



Methylene Blue-Mediated Photodynamic Therapy in Combination With Doxorubicin: A Novel Approach in the Treatment of HT-29 Colon Cancer Cells

Nima Rastegar-Pouyani^{1,2} , Jaber Zafari³, Alireza Nasirpour⁴, Hossein Vazini⁵, Nabaa Najjar⁶, Seyedeh Zohreh Azarshin¹, Fatemeh Javani Jouni⁷

¹Laser Application in Medical Sciences Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

²Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

³Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran

⁴Department of Electrical and Computer Engineering, Batten College of Engineering, Old Dominion University, Norfolk, VA, United States

⁵Nursing Department, Basic Sciences Faculty, Islamic Azad University, Hamedan Branch, Hamedan, Iran

⁶Basic Medical Science Research Center, Zist Pajooch Afra Company, Tehran, Iran

⁷Department of Biochemistry and Biophysics, Faculty of Advanced Sciences and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran

*Correspondence to

Fatemeh Javani Jouni,
Email: Javani_f@yahoo.com

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Abstract

Introduction: With an alarmingly growing number of patients diagnosed with colorectal cancer, adopting innovative anti-cancer approaches has recently garnered great attention. One interesting concept is the co-administration of cytotoxic agents and safer modalities such as photodynamic therapy (PDT), which can subsequently improve therapeutic efficacy and potentially reduce the risks of severe adverse effects and drug resistance. In the course of PDT, a locally injected photosensitizer (PS) is irradiated with a light source, which subsequently generates reactive oxygen species (ROS) and induces programmed cell death in tumor cells.

Methods: In this study, to evaluate the potential anti-cancer effects of chemotherapy combined with PDT, in comparison to each alone, we employed PDT, comprising methylene blue (MB) and diode lasers at 630 and 810 nm wavelengths, in conjunction with the chemotherapeutic agent doxorubicin (DOX).

Results: The MTT assay showed that the viability of colorectal cancer HT-29 cells decreased significantly following DOX+PDT treatment. Similarly, lactate dehydrogenase (LDH) release and lipid peroxidation rates were substantially higher in DOX+PDT treatment groups. Lastly, the catalase (CAT) assay indicated that the combination reduced the ability of CAT in the detoxification of H₂O₂.

Conclusion: Our study suggests that MB-mediated PDT combined with chemotherapy might provide a promising avenue to improve therapeutic efficacy and potentially reduce the risk of adverse effects and drug resistance. Without a doubt, further investigations need to delve into the pharmacological advantages and disadvantages of PTD-based combination therapy and optimize its administered doses along with other modalities.

Keywords: Cancer; Combination therapy; Photodynamic therapy; Doxorubicin; Methylene blue.



Introduction

Known as the second deadliest type of cancer, colorectal carcinoma (CRC) is deemed to alarmingly claim a much greater number of lives in the near future, which urgently calls for therapeutic alternatives to the ones currently adopted.¹ If diagnosed at early stages, CRC usually has a better prognosis after performing surgery and adjuvant therapy, mostly chemotherapy and/or radiotherapy,²

while cytoreductive surgery, hyperthermic intraperitoneal chemotherapy (HIPEC), as well as other conventional modalities mentioned earlier, may be adopted against this malignancy at late stages.^{3,4} Moreover, like many other types of cancer, therapeutic measures against CRC may undesirably meet resistance, an event leading to the failure of therapy.^{5,6} As the majority of conventional anti-cancer modalities lack a wide therapeutic index,

administering higher doses thereof is concurrent with the emergence of deleterious effects, making them unfeasible,⁷ which prompts researchers to develop novel solutions to tackle this daunting challenge.^{8,9} One such strategy is to combine conventional therapies with novel modalities owning satisfying safety so much so that the required doses of toxic agents can be reduced subsequently.¹⁰ Photodynamic therapy (PDT) is a non-invasive outpatient procedure that offers a wide range of usage in clinics, from skincare and dental applications to the treatment of certain malignancies.^{11,12} PDT basically requires three factors, namely a photosensitizer (PS), a light source, and oxygen; following absorption of the light with a proper wavelength, the excited PS becomes able to generate reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), superoxide radical (O₂⁻), and singlet oxygen (¹O₂), which, upon generation, harshly damage any organic compounds nearby, hence exhibiting striking cytotoxicity.^{13,14}

