Evaluation of the Anticancer effect of Doxorubicin-loaded on pegylated copper oxide magnetic nanoparticles on MCF-7 breast cancer cell line

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ABSTRACT

Breast cancer is one of the most widespread cancers in Iran. Currently, more than forty thousand people in the country are suffering from this disease. One of the ways to treat it is chemotherapy. Doxorubicin is one of the common drugs used in the treatment of breast cancer. There are two proposed mechanisms that show how this drug works. In the first mechanism, it causes DNA interference and disruption of DNA repair mediated by topoisomerase II, and in the second mechanism, it causes the production of free radicals and damage to cell membranes, DNA and proteins. Recently, the effectiveness of nanoparticles for the treatment of cancer, including breast cancer, has been considered. In the present study, the anticancer the effect of doxorubicin-loaded pegylated copper oxide magnetic nanoparticles was investigated on MCF-7 human breast cancer cells. First, the cells were cultured in a culture medium containing 10% fetal bovine serum and after 24 hours, they were treated with equal concentrations of the compounds (free doxorubicin - free nanoparticle - doxorubicin loaded on the aforementioned nanoparticle). The MTT test was used to examine the viability of the aforementioned cells. Based on the results obtained from this test, which were the average of 3 independent repetitions of the treatments with a significant level of $P<0.01$, all compounds caused a significant decrease in growth in the treated cells, with this decrease being the highest for the cells treated with the doxorubicin compound loaded on the pegylated copper oxide magnetic nanoparticle and the lowest for the free doxorubicin compound.

Keywords: Breast cancer, Copper oxide nanoparticles, Fe3O4, Doxorubicin.

1. INTRODUCTION

Cancer is the second leading cause of death worldwide, accounting for approximately one in six deaths globally. According to global cancer statistics, the number of cancer cases is expected to increase from 19.3 million in 2020 to 28.4 million by 2040 [1]. Among various cancer types, breast cancer is the most common malignancy and the second leading cause of cancer-related death among women. In 2018, breast cancer was reported as the most frequently diagnosed cancer in women worldwide, with approximately 1.2 million new cases and an annual increase rate of 1.3%, along with more than 620,000 deaths per year [2].

Based on global cancer statistics in 2020, breast cancer surpassed lung cancer to become the most commonly diagnosed cancer worldwide, with an estimated 2.3 million new cases, representing 11.7% of all cancer diagnoses and ranking first in terms of cancer incidence. Breast cancer is a heterogeneous disease and is classified into several molecular subtypes. Depending on the subtype, systemic treatment strategies include hormonal therapy, targeted therapy, and immunotherapy, which are often used in combination with conventional chemotherapy [3].

Doxorubicin is an anthracycline antibiotic derived from the bacterium Streptomyces peucetius and has been widely used as a chemotherapeutic agent since the 1960s. As a member of the anthracycline class, doxorubicin

is commonly used in the treatment of a wide range of malignancies, including breast, ovarian, bladder, thyroid, and lung cancers, as well as soft tissue and bone sarcomas. Additionally, it is used in the treatment of hematological malignancies such as acute lymphoblastic leukemia, acute myeloblastic leukemia, and Hodgkin's lymphoma. Despite its broad antitumor activity, the clinical application of doxorubicin is limited by dose-dependent toxicity and adverse side effects [4].

Nanoparticles are materials with at least one dimension in the nanoscale range (typically 1–100 nm). Due to their unique physicochemical properties, including high surface-to-volume ratio, size-dependent behavior, and quantum effects, nanomaterials have attracted considerable attention in various fields such as mechanics, electronics, optics, magnetism, and catalysis. In recent years, magnetic nanomaterials have emerged as particularly promising candidates in biomedical applications, including drug delivery systems and biological molecular probes [5].

Copper oxide (CuO) nanoparticles have demonstrated significant anticancer activity by inducing mitochondrial membrane potential disruption, accumulation of reactive oxygen species, and interference with normal cellular processes. Moreover, increased messenger RNA (mRNA) expression of apoptosis-related genes, including p53, Bax, caspase-3, and caspase-9, in MCF-7 breast cancer cells has confirmed the potential anticancer properties of these nanoparticles [6].

