

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**PHOTODYNAMIC THERAPY EFFECTS OF NIR-ABSORBING
NOVEL PHTHALOCYANINES**

EDA GAZEL PEHLIVAN
A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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GEBZE

2022

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DOKTORA TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

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SUMMARY

Photodynamic therapy (PDT) is a new approach developed as an alternative to traditional cancer treatment methods such as surgery, chemotherapy, or radiotherapy that are widely used today. The aim of this study is to investigate the photodynamic effects of specific phthalocyanines, which will be used for the first time in biomedical research, on human cancer cell lines. Since the phthalocyanines to be studied are with near-infrared (NIR) absorption, the activating light will be able to reach tumors in the human body at a long distance. This study is divided into two parts. In the first part, the heavy atom effect of the palladium was evaluated via the difference between palladium-containing phthalocyanine versus its zinc metal-containing phthalocyanine counterpart. Phthalocyanine with palladium is expected to be more efficient since its relatively higher singlet oxygen-producing capacity. In the second part, the photodynamic effect of substitution bulkiness of the phthalocyanines was studied with the expectation of more solubilization of the substituted compound. Cytotoxicity caused by photodynamic therapy of the subjected compounds was analyzed by MTT cell viability assay. The uptake of the compounds was analyzed by cell uptake assay. Morphological changes in the cells and nuclei of the cells caused by PDT were also studied. And finally, the colony formation capacities of the PDT-treated cells were evaluated. It was seen that palladium causes more effective photodynamic therapy than zinc. After PVP formulation, bulky substituted phthalocyanine is more beneficial for the treatment.

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Key Words: Photodynamic Therapy, NIR, Phthalocyanine, PVP, Aggregation, Cancer

ÖZET

Fotodinamik terapi (FDT), günümüzde yaygın olarak kullanılan cerrahi, kemoterapi veya radyoterapi gibi geleneksel kanser tedavi yöntemlerine alternatif olarak geliştirilmiş yeni bir yaklaşımdır. Bu çalışmanın amacı, biyomedikal araştırmalarda ilk kez kullanılacak olan spesifik ftalosiyaninlerin insan kanser hücre hatları üzerindeki fotodinamik etkilerini araştırmaktır. İncelenecek olan ftalosiyaninler yakın kızıl ötesi (NIR) absorpsiyonlu olduklarından, aktive edici ışık insan vücudundaki tümörlere uzak mesafeden ulaşabilecektir. Bu çalışma iki bölüme ayrılmıştır. Birinci bölümde, paladyum içeren ftalosiyanin ile çinko metal içeren ftalosiyanin karşılığı arasındaki fark üzerinden paladyumun ağır atom etkisi değerlendirilmiştir. Paladyumlu ftalosiyaninin, nispeten daha yüksek singlet oksijen üretme kapasitesinden dolayı daha verimli olması beklenmektedir. İkinci bölümde, ftalosiyaninlerin süstitüsyon hacminin fotodinamik etkisi, süstitüe edilen bileşiğin daha az agregre olması beklentisiyle incelenmiştir. Söz konusu bileşiklerin fotodinamik tedavisinin neden olduğu sitotoksite, MTT hücre canlılığı tahlili ile analiz edilmiştir. Bileşiklerin hücre içine alımı, hücre alım tahlili ile analiz edilmiştir. FDT'nin hücrelerde ve hücre çekirdeklerinde meydana getirdiği morfolojik değişiklikler incelenmiştir. Son olarak, FDT'nin hücrelerin koloni oluşturma kapasiteleri üzerindeki etkisi değerlendirilmiştir. Paladyumun daha etkili fotodinamik tedaviye neden olduğu görülmüştür. PVP formülasyonundan sonra, FDT için hacimli süstitüe ftalosiyaninin daha etkili olduğu görülmüştür.

Bu çalışma Gebze Teknik Üniversitesi BAP (G.T.Ü. BAP 2017-A102-20) tarafından desteklenmiştir.

Anahtar Kelimeler: Fotodinamik Terapi, NIR, Ftalosiyanin, PVP, Agregasyon, Kanser

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LIST OF ABBREVIATIONS AND ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
µg	: Microgram
µl	: Microliter
a.u.	: arbitrary unit
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl sulfoxide
FBS	: Fetal Bovine Serum
MW	: Molecular Weight
nm	: nanometer
PBS	: Phosphate Buffered Saline
Pc	: Phthalocyanine
PVP	: Polyvinylpyrrolidone
Pc	: Phthalocyanine
a.u.	: arbitrary unit
nm	: nanometer
MW	: Molecular Weight
PDT	: Photodynamic Therapy
h	: Hour
MTT	: Methyl-thiazolyl-tetrazolium
J/cm ²	: Joules per centimeter square
Zn	: Zinc
Pd	: Palladium
MCF-7	: Michigan cancer foundation
MDA-MB-231	: M.D. Anderson - Metastatic Breast 231
°C	: Celcius Degree
ROS	: Reactive oxygen species

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1. INTRODUCTION

Cancer is one of the most critical health problems of today. It is among the leading causes of death in the world. According to the World Health Organization (WHO) report in 2020, there were nearly 10 million cancer-related deaths worldwide [Web 1].

According to the Turkish Statistical Institute (2017), the total number of people with cancer in Turkey is 180.288 [Web 2]. The WHO report indicates that 14 million people worldwide were diagnosed with cancer for the first time in the same year. In the next 20 years, the annual number of people diagnosed with cancer for the first time is expected to rise to 22 million [World Cancer Report, 2014].

The disadvantages of traditional cancer treatment methods (surgery, chemotherapy, and radiotherapy) have made scientists even more ambitious to find alternative treatment methods. Photodynamic therapy (PDT) has been one of the important methods used in cancer treatment because it has fewer side effects than other methods and is expected to be used widely in the future. PDT basically consists of three non-toxic components: photosensitive substance (photosensitizer), light, and oxygen. Photosensitizer, which is applied intravenously or topically to the patient in a dark environment, selectively accumulates in cancerous cells and is excreted from healthy cells. The photosensitizer, which is then activated by the effect of light at a specific wavelength, causes the cells to die in the presence of oxygen [Brown et al., 2004].

The phthalocyanines are unique photosensitizers. One of the properties of phthalocyanines is that they can be excited at the near-infrared (NIR) wavelength, allowing the light to travel more easily within the tissue without scattering and not losing its energy without being absorbed. In this way, its energy does not decrease; light can travel long distances in the human body and reach distant tumors. But yet, there is no ideal photosensitizer with these features for PDT in the literature [Nyokong, 2011].

One of the most studied application areas in recent years is the use of phthalocyanine molecules, which are second-generation photosensitizers, in PDT applications due to their favorable photophysical and photochemical properties. The photophysical properties of phthalocyanines depend highly on the presence and type

of core metal. Therefore, depending on the metal used, the singlet oxygen generation efficiency of phthalocyanines can be increased. In addition, their low toxicity for cells in the dark is another reason for their use as photosensitizers [Chin et al., 2014].

1.1. The Purpose, Contribution and Content of the Thesis

This doctoral study aims to investigate the photodynamic efficiency of specific phthalocyanines on human cancer cell lines that can be used for phototherapeutic purposes with near-infrared (NIR) absorption, which has not been studied before in the literature. For a more efficient PDT treatment, introducing heavy atom and inhibiting aggregation by bulky substituents are the two main ways the modifications can be made with phthalocyanines to increase singlet oxygen generation capability. In this context, this thesis consists of two parts. Four different phthalocyanines in two sets, Set 1 and Set 2, were investigated with different experimental aspects. In the first part (Set 1), the heavy atom effect of palladium (PdPc-4PEG) was examined against its counterpart, zinc-phthalocyanine (ZnPc-4PEG). In the second part (Set 2), the effect of substitution bulkiness was studied between ZnPc-tBu and ZnPc-nBu. ZnPc-tBu was the bulky phthalocyanine; the other one was the non-bulky phthalocyanine. Two cell lines were used in the *in vitro* experiments; MCF7 and MDA-MB-231. Within the scope of this thesis,

Uv-vis absorption of the phthalocyanines in different solvents and PVP-formulated phthalocyanines in water were recorded.

Dark and Phototoxicity of the phthalocyanines in DMSO were performed, and cell viabilities were analyzed by MTT assay. For Set 2, MTT assay was also conducted with PVP-formulated Set 2 phthalocyanines in water.

Uptake of the phthalocyanines was analyzed by cell uptake experiments with the cell lysis method, and their fluorescence integrity was evaluated with their emission spectra.

Nuclear morphology analysis was performed to evaluate the morphologic changes in the nuclei of PDT-treated cells.

Wound healing assay was used to investigate the morphologic changes as well as the migration capability of the PDT-treated cells.

Colony formation assay was used to determine the potentially lethal- and sublethal damage repair of the PDT-treated cells.

2. LITERATURE REVIEW

2.1. Phototherapeutic Window in Biological Tissue

The wavelength range of light from 650 nm to 1350 nm is called the Phototherapeutic Window (NIR window). This long wavelength allows the light to travel more easily through the tissue without scattering and not to lose its energy without being absorbed [Smith et al., 2009].

While short-wavelength light travels through the tissue, it is scattered by tissue components such as cell membrane, nucleus, or mitochondria. It cannot penetrate deeper and is absorbed by biological chromophores such as melanin, hemoglobin, and bilirubin. However, high-wavelength light is less scattered by tissue components and less absorbed by biological chromophores, as displayed in Figure 2.1 [Wainwright, 2010]. Therefore, light, whose energy has not decreased, can travel long distances in the human body, reach tumors in distant regions, and activate the photosensitizers accumulated there [Nyokong and Ahsen, 2012, p. 11]. For example, Photofrin, one of the widely used photosensitizers, is activated by light of 630 nm wavelength. However, there are studies in the literature reporting that light at this wavelength is absorbed by hemoglobin, melanin, cytochromes, and flavoproteins [Arnfield et al., 1993], [Lee et al., 1995]. In addition, changing hemoglobin concentration during PDT may reduce administration efficiency [Chen et al., 1997]. Therefore, obtaining a photosensitizer capable of producing singlet oxygen by being activated in the NIR window is of great importance for photodynamic therapy. The wavelengths of 650 nm and 950 nm, the first optical window, are the minimal absorption region, are less scattered than the visible wavelength range, and are used in many medical imaging techniques [Sordillo et al., 2014].

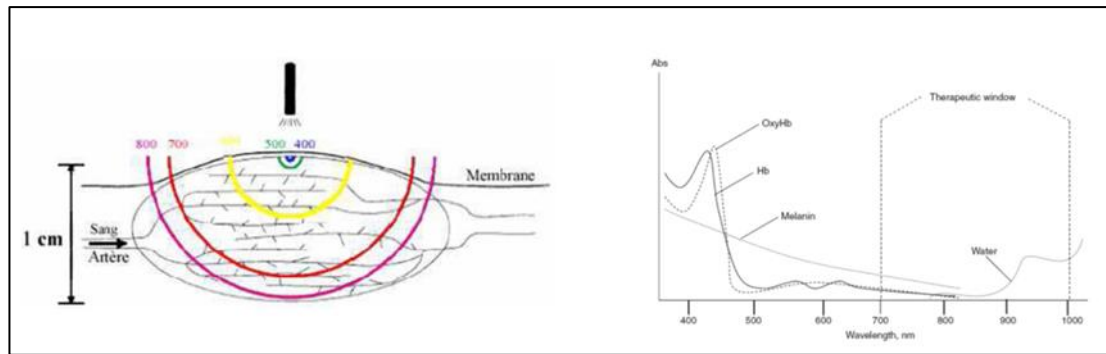


Figure 2.1: Phototherapeutic window.

2.2. Photodynamic Therapy

Photodynamic Therapy (PDT) is a new approach developed as an alternative to surgery, radiotherapy, and chemotherapy, which are the traditional cancer treatment methods widely used today. PDT basically consists of three non-toxic components: photosensitive substance (photosensitizer), light, and oxygen. Once exposed to light, the photosensitizer triggers the cell death response in the presence of oxygen [Brown et al., 2004].

In this application, the photosensitizer applied intravenously or topically to the patient selectively accumulates in cancer cells and tumors, not in healthy tissues. The photosensitizer, which is stimulated by light of a specific wavelength, activates and creates singlet oxygen or free radicals in the cancer cell, and as a result, cancer cells and tumors are destroyed without damaging healthy tissues [Mroz et al., 2011]. Figure 2.2 shows the general mechanism of action of Photodynamic Therapy [Web 3].

PDT has been approved for use in the treatment of various cancers by health institutions in many countries such as America, Germany, Japan, England, France, the Netherlands, and Canada [Dolmans et al., 2003]. Among all countries, Russia is the fastest country in terms of starting PDT applications. In 1990, they developed a drug called Photogem, similar to Hematoporphyrin. The clinical studies of the drug, which was approved by the Russian Ministry of Health, were conducted between 1992-1996; curative effects were observed in 92% of 1500 patients using Photogem, of which the tumor disappeared completely in 62% and partial recovery was achieved in 25% [Mironov, 2000].

The conditions in which PDT is most effective are Actinic keratosis, Bowen's disease (Squamous cell carcinoma), and Basal cell carcinomas [Wan and Lin, 2014]. Recently, it has been used in skin diseases such as Hirsutism, Alopecia Areata, and Acne Vulgaris [Han et al., 2005], [Wan and Lin, 2014]. Besides skin diseases, successful studies have also been reported on bladder, esophagus, lung, head, and neck cancers. In the field of eye diseases, it is used to treat age-related macular degeneration. In addition, it has potential applications in the future treatment of coronary artery diseases, infectious diseases, and hematological cancers. It is also widely used for diagnostic purposes due to the selective uptake of tumor tissues [Dolmans et al., 2003], [Brown et al., 2004], [Bown, 2013].

Although PDT has a broad spectrum of action, its effect is limited to the application area and does not cause any permanent side effects or develop resistance. With PDT, more positive results are obtained in aesthetics, and repetitive applications can be performed safely since the level of treatment-related mortality is low. It is predicted that PDT will be widely used in medicine in the future as it does not require a unique treatment environment for this low-cost application and can be applied to patients of all age groups and health conditions [Vernon et al., 2006].

Hematoporphyrin derivatives (Hematoporphyrin Derivative-HPD) are the first-generation photosensitizers used in PDT. However, their disadvantages have led to synthesizing second-generation photosensitizers, including phthalocyanines. Although Photofrin, aminolevulinic acid (ALA), and Visudyne are FDA-approved photosensitizers used in various cancer types worldwide, they have some non-permanent, mild side effects and cannot fully meet the ideal photosensitizer criteria. Phthalocyanines are second-generation photosensitizers and have a high potential to be used in PDT due to their photochemical and photophysical properties [Okura, 2000], [Bown, 2013], [Mroz et al., 2011].

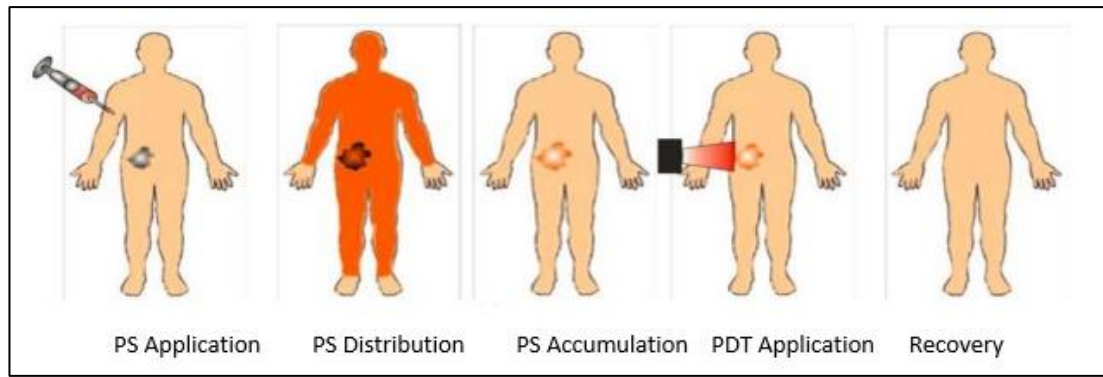


Figure 2.2: General mechanism of action of photodynamic therapy.
PS: Photosensitizer, PDT: Photodynamic Therapy.

In the mechanism of action of PDT, the basal state (ground state) photosensitizer is stimulated to a higher triplet state after light absorption. As a result of this stimulation, two different pathways can be followed: Firstly, it reacts with the substrates in the cell to form free radicals such as reactive oxygen molecules, peroxide, or superoxide (Type 1 Reaction); In a second way, it creates reactive singlet oxygen ($^1\text{O}_2$) by transferring its energy to molecular oxygen (Type 2 Reaction). As a result of both pathways, target cell death occurs. Type 2 reaction is more dominant than Type 1 in clinical PDT. The mechanism of cytotoxicity, photophysical and photochemical reactions is illustrated by the modified Jablonski diagram in Figure 2.3 [Feng et al., 2020].

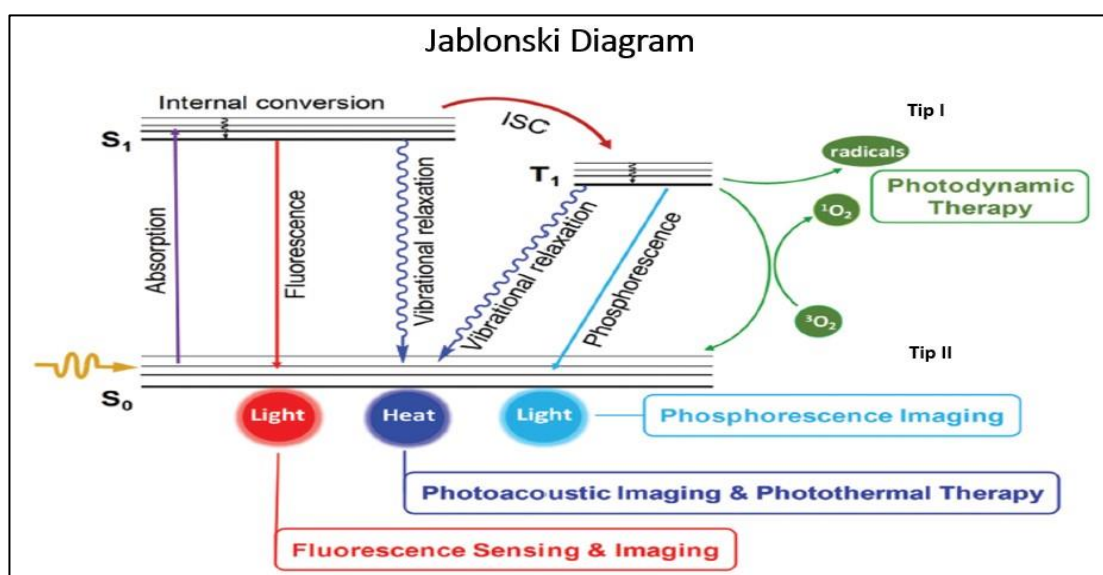


Figure 2.3: Jablonski diagram.

Reactive singlet oxygen is extremely dangerous for cell viability, causing apoptosis and vascular endothelial damage in the target tissue [Dolmans et al., 2003]. Cellular damage is of lysosomal membrane and mitochondrial origin [Babilas et al., 2005]. Therefore, the presence of sufficient molecular oxygen in the target region is important for the success of the PDT application [Moan and Berg, 1991]. It has been shown *in vivo* and *in vitro* that the effect of PDT may be reduced or lost as a result of low oxygen concentration [Moan and Peng, 2003].

PDT is preferable because it will not cause further immunosuppression in immunocompromised patients. Radiotherapy is the most common treatment method for Kaposi's sarcoma, which develops secondary to AIDS. Therefore, the effectiveness of PDT on the disease was wanted to be investigated by Schweitzer and Visscher (1990). In the study conducted, 4 of 7 patients showed complete recovery, while three patients showed partial recovery.

PDT is an alternative method that can be applied to patients who cannot undergo surgery or have received radiotherapy. In the study of Rhodes et al. (2007), 50 patients with Nodular Basal Cell Carcinoma were followed for five years. PDT with local methyl aminolevulinate was applied to the disease lesions, followed by a red light with a wavelength of 570-670 nm. As a result of the study, a 5-year recurrence of the disease rate was found to be 14%.

The efficacy of PDT can be enhanced by various adjuvants. These methods are; penetration enhancers such as dimethylsulfoxide (DMSO) and ethylenediaminetetraacetic acid (EDTA). It is recommended to add 3% desferrioxamine or 2% DMSO + 2% EDTA to the ALA photosensitizer used for deep tumors such as Nodular Basal Cell Carcinoma to respond better to PDT [Gold and Goldman, 2004]. As a result of 2% DMSO + 2% EDTA added to 20% ALA, it was determined that this mixture gave more fluorescence and increased the PDT efficiency to 77% [Babilas et al., 2005].

2.3. Phthalocyanines

One of the most studied application areas today is the use of phthalocyanine compounds as photosensitizers in photodynamic therapy. Over other types of photosensitizers, phthalocyanines have the advantage of being excited by near-infrared

(NIR) wavelengths. The general structure of phthalocyanines, where M is the central metal ion (Al, Zn, Co, etc.), can be seen in Figure 2.4 [Dumoulin et al., 2010].

Phthalocyanines are stable structures, absorption at high wavelengths (around 700 nm), high triplet state quantum yields, long residence times in triplet state, capacity to generate singlet oxygen effectively, selectively accumulate in the tumor, reach the highest concentration in the tumor in a short time (1 -3 hours) and due to the absence of dark toxicity, they are target molecules that can be used in cancer treatment by photodynamic therapy method [Kenney et al.,1992], [Brown et al., 2004], [Gold and Goldman, 2004].

Phthalocyanines are synthetic molecules that can penetrate deeper into the target tissue by absorbing up to 100 times more intensely than porphyrins since they can be stimulated at the NIR wavelength [Okura, 2000], [Atsay, 2009]. If its maximum absorption is usually around 700 nm, appropriate substitutions allow the absorption to rise to 800 nm, conforming to the phototherapeutic window [Topal et al., 2014], [Machacek et al., 2015].

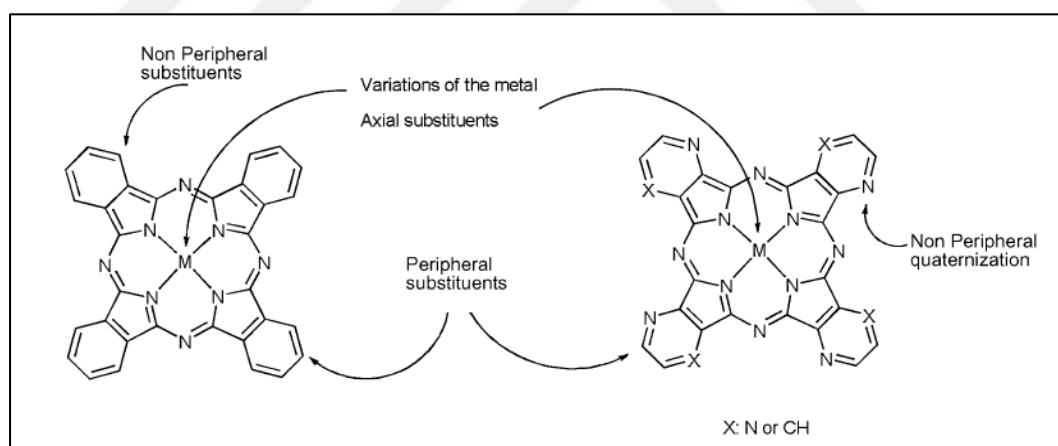


Figure 2.4: General structure of phthalocyanines. M is the central metal ion (Al, Zn, Co, etc.).

Phthalocyanines can form complexes with almost all metals in the periodic table. For effective photosensing, the triplet state lifetime must be prolonged. This property can be achieved by incorporating a diamagnetic atom such as zinc into the phthalocyanine macromolecule [Chin et al., 2014]. For example, Zinc(II) phthalocyanine Tetrasulfonic Acid (ZnPcS₄) is one of the model PDT photosensitizers

whose photophysical and photochemical properties are well known [Putz, 2012, p. 414].

ZnFPc is a zinc phthalocyanine containing six Fluorine atoms. It is thought that this hydrophobic molecule, which is known to accumulate selectively in the tumor, has low PDT activity due to its aggregation tendency. Therefore, two Fluorines were removed from the ZnFPc structure to reduce the aggregation tendency, and carboxyl groups were added to increase its hydrophilic property. Later, in a study with a halogen lamp with a luminous power of 31 J/cm^2 , it was observed that HeLa cells incubated with $1 \text{ }\mu\text{M}$ concentration of this new molecule died after 20 hours of light treatment [Oda et al., 2000].

Formulation tools are used to prevent phthalocyanines from entering the cell as aggregates due to their hydrophobic character. In a study using 'Pluronic®-structured triblock copolymer micelles', it was observed that the studied phthalocyanines were efficiently taken up into cells. It has been reported that one of the phthalocyanines, which is the subject of the research, destroyed the colon cancer tumors formed in 20% of the mice in an *in vivo* study; the tumors disappeared for a year, and recurrence was not observed [Pucelik et al., 2016].

2.4. Singlet Oxygen Generation

Photodynamic therapy involves using photosensitizers, visible light, and molecular oxygen to generate reactive oxygen species (ROS). These ROS kill cancer cells by apoptosis or necrosis, inhibit the growth of tumor microvasculature, and stimulate the immune system of the host. It is widely accepted that the excited singlet state of oxygen ($^1\text{O}_2$) is the primary source of cytotoxic reactive oxygen species (ROS) generated during photodynamic therapy (PDT) for the vast majority of photosensitizers that are currently used in clinical practice as well as for a large number of new agents that are currently under investigation. As a result, increasing its production level within tumor cells and tissues can improve the therapeutic response [Callaghan and Senge, 2018].

Since the lifetime of reactive singlet oxygen in biological systems is $0.04 \text{ }\mu\text{s}$, and the effect radius is $0.02 \text{ }\mu\text{m}$, the damage to the surrounding tissues is prevented, and the effect of the application is limited to the targeted region [Komerik et al., 2000].

One of the most important characteristics of metal-based phthalocyanines for a more effective PDT treatment is the increased singlet oxygen production capability of these phthalocyanines. This capability of phthalocyanines can be improved by making a few adjustments to the chemical structure [Nyokong and Ahsen, 2012, p. 6-11], [Lo et al., 2020]. The primary two ways in which alterations can be achieved with phthalocyanines are the introduction of heavy atoms [Gorman et al., 2004] [Obata et al., 2009] and the suppression of aggregation by bulky substituents [Nyokong, 2007], [Tillo et al., 2015]

2.5. Amphiphilicity

Different studies have shown the mechanism of aggregation, which is the preferential association of phthalocyanine rings to form dimers, the effect of central metal ions on the aggregation properties of phthalocyanine, and the effect of the solvent used to dissolve phthalocyanine. Phthalocyanines tend to dimerize in non-polar solvents such as toluene, while in polar solvents, they remain essentially monomeric. Aggregation causes a decrease in the molar absorptivity coefficient of Pcs and a decrease in the fluorescence quantum yield [Mayildurai et al., 2017]. Amphiphilic photosensitizers have dual nature, which enables the required water solubilization. In the literature, several studies have shown that this nature of the compounds promotes tumor accumulation and tissue distribution, which increases their cellular uptake. Also, their self-assembly capabilities help them to either minimally aggregate intracellularly or disaggregate upon entering the cell, maximizing $^1\text{O}_2$ generation during photoirradiation [Desgranges et al., 2022]. If a solubility problem is encountered in water/biological environments after these studies, formulation studies would be carried out to increase the solubility. Numerous polymer structures are used as potential carrier systems to improve the stability of phthalocyanine and increase cell uptake under physiological conditions [Penjweini et al., 2014].

2.6. Polyvinylpyrrolidone (PVP) As a Formulation Agent

Polyvinylpyrrolidone (PVP), one of the widely used polymers approved by the FDA and a food additive approved as E1201, forms a single macromolecular network that allows guest compounds to enter the polymer matrix. It is a non-toxic amphiphilic

polymer made from the monomer N-vinylpyrrolidone, a linear polymer of 1-vinyl-2-pyrrolidone monomers, for humans and the environment. The molecular weight of the polymer ranges from 10,000 to 700,000 [Nair, 1998]. It can be solubilized in water and many polar organic solvents (methanol, ethanol, amines, acids, etc.). PVP is non-ionic, inert, temperature-resistant, pH-stable, biocompatible, and has a high affinity for hydrophilic and hydrophobic drugs. As a result, PVP has gained a useful role in the pharmaceutical, biomedical, cosmetics, and food industries. The general PVP structure can be seen in Figure 2.5 [Marani et al., 2016].

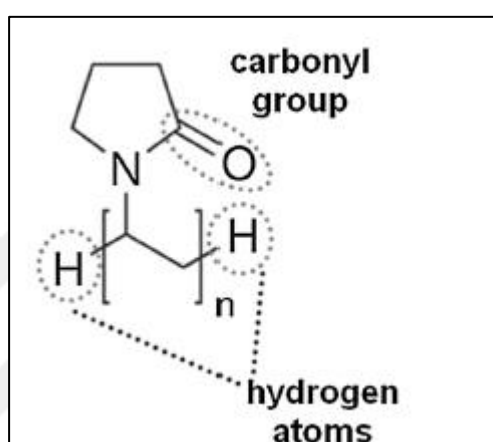


Figure 2.5: General polyvinylpyrrolidone structure.

The weight-average molecular weights obtained from kinematic viscosity measurements are used to identify PVP polymers (K-values). PVP grades K-25 and higher are in compliance with the minimum weight-average molecular weight requirements of the EU specification for E 1201 [Younes et al., 2020].

In vitro, photodynamic therapy studies with PVP have investigated the effectiveness of this formulation with various photosensitizers. PVP has been used as the delivery system for chlorine e6 (Photolon) in clinical studies [Chin et al., 2008], [Lee et al., 2010]. In the study conducted with chlorine e6 (Ce6, Fotolon), it was stated that the tumor selectivity of Ce6-PVP was higher than in other formulations. *In vitro*, PDT results showed that Ce6-PVP increases selective phototoxicity in leukemic cells compared to peripheral mononuclear blood cells. In addition, after 1 hour of incubation *in vivo*, although there was no toxicity in the animal, an increase in tumor necrosis was observed. In human experiments, selective Ce6 - PVP accumulation was observed in angiosarcoma lesions compared to normal skin after intravenous administration. As a

result, it was shown in the study that PVP significantly increased Ce6 concentration and did not cause any significant side effects in animals and humans [Chin et al., 2008].

In another study in which Ce6-PVP was used as the delivery system, patients with recurrent bladder cancer that did not reach the muscle were studied, and it was stated that Ce6-PVP is a compound with the potential to prevent the recurrence of this type of cancer [Lee et al., 2010].

It has been suggested that PVP-Hypericin targets membrane structures in the cytoplasm and on the cell surface via the endoplasmic reticulum, and it has been shown to have a significantly higher affinity for cancer cells than hypericin dissolved in DMSO [Kubin et al., 2008]. In a study showing the transport and accumulation of PVP-Hypericin in normal cells and cancer cells, attention was drawn to the fact that the accumulation was constant and the transport was slow between organelles. It was stated that this could cause maximum death of cancerous cells and thus protect healthy cells from the drug [Penjweini et al., 2014]. In a study conducted in a lung cancer cell line, it was stated that PVP Hypericin was suitable for photodynamic diagnosis (PDD) when treated with low-dose light (blue LED) [Penjweini et al., 2012]. In the following study, significant cell death was observed in the same cell line using the standard photodynamic therapy method [Penjweini et al., 2013].

Nanographene oxide is a new type of nanomaterial that has been used for the combination of photothermal therapy and chemotherapy. It is used as a cancer drug delivery system and causes photothermal tumor ablation by near-infrared radiation. In a study, folic acid was added to the structure after its formulation with PVP, and thus an increase in the photothermal efficiency of the material was observed *in vitro*. It has been shown that the drug delivery feature of the system loaded with doxorubicin was also high [Qin et al., 2013].

In a study in which silver nanoparticle (Ag Np) was formulated with PVP, it was shown that Ag NP coated with PVP had a greater effect than citrate-coated Ag NP in promoting cell death, cell size, and tyrosine hydroxylase activity in PC12 cells [Powers et al., 2011]. In another study, the yields of PVP-Ag Np and silver ions were compared *in vitro* in THP-1 monocyte cells, and it was stated that both increased reactive oxygen species, apoptosis, and necrosis in the cell [Foldbjerg et al., 2009].

