# Estimation The Median Lethal Dose and Inhibitory Concentration of $TiO_2$, $SiO_2$, ZnO and CuO Nanoparticles on Human Hepatoma HEPG2 Cells

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## ABSTRACT

In the introduced research, the metal oxides nanoparticles (CuO, ZnO, $SiO_2$ and $TiO_2$) were prepared and described with X-ray diffraction (XRD) and transformation electron microscope (TEM). The half maximal inhibitory concentration (MIC50) was detected for each nanoparticle as anti- cancer vitality versus HepG2 cells. The median lethal dose (LD50) was estimated as mg/ kg animal body weight via intra-peritoneal administration. The outcomes demonstrated that MIC for (CuO, ZnO, $SiO_2$ and $TiO_2$) was (5. 99± 0. 07 g/mL, 14±0. 48, 22. 7±1. 25 and 290±29. 10), respectively. Also, the lethal dose (LD50) was estimated as (2012, 2225, 2775 and 3000) for (CuO, ZnO, TiO2 and SiO2) respectively. In a conclusion, the research had proved that the inorganic nanoparticles were ready to inspire cytotoxicity and apoptosis in distinctive cancer cells. In addition, the effect of the metal oxide nanoparticles as anti-cancer viability had relied upon the kind and the concentrations of the nanoparticles. However, the action of the nanoparticles is not truly comprehended yet.

**Key Words:** Anticancer activity; human Hepatoma HepG2 model cells; Degradation; Inorganic nanoparticles.

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## INTRODUCTION

Cancer is one of the most challenges threatens the public health, and between all the types of cancer, hepatocellular carcinoma considered as one of the most widespread malignancy, attack about one million people worldwide annually. It comes in the third rank of cancer-related deaths, causing more than 600,000 deaths annually around the world [1]. The influenced hepatocellular carcinoma

inhabitants are mainly in Asia and Africa. However, current reports have revealed an increasing of the incidence rate of primary liver cancer in the Europe and United States [2]. Such an increase is prospective to continue for several decades, which make hepatocellular carcinoma a more menacing disease to human beings.

Nowadays, nano-science is going to affect all aspects of life. It has been shown that the chemically synthesized nano-particles (NPs) have anti-activity effects on different types of cancer cells [3]. Recently, organic and biopolymers nanoparticles has been produced which is completely non-toxic, biodegradable and nimble in the way of drug delivery [4]. Nano forms of metals, metal oxides are being used in several applications including diagnosis, drug delivery, etc. Zinc oxide nanoparticles (ZnO NPs) are one of the most abundantly used nanomaterials in the food

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industry as additives and in packaging due to their antimicrobial properties [5,6]. They are also being explored for their potential use as fungicides in agriculture [7]. As anticancer drugs and as contrast agents in biomedical imaging applications [8,9].

Among the synthesized nanoparticles, copper oxide (CuO) NPs are used in batteries, solar energy converters, industrial catalysis, and superconductors and is components of gas sensors [10]. CuO NPs have antimicrobial activity and are used to disposable textiles and food containers [11,12]. Titanium dioxide nanoparticles (nano-$TiO_2$) have been used as an additive to sunscreens, paints, toothpastes and food coloring due to their small size, white pigmentation, resistance to degradation and high refractive index [13,14]. Nano-$TiO_2$ has produced anomalous biological changes in vitro and in vivo. The greater part of studies has found that the Titanium dioxide nanoparticles- cultured in human cells have induced cytotoxicity, genotoxicity, inflammation and reactive oxygen species (ROS) production [15]. However, other studies have showed that nano-$TiO_2$ did not induce DNA damaging in human lung cells and lymphocytes [16]. Silicon dioxide ($SiO_2$) nanoparticles have been widely used as chemical mechanical polishing, additives for drugs, cosmetics, printer toners and varnishes [17]. Recently, the use of $SiO_2$ nanoparticels have been outspread to the biotechnological and biomedical fields, such as biosensors for simultaneous assay of glucose, lactate, L-glutamate and hypoxanthine levels in rat striatum [18]. biological marker for cellular imaging [19]. cancer therapy [20]. DNA and drug delivery [21]. And enzyme immobilization [22]. Nanoparticles biological response depends on their size, type, and the specific surface area. Recent studies into biomedical systems stated the Cytotoxicity of nanoparticles depend on their type and size. Also, it has been reported that the particles size plays a significant role in the cellular uptake of nanoparticles and so the bioactivity [23,24]. In this study, the impact of inorganic nanoparticles with different sizes and types was investigated as anti-cancer viability on HepG2 cells. We select the HepG2 cell line because; hepatoma is one of the common tumors spread all over the world and a main malignancy of the liver [25,26].

