J Nanostruct 16(1): 353-364, Winter 2026

RESEARCH PAPER

Zinc Oxide Nanoparticles Conjugated with Methotrexate: Anti-Proliferative Impacts via P53 and Cox2 Pathway Control in MCF7 Breast Cancer Cells

*Ali M. Ahmed [1], Russul M. Shehab [2], Mustafa Adnan Zaidan [3], Noor M. Saadoon [4] **

[1] College of Medicine, Mustansiriyah University, Baghdad, Iraq
[2] Electrical Engineering Technical College, Middle Technical University, Baghdad, Iraq
[3] Medical Technical College, Al-Farahidi University, Baghdad, Iraq
[4] Centre of Nanotechnology and Advanced Material, University of Technology, Iraq

ARTICLE INFO

Article History:
Received 05 August 2025
Accepted 27 November 2025
Published 01 January 2026

Keywords:
Breast cancer
Cox2
Methotrexate
P53
Zinc oxide nanoparticles

ABSTRACT

Treated in breast cancer depends on how advanced the cancer is, so the way the treatment of the cancer may use drugs, operation, or radiation treatment. However, all these lead to unwanted effects including chemotherapy and radiation. It is critically essential to recognize new therapeutic targets and produce efficient, targeted therapies. The development of methotrexate-linked zinc oxide nanoparticles (MTX-ZnONPs) and their potent anti-breast cancer cell properties are investigated in this study. Using various method such as UV visible spectroscopy, Dynamic light scattering (DLS), Scanning electron microscopy (SEM) and Scanning electron microscopy (SEM) we have confirmed the linking of ZnONPs with MTX. We also treated MCF7 cells with MTX-ZnONPs in a laboratory setting in order to test the effectiveness of MTX-ZnONPs, and found out how it behaves using the MTT assay. Then, to confirm the cytotoxic effect, further investigation is needed to investigate the cytotoxic effects and the expression levels of two key genes, P53 and COX-2. MTX and MTX-ZnONPs were expressed at different levels in both genes and compared to expression of the control group. As indicators of oxidative stress, the malondialdehyde (MDA) level was also measured. The MTT assay findings demonstrated that both free MTX and MTX-ZnONPs were efficient at causing cytotoxic effects on MCF7 cells. Increased gene expression of P53 and COX-2 was noted in cells when treated with MTX-ZnONPs or MTX instead of the control group, possibly indicating potential therapy. As a marker of oxidative stress, levels of malondialdehyde (MDA) were also measured and there was a significant increase in MTX-ZnONPs and MTX compared to controls, MTX and MTX-ZnONPs, respectively. As a result, our findings demonstrated the excellent efficacy of MTX-ZnONPs, suggesting that ZnONPs function as nanocarriers to deliver the medication intracellularly.

How to cite this article
Ahmed A., Shehab R., zaidan M., Saadoon N. Zinc Oxide Nanoparticles Conjugated with Methotrexate: Anti-Proliferative Impacts via P53 and Cox2 Pathway Control in MCF7 Breast Cancer Cells. J Nanostruct, 2026; 16(1):353-364. DOI: 10.22052/JNS.2026.01.031

* *Corresponding Author Email: mae.visit.04@uotechnology.edu.iq*

INTRODUCTION

Breast cancer is the most common malignancy diagnosed in women all over the world [1–3]. That was the year, 2020, when overall breast cancer incidence totaled 2.3 million new cases and 685,000 deaths. The need for better treatments of this illness [1] is critical and immediate; by 2040 almost 3 million new cases and 1 million deaths per year are predicted. Although it is increasing globally [3], deaths associated with breast cancer seem to be declining in wealthy nations. Although fewer, the deaths of countries with lesser income and middle classes are more than that of wealthier nations [4].

Breast cancer treatment depends on the stage of cancer and includes medications, surgery, and possibly radiation therapy [1].Currently in practice around the world, treatments include hormone therapy for hormone positive diseases that targets the hormone receptors to slow or stop the cancer cell growth, antiHER2 treatment for HER2 positive disease to target the HER2 protein in cancer cells, immunotherapy uses the bodies immune system to fight cancer and chemotherapy uses drugs to kill cancer cells [3]. The breast cancer cells determine how one would select the best treatment options. Some of these factors are: genomic alterations, immune environment markers, hormone receptors and the activation of HER2 (6Doctors have categorized the key medications used to treat breast cancer as essential by the World Health Organization, or WHO. The list of these key medicines comprise of doxorubicin, cyclophosphamide, paclitaxel, docetaxel, tamoxifen, aromatase inhibitors and trastuzumab [7]. To achieve the best results in treating breast cancer, it is crucial to diagnose the disease early and to use a thorough and team-based approach for patient care, highlighting the need for teamwork in managing patients and the important roles that we each have in this process[2]. The aim of treatment is to reduce deaths from breast cancer and reduce the impact of the cancer overall [1]. In many cases, local therapies such as surgery and radiation, along, if necessary, systemic treatments, can treat early breast cancer successfully [6]. For stage IV breast cancer, the objective is to manage the disease for as long as possible, primarily using medications [1]. Palliative care focuses on managing patients with metastatic disease and should be introduced soon after diagnosis, including pain management, as well as psychosocial and spiritual support [2].

Current treatments for breast cancer include surgery, radiation, and systemic therapies, namely, hormone therapy, chemotherapy, targeted therapy, and immunotherapy [3,7] and despite the positive effect of treatment on survival, its effectiveness is far from perfect. This points to an equally pressing and urgent need for new, more effective ways to fight breast cancer in this field, an infographic.

Limitations of Current Treatments

Chemotherapy and radiation can deliver side effects like neutropenia, lymphedema, hair loss, nausea and cognitive impairment [2, 8].

Breast cancer cells can develop resistance to endocrine therapy, anti-HER2 therapy, and chemotherapy [9, 10]. Mechanisms that cause drug resistance lead to the development of vital new treatments. However, these mechanisms have complexities that make the challenge of fighting breast cancer more difficult.

Therefore, Triple-negative breast Cancer (TNBC) has ill defined molecular target limiting effective use of targeted therapies. TNBC has higher metastasis and recurrence rates [11], so currently chemotheraphy is the primary systemic treatment.

Unfortunately, current treatments have some disadvantages, such as incomplete surgical removal of tumors, toxic effects to healthy tissues, inadequate drug concentration at the tumor site, and poor drug penetration into tumors because of abnormal blood vessels [12].

Access to essential medicines and treatment facilities is limited in low- and middle-income countries (LMICs), which adversely affects breast cancer survival for these patients [2].

