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RESEARCH ARTICLE

ANTI-CANCER POTENTIAL OF COPPER OXIDE NANOPARTICLES AGAINST MURINE MAMMARY ADENOCARCINOMA (AMN-3) CELLS

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Abstract

This study was designed to evaluate the cytotoxic effect of copper nanoparticles in murine adenocarcinoma cells (AMN-3). The exposure period of cell line was performed at 24 hr in a microtitration plate under complete sterile conditions. Different concentrations were used started from 0.39 $\mu gmL^{-1}$to 50 μgmL^{-1} in three independent experiments. First, the cells were stained by MTT and the absorbance were measured using Elisa reader at 492 nm. The treatment with nanoparticles showed a significant inhibition ($P< 0.05$) on cells and the effect was concentration dependent, the highest inhibition was 88% at 50 μgmL^{-1}, while was 15% at 0.39 μgmL^{-1} and the inhibitory concentration 50 was 1.5 μgmL^{-1}. The cell death was evaluated in cell line after a treatment with CUNPs through two types of assessments, which were mitochondrial membrane potential assay and acridine orange- ethidium bromide dual staining assays. Results revealed that the tested substances showed a potent inhibitory cytotoxic effect against the proliferation of AMN-3 cells through apoptosis.

Introduction:-

Nanotechnology and nanobiotechnology are now almost an equal term in the sense of utilizing nanomaterial synthesized by physical or chemical procedures in biological applications. Inorganic nanoparticles demonstrate substances that possess suitable characteristics to apply them in many biological applications [1].

When materials are engineered to nano-size (100 nanometers or less), they acquire unique physical and chemical properties. Hence these materials are increasingly being used in the commercial manufacture of fillers, opcifiers, catalysts, semiconductors, cosmetics, microelectronics, and drug carriers [2]. Just as the nano-scale fabrication has enabled several commercial applications; it can also lead to potential risks [3].

The many industrial uses as well as the direct contact of the nanoparticles to human skin increase the chances of nanoparticles entering the body easily through respiratory, gastrointestinal and dermal passages [4].

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Copper nanoparticles are now being widely manufactured and are available commercially to be used in applications such as facial spray, lubricants, anti-oxidants and anode materials for chlithium ion batteries [5]. In the biological system, copper is an essential trace mineral critical for energy production in the cells. Copper is required for the formation of cupro proteins, like ceruloplasmin and for the activity of enzymes such as lisyl oxidase, cytochrome-c oxidase, superoxide dismutase, and tyrosinase [6]. The brain contains high levels of copper where it stimulates production of the neurotransmitter's epinephrine and norepinephrine. In the human body, copper is maintained in homeostasis [7] since it becomes toxic when it is in excess and not properly bound. Under toxic conditions, its redox reactivity can lead to the formation of reactive oxygen species (ROS) such as, superoxide anion, hydrogen peroxide, and hydroxyl radical. Accumulation of ROS leads to cell damage through oxidative modifications of proteins, lipids, and nucleic acids, adversely affecting their structures and functions [8]. In addition, copper can be toxic by directly binding to free thiols of cysteines and sulfhydryl groups in proteins, resulting in enzyme inactivation or alteredprotein conformation [9].

Many reports are available on the biogenesis of cupper nanoparticles using several plant extracts, particularly Magnolia kobus leaf extract [10], HibicusRosasinensis [11] ,Ocimumsantanum leaf extract [12] , and Syzygiumaromaticum [13]. However, potential of the plants as biological materials for the synthesis of nanoparticles is still under utilization.

Olive (Olea europaea) tree leaves contain many potentially bioactive compounds that may haveantioxidant [14], antihypertensive [15], antiatherogenic [14], antirheumatic [16], and anti-inflammatory properties [17]. The primary medical constituents contained in olive leaf is believed that antioxidants such as oleuropein and hydroxytyrosol, as well as other flavonoids, which are the most common group of polyphenolics in the human diet. The antioxidant potentials of olive leaves are protect the body from the continuous activity of free radicals [18]. Here in, we report for the first time synthesis of copper nanoparticles (CUNPs) using an aqueous extract of O. europaea as a reducing agent. The anti-proliferative potentials of murine mammary adenocarcinoma (AMN-3) cell line was evaluated.