## 2. MATERIALS AND METHODS

Precursor zinc nitrate, precipitating agent KOH, Copper (II) chloride dehydrates and sodium hydroxide pellets were purchased. Explanatory reagents graded chemicals were utilized within the analysis without purification. Deionized water was used for washing.

### Nanoparticles Preparation:

ZnO nanoparticles were synthesized by direct precipitation method using precursors zinc nitrate and KOH. In this work, KOH (0. 4 M) was added to watery (0. 2 M) zinc nitrate (Zn $(NO_3)_2$. $6H_2O$) under strong attractive mixing until shaping a white suspension. The suspension was centrifuged at 5000 rpms for 20 minutes, washed three times with refined water, calcined at 500 °C for 3 hrs. Copper oxide (CuO) nanoparticles were synthesized with standard chemical precipitation technique [27]. Copper chloride was utilized as a portent and NaOH as a stabilizing agent. Silicon nanoparticles were prepared by adding 2 .5% of HCl acid to sodium silicate till a shady colloidal precipitate shaped. The gotten precipitated was centrifuged at 5000 rpms for 20 minutes, filtered, washed three times and dried at100 °C for 24 hours. The titanium oxide nanoparticles were purchased.

### Nanoparticles Characterizations:

The synthesized nanoparticles were characterized with two techniques. X-ray diffraction investigation was performed at room temperature (29 °C) and (20-80 theta degree) with CuK radiation. The transmission electron microscope instrument which worked at accelerating voltage 80 KV was used to characterize the size of the nanoparticles.

### Cell Culture:

HepG2 (ATCC Number HB-8065) cell line was obtained from VACSERA, Cairo, Egypt. The cells were maintained in culture flask 75-$cm^2$ surface area containing DMEM culture medium, 10% fetal bovine serum (FBS) and glutamine (200 mM). The medium was renewed twice per week. The cell layer was detached from the flask by washing the cells twice with sterile phosphate buffered solution (PBS). The cells were incubated with trypsin-EDTA solution (0.25% (w/v) Trypsin- 0.53 mM EDTA) for 5-15 minutes in a humidified atmosphere of 5% $CO_2$ and 37°C.

### Half maximal Inhibitory Concentration (IC50) determination:

The half inhibitory concentration ($IC_{50}$) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function of the cells after an overnight incubation [28]. The ($IC_{50}$) was affirmed for each incorporated nanoparticles. A stock convergence of 1 mg/ml was set up from each nanoparticle and debilitated to various concentrations (500, 250, 125, 62. 5, 31. 25, 15. 6, 7. 8, 3. 9 and zero µg/ml). HepG2 cells were treated with these concentrations; the cytotoxicity was measured by MTT test.

### MTT assay for measuring cell viability:

The MTT assay is a colorimetric assay for assessing cell metabolic activity. Preparations and tests were performed as described [29]. The cells were counted as ($10^5$ cells/ml), plated onto a 96-well plate in 100 µl of culture medium and incubated for 24 hrs. The tested nanoparticles were added to each well at different concentrations and the cells with tested nanoparticles were incubated for extra 48 hrs, 25 µl of 5 mg/ml tetrazolium dye MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide were added to each well, the cells were incubated for extra 4 hrs. The formed supernatant was removed and 100 µl of DMSO were added to each well. The absorbance of the formed insoluble formazan was measured with ELISA

assay. The procedures were performed in a triplicate for each concentration.