Among various nanomaterials, magnetic nanoparticles—particularly iron oxide nanoparticles such as Fe_3O_4 (magnetite) and Fe_2O_3 (maghemite)—have received extensive attention due to their superparamagnetic properties. Superparamagnetic iron oxide nanoparticles (SPIONs) are especially attractive for biomedical applications because of their biocompatibility and magnetic responsiveness [7]. These nanoparticles have been widely investigated for applications including cell separation, targeted drug delivery, magnetic resonance imaging (MRI), cellular imaging, and cancer hyperthermia [8]. Recently, SPION-based nanocarriers have gained significant interest for their multifunctional potential in imaging, gene delivery, hyperthermia, and controlled drug delivery systems [9].

2. METHODS AND MATERIALS

2.1 materials

Doxorubicin hydrochloride 5mg/10ml (from EBEWE PHARMA AUSTRIA, PEG2000, dialysis bag(14kDa), HCl, NaOH, $FeCl_3.7H_2O$, $FeSO_4.7H_2O$ and $Cu(NO3)_2.6H_2O$ from Merck.

2.2 Synthesis of nanoparticles by co-precipitation method

Co-precipitation is a simplest and most efficient chemical method for the synthesizing of magnetic nanoparticles (metal oxides and ferrites). The main advantage of co-precipitation is its ability to synthesize a large number of nanoparticles. The co-precipitation reaction has two stages: a) Co-precipitation stage b) Ferrite formation stage

In the first stage, metal hydroxides are formed in an alkaline environment. Schematically, the following reaction takes place, in which different elements can be present, including Zn^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+}.

1. $M^{2+} + 2Fe^{3+} \rightarrow M(OH)_2.\ 2Fe(OH)_3$

In the second stage, by exposure to an alkaline solution, the metal hydroxides are converted to the desired ferrite. The reaction in this stage is as follows:

2. $M(OH)_2.\ 2Fe(OH)_3 \rightarrow MFe_2O_4.\ nH2O + (4\ n)\ H_2O$

For the production of magnetite in the above equations, M^{2+} is equivalent to Fe^{2+} and the overall reaction is as follows

3. $Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4 + 4H_2O$

Furthermore, Fe_3O_4 can be oxidized to maghemite (γ- Fe_2O_3).

4. $Fe_3O_4 + 0.25\ O_2 + 4.5\ H_2O \rightarrow 3Fe(OH)_3$

5. $Fe_3O_4 + 2H+ \rightarrow \gamma\text{-}Fe_2O_3 + Fe^{2+} + H_2O$

2-2-1 Synthesis of magnetite by co-precipitation method

Briefly, 3.5 g of Iron III chloride hexahydrate and 2 g of Iron sulfate heptahydrate were dissolved in 500 ml of distilled water at 60 °C under magnetic stirring. Then, sodium hydroxide was added dropwise using a burette until the pH of the solution reached 11, and at this acidity, iron oxide nanoparticles precipitated. Then, they were washed several times with distilled water until the acidity reached neutral (pH=7). The resulting nanoparticles were filtered and dried in an oven at 60 °C for 24 hours, followed by calcination at 350 °C for 4 hours [10].

2-2-2 Synthesis of pegylated copper oxide magnetic nanoparticles:

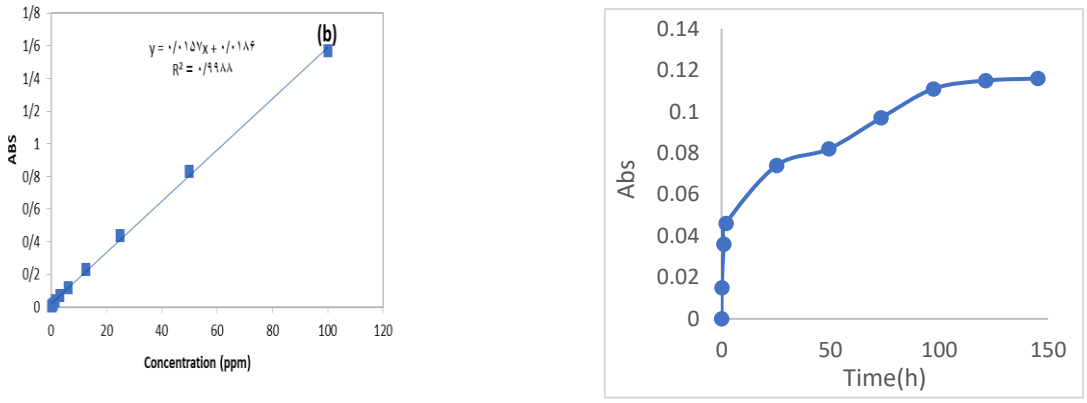
The synthesis was carried out in two main steps. In td-he first step, Iron oxide (0.6 g) and copper nitrate (2.5 g) were dissolved in 60 ml of distilled water at 50 °C under magnetic stirrer for one hour. Then, sodium hydroxide solution was added dropwise until the pH of the solution reached 12, leading to the formation of precipitates. Then, precipitates were washed several times with distilled water to adjust the pH to 7.5. Then, the nanoparticles were separated from the solution using filter paper and dried in an oven at 60 °C. The nanoparticle shapes were calcined at 350 °C for 3 hours to remove excess organic matter from the nanoparticles.

In the Second stage, 0.6 g of the prepared $CuO@Fe_3O_4$ nanoparticles were dissolved in 100 ml of water, and polyethylene glycol 2000 solution was added dropwise under continuous stirring. the pH was adjusted to pH=6.5 using diluted acid. The pegylated nanoparticles were separated by filtration and were calcined at 350 °C for 3 hours.

2.3 Loading and release of doxorubicin drug on pegylated copper oxide magnetic nanoparticles

After the synthesis of pegylated copper oxide magnetic nanoparticles, doxorubicin was prepared at a concentration of 20 ppm and added to 0.1 g of nanoparticles. The mixture was stirred for several hours to allow drug loading. The suspension was centrifuged and the supernatant was absorbed. The drug loading was determined to approximately 70%.

For the drug release study, phosphate-buffered saline (PBS; pH= 5.4) was prepared, then amount of doxorubicin loaded on pegylated copper oxide magnetic nanoparticles was placed into a dialysis bag containing phosphate saline buffer (pH = 5.4.) It was removed from the buffer at different intervals and its release was read, and the drug release was approximately 65%.



A *B*

Fig. 1. *A) Calibration graph of doxorubicin in PBS buffer, pH= 5.4 and B) Release of doxorubicin loaded on nanocarrier in PBS buffer, pH= 5.4*

2.4 Preparation of phosphate buffered saline (PBS)

Initially, 100 mL of distilled water was exposed to ultraviolet light for 20 minutes to eliminate bacterial contamination. Subsequently, 3.581 g of disodium hydrogen phosphate was accurately weighed using a digital balance and gradually added to the sterilized distilled water under continuous stirring until completely dissolved. The final pH of the solution was then adjusted to the desired value using hydrochloric acid or sodium hydroxide, as monitored by a calibrated pH meter.

3. CULTURING Of MCF-7 BREAST CANCER CELLS

3.1 Materials and equipment required for cell culture

The materials and equipment used for cell culture included a laminar flow hood, Memmert incubator (USA), inverted light microscope (HUND, Germany), centrifuge (Sigma, Germany), autoclave (Germany), sterile T25 and T75 cell culture flasks (Biophilia, Germany), RPMI-1640 cell culture medium (Gibco, UK), fetal bovine

serum (FBS, UK), penicillin–streptomycin antibiotics (Gibco, UK), phosphate-buffered saline (PBS), and dimethyl sulfoxide (DMSO) (Scharlau, Spain).
To prepare a complete culture medium containing 10% FBS, 44.5 mL of RPMI-1640 medium was poured into a 50 mL Falcon tube, followed by the addition of 5 mL of heat-inactivated FBS and 0.5 mL of penicillin–streptomycin solution, resulting in a final volume of 50 mL. Heat inactivation of FBS was performed by incubating the serum at 60–65 °C for 30 minutes.