Materials and Methods:-

Synthesis and characterization of copper nanoparticles

The preparation of plant extract, synthesis and characterization of copper nanoparticles were performed according to previously method described by Sulaiman and his co workers [19]

Cancer cell line

The murine mammary adenocarcinoma (AMN-3) cell line was used in this study was provided by the Iraqi Center for Cancer and Medical Genetic Research (ICCMGR), Mustansiriyah University, Baghdad, Iraq. This rest of the cell line (AMN-3) was maintained in RPMI-1064 medium with 10% fetal calf serum and supplemented with 2 mM glutamine, 100 mL^{-1} penicillin, and 100 mL^{-1} streptomycin (SDI, Iraq). Cell was cultivated and ex-posed to copper nanoparticles in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C.

MTT assay

Cell viability was determined with Cell Viability QMTT Assay kit (US Biological, USA). Ten concentrations of copper nanoparticles were used in this study; they were 0.39, 0.7, 1.5, 3.1, 6.3, 12.5, 25, 50, 100 and 200 µg mL^{-1}. Suitable growth media free of copper nanoparticles was used in untreated control cells treatments. The treated cells were incubated fro 24 hr at 37°C [20].

Mitochondrial membrane potential assay

Mitochondrial potential disruption was assayed using the procedure of Ali et.al, [21] on adherent cells with minor modifications using Apoptosis Detection Mitochondria Bioassay Kit (US Biological, USA). Cells imaged with CCD camera (Micros, Switzerland) under fluorescence microscope (Micros, Switzerland) and images for treated to 1.5 µg/ml Cu nanoparticles and control untreated cells analyzed suing ImageJ® analyzing software (NIH, USA).

Acridine orange and ethidium bromide (AO/Eth) assay

In order to confirm the event of apoptosis, the treated and control cellswas stained with fluorescent dyes to determine the cells morphology andnucleus shape. Cells were grown in 96-microwell plate until monolayer was achieved. Cells was exposed to 1.5 μgmL^{-1}CUNPs in serum free media for 16 hr and incubated at 37 °C with 5% CO After the time of incubation was over media was discarded and cells was washed with PBS, AO/Eth stain mixture (μgmL^{-1}, 10 µl) was added over the cells and cover slip was laid. Cells were observed under fluorescent

microscope (Olympus, Japan) at 200× magnification. Microscopic fields were photographed with digital camera (Lumenira corporation, Austria) [22].

Statistical Analysis

The grouped data were statistically performed using ANOVA with SPSS program (SPSS/14.0; SPSS Inc., Chicago, IL, USA). Values were presented as the mean ± S.D. of the three replicates of each experiment [23].

Results and Discussion:-

The characterization results were introduced in details at the published study of Sulaiman and his co workers [19]. For AMN-3 cell line, the results illustrated that treatment with Cu nanoparticlesinhibited the growthcells significantly ($P \leq 0.05$) as compared to those of control cultures and the reduction was concentration dependent. The highest inhibition (88%) was found at concentration of 50 μgmL^{-1} of CUNPs, while at 0.39 μgmL^{-1} concentration 15% cells were dead and the inhibitory concentration value (IC_{50}) was1.5 μgmL^{-1}**(Figure 1).**

To investigate if the apoptosis induced in treated cells to CUNPs nanoparticles and in order to explore the mechanism of this induction, mitochondrial membrane potential disruption was determined. Results indicated that apoptosis take place in the AMN-3 treated cells just after 4hrs of incubation time through distraction of mitochondrial membrane (**Figure 2**). The intensity of green color fluoresces was much higher in treated cells after this time of incubation with AMN-3 compared to control untreated cells. As indicated using ImageJ ® software AMN-3 cell line treated with IC_{50} 1.5 μgmL^{-1}concentration revealed loss in mitochondrial membrane integrity and apoptotic induction with green nuclei when compared with red nuclei control. The cells with green stain of their nucleus indicating the early stage of apoptosis and this effect were associated with low cell viability. The results of present study suggest that CUNPs may induce apoptosis through the changes in the mitochondrial mediated apoptosis pathway. Thus, the induction of cancer cell apoptosis is a crucial mechanism for an anti-cancer compound [24]