**Calculation the LD50 of the Nanoparticles:**

The median lethal dose measurement for each tested nanoparticle was assessed using Karber method and Hodge and Sterner toxicity scale. Where, in this technique; distinctive doses for each tested nanoparticle were administrated to different gatherings. Six gatherings for each tried nanoparticle and each gathering included eight male Albino mice (25 - 30 g). All mice were dealt with intra-peritoneal once and distinctive doses (Control, 3500, 3300, 3100, 2900, 2700, 2500, mg/kg), the first group of mice was administered with the vehicle in which the test nanoparticle was dissolved (normal saline), while, from the second gathering forward gets diverse doses of the tried nanoparticles. The increment dose was (200 mg/kg) advances from gathering to gathering. For each mouse, the perception was made for 24 hrs and indications of toxicity and rate of mortality in each gathering were noted. Toward the finish of study period, terminated creatures were meant the computation of LD50. The assurance of LD50 was by using the arithmetic method of Karber as follow:

$LD50=LD100 -\sum (a \times b) / n)$

Where, LD50 = Median lethal dose, LD100 = Least dose required to kill all test animals 100%, a = the difference between two successive doses of administered extract/substance, b = the average number of dead animals in two successive doses and n = total number of animal in a group.

**Statistical analysis:**

The value of viability percent for each concentration was measured and represented as mean ± SD. All experiments were conducted 3 times. The regression test was used to determine the $IC_{50}$ values and the correlation between cell viability and the concentration of the test metal oxides.

## 3. RESULTS AND DISCUSSION

The transmission electron microscope measured the size of the synthesized inorganic nanoparticles and its distributions. The TEM images in the figures (1-4) showed a bimodal profile of the particle size distribution, the aggregation occurred in the all synthesized nanoparticles. The size of CuO nanoparticles was around 47 nm in diameter, 20 nm for $SiO_2$ and 32 nm for ZnO and for $TiO_2$. X-ray diffraction analysis clarified the phase formation of the nanoparticles at room temperature (29 °C) in the range (20-80 degree) with CuK radiation. Figures (5-8) show the x-ray diffraction patterns of the calcinated nanoparticles at 600 °C. The presented XRD patterns of the synthesized nanoparticles exhibit a typical phase composition for each type and theses patterns are close to those in the JCPDS cards (CuO, ZnO and $TiO_2$) 04-006-2679, 01-089-7102 and 03-065-5714, respectively. All the synthesized nanoparticles have pure structures without extra phases, while $SiO_2$ showed no peak because of its amorphous nature.