Zinc oxide nanoparticles (ZnO NP) formulated with PVP were used in a study comparing the effect of phosphate-containing and non-phosphate DMEM on cell viability in the presence of ZnO NP [Everett et al., 2014]. The contribution of PVP to

the effectiveness of nanoparticles in terms of magnetic resonance imaging (MRI) has also been investigated in the literature. Analyzes show that PVP-coated magnetite (Fe_3O_4) nanoparticles (MNPs) have great potential for use as T2 contrast agents in MRI [Arsalani et al., 2010]. In another study, *in vitro* and *in vivo* studies performed after coating uniform-sized manganese oxide nanoparticles (oleic-capped MnO NPs) with PVP found that the compound could be used as low cytotoxicity, biocompatible, and T1-positive contrast agent for passively targeted MRI. In addition, *in vivo* studies have shown that the compound can pass through the blood-brain barrier [Hu et al., 2013].



3. MATERIALS

Table 3.1: Cell lines.

Name	Catalogue Number	Company
MCF7	-	Prof. Dr. Tamer Yağcı
MDA-MB-231	-	Prof. Dr. Nuri Öztürk

Table 3.2: Phthalocyanines.

Name	Synthesized by
ZnPc-4PEG	Yıldız Ek, under the supervision of Assoc. Prof. Dr. Fabienne DUMOULIN
PdPc-4PEG	Dr. Öğr. Üyesi ZEYNEL ŞAHİN, under the supervision of Assoc. Prof. Dr. ÜMIT İŞÇİ
ZnPc-tBu	Dr. Öğr. Üyesi ZEYNEL ŞAHİN, under the supervision of Assoc. Prof. Dr. Ümit İŞÇİ
ZnPc-nBu	Tuğba Korkmaz Küçük, under the supervision of Assoc. Prof. Dr. Fabienne DUMOULIN

Table 3.3: Chemicals and reagents.

Name	Catalogue Number	Company
DMEM	41966029	Gibco
FBS	26140-111	Gibco
DPBS	14190094	Gibco
Penicillin/Streptomycin	SV30010	HyClone
DMSO	116743	Merck
Trypsin 0.05% EDTA	25300054	Gibco
Absolute Ethanol	32221	Sigma-Aldrich
Trypan Blue	T10282	Invitrogen
Hoechst 33342	H1399	Invitrogen
MTT	M6494	Thermo Fisher

Table 3.3 (Continue): Chemicals and reagents.

Name	Catalogue Number	Company
Propidium iodide	P4170	Sigma-Aldrich
Methanol	34885-2.5L-R	Sigma-Aldrich
Acetone	W332615	Sigma-Aldrich
Acetic acid	A6283	Sigma-Aldrich
Crystal Violet	1.014.080.100	Merck
Polyvinylpyrrolidone K25	-	Prof. Kristjan Plaetzer

Table 3.4: Glassware, plastics, and consumables.

Name	Catalogue Number	Company
T25 Flasks	90026 90076	TPP
T75 Flasks	90076	TPP
Petri Dishes (100×20 mm)	93100	TPP
6 Well Plates	92006	TPP
15 ml Falcon Tubes	91015	TPP
50 ml Falcon Tubes	91050	TPP
25 cm ² flask	TCF-25V	SUNUB
Neubauer Counting Chamber	900505	Marienfeld
Cryo Tubes	89012	TPP
Freezing Container	5100-0001	Nalgene
96 well plate	92096	TPP
Syringe Filters (0.22 µm)	99722	TPP
50 ml centrifuge tube	5100050C	CAPP
15 ml centrifuge tube -	5100015C	CAPP
1.5 ml Micro Centrifuge tube	5101500C	CAPP
96 well black plate	P8366	Nunc
1.5mL Black Microcentrifuge Tubes	15386548	Fisherbrand
96 well black plate	655076	Greiner Bio-one

Table 3.4 (Continue): Glassware, plastics, and consumables.

Name	Catalogue Number	Company
Eppendorf Tubes	MCT-060-C / 150-C	Axygen
Filter Tips 10 µl	PPU-10	SUNUB
Filter Tips 20 µl	PPU-20	Axygen
Filter Tips 200 µl	PPU-200	Axygen
Filter Tips 1000 µl	PPU-1000	Axygen
Parafilm	058.01.001	Isolab
10 uL tip, non steril	5030010C	CAPP

Table 3.5: Equipment.

Name	Catalogue Number	Company
LED Lamp	-	-
Inverted Fluorescence Microscope	Eclipse 80i	Nikon
CO ₂ Incubator	HF90	Heal Force
Water Bath	0400702135J036	Wisd Lab. Ins.
Liquid Nitrogen Tank	LS4800	Taylor Wharton
Inverted Light Microscope	Eclipse TS100	Nikon
Shaker	S2025-B-230V	Labnet
Multi-Rotater	Multi RS-60	Biosan
Varioskan Plate Reader	3001-1571	Thermo Scientific
Cell Culture Hood	Puricube 1200	Cryste
Chemi Doc XRS+	721BR04545	Biorad
Confocal Microscope	LSM 880	Zeiss
Centrifuge	1-14	Sigma
Vortex	444-1372	VWR International

4. METHODS

4.1. Photosensitizers

Phthalocyanines used in this study were synthesized in the Chemistry Department of Gebze Technical University (Table 3.2). Two sets of phthalocyanines, each has two compounds with different properties, were used. In the first set, PdPc-4PEG and ZnPc-4PEG, the heavy atom effect was investigated between Zn and Pd. In the second set, ZnPc-tBu and ZnPc-nBu, substitution bulkiness was examined between s-butyl and n-butyl.

4.2. Preparation of the PVP formulation

In formulation studies with PVP, Kubin et al. (2008) proposed protocol was used. According to the protocol, the formulation was made using the ratio of 1 μ mol photosensitizer (PS): 100 mg PVP. PVP K25 was used in the formulation studies of this thesis.

Accordingly, the desired amount of photosensitizer was dissolved in ethanol and sonicated for 2 minutes. Then, PVP and ethanol were added to the medium at the required rate, and the mixture was heated at 70 °C for 5 minutes. Next, extra ethanol was added, and the mixture was stirred vigorously for 10 minutes. Then, the solvent was carefully removed from the rotary evaporator, and the formulation process was finalized.

It has been reported that different amounts of PVP have various effects on the solubility of photosensitizers [Kubin et al., 2008], [Everett et al., 2014]. In this context, PS:PVP formulation was made at different ratios to increase the solubility of aggregated compounds. 1 μ mol PS and 100, 200, 300, 400, 500, and 1000 mg PVP were used for ZnPc-4PEG formulations. 1 μ mol PS and 100, 150, 200, 250, and 300 mg PVP were used for ZnPc-tBu and ZnPc-nBu formulations.

4.3. Photosensitizer Stock Solutions

Stock solutions of all compounds but not formulated ones were prepared by dissolving them in DMSO, sonicated in an ultrasound bath, and vortexed vigorously. Formulated compounds were dissolved in water. In all *in vitro* experiments, working concentrations of the compounds were 1X, and stock solutions were prepared as 2X.

4.4. UV-vis Absorption

UV-vis absorption spectra were measured with a Shimadzu UV-1800 UV-vis spectrophotometer. 1, 2, 4, 6, 8, and 10 μ M dilutions from stock solutions in different solvents (water or DMSO) were recorded. Different ratios of PS:PVP and different dilutions of PVP in water were also recorded.

4.5. Light Source

In this study, a handmade LED lamp was used as the light source and was made as previously described [Pieslinger et al., 2006]. Controlling the power output is achieved by using an adjustable power supply. In order to illuminate attached living cells from the bottom of culture dishes or plates, the lamp has been purpose-built to deliver consistent and homogenous fluency across an illumination region, making it possible for the device to fulfill its intended function. 680 nm – 700 nm LED lamps were used in the conducted *in vitro* assays of this study.

4.6. Cell Culture

Two different breast cancer cell lines, MCF7 and MDA-MB-231 were used. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose, Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin. Cells were maintained in 75 cm² flasks at 37°C in a humidified chamber with 5% CO₂.

4.7. Dark Toxicity Assay

For the determination of dark toxicities of phthalocyanine molecules, cell lines were seeded in at least three wells in 96-well plates with 10^4 cells/100 μ l medium. After 24 hours of incubation in the incubator (37°C, 5% CO₂), new media containing different 2X concentrations of each phthalocyanine were added to each well as 100 μ l. Target concentrations of 0.0625, 0.125, 0.25, 0.5 and 1 μ M were used for PdPc-4PEG and ZnPc-4PEG. For ZnPc-tBu and ZnPc-nBu, target concentrations of 0.125, 0.25, 0.5, 1, 5 and 10 μ M were used. Incubation time with the compounds was 1 and 24 hour. As a negative control, cells to which only DMSO-containing medium without phthalocyanine was added. A target concentration of 10 μ M was used for ZnPc-tBu and ZnPc-nBu with an incubation time of 24 hour and 48 hour, in which PVP formulations were made at different rates. As a negative control, cells to which only water-containing medium without phthalocyanine was added. At this stage, where phthalocyanines are added, the cells are in the exponential phase, and the 'confluency' rate should not be more than 70%. At the same time, cells containing only fresh medium (without a 'formulator' or phthalocyanine) were used to control the ambient conditions. After 1 and 24 hours of incubation (37°C, 5% CO₂), the medium was removed, washed once with 250 μ l of 1X PBS, and then added 200 μ l of fresh medium was. At the end of 24 hours of incubation (37°C, 5% CO₂), whether phthalocyanines have a toxic effect on each cell line in the dark was determined by the MTT Test.

4.8. Phototoxicity Assay

In order to investigate phototoxicity caused by phthalocyanines in cells due to photodynamic therapy application, cells were seeded into 96-well plates in at least three wells with 10^4 cells /100 μ l medium. After 24 hours of incubation in the incubator (37°C, 5% CO₂), new media containing different 2X concentrations of phthalocyanines were added to each well as 100 μ l. Working concentrations of 0.0625, 0.125, 0.25, 0.5, and 1 μ M were used for PdPc-4PEG and ZnPc-4PEG. For ZnPc-tBu and ZnPc-nBu, working concentrations of 0.125, 0.25, 0.5, 1, 5 and 10 μ M were used. Incubation time with the compounds was 1 and 24 hour. As a negative control, cells to which only DMSO-containing medium without phthalocyanine was added. A working

concentration of 10 μM was used for ZnPc-tBu and ZnPc-nBu with an incubation time of 24 hour and 48 hour, in which PVP formulations were made at different rates. As a negative control, cells to which only water-containing medium without phthalocyanine was added. After incubation in the incubator (37°C , 5% CO_2), the medium was removed, washed once with 250 μl of 1X PBS, and then 200 μl of fresh medium was added.

Cells with unformulated phthalocyanine were exposed to light with appropriate wavelength (680 nm-700 nm) at a power (F) of 1.8 J/cm^2 , 3.6 J/cm^2 , 7.2 J/cm^2 , and 14.4 J/cm^2 (Fluence rate: 10 mW/cm^2). Cells with formulated phthalocyanine were exposed to light at a power of 14.4 J/cm^2 (F). How long the cells will be exposed to these light doses is calculated according to the following formulas, 4.1 and 4.2 (Faizal Asan et al., 2021):

$$\text{Time (seconds)} = \frac{\text{Energy received by cells (F)}}{\text{Intensity of radiation (I)}} \quad (4.1)$$

$$\text{Intensity of radiation (I)} = \frac{\text{Diode laser power}}{\text{Area of the irradiated region}} \quad (4.2)$$

After the light energy was given, the plates were put back into the incubator. The phototoxicity of phthalocyanine molecules was determined by MTT test after 24 hours of incubation in the incubator (37°C , 5% CO_2).

4.9. MTT Cell Viability Assay

Measurement of cell viability using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay was carried out for each of the compounds. First, the MTT reagent (5 mg/ml) was dissolved in PBS and added to the wells. The final concentration at each well was 0.5 mg/ml. After 3 hours of incubation in the incubator (37°C , 5% CO_2), old media were removed, and 100 μl DMSO was added to each well to dissolve the formed formazan crystals. Plate shakers were used to shake the plates for 15 minutes at room temperature gently. A plate reader was used to measure the absorbance at 570 nm (Varioskan - Thermo Fisher Scientific, Vantaa, Finland).

4.10. Cellular Uptake

Cell lysis was performed to determine the uptake potential of phthalocyanine molecules. Cells were seeded into 6-well plates as 25×10^4 cells/2 ml medium. After 24 hours of incubation in the incubator (37°C, 5% CO₂), old media were removed from the wells, and new media containing 1 µM PdPc-4PEG and ZnPc-4PEG at working concentrations, reduced 1X in 2ml from 2X stock solutions, were added. As a negative control, cells to which only DMSO-containing medium without phthalocyanine was added. After 1 and 24 hours of incubation with the compounds in the incubator (37°C, 5% CO₂), old media were removed and washed once with 2 ml of 1X PBS. Next, PBS was removed, and 500 µl of trypsin was added to each well; Cells were incubated for 30 seconds at 37°C, 5% CO₂ in the incubator to allow faster separation of cells from each other and from the wells. After incubation, 800 µl of fresh medium was quickly added to the wells, all wells were carefully washed, and cells were collected. The solution of each well was transferred to 1.5 ml black eppendorfs separately. For cell counting, 10 µl of medium from each eppendorf was taken and counted with trypan blue. The residues in the eppendorfs were centrifuged at 1500 RPM for 5 minutes. After centrifugation, the supernatants were removed, the pellets in the eppendorfs were first vortexed, and then 1 ml of PBS was added and again vortexed. Cells were centrifuged at 1500 RPM for another 5 minutes. After centrifugation, the supernatant was removed, the pellet was vortexed, and 300 µl of DMSO was added to each eppendorf and vortexed again. Cells were sonicated in a water bath for 10 minutes and centrifuged at 14000 RPM for 20 minutes to eliminate cellular membrane fragments. A new calibration curve was drawn with different concentrations of phthalocyanines in each new experiment. After centrifugation, 100 µl of the supernatant was transferred to 96-well black plates in triplicate. In addition, only 100 µl of DMSO was placed in triplicate as blank on these plates. Readings were made using the 690 nm excitation and 720 nm emission wavelengths of phthalocyanines in the spectrofluorometer, Varioskan - Thermo Fisher Scientific, Vantaa, Finland. The phthalocyanine uptake graph of the cells was drawn in Microsoft Excel using the calibration curve.

4.11. Nuclear Morphology Analysis

Hoechst 33342/Propidium iodide double staining without fixation was performed to observe morphological changes in the nuclei of PDT-treated cells. 50000 cells/well were seeded in 24 well plates and treated with 1 μ M of PdPc-4PEG and ZnPc-4PEG and 10 μ M of ZnPc-tBu and ZnPc-nBu for 1 h and 24 h as described before. Six hours after PDT treatment, cells were washed with PBS twice and incubated with 1 μ g/ml for each reagent for 15 minutes at 37°C in the dark. Afterward, the fluorescent dyes containing mediums were removed, and cells were washed again with PBS, followed by observation under Zeiss LSM 880 Confocal Microscope (Oberkochen, Germany).

4.12. Wound Healing Assay

Migration of MCF7 and MDA-MB-231 cells was investigated after PDT treatment with PdPc-4PEG, ZnPc-4PEG, ZnPc-tBu, and ZnPc-nBu by a wound healing assay. First, 5×10^5 cells were seeded in a 6-well plate and incubated for 24 hours. After incubation, cells were treated with 1 μ M PdPc-4PEG and ZnPc-4PEG; 10 μ M ZnPc-tBu and ZnPc-nBu for 1 hour and 24 hours. Then, old media were removed, and wells were washed once with excessive PBS and illuminated by the hand-made LED lamp with a light dose of 14.4 J/cm² (Fluence rate: 10 mW/cm²). Immediately after treatment, wounds were stimulated by using a yellow sterile pipette tip to make scratches. Then, the old media were removed, and wells were washed once with PBS. After the washing step, complete medium was added to the wells, and the plates were returned to the incubator. Cell migration was controlled by an Inverted Fluorescence Microscope Eclipse 80i, Nikon. Images were taken at 0 h, 24 h, and 48 h time points.

4.13. Colony Formation Assay

In order to test the cytotoxicity from PDT treatment, a colony formation assay was performed. 5×10^5 MCF7 and MDA-MB-231 cells were seeded in a 6-well plate and incubated for 24 hours. After incubation, cells were treated with 1 μ M of PdPc-4PEG and ZnPc-4PEG; 10 μ M of ZnPc-tBu and ZnPc-nBu for 1 hour and 24 hours.

After PDT treatment with the light dose of 14.4 J/cm², cells were trypsinized, counted, and seeded as 1500 cells per well into 6-well plates for colony formation assay. Cells were incubated for 10 days at 37°C and 5% CO₂, and the medium was not changed. Cells were then fixed with cold methanol at -20°C for 10 min and stained with (0.5% w/v) for 20 minutes. Images were taken by using Chemi Doc XRS+ imaging system.

4.14. Statistical Analysis

The statistical significance of the treatment outcomes was assessed by performing a two-way analysis of variance or one-way analysis of variance followed by either Dunnett's multiple comparisons test and Student's t-test on GraphPad Prism version 7.04 (GraphPad Software, La Jolla, California, United States). $p < 0.05$ was considered statistically significant.

5. RESULTS

5.1. Heavy Atom Effect

Set 1 phthalocyanines, ZnPc-4PEG and PdPc-4PEG, were investigated in order to evaluate the heavy atom effect between Zn and Pd. The molecular weight of ZnPc-4PEG is 1226, and the molecular weight of PdPc-4PEG is 1267. The geometric structures of the phthalocyanines are in Figure 5.1. In this part of the study, Set 1 phthalocyanines were solved in DMSO, and their stock solutions for the conducted *in vitro* assays were prepared in DMSO.

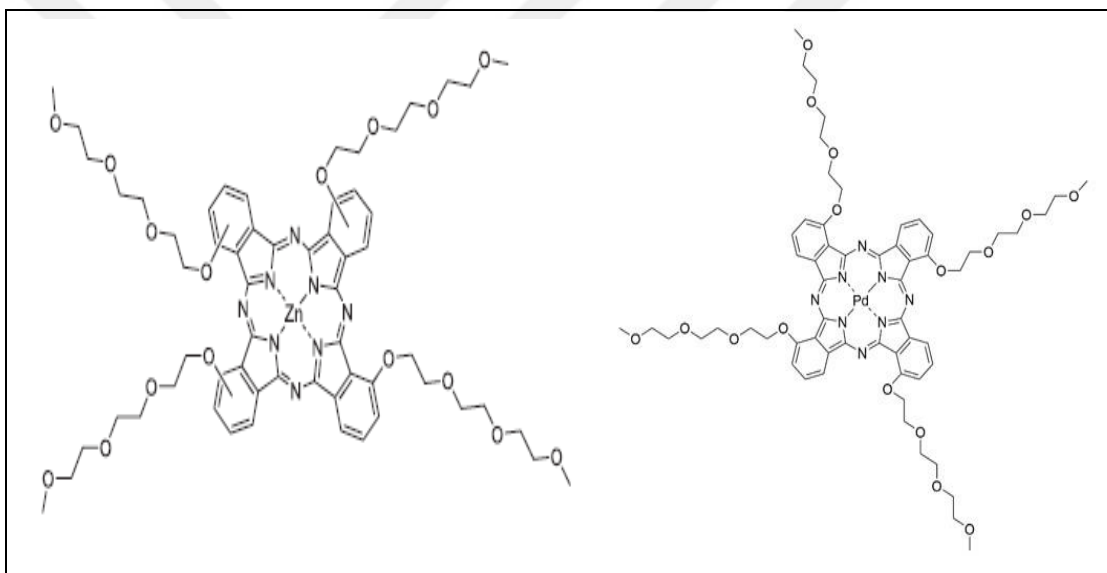


Figure 5.1: Chemical structures of ZnPc-4PEG and PdPc-4PEG, respectively.

5.1.1. UV-vis Absorption of Set 1

UV or visible light absorbed by or transmitted through a sample in contrast to a reference or blank sample is measured by UV-Vis spectroscopy. The sample composition impacts this feature, potentially revealing what is in the sample and at what concentration. In the context of this study, UV-vis absorption spectra were recorded to determine; the wavelengths at which absorption occurs, the degree of the absorptions at each wavelength, the maximum absorption wavelength and to evaluate the solubilization capability of the phthalocyanines. Firstly, Set 1 phthalocyanines

were dissolved in DMSO. Then, stock solutions were diluted to 1, 2, 4, 6, 8, and 10 μM in DMSO. Their UV-vis absorption spectra were measured (Figure 5.2). The maximum Q bands of ZnPc-4PEG and PdPc-4PEG were 704 nm and 692 nm, respectively. Corresponding values of the $\log \epsilon$ were also calculated for ZnPc-4PEG was 5.34 and for PdPc-4PEG was 5.17. It was seen that Set 1 had dissolved and not aggregated in DMSO.

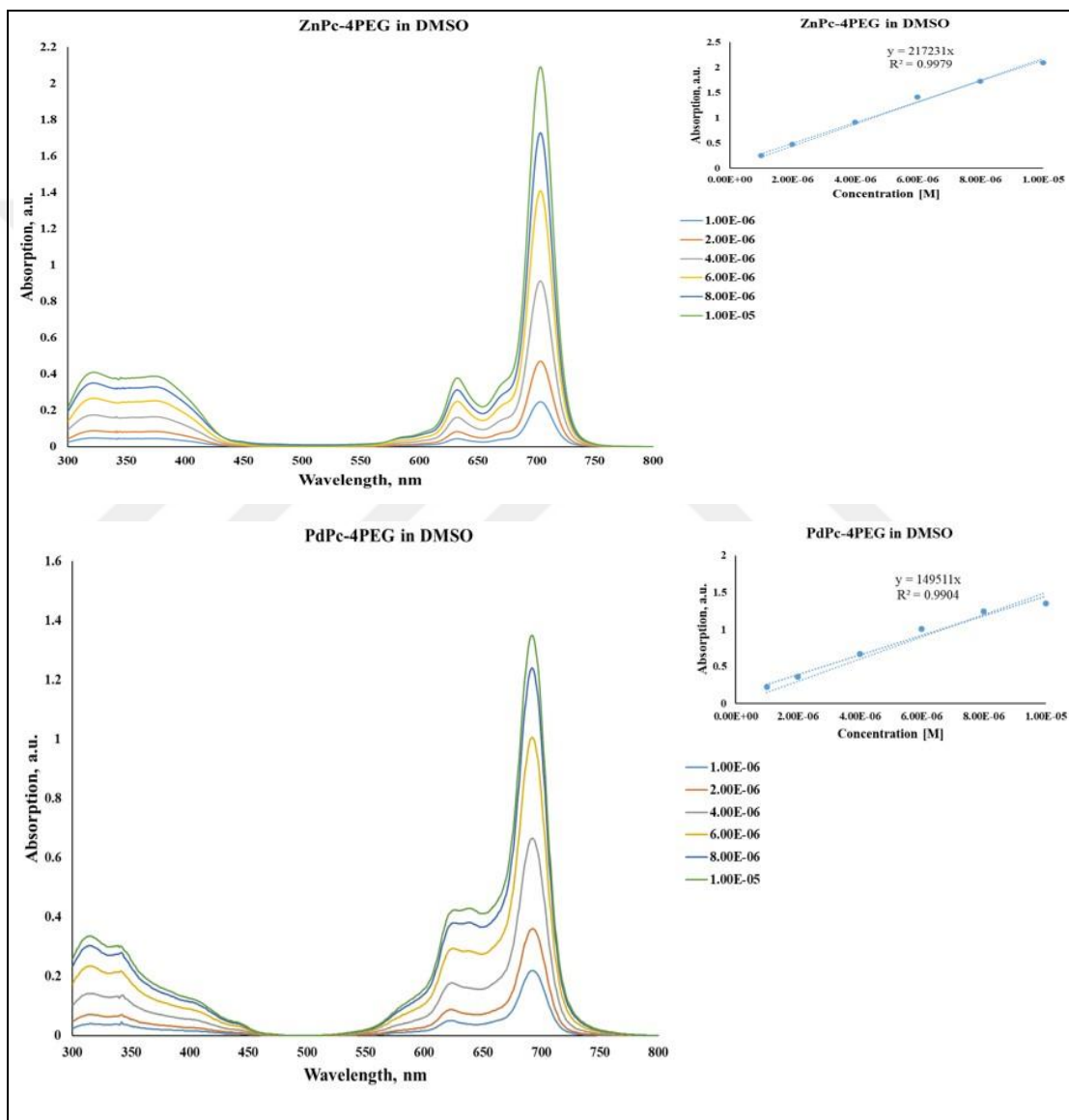


Figure 5.2: Absorption spectra of Set 1 in DMSO.

In order to evaluate the degree of absorptions, Uv-vis spectra of 10 μM of the compounds in DMSO were also compared. According to the spectrum of each

compound, it was seen that the degree of absorption of PdPc-4PEG is lower than ZnPc-4PEG (Figure 5.3).

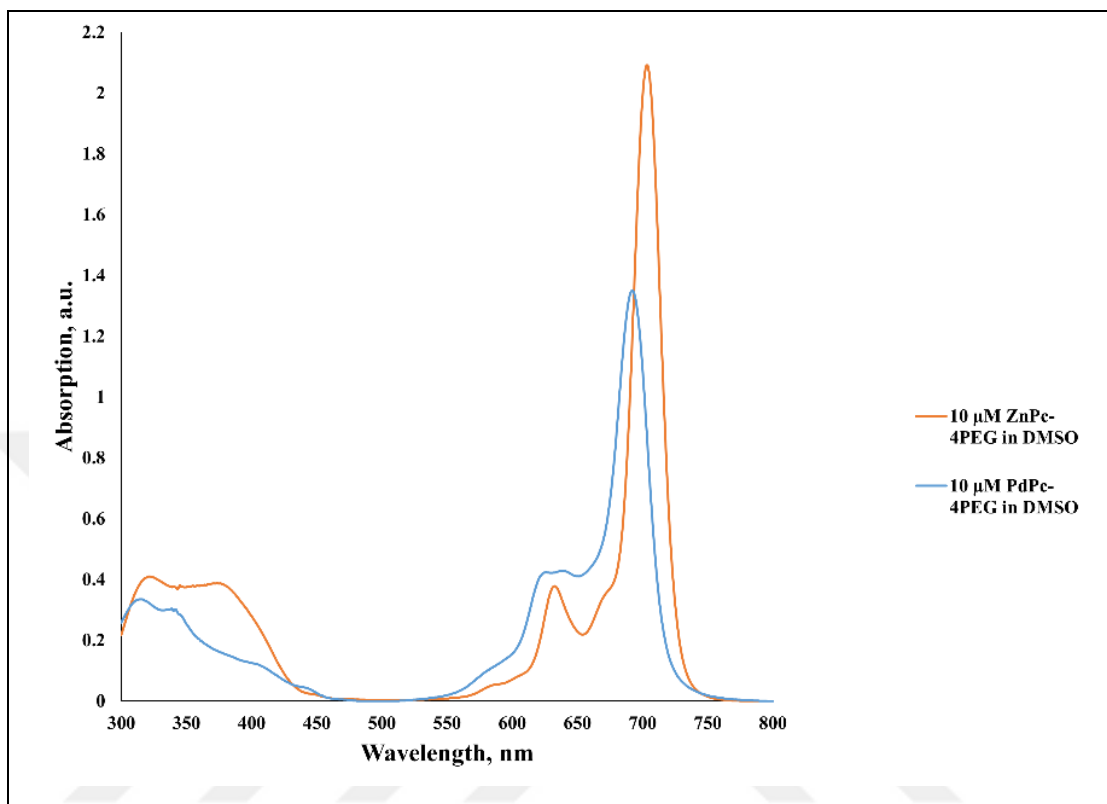


Figure 5.3: Comparison of UV-vis Absorption of Set 1 in DMSO at 10 μ M.

Secondly, compounds were dissolved in water to record their UV-vis absorption spectra. Stock solutions were diluted to 1, 2, 4, 6, 8, and 10 μ M in water. Their UV-vis absorption spectra were measured (Figure 5.4). The maximum Q bands of ZnPc-4PEG and PdPc-4PEG were lower than they were in DMSO and were recorded as 668 nm and 619 nm, respectively. Corresponding values of $\log \epsilon$ were also lower than DMSO; for ZnPc-4PEG was 4.75 and for PdPc-4PEG was 4.48. Dissolving in water is a valuable feature for a photosensitizer candidate, and it was seen that Set 1 dissolves in DMSO better than in water, but still, it can be dissolved in water.

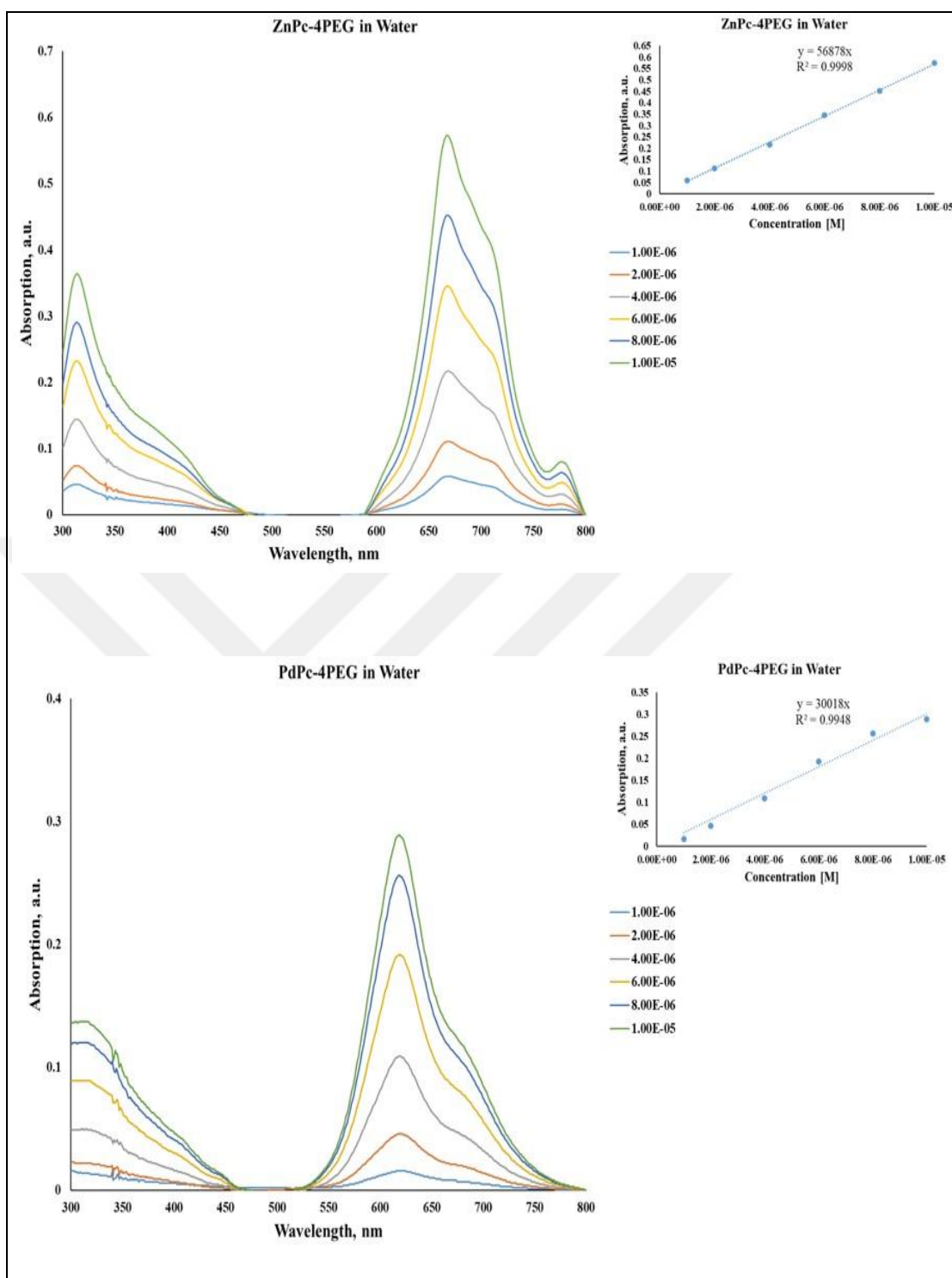


Figure 5.4: Absorption spectra of Set 1 in water.