It revealed a higher level of COX2 mRNA in breast cancer and various other solid tumors in humans. These include cancers of the colorectal region, lung, stomach, bladder, pancreas, uterus, and prostate [13]. The overactivity and high levels of COX2 have been proven in research rats with breast cancer caused by carcinogens, as well as in genetically modified mice that produce too much HER2 [14]. The most known targets of NSAIDs are the cyclooxygenase (cox) enzyme. It is this enzyme that shrolls prostaglandins (PGs) from arachidonic acid. Two isoforms of Cox gene have been identified. Cyclooxygenase1 (cox1) has been proven in normal tissues to be in cell membranes and to participate in many of the physiological

roles, such as protecting the stomach and regulating platelet aggregation. On the other hand, cyclooxygenase2 (cox2) is located in the cytoplasm and is not usually found in most normal tissues; it only appears when there is inflammation or other stimulating factors. Additionally, the expression of cox2 can be increased by oncogenes, growth factors, cytokines, and tumor promoters, and it has been shown to prevent apoptosis. In various tumors, including those of the colon, esophagus, stomach, cervix, and breast, cox2 is associated with cancer development and the progression of tumors [6]. COX2 is an enzyme that promotes inflammation. It is commonly associated with the expression of NOS2 in situations where there is inflammation. Additionally, COX2 has been connected to negative results in cancer [15].

The tumor suppressor p53 is one of an essential transcription factor that controls numerous cellular function. Although there are many conditions that $p5_3$ can stop cells from growing in cancer, should they be triggered, p53 limits cell growth helping to prevent tumours developing. This function causes it to be called 'the guardian of the genome'. p53 undergoes several changes after translation when cells are stressed to including 1) phosphorylation which stabilizes p53 by changing its interaction with MDM2, 2) DNA binding to specific sites, and 3) transactivation by p53 via gene either manipulation, stimulation, or inhibition of gene that regulate the cell cycle, DNA repair, and aging and how cells die [16].

Need for New and Effective Therapies

Currently, there is no good treatment strategy for metastatic breast cancer with a good survival rate, which is not sufficient.16 Metastatic TNBC needs new solutions [10]. Future therapeutic concepts are designed around de-escalation or escalation of treatment based on cancer biology and early response to therapy and to individualize therapy. Treatment de-escalation refers to reducing the intensity of treatment, if the cancer responds well, whereas treatment escalation implies increasing the intensity of treatment if the cancer does not respond well [3]. However, new therapeutic targets and effective targeted agents are urgently needed, especially for TNBC [11]. To identify emerging treatments and repurposed drugs, new drugs are used to mark tumor driving pathways in TNBC [17]. Novel therapeutic agents with diverse modes of action are needed to treat HR+ breast cancer more professionally and to overcome resistance [8].

Such new methods include antibody drugs conjugates, immunotherapy, and the treatment in distant pathways [18]. In the future, effective therapeutic methods include caspase independent cell death programs and lysosome dependent cell death (LDCD) [19]. For patients with metastatic BC, the clinical trials check out the PROTAC system, which represents proteolysis targeting chimera. It can improve current strategies for treatment of cancer [20].

Methotrexate, usually known as MTX, interrupts the creation of nucleic acids and reduces the development of cancer cells. Is a chemotherapy medication frequently utilized in the treatment of diverse forms of cancer. This drug works well because it blocks the role of dihydrofolate reductase or DHFR. MTX works by binding to folate receptors located on the outer layers of tumors and cancerous cells [21].

But it has drawbacks that include severe toxicity on healthy cells [22, 23], non specific delivery of drugs [21], drug resistance [22], low permeability and solubility [24], drug efflux from cells, hemolytic toxicity [24] and short blood circulating half life [25]. Therefore, researchers have explored the use of nanoparticles (NPs) for targeted drug delivery which would help in overcoming these restrictions [26]. Conjugating MTX with NPs enhances toxicity of even lower doses of MTX than free MTX [21].

ZnONPs have attracted attention for their potential use in medicine due to their physical and chemical properties. These uses of interest to the practitioner are antibacterial, regenerative, mechanical antiinflammatory, anti demineralizing and remineralizing and may also empower antitumor uses [27]. There are various techniques by which zinc oxide nanoparticles are made, including chemical, physical and green (biological). The United States Food and Drug Administration considers ZnO generally safe. They are considered less toxic than other metal oxide nanoparticles [28]. ZnO nanoparticles have customized properties relating to optics, magnetism, shape, electricity, catalysis, mechanics, and photochemistry that can be tailored for particular uses. A large surface area is derived from small size of nanoparticles which makes them useful for carrying drugs and engage with biological systems [27]. Zinc oxide nanoparticles can trigger the formation of reactive oxygen species, which plays a vital role in their

properties that fight bacteria and cancer [29]. Zinc oxide nanoparticles are more likely to dissolve in low-pH environments. This property makes them ideal for delivering drugs directly to tumors and releasing them inside cells within the acidic conditions in tumor microenvironments [28]. ZnO nanoparticles have great potential for delivering drugs directly to certain areas. This is because they can move through tiny blood vessels, be absorbed by cells, and help concentrate medicines effectively where needed [28]. The ability of these materials to break down naturally supports the gradual release of medications [29]. Essential features of ZnO nanoparticles in precise drug delivery consist of:

Zinc oxide nanoparticles can be combined with a variety of treatment substances, such as cancer medications, active biological molecules, genetic material, proteins, and agents used for imaging [28]. To improve how ZnO NPs deliver substances to specific cells and get absorbed, their surface can be changed by adding ligands, linker chains, medications, and markers that connect with receptors found on the intended cells, like cancer cells [27]. As an example, ZnO nanosheets that have folic acid attached have been utilized for the directed delivery of doxorubicin to cells in breast cancer [28]. Encapsulating drugs in ZnO nanoparticles can enhance their efficacy, stability, and bioavailability in the body because these drugs can be designed to release their medication under specific conditions, such as acidic pH in the tumor vicinity, thereby reducing damage to normal cells [28].

Here, we examine the use of methotrexate zinc oxide nanoparticles (MTXZnO NPs) as a directed treatment for breast cancer. Molecular and cellular reaction involving nanoparticulate are assessed during this study to see how these nanoparticles affect MCF7 cell line. In order to improve our understanding of how MTXZnO nanoparticles influence the level of cancer cell growth and death, the first part of the study looks at how MTXZnO nanoparticles affect the level of the genes P53 and Cox2, plays a crucial role in controlling tumor. In addition, the research has studied the obtained oxidative stress by these nanoparticles and have measured the amount of malondialdehyde (MDA) by using ELISA method. In addition we have studied the MTT assay to analyze cell viability, viability inhibition, and the amount of growth restrained by MTXZnO nanoparticles. This research aims at building more effective and targeted therapies for breast cancer with the aim to improve patient outcomes by focusing on these objectives.