In the present study, to appraise the potential of PDT combined with chemotherapy in the treatment of CRC, we employed methylene blue (MB) as the PS, low-energy 630 and 810 nm diode lasers as the light source, and doxorubicin (DOX), a well-known chemotherapeutic agent belonging to anthracyclines. DOX manifests its cytotoxic effects by interrupting topoisomerase-II-dependent DNA repair, as well as free radical-induced membrane damage,¹⁵ and has been utilized in several research studies on CRC.^{16,17} Nevertheless, DOX-induced cardiomyopathy is a daunting challenge observed among a considerable number of patients under treatment with even routinely administered doses of DOX¹⁸; combination therapy using PDT seems practically feasible to tackle it. First, the cell viability of different treatment groups of CRC cell line HT-29 was assessed by the MTT assay. Afterwards, to evaluate oxidative damage in different groups, we performed lipid peroxidation and lactate dehydrogenase enzyme (LDH) assays. Lastly, the activity of the catalase (CAT), which highly contributes to the detoxification of H₂O₂,¹⁹ underwent inspection among different treatment groups.

Materials and Methods

Cell Culture

The CRC cell line (HT-29) was purchased from the National Cell Bank of Iran (NCBI). HT-29 cells were cultured in Dulbecco's modified eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (P/S; Gibco) in a humidified atmosphere at 37 °C and 5% CO₂. Cultured cells were observed with a light microscope every 48 hours up to 70%-80% confluency.

Cell Treatments

HT-29 cells were treated with 20 µM MB for 2 hours

with or without continuous mode 630 (L630 nm; Pioon) or 810 nm (L810 nm; Elexxion) diode laser irradiation (6 J/cm²) (Table 1); subsequently, fresh medium was substituted for the already used medium containing MB, and the cells were incubated for yet another 46 hours. In groups with DOX treatment, the cells were treated with different concentrations (0.1, 0.4, 0.8, 1.6, 3.2 µg/mL) for 48 hours. Moreover, it is important to note that in groups where the cells received both PDT and DOX, first, they underwent PDT (MB: 2 hours and laser: 30 seconds); afterwards, a medium containing the half-maximal inhibitory concentration (IC₅₀) value of DOX measured for the stand-alone DOX treatment group was substituted for the already used medium containing MB, and then, incubated for next 46 hours.

MTT Assay

The MTT assay was performed to evaluate the toxicity of mono- and combination therapy of PDT and DOX at different wavelengths and concentrations. First, the impact of PDT on cell viability was to be evaluated; to do so, we divided the cells into 6 groups (Control, MB, L630 nm, L810 nm, MB+L630 nm, and MB+L810 nm) and treated them with MB with or without L610 nm or L810 nm for 2 hours, and the other 46 hours with a fresh medium. Later, to investigate the toxic effects of DOX, we treated the cells with the above-mentioned concentrations of DOX for 48 hours. Moreover, the cytotoxicity of the combination of DOX and diode lasers with and without MB was also inspected. Finally, the IC₅₀ value of DOX in each group was calculated by the four-parameter logistic (4PL) equation, a sigmoidal function, used to model dose-response relationships, where, Y: percentage of viability compared to the control, X: Log-transformed concentration of DOX, Mini: minimum viability, Max: maximum viability, LogIC₅₀: logarithm of the IC₅₀, and Hill Slope: steepness of the curve.²⁰

$$Y = \text{Min} + \frac{\text{Max} - \text{Min}}{1 + 10^{(X - \text{LogIC}_{50}) \cdot \text{Hill Slope}}}$$

Measurement of Membrane Lipid Peroxidation

The concentrations of malondialdehyde (MDA) were measured by means of the lipid peroxidation assay kit (Kia-Zist). After each treatment followed by incubation, the cell suspension was collected and centrifuged at 1230 rpm for 5 minutes to acquire the cell pellet, which was