The degree of absorptions in water was also evaluated, and Uv-vis spectra of 10 μM of the compounds in water were compared with each other. It was seen that the degree of absorption of PdPc-4PEG is lower than ZnPc-4PEG as it was in DMSO, but also, the values of each absorption in water were lower than DMSO (Figure 5.5).

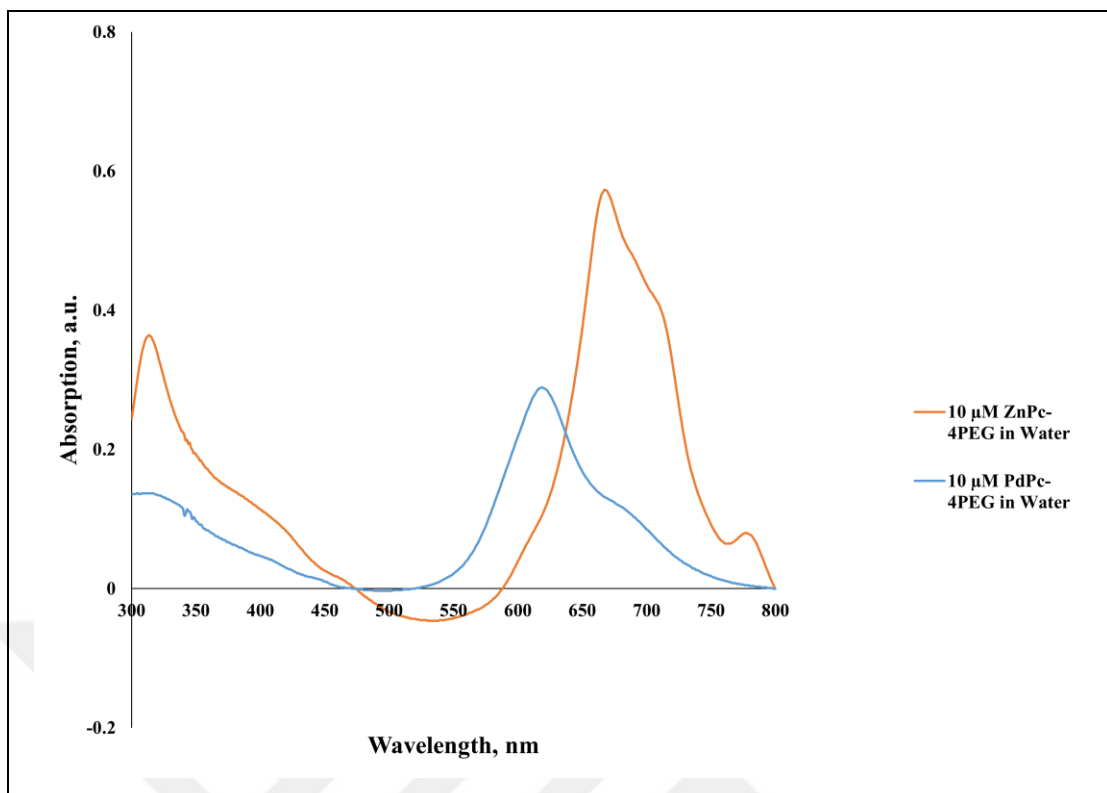


Figure 5.5: Comparison of UV-vis Absorption of Set 1 in water at 10 μ M.

Thirdly, PVP formulation with ZnPc-4PEG was performed for further examinations. For that purpose, firstly, the compound was formulated with PVP in different ratios. 1 μ mol phthalocyanine was formulated with 100, 200, 300, 400, 500, and 1000 mg PVP. Then the formulated compounds were dissolved in water, diluted to 10 μ M, and their UV-vis spectra were recorded (Figure 5.6). Their maximum Q bands and corresponding values of $\log \epsilon$ for ZnPc-4PEG-PVP of each formulation can be seen in Table 5.1.

Table 5.1: Maximum Q bands and related values of $\log \epsilon$ for ZnPc-4PEG-PVP of each formulation.

PVP RATIO:	1:100	1:200	1:300	1:400	1:500	1:1000
λ_{max} (nm)	677	679	679	680	680	680
$\log \epsilon$:	4.58	4.63	4.62	4.62	4.62	4.6

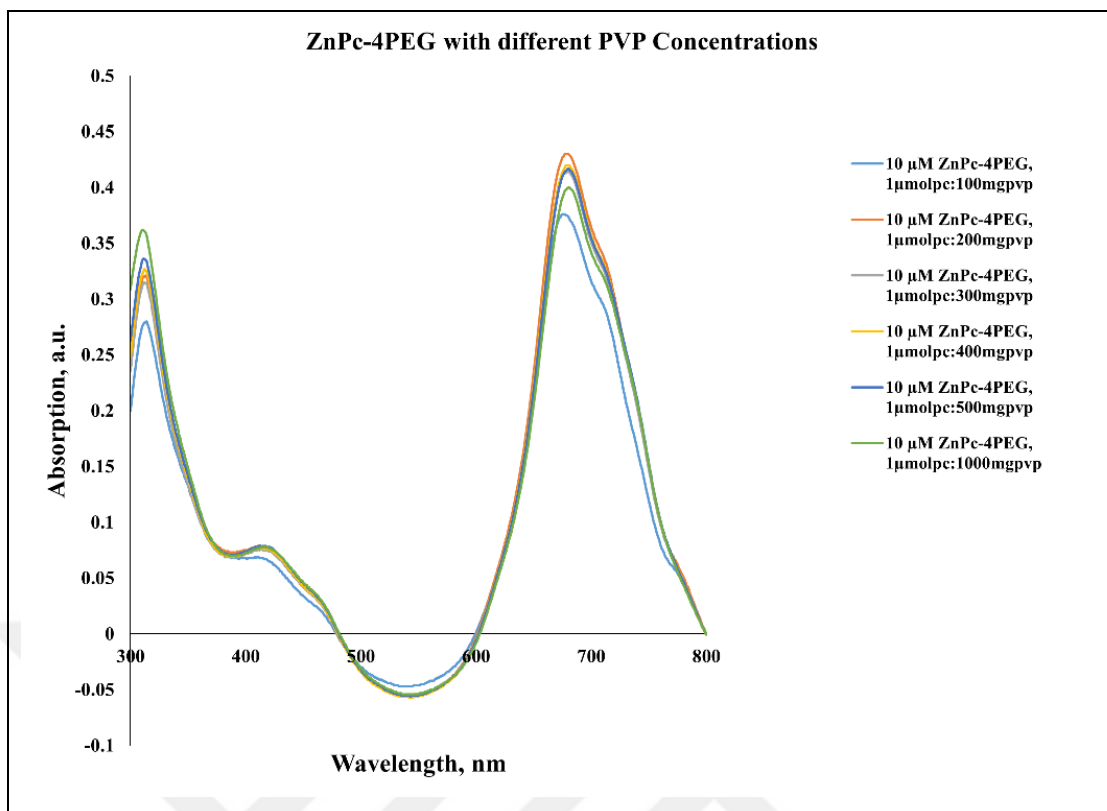


Figure 5.6: Absorption spectra of ZnPc-4PEG with PVP formulation in different ratios in water.

After the comparison of the PVP ratios, one of the ratios was chosen for further biological examinations. As it was seen in Figure 5.6, the maximum absorption wavelengths of the PVP ratios and their degrees were similar. So, for economic reasons, the 1:100 ratio was chosen. After that evaluation and choice, ZnPc-4PEG with 1:100 PVP ratio was dissolved in water, diluted to 1, 2, 4, 6, 8, and 10 μM in water, and their UV-vis spectra were recorded (Figure 5.7). The maximum of the Q band was 675, and the corresponding value of $\log \epsilon$ was 4.6 for ZnPc-4PEG-PVP. The maximum Q band was slightly higher; $\log \epsilon$, and the degrees of absorption were lower for PVP formulated ZnPc-4PEG than ZnPc-4PEG in water without formulation. UV-vis spectrum of only PVP (4,843.75 mg/ml PVP) in water was also recorded; no absorption of PVP itself was observed (Appendix B).

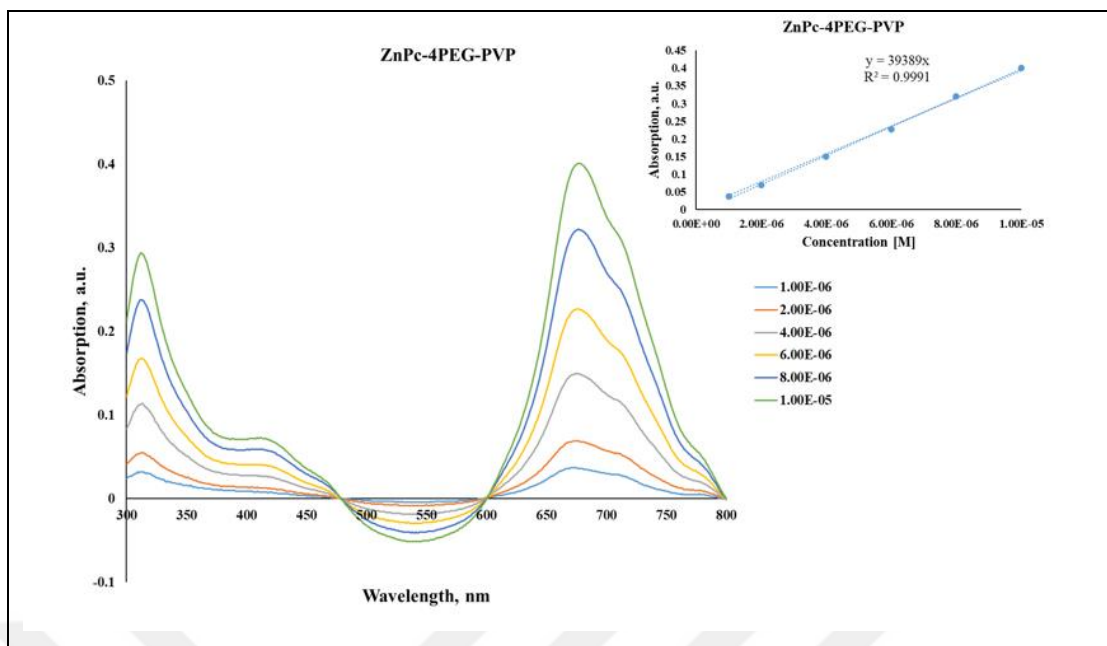


Figure 5.7: Absorption spectra of ZnPc-4PEG-PVP in water.

5.1.2. Dark Toxicity and Phototoxicity of Set 1

Phthalocyanines used in PDT should be non-toxic while not exposed to light but should eradicate cells when activated by light. To assess the performance of a photosensitizer, firstly, its dark toxicity is evaluated, and then phototoxicities in different light doses are studied. The optimum conditions mainly depend on the solubilization capacity of the compound to reach maximum performance.

Before biological investigations and their comparisons of Set 1, in order to examine the effect of solvent and the PVP formulation, dark and phototoxicity assays were conducted with ZnPc-4PEG on the MDA-MB-231 cell line (Figure 5.8). ZnPc-4PEG was dissolved in DMSO (A), in water (B) and formulated with PVP in 1:100 ratio, and again dissolved in water (C). Stock solutions were diluted to 0.125, 0.25, 0.5, and 1 μM in their appropriate solvents. After incubation of MDA-MB-231 cells with compounds in 1 hour and 24 hour time points, the subjected cells were exposed to 3.6 J/cm² light dose.

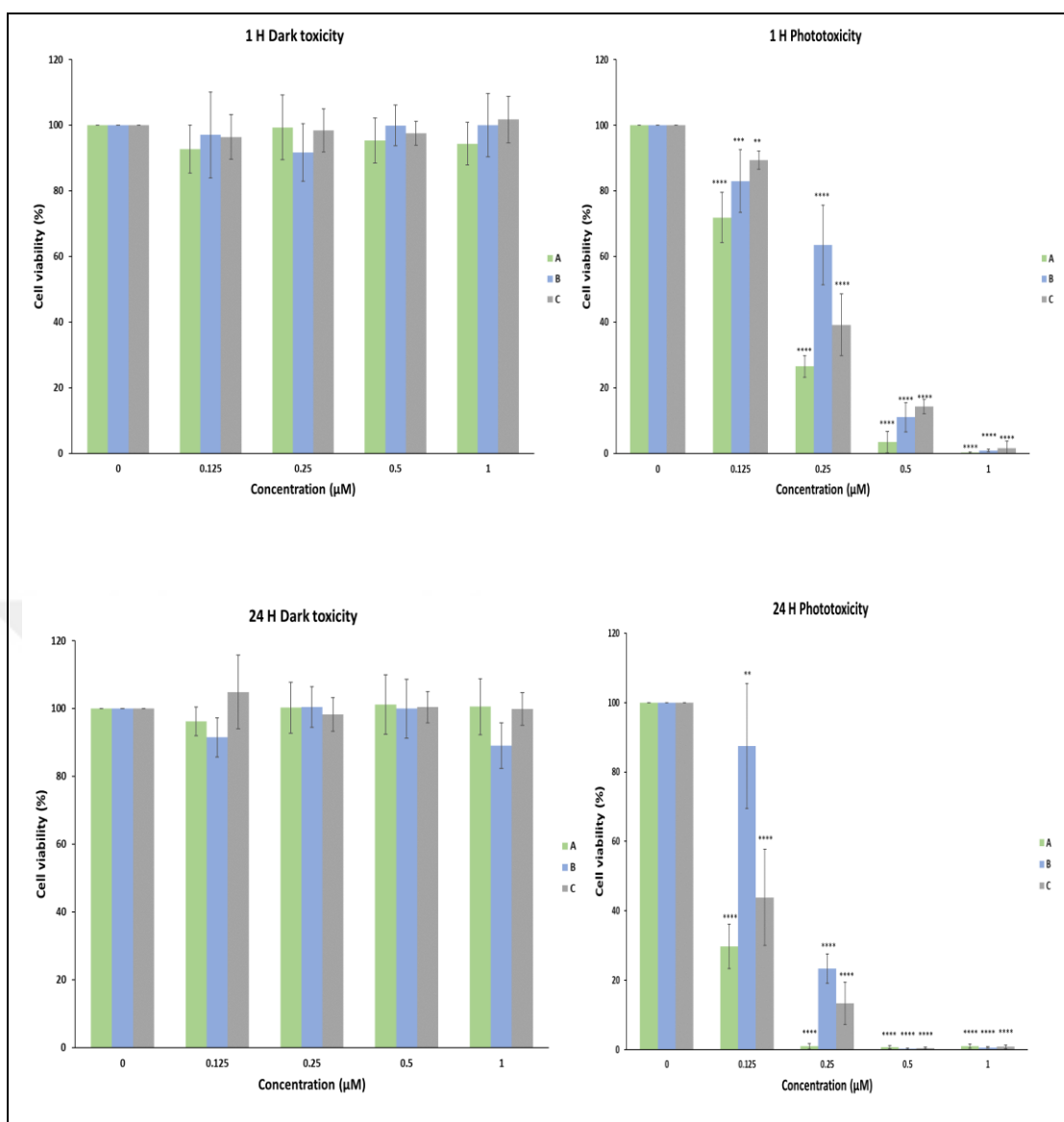


Figure 5.8: Dark and photocytotoxicity of ZnPc-4PEG incubated for 1 hour and 24 hour in different solvents in MDA-MB-231 cells. In DMSO (A), in water (B), and with PVP formulation in water (C). Light exposure is 3.6 J/cm^2 . Data are the mean $\pm$ SD of at least three independent experiments performed in triplicates. **p < 0.01, ***p < 0.005 and ****p < 0.001 compared to the control non-treated cells using two-way ANOVA with Dunnett's post-test.

Table 5.2: IC₅₀ and IC₉₀ values of phototoxicity (μM) for A, B, and C after 1 and 24 h of incubation with MDA-MB-231 cells.

Incubation time		A	B	C
IC ₅₀	1 h	0.17	0.28	0.23
	24 h	0.11	0.19	0.11
IC ₉₀	1 h	0.37	0.59	0.48
	24 h	0.16	0.31	0.28

For 1 hour incubation time point, the lowest IC₅₀ value was 0.17 μ M of A, and the highest IC₅₀ value was 0.28 μ M of B. For 24 hour incubation time point, the lowest IC₅₀ value was 0.11 μ M for both A and C; the highest IC₅₀ value was 0.19 μ M for B (Table 5.2). With increasing time, IC₅₀ values were decreased, and the compound could reach its maxima either in DMSO or water. But in general, it was seen that the phototoxicity results of DMSO were better than water or PVP formulation. Yet, the results of PVP formulation were also promising and can be used in further examinations. There was no dark toxicity of the compounds. And also, LED lamp caused no toxicity to the cells [Pehlivan et al., 2019].

After evaluating the solvent effect and PVP formulation, the cytotoxic effect of photodynamic therapy with the Set 1 compounds at different concentrations on MCF7 and MDA-MB-231 cell lines was investigated. DMSO was chosen as the solvent for the compounds. The compounds were incubated with the cells for 1 hour and 24 hour. Five different concentrations were studied as 0.0625, 0.125, 0.25, 0.5 and 1 μ M. Culture medium only with 0.05 % DMSO was used as control, 0 μ M. Four different light doses were used 1.8 J/cm², 3.6 J/cm², 7.2 J/cm², and 14.4 J/cm² by a handmade LED lamp, and 3, 6, 12, and 24 minutes took time for irradiation, respectively.

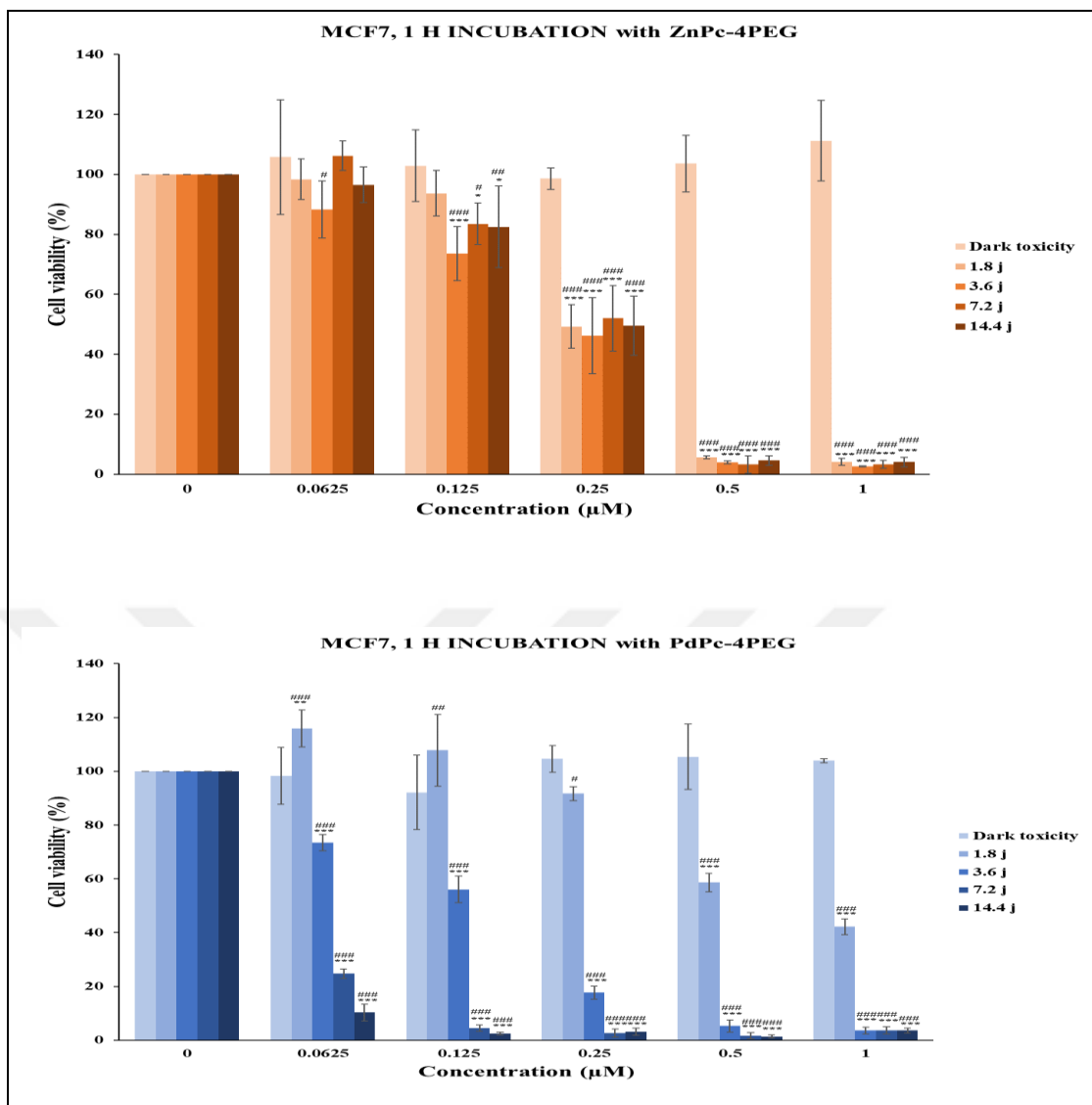


Figure 5.9: Dark and phototoxicity of Set 1 incubated for 1 hour with different concentrations in MCF7 cells. Data are the mean $\pm$ SD of at least three independent experiments performed in triplicates. * $P < 0.05$ compared to the control non-treated cells (0). # $P < 0.05$ compared to dark toxicity. ** $p < 0.01$, *** $p < 0.001$ compared to the control non-treated cells using two-way ANOVA with Dunnett's post-test.

Table 5.3: IC values of ZnPc-4PEG with different light doses on MCF7 cells; incubation time with compounds is 1 hour.

	1.8 J/cm ² ZnPc-4PEG	3.6 J/cm ² ZnPc-4PEG	7.2 J/cm ² ZnPc-4PEG	14.4 J/cm ² ZnPc-4PEG
IC50	0.2481	0.2066	0.2447	0.2354
IC90	0.4362	0.558	0.4807	0.5078
IC10	0.1411	0.07648	0.1246	0.1091

Table 5.4: IC values of PdPc-4PEG with different light doses on MCF7 cells; incubation time with compounds is 1 hour.

	1.8 J/cm² PdPc-4PEG	3.6 J/cm² PdPc- 4PEG	7.2 J/cm² PdPc- 4PEG	14.4 J/cm² PdPc- 4PEG
IC50	0.7468	0.1246	0.04015	0.01746
IC90		0.4084	0.09568	0.06394
IC10	0.2372	0.03802	0.01685	0.004766

IC values were calculated and evaluated after 1 hour of incubation with Set 1 on MCF7 cells (Table 5.3 and Table 5.4). A decrease in the values of PdPc-4PEG was observed, but there was not a noticeable decrease in the values of ZnPc-4PEG. The lowest IC₅₀ value belonged to PdPc-4PEG, which was 0.01746 μM at 14.4 J/cm². The highest IC₅₀ value also belonged to PdPc-4PEG as 0.7468 μM at 1.8 J/cm². When Tables 5.3 and 5.4 are compared, it was seen that the IC₅₀ value of ZnPc-4PEG is lower than PdPc-4PEG only at a light dose of 1.8 J/cm². As seen in Figure 5.9, PdPc-4PEG performs better even at lower concentrations. Increasing the light dose leads to more effectiveness of PdPc-4PEG than ZnPc-4PEG, especially at the lowest concentrations, 0.0625, 0.125, and 0.25 μM . No significant change was observed between compounds at 0.5 and 1 μM , except 1.8 J/cm². There was no significant dark toxicity of the compounds at any concentration. At two concentrations of PdPc-4PEG with 1.8 J/cm², 0.0625, and 0.125 μM , increasing cell proliferation was observed compared to the dark toxicity, which the stress may cause due to the activated phthalocyanine at a low light dose.

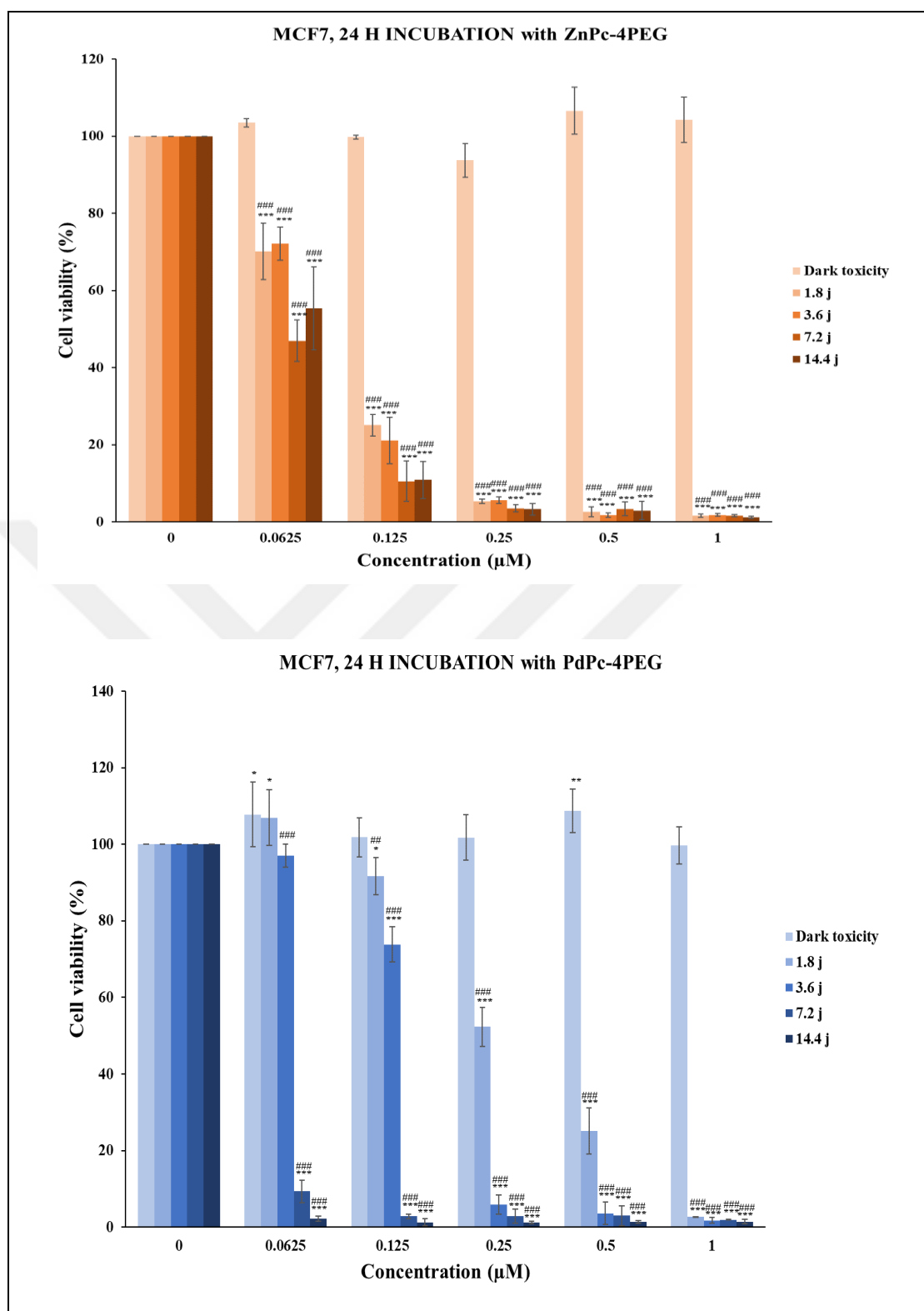


Figure 5.10: Dark and phototoxicity of Set 1 incubated for 24 hour with different concentrations in MCF7 cells. Data are the mean $\pm$ SD of at least three independent experiments performed in triplicates. * $P < 0.05$ compared to the control non-treated cells (0). # $P < 0.05$ compared to dark toxicity. ** $p < 0.01$, *** $p < 0.001$ compared to the control non-treated cells using two-way ANOVA with Dunnett's post-test.

Table 5.5: IC values of ZnPc-4PEG with different light doses on MCF7 cells; incubation time with compounds is 24 hour.

	1.8 J/cm ² ZnPc-4PEG	3.6 J/cm ² ZnPc-4PEG	7.2 J/cm ² ZnPc-4PEG	14.4 J/cm ² ZnPc-4PEG
IC50	0.08492	0.08389	0.0596	0.06669
IC90	0.1881	0.1678	0.1318	0.131
IC10	0.03833	0.04194	0.02695	0.03396

Table 5.6: IC values of PdPc-4PEG with different light doses on MCF7 cells; incubation time with compounds is 24 hour.

	1.8 J/cm ² PdPc-4PEG	3.6 J/cm ² PdPc-4PEG	7.2 J/cm ² PdPc-4PEG	14.4 J/cm ² PdPc-4PEG
IC50	0.2873	0.1514	0.01664	0.01244
IC90	0.7105	0.2279	0.06094	0.0324
IC10	0.1161	0.1005	0.004544	0.004774

IC values can be seen in Table 5.5 and Table 5.6 of 24 hour incubation with Set 1 on MCF7 cells, as they are much lower compared to the 1 hour incubation. The lowest IC₅₀, 0.01244 μ M, belonged to PdPc-4PEG at 14.4 J/cm². The highest IC₅₀, 0.2873 μ M, also belonged to PdPc-4PEG at 1.8 J/cm². The IC₅₀ value of ZnPc-4PEG is lower than PdPc-4PEG at light doses of 1.8 J/cm² and 3.6 J/cm². But with increasing light doses, PdPc-4PEG shows a much better performance. No significant change was observed between compounds at 0.5 and 1 μ M, except 1.8 J/cm² at 0.5 μ M of PdPc-4PEG. Dark toxicity was not observed with ZnPc-4PEG; at two concentrations of PdPc-4PEG, 0.0625 and 0.5 μ M, a slightly increasing cell proliferation was observed. Also, with 0.0625 μ M of PdPc-4PEG at 1.8 J/cm² light dose, there was an increase in cell proliferation (Figure 5.10).

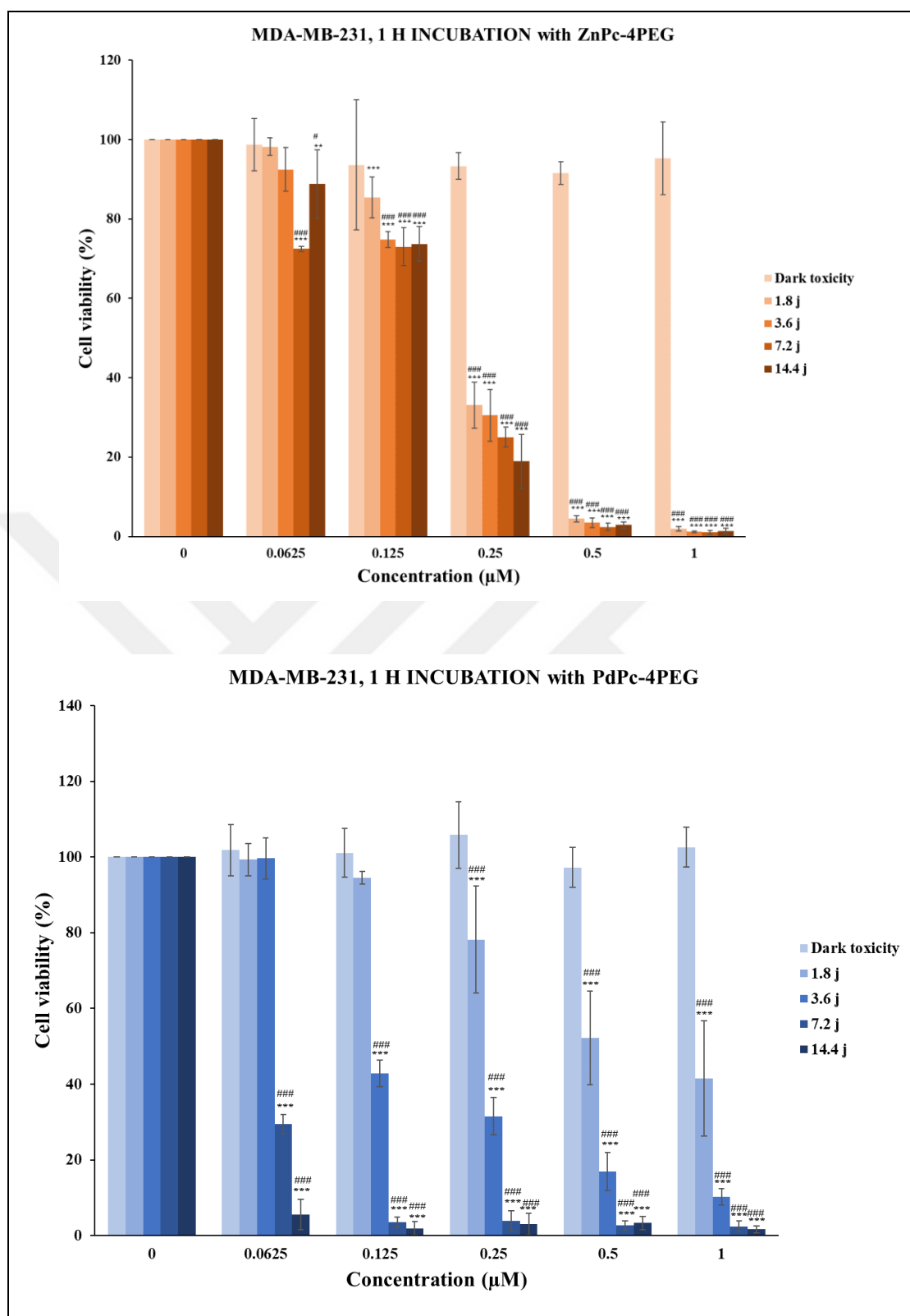


Figure 5.11: Dark and phototoxicity of Set 1 incubated for 1 hour with different concentrations in MDA-MB-231 cells. Data are the mean $\pm$ SD of at least three independent experiments performed in triplicates. * $P < 0.05$ compared to the control non-treated cells (0). # $P < 0.05$ compared to dark toxicity. ** $p < 0.01$, *** $p < 0.001$ compared to the control non-treated cells using two-way ANOVA with Dunnett's post-test.

