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Oxidative Stress-Based Photodynamic Therapy with Synthetic Sensitizers and/or Natural Antioxidants

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Abstract

Photodynamic therapy (also called PDT, photoirradiation therapy, phototherapy, or photochemotherapy) as a new modality for cancer treatment involves the combination of light with a photosensitizing drug in an oxygen-rich environment. PDT consists in the systemic administration of a photosensitizer (PS), which is selectively retained by tumor cells and the subsequent irradiation with visible light of the affected area. Basically two types of reactions can occur after photoactivation of the photosensitizer: the first involves the generation of free radicals (type-I photochemical reaction) and the other the production of singlet molecular oxygen, (type II) as the main species responsible for cell inactivation. The mechanism of PDT effects may involve a direct tumor cell injury and also an indirect cell killing via microcirculatory changes resulting in reduced blood flow in the tumor. Also, PDT may be considered an oxidative stress that induces cellular death in different types of cancerous cells both in vitro and in vivo. Oxidation or oxidative stress leads to the production of free radicals. Generated reactive oxygen species (ROS) cause oxidative stress in the cells targeting mainly at cellular macromolecules, such as lipids, nucleic acids and proteins. These oxidizing agents can damage cells by starting chain reactions such as lipid peroxidation, or oxidation of proteins or DNA, causing mutations and even major diseases, while protein oxidation can lead to distortion and degradation. To evaluate the presence of oxidative stress in PDT, some methods could be used: detection of malondialdehyde (MDA), the carbonylated proteins (CP), the hydrogen donating capacity (HDA), detection of the -SH groups. By reducing basal levels of ROS in cells may facilitate the therapeutic effects of oxidative stress-based therapies. Natural

antioxidants may also protect healthy tissues and lower the incidence of treatment-related side effects, and the chapter shows some results about hesperidin, hesperitine, diosmin and also, some tea extracts based on tea leaves and fruits. The Green tea polyphenols have been shown to have a protective effect in different forms of cancer in a variety of pre-clinical animal models. Green tea is composed of several catechins, including (-)-Epigallocatechin-3-gallate (EGCG), epicatechin (EC), epicatechin-3-gallate (ECG), and epigallocatechin (EGC). Among them, (-)-Epigallocatechin-3-gallate (EGCG), the major catechin found in green tea, has been recognized as a potential therapeutic agent. In the context where many clinical studies with respect to the application of antioxidants as sensitizers are lacking, this chapter shows a systematic review by putting into evidence the antioxidant action in photochemotherapy and their comparison with synthetic sensitizers (porphyrins and phthalocyanines). Some up-to-date results of photodynamic therapy with synthetic sensitizers and/or coupled with some natural antioxidants are shown and discussed.

Keywords: photodynamic therapy, sensitizers, natural compounds, antioxidants

1. Introduction

Photodynamic therapy (PDT) (also known as photoirradiation therapy, phototherapy or photochemotherapy) is a new modality for cancer treatment and involves the combination of light with a photosensitizing drug in an oxygen-rich environment [1, 2]. The photodynamic therapy has been successfully used in the last decade for the therapy of solid malignant tumors and non-tumoral diseases such as psoriasis, age-related macular degeneration, actinic keratosis, ageing [3]. The PDT mechanism could be a direct one (by apoptosis and necrosis), or an indirect one (by immune response on tumor vasculature). An ideal sensitizer should have a defined pure chemical structure, a proper absorption in red/near-infrared region, and a good capacity of singlet oxygen generation. After irradiation, the photosensitizer (PS) passes into the singlet oxygen excited state and afterwards it can pass into the triplet state; in this state it can react with molecular oxygen, generating singlet oxygen or it can react with different molecules from the tissue generating radical forms of PS [4]. This final form can react with oxygen, leading to the formation of different reactive oxygen species such as the hydroxyl radical, hydrogen peroxide and the superoxide anion, which in their turn may oxidize the macromolecular cellular components, resulting in cellular death through either apoptosis or necrosis. PDT induces oxidative stress at specific subcellular sites through the light activation of organelle-associated photosensitizers, and is used in the destruction of tumor cells.

This chapter offers the most up-to-date results of photodynamic therapy with synthetic sensitizers and/or coupled with some natural antioxidants, by using our data correlated with literature reports.

2. General concept of photodynamic therapy

Photodynamic therapy (PDT) is a relatively new type of treatment for cancer, and makes use of a photosensitizer, visible light and molecular oxygen. Reactive oxygen species (ROS) are generated, causing the death of tumor cells by apoptosis or necrosis. Significant research efforts are nowadays focused on finding new photosensitizers with antineoplastic activity.