After centrifugation and sedimentation of the cells, the supernatant was discarded and 1 mL of sterile culture medium was added to the cell pellet. The cells were gently resuspended until a homogeneous suspension was obtained. Subsequently, 20 µL of the cell suspension was transferred to a well of a 96-well plate, mixed with 20 µL of trypan blue solution, and immediately loaded into a Neubauer hemocytometer by gently injecting the mixture between the slide and the coverslip. The cells were then counted under an inverted microscope.
In trypan blue staining, dead cells appear blue or wrinkled due to membrane damage, whereas viable cells remain colorless. The number of cells per milliliter was calculated using the following formula:
To determine cell viability, 10 µL of the cell suspension was mixed with 10 µL of 0.1% trypan blue solution and incubated at room temperature for 2–3 minutes. Viable cells actively exclude trypan blue from their cytoplasm through active transport mechanisms, while non-viable cells are unable to exclude the dye and therefore appear blue [11].

3.2 Evaluation of cell proliferation and viability using the MTT method
The MTT assay was used to evaluate cell proliferation and cytotoxicity of the tested compounds. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazolium salt that is taken up by metabolically active cells. In the mitochondria of viable cells, MTT is reduced by dehydrogenase enzymes to form insoluble purple formazan crystals. These crystals are subsequently dissolved in an appropriate solvent, such as dimethyl sulfoxide (DMSO), and the absorbance is measured using an ELISA reader. The intensity of the purple color is directly proportional to the number of living cells [12].

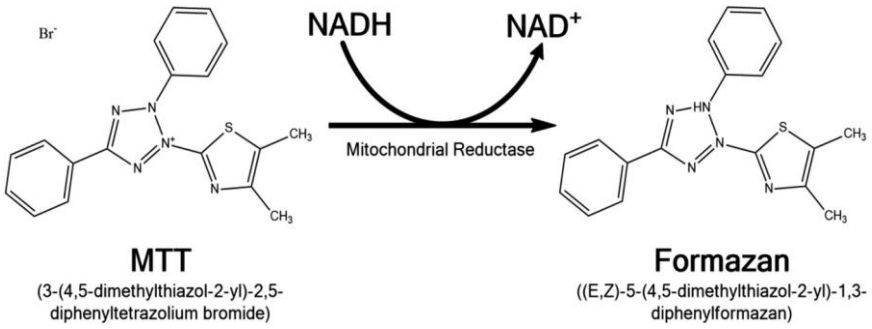


Fig. 2. *Formation of formazan in the MTT assay through the action of dehydrogenase enzymes accompanied by the consumption of NAD^+ and production of NADH.*

3.3 Preparation of the desired concentration of pegylated magnetic copper oxide nanoparticles
Following the synthesis of pegylated magnetic copper oxide nanoparticles, an appropriate amount of the nanoparticle powder was accurately weighed using a digital analytical balance and dissolved in sterile PBS to prepare a stock solution. Subsequently, the desired working concentration of nanoparticles (2 µg/mL) was prepared by appropriate dilution with PBS.

3.4 Preparation of the desired concentration of doxorubicin
A stock solution of doxorubicin was prepared and diluted with sterile solvent to obtain a final concentration of 2 µg/mL for experimental use.

3.5 Investigation of the cytotoxic effects of pegylated magnetic copper oxide nanoparticles on the performance of doxorubicin using MTT
After cell counting and determination of cell viability using trypan blue staining, 6,000 viable cells were seeded into each well of a 96-well plate in a final volume of 50 µL of culture medium containing 10% FBS.

Various concentrations of nanoparticles and/or doxorubicin were then added to each well in an additional volume of 50 µL. The first row of the plate was considered as the untreated control group.

