution analysis shows that the most of the particles had a size of 60 nm.

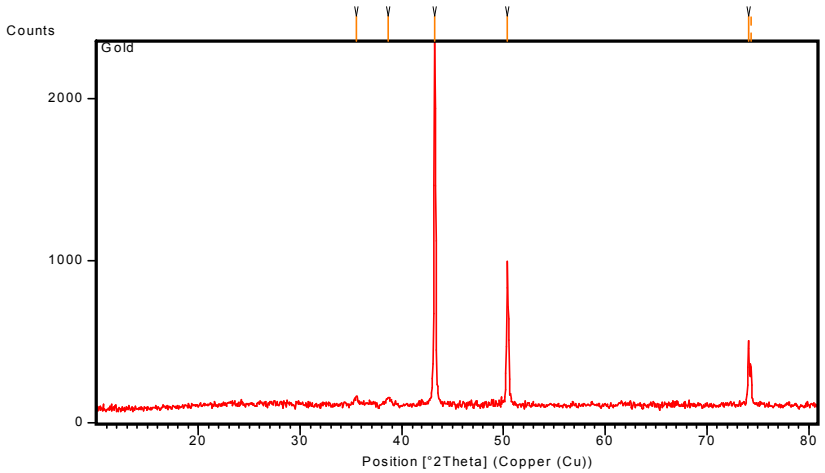


Fig. 3: XRD of copper nanoparticles.

Pos. [°2Th.]	Height [cts]	FWHM Left [°2Th.]	d-spacing [Å]	Rel. Int. [%]
35.4837	49.36	0.2952	2.52991	2.52
38.6595	42.89	0.3936	2.32909	2.19
43.3087	1957.08	0.1476	2.08922	100.00
50.4354	856.10	0.1968	1.80946	43.74
74.0686	390.50	0.1200	1.27894	19.95
74.3213	237.67	0.1200	1.27839	12.14

Table 1 : X ray diffraction angle of copper nanoparticles.

Invitro cytotoxic effect

MCF7

E 206	**Conc**	**3.125 µg**	**6.25 µg**	**12.5 µg**	**25 µg**	**50 µg**	**Cont**
	ABS	0.342	0.315	0.293	0.177	0	0.345
		0.338	0.323	0.277	0.164	0	0.326
		0.334	0.323	0.278	0.204	0	0.33
	Avg	**0.338**	**0.320333**	**0.282667**	**0.181667**	**0**	**0.333667**

Conc (µg/ml)	**% Cell Inhibition**
3.125	-1.2987
6.25	3.996004
12.5	15.28472
25	45.55445
50	100

IC 50	**24.97 µg/ml**
R^2	**0.9786**

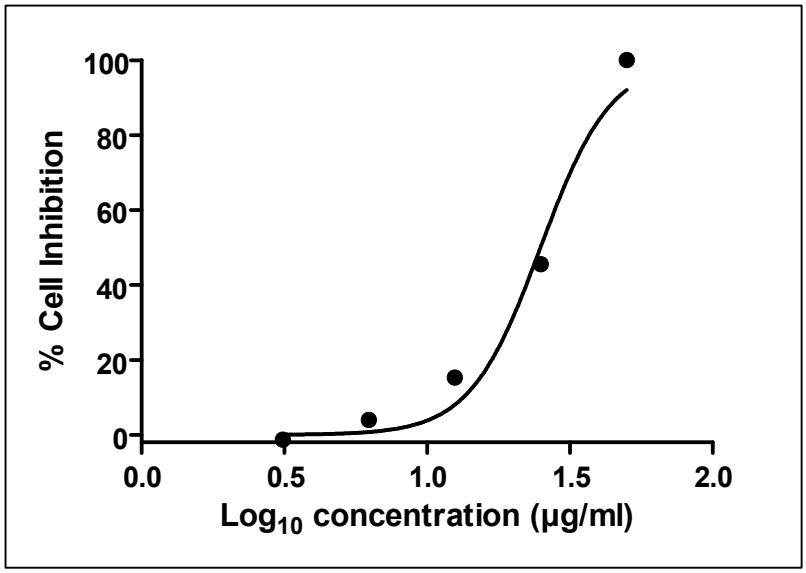
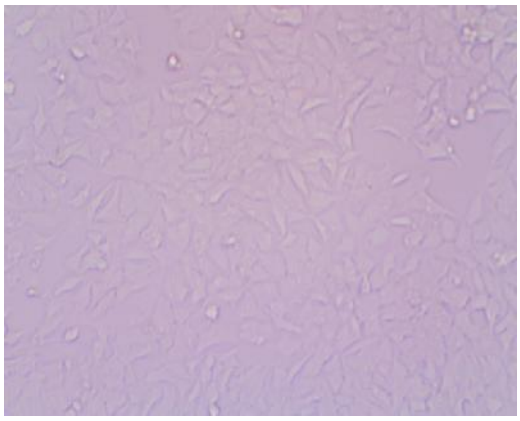


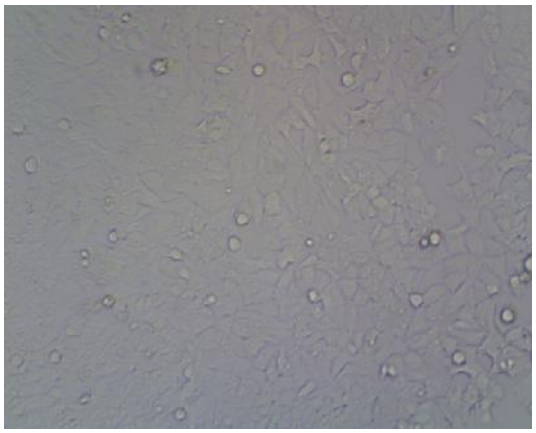
Table 2: IC_{50} of MCF7 Cells treated with copper nanoparticles at various concentrations.