MATERIALS AND METHODS

Synthesis of Zinc Oxide Nanoparticles (ZnONPs)

To create ZnONP, we began with 100 ml of deionized water and dissolved 0. 1 grams of zinc nitrate in it. After that, we mixed in 1% of bacterial supernatant into the solution we had prepared. This combination was incubated at 60 degrees Celsius for 5 hours while stirring at a speed of 200 RPM until it completely developed. Following this, we rinsed it multiple times with distilled water and ethanol to eliminate any leftover substances. Subsequently, it was placed in a muffle furnace and calcined for 2 hours at a temperature of around 350 °C±10 °C. , during which zinc nitrate decomposed thermally according to the Eq. 1:

$$Zn(NO_3)_2 \cdot 6H_2O + Heat \rightarrow ZnO + 2NO_2\uparrow + \tfrac{1}{2}O_2\uparrow + 6H_2O\uparrow \quad (1)$$

In the end, a white powder of ZnONPs was produced [30].

Conjugation of MTX with ZnONPs

MTX and ZnONPs were connected. MTXZnONPs are created by this linking procedure using an EDC coupler that binds to the amino group of MTX and the carboxyl groups of ZnONPsThe reaction mixture consisted of 800µL of ZnONP suspension, 50 mM HEPES buffer, MTX (3 mg/ml), and 5 mM EDC in a volume of 1 mL.For five hours, this mixture had to stay at 30° C. Therefore, centrifugation at 10,000 rpm for 10 minutes was used to isolate MTXZnONPs.

Characterization of MTX-conjugated ZnONPs

A particle size analyzer (Zetasizer NanoZS, Model ZEN3600, Malvern Instruments Ltd., Malvern, UK) was used to determine the zeta potential and particle size (distribution) of MTXZnONP. MTXZnONPs [31] were characterized with a Shimadzu dual beam spectrophotometer (resolution, 1 nm), UV1601 PC, Kyoto, Japan, which is a UVvis spectrophotometer. The size of the inorganic core was measured using a scanning electron microscope (Tecnai™ G2 Spirit BioTWIN, FEI, Hillsboro, United States) running at an accelerating voltage of 80 kV [32].

Cell culture

MCF7 cells (from ATCC) were kept in Dulbecco Modified Eagle Medium (DMEM) mixed with 10% Fetal Bovine Serum (FBS) and a combination of Penicillin at 100 units/mL and Streptomycin at 100 ug/mL (Invitrogen) [DMEM 10% FBSPenStrep]. These cells were grown in an environment with 5% CO_2 at 37°C.

Calculation of Cytotoxicity

MTX was analyzed by the MTT tetrazolium dye test to determine its effect on cells' growth and survival. Costar cells in Cambridge MA were seeded into a 96 well microplate at a concentration of 4 x 10^4 cells/ml. They were allowed to stick to the plate overnight and then treated with different drug levels for either 2 hours or 24 hours. After treatment, the drug was taken out by gently flipping the microplate, and the cells were rinsed two times with a fresh complete medium warmed to 37 degrees Celsius. The cells were kept in a drug-free medium for an extra 72 hours before undergoing a 4-hour incubation in an MTT solution, with a concentration of 2 mg MTT/ml in PBS, while kept in low light. The formazan blue compound formed by mitochondrial succinate dehydrogenase activity was extracted from the cells once the MTT solution was removed and DMSO was added. An EL800 auto microplate reader from Burlington, Vermont-based BIOTEK Instruments Inc. was used to measure the absorbance at 490 nm.

Quantitative real time PCR and RT-PCR

Total RNA was extracted from the collected cells with the help of TRIzol reagent from Invitrogen. This RNA was then converted into cDNA using an oligo(dT) primer, dNTPs sourced from Bioneer in Korea, and MMLV reverse transcriptase and RT buffer from Promega in the USA. The cDNA underwent amplification via PCR, which involved a mixture of cDNA, specific primers, dNTPs, and Top DNA polymerase. All primers and reagents used in this process were obtained from Bioneer. The PCR products were analyzed through electrophoresis on 1% agarose gels that contained ethidium bromide. The following primers were used: F: ACAGCGTGGTGGTACCGTAT and R: GGAGCTGTTGCACATGTACT for p53; F: AGCTTGTGTGTGAGTGGTAG and R: TGAGAGATGGGCTGTTGTGT for COX2; F: AAG TTC AAC GGC ACA GTC AAG G and R: CAT ACT CAG CAC CAG CAT CACC for GAPDH. According to the manufacturer's instructions, the quantitative real-time PCR was amplified using Power SYBR Green PCR Master Mix on an ABI StepOnePlus RealTime PCR thermal cycler (Applied Biosystems, USA). After adjusting the target's mRNA levels with respect to GAPDH, the mRNA expression levels were calculated in relation to this normalization.

Statistical analysis

The information is shown as the average ± SEM from three experiments conducted in triplicate. One-way ANOVA with Dunnett's post hoc test, two-way ANOVA with Bonferroni's post hoc test, and paired t-test (using GraphPad Prism 8. 0) were used to evaluate significance across various treatment groups. (Significant differences between the mean values of different treatment groups are indicated by * $p < 0.05$, * * $p < 0.01$, and * * * $p < 0.001$.)

RESULTS AND DISCUSSION

Characterization of MTX.Zn-NP conjugates

The measurement of MTX.ZnONP nanoparticles were performed using dynamic light scattering (DLS). The size of nanoparticles is 56.63nm was observed from the count distribution graph. In that graph, a peak means uniform distribution of nanoparticles and no aggregation at least in the sample. Therapeutic effect is enhanced by increased percentage of nanoparticles entering the cells (Fig. 1A), due to a uniform distribution of nanoparticles. The analysis of the conjugation between MTX and ZnONPs involved examining the spectra of the drug and nanoparticle solution, which revealed a shift in the ZnONPs' particular wavelength. The main peak is at 339 nm (Fig. 1B). For MTX, the zeta potential was measured. The ZnONPs value was -19.9 mV, suggesting that the drug nanoconjugate is stable (Fig. 1C). The intense and main peak in X-ray diffraction (XRD) is located around 36-38 degrees (Fig. 1D). Scanning electron microscopy (SEM) was used to verify the MTX.ZnONPs' average size and shape. The size distribution graph showed that the drug nanoparticle conjugates had an average size of 55.5 nm (Fig. 1E).

Effect of MTX.ZnNP on MCF7 Cell Viability After 72 Hours

The viability of the MCF7 control group and the cells that received sham treatment is at its peak, nearly 100%. This demonstrates the original

growth ability of the untreated MCF7 cells and indicates that the sham treatment does not cause any notable toxicity. Treatment of cells with MTX 50 causes a decrease in cell viability compared to the control group. An apparent reduction in cell viability depends on the amount of Met. ZnNP used can be seen in the different concentrations:

Viability decreases are not significant at lower levels (3. 12 and 6. 25 µM) and are also similar to the untreated controls. In their nature, a more abrupt decrease in cell viability indicates increased toxicity at the moderate level (12.5 µM). Viability is greatly decreased with higher amounts (25 and 50 µM) and, especially with 50 µM Met. The ZnNP group, which is most reduced (Fig. 2).