Table 1. Technical Specifications of Different Lasers

Name of the Laser Company	Pioon	Elexxion Nano
Wavelength (nm)	610 ± 20	808 ± 10
Power output	200 ± 20 mW	0-15 W
Dose (J/cm ²)	6	6
Time (s)	30	30
Operation	Continuous	Pulse (16 µs/CW)

later resuspended on ice in 300 μL of MDA lysis buffer with 3 μL of butylated hydroxytoluene (BHT) 100X. After being centrifuged at 6000 g for 10 minutes, 200 μL of each group was mixed with 600 μL thiobarbituric acid (TBA), incubated at 95 $^{\circ}\text{C}$ for 60 minutes, cooled at room temperature for 10 minutes, and then analyzed for MDA levels using Colorimetry. Via an ELISA plate reader, the absorbance of each sample was read at 532 nm. The average absorbance of the blank was subtracted from the absorbance of each sample and standard. Afterwards, the MDA concentrations were measured from the standard curve drawn based on the manufacturer's instructions.

LDH Leakage Assay

The LDH leakage rate, as a prominent indicator for cytotoxicity, was calculated by means of the LDH cytotoxicity assay kit (Pars-Azmoon). Twenty-four hours after the treatment, 100 μL of the medium from each well was transferred with particular care to a new 96-well plate. Subsequently, 100 μL of LDH reaction solution, which was prepared according to the instructions of the LDH cytotoxicity assay kit, was added to each well. Next, the plate was shaken at 37 $^{\circ}\text{C}$ for 30 minutes, and later, the absorbance was measured at 496 nm by using an ELISA plate reader. The final results were expressed as percentages of the control group.

Catalase Analysis

The antioxidant enzyme CAT is commonly found in aerobic cells and is able to detoxify H_2O_2 (15). In the present investigation, following the treatment, the CAT activity measurement of groups was performed by a kit (Teb Pajohan Razi), depending on the CAT reaction with methanol in a proper concentration of H_2O_2 . The chromogenic reagent forms a heterocyclic ring with formaldehyde produced throughout this reaction, which shifts from colorless to purple in the course of oxidation. Eventually, formaldehyde concentrations generated during the reaction were measured using colorimetry.

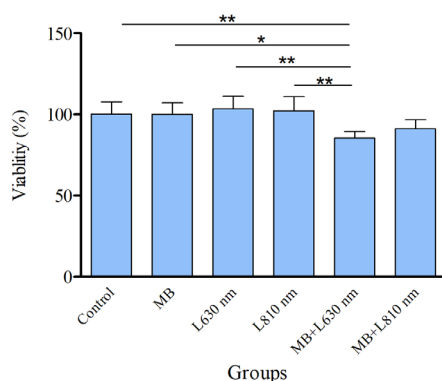


Figure 1. Comparative Analysis of the Effects of Different Constituents of PDT on the Viability of HT-29 Cells (* $P \leq 0.05$, ** $P \leq 0.01$; * Compared to MB + L630 nm). Data are expressed as mean $\pm$ SD

Statistical Analysis

The results of the present study were reported as the mean $\pm$ standard deviation (SD) value of three independent experiments. Data analysis was conducted with a one-way analysis of variance (ANOVA) and Tukey post hoc test via GraphPad Prism[®] version 5.01 software (GraphPad Software, USA). $P < 0.05$ was regarded as being statistically significant.