After 24 hours of incubation, 10 µL of MTT solution was added to each well and the plate was incubated for an additional 4 hours at 37 °C. Subsequently, 100 µL of pure DMSO was added to each well to dissolve the formazan crystals. After 20 minutes of incubation at room temperature, the absorbance was measured at a wavelength of 570 nm using an Elasangar ELISA reader.

Cell viability was calculated based on the absorbance of treated wells relative to the control wells. To evaluate the effect of copper oxide/iron oxide/polyethylene glycol nanoparticles on the cytotoxic performance of doxorubicin, a nanoparticle concentration of 2 µg/mL was selected due to its relatively low intrinsic cytotoxicity. In a separate 96-well plate, cells were treated with a combination of 2 µg/mL doxorubicin and 2 µg/mL nanoparticles. The first row was again considered as the control group, and cell viability was determined using the same method described above [13].

4- RESULTS and DISCUSSION

4.1 Dynamic Light Scattering (DLS) Analysis

Dynamic light scattering (DLS) is a physical technique used to determine the size distribution of particles in solutions and suspensions. This non-destructive and rapid method is based on the interaction of incident light with particles undergoing Brownian motion and is widely applied for measuring particle sizes ranging from a few nanometers to several micrometers.

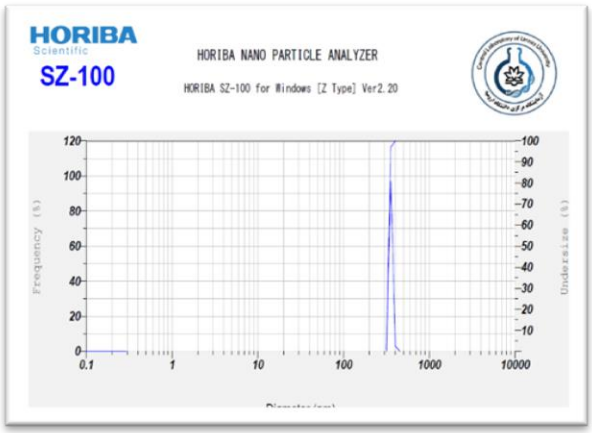


Fig. 3. *The Graph of DLS Analysis*

Table 1. *Result of DLS Analysis*

Average particle size	Surface area ratio of S/P	Median	Different in the average partical size	Peak number
336/4 nm	1/00	335/7 nm	7/5 nm	1
336/4 nm	1/00	335/7 nm	7/5 nm	Total particle size

4.2 FT-IR Analysis

Fourier Transform Infrared (FT-IR) measurements were performed to investigate possible interactions between doxorubicin, copper oxide, iron oxide, and polyethylene glycol 2000. To perform this analysis, a FT-IR spectrophotometer (BRUKER 27) was used in the wavelength range of 500-4000 cm $^{-1}$ to record the functional groups of doxorubicin, copper oxide, polyethylene glycol -2000 and magnetite nanoparticles, which are the peaks at 3629 and 3282 cm^{-1} related to O-H stretching, the peak at 2935cm^{-1} related to C-H stretching,

the peak at1618 cm^{-1} related to C=C, the peak at 1376cm^{-1} related to C-H bending, the peak at 1053cm^{-1} related to C-O, and the peaks at 714 and 1889 cm^{-1} related to C=C(alkene), and 714 and 889 cm^{-1} related to C=C (alkene) in the drug doxorubicin.

The peak in the 3439 cm^{-1} is related to the O-H bond, the peak in the 1627 cm^{-1} is related to the C=O bond, and the peaks in the 500 and 600 cm^{-1} are related to the Cu-O bond in CuO nanoparticles. The peak in the 500 region is related to Fe-O, the peak in the 1637 cm^{-1} is related to the O-H, and the peak in the 3435 cm^{-1} is related to the O-H in Fe3O4 nanoparticles. The peak in the 3448 region is related to the N-H of doxorubicin, and the O-H of magnetite and doxorubicin. The peak in the 1618 region is related to the C=O and C=C bonds of doxorubicin. The peak in the 1170 region is related to the C-O bond of doxorubicin.