Table 5.7: IC values of ZnPc-4PEG with different light doses on MDA-MB-231 cells; incubation time with compounds is 1 hour.

	1.8 J/cm² ZnPc-4PEG	3.6 J/cm² ZnPc-4PEG	7.2 J/cm² ZnPc-4PEG	14.4 J/cm² ZnPc-4PEG
IC50	0.2052	0.1833	0.1561	0.165
IC90	0.3826	0.41	0.4333	0.3266
IC10	0.1101	0.08196	0.05624	0.08333

Table 5.8: IC values of PdPc-4PEG with different light doses on MDA-MB-231 cells; incubation time with compounds is 1 hour.

	1.8 J/cm² PdPc-4PEG	3.6 J/cm² PdPc-4PEG	7.2 J/cm² PdPc-4PEG	14.4 J/cm² PdPc-4PEG
IC50	0.6589	0.1504	0.04764	0.01435
IC90		0.5066	0.09416	0.04671
IC10	0.1266	0.04466	0.0241	0.004405

After 1 hour of incubation with Set 1 on MDA-MB-231 cells, it was seen that the IC₅₀ value of ZnPc-4PEG is lower than PdPc-4PEG only at a light dose of 1.8 J/cm² (Table 5.7 and Table 5.8). But at the other light doses, PdPc-4PEG shows a better performance, especially at 7.2 and 14.4 J/cm². PdPc-4PEG had the lowest IC₅₀ value of 0.01435 μ M at 14.4 J/cm² and the highest IC₅₀ value of 0.6589 μ M at 1.8 J/cm². Again, at lower concentrations, PdPc-4PEG causes significantly lower cell viability. As seen in Figure 5.11, incubation with PdPc-4PEG for 1 hour shows more effectiveness starting from 0.0625 μ M at 7.2 J/cm². There was no dark toxicity at any range of concentration of the compounds.

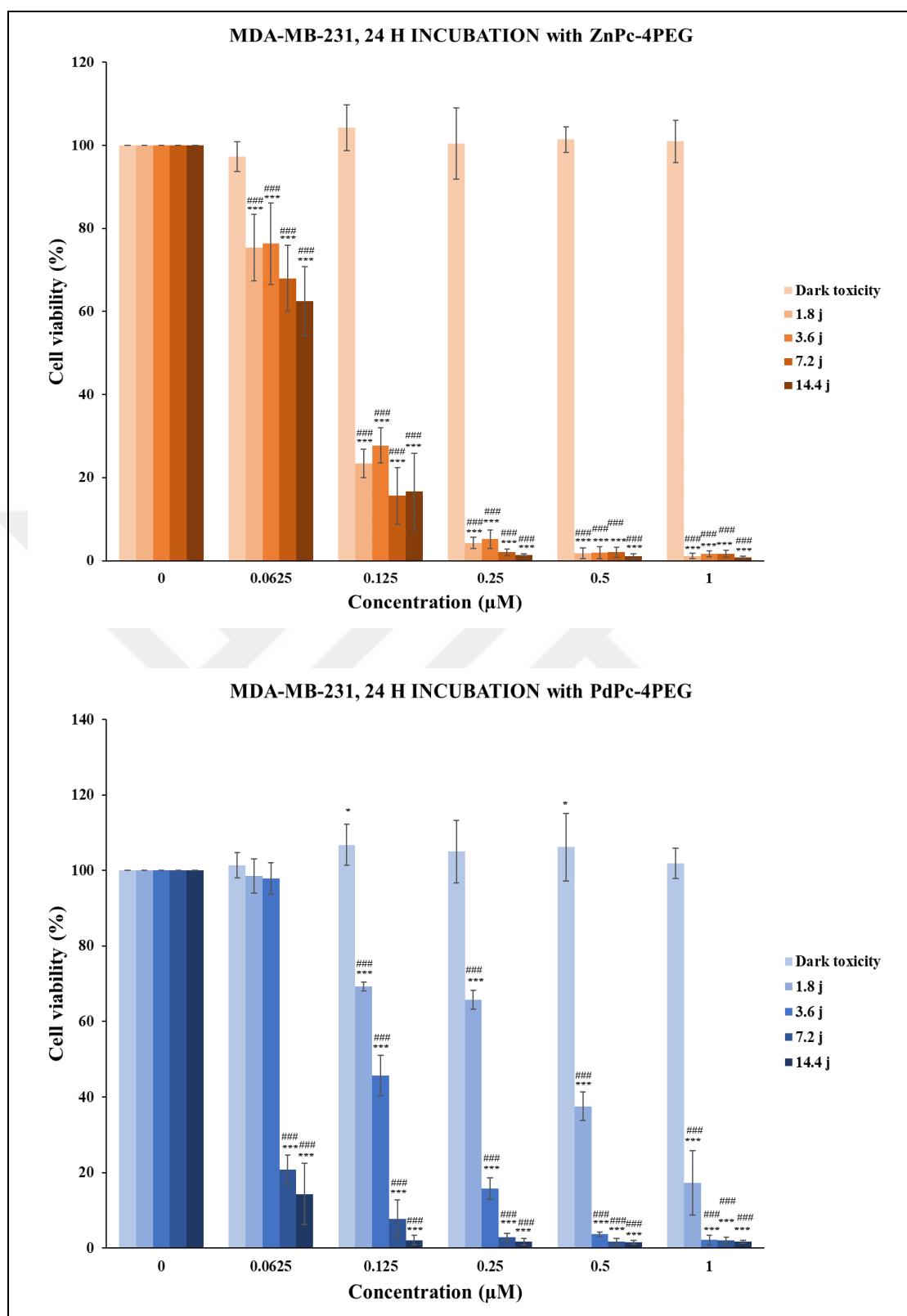


Figure 5.12: Dark and phototoxicity of Set 1 incubated for 24 hour with different concentrations in MDA-MB-231 cells. Data are the mean $\pm$ SD of at least three independent experiments performed in triplicates. * $P < 0.05$ compared to the control non-treated cells (0). # $P < 0.05$ compared to dark toxicity. ** $p < 0.01$, *** $p < 0.001$ compared to the control non-treated cells using two-way ANOVA with Dunnett's post-test.

Table 5.9: IC values of ZnPc-4PEG with different light doses on MDA-MB-231 cells; incubation time with compounds is 24 hour.

	1.8 J/cm ² ZnPc-4PEG	3.6 J/cm ² ZnPc-4PEG	7.2 J/cm ² ZnPc-4PEG	14.4 J/cm ² ZnPc-4PEG
IC50	0.08778	0.0917	0.07747	0.07384
IC90	0.172	0.1899	0.1452	0.1509
IC10	0.0448	0.04427	0.04132	0.03613

Table 5.10: IC values of PdPc-4PEG with different light doses on MDA-MB-231 cells; incubation time with compounds is 24 hour.

	1.8 J/cm ² PdPc-4PEG	3.6 J/cm ² PdPc-4PEG	7.2 J/cm ² PdPc-4PEG	14.4 J/cm ² PdPc-4PEG
IC50	0.3427	0.1253	0.02654	0.03141
IC90		0.2548	0.1077	0.0728
IC10	0.07168	0.06164	0.00654	0.01356

For 24 hour incubation with Set 1 compounds on MDA-MB-231 cells, the IC50 value of ZnPc-4PEG is lower than PdPc-4PEG only at the dose of 1.8 and 3.6 J/cm². But at the higher light doses, 7.2 and 14.4 J/cm², PdPc-4PEG shows a better performance. Again, at lower concentrations, PdPc-4PEG causes significantly lower cell viability. At 0.25, 0.5, and 1 µM concentration, there is no significantly high difference between compounds except 1.8 J/cm². The lowest and the highest IC50 values belong to PdPc-4PEG as 0.02654 at 7.2 J/cm² and 0.3427 µM at 1.8 J/cm², respectively (Table 5.9 and 5.10). No dark toxicity was observed with compounds, but slightly higher cell viability was observed at 0.125 and 0.5 µM concentrations of PdPc-4PEG. Those concentrations might cause stress on the cells, which leads to cell accumulation (Figure 5.12).

5.1.3. Cellular Uptake of Set 1

PDT is based on the idea that cancer cells take up and accumulate PS before it is exposed to light treatment. The rising fluorescence intensity of the emission spectra was found to be a valuable indicator of accumulation over time. Uptake experiments were done with MCF7 and MDA-MB-231 cell lines with Set 1 compounds at 1 µM

for 1 hour and 24 hour incubation times (Figure 5.13). Cells were lysed, and their drug intake was determined by fluorescence measurements. The calibration curve of each compound was used to draw the graphs and for statistical analysis. A new calibration curve was drawn in each experimental trial. Sample calibration curves of compounds in DMSO are in the appendices (Appendix A).

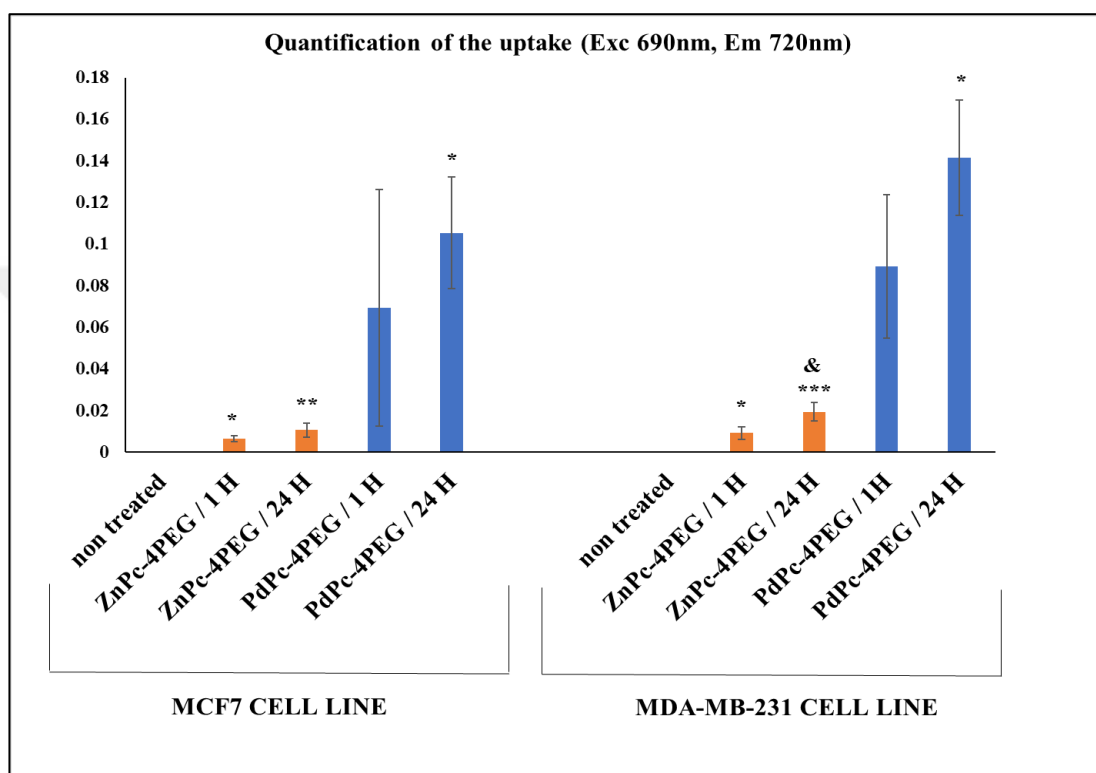


Figure 5.13: Cell uptake experiment of Set 1 at 1 μ M, 1 hour and 24 hour of incubation with MCF7 and MDA-MB-231 cells. *P < 0.05 compared to the control non-treated cells. &P < 0.05 comparison between 1 hour and 24 hour cell uptake of each compound inbetween. ***p < 0.001, *p < 0.05 compared to the control non-treated cells using one-way ANOVA with Dunnett's post-test. P values < 0.05 were considered to be statistically significant.

Cell uptake of the compounds was evaluated against non-treated cells, which are only treated with the appropriate amount of DMSO and between 1 hour and 24 hour uptake capacity of each compound. At the 1 hour time point, uptake of PdPc-4PEG was not significant on both of the cell lines. It is probably due to the weak fluorescent signal of PdPc-4PEG; it may inhibit the detection of the compound. However, uptake of ZnPc-4PEG was significant at this time point on both of the cell lines, at 0.0064 a.u. on MCF7 and 0.0092 a.u. for MDA-MB-231 cells. For 24 hour time point on MCF7

cells, ZnPc-4PEG has 0.0107 a.u. and PdPc-4PEG has 0.1054 a.u.; on MDA-MB-231 cells, ZnPc-4PEG has 0.0194 a.u. and PdPc-4PEG has 0.1416 a.u. With increasing incubation time, the accumulation of ZnPc-4PEG was observed. It was seen that in both cell lines, PdPc-4PEG accumulated more in the cell than ZnPc-4PEG at the 24 H time point. 1 H and 24 H in between comparisons were only significant in MDA-MB-231 cells with ZnPc-4PEG, and it was seen that with increasing time, the uptake of the compound is also increasing.

Comparison of uptakes of the compounds with each other was also evaluated by Student's t-test (not shown in Figure 5.13). On MDA-MB-231 cell line, PdPc-4PEG and ZnPc-4PEG uptake capacities at 1 and 24 hours were statistically significant to each other. However, on MCF7 cells, the comparison between the two compounds was significant only at 24 hour time point, not at 1 hour time point.

Since the characteristics of the cell lines are different, it is expected that the uptake capacity would be different for the cells. On MCF7 cell line, the highest uptake was 0.1054 a.u. which belonged to PdPc-4PEG at 24 hour time point, and on MDA-MB-231 cell line, the highest uptake was 0.1416 a.u., which again belonged to PdPc-4PEG. A similar pattern related to the uptake capacity of the cells was observed at all time points with both of the compounds. MCF7 cell line is not very aggressive; estrogen and progesterone receptors positive, HER2 negative. MDA-MB-231 are triple negative, metastatic, and more aggressive relative to MCF7 [Holliday and Speirs, 2011]. Two intracellular uptake mechanisms were mainly reported as endocytosis/phagocytosis and nanopenetration [Moor, 200], [Sahoo et al., 2011], [Jokar et al., 2019]. When the values were compared, it can be said that the uptakes of the compounds were higher in MDA-MB-231 cells. As mentioned before, this cell line is metastatic, and the obtained cell uptake results are compatible with the literature [Saulite et al., 2018], [Jokar et al., 2019].

5.1.4. Nuclear Morphology Analysis of Set 1

In relation to photodynamic treatment, cells show altered morphology in the cell cytoplasm, membrane, or nuclei. By nuclear morphology analysis, early apoptotic, necrotic cells, and late apoptotic cells can be observed [Moor, 2000], [Prasad et al., 2017]. For the first trial, 0.5 μ M ZnPc-4PEG was used against MDA-MB-231 cells at 1 hour and 24 hour incubation time points. Confocal pictures can be seen in Figure

5.14. This concentration was chosen due to the MTT results; while it was the first concentration tried, the most cell death was seen. Control cells were treated only with the appropriate amount of DMSO containing media. After PDT treatment with 3.6 J/cm^2 , all three nuclear morphologic changes, early apoptotic, necrotic cells, and late apoptotic nuclear cells, were observed in cells at this concentration compared to the normal nuclei of non-treated control cells. Fragmented or highly condensed nuclei were observed in early apoptotic cells, stained as blue. Necrotic cells displayed normal nuclei, but the staining method revealed their status by staining them pink. Finally, late apoptotic cells also have highly fragmented or fragmented chromatin, but they were stained pink. According to the MTT results, $1 \mu\text{M}$ of the compounds of Set 1 was the concentration that was the most effective in both cell lines. So, Set 1 compounds were administered at $1 \mu\text{M}$ concentration to MCF7 and MDA-MB-231 cells at 1 hour and 24 hour incubation time points. A light dose of 14.4 J/cm^2 was used as PDT treatment. Control cells were treated only with 0.05% DMSO. Only late apoptotic cells were observed in both cell lines. Confocal pictures can be seen in Figure 5.15. In untreated controls, nuclear morphology was not affected by light or DMSO alone (data not shown).

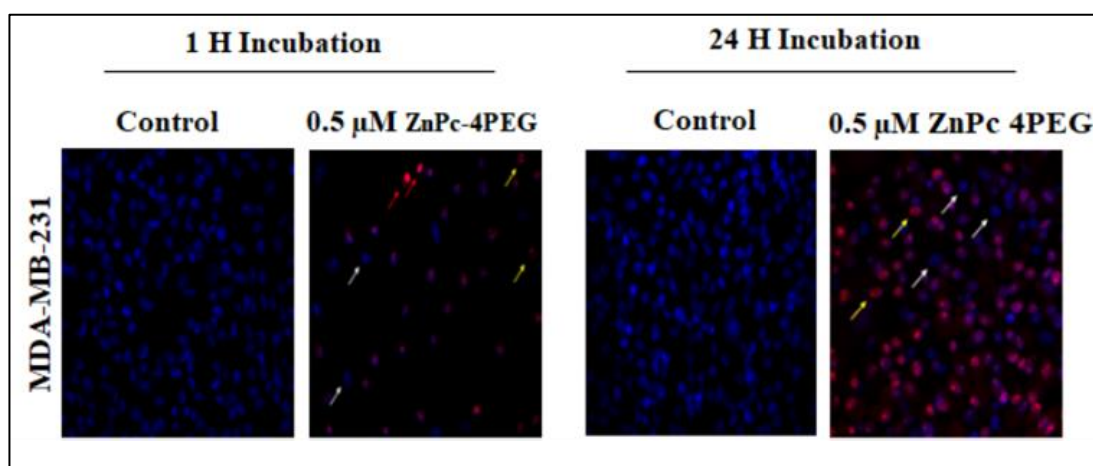


Figure 5.14: Nuclear morphology analysis of ZnPc-4PEG at $0.5 \mu\text{M}$ after 1 hour and 24 hour incubation on MDA-MB-231 cells. Light dose was 3.6 J/cm^2 . Green arrows show cells with normal nuclei. White arrows show early apoptotic cells with highly condensed or fragmented blue nuclei. Red arrows show necrotic cells with normal nuclei with bright pink chromatin. And yellow arrows show late apoptotic cells with highly condensed or fragmented bright pink chromatin.

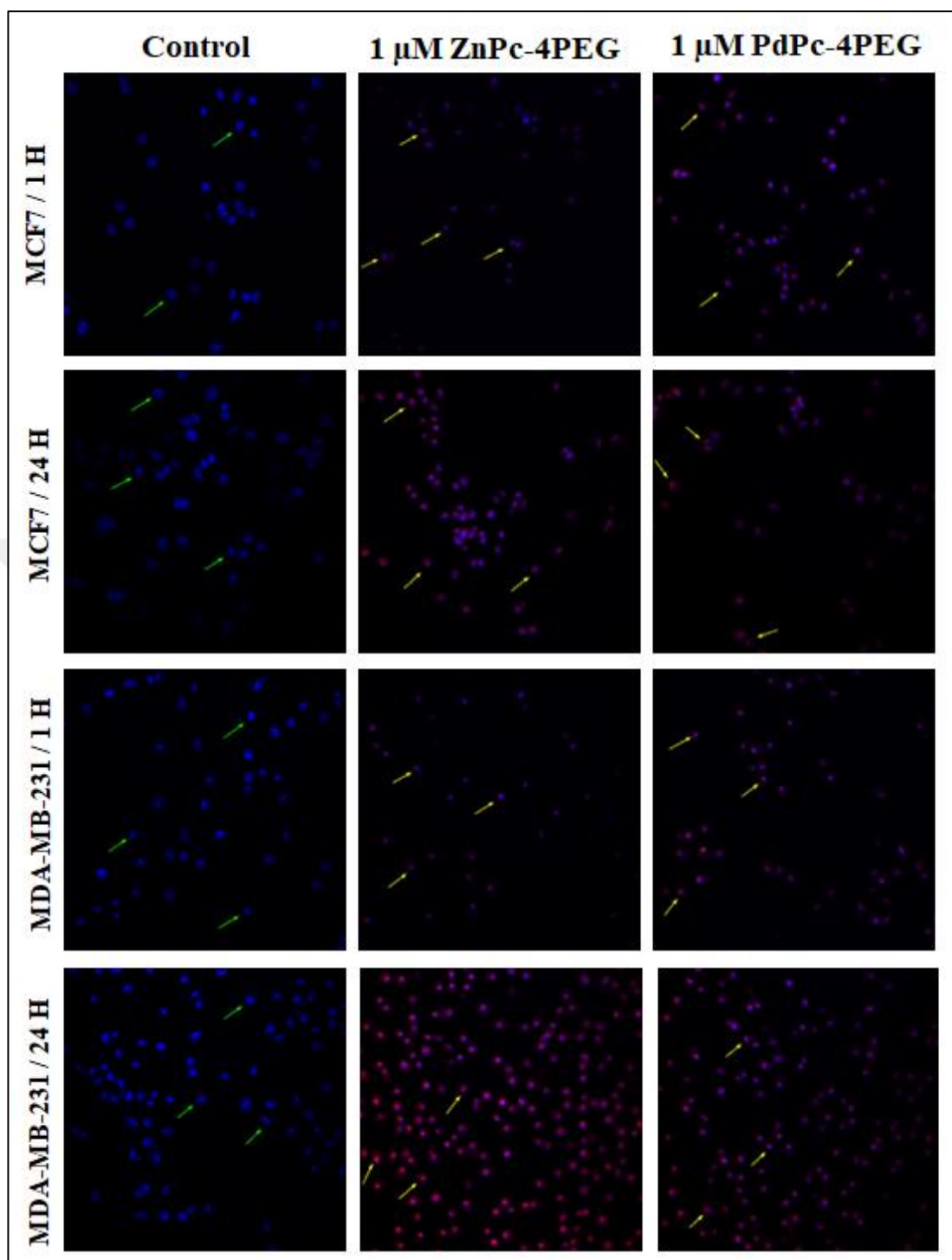


Figure 5.15: Nuclear morphology analysis of Set 1 at 1 μ M after 1 hour and 24 hour incubation on MCF7 and MDA-MB-231 cells. Green arrows show cells with normal nuclei. Yellow arrows show late apoptotic cells with highly condensed or fragmented bright pink chromatin.

5.1.5. Wound Healing Assay of Set 1

Photodynamic therapy causes morphologic changes, including swollen membrane, irregular cytoplasm, blebbing, chromatin condensation, and nucleus fragmentation [Savill and Fadok, 2000]. The drug used will determine the sensitivity levels of MCF7 and MDA-MB-231 cell lines. MDA-MB-231 cells are metastatic. Therefore, cell migration and invasion experiments performed *in vitro* are excellent ways to measure the level of activity produced by these cells. Because MCF7 cells do not have the ability to metastasize, they are suitable for use as a negative control in *in vitro* cell migration and invasion tests [Holliday and Speirs, 2011], [Dos Santos et al., 2017]. Wound healing assay can be used to investigate these morphologic changes as well as the migration capability of the cells. In accordance with the nuclear morphology assay, 1 μM of the compounds of Set 1 was used as the concentration, which was the most effective in both cell lines (Figure 5.16, Figure 5.17, Figure 5.18, and Figure 5.19). So, Set 1 compounds were administered at 1 μM concentration to MCF7 and MDA-MB-231 cells at 1 hour and 24 hour incubation time points. A light dose of 14.4 J/cm^2 was used as PDT treatment. After PDT treatment, it was seen that there was no cell migration with treated cells which is affirmative to the MTT assay. However, in the controls, cell migration was observed. Differences in cell morphologies between control cells and the treated cells were significant. Treated cells showed the characteristic features of apoptosis with membrane blebbing, cell shrinkage, swollen membranes, or irregular cytoplasm. Recovery was faster in control cells of MDA-MB-231 cell line as it is more aggressive than MCF7.

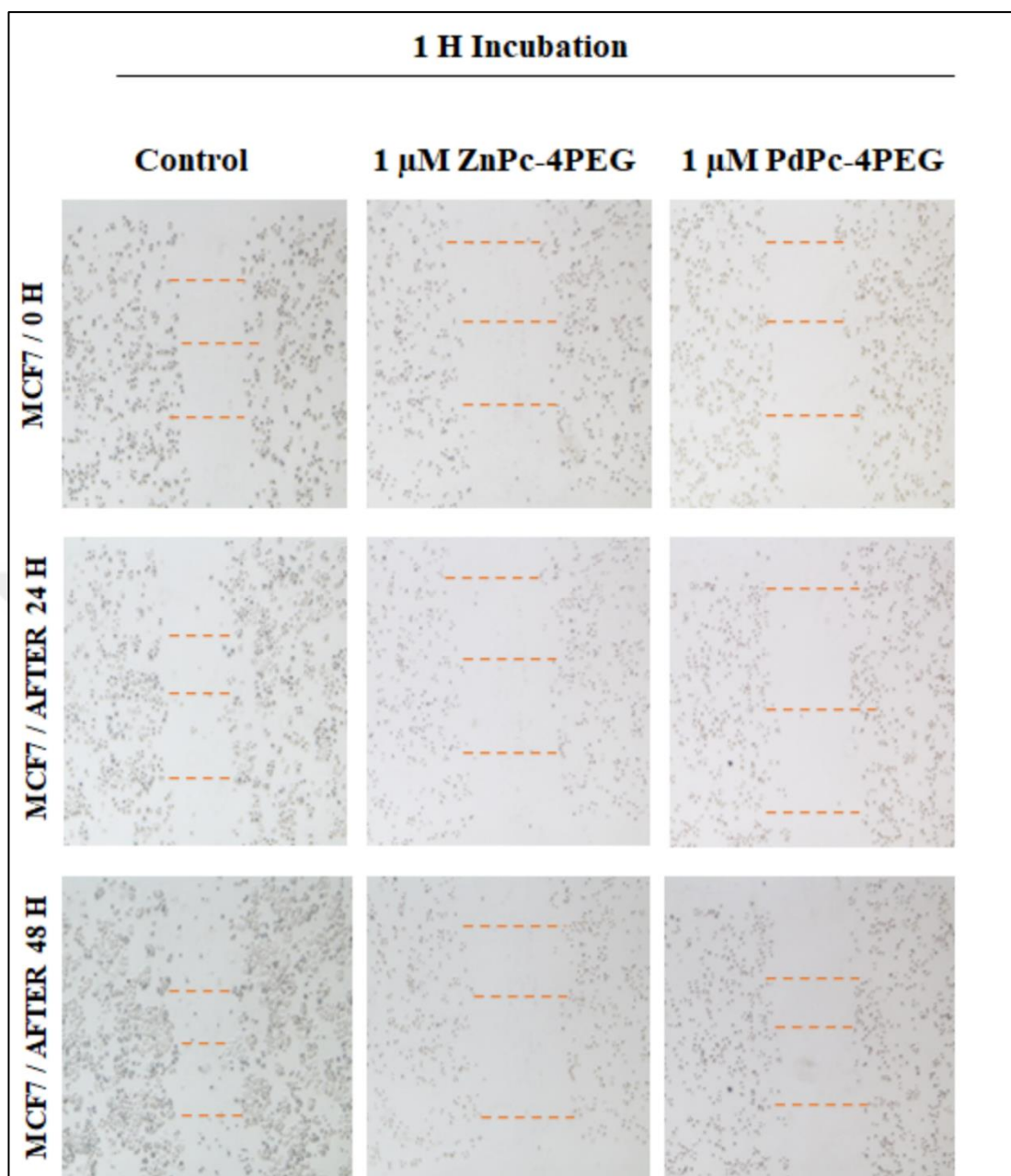


Figure 5.16: Wound healing assay of Set 1 at 1 μ M after 1 hour incubation on MCF7 cells. Pictures were taken directly after treatment (0 H), 24 hours later, and 48 hours later.

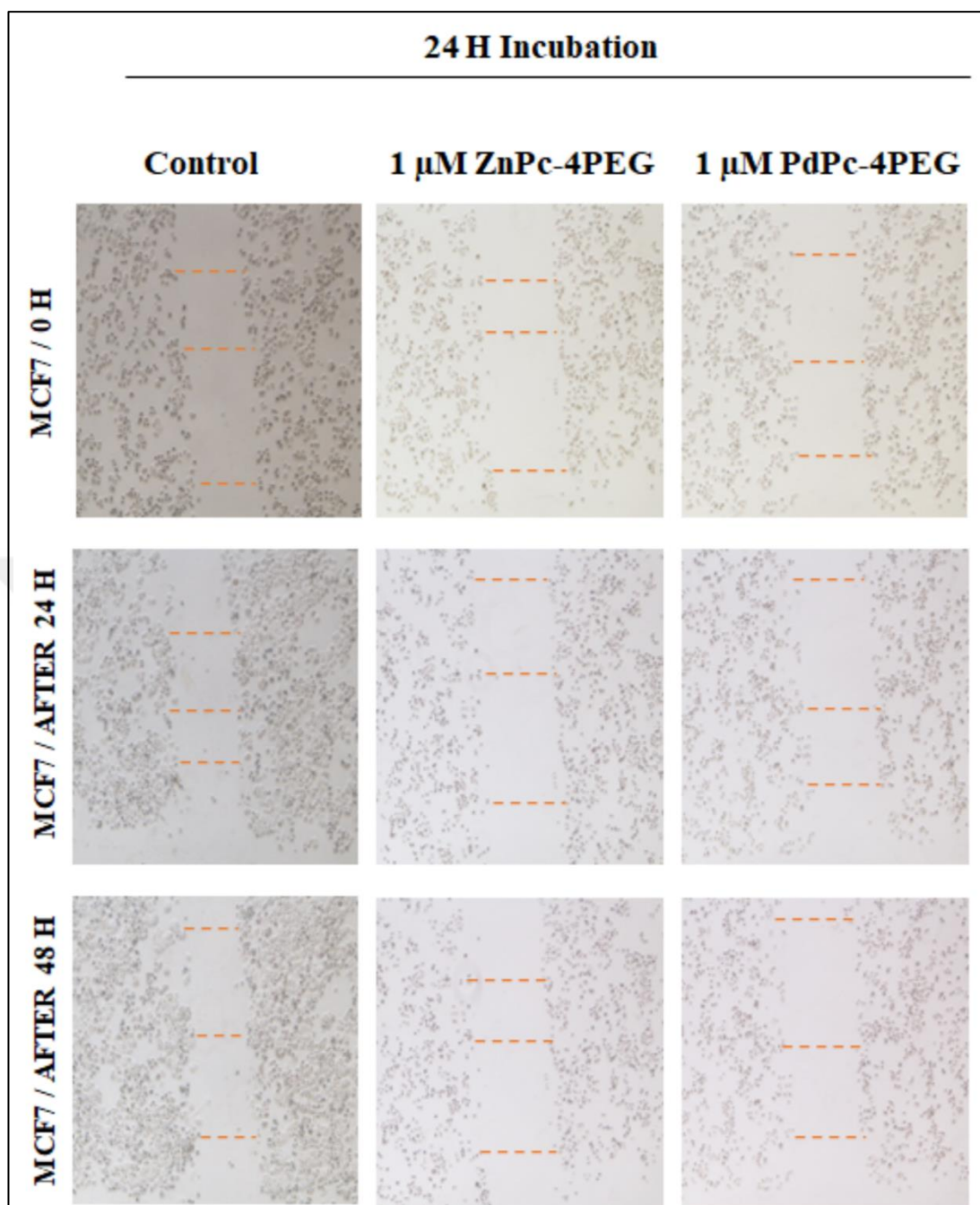


Figure 5.17: Wound healing assay of Set 1 at 1 μ M after 24 hour incubation on MCF7 cells. Pictures were taken directly after treatment (0 H), 24 hours later, and 48 hours later.