Increased oxidative stress and lipid peroxidation in MCF7 cells treated with methotrexate-zinc oxide nanoparticles

Fig. 3 shows the treated cells with Met. The

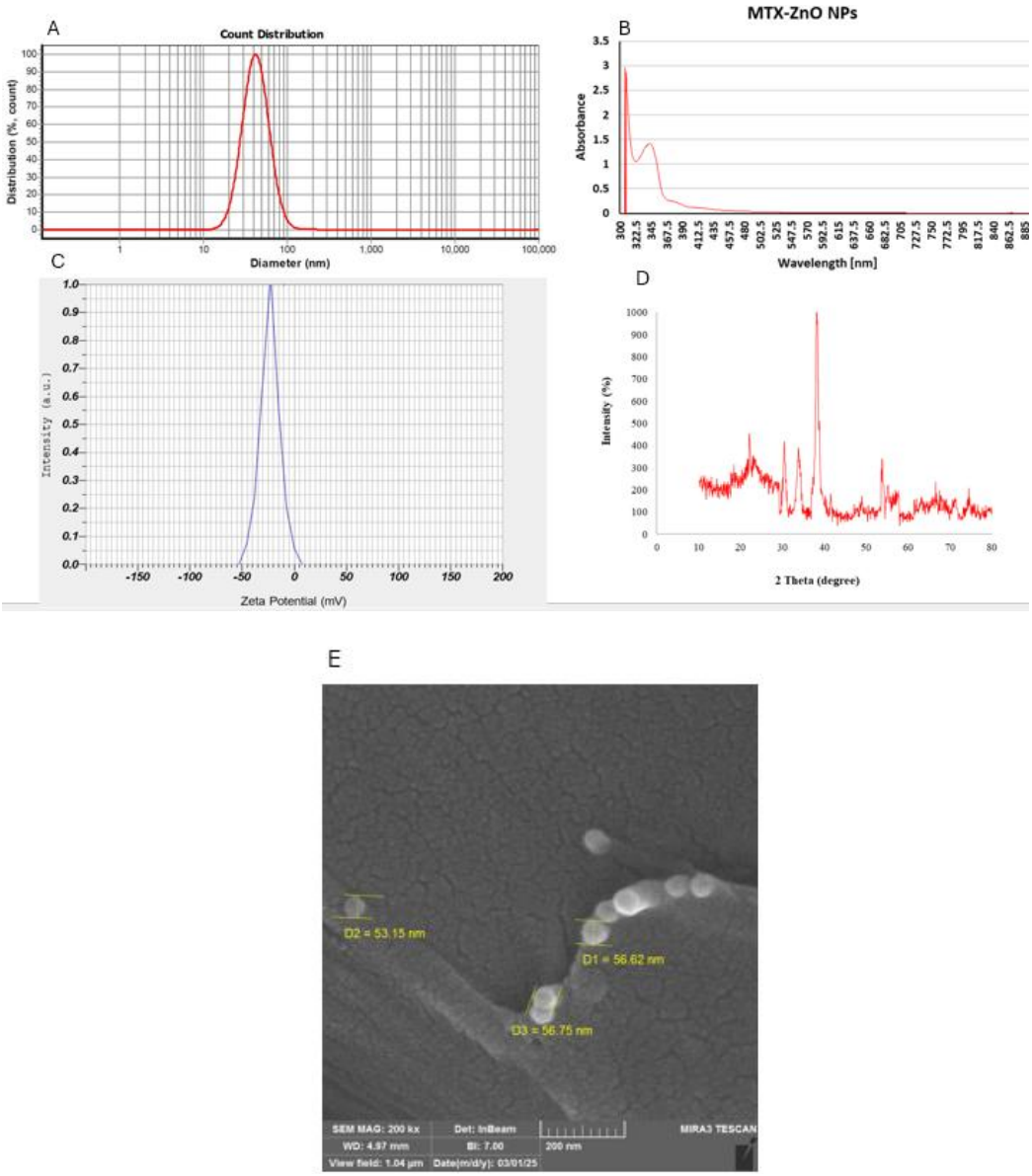

Fig. 1. MTX.ZnONPs' characteristics under (A) hydrodynamic diameter (56.63 nm), (B) UV-visible spectra (339 nm), (C) zeta potential (−20.9 mV), (D) XRD pattern of MTX. ZnO in the 2θ range of 10 to 80 degrees, (E) Scanning electron microscopy (size 55.5 nm).

results show that Zn.NP possesses highest level of MDA, which suggests high level of oxidative stress. When this group is compared with the two groups MCF7 and Sham, we see that they show a statistically significant difference ($p < 0.0001$). The Met group also shows a considerable difference compared to the two groups, MCF7 and Sham ($p < 0.01$). The Sham group has the lowest level of MDA and shows no significant difference compared to the control group. The two groups Met. Zn.NP and Met also have ($p < 0.05$), indicating a statistical difference.

Increased expression of the P53 gene in treatment with MTX.ZnNP$_s$ in MCF7 breast cancer cells

The MCF7 and Sham were the lowest two groups showing expression levels. A score of > 15 was significantly increased ($p <0.01$) relative to the control and sham groups. The Met.Zn.NP group also showed a significant increase ($p < 0.001$) compared to the two groups, MCF7 and Sham. No statistically significant difference exists between Met and Met. Zn.NP groups. There is no significant difference between the two groups, MCF7 and Sham.

The effect of MTX and MTX.ZnNP$_s$ on COX-2 gene expression in MCF7 cancer cells

The COX2 gene expression in the Met group is dramatically higher than control ($p<0.05$). Compared to control, the increase of the Met. Zn.NP group was much higher, $p<0.001$. There was no significant difference between Met and Met. Zn.NP groups. The difference between these two groups and the Sham group was also substantial (* $p<0.05$ and *** $p<0.001$).