Results

MTT Analysis

As shown in Figure 1, the results of the MTT assay with the constituents of the adopted PDT indicated that while the difference between the MB + L630 nm and MB + L810 nm groups was not statistically significant, the MB + L630 nm group had significantly highest cytotoxicity in comparison to the rest of the groups, highlighting the considerably increased cytotoxicity of MB in conjunction with L630 nm irradiation. Regarding the cytotoxicity of DOX (Figure 2), concentrations greater than or equal to 0.4 $\mu\text{g/mL}$ were found to substantially reduce the cell viability among HT-29 cells; the IC_{50} value of DOX was determined to be 1.349 $\mu\text{g/mL}$, which was to be employed for subsequent assays as discussed later. Although the combination of DOX + L630 or 810 nm had no substantial impact on the DOX-induced cytotoxicity in HT-29 cells (Figure 3), adding MB to this combination (DOX + MB + 630 or 810 nm) interestingly managed to increase the anticancer effects as the group treated with 0.1 $\mu\text{g/mL}$ of DOX (Figure 4), unlike its counterpart in the stand-alone DOX treatment, significantly resulted in lower percentages of cells in comparison to the control. As presented in Table 2, a decreasing trend is noticeable in the measured IC_{50} values of DOX from the stand-alone DOX group to the DOX + MB + L630 nm group. Taken altogether, the data suggested that combining PDT with DOX would increase cytotoxic efficacy.

The Membrane Lipid Peroxidation Rate Measurement

The concentration of the final product of lipid

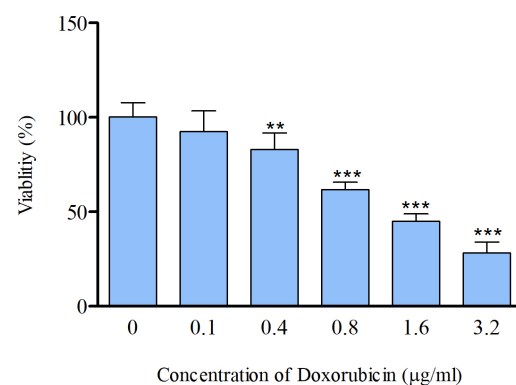


Figure 2. Comparative Analysis of the Effects of Different Concentrations of DOX on the Viability of HT-29 Cells (* $P \leq 0.05$, ** $P \leq 0.01$; * Compared to the control). Data are expressed as mean $\pm$ SD

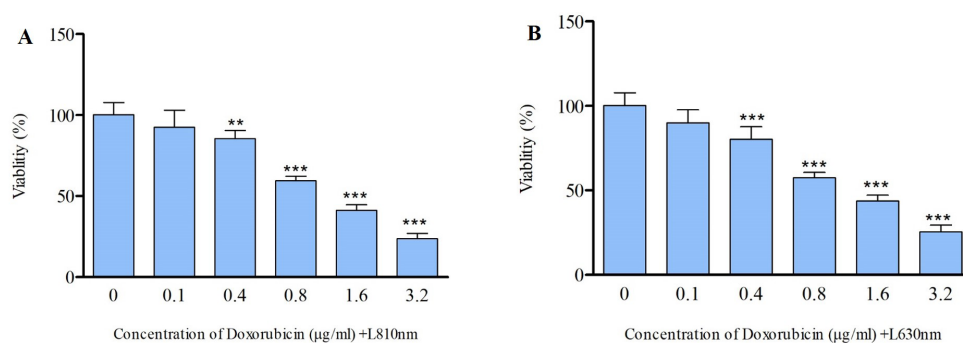


Figure 3. Comparative Analysis of the Effects of Different Concentrations of DOX+Diode Lasers at 810 (A) and 630 (B) nm Wavelengths on the Viability of HT-29 Cells (** $P \leq 0.01$, *** $P \leq 0.001$; * Compared to the control). Data are expressed as mean $\pm$ SD