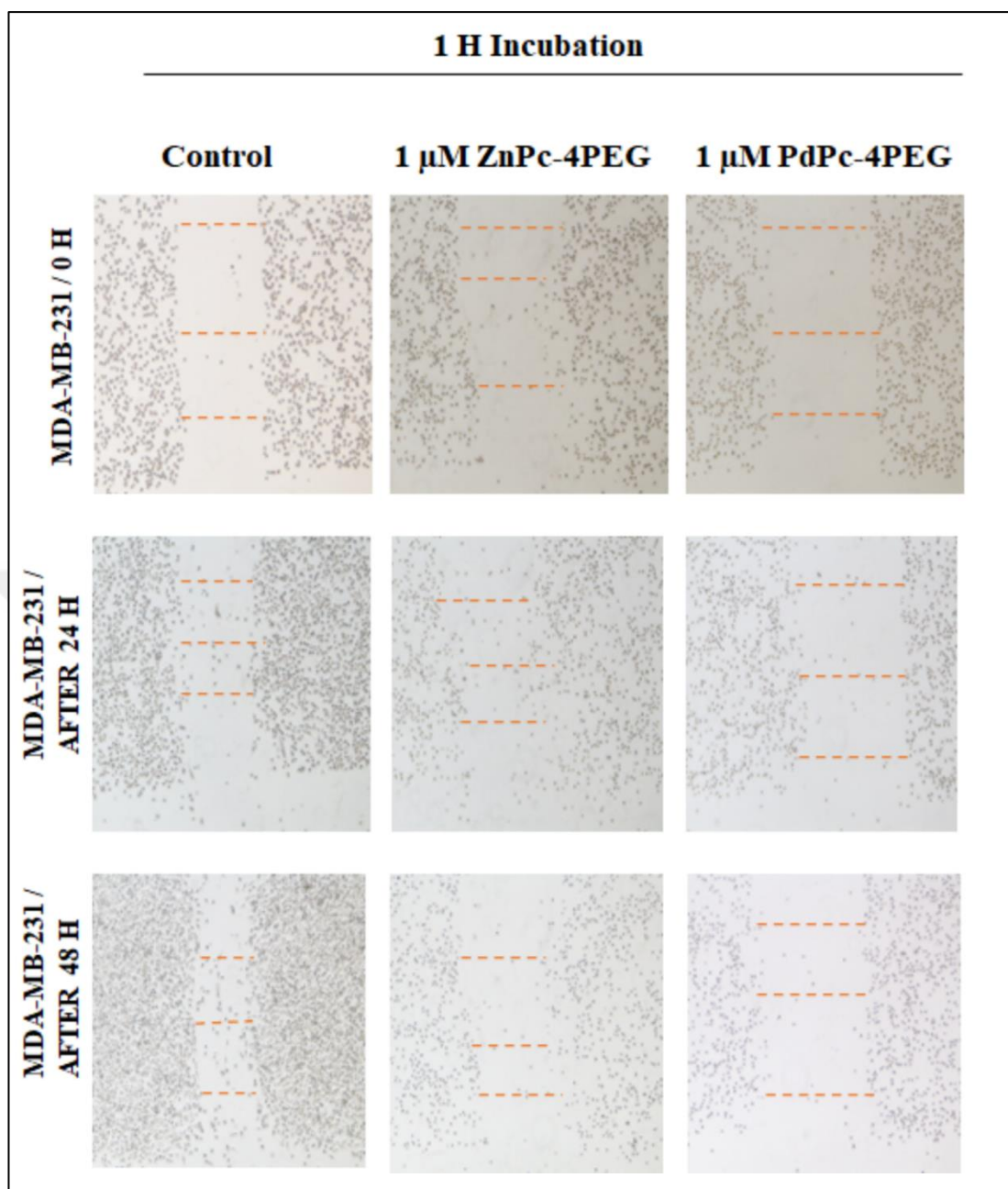


Figure 5.18: Wound healing assay of Set 1 at 1 μ M after 1 hour incubation on MDA-MB-231 cells. Pictures were taken directly after treatment (0 H), 24 hours later, and 48 hours later.

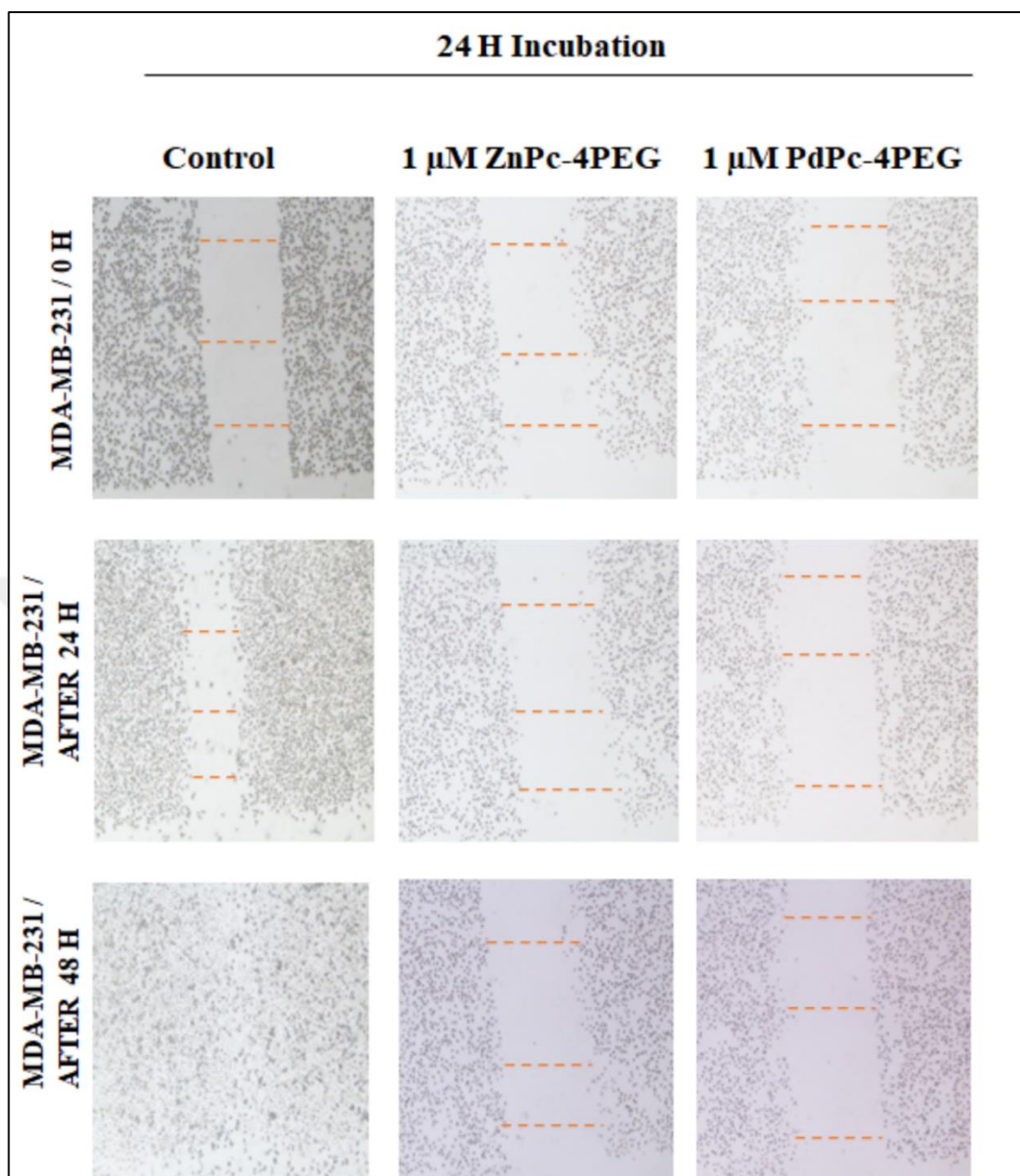


Figure 5.19: Wound healing assay of Set 1 at 1 μ M after 24 hour incubation on MDA-MB-231 cells. Pictures were taken directly after treatment (0 H), 24 hours later, and 48 hours later.

5.1.6. Colony Formation Assay of Set 1

There are two options for performing colony formation assay. In the first one, cells are plated first and then treated subsequently. The treatment must be performed before cells start proliferating, which misleads the colony formation, yielding more colonies. This option is used for quick screening of the sensitivity of cells to different treatments. In the second option, at first, cells are treated, then harvested and plated

again. This option is used for the determination of potentially lethal- and sublethal damage repair [Franken et al., 2006]. In this study, the second option was chosen to perform a colony formation assay. In accordance with the nuclear morphology assay, 1 μM of the compounds of Set 1 was administered to MCF7 and MDA-MB-231 cell lines for 1 hour and 24 hour incubation (Figure 5.20 and Figure 5.21). Only 0.05% DMSO applied cells were used as non-treated control. A light dose of 14.4 J/cm^2 was used as PDT treatment. After the treatment, there were no colonies of the treated cells with Set 1 compounds after 10 days of incubation. Instead, colonies were observed with non-treated control cells. There were more colonies with MDA-MB-231 cells than MCF7 cells, as the first one is a more aggressive breast cancer cell line.

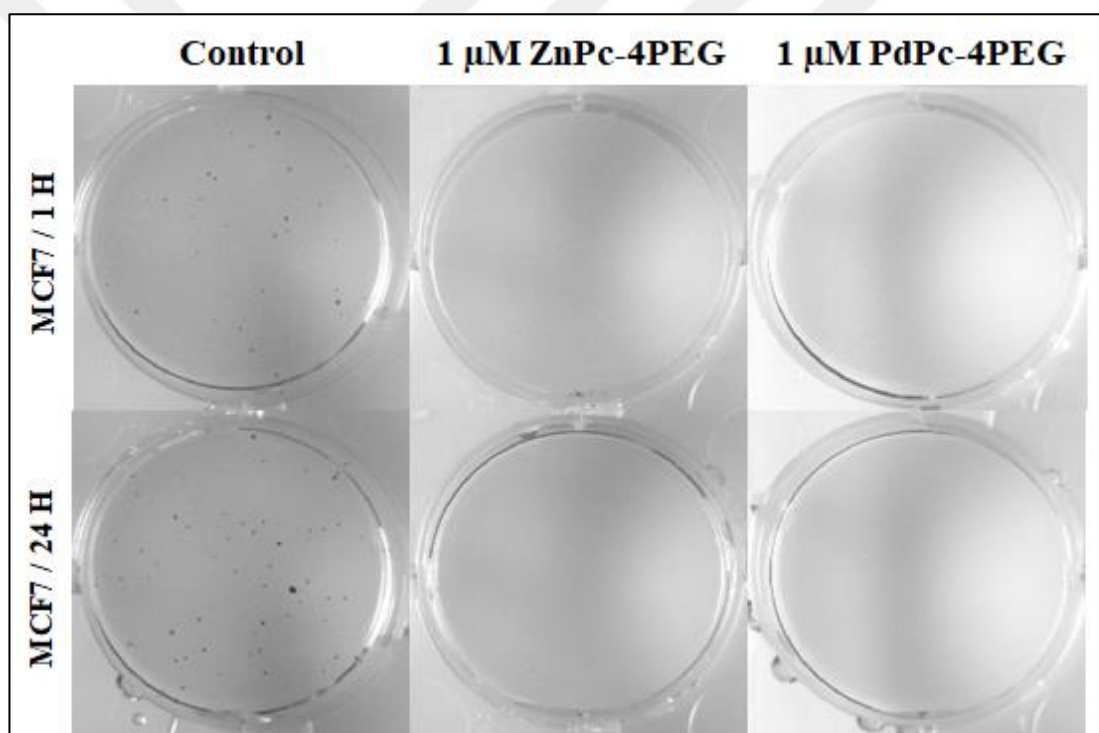


Figure 5.20: Colony formation assay of Set 1 at 1 μM after 1 hour and 24 hour incubation on MCF7 cells.

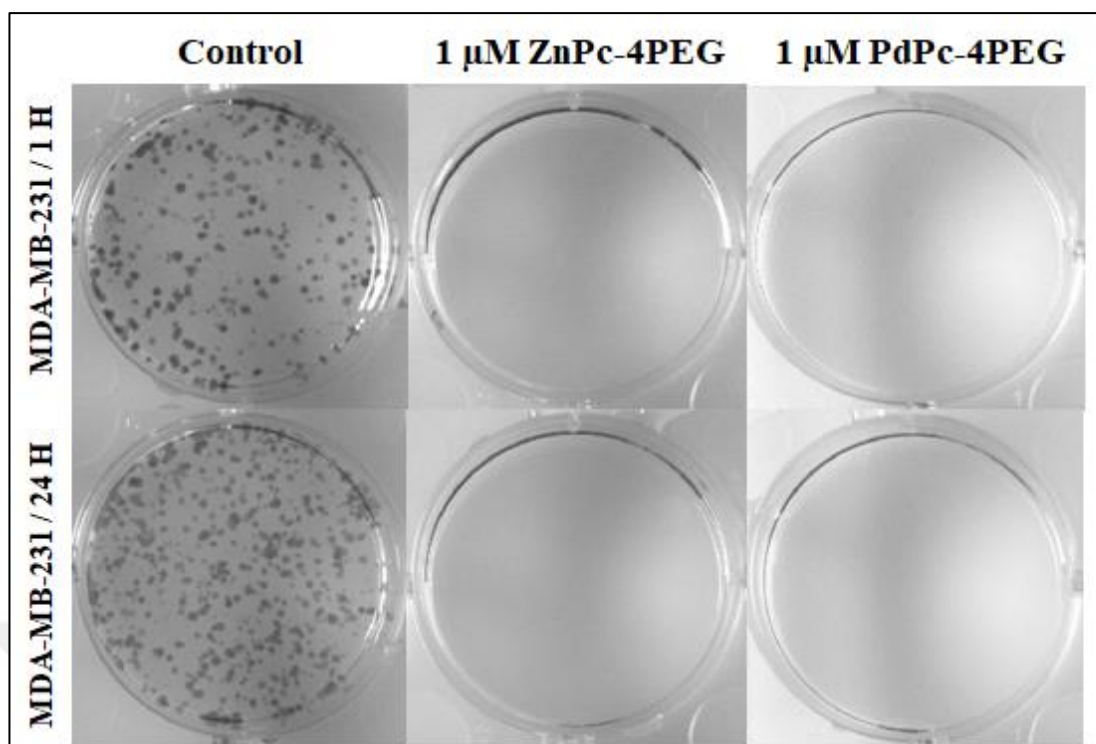


Figure 5.21: Colony formation assay of Set 1 at 1 μ M after 1 hour and 24 hour incubation on MDA-MB-231 cells.

5.2. Effect of Substitution Bulkiness

In this part of the study, Set 2 phthalocyanines, ZnPc-tBu and ZnPc-nBu, were examined to determine the effect of substitution bulkiness. ZnPc-tBu was the bulky phthalocyanine, and ZnPc-nBu was the non-bulky phthalocyanine. The molecular weight of both of the phthalocyanines was 930.588. The geometric structures of the phthalocyanines are in Figure 5.22. In this part of the study, firstly, Set 2 phthalocyanines were solved in DMSO, and their stock solutions for the conducted *in vitro* assays were prepared in DMSO. Then, Set 2 was formulated with PVP, dissolved in water, and their stock solutions for the examined *in vitro* assays were prepared in water.

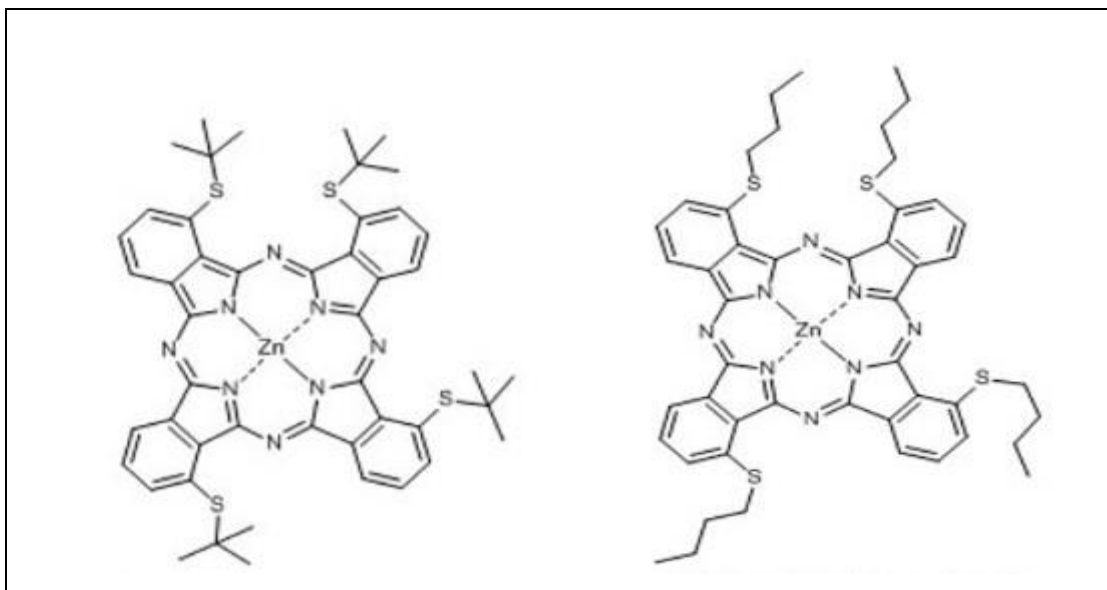


Figure 5.22: Geometric structures of ZnPc-tBu (bulky Pc), and ZnPc-nBu (non-bulky Pc), respectively.

5.2.1. Uv-vis Absorption of Set 2

As mentioned in the first part of the study, UV-vis absorption spectra of the compounds were recorded to reveal specific features such as absorption wavelengths, absorption degree at each wavelength, solubilization capacity, and maximum absorption wavelength. ZnPc-tBu and ZnPc-nBu were dissolved in DMSO. 1, 2, 4, 6, 8, and 10 μM of each compound were prepared in DMSO. Their UV-vis absorption spectra (Figure 5.23) were measured, and $\log \epsilon$ values were calculated. The maximum Q bands of ZnPc-tBu and ZnPc-nBu were 692 nm and 717 nm, respectively. Corresponding values of $\log \epsilon$ were also calculated for ZnPc-tBu was 5.18 and for ZnPc-nBu was 5.3. No aggregation of the compounds was observed.

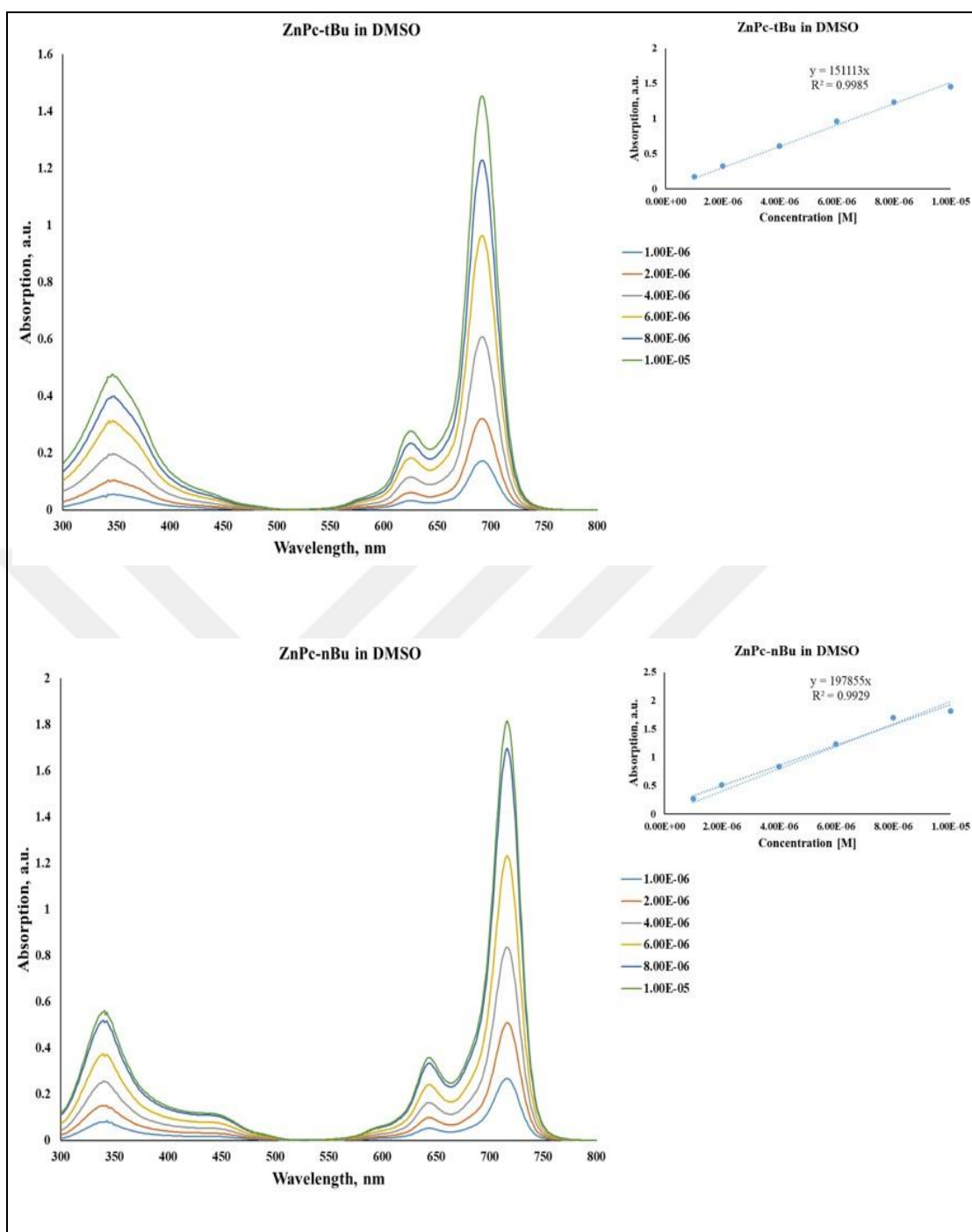


Figure 5.23: Absorption spectra of Set 2 in DMSO.

To assess the absorption degree of compounds, Uv-vis spectra of 10 μ M of the compounds in DMSO were also compared. It was seen that the absorption degree of ZnPc-tBu was lower than ZtPc-nBu (Figure 5.24).

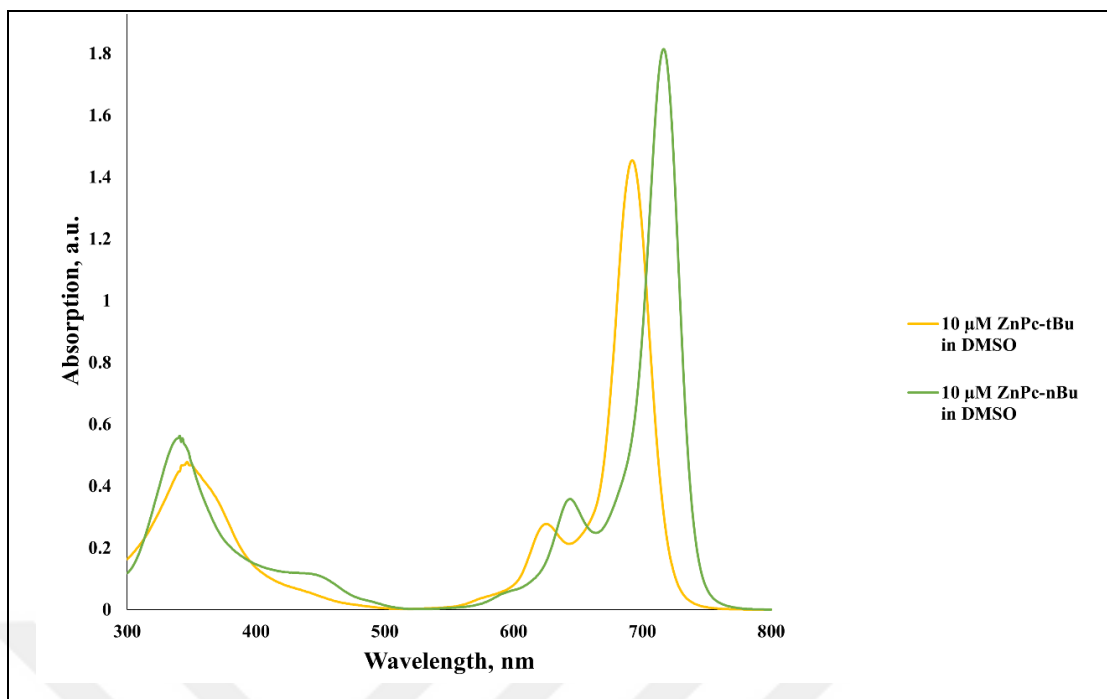


Figure 5.24: Comparison of UV-vis Absorption of Set 2 in DMSO at 10 μ M.

Then, Set 2 was also tried to be dissolved with water, but it aggregated and did not solve. Due to high aggregation, PVP formulation was performed for Set 2 to achieve dissolving them. The formulation in different PVP ratios was examined. 1 μ mol phthalocyanine was formulated with 100, 150, 200, 250, and 300 mg PVP. Formulated compounds were dissolved in water, diluted to 10 μ M, and their UV-vis spectra were recorded (Figure 5.25). The maximum Q bands and corresponding values of $\log \epsilon$ for each formulated compound can be seen in Table 5.11 and Table 5.12. It was seen that formulation of ZnPc-nBu has highly decreased the absorption wavelength of the compound. According to the UV-vis spectra of each phthalocyanine, the 1:100 ratio was chosen for further examination. UV-vis spectrum of only PVP (4,843.75 mg/ml PVP) in water was also recorded; no absorption of PVP itself was observed (Appendix B).

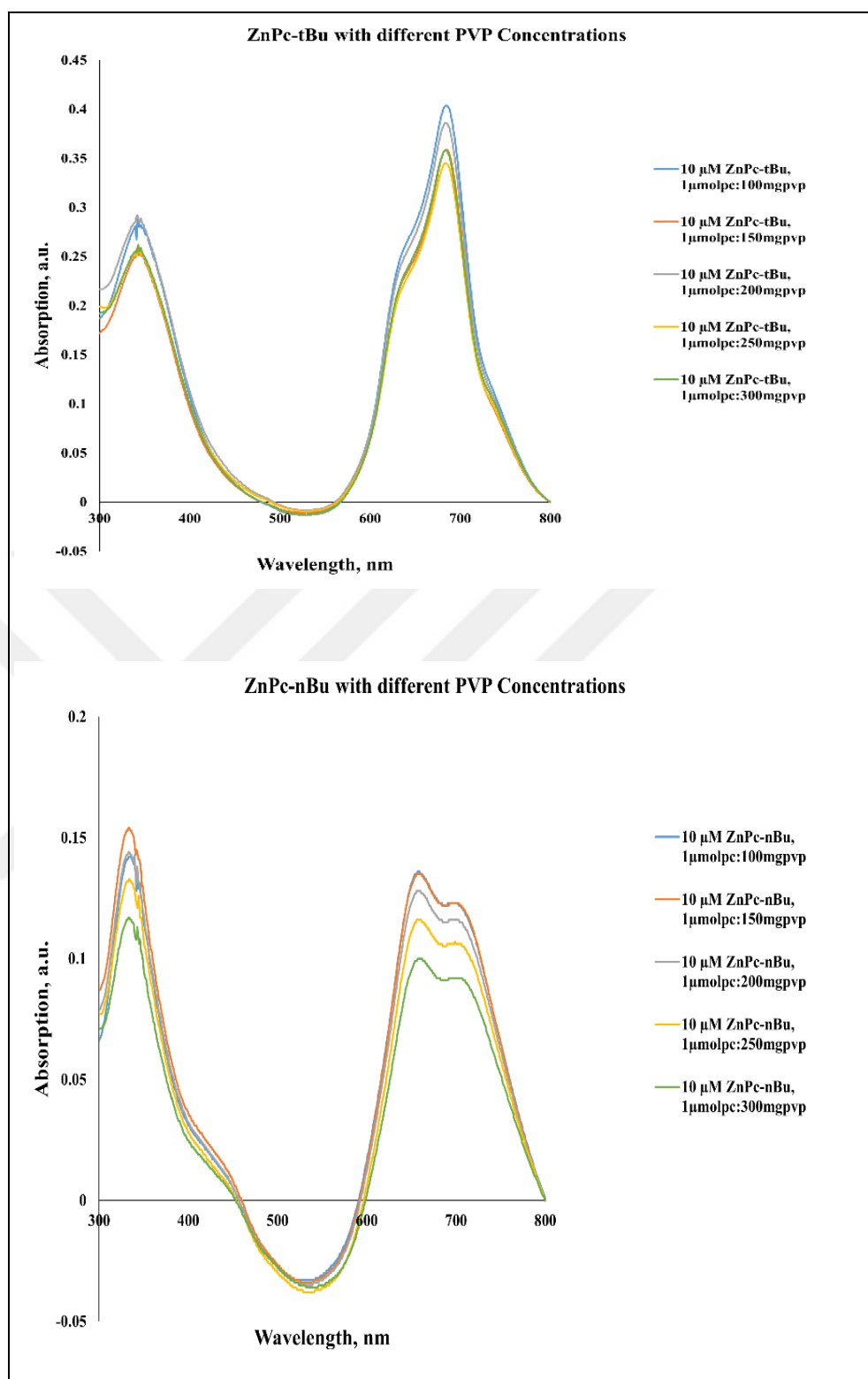


Figure 5.25: Absorption spectra of Set 2 with PVP formulation in different ratios in water.

Table 5.11: Maximum of Q band and related values of $\log \epsilon$ for ZnPc-tBu of each formulation.

PVP RATIO:	1:100	1:150	1:200	1:250	1:300
λ_{max} (nm)	685	685	685	685	685
$\log \epsilon$:	4.61	4.56	4.59	4.54	4.55

Table 5.12: Maximum of Q band and related values of $\log \epsilon$ for ZnPc-nBu of each formulation.

PVP RATIO:	1:100	1:150	1:200	1:250	1:300
λ_{max} (nm)	659	661	660	661	662
$\log \epsilon$:	4.13	4.13	4.11	4.06	4

ZnPc-tBu-PVP and ZnPc-nBu-PVP, which were in 1:100 ratio as mentioned before, were dissolved in water. 1, 2, 4, 6, 8, and 10 μM of each compound were prepared in water (Figure 5.26). The maximum Q bands of ZnPc-tBu and ZnPc-nBu were 685 nm and 661 nm, respectively. Corresponding values of $\log \epsilon$ were also calculated for ZnPc-tBu was 4.62 and for ZnPc-nBu was 4.15. There was no aggregation of the compounds.