Breast cancer is a varied condition that includes different types of tumors. Triple-negative breast cancer (TNBC) makes up 15% of cases, is very aggressive, and shows strong resistance to

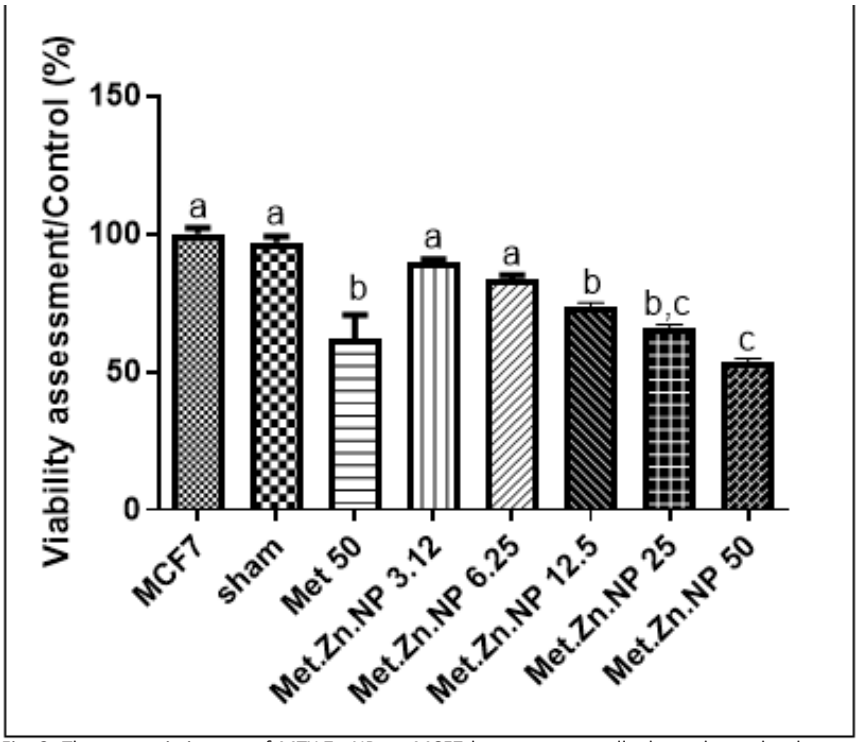

Fig. 2. The cytotoxic impact of MTX.Zn-NP on MCF7 breast cancer cells depends on the dosage, experimental after 72 hours of incubation. Variations in letters signify statistically meaningful differences among the groups ($p < 0.05$). above the bars represent statistical comparisons between different groups: Groups labeled "a" are statistically alike, meaning there is no vital difference in viability. Groups labeled "b" have a significantly lower viability than the control. The most negative effects of MTX.Zn-NP are shown by the groups identified as "c", with a great potential of toxicity associated with the raised MTX.Zn-NP concentrations.

treatment (15). Inflammation is a complicated process involving biochemistry and the immune system in blood vessel tissues when faced with different harmful triggers, such as germs, abnormal cells, or irritants. The primary objective is to set the body into defensive mode against infections or injury, thereby helping the body to heal. Prostaglandins, essential to inflammation, are produced by COX2 and cause blood vessels at the injury location to react, starting and keeping the inflammatory process [13].

Combining traditional chemotherapeutic medications with a carrier or delivery agent that reduces their side effects can help reduce their dosage because these drugs often have severe side effects. By combining these drugs with a carrier, the medication can be directed straight to the cancer cells [33]. In this context, nanotechnology provides excellent options for delivering drugs. The impressive chemical and physical properties of ZnONPs and their compatibility with living tissue show great potential for identifying and treating various forms of cancer[11]. In the research, biological method of creating ZnONPs and combining the antifolate drug MTX with the skill to terminate breast cancer cells were evaluated. Here we demonstrated the combination of ZnONPs and MTX by means of UV-vis spectroscopy, DLS, XRD, and SEM. MTX was then linked with ZnONPs using EDC chemistry. Since the amide bond of MTX with ZnONPs is very stable, the drug's and nanoparticles' connection was very stable.

The study built on the success of MTX and biologically produced zinc oxide nanoparticles, which was found as a potential carrier for the medication. UVvis spectroscopy, DLS, XRD, and SEM analyses were used to verify this. The amide bond formed between MTX and ZnONPs ensures stability of the drug nanoparticle conjugates and restricts destabilization by surface adsorption of acidic molecules.

The results of the MTT test presented that Met. Zn.NP has a lethal effect on MCF7 breast cancer cells. While the control and sham groups, which did not receive the drug, survived. Met 50, which is a chemotherapy drug, has also caused cell

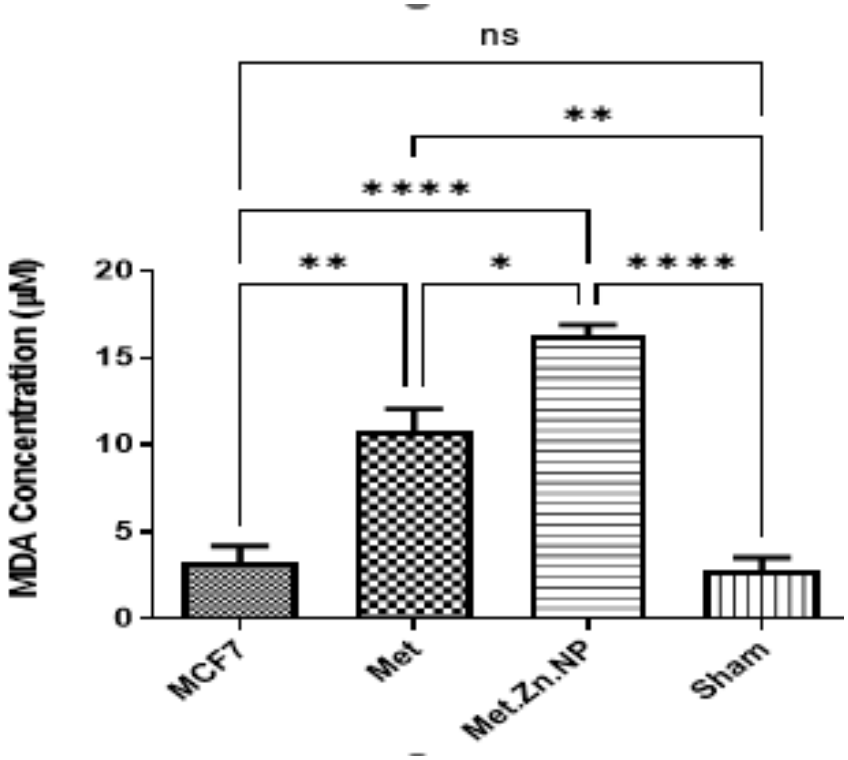

Fig. 3. The malondialdehyde (MDA) concentration in the MCF7, Met, Met.Zn.NP at a concentration of 50 ng/ml and Sham groups. The results are presented as mean ± standard deviation (* p<0.05, ** p<0.01 and **** p<0.0001).