The visualization of AMN-3 cells damage as a result of its exposure to Cu nanoparticles was carried out using fluorescent stains mixture of acridine orange and ethidium bromide. The cells suffered from aggressive membrane disintegration when exposed to IC_{50} 1.5 $\mu gmL^{-1}$of copper nanoparticle for 16hr (Figure 3). Acridine Orange, can pass through viable cell membranes stains the DNA of live cells and emits green fluorescence if interrelated into double stranded nucleic acid (DNA) or red fluorescence if bound to single stranded nucleic acid (RNA). Ethidium Bromide, on the other hand is excluded from the cells having intact plasma membrane and stains the DNA of dead cells itis taken up only by nonviable cells and emits orange fluorescence by intercalation into DNA [25]. Thus the morphological changes observed that reveal Cu nanoparticle induces only cell death through apoptosis rather than through necrosis. However, similar findings were also reported by Subarkhan et al., and Shafagh al., [26, 27] who observed that the copper nanoparticles can induce apoptotic cell death in MCF-7 and K562 cancer cells, respectively.

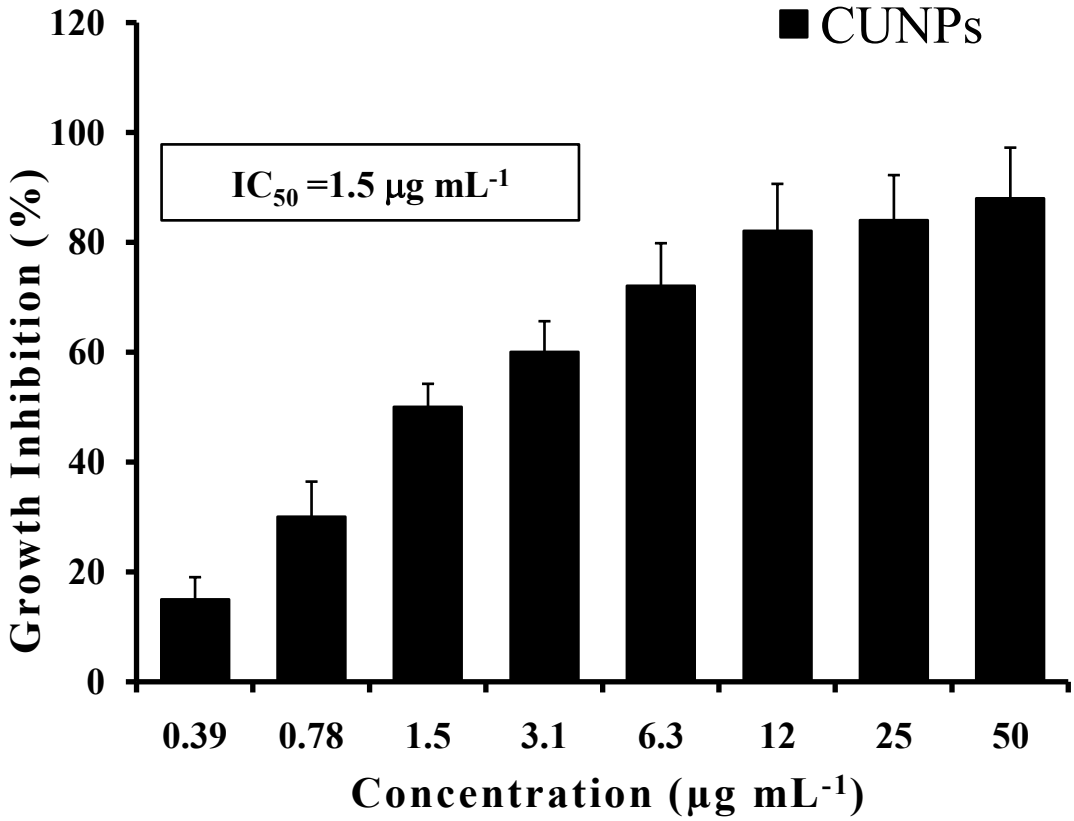


Figure 1:- Growth inhibition of AMN-3 cell line: The cells were seeded in 96 well plates (10.000 cells well $^{-1}$) and different concentrations (0.39, 0.78, 1.5, 3.1, 6.3, 12, 25 and 50 µg mL^{-1}) of CUNPs were added and then performed using MTT assay after 24 h of treatment. The values were the M ± S.D from three independent experiments expressed as percent with control. IC_{50} value of CuNPs was 1.5 µg mL^{-1}.