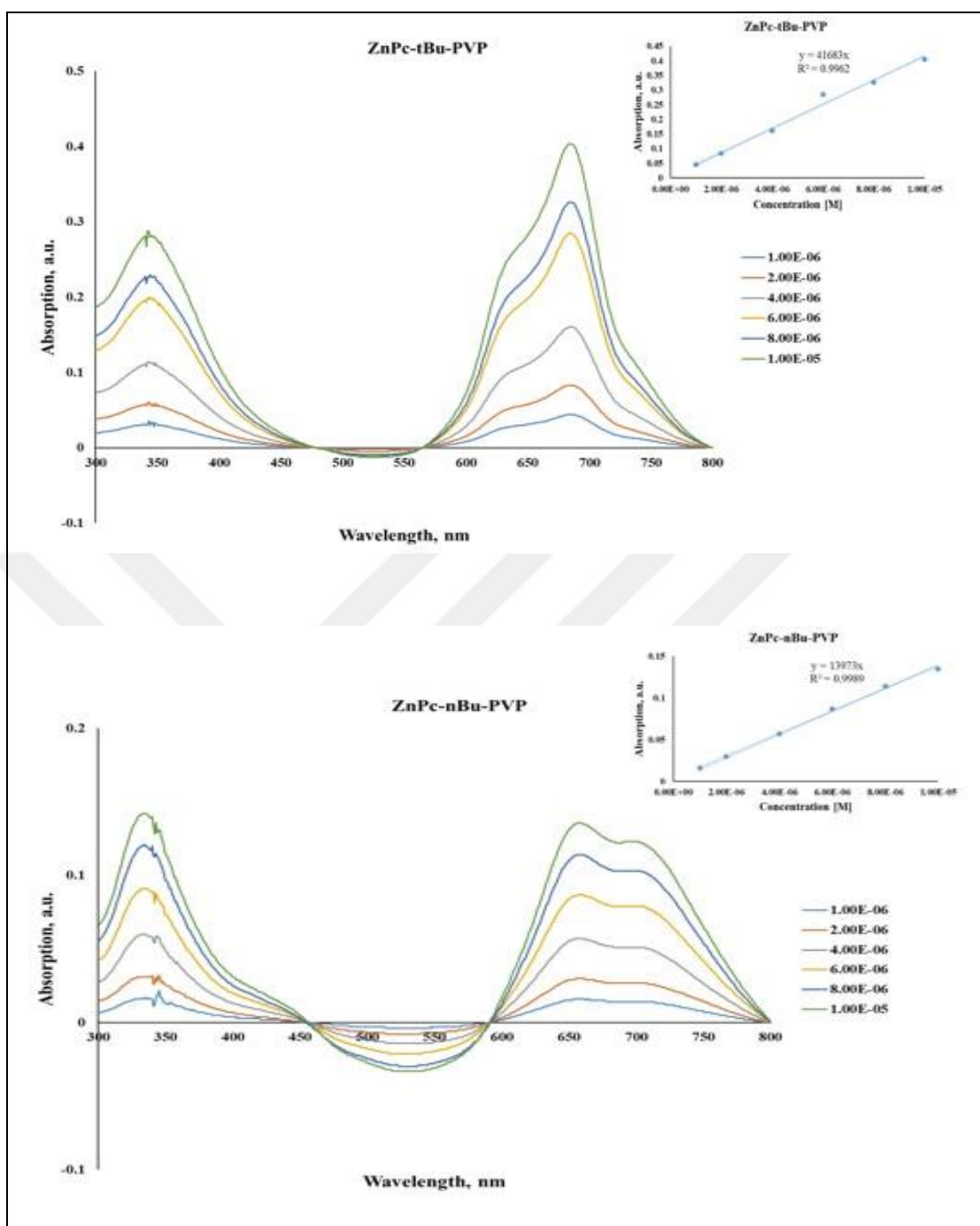


Figure 5.26: Absorption spectra of PVP formulated Set 2 in water.

To assess the absorption degree of compounds, Uv-vis spectra of 10 μM of the compounds in water were also compared. It was seen that the absorption degree of ZnPc-nBu-PVP was lower than ZnPc-tBu-PVP (Figure 5.27).

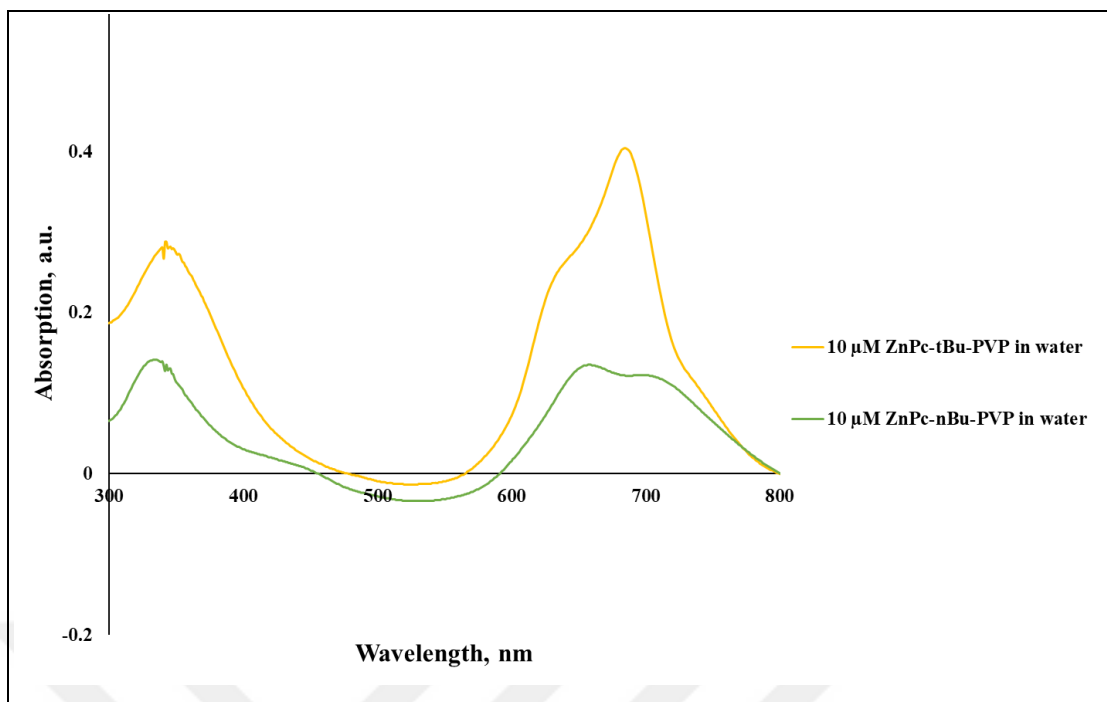


Figure 5.27:: Comparison of UV-vis Absorption of PVP formulated Set 2 in water at 10 μ M.

5.2.2. Dark Toxicity and Phototoxicity of Set 2

In PDT studies, it is expected that the compounds should not be toxic to the cells when there is no light. According to the cell viability assay results, Set 2 phthalocyanines in DMSO were not toxic in the dark but also not in the light. Subjected phthalocyanines at different concentrations on MCF7 and MDA-MB-231 cell lines were examined (Figure 5.28, Figure 5.29, Figure 5.30, Figure 5.31). Six different concentrations were used as 0.125, 0.25, 0.5, 1, 5, and, 10 μ M. Four different light doses were used 1.8 J/cm², 3.6 J/cm², 7.2 J/cm², and 14.4 J/cm² by a handmade LED lamp, and 3, 6, 12, and 24 minutes took time for irradiation, respectively. DMSO was chosen as the solvent for the compounds.

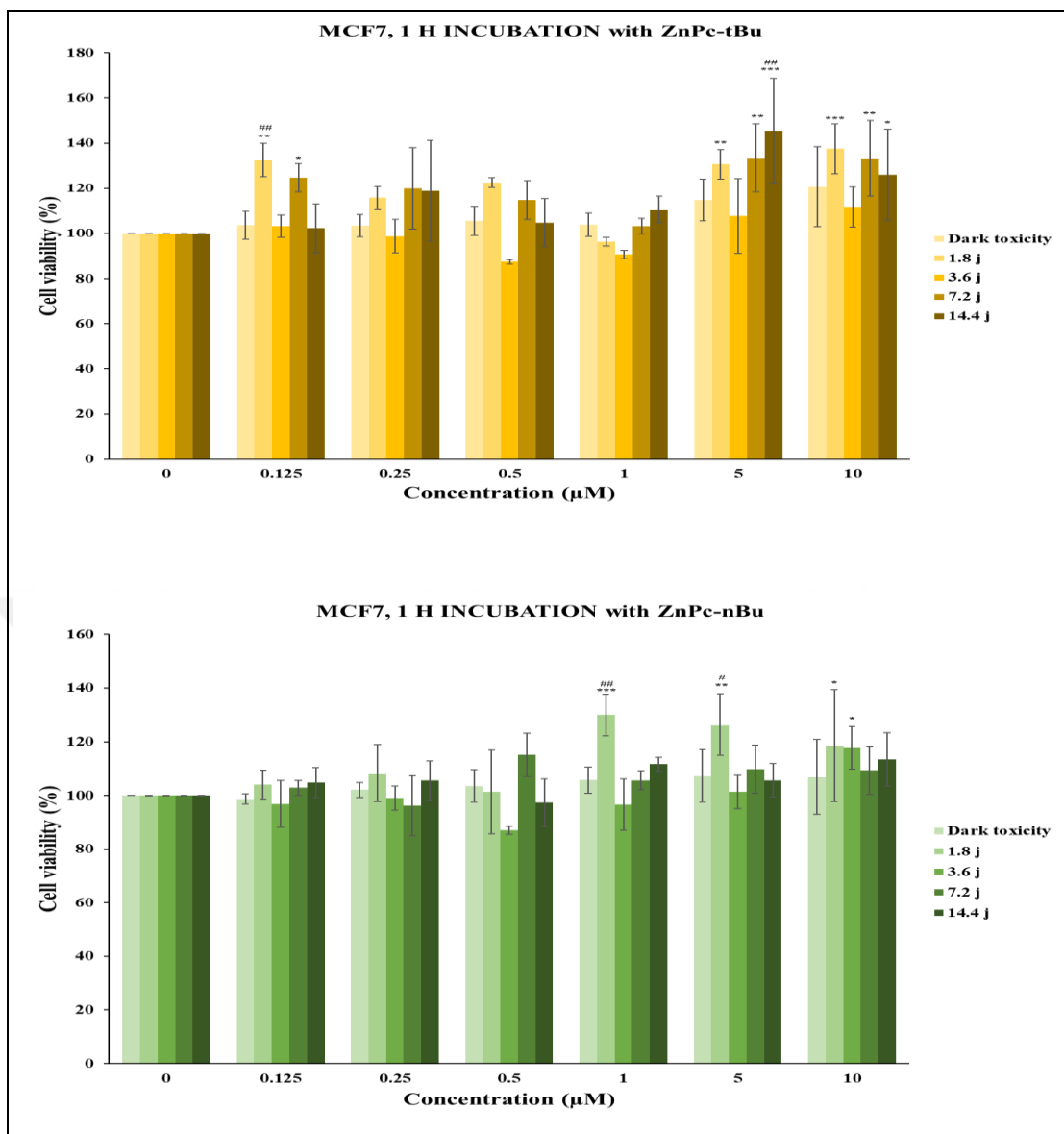


Figure 5.28: Dark and phototoxicity of Set 2 incubated for 1 hour with different concentrations in MCF7 cells. Data are the mean $\pm$ SD of at least three independent experiments performed in triplicates. * $P < 0.05$ compared to the control non-treated cells (0). # $P < 0.05$ compared to dark toxicity. ** $p < 0.01$, *** $p < 0.001$ compared to the control non-treated cells using two-way ANOVA with Dunnett's post-test.

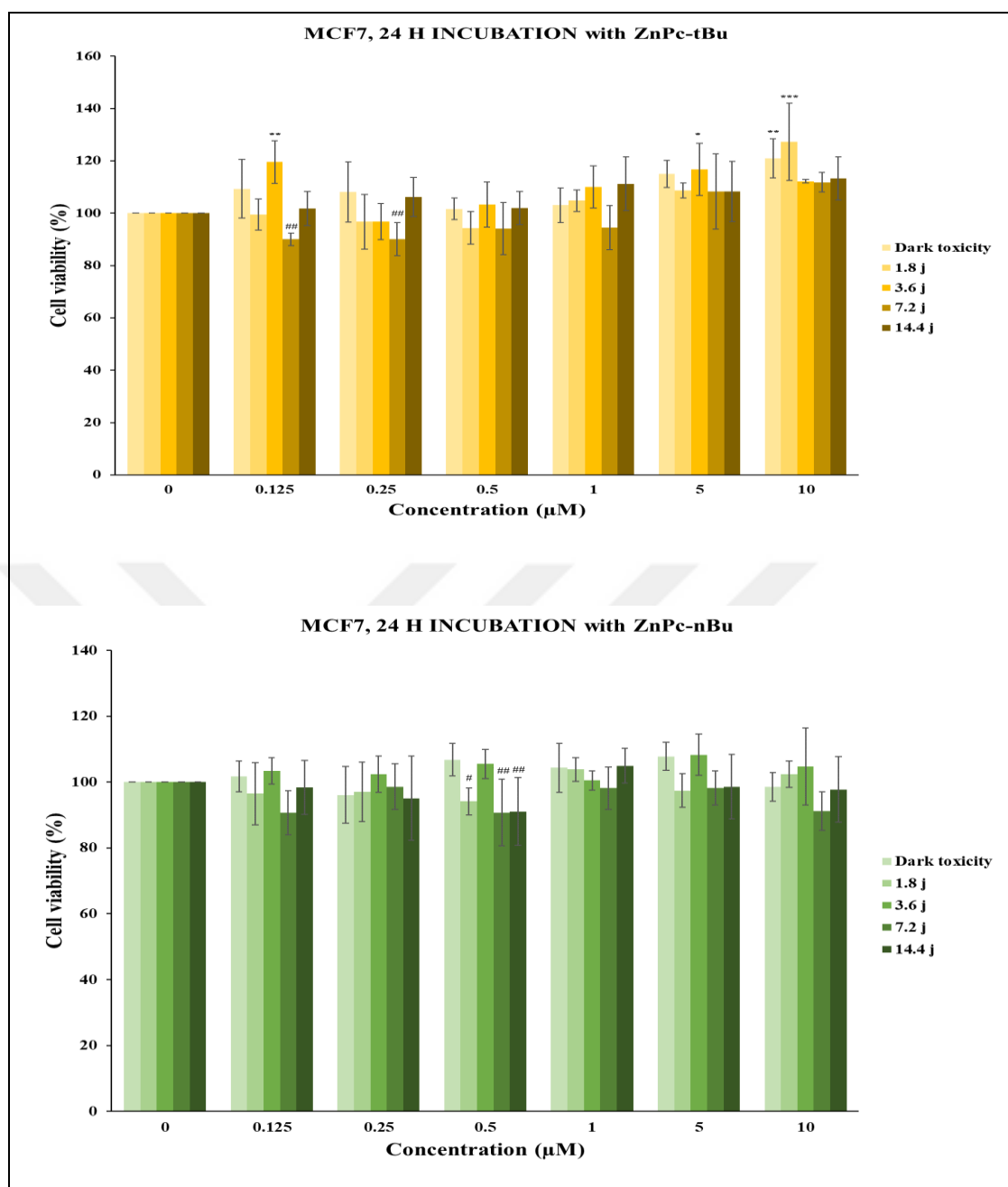


Figure 5.29: Dark and phototoxicity of Set 2 incubated for 24 hour with different concentrations in MCF7 cells. Data are the mean $\pm$ SD of at least three independent experiments performed in triplicates. * $P < 0.05$ compared to the control non-treated cells (0). # $P < 0.05$ compared to dark toxicity. ** $p < 0.01$, *** $p < 0.001$ compared to the control non-treated cells using two-way ANOVA with Dunnett's post-test.

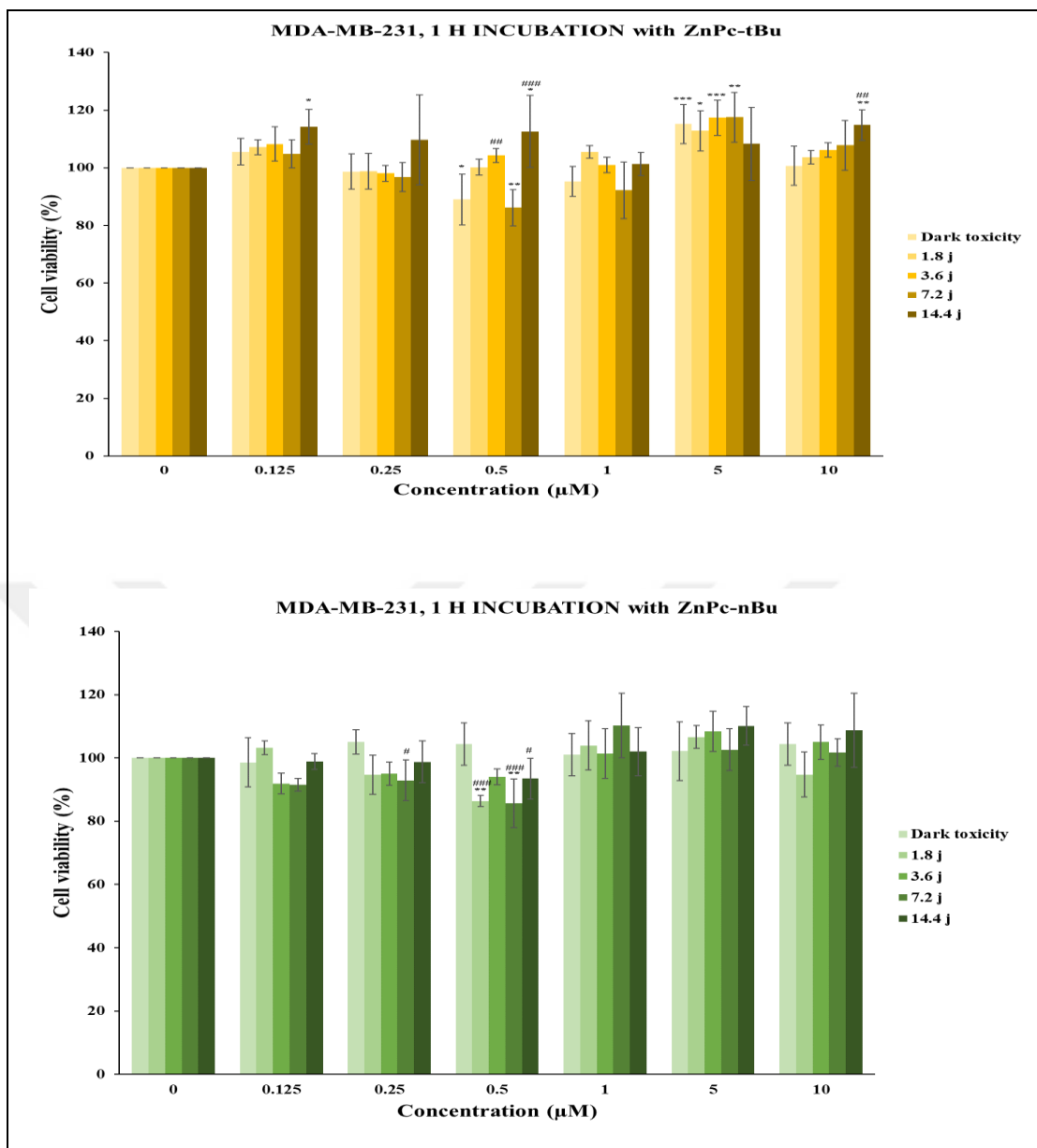


Figure 5.30: Dark and phototoxicity of Set 2 incubated for 1 hour with different concentrations in MDA-MB-231 cells. Data are the mean $\pm$ SD of at least three independent experiments performed in triplicates. * $P < 0.05$ compared to the control non-treated cells (0). # $P < 0.05$ compared to dark toxicity. ** $p < 0.01$, *** $p < 0.001$ compared to the control non-treated cells using two-way ANOVA with Dunnett's post-test.

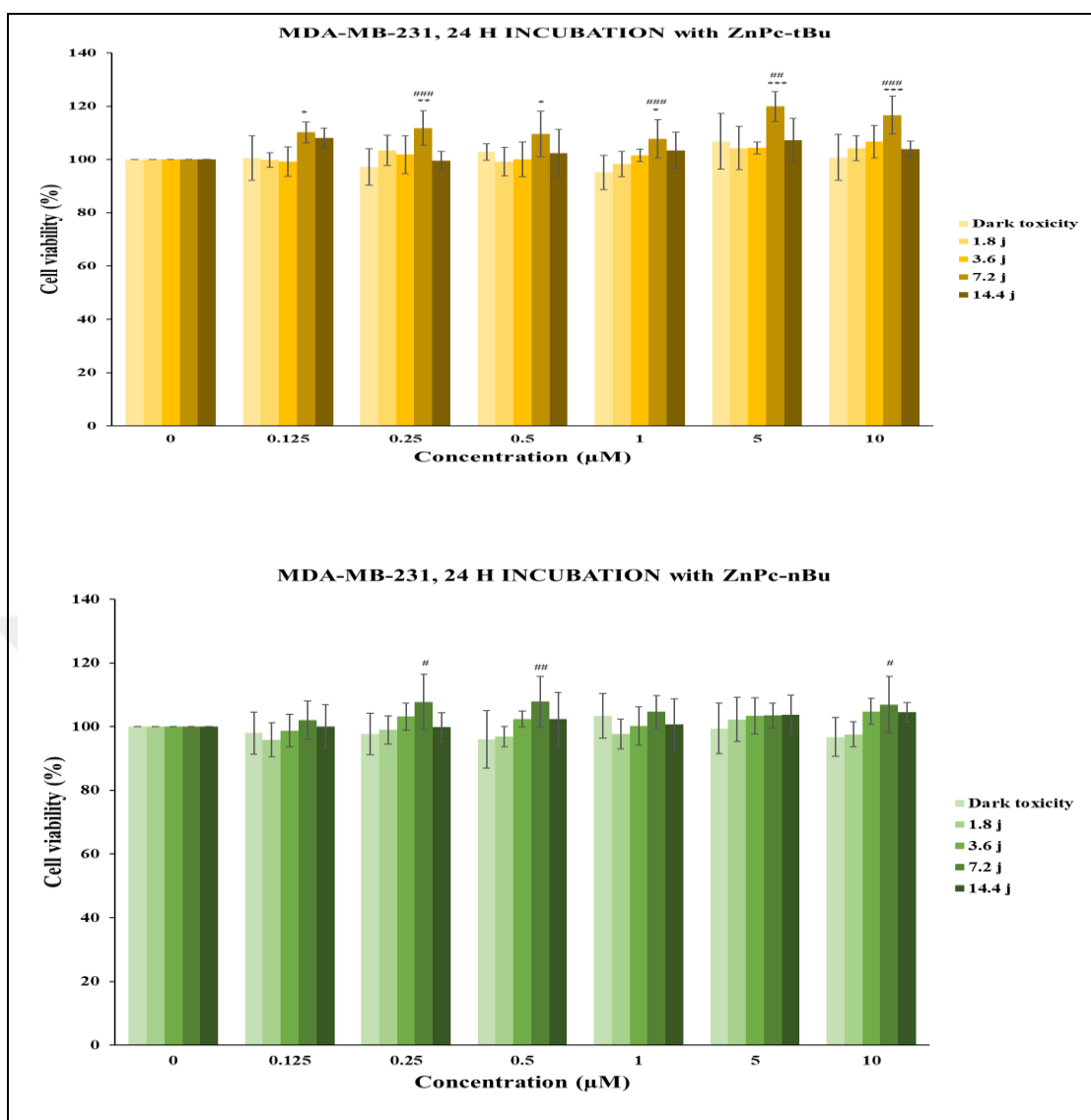


Figure 5.31: Dark and phototoxicity of Set 2 incubated for 24 hour with different concentrations in MDA-MB-231 cells. Data are the mean $\pm$ SD of at least three independent experiments performed in triplicates. * $P < 0.05$ compared to the control non-treated cells (0). # $P < 0.05$ compared to dark toxicity. ** $p < 0.01$, *** $p < 0.001$ compared to the control non-treated cells using two-way ANOVA with Dunnett's post-test.

5.2.3. Cellular Uptake of Set 2

An uptake experiment was performed with MCF7 and MDA-MB-231 cell lines with Set 2 compounds at 10 μM for 1 hour and 24 hour incubation times (Figure 5.32). Cells were lysed, and their drug intake was determined by fluorescence measurements. The calibration curve of each compound was used to draw the graphs and for statistical

analysis. A new calibration curve was drawn in each experimental trial. Sample calibration curves of compounds in DMSO are in the appendices (Appendix A).

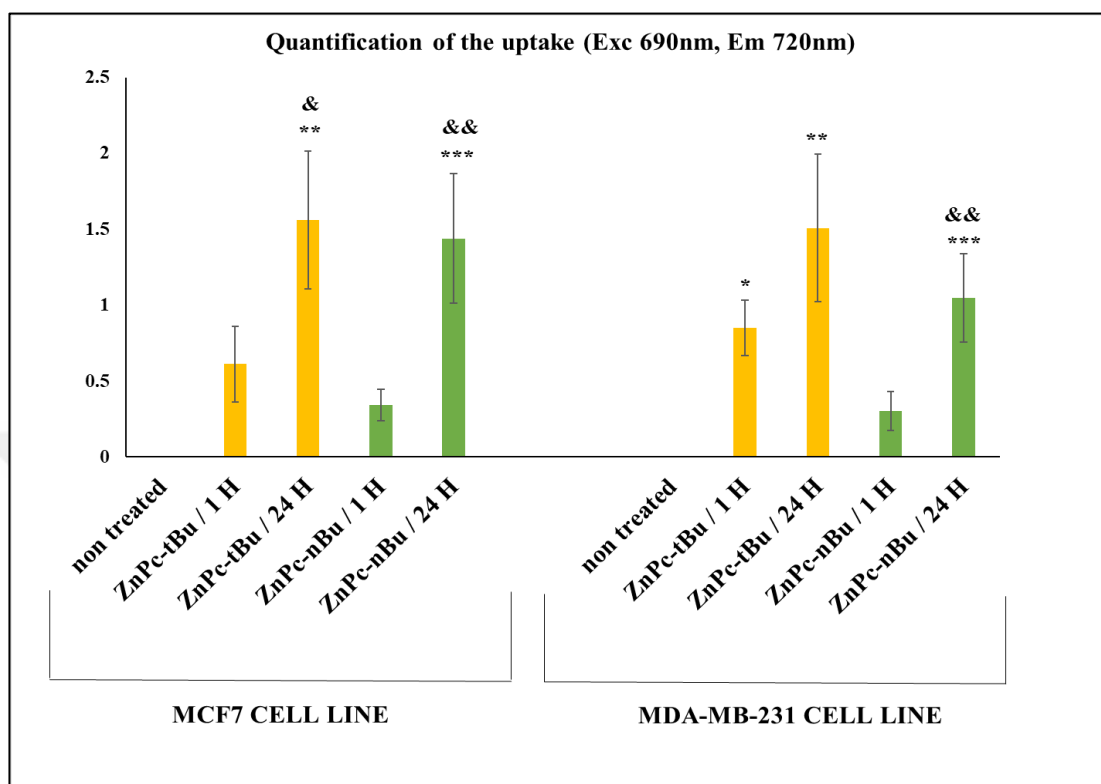


Figure 5.32: Cell uptake experiment of Set 2 at 10 μ M, 1 hour and 24 hour of incubation with MCF7 and MDA-MB-231 cells. &P < 0.05 comparison between 1 hour and 24 hour cell uptake of each compound inbetween. ***p < 0.001, *p < 0.05 compared to the control non-treated cells using one- way ANOVA with Dunnett's post-test. P values < 0.05 were considered to be statistically significant.

Cell uptake of the compounds was performed against non-treated cells and between 1 hour and 24 hour uptake capacity of each compound in itself. Results of the cell viability assay showed that ZnPc-tBu and ZnPc-nBu could not be activated in the cells when they were dissolved in DMSO. Results of cell uptake studies showed that at 24 hour time point, two of the phthalocyanines were entering both of the cell lines, and at 1 hour time point, ZnPc-tBu was also entering MDA-MB-231 cells. ZnPc-tBu has 1.5603 a.u. and ZnPc-nBu has 1.4396 a.u. when administered to MCF7 cells, and they have 1.5076 a.u. and 1.0474 a.u. on MDA-MB-231 cells, respectively, for 24 hour time incubation. ZnPc-tBu has 0.8499 a.u. value at the end of the 1 hour incubation of uptake assay on MDA-MB-231 cells. The detected fluorescence signals may also be

caused by compounds attached to the cell membrane. In the technique, membranes of the cells are lysed and removed, but still, it may affect the results.

5.2.4. Nuclear Morphology Analysis of Set 2

Nuclear morphology analysis was conducted with 10 μM of each Set 2 phthalocyanines in DMSO for 1 hour and 24 hour incubation time on MCF7 and MDA-MB-231 cells. Confocal pictures can be seen in Figure 5.33. A light dose of 14.4 J/cm^2 was used as PDT treatment. No significant change in the nuclear morphologies was observed against non-treated cells at any of the time points. This morphologic analysis also confirmed the MTT results of Set 2 in DMSO.

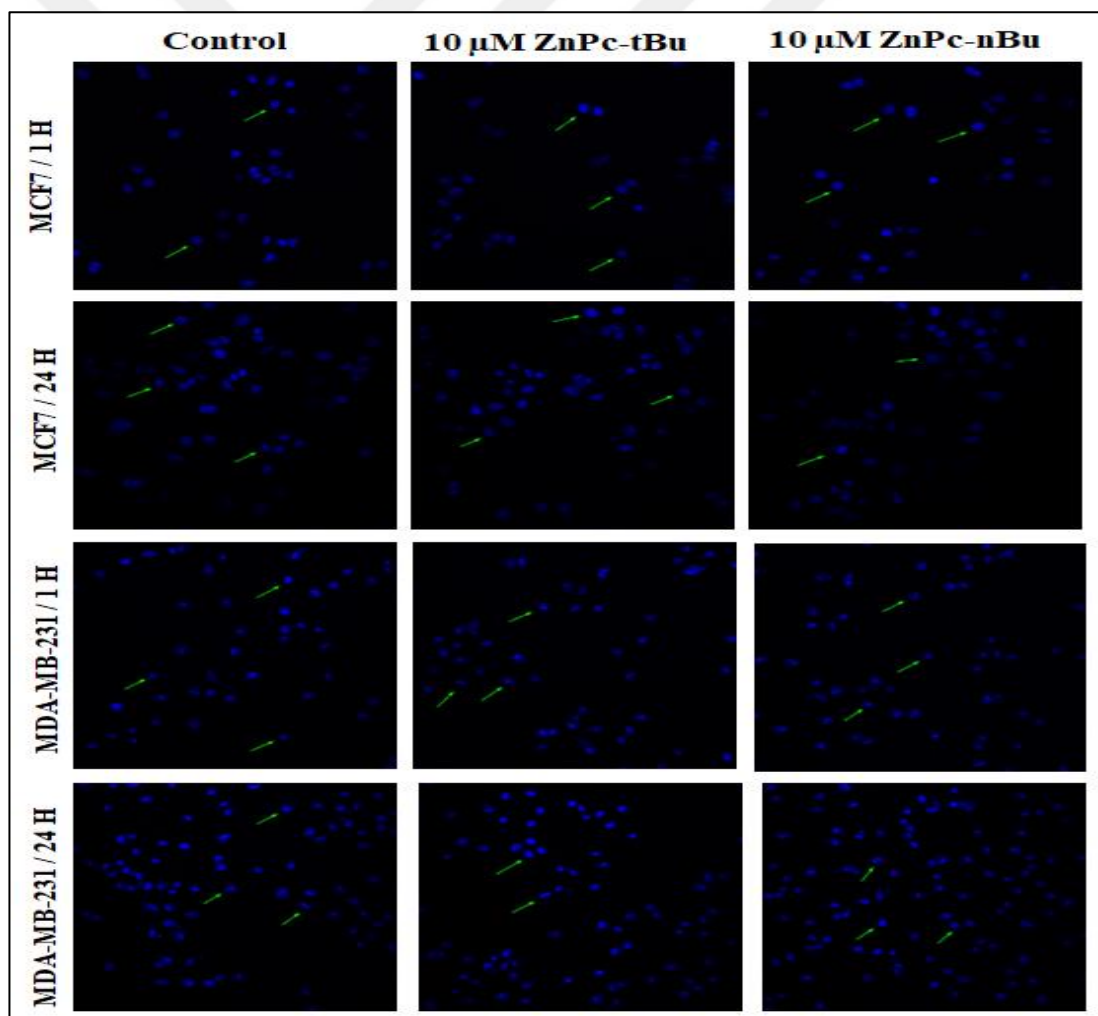


Figure 5.33: Nuclear morphology analysis of Set 2 at 10 μM after 1 hour and 24 hour incubation on MCF7 and MDA-MB-231 cells. Green arrows show cells with normal nuclei.