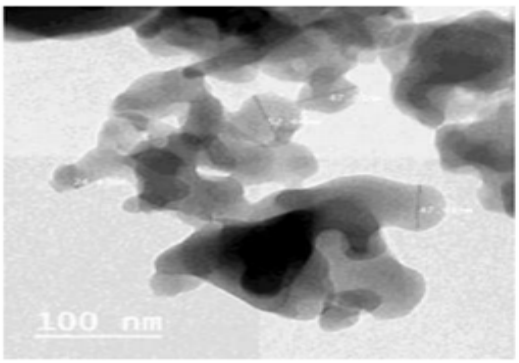

Fig (1) TEM image of CuO nanoparticles

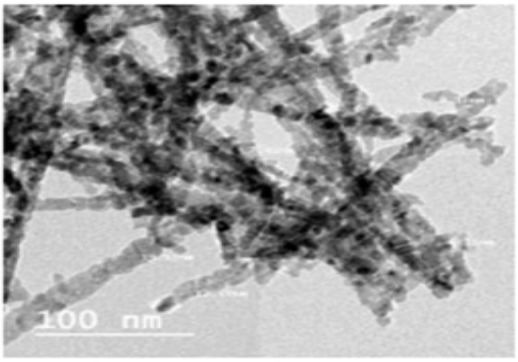

Fig (2) TEM image of $SiO_2$ nanoparticles

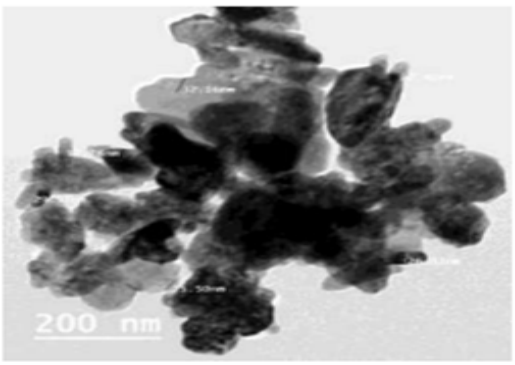

Fig (3) TEM image of ZnO nanoparticles

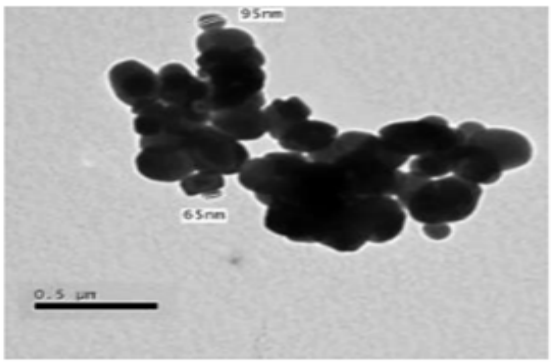

Fig (4) TEM image of $TiO_2$ nanoparticles

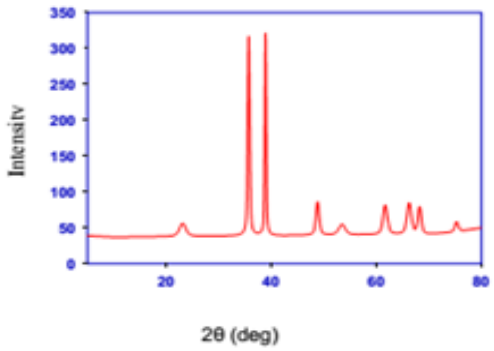


Fig (5) XRD of CuO nanoparticles

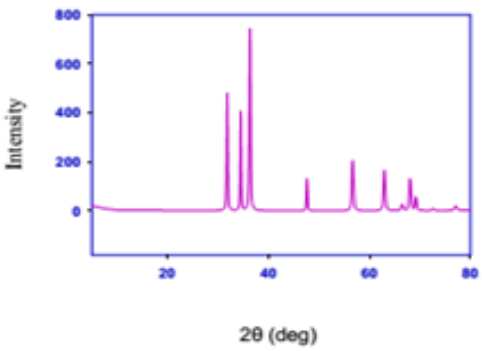


Fig (6) XRD of ZnO nanoparticles

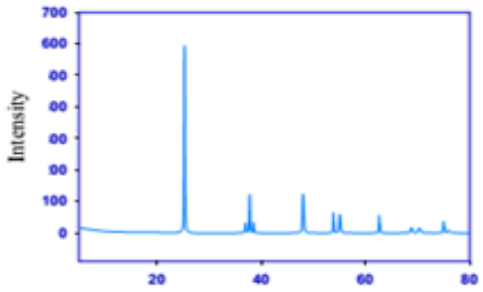


Fig (7) XRD of $TiO_2$ nanoparticles

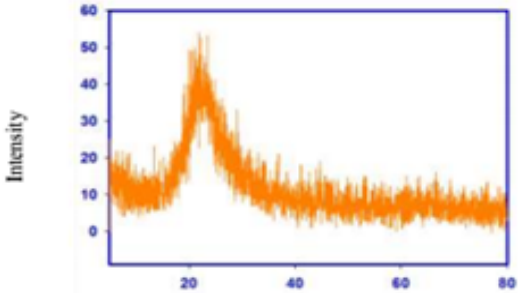


Fig (8) XRD of $SiO_2$ nanoparticles

In this report, the cytotoxicity of varying types of nanoparticles was tested in vitro. The results of the cell viability and the inhibitory concentrations of the all synthesized inorganic nanoparticles within 24 hrs showed an effective reduction of the cell viability. All the synthesized nanoparticles reduced the cell viability even at low concentrations except the $TiO_2$ nanoparticles; the reduction was at a high concentration as shown in the (Fig 9). The same figure showed a distinction difference among the synthesized nanoparticles at the same concentrations. The results revealed that IC50 values for (CuO, ZnO, $SiO_2$ and $TiO_2$) nanopowder were (5.99± 0.07 µg/mL, 14±0.48 µg/mL, 22.7±1.25 µg/mL and 290±29.10 µg/mL), respectively.

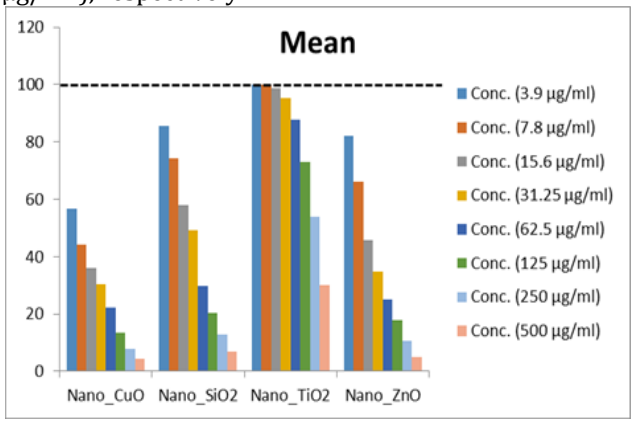


**Fig (9)** MTT activities of HepG2 cells (treated with different metal oxide NPs of various concentrations for 48 hours (replicated three). Figure only reflects the data points of effective concentrations.