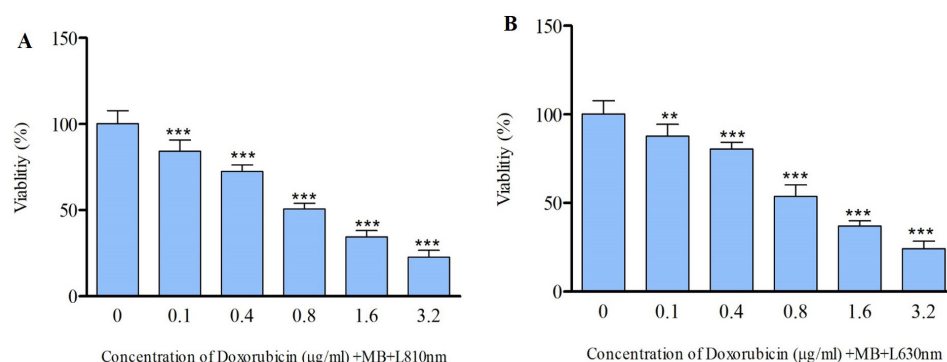


Figure 4. Comparative Analysis of the Effects of Different Concentrations of DOX+PDT (A: 810 nm, B: 630 nm) on the Viability of HT-29 Cells (** $P \leq 0.01$, *** $P \leq 0.001$; * Compared to the control). Data are expressed as mean $\pm$ SD

Table 2. The IC_{50} Values of DOX

Groups	IC_{50} (μg/mL)
DOX	1.349 $\pm$ 0.92
DOX+L810 nm	1.215 $\pm$ 1.23
DOX+L630 nm	1.197 $\pm$ 0.74
DOX+MB+L810 nm	1.043 $\pm$ 1.04
DOX+MB+L630 nm	0.87 $\pm$ 0.28

peroxidation, MDA, was comparatively evaluated in different groups as shown in Figure 5. The data showed that after irradiation of both lasers, the groups treated with stand-alone DOX treatment and DOX+L630/810 nm had higher levels of MDA compared to the control, although the difference between them was not statistically significant. More importantly, the groups treated with DOX+MB+L630/810 nm had the highest levels of MDA compared to any other groups. Overall, the results point to the important role of PDT in increasing the cytotoxicity of DOX in HT-29 cells.

LDH Release Rate Measurement

The LDH release rate in different treatment groups was comparatively evaluated as a percentage of control (Figure 6). The data showed that after irradiation of both lasers, the groups treated with stand-alone DOX

treatment and DOX+L630/810 nm had higher rates of the LDH release compared to the control; however, the difference between them was not statistically significant. Interestingly, the MB+L630 nm group, unlike MB+L810 nm, remarkably increased the LDH release rate compared to the control. Moreover, the LDH levels in the DOX+MB+L630/810 nm groups were the highest compared to any other counterpart groups, although the difference between DOX+MB+L810 nm and DOX+L810 nm was not statistically significant.

CAT Activity Analysis Results

As presented in Figure 7, the results suggest that both DOX+L630/810 nm and DOX+MB+L630/810 nm significantly reduced the CAT activity compared to that of the control group; however, the difference between DOX+L630/810 nm and stand-alone DOX was not statistically significant.

Discussion

Novel approaches have been adopted to outmaneuver cancer resistance and progression.^{21,22} Recently, PDT, combined with chemotherapeutic agents, has emerged as a promising approach in cancer therapy as it satisfactorily minimizes the required doses of cytotoxic drugs and reduces the possibility of adverse effects while maintaining

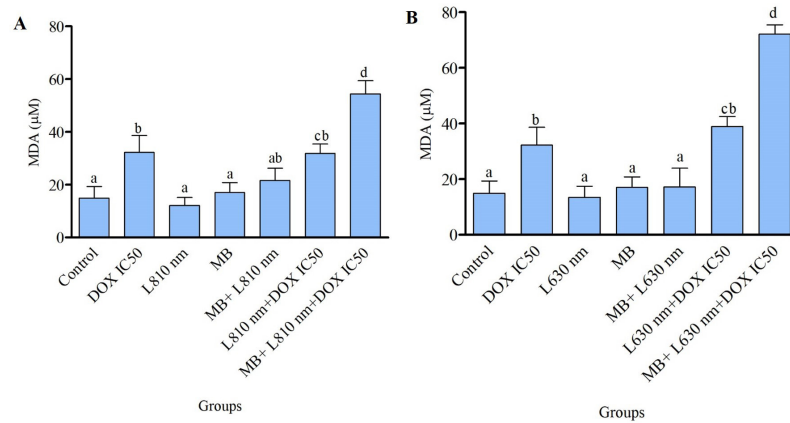


Figure 5. The Membrane Lipid Peroxidation rates in Different Treatment Groups (A: laser 810 nm, B: laser 630 nm). Data are expressed as mean $\pm$ SD. Means followed by the same letter are not significantly different at the $P < 0.05$ level