death. Met.Zn.NP at a dose of 50 has a higher cytotoxic effect compared to Met ($p < 0.05$). The results of the MTT test presented that Met. Zn.NP has a lethal effect on MCF7 breast cancer cells. While the control and sham groups, which did not receive the drug, survived. Met 50, which is a chemotherapy drug, has also caused cell death. Met.Zn.NP at a 50 ng/ml dose has a higher cytotoxic effect than Met ($p < 0.05$). The increased activity of MTXZnONPs may be due to increased MTX entering the cell. This means drug delivery has been carried out correctly, and cancer cells are suppressed. Studies have also shown that human folate receptors tend to absorb MTXZnONPs better than MTX. These nanoparticles are also released in the acidic environment of lysosomes [34]. Because the folate receptor is highly expressed in cancer cells, MTXZnONPs may be taken up by these cancer cells easily and effectively. Our findings matched those of an earlier study in which MTX was linked to iron oxide nanoparticles (IONPs) functioning as a drug delivery method to examine its harmful effects on cancer cells [35]. ZnONPs by themselves greatly reduced the survival of A549 cells when used at a much larger concentration, specifically between 10 and 80 µg/mL. In comparison, MTXZnONPs and MTX showed less effect. This indicates that ZnONPs possess inherent toxic qualities toward lung cancer cells when present in higher amounts [31]. When MTX is combined with ZnONPs, it has greater lethality compared to MTX alone. The findings also indicate that ZnONPs are not cytotoxic and are a suitable nanocarrier for drugs [31].

In a study on rats, it was shown that there was an increase in MDA and a decrease in the antioxidants superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT). In these rats that were treated with MTX, an increase in lipid peroxidation in the liver was observed [36]. Our results also showed that the Met and Met significantly increased the malondialdehyde (MDA) level as an oxidative stress indicator. Zn.NP groups are significantly lower than MCF7 ($p < 0.01$) and sham groups ($p < 0.0001$). Especially the Met. The level of MDA in MCF7 cells increased further

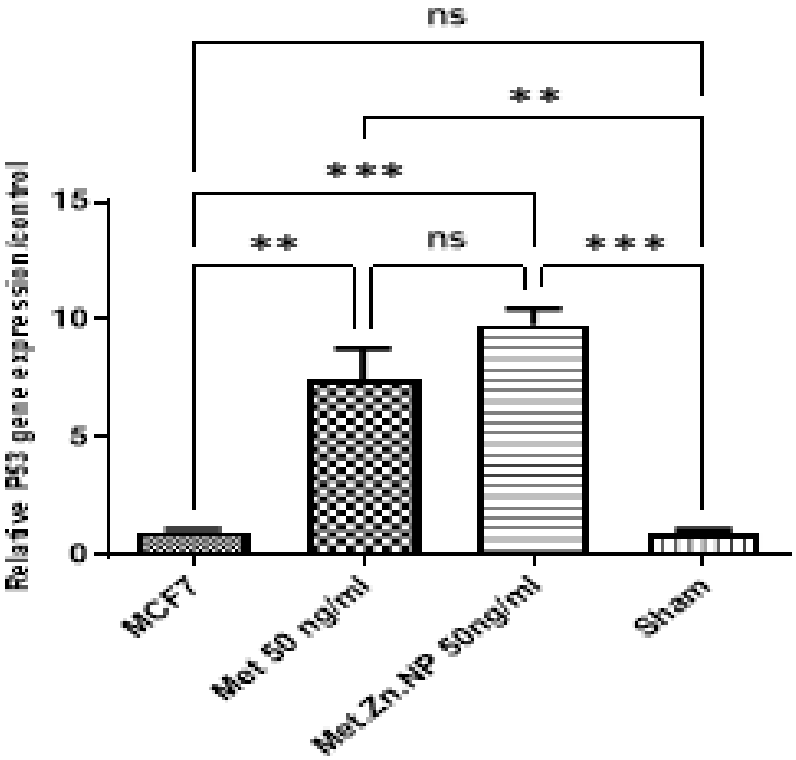

Fig. 4. P53 gene expression in breast cancer cells in 4 groups: MCF7, Met, Met. Zn.NP at a 50 ng/ml concentration and Sham. The results are presented as mean ± standard deviation (** $p<0.01$ and *** $p<0.001$).

with Zn.NP group, revealing more oxidative stress and lipid peroxidation.

The phosphorylation of p53 at Ser15 and its acetylation at Lys373/382 was enhanced, and the more stable and more expression was achieved. Apoptosis and reduced cell viability in response to MTX was dependent on p53 and to some extent p21 [37]. The treated cells include cells with MTX (50 ng/mL) and Met. Zn.NP (50 ng / mL) significantly exceeded that measured in the control group (MCF7 without treatment) ($p < 0.01$ and $p < 0.001$). This indicates that both treatments increase P53 expression and enhance apoptosis. The average expression of P53 in the group treated with Met.Zn-NP was slightly higher than in the pure MTX group, but this difference was insignificant (ns). This indicates that at a concentration of 50 nanograms per milliliter, Met. Zn-NP does not have a greater effect compared to pure MTX, and it might be better to test higher concentrations. These results are consistent with our hypothesis that Met. Zn.NP acts as a potential therapeutic strategy in breast cancer through the initiation of the P53 pathway. Given the key role of P53 in persuading apoptosis, these results recommend that Met. Zn.NP could be a promising approach for inhibiting tumor growth.

Cox2 is recognized for its role in tumor processes. Research indicates that while COX2 usually is absent in breast epithelial tissue, its expression rises in 63 to 85 percent of premalignant breast cancer cases like ductal carcinoma in situ. Likewise, around 40 percent of breast cancers in humans show higher levels of COX2, although this percentage can range from 5 to 100 percent based on the research methods used [5]. These processes include the growth and development of tumors, suppression of the immune system, the formation of blood vessels, and prevention of cell death, all through the production of prostaglandins. The importance of COX2 overexpression in predicting outcomes in breast cancer is notable. There was a strong link