Over the last decade, there is an increasing interest in photosensitization mechanisms in biological systems, in relation to the therapeutic aspects of this phenomenon [5, 6]. The most used photosensitizers in photodynamic therapy are porphyrins, phthalocyanines and related compounds (Figure 1). These compounds are capable of generating a long-lived triplet excited state, responsible for facile energy transfer to molecular oxygen, leading to the formation of singlet oxygen. The photosensitization reactions include free radical reactions (type I) and singlet oxygen reactions ($^1\text{O}_2$) (type II) (Figure 2). The mechanism of PDT may lead to a direct tumor cell injury and also an indirect cell death via microcirculatory changes, resulting in reduced blood flow to the tumor [7-10].

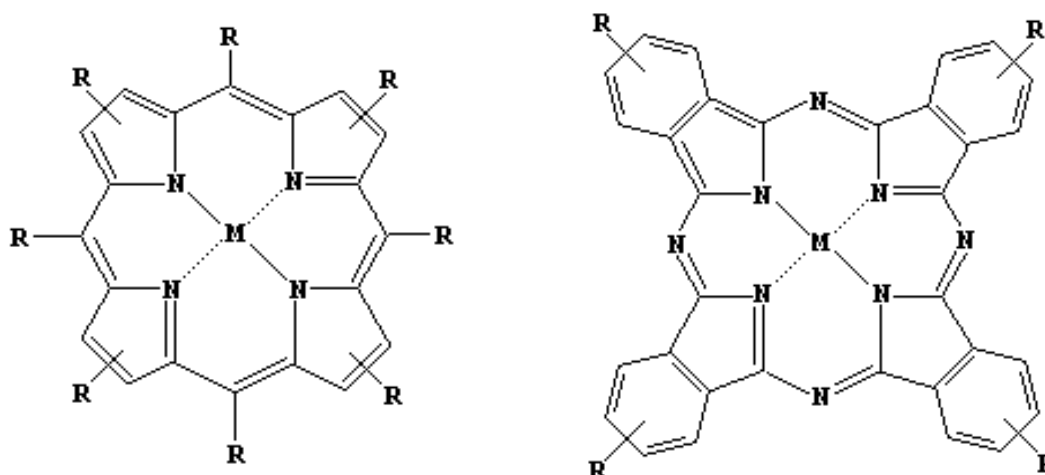


Figure 1. The structure of porphyrin (left) and phthalocyanine (right). (R=meso-substituent; M=central metal)

The study of radical-induced damage in living systems is a topic of great interest in biology and medicine. Attention has been increasingly focused on the role of free radicals in normal physiological conditions and in different pathologies, with an essential role in cellular processes resulting in damages [11].

3. Mechanisms of photodynamic therapy

3.1. Photophysical and photochemical mechanism

Porphyrins are used in medical and biological applications as they can generate new sensitizer structures with extensive and versatile photophysical and photochemical properties. Synthetic

