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Photosensitivity and Radiosensitivity of Methylene Blue (MB) With Gold Nanoparticles Coated By Thioglucose (Gnps-Tio): An In Vitro Study

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ABSTRACT

Introduction: Multifunctional of cancer-specific tumor biomarkers is a potent therapeutic approach to treat cancer diseases with high efficacy. Among these methods that can be mentioned are the composition and design of nanoparticles and photosensitizers (PS). The purpose of this study is to investigate the effect of gold nanoparticles (GNPs) coated thioglucose (Tio) combined with methylene blue photosensitizer to enhance the efficacy of hybrid therapy (photodynamic and radiation therapy).

Material and Methods: First, GNPs-Tio was synthesized. Next, the toxicity of GNPs-Tio, MB, and their combinations was determined on the MCF-7 cell line to achieve their optimal concentrations. In the next step, the efficacy of combination therapy was evaluated using hybrid therapy. For this purpose, an optical dose of 15.6 J/cm^2 and 2 Gy for radiation therapy were delivered. Cell viability was evaluated using MTT and colony assays.

Results: According to the MTT assay, the combined photodynamic and radiation treatment of GNPs-Tio did not cause significant cell death. But this induced significant cell death by using GNPs-Tio + MB while the cell survival rate was almost zero**.** Combined therapy caused significant cell death in the presence of each of the pharmacological agents alone and their combination in colony assay.

Conclusion: The difference in treatment results between the MTT and the colony assay can be due to the more accurate colony assay for cell death detection. Significant cell death was achieved in the combination of photodynamic and radiotherapy in the presence of MB and MB + GNP-Tio.

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Introduction

Cancer remains a leading cause of mortality worldwide. Current clinical interventions for cancer comprise a multifaceted approach that may include surgical intervention, radiation therapy, chemotherapy, and, in recent years, the emergence of immunotherapy and other targeted modalities that are often used in combination with the aforementioned strategies [1]. Furthermore, surgical removal of some tumors does not prevent recurrence, and the cumulative radiation dose severely limits radiotherapy. Therefore, although refinement of the usual anti-cancer method is important, developing new therapeutic approaches that are safe, powerful, and cost-effective seems essential. One of these new approaches is photodynamic therapy, which is related to the fluorescence of the dye [2]. The most important advantage of PDT over conventional anti-cancer therapies is that it limits the toxic effects of PS and light on biological tissues, thus protecting natural tissues. In addition, the combination of PDT with other chemotherapeutic drugs, due to their possible synergistic effects, may help control long-term tumors [2]. Methylene blue, a photosensitizer with absorbance ranging between 650-670 nm and a high extinction coefficient in the Near-Infrared Region I (NIR-I: 600–900 nm) spectrum, finds extensive use across various applications. Its applications include anti-bacterial treatments, treatment of toenail onychomycosis, and several therapeutic interventions in cancer treatment. Which can be effectively activated in a deeper area. Some properties of methylene blue make this photosensitizer capable of being an almost ideal photodynamic agent. The low cost, accessibility of this photosensitizer, and low dark toxicity can be considered as one of the advantages of its clinical application. High purity, stable formulation are the most suitable physical and chemical characteristics, which make it distinguished from other photosensitizers. Moreover, certain research studies have indicated that a combination of Methylene blue