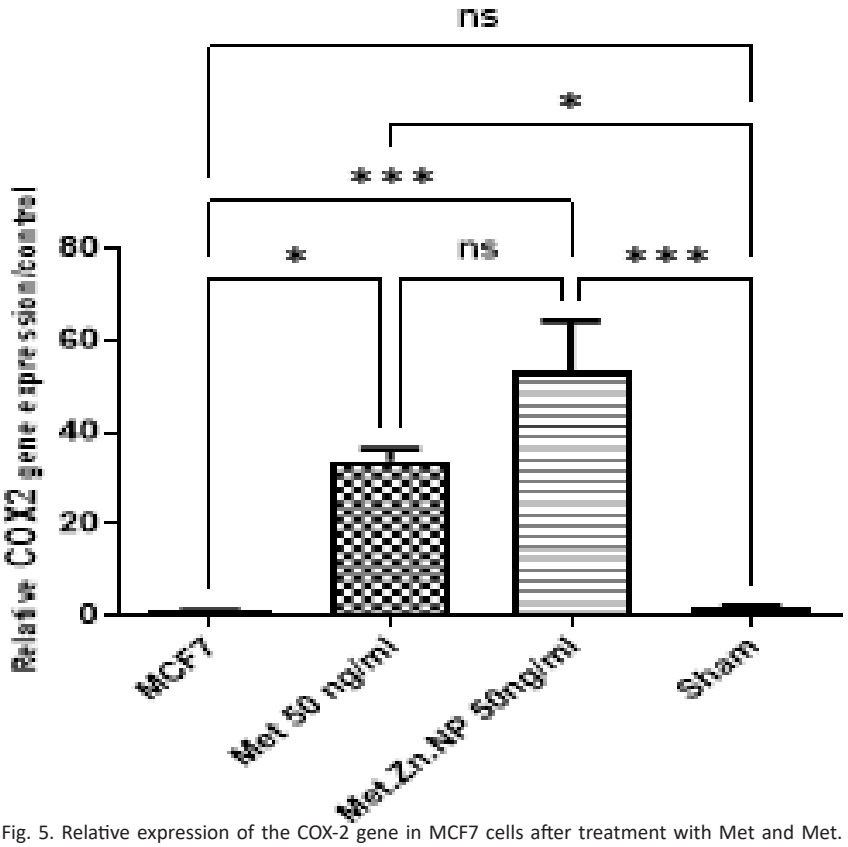

Fig. 5. Relative expression of the COX-2 gene in MCF7 cells after treatment with Met and Met. Zn.NP at a concentration of 50 ng/ml. The results are presented as mean ± standard deviation (* $p<0.05$ and *** $p<0.001$).

between COX2 overexpression and both luminal B and triple-negative breast cancer types [18]. The relative expression of COX-2 in the group treated with methotrexate at 50 ng/ml significantly increased compared to the control group (untreated MCF7, $p < 0.05$). COX-2 expression in Met.Zn-NP (50 ng/ml) group was higher than met.MX group (ns). This finding indicates. In MCF7 cells, the Sham group had the lowest level of COX-2 expression, which corresponded this gene's baseline level. This confirms that both types of treatment did increase COX-2 expression compared to the control state and that there was a significant difference between both treatment groups and the Sham ($p < 0.001$).

About because traditional chemotherapy drugs usually result in significant side effects, their doses can be reduced by combining them with a delivery agent or carrier. The combination helps the medication to take aim more directly at cancer cells. This means that a smaller amount of the drug can work better than a normal dose used for treatment [39]. Because of their remarkable physicochemical properties and biocompatibility, nanotechnology has become a useful modality for drug delivery and has significantly increased MTX within cells [40].

ZnO encasing of MTX can improve the efficacy, stability, and bioavailability of MTX in the body. Encapsulating MTX in ZnO can enhance its efficacy, stability, and bioavailability in the body because MTX can be designed to release its drug under specific conditions, such as acidic pH in the tumor vicinity, thereby inhibiting the growth of cancer cells [34]. Because the folate receptor is highly present in the cancer cells we are targeting, MTXZnONPs may be taken up by these cancer cells quickly and effectively. Our findings matched those of earlier research that involved linking MTX to iron oxide nanoparticles to deliver drugs, which aimed to examine its harmful impact on cancer cells [41].

CONCLUSION

The study combined MTX with zinc oxide nanoparticles in the concentration ranging from 1 to 50 ng/ml, which resulted in varying cytotoxicity considering the concentration: for 50 ng/ml the cytotoxicity was increased, but at concentrations below 50 ng/ml the cytotoxicity decreased. This shows that the dosage of MTX-ZnONPs is more cytotoxic than the medicinal dosage of MTX. Our findings indicate that the MTX-ZnONPs might be effective since they can translocate MTX inside the cells effectively. Finally, we showed effective delivery of methotrexate to breast cancer cells using ZnONPs as the carriers for the drug, thus our findings.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

REFERENCES

1. Arnold M, Morgan E, Rumgay H, Mafra A, Singh D, Laversanne M, et al. Current and future burden of breast cancer: Global statistics for 2020 and 2040. The Breast. 2022;66:15-23.
2. Rossi L, Mazzara C, Pagani O. Diagnosis and Treatment of Breast Cancer in Young Women. Curr Treat Options Oncol. 2019;20(12).
3. Smolarz B, Nowak AZ, Romanowicz H. Breast Cancer—Epidemiology, Classification, Pathogenesis and Treatment (Review of Literature). Cancers (Basel). 2022;14(10):2569.
4. Barrios CH. Global challenges in breast cancer detection and treatment. The Breast. 2022;62:S3-S6.
5. Young JP. Excellent summary of breast cancer recommendations. Can Fam Physician. 2021;67(3):157.152-159.
6. Hong R, Xu B. Breast cancer: an up-to-date review and future perspectives. Cancer Commun. 2022;42(10):913-936.
7. Mutebi M, Anderson BO, Duggan C, Adebamowo C, Agarwal G, Ali Z, et al. Breast cancer treatment: A phased approach to implementation. Cancer. 2020;126(S10):2365-2378.
8. Mayrovitz HN. Measuring Breast Cancer-Related Lymphedema. Breast Cancer: Exon Publications; 2022. p. 63-82. http://dx.doi.org/10.36255/exon-publications-breast-cancer-lymphedema
9. Barzaman K, Karami J, Zarei Z, Hosseinzadeh A, Kazemi MH, Moradi-Kalbolandi S, et al. Breast cancer: Biology, biomarkers, and treatments. Int Immunopharmacol. 2020;84:106535.
10. Ahmed AM, Omran ZH, Taha SY, Mohammed Yahya A, Jihad MA, Shehab RM, et al. Dark matter subhalo disturbance in the tidal field: Towards a new simulations accuracy. Materials Today: Proceedings. 2023.
11. Gupta GK, Collier AL, Lee D, Hoefer RA, Zheleva V, Siewertsz van Reesema LL, et al. Perspectives on Triple-Negative Breast Cancer: Current Treatment Strategies, Unmet Needs, and Potential Targets for Future Therapies. Cancers (Basel). 2020;12(9):2392.
12. Bhushan A, Gonsalves A, Menon JU. Current State of Breast Cancer Diagnosis, Treatment, and Theranostics. Pharmaceutics. 2021;13(5):723.
13. Russul MS, Alwan MA. Improved the Sensitivity and Limit of Detection of Surface Alloying SERS Sensors by Controlling Mixing Ratio of Trimetallic (Ag-Au-Pd) Nanoparticles. International Journal of Nanoelectronics and Materials (IJNeaM). 2024;16(2):359-370.
14. Barisik NO, Bozkurt S, Gumus M, Kaygusuz I, Karadayi N, Bas E, et al. Expression and prognostic significance of cox-2 and p-53 in hodgkin lymphomas: a retrospective study. Diagn