5.2.5. Wound Healing Assay of Set 2

Cell migration capacity was evaluated by wound healing assay. Also, cell morphology changes due to the PDT treatments would be observed by this technique. MCF7 and MDA-MB-231 cells were treated with ZnPc-tBu and ZnPc-nBu in DMSO at 10 μ M concentration; there was no difference against the non-treated control cells at 24 hour or 48 hour time points; treated cells were migrated and proliferated as control cells (Figure 5.34, Figure 5.35, Figure 5.36, and Figure 5.37).

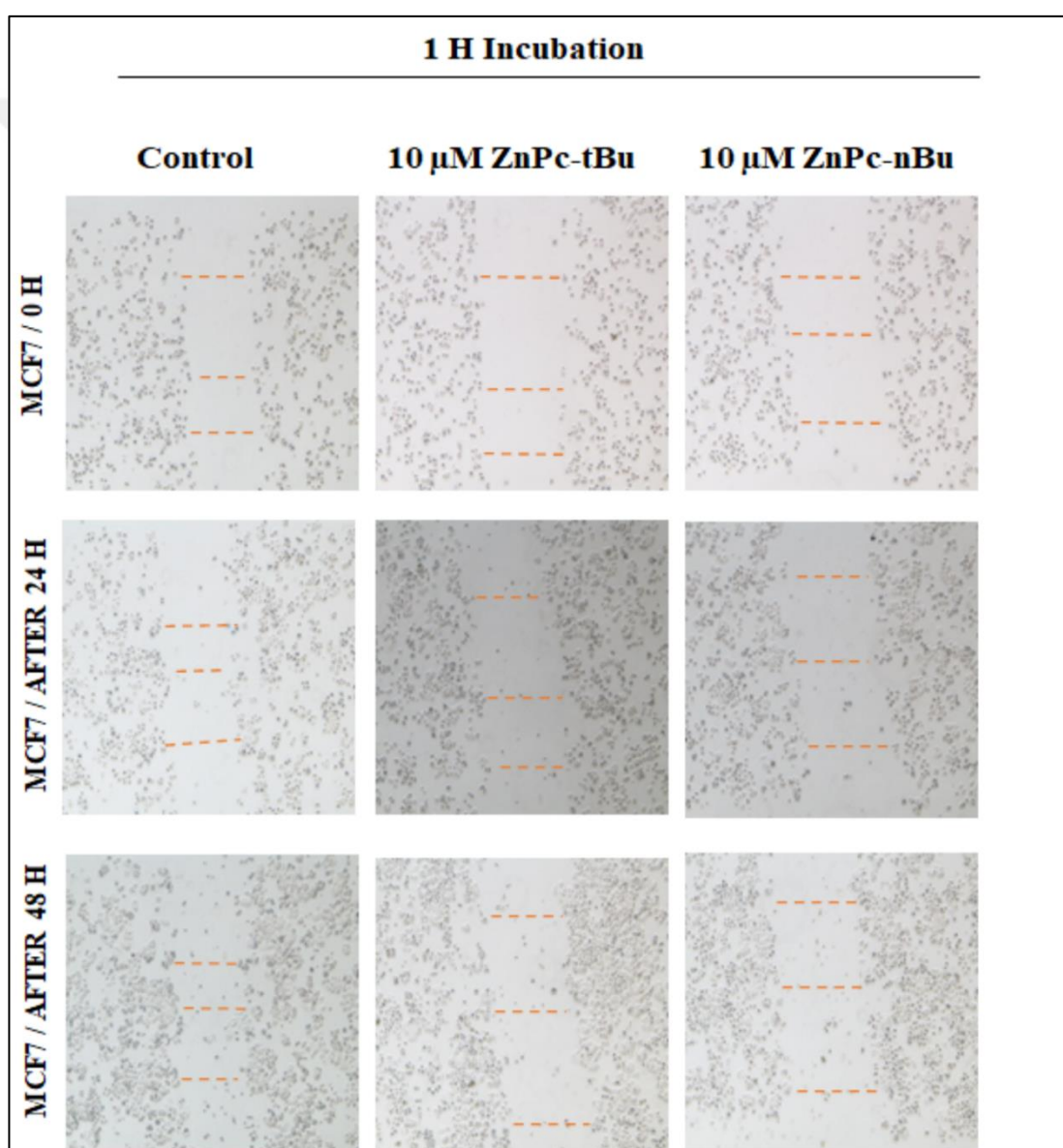


Figure 5.34: Wound healing assay of Set 2 at 10 μ M after 1 hour incubation on MCF7 cells. Pictures were taken directly after treatment (0 H), 24 hours later, and 48 hours later.

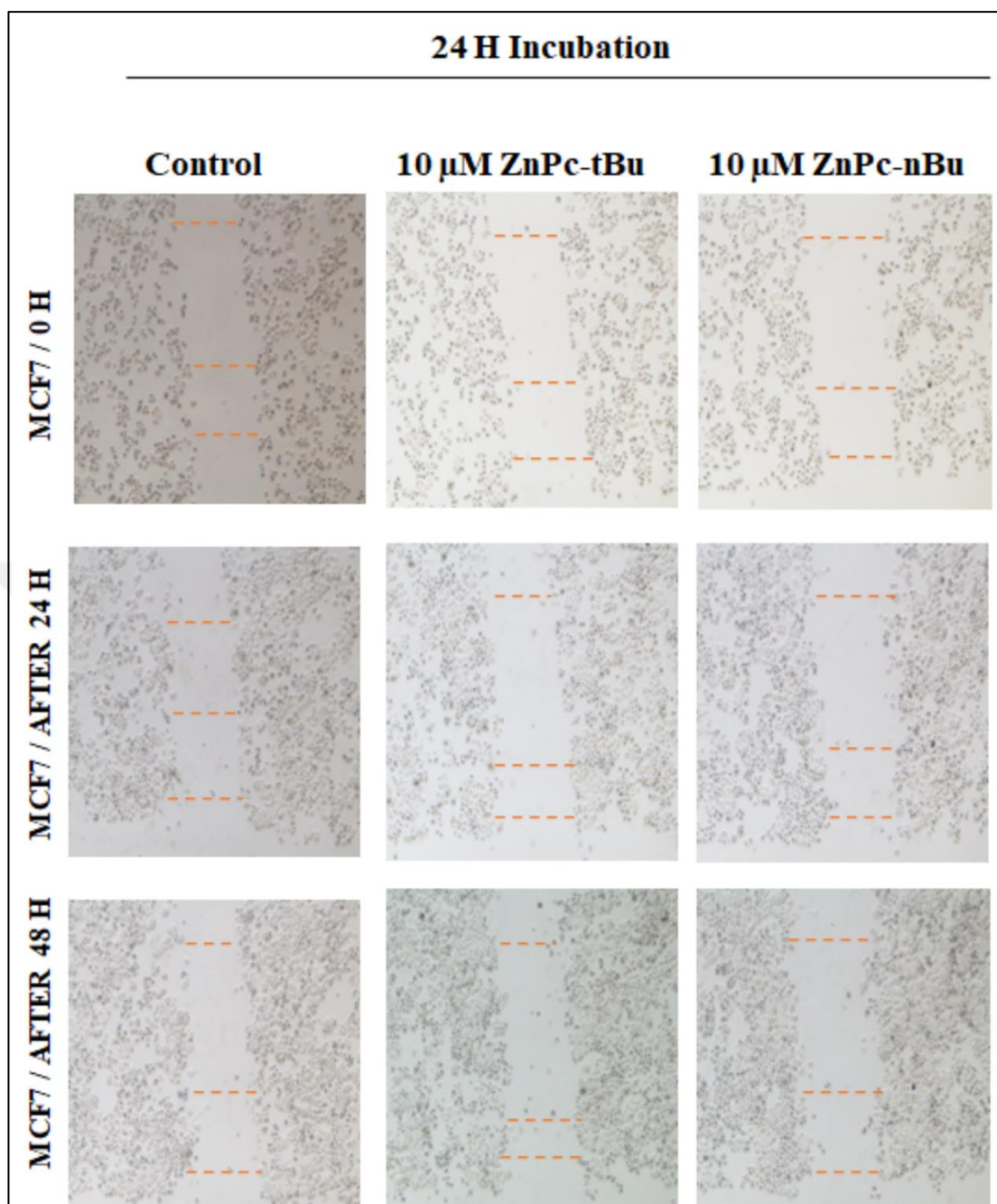


Figure 5.35: Wound healing assay of Set 2 at 10 μ M after 24 hour incubation on MCF7 cells. Pictures were taken directly after treatment (0 H), 24 hours later, and 48 hours later.

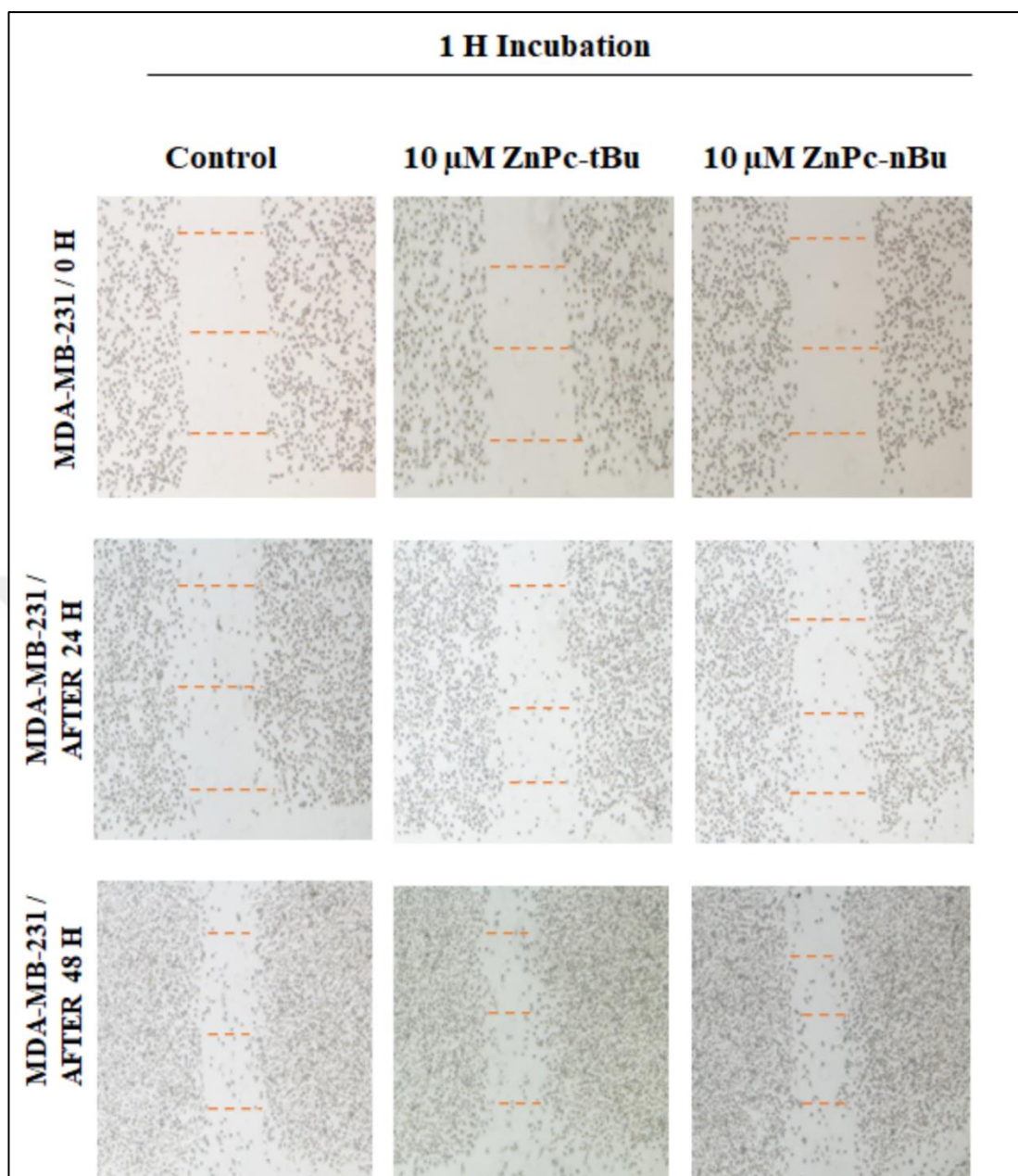


Figure 5.36: Wound healing assay of Set 2 at 10 μ M after 1 hour incubation on MDA-MB-231 cells. Pictures were taken directly after treatment (0 H), 24 hours later, and 48 hours later.

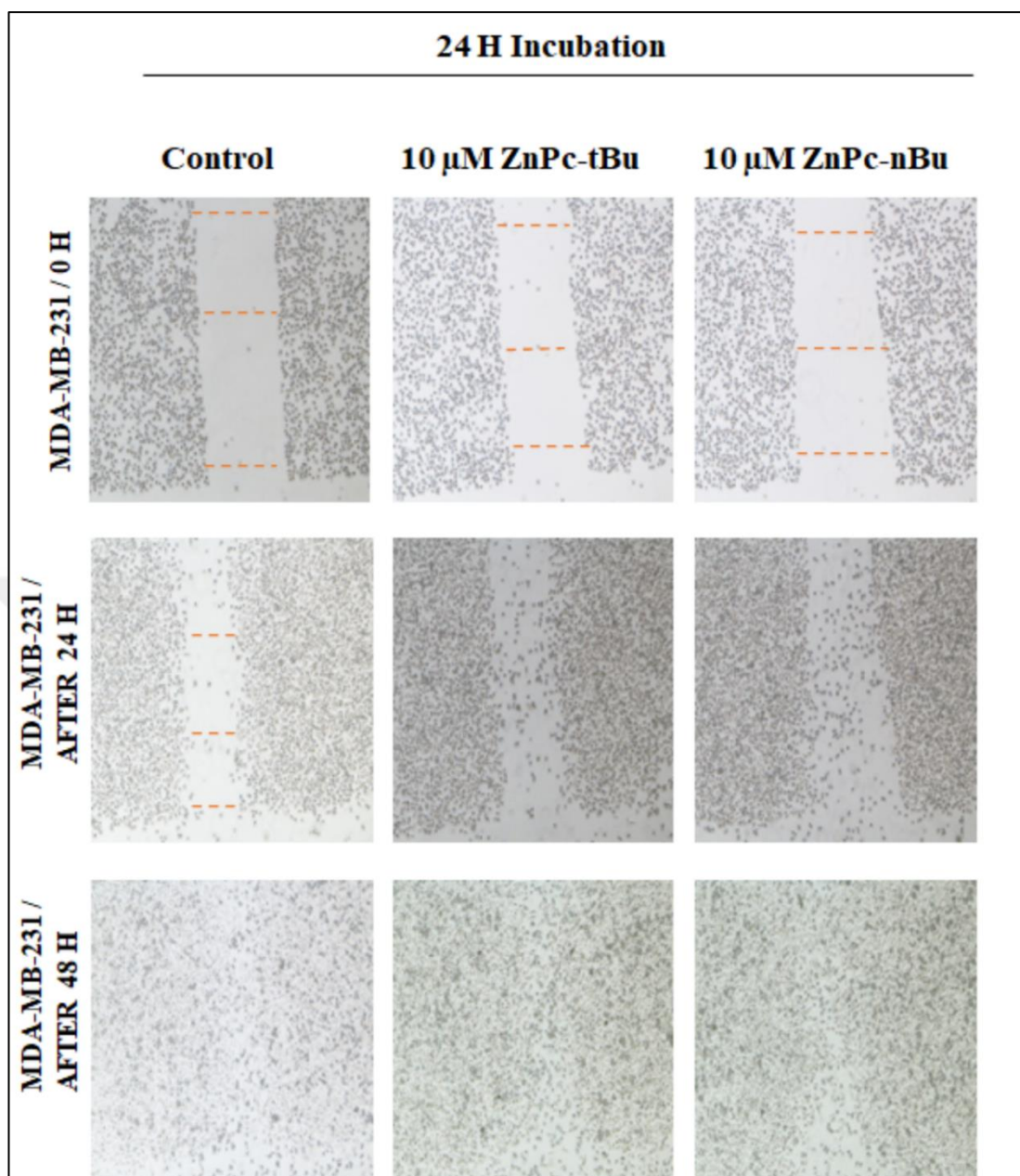


Figure 5.37: Wound healing assay of Set 2 at 10 μ M after 24 hour incubation on MDA-MB-231 cells. Pictures were taken directly after treatment (0 H), 24 hours later, and 48 hours later.

5.2.6. Colony Formation Assay of Set 2

In order to determine the colony formation capacities of the phthalocyanines, MCF7 and MDA-MB-231 cells were treated with 10 μ M of ZnPc-tBu and ZnPc-nBu in DMSO for 1 hour and 24 hour incubation times (Figure 5.38 and Figure 5.39). A light dose of 14.4 J/cm² was used as PDT treatment. After incubation of the cells for 10 days, there was no difference between the treated cells of both cell lines and the control cells; both formed colonies in a similar way.

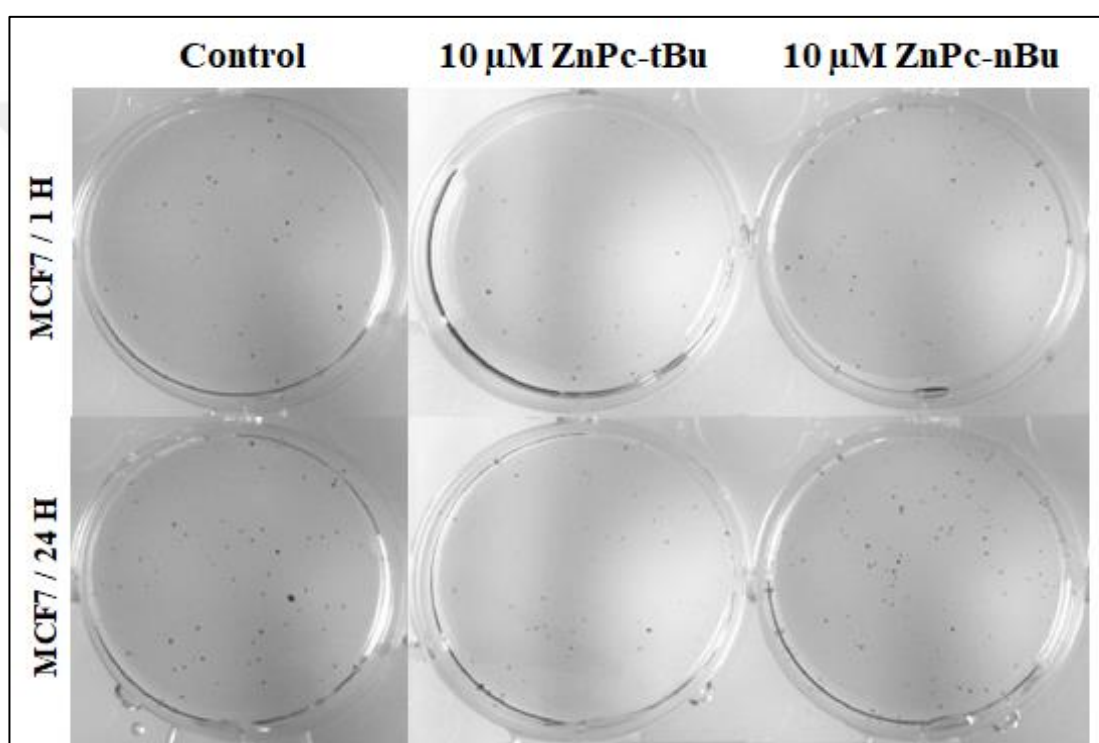


Figure 5.38: Colony formation assay of Set 2 at 10 μ M after 1 hour and 24 hour treatment on MCF7 cells.

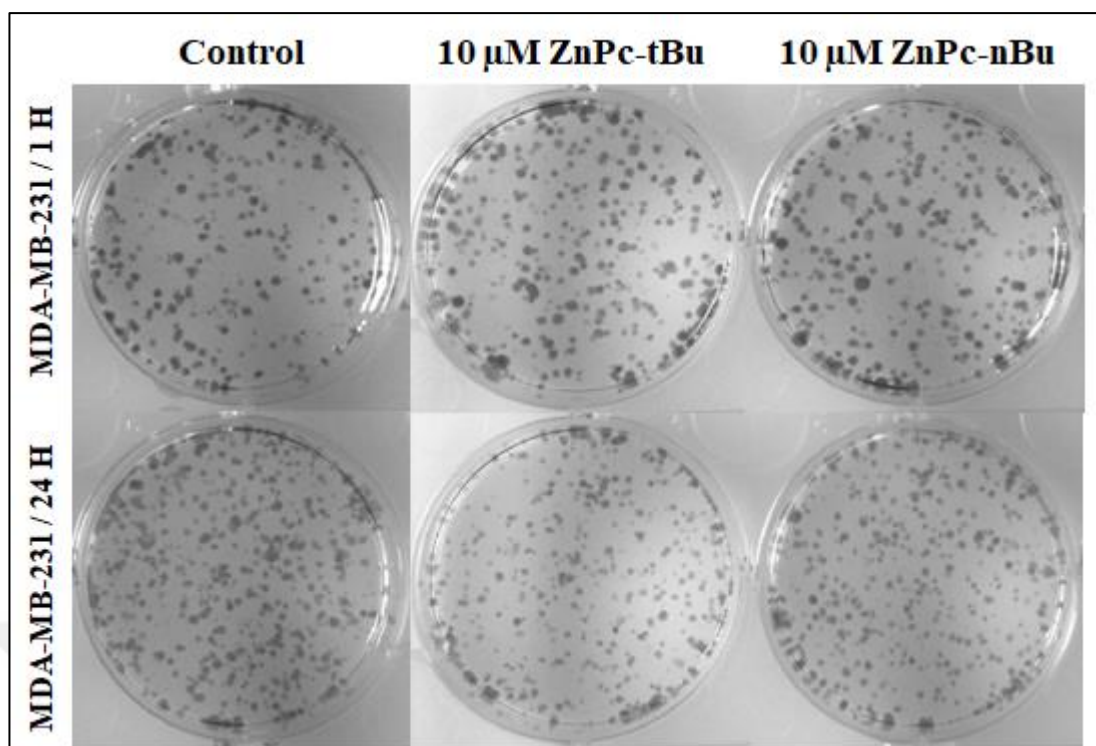


Figure 5.39: Colony formation assay of Set 2 at 10 μ M after 1 hour and 24 hour treatment on MDA-MB-231 cells.

5.2.7. Dark Toxicity and Phototoxicity of PVP Formulated Set 2

The dissolving capacities of Set 2 phthalocyanines were adequate in DMSO, but in dark and phototoxicity assay examinations, they could not fulfill the task. In order to achieve better solubilization with Set 2, formulation studies were done with different PVP ratios. Both formulated phthalocyanines showed no toxicity in the dark (Figure 5.40 and Figure 5.41). Phototoxicity assay was conducted with 14.4 J/cm² for 24 hour and 48 hour incubation time points on MCF7 and MDA-MB-231 cell lines with 10 μ M of the compounds (Figure 5.42 and Figure 5.43). Formulated ZnPc-tBu results showed very significant and high cell death with phototoxicity assay, and also cell death was higher at 48 hour time point than 24 hour time point, but formulated ZnPc-nBu was again not toxic for the cells in the light. However, increasing cell proliferation was conspicuous, which may be meant that there was stress on the cells that caused cell number to increase. Only an appreciated volume of water-containing media was used to treat control cells. PVP was also examined in the lowest and the highest amounts; no toxicity was seen either in the light or in the dark, which is also confirmative of the mentioned cell stress.

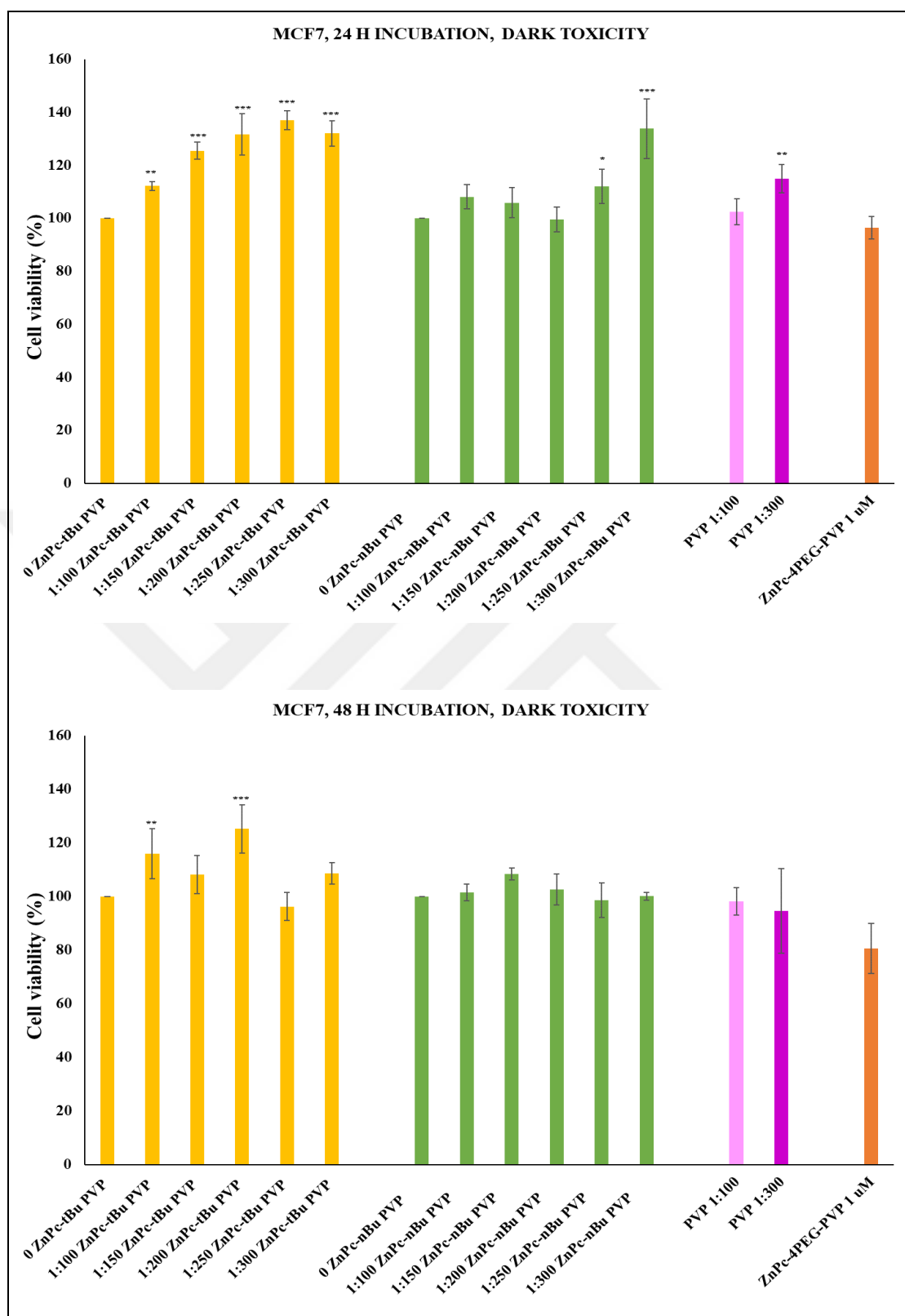


Figure 5.40: Dark toxicity of Set 2 incubated for 24 hour and 48 hour with formulated with different ratios of PVP at 10 μ M in MCF7 cells. Light dose was 14.4 J/cm². *P < 0.05 compared to the control non-treated cells (0). **p < 0.01, ***p < 0.001 compared to the control non-treated cells using two-way ANOVA with Dunnett's post-test.

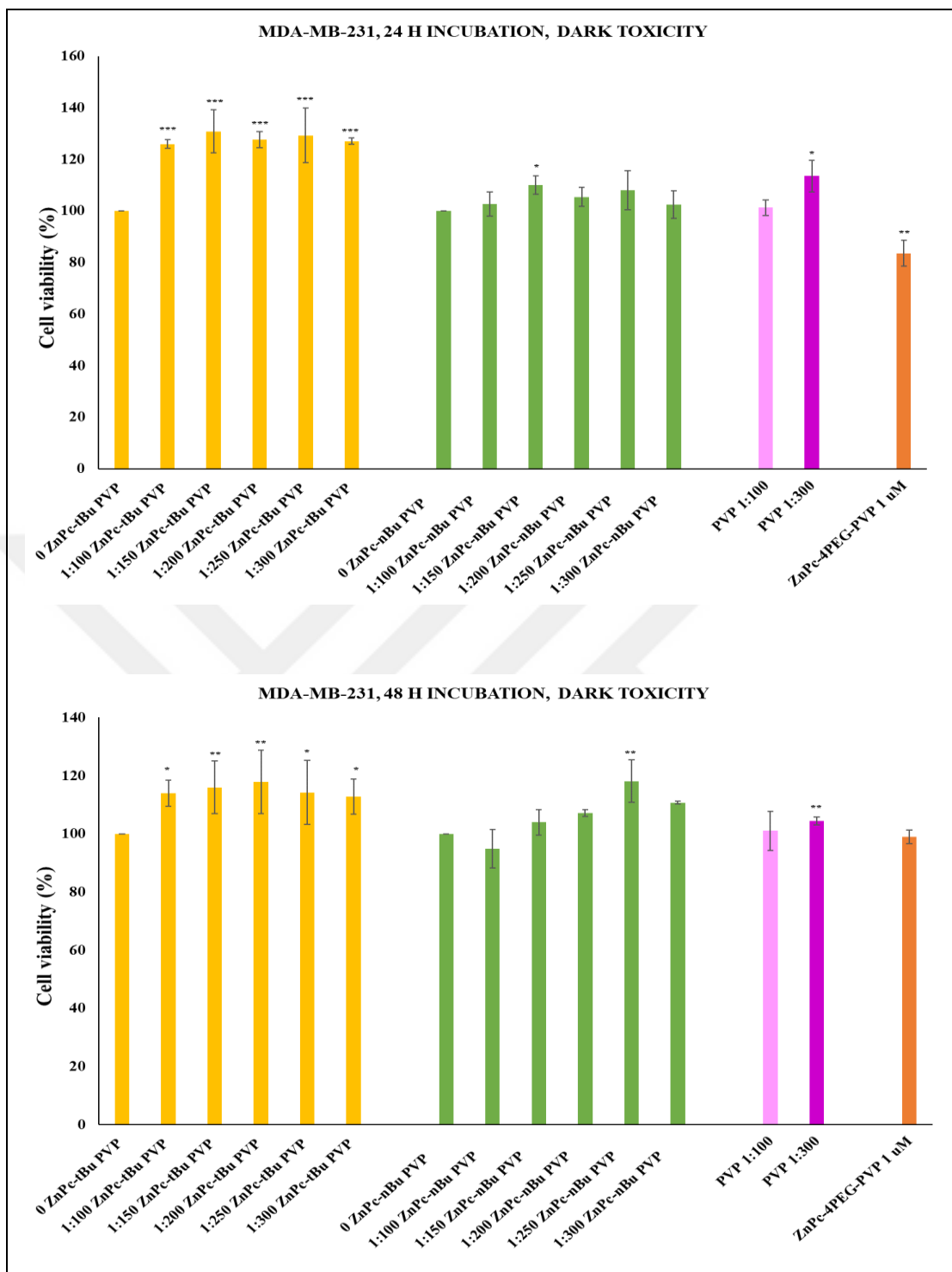


Figure 5.41: Dark toxicity of Set 2 incubated for 24 hour and 48 hour with formulated with different ratios of PVP at 10 μ M in MDA-MB-231 cells. Light dose was 14.4 J/cm². *P < 0.05 compared to the control non-treated cells (0). **p < 0.01, ***p < 0.001 compared to the control non-treated cells using two-way ANOVA with Dunnett's post-test.