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(MB) and photodynamic therapy (PDT) can be a moderately effective therapeutic approach capable of inducing programmed cell death through apoptosis, a less severe and controlled form of cell death, rather than necrosis. MB and PDT have demonstrated the ability to generate a high singlet oxygen quantum yield and selectively target specific tumor cells. However, the poor cellular uptake of MB due to its inability to penetrate through the cell membrane remains a challenge. In this context, the use of drug carriers that can enhance cellular uptake may provide a suitable solution [3]. Targeting deep tumors and internal organs will be challenging due to the absorption of light used in photodynamic therapy by the tissue. Studies show that in order to optimize the depth of treatment, radiation therapy (RT) can be used alongside photodynamic therapy. In radiation therapy, healthy cells, in addition to tumor cells, are damaged by receiving ionizing radiation doses. Therefore, many studies have been performed to increase the efficiency of this method and thus the possibility of using lower doses to reduce the side effects of healthy cells. Because the optical-sensing and radiation-sensing factors are usually different, the design of a composite structure that simultaneously combines both of these properties can be effective. In modern research, including nanotechnology, gold nanoparticles due to their low toxicity, high biocompatibility, and aggravating biological damage caused by radiation can be studied more carefully[4, 5]. Gold nanoparticles have a high adsorption cross-section and their biological compatibility allows their surface to be operated. In addition, it has been widely used for diagnostic applications because of its dispersion and adsorption properties. The maximum absorption wavelength and dispersion of gold nanoparticles can vary depending on their size and shape. Due to their high biocompatibility and surface performance, gold nanoparticles have recently been considered as a suitable transfer agent in the fields of diagnosis, biological imaging, and cancer treatment. Gold nanoparticles are also widely used to transfer PS drugs to the target area. Cationic thiazine dyes, such as MB, interacted strongly with GNP, increasing UV-visible absorption. As a result, this combination agent can be used as a targeted drug delivery system for radiotherapy and photodynamic synergetic therapies (6,7).Heparin-coated gold nanoparticles (AuNPs) were developed as carriers of PS drugs for the treatment of PDT. Heparin was used to increase the water solubility of gold nanoparticles, biocompatibility, and colloidal stability [6]. AuNPs have a large interaction cross section with X-ray radiation up to about 1 MeV as well as with ion radiation [7, 8].

In 2012, Shakir Khan and et al. studied the MB conjugate effect with gold nanoparticles to treat PDT[6]. In this study, gold nanoparticles were used as a carrier of anti-tumor drugs, antibodies, antibiotics, and other drugs to selectively kill diseased cells and microbes. The distinctive chemical and physical properties inherent to nanoparticles render them efficacious as carriers and enhancers [6]. In 2010, a study was conducted by Camerin et al. In which the in vivo use of gold nanoparticles was reported. In this study, the efficacy of photodynamic therapy was investigated using gold-plated phthalocyanine. Cell line B78H1 derived melanoma was used and the drugs were injected intramuscularly into the tumors [9].

In addition to photodynamic therapies, MB combined with gold nanoparticles is predicted to be effective in imaging and identifying tumor sites. By increasing the sensitivity of the beam to the dose below the dose of ionizing radiation, it increases the efficiency of radiation therapy, which in turn reduces the dose received by the patient and reduces the side effects of treatment. In the present study, gold nanoparticles coated with thioglucose [due to the high metabolism of cancer cells, inhibition of the aggregation phenomenon, and increased uptake of gold nanoparticles by the cell] together with methylene blue were used to evaluate the efficacy of radiation therapy and photodynamic therapy[7].

Materials and Methods

Synthesis of GNPs-Tio

The synthesis of gold nanoparticles coated with thioglucose was performed based on the following steps[8]:

A) First, a gold chloride solution was made with 0.03 g of gold chloride and 3.2 ml of deionized water. After that, sodium barohydrate solution was obtained using 0.004 g of sodium barohydrate and 4 ml of deionized water. Then, a thioglucose solution using 0.05 g of thioglucose and 12 ml of deionized water was prepared.

B) In the container, 60 mL of deionized water and ice were prepared. After that, it was mixed at a speed of 400 to 500 rpm [the water temperature on the mixer should be 0 °C]. The sodium barohydrate solution and thioglucose solution were then slowly added to zero-degree water and mixed for ten minutes. The color of the synthesized gold nanoparticles is purple.

Characterization of GNPs-Tio

The absorption spectrum of GNPs-Tio was recorded by a spectrophotometer (Shimadzu Model UV-1700, Japan). The size distribution of gold nanoparticles and their zeta potential were determined using a particle size analyzer (Malvern Instruments, Southborough, MA). Transmission electron microscopy (TEM) (Leo 912-ab, Zeiss Germany) was used to investigate the morphology of the GNPs.

Cell line

There are currently several cell types used as experimental models for research in the biological and pharmacological fields of breast tumors. These cell categories are derived from different human tumors and

have different morphological, cytogenetic, and biochemical characteristics. Among these, the MCF-7 cell line obtained from human adenocarcinoma tumors is one of the most widely used in the field of external breast cancer research. In some ways, this cell line is similar to that of estrogen receptors in the differentiated epithelial cells of mammals. The cell line MCF-7 was prepared by the Pasteur Institute of Iran.