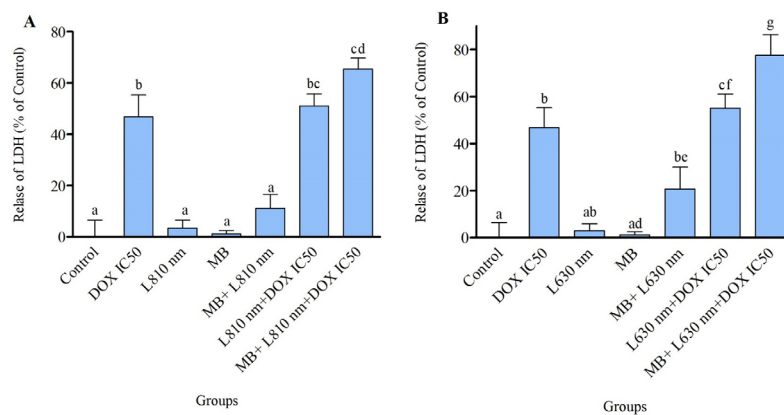


Figure 6. The LDH Release Rates in Different Treatment Groups (A: laser 810 nm, B: laser 630 nm). Data are expressed as mean $\pm$ SD. Means followed by the same letter are not significantly different at the $P < 0.05$ level

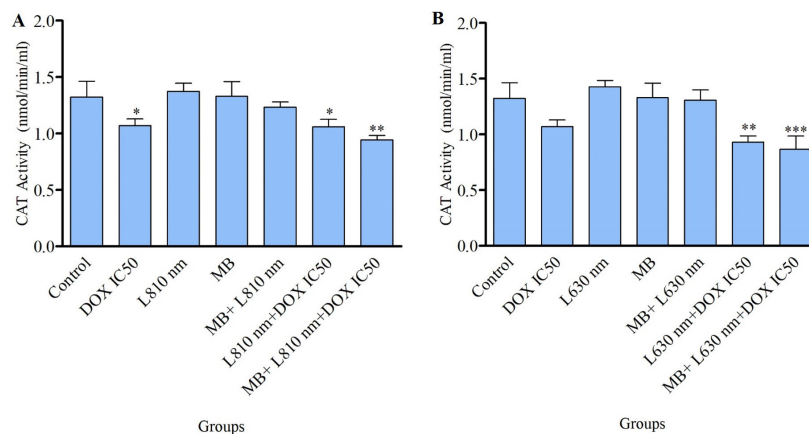


Figure 7. Analysis of CAT Activity in Different Treatment Groups (A: laser 810 nm, B: laser 630 nm). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

therapeutic efficacy.^{23,24} MB is a salt, famously used for several medical purposes, such as methemoglobinemia,²⁵ ifosfamide neurotoxicity,²⁶ isobutyl nitrite toxicity,²⁷ and cyanide poisoning.²⁸ Owing to its light absorption properties, it can also act as a PS in PDT.²⁹ In a study by dos Santos et al, MB-mediated PDT differentially increased

cell death among human breast cancer cells, while the normal cells were relatively unharmed.³⁰ In another study, Le et al reported that MB-based PDT downregulated the expression levels of matrix metalloproteinases in oral carcinoma,³¹ a group of proteases, which play an important role in cancer invasion, migration, and metastasis.³²