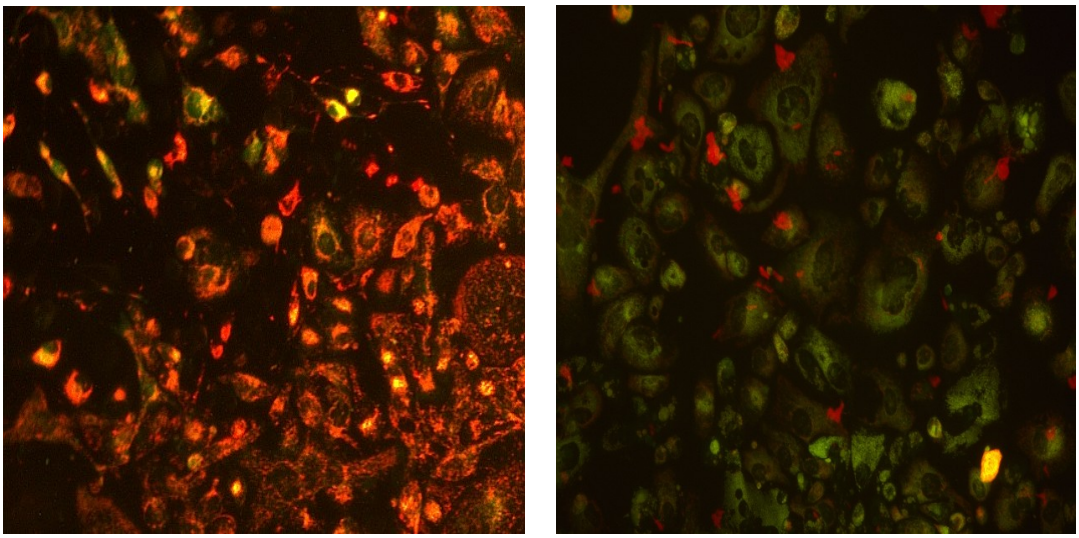
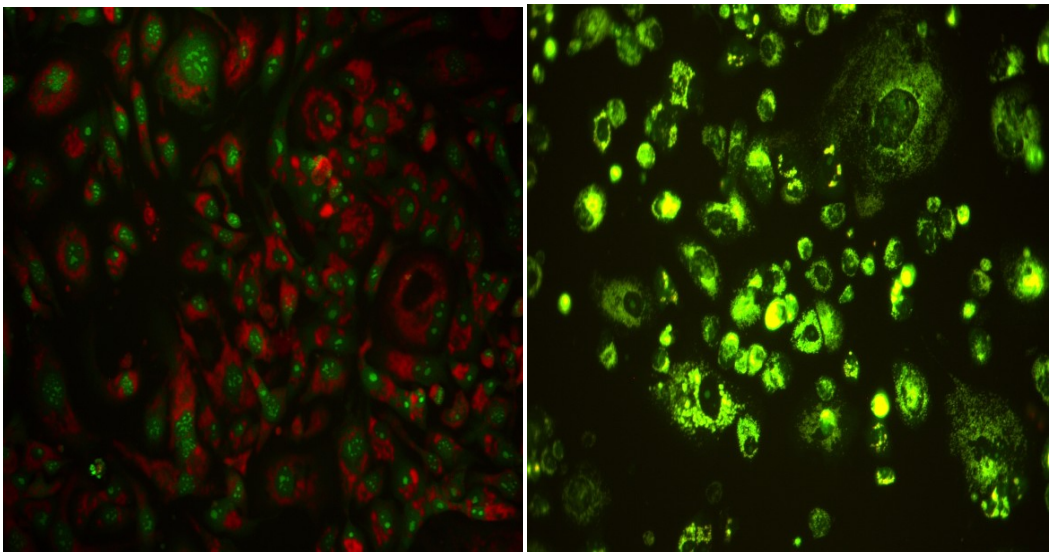


Figure 2:- Fluorescence intensity of red and green color as detected under fluorescent microscope, (**left lane**) untreated AMNO-3 cells, (**right lane**) AMN-3 cells incubated with 1.5 µgmL^{-1}of copper nanoparticles for 4 hrs, Images analyzed with ImageJ® software.



Figurer 3:- AMN-3 cell line incubated for 16 hr without copper nanoparticles (**left lane**) or with 1.5 µgmL^{-1}of copper nanoparticles synthesized by leaf extract of Olea europaea(**right lane**).

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