Cytotoxicity determination of the pharmaceutical agents

In order to determine the maximum non-toxic concentration of the pharmaceutical agents, after trypsin and cell counting by the Trypan Blue method, a cell suspension with a density of 104 cells per CC was prepared in RPMI culture medium with 10% FBS and was cultured in 96 plates and incubated for 24 hours in the incubator (370 C and 5% CO2). In the next step, after washing the wells, different concentrations of MB (6, 12, 18, 24 (µg/ml)) and GNPs-Tio (20, 40, 60, 80 (µg/ml)) were added for 2 hours. It should be noted that drug suspensions were prepared in RPMI culture medium with 3% FBS to create suitable conditions for cellular uptake of drugs. Then, after discharging, culture medium was added to each well with 10% FBS and re-incubated for 24 hours. Finally, the MTT assay was used to determine the percentage of cellular survival and cell toxicity assessment. IC50 and IC10 of GNPs-Tio and MB were determined for treatment application.

Indicators calculated in the colony assay

$$\text{Plate efficiency } (P = \frac{\text{Number of coloneis in the control group}}{\text{Number of cultured cells}}$$

$$\text{Cell survival fraction} = \frac{\text{Number of colonies in the experimental group}}{\text{Number of cells in the experimental group} \times \text{PE}}$$

Experimental groups

After determining the optimal concentrations of GNPs-Tio combined with MB, the cells were divided into different groups to evaluate the simultaneous effects of PDT and RT on cell survival. The concentration of GNPs-Tio and MB was selected at 80 and 18 µg/ml, respectively. For this purpose, the cells were incubated with the pharmaceutical agents [MB, GNPs-Tio, and GNPs-Tio combined with MB for 2 h, separately. After washing the cells, a fresh culture medium was added to each well, and PDT and RT were performed. A surface X-ray tube with a radiation condition of 100 kVp and 80 mA was used to deliver a dose of 2 Gy at a 1229.5 cGy/min dose rate (during radiation, all samples were placed on ice). A Luma-Care source equipped with the fiber optic probe at a wavelength of 670 nm, a 30 nm band width, and a power density of 65 mW/cm2 was used to deliver an optical dose of 15.6 J/cm2. Then, treated cell survival was assayed by MTT and colony assay after 24 h and 12 d, respectively. To confirm the results, each test was repeated at least three times.

Judgment criteria and data analysis

The indicators used to evaluate the results in this study are IC10, IC50, and ED50.

IC10: the concentration of the agent (MB or GNPs-Tio) required to cause 10% cell death.

IC50: the concentration of the agent (MB or GNPs-Tio) required to cause 50% cell death.

ED50: The amount of exposure required to cause 50% cell death.

ED50 was calculated by examining the chart of the average percentage of cell survival relative to exposure [energy per unit area] in each case.

In statistical analysis, the data was analyzed by SPSS software. The data were first checked for normality using the Kolmogorov-Smirnov test, and then statistical analysis was performed using the One-Way ANOVA and Tukey tests.

The percentage of cell viability in directly irradiated and control cell groups was a mean of at least three independents.

Results

Characterization of GNPs-Tio

The absorption spectrum of GNPs

The absorption spectrum of GNPs in the ultraviolet-visible region, is shown in Figure 1 the peak absorption of GNPs was recorded at 553 nm.

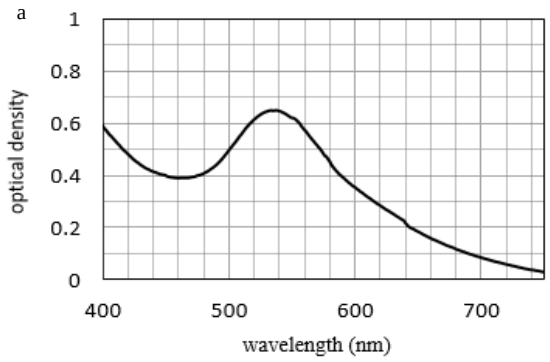

Figure. 1. UV-Vis spectrum of GNPs-Tio Glucose

Size distribution and Zeta potential (mV) of GNPs-Tio

The size distribution of gold nanoparticles coated with thio-glucose was studied using a particle size analyzer. The highest frequency of GNPs-Tio was determined by the Z-average size and polydispersity index (PdI) of 53.9 nm and 0.408, respectively. A Zeta potential of GNPs-Tio (-35 mV) has been reported.