Diode lasers, which radiate coherent light at certain wavelengths, have been successfully applied in several medical conditions, functioning as a valued light source for PDT.^{33,34} Likewise, diode lasers at two wavelengths of 630 and 810 nm were employed in the present study. Although the 610 nm laser obviously had a higher energy level in comparison to the 830 nm laser, no significant difference, in most cases, was found between the counterpart groups exposed to each one of them ([Supplementary file 1](#)). Previous findings have reported that a 664 nm diode laser in conjunction with talaporfin sodium, as a PS, can show promising results after unsuccessful chemoradiotherapy in esophageal cancer patients.³⁵ Other findings have demonstrated that a combination of a low-power diode laser with a wavelength of 810 nm and curcumin, a well-known antioxidant, synergistically reduced the proliferation rate of breast cancer cells in comparison with stand-alone curcumin treatment.³⁶ Furthermore, a study on lung cancer by Cacaccio et al reported that a combination therapy comprising PDT and DOX showed significant synergism and had substantially higher cytotoxicity compared to each of them alone.³⁷ The same has been reported by Chilakamarthi and colleagues' study, where porphyrin-based PDT combined with low-dose DOX resulted in better outcomes against colon cancer.³⁸ Similarly, the data from the MTT assay showed that MB-based PDT increased the cytotoxicity of DOX as the viability of HT-29 cells was significantly reduced in groups treated with 0.1 µg/mL DOX combined with PDT compared to the control. In contrast, such was not the case in the stand-alone DOX treatment group. As seen in [Table 2](#), there was a decreasing trend in the IC₅₀ values of DOX, with the stand-alone DOX treatment being the highest and the DOX + MB + L610 nm being the lowest.

As both DOX and PDT generate free radicals, the levels of lipid peroxidation and its products such as MDA could increase in cells, which give rise to cell membrane disruption.³⁹ The MDA level is a prominent marker of oxidative stress in cancer patients.⁴⁰ The result of our study showed that the groups treated with DOX + PDT had the highest levels of MDA resulting from stronger oxidative stress caused by them combined. LDH leakage is a well-known marker for apoptotic cell death.⁴¹ Several studies have shown that PDT via different mechanisms, including ROS generation, damage to cellular components (for example lipids, proteins, and nucleic acids) and, as a result, disrupting the integrity of the cell membrane, and induction of apoptosis can increase the LDH levels.^{42,43} In the present study, the results strongly suggest that the groups treated with DOX + MB + L 610/830 nm had the highest rates of LDH release. Likewise, a study by Aniogo et al indicated that DOX combined with Sulfonated zinc phthalocyanine (ZnPcS)-based PDT led to a significantly higher rate of LDH leakage compared to each of them alone.⁴⁴ Moreover, CAT is one of the main H₂O₂-

removing enzymes, ubiquitously expressed in human tissues.¹⁹ In the field of cancer, the antioxidative activity of CAT is crucial for the protection of cancer cells against oxidative stress. Studies have reported that ¹O₂ generated by PDT can reduce CAT activity.⁴⁵ Similarly, the results of our study demonstrated that DOX combined with PDT at both wavelengths managed to significantly reduce CAT activity.

Conclusion

In conclusion, our study shows that the employed combination resulted in significantly higher cytotoxicity and oxidative stress, as evidenced by a reduction in cell viability and CAT activity, as well as an increase in lipid peroxidation and LDH release. MB-mediated PDT in combination with DOX may hold promise for future clinical translations with improved cancer treatment efficacy and lower risks of adverse effects and drug resistance. Undoubtedly, to fulfill this goal, further investigations need to expand the anti-tumor properties of this synergistic approach against cancer and optimize its administered doses.

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Authors' Contribution

Conceptualization: Fatemeh Javani Jouni.

Data curation: Alireza Nasirpour.

Formal analysis: Jaber Zafari.

Investigation: Nabaa Najjar.

Methodology: Hossein Vazini.

Resources: Jaber Zafari.

Software: Seyedeh Zohreh Azarshin.

Supervision: Jaber Zafari.

Validation: Fatemeh Javani Jouni.

Visualization: Nabaa Najjar.

Writing—original draft: Nima Rastegar-Pouyani.

Writing—review & editing: Nima Rastegar-Pouyani.

Competing Interests

None declared.

Ethical Approval

This research was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (reference number: IR.SBMU.RETECH.REC.1401.156).

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Supplementary Files

Supplementary file 1 contains Figures S1-S5.

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