MCF7 cells treated with copper nanoparticles

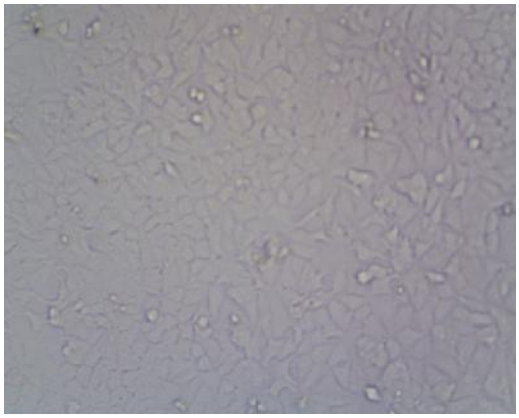
Control



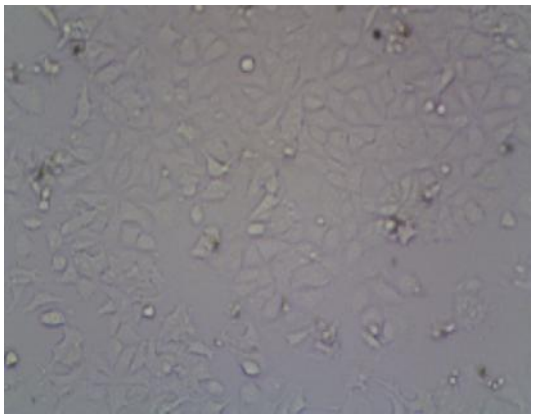
3.125µg/ml



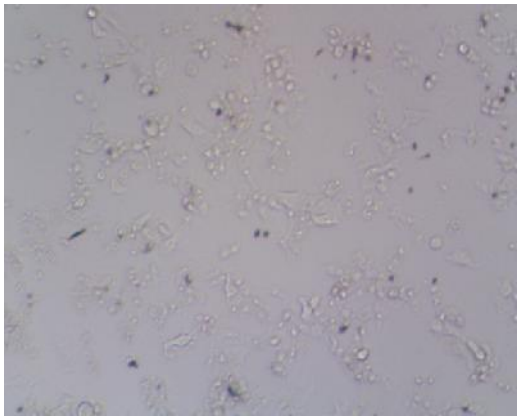
6.25 µg/ml



12.5 µg/ml



25 µg/ml



50 µg/ml

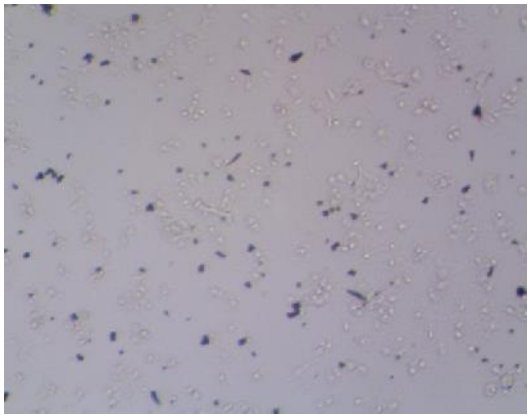


Figure 3 : Photograph showing MCF7 Cells treated with copper nanoparticles at various concentrations.

Cu NPs appeared to have a high cytotoxicity in the cell type, the MCF-7 cells are more sensitive and IC $_{50}$ value was found to be24.97µg/ml. The data obtained showed that the nanoparticles arecytotoxic and it can also biocompatible and copper nanoparticles can be used as a vehicle for drug delivery system .

Over 95% of copper (both Cu(II) and Cu(I) that is present in serum is bound to ceruloplasmin (ferroxidase). However, it is not responsible for transporting copper inside the cell. Before they enter the cell, copper(II) ions are reduced to copper(I) by maetaloreducatases located on the cell's surface. Cu^{+} ions are transported into the cell mainly by a specific copper transporter (hCtr). The independent system of entering the cell, enables biologically active copper compounds to penetrate the cell surface without binding to other agents as opposite to coordination compounds of other metals (Puig and Thiele 2002).

Anticancer activity of copper complex compounds is related to their ability to produce reactive oxygen species (ROS). Copper(I) ions can reduce hydrogen peroxide to hydroxyl radical. Copper(II) ions may in turn be reduced to Cu(I) by superoxide anion($O_2^{\bullet-}$), or glutathione.

Superoxide anion **($O_2^{\circ-}$)** is the product of reduction of the molecular oxygen that occurs in many biological processes. It is converted into hydrogen peroxide through dismutation. Both of these forms of ROS lead to the formation of another type of reactive oxygen species – the hydroxyl radical (OH°). It occurs in a reaction catalysed by copper (or iron) ions. This radical is believed to be the main factor causing DNA damage in cells under oxidative stress (Scibior *et. al*.,2009).

Conclusion

It is evident that metal based nanoparticles due to their biological and physiochemical properties are promising as a therapeutic agents. They can be used to address a number of challenges in the field of nanomedicine. But it must be remembered that they can also possibly cause adverse biological effects at the cellular and subcellular levels. It is clear that metallic nanoparticles have great potential in many different industries. The need for a process to synthesis such nanoparticles in a reliable and green way is becoming more pressing.

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