The transmission electron microscopy (TEM) image of GNPs

Figure 2 shows the transmission electron microscopy (TEM) image of GNPs.

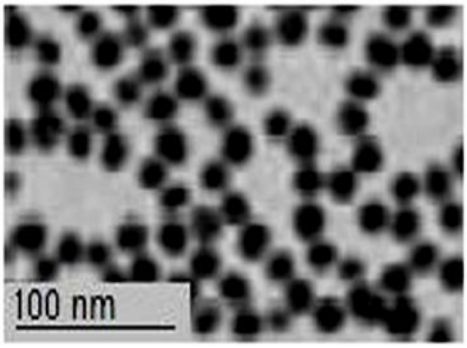

Figure.2. Transmission electron microscopy (TEM) image of the GNPs

Cytotoxicity of the pharmaceutical agents

In order to achieve the optimal concentration for the treatments, the toxicity of MB, GNPs-Tio, and the combination of MB with GNPs-Tio were determined at several different concentrations. Cell toxicity of MB on the MCF-7 cell line was evaluated at concentrations of 6, 12, 16, 18, and 24 (µg /ml) (Fig.3a). According to the findings, the control group's mean percentage of cellular survival differs significantly from the concentration of 18 µg/ml (P-Value<0.05). The cellular toxicity of GNPs coated with thioglucose [53.9 nm] was studied at different concentrations (Fig.3b). According to statistical analysis, the control group showed a significant difference with concentrations of 60 and 80 µg/ml. In these experiments, the cellular toxicity of 18 µg/ml concentration of MB combined with different concentrations of thioglose-coated GNPs for MCF-7 cells was investigated. The results show that there was a significant difference only between control and 80 µg/ml concentration (Fig.3c).

Results of X-ray therapy and photodynamic therapy simultaneously

Photodynamic and radiation therapy using the sensitized agents was performed simultaneously with MB and GNPs-Tio at an optical dose and radiation dose of 15.6 J/cm^2 and 2 Gy, respectively. As shown in Fig. 4, according to the MTT assay, the combination of optical and radiation dose without any agents did not have a significant effect on cell survival. The presence of MB and cell death of almost 92% was observed by using a combination of optical and radiation dose. However, this treatment using GNPs-Tio did not have a significant effect on cell survival. The simultaneous presence of GNPs-Tio and MB with an optical dose of 15.6 J/cm^2 and a radiation dose of 2 Gy reduced cell survival to 4%. As shown in Fig.5, according to the colony assay, the combination of optical and radiation without any agents showed a significant effect on cell survival. The presence of MB and GNPs-Tio combined with MB by using an optical dose of 15.6 J/cm^2 and a radiation dose of 2 Gy reduced cell survival to 2% and zero, respectively.