Pathol. 2010;5(1).
15. Sulaiman D, Wali LA, Alwan AM. Development of Flexible SERS Sensors via Trimetallic Nanoparticles/MWCNTs for Carbaryl Pecticide Detection. Plasmonics. 2025;20(8):6359-6370.
16. Oreibi I, Habeeb MA, Hamza RSA. Tailoring the Structural and Optical Features of PVA/SiO2-CuO Polymeric Nanocomposite for Optical and Gamma Ray Shielding Applications. Silicon. 2023;16(4):1407-1419.
17. Xu L, Xu P, Wang J, Ji H, Zhang L, Tang Z. Advancements in clinical research and emerging therapies for triple-negative breast cancer treatment. Eur J Pharmacol. 2025;988:177202.
18. Ferro A, Campora M, Caldara A, De Lisi D, Lorenzi M, Monteverdi S, et al. Novel Treatment Strategies for Hormone Receptor (HR)-Positive, HER2-Negative Metastatic Breast Cancer. Journal of Clinical Medicine. 2024;13(12):3611.
19. Shiralipour A, Khorsand B, Jafari L, Salehi M, Kazemi M, Zahiri J, et al. Identifying Key Lysosome-Related Genes Associated with Drug-Resistant Breast Cancer Using Computational and Systems Biology Approach. Iranian Journal of Pharmaceutical Research. 2022;21(1).
20. Mohammed Yahya A, Taha SY, Shehab RM, Omran ZH, Jihad MA, Ali Al-Hilali A, et al. The zinc Lysinate-riboflavinate structure colloid particles are a kind of colloid particles. Materials Today: Proceedings. 2023.
21. Mahmood AI, Shehab RM, Khalid EA, Alwan AS. Simulation and experimental of Nd-YAG laser surface treatment of biomaterials type 316 L stainless steel. AIP Conference Proceedings: AIP Publishing; 2024. p. 040003.
22. Chaudhari R, Patel P, Meghani N, Nasra S, Kumar A. Fabrication of methotrexate-loaded gold nanoconjugates and its enhanced anticancer activity in breast cancer. 3 Biotech. 2021;11(4).
23. Hooshyar VA, Mohammadi A, Ghorbani Y, Ebrahimi HA, Danafar H, Nosrati H, et al. Methotrexate conjugated polymeric prodrug encapsulating gold nanoparticles for combined chemo/radiation therapies. Appl Organomet Chem. 2024;38(6).
24. Rahman M, Khan JA, Kanwal U, Awan UA, Raza A. Methotrexate-loaded PEGylated gold nanoparticles as hemocompatible and pH-responsive anticancer drug nanoconjugate. J Nanopart Res. 2021;23(8).
25. Alwan AM, Mohammed MS, Shehab RM. Modified laser-etched silicon covered with bimetallic Ag–Au Alloy nanoparticles for high-performance SERS: laser wavelength dependence. Indian Journal of Physics. 2020;95(9):1843-1851.
26. Orrantia-Borunda E, Acuña-Aguilar LE, Ramírez-Valdespino CA. Nanomaterials for Breast Cancer. Breast Cancer: Exon Publications; 2022. p. 149-162.
27. Zakinyan A, Ahmed AM, Kononenko D. Local field and deformation of droplets in emulsions. J Mol Liq. 2025;427:127406.
28. Anjum S, Hashim M, Malik SA, Khan M, Lorenzo JM, Abbasi BH, et al. Recent Advances in Zinc Oxide Nanoparticles (ZnO NPs) for Cancer Diagnosis, Target Drug Delivery, and Treatment. Cancers (Basel). 2021;13(18):4570.
29. Al-darwesh MY, Ibrahim SS, Mohammed MA. A review on plant extract mediated green synthesis of zinc oxide nanoparticles and their biomedical applications. Results in Chemistry. 2024;7:101368.
30. Gad El-Rab SMF, Abo-Amer AE, Asiri AM. Biogenic Synthesis of ZnO Nanoparticles and Its Potential Use as Antimicrobial Agent Against Multidrug-Resistant Pathogens. Curr Microbiol. 2020;77(8):1767-1779.
31. Mishra P, Ali Ahmad MF, Al-Keridis LA, Saeed M, Alshammari N, Alabdallah NM, et al. Methotrexate-conjugated zinc oxide nanoparticles exert a substantially improved cytotoxic effect on lung cancer cells by inducing apoptosis. Front Pharmacol. 2023;14.
32. Khan S, Ahmad K, Ahmad A, Raish M, Jan BL, Khan A, et al. Biogenic pentagonal silver nanoparticles for safer and more effective antibacterial therapeutics. International Journal of Nanomedicine. 2018;Volume 13:7789-7799.
33. Zakinyan AR, Ahmed AM. Instability of ferrofluid interfacial surface in simultaneously acting magnetic and electric fields. Chem Phys Lett. 2024;841:141169.
34. Alwan AM, Mohammed MS, Shehab RM. Optimization of an ultra-sensitive Ag core-Au shell nanoparticle/Si Surface-enhanced Raman scattering (SERS) sensor. AIP Conference Proceedings: AIP Publishing; 2020. p. 020005.
35. Al-Majeed MA, Ahmed AM, Abdulmohsin AS, Mohammed MQ, Al-Hilali AA. Information of silicon nanoparticles by pulsed laser. AIP Conference Proceedings: AIP Publishing; 2023. p. 050040.
36. Ghoneum M, El-Gerbed MSA. Human placental extract ameliorates methotrexate-induced hepatotoxicity in rats via regulating antioxidative and anti-inflammatory responses. Cancer Chemother Pharmacol. 2021;88(6):961-971.
37. Zhao Q, He W, Liu Z, Huang L, Yang X, Liu Y, et al. LASS2 enhances p53 protein stability and nuclear import to suppress liver cancer progression through interaction with MDM2/MDMX. Cell Death Discovery. 2023;9(1).
38. Kennedy BM, Harris RE. Cyclooxygenase and lipoxygenase gene expression in the inflammogenesis of breast cancer. Inflammopharmacology. 2018;26(4):909-923.
39. Zhang T, Chen Y, Ge Y, Hu Y, Li M, Jin Y. Inhalation treatment of primary lung cancer using liposomal curcumin dry powder inhalers. Acta Pharmaceutica Sinica B. 2018;8(3):440-448.
40. Deng X, Luan Q, Chen W, Wang Y, Wu M, Zhang H, et al. Nanosized zinc oxide particles induce neural stem cell apoptosis. Nanotechnology. 2009;20(11):115101.
41. alnada faris Husham K, khdier HM, Salih WM. Eco-friendly epoxy composites for high-performance skateboards. Discover Materials. 2025;5(1).