The cytotoxicity percent varied in a concentration-dependent manner as shown in the (Fig 10). The inhibitory percent at IC50 was (55.73%, 54, 17%, 49, 31% and 46, 24%) for (CuO, ZnO, $SiO_2$ and $TiO_2$), respectively. The cellular viability decreased by (95.68%, 94.98%, s 93.23%) for (CuO, ZnO, $SiO_2$) at the same concentration 500 µg/l, while it was 70.01% for $TiO_2$ as shown in the (Fig 11). The results revealed that HepG2 cells were sensitive to the synthesized metal oxide nanoparticles, and the experiments confirmed that these nanopowders have anti-cancer activity in HepG2 cells. Johnston et al. attributed the significant differences in the cytotoxicity between the nanoparticles to the nanoparticles dimensions [30]. Another factor can affect the nanoparticles cytotoxicity is the dispersion of the nanoparticles inside the cells, [31] showed that the solution in which the NPs suspend has also effect. The mechanism of the cytotoxicity of NPs is complex; so it is important to mention that, this research presented the first report, another research will study the different mechanisms of the cytotoxicity of the synthesized nanoparticles. The mechanisms of the cytotoxicity that will be studied are formation of the oxidative stress, formation of the reactive oxygen species, inducing the lysosomes membrane permeabilization and changing the action potential of the mitochondrial membrane,

the flow cytometry assay will clarify whether metal oxide NPs induce apoptosis or not.

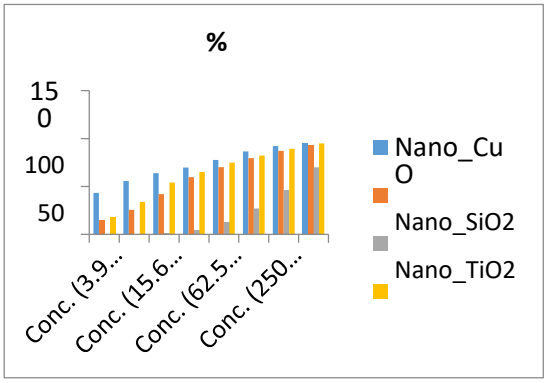


**Fig (10)** Inhibitory percent of HepG2 cells (treated with different metal oxide NPs of various concentrations for 48 h (replicate number 3). Figure only reflects the data points of effective concentrations.