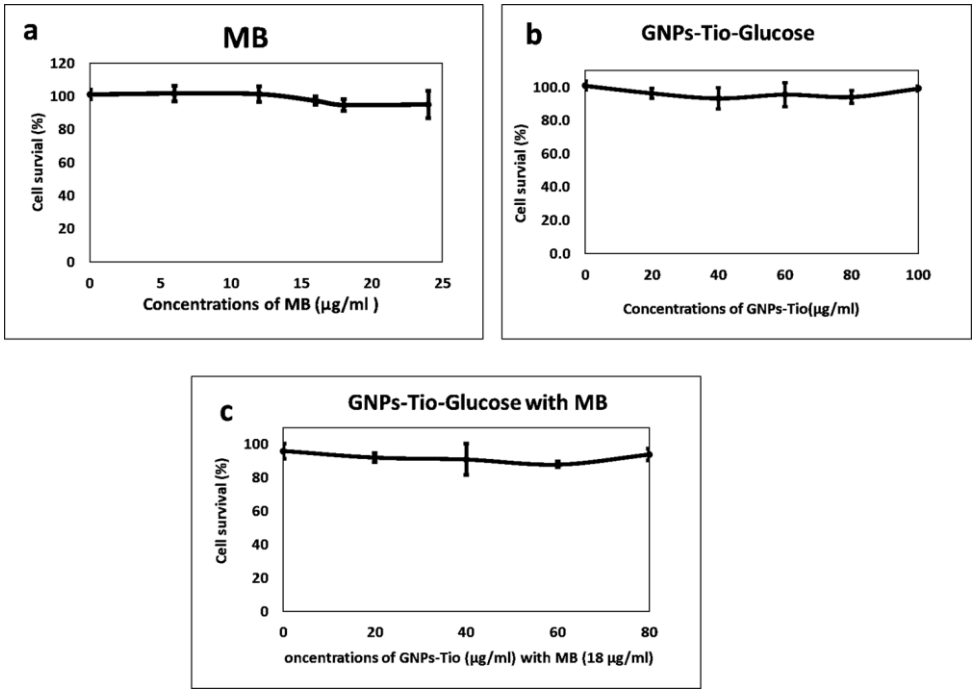

Figure 3. Cell survival percentages in the presence of various concentrations of a) MB with a 1h incubation time, b) GNPs-Tio-Glucose with a 2h incubation time, c] GNPs-Tio combined with MB (with an 18-µg/ml concentration of MB) with a 2h incubation time. The data represents the mean ± standard deviation obtained from three experiments.

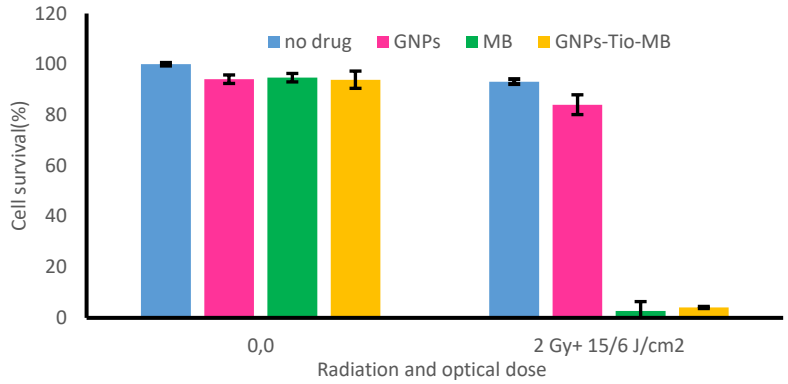

Figure 4. Percentage of cell survival in the presence of MB, GNPs-Tio, and a combination of MB with GNPs-Tio by MTT assay with optical and radiation doses of 15.6 J/cm2 and 2 Gy for 24 h and 12 h, respectively, after treatment. The data represents the mean ± standard deviation obtained from three experiments.

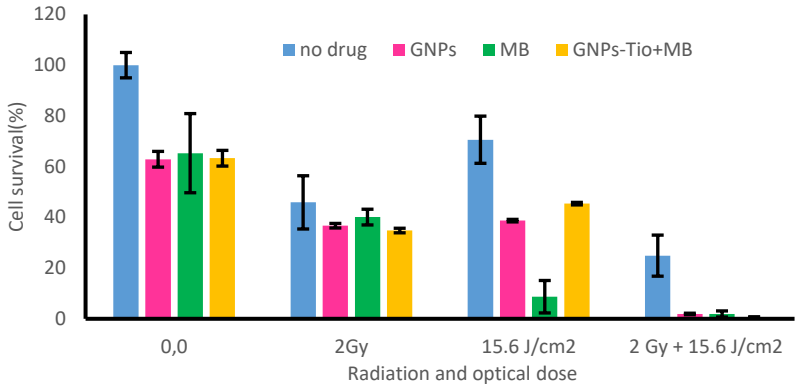

Figure 5. Percentage of cell survival in the presence of MB, GNPs-Tio, and a combination of MB with GNPs-Tio with an optical dose of 15.6 J/cm2 and a radiation dose of 2 Gy, alone with each other and 12 days after treatment. The data represents the mean ± standard deviation of three experiments (conducted using the colony assay).