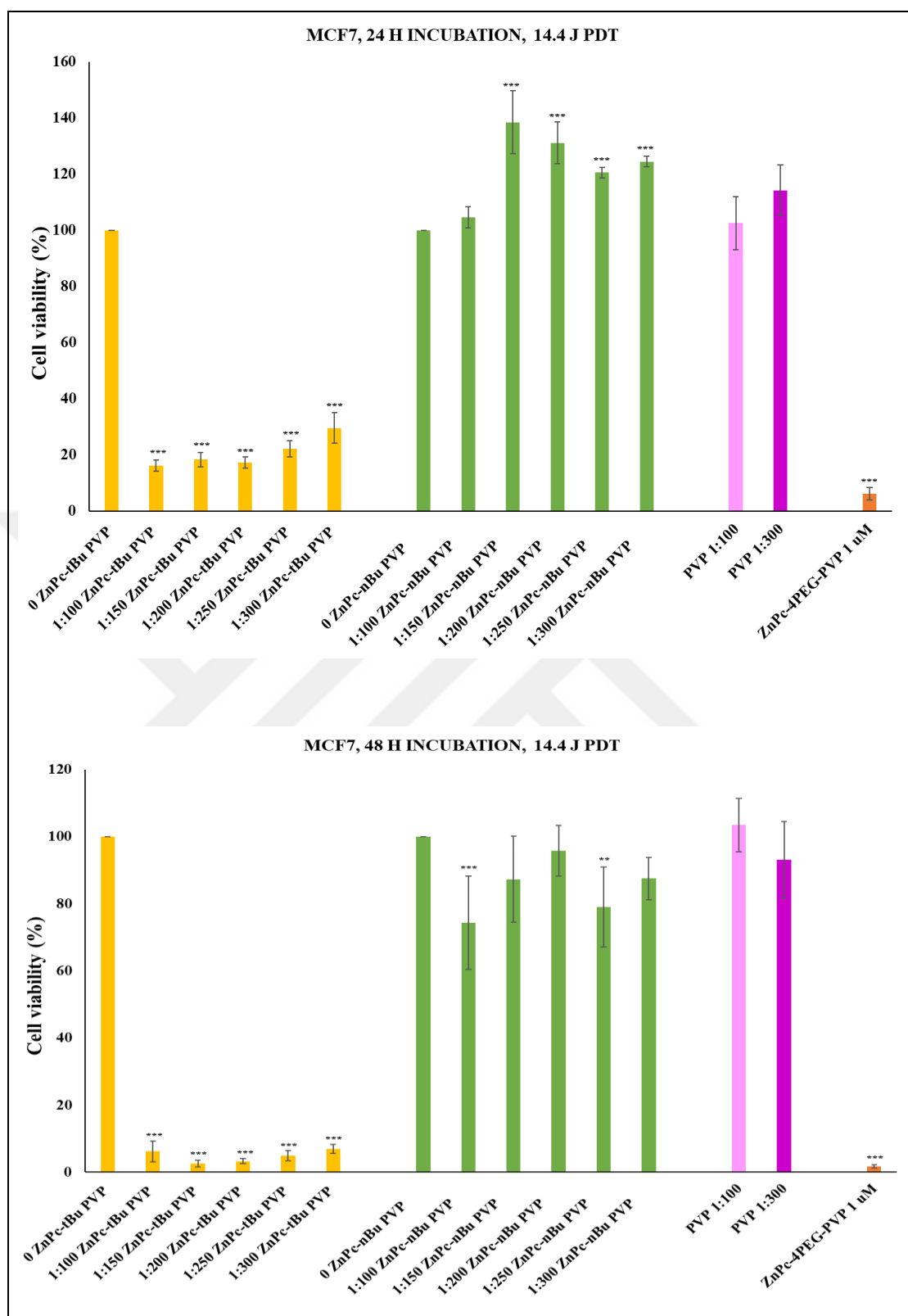


Figure 5.42: Phototoxicity of Set 2 incubated for 24 hour and 48 hour with formulated with different ratios of PVP at 10 μ M in MCF7 cells. Light dose was 14.4 J/cm². *P < 0.05 compared to the control non-treated cells (0). **p < 0.01, ***p < 0.001 compared to the control non-treated cells using two-way ANOVA with Dunnett's post-test.

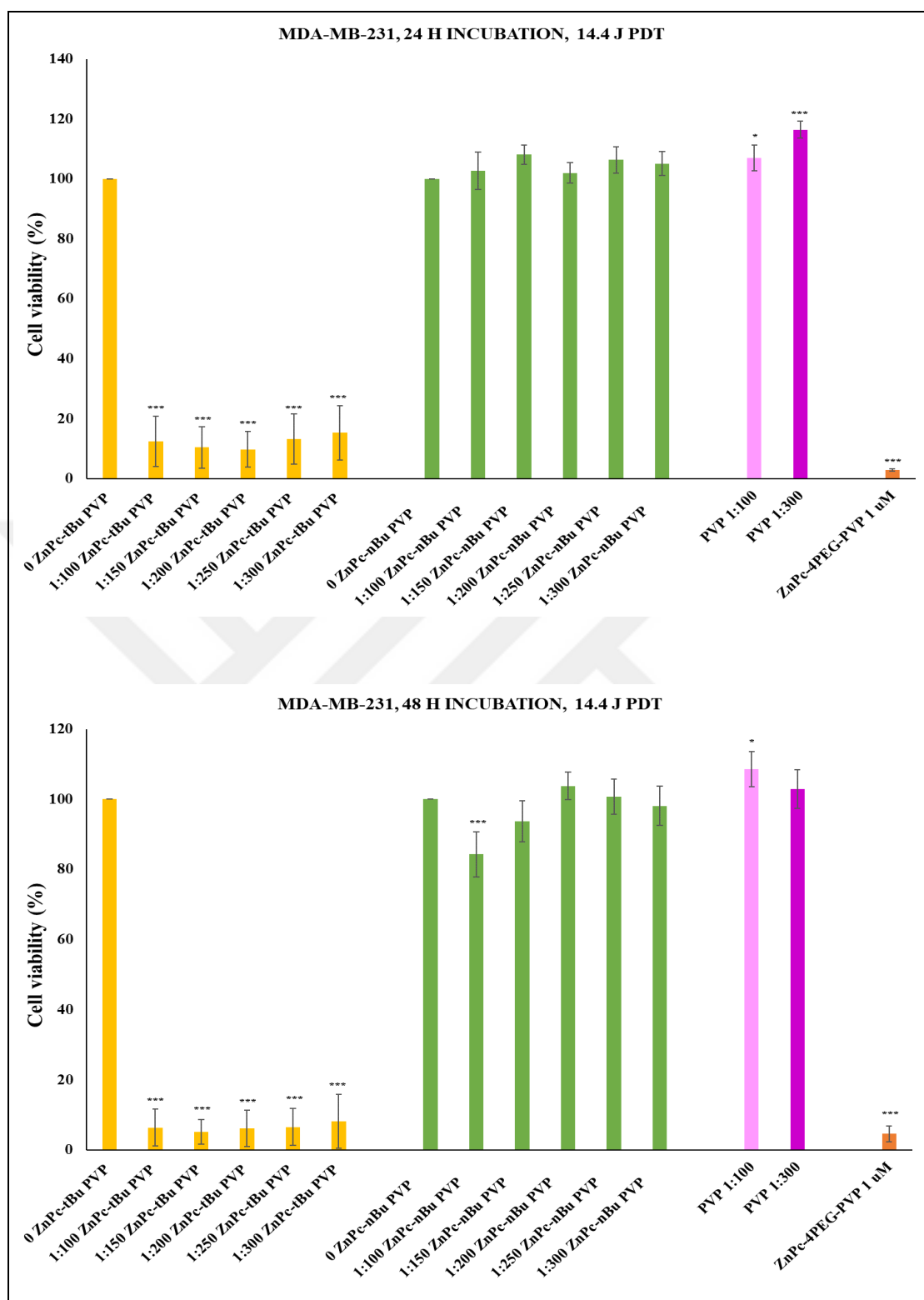


Figure 5.43: Phototoxicity of Set 2 incubated for 24 hour and 48 hour with formulated with different ratios of PVP at 10 μ M in MDA-MB-231 cells. Light dose was 14.4 J/cm². *P < 0.05 compared to the control non-treated cells (0). **p < 0.01, ***p < 0.001 compared to the control non-treated cells using two-way ANOVA with Dunnett's post-test.

6. DISCUSSION

During photodynamic therapy, the photosensitizer is administrated intravenously or topically to the patient before laser treatment at an appropriate wavelength is delivered to the cancerous site to activate the photosensitizer. The activated photosensitizer will react with oxygen typically present in the cancerous tissue to produce reactive oxygen species for the eradication of the cancerous tissue.

A series of metal-based phthalocyanine complexes have been discovered that may be used as a drug or photosensitizer in photodynamic therapy for the treatment of cancers [Nyokong, 2007], [Mayildurai et al., 2017]. The photodynamic therapy (PDT) effect of these metal-based phthalocyanines also relies on their stability, peak absorption wavelength, phthalocyanine uptake, phthalocyanine concentration, localization, and the light dose that is administered during treatment [Nyokong, 2011], [Sekkat et al., 2011].

Increased singlet oxygen generation capability of metal-based phthalocyanines is one of their key features for a more efficient PDT treatment. Thanks to several modifications, this capacity of phthalocyanines can be optimized [Nyokong and Ahsen, 2012, p. 6-11], [Lo et al., 2020]. Introducing heavy atom [Gorman et al., 2004], [Obata et al., 2009] and inhibition of aggregation by bulky substituents [Nyokong, 2007], [Tillo et al., 2015] are the two main ways the modifications can be made with phthalocyanines. In this context, this thesis consists of two parts. Four different phthalocyanines in two sets, Set 1 and Set 2, were investigated with different experimental aspects. In the first part, with two phthalocyanines as Set 1, the heavy atom effect of palladium (PdPc-4PEG) was examined against its counterpart, zinc-phthalocyanine (ZnPc-4PEG). In the second part, with two phthalocyanines as Set 2, the effect of substitution bulkiness was studied between two zinc-phthalocyanines, as ZnPc-tBu and ZnPc-nBu. ZnPc-tBu was the bulky phthalocyanine; the other one was the non-bulky phthalocyanine.

One of the most critical needs is the capability to absorb light in the PDT window, which is the range of wavelengths from 650 to 950 nm. This capability allows for good light penetration into deep tissues as well as a suitable amount of energy to activate oxygen molecules [Sordillo et al., 2014]. In this respect, UV-vis absorptions of the Set 1 in DMSO and water were recorded. Also, ZnPc-4PEG was formulated

with PVP K25 in order to achieve more solubilization in water. PVP is an FDA-approved food additive approved as E1201 and is used for formulation studies in the literature [Kubin et al., 2008], [Marani et al., 2016]. Uv-vis absorption studies showed that ZnPc-4PEG has a higher absorption peak (704 nm) than PdPc-4PEG (692 nm) in DMSO. When they were dissolved in water, their absorption peaks were decreased because of the aggregation; and ZnPc-4PEG (668 nm) had a higher peak than PdPc-4PEG (619 nm). Since the absorption degrees of the compounds were better in DMSO, it was chosen as the solvent for *in vitro* assays. Finally, ZnPc-4PEG was formulated with PVP in different ratios. Again, absorption peaks were lower than in DMSO, but it was promising for further studies. For the formulation of the compounds, 1 micromolar phthalocyanine: 100 milligram PVP ratio was chosen for *in vitro* assays in this thesis.

In PDT, it is expected that the subjected photosensitizers should not be toxic to the cells when they are inactive in the dark. However, they should be activated with light at a suitable wavelength and eradicate cancerous cells. Before *in vitro* assays of Set 1, the effect of solvent and the PVP formulation, dark and phototoxicity assays were examined with ZnPc-4PEG on the MDA-MB-231 cell line. It was seen that the phototoxicity results of DMSO were better than water or PVP formulation. Still, the results of PVP formulation were also promising and can be used in further examinations. Then, MTT assay was performed with Set 1 compounds in DMSO on MCF7 and MDA-MB-231 cell lines after dark and phototoxicity assay. Four light doses were used; 1.8, 3.6, 7.2, and 14.4 J/cm². There was no dark toxicity of the compounds at any concentration for 1 hour and 24 hour incubation time points. In phototoxicity assay for 1 hour incubation with MCF7 cells, the IC₅₀ value of ZnPc-4PEG was lower than PdPc-4PEG only at a light dose of 1.8 J/cm², and PdPc-4PEG had the lowest IC₅₀ value, 0.01746 µM at 14.4 J/cm². In this incubation time, PdPc-4PEG showed a better performance even at lower concentrations. Increasing light dose caused more cell death with PdPc-4PEG than ZnPc-4PEG, especially at the lowest concentrations, 0.0625, 0.125, and 0.25 µM. A decrease in IC₅₀ values with increasing light dose was observed with PdPc-4PEG, but with ZnPc-4PEG, it was not noticeable. With increasing incubation time, IC₅₀ values were significantly decreased. For 24 hour incubation on MCF7 cells, the lowest IC₅₀, 0.01244 µM, belonged to PdPc-4PEG at 14.4 J/cm². A similar pattern with IC₅₀ values of PdPc-4PEG was also observed, which was higher than ZnPc-4PEG at light doses of 1.8 J/cm² and 3.6 J/cm².

Phototoxicity assay was also performed with MDA-MB-231 cells. PdPc-4PEG had the lowest IC₅₀ value at 0.01435 μM at 14.4 J/cm² at 1 hour incubation time. Again, the lowest IC₅₀ value belongs to PdPc-4PEG as 0.02654 at 7.2 J/cm² for 24 hour incubation time on MDA-MB-231 cells. It was seen that in Set 1 heavy metal effect is conspicuous. PdPc-4PEG was leading to more cell death in two cell lines than ZnPc-4PEG in certain conditions. Especially with increasing the light dose, PdPc-4PEG effect gets higher.

Cell uptake of the compounds was evaluated by cell uptake assay with the cell lysis method. Again, 1 hour and 24 hour incubation times were used, with one concentration of 1 μM . When the incubation time was longer, the accumulation of ZnPc-4PEG was higher on both cell lines. However, at 1 hour point, PdPc-4PEG could not be detected, which is probably due to the low fluorescence signal of the compound. It was seen that in 24 hours of cell uptake experiments, Pd enters cells more than ZnPc-4PEG in both MCF7 and MDA-MB-231 cells. The uptake of phthalocyanines depends on not only the features of the compound itself but also cell type. As indicated before, phthalocyanines tend to accumulate more in metastatic cells such as MDA-MB-231 [Saulite et al., 2018], [Jokar et al., 2019]. In this perspective, uptake results showed that compounds are accumulated in MDA-MB-231 cells more than in MCF7 cells. Two intracellular uptake mechanisms were reported as endocytosis/phagocytosis and nanopenetration [Moor, 2000], [Sahoo et al., 2011]. As indicated in the literature, unlike MCF7 cells, MDA-MB-231 cells may have more than one uptake mechanism, which may also differ in cell membrane composition [Jerath et al., 2020].

PDT treatment can lead to three forms of cell death, namely apoptosis, necrosis, and autophagy [Kessel, 2006]. Apoptosis and necrosis can be observed by nuclear morphology assay [Moor, 2000], [Prasad et al., 2017]. In the lower concentration of the ZnPc-4PEG, 0.5 μM , cells showed more different nuclear morphologies. Nevertheless, when treated with the higher concentration of the Set 1, 1 μM , with 1 hour and 24 hour incubation time at 14.4 J/cm² light dose, both compounds led to one morphology in the cells, which was the late apoptotic nucleus.

The conducted wound healing assay also confirmed MTT results. There was no cell migration caused by treated cells in both cell lines with 1 μM of Set 1 at 14.4 J/cm² light dose for 1 hour and 24 hour incubation. This result also showed that resulting cell death after treatment is inevitable, and cells can not survive, while the assays take more time with the treated cells, as the wound healing assay took 48 hours. In addition,

specific morphologic changes in the cell caused by PDT were observed, such as swollen membrane, irregular cytoplasm, blebbing, and nucleus fragmentation [Savill and Fadok, 2000], [Holliday and Speirs, 2011], [Dos Santos et al., 2017].

Colony formation assay was used for the determination of potentially lethal- and sublethal damage repair [Franken et al., 2006]. This method also confirmed previously performed *in vitro* assays. There were no colonies caused by treated cells in both cell lines with 1 μM of Set 1 at 14.4 J/cm² light dose for 1 hour and 24 hour incubation. As the assay took 10 days, it was clear evidence that treated cells could not survive PDT treatment in both cell lines.

In the second part of the thesis, Set 2 phthalocyanines, two phthalocyanines were examined to determine the effect of substitution bulkiness. ZnPc-tBu was the bulky phthalocyanine, and ZnPc-nBu was the non-bulky phthalocyanine. Uv-vis absorption studies showed that ZnPc-nBu has a higher absorption peak (717 nm) than ZnPc-tBu (692 nm) in DMSO. Compounds of Set 2 could not achieve to cause phototoxicity on treated cells when they dissolved in DMSO. Set 2 in DMSO also could not show any differences against the non-treated control cells in other conducted *in vitro* assays within the scope of this thesis. However, uptake experiments showed that the compounds also enter into cells. This high fluorescence observation of the Set 2 compounds in uptake experiments may be explained by their aggregation of them on the cell membranes or the compounds enter the cell but can not be activated. To ensure the performance of the compounds, they were formulated with PVP in different ratios. 1 micromolar phthalocyanine: 100 milligram PVP ratio was chosen for *in vitro* cell viability assays of Set 2. After 1 hour and 24 hour incubation with formulated Set 2 compounds at 10 μM , cells were subjected to 14.4 J/cm² light dose. And the results showed that PVP helped one of the compounds, ZnPc-tBu, to cause significantly high cell death on both of the cell lines in all subjected PVP ratios.

Overall, the results of the ZnPc-4PEG, PdPc-4PEG, and PVP-formulated ZnPc-tBu are very promising, which can lead this study to further investigations, such as cell line-derived xenograft studies and animal studies.

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BIOGRAPHY

Eda Gazel Pehlivan received her B.Sc. degree in the department of Biology Teaching at Hacettepe University in 2010. She then joined the MSc. program at the department of Biology at Middle East Technical University and received her M.Sc. degree in 2013. She has been a part of a Ph.D. program in Molecular Biology and Genetics at Gebze Technical University under the supervision of Prof. Dr. Uygur Halis Tazebay.



APPENDICES

Appendix A: Calibration Curves Samples of Set 1 and Set 2

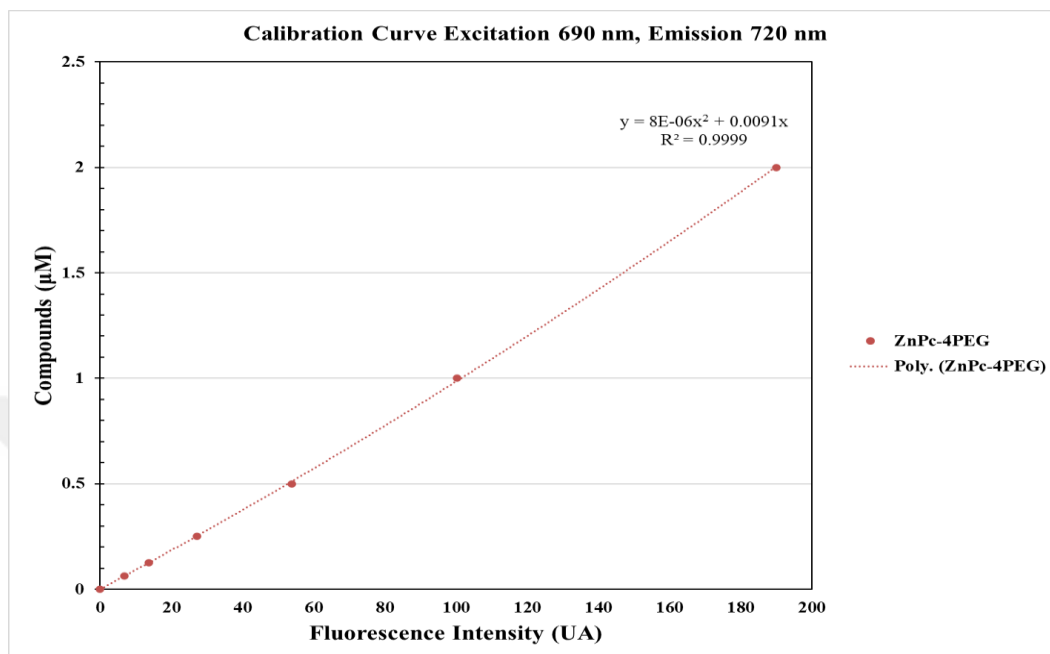


Figure A1.1: Calibration curves sample of ZnPc-4PEG in DMSO.

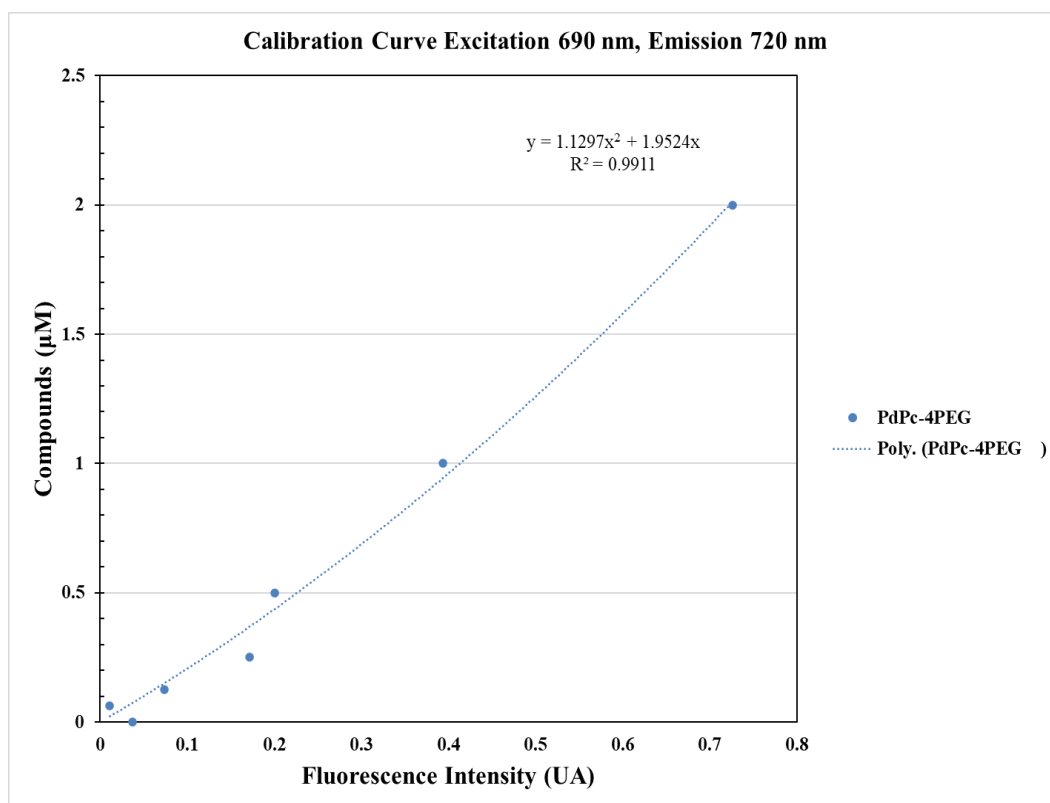


Figure A1.2: Calibration curve sample of PdPc-4PEG in DMSO.

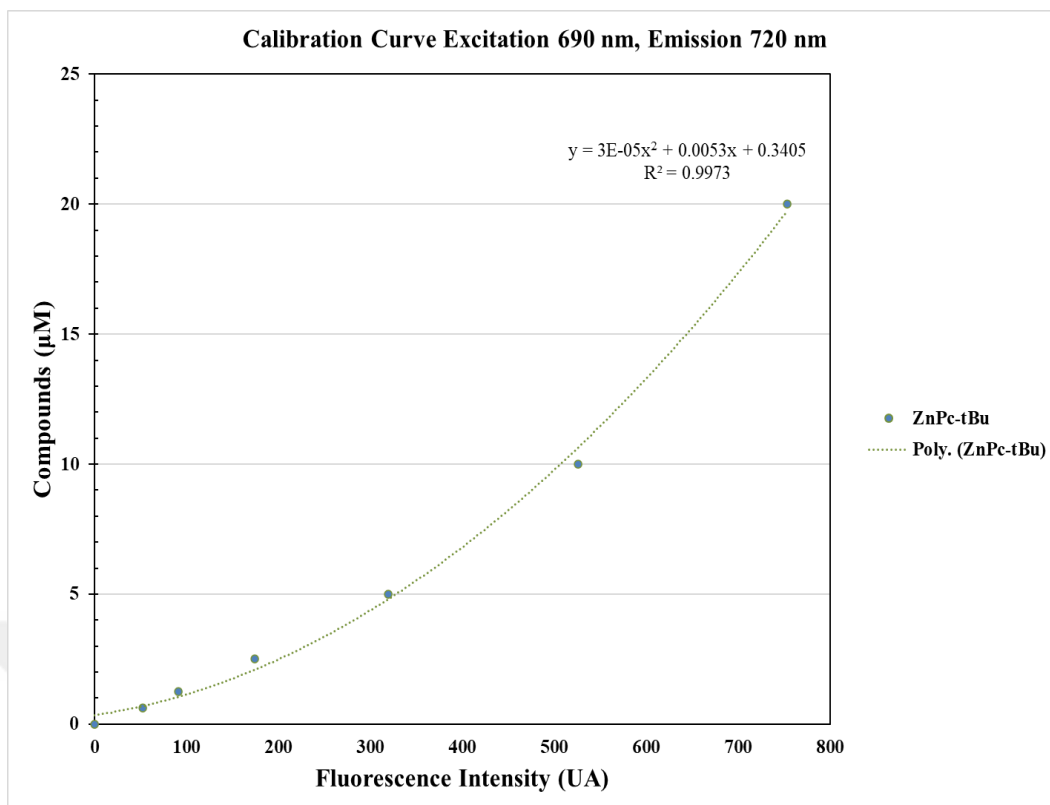


Figure A1.3: Calibration curve sample of ZnPc-tBu in DMSO.

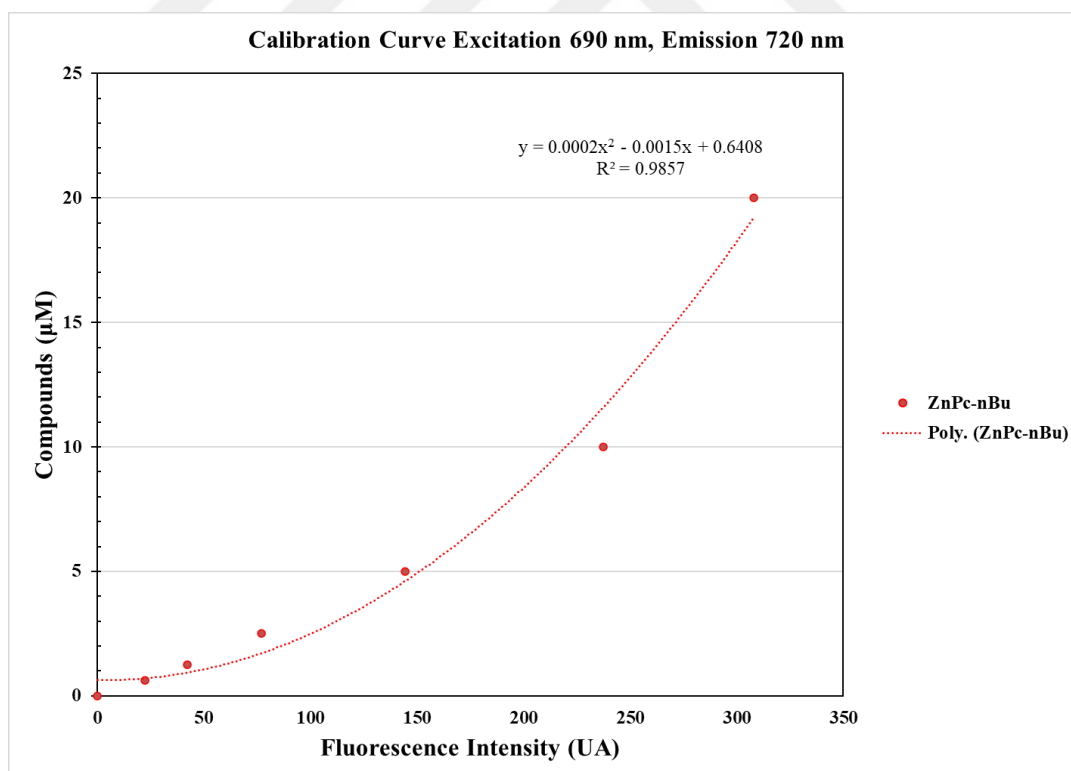


Figure A1.4: Calibration curve sample of ZnPc-nBu in DMSO.

Appendix B: UV-vis Absorption Spectra of PVP in Water

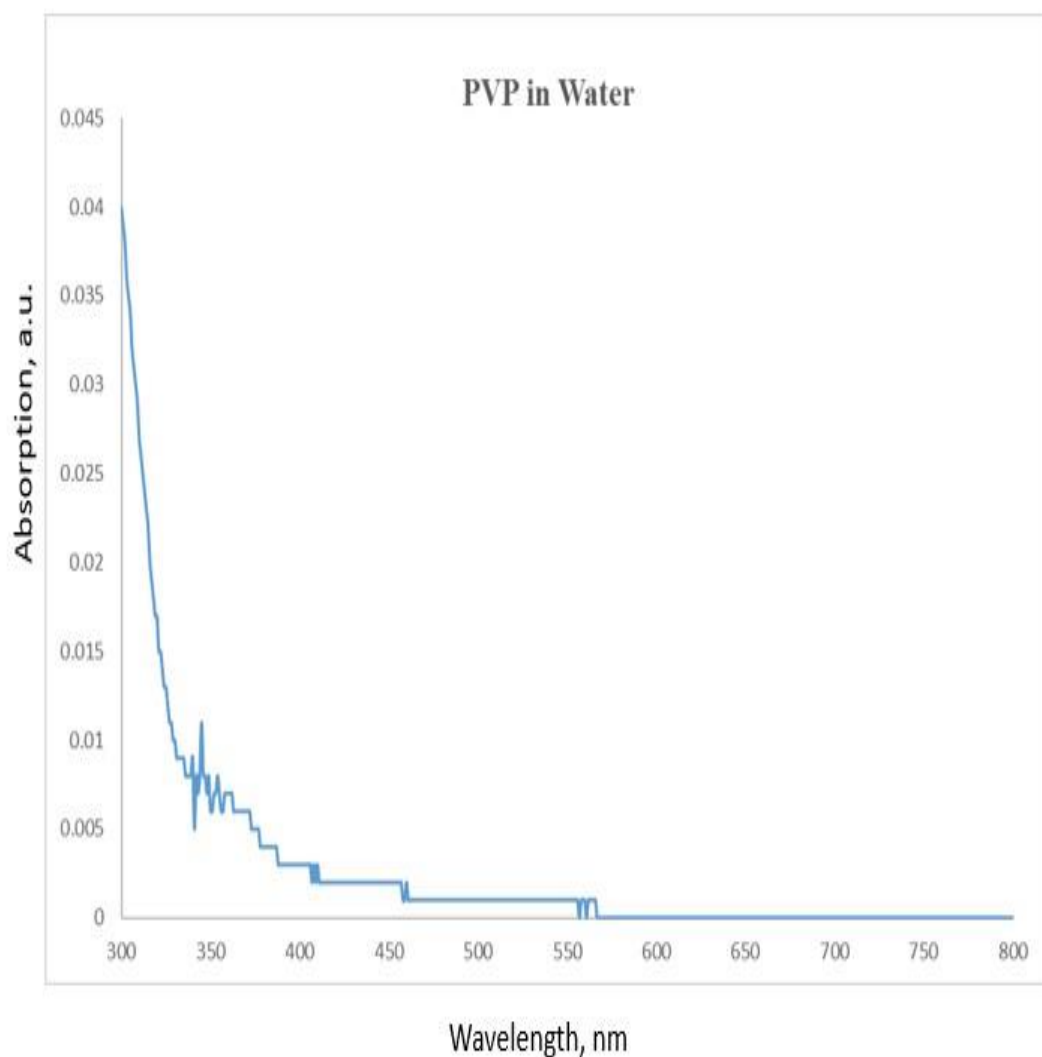


Figure B1.1: UV-vis absorption spectra of PVP in water (4,843.75 mg/ml PVP).

Appendix C: The Paper Published within the Scope of the Thesis

Pehlivan E. G., Ek Y., Topkaya D., Tazebay U. H., Dumoulin F. J., (2019), "Effect of PVP formulation on the *in vitro* photodynamic efficiency of a photosensitizing phthalocyanine", J. Porphyrins Phthalocyanines, 23, 1587-1591.