The peak in the 1365 region is related to the C-H bond of doxorubicin. The peak in the 2853 region is related to the C-H bond of the drug doxorubicin, the peak in the 2925 cm^{-1} is related to the O-H bond of magnetite, and the peak in the 531 region is related to Fe-O and Cu-O [14].

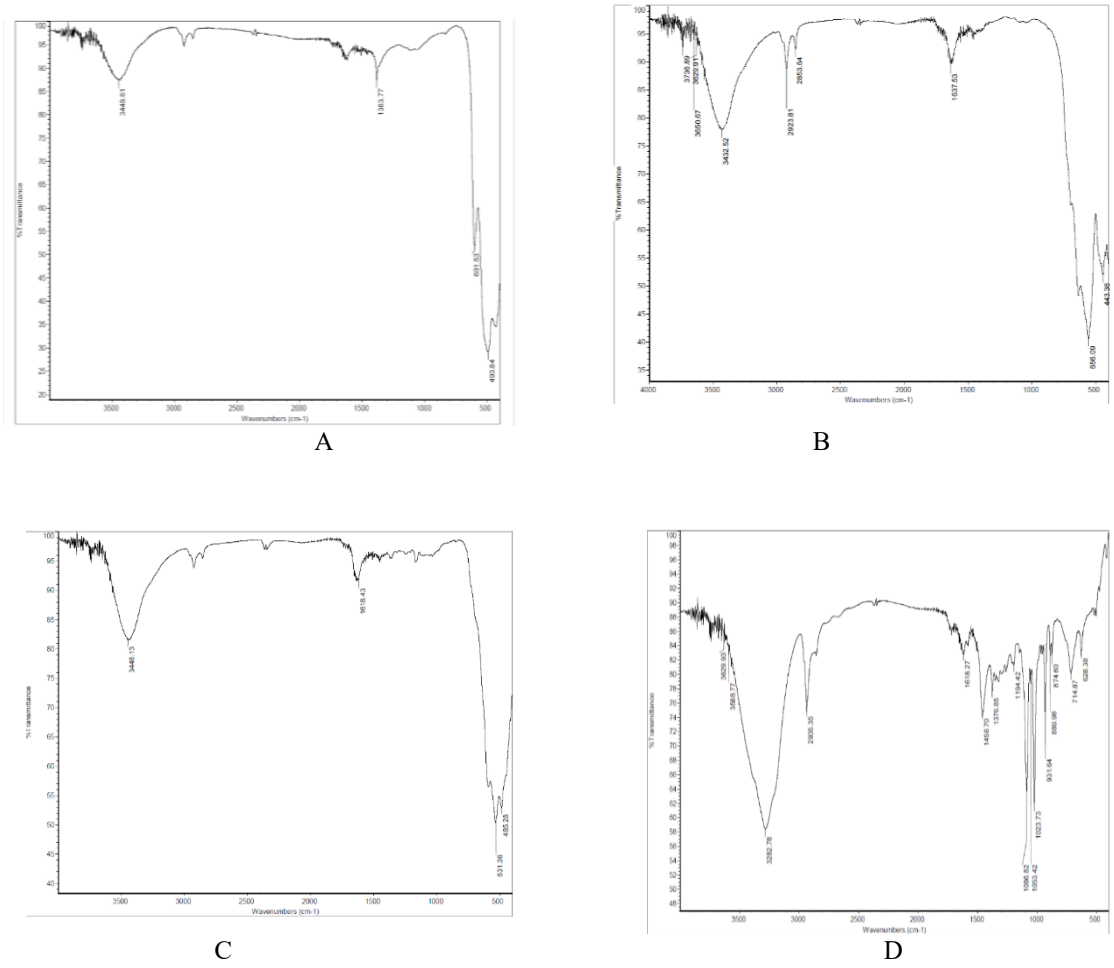
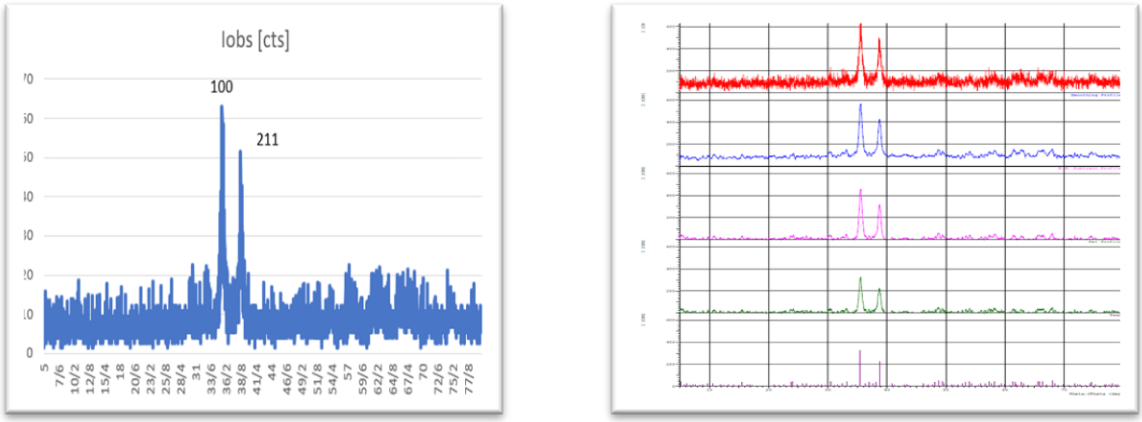