Discussion

Methylene blue (MB), a photosensitizer based on phenothiazinium, finds widespread use across various applications including anti-bacterial treatments, treatment of toenail onychomycosis, and several therapeutic interventions in cancer treatment [10, 11]. Its absorption range is 650–670 nm, which is coherent with the NIR-I window (600–900 nm)[12]. Therefore, it can be effectively activated in a deeper area [2]. Many studies confirm the low toxicity of methylene blue. In an in vivo study, Paulo et al. (2005) showed that MB did not cause significant toxicity [13]. The current study revealed low toxicity of Methylene blue (MB) on the MCF-7 cell line. Furthermore, certain researchers have reported that a combination of MB and photodynamic therapy (PDT) is a moderately effective therapeutic approach capable of inducing programmed cell death through apoptosis, a less severe and controlled form of cell death, rather than necrosis [14]. Despite its various advantages, Methylene blue (MB) has certain limitations that impede its clinical application as a photosensitizer. Firstly, low accumulation in tumors resulting from intravenous injection or direct injection into brain tumors can considerably reduce its effectiveness [15]. Second, the optical absorption coefficient of MB decreases in the biological and cellular environment. This effect can be reduced by loading MB in a nanostructure and conjugating it with a targeting group such as antibodies or specific tumor peptides [16]. Also, poor cellular uptake of dye can be enhanced by drug carriers [17]. Nanoscale materials can not only prolong the circulation time in body but also enter the cells through endocytosis [18]. Furthermore, EPR [enhanced permeability and retention] allows nanoparticles to easily enter and accumulate in cells [19]. Nanoparticles are also suitable for cancer therapy. Among different nanoparticles, gold nanoparticles have unique features such as small size, good biological adaptability, and low toxicity[20]. In this study, since the GNPs-Tio glucose showed less than 10% toxicity, it is predictable that the IC_{50} concentration for gold nanoparticles to reduce MCF-7 cell survival is greater than 100 µg /ml. Due to the low toxicity of the nanoparticle, 80 µg/ml was selected for treatments that were related to IC_{10} concentrations of GNPs-Tio glucose. Many researchers have even reported the non-toxicity of gold nanoparticles in in-vivo studies. Among them, Zhang et al. examined the toxicity of gold nanoparticles in three methods: intravenous injection, intraperitoneal injection, and swallowing by weighing the mice up to 28 days after injection. The results showed that intravenous injection was the least toxic Toxicologic effects of gold

nanoparticles in vivo by different administration routes. International Journal of Nanomedicine [21].

More studies, including in vitro and in vivo studies, have shown that the presence of gold nanoparticles increases the effectiveness of radiation therapy. Most of this research has been done with small gold nanoparticles [22, 23]. Increase efficiency and increasing the depth of photodynamic treatment, reducing the dose required in radiation treatments and following that reducing side effects were important aim of this study [24]. Therefore, in this study, the efficacy of optical and radiation therapy of MB and MB with GNPs-Tio as a drug carrier [through adsorption] for tumor cells was evaluated. The MTT assay results show a significant difference between the control group and the MB/MB with GNPs-Tio groups that received optical and X-ray doses. According to the MTT results, after hybrid therapy, using MB combined with GNPs-Tio could not provide a significant difference compared to the group that received only MB. But the groups that received MB or two drug agents (MB combined with GNPs-Tio) were significantly different from the control group as well as the group that received thioglucose-coated gold nanoparticles. The colony assay has shown relatively similar results, except that the combination therapy in the presence of any pharmaceutical agent only has the same therapeutic efficacy. The drug-free groups treated had significant cell death compared to the control group. It should be noted that there is a significant difference in the effectiveness of treatment between the results of MTT tests and cloning in treatment groups without MB. But the treatment groups that received MB showed more treatment efficiency in the colony assay. It is based on lower colonization potential and higher efficiency by using combination therapy. Since, MTT and colony assays affect metabolic and colonization respectively, Therefore, it is predicted that the presence of MB further affects the clonogenic potential of the cells compared to its metabolic activity. The calculation of the incremental index in the present of GNPs-Tio with and without MB by using the MTT assay is 0.99. This index was 1.02 by using a colony assay. Thus, it can be predicted that GNPs-Tio with the presence of MB could not provide a more effective therapeutic response in combination therapy. A similar calculation for the GNPs-Tio without MB is 2.28 for the MTT assay and 1.43 for colonization findings. This information indicates that GNPs-Tio has acted as an effective agent to increase the efficiency of this combination therapy. A similar study to compare with our findings section has not been reported.

Conclusion

According to the findings of this study, MB combined with thioglucose coated GNPs has greatly increased the optical and radiation sensitivity of the MCF-7 cell line.

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