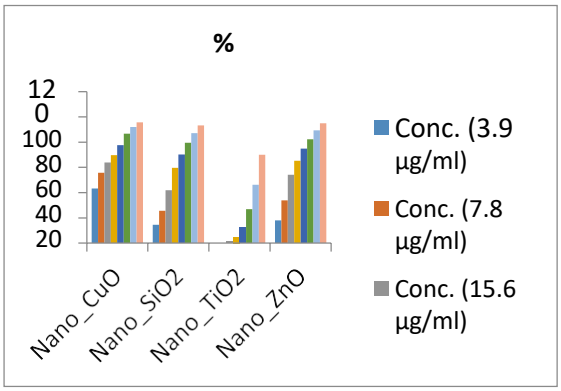


**Fig (11)** Inhibitory percent of HepG2 cells for each type of nanoparticles showing the significant differences and concentration dependant manners

Acute toxicity is defined as the unwanted effect (s) that occurs either immediately or at a short time interval after a single or multiple administrations of such substance within 24 hours. To test the acute cytotoxicity of the synthesized nanoparticles; the lethal dose (LD50) (the dose that kills 50% of the tested animal's population) was a major parameter in measuring acute cytotoxicity. Based on Karber method [32]. the estimated lethal dose (LD50) was (2012, 2225, 2775 and 3000) for (CuO, ZnO, $TiO_2$ and $SiO_2$), respectively, all tested nanoparticles were slight toxic (500-5000 mg/kg) according to Hodge and Sterner toxicity scale [33]. Behavioral patterns like salivation sleep cycle and corner sitting of the treated animals enhanced.

The utmost cytotoxicity of CuO nanoparticles was the main result in this study. Although free copper quenches oxidative stress; as it is implicated in the metabolic removal of reactive oxygen species, such as the superoxide radical through Cu-Zn dependent superoxide dismutase [34]. The transition metal nature of Cu, CuO nanoparticles have surface properties and thus surface reactivity and CuO nanoparticles concede Cu-ions that are toxic to the cells are the reasons of the high in vitro toxicity of the CuO nanoparticles. The current study attributed the cytotoxicity of CuO nanoparticles to Cu ions and agreed with [35]. Whereas another study showed that dissolution of Cu nanoparticles was insufficient to explain the cytotoxicity [36].

ZnO nanoparticles also have exhibited cytotoxicity in this study. Several studies have reported on the cytotoxicity of ZnO.[37,38]. An Ecotoxicological study showed the cytotoxicity depends on soluble Zn ions [39]. The synthesized crystalline silica nanoparticles activated their cytotoxicity through either oxidative stress by increasing $H_2O_2$ concentrations or generation of the reactive oxygen species. So, our study showed that there was a high fluctuation among different nanoparticles regarding their competency to cause cytotoxicity.

## CONCLUSION

In this research, distinctive inorganic nanoparticles have been synthesized and tried for inhibitory concentration activity against HepG2 cells and median lethal dose estimation. The outcomes demonstrated that the tested nano-powders had convenient anti-HepG2cancer cells activity. Additionally, the $IC_{50}$ was significantly distinctive between the different sorts of nanoparticles. The CuO nanoparticles have the most minimal $IC_{50}$ esteem while the $TiO_2$ nanoparticles have the most noteworthy esteem. Nano-CuO showed the most noteworthy cytotoxicity among the four metal oxide NPs, with 55.73%, at 5.99± 0.07 µg/mL. Furthermore, all the tried nanoparticles were marginally lethal when administrated intra-peritoneal.



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