Fig. 4. *FT-IR; A) CuO, B) Doxorubicin, C) Doxorubicin/CuO/Fe3O4 and D) Fe_3O_4*

4.3 XRD Analysis

Since the discovery of X-rays at the end of the 19th century and the first discoveries about X-ray diffraction by crystals, there have been great advances in the application of these methods to the characterization of materials [15].

X-ray diffraction analysis was performed to confirm the synthesized Fe3O4/CuO nanoparticles. For CuO, peaks were observed at 2Θ= 6.46, 8.38, 2.36, 8.51, 6.59, and 4.67 with a Miller index of 100, but the index peaks (sharps) were at (100)2.36 = 2Θ and (211)8.38 = 2Θ. For Fe3O4, peaks were observed at 57, 4.54, and 44 and (100)2.36 and 6.33 = 2Θ [16].

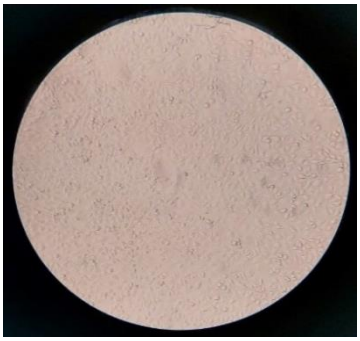


A *B*

Fig. 5. *A and B showed the graphs of XRD Analysis*

4.4 Microscopic Results

The appearance of the treated cells after 24 hours of treatment with the calculated concentration of doxorubicin, pegylated copper oxide magnetic nanoparticles, and doxorubicin loaded on the nanoparticles was observed and photographed by microscope. The observations indicated that treatment with the drug caused a change in the appearance of the cells and the elimination of the regular shape in the morphology of the cells.



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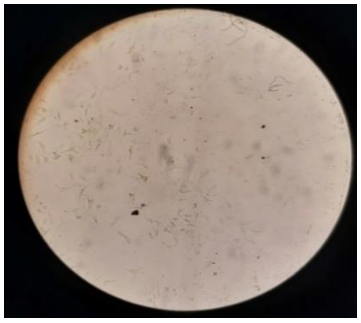

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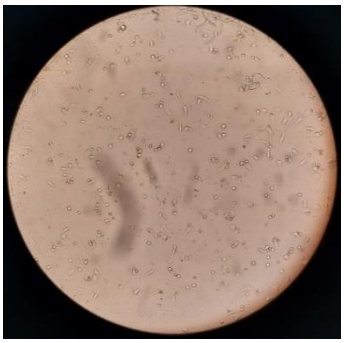

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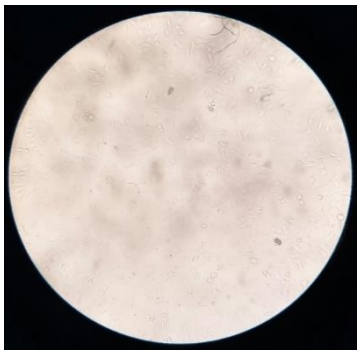

D

Fig. 6*. Microscopic images of A) control MCF-7 cells, B) cells treated with pegylated magnetic copper oxide nanoparticles, C) cells treated with doxorubicin, D) cells treated with doxorubicin-loaded pegylated magnetic copper oxide nanoparticles after 24 hours of incubation.*

4.5 Investigation of Biochemical Factors Involved in Oxidative Stress

Oxidative stress is one of the major mechanisms involved in cancer cell death. Therefore, to further investigate the effects of doxorubicin, pegylated magnetic copper oxide nanoparticles, and doxorubicin loaded onto the nanoparticles on sensitizing MCF-7 cells, intracellular oxidative stress parameters were evaluated.

4-5-1 Investigation of Lipid Peroxidation and Malondialdehyde (MDA) Production

The generation and accumulation of reactive oxygen and nitrogen species lead to oxidative damage of cellular macromolecules, particularly membrane lipids, resulting in lipid peroxidation and the formation of stable peroxide radicals. Malondialdehyde (MDA), a final product of lipid peroxidation, is commonly used as a biomarker to assess intracellular oxidative stress.

MCF-7 cells were treated with a concentration of 2 µg/mL of doxorubicin, pegylated magnetic copper oxide nanoparticles, and doxorubicin-loaded nanoparticles. The results demonstrated that, compared to control cells, all treatments led to a significant increase in MDA levels, indicating enhanced lipid peroxidation. Among the treated groups, cells exposed to doxorubicin-loaded nanoparticles showed the highest increase in MDA concentration, while cells treated with doxorubicin alone exhibited the lowest increase. These findings suggest that loading doxorubicin onto nanoparticles intensifies oxidative stress-mediated cytotoxicity in MCF-7 cells.

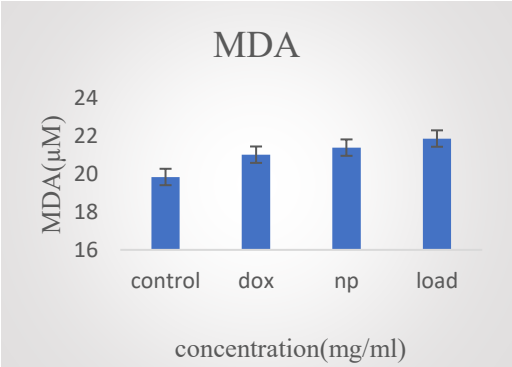

Fig. 7. *Measurement of malondialdehyde (MDA) levels in MCF-7 cells treated with doxorubicin, pegylated magnetic copper oxide nanoparticles, and doxorubicin-loaded nanoparticles.*

5. CONCLUSION

In the present study, the cytotoxic effects of doxorubicin, pegylated magnetic copper oxide nanoparticles, and doxorubicin-loaded nanoparticles on MCF-7 breast cancer cells were evaluated. The results demonstrated that the combination of doxorubicin loaded onto nanoparticles exhibited the highest cytotoxicity, whereas nanoparticles alone showed the lowest cytotoxic effect. In all treated groups, cell viability was significantly reduced compared to the control group.

The enhanced anticancer activity observed for the combined formulation can be attributed to improved cellular uptake and increased induction of oxidative stress. Overall, the findings suggest that the combination of doxorubicin with pegylated magnetic copper oxide nanoparticles is more effective than either doxorubicin or nanoparticles alone. Therefore, this nanoparticle-based drug delivery system may serve as a promising candidate for more effective breast cancer treatment.

6. ACKNOWLEDGMENTS

We sincerely acknowledge and appreciate the valuable contributions of our esteemed colleagues involved in this project, which represents a collaborative effort among the Faculties of Applied Chemistry, Biology, and Veterinary Medicine at Urmia University.

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