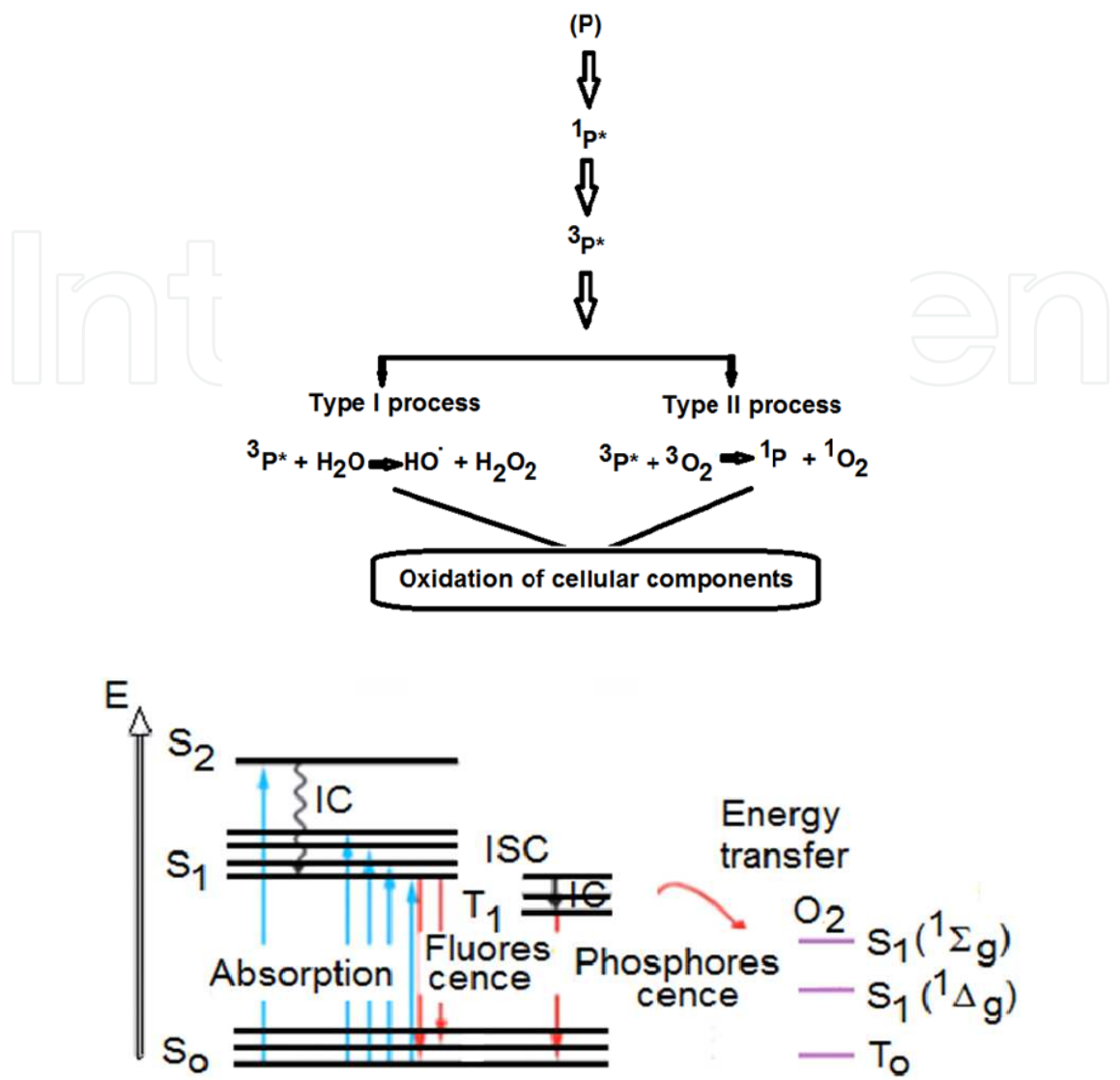


Figure 2. The dual (up) and singlet mechanism (down) of PDT

porphyrins and phthalocyanines appear to be good models in order to create new efficient drugs for photodynamic therapy of cancer [12]. The photodynamic therapy of cancer is based on the ability of some sensitizers to be retained in larger amounts and longer time in neoplastic than in normal cells as well as on the possibility to generate singlet oxygen after the activation of the porphyrin with red light [13].

In our previous investigations on PDT sensitizers [14, 15], we have used several spectroscopic methods to evaluate the efficiency of triplet state generation of some sensitizers [16]. The absorption spectra of the dye incorporated into the cells provide information with low accuracy because of small absorption amplitudes and perturbation of the spectra by light scattering. Therefore fluorescence methods are widely used [17]. The fluorescence of photosensitizers observed under a confocal microscope enables to establish the localization of dye in the cells [18–20]. Changes in the fluorescence of cell material due to illumination of samples depend on

the sensitizer localization in the cells, but for a group of similar dye molecules, the cells, stained to a higher degree, usually show higher fluorescence intensity [21].

3.2. Photobiological mechanism

Within PDT, a sensitizer, light and oxygen are used to cause photochemically induced cell death via apoptotic pathways. In vitro and in vivo photosensitizing efficacy of certain synthesized photosensitizers are usually investigated by their interaction with some proteins (human serum albumin (HSA) (Site II) for binding affinity, intracellular localization and DNA attack [22, 23].

The photobiological mechanisms by which some sensitizers induce tumor necrosis are very different: they strongly associate with serum albumins and it is assumed that they kill neoplastic cells indirectly by damaging blood vessels and interrupting the supply of oxygen [24]. A good example is water-soluble porphyrins, tetrakis (4-sulfonatophenyl) porphyrin (H_2TSPP) as one of the best tumor localizer. It has a very low affinity for human plasma lipoproteins but binds strongly to human serum albumin (HSA), Figure 3. The association between H_2TSPP and HSA has a spectacular effect on the singlet-state lifetime of porphyrins: it decreases from 5.2 ns to about 1.6 ns [25]. This marked decrease is due to the enhancement of one of the molecular processes involved in the deactivation of the singlet state. The shortening of the singlet state lifetime causes an important decrease of the quantum yield of triplet state (ϕ_T). This assumption is in agreement with the result of Davila and Harriman [26] that measured the ϕ_T of the complex H_2TSPP -HSA and reported a value of 0.70. The shortening of the singlet state lifetime of this complex is compensated by a corresponding increase of the intersystem crossing rate. The photosensitizing efficiency of bound H_2TSPP has a reduced mobility, characteristic of molecules bound deeply into the protein structure. Such molecule is expected to be less accessible by oxygen and thus less efficient in producing singlet oxygen. The number of binding sites per protein molecule is greater than unity ($n=3$ and $k=1.5 \times 10^6 M^{-1}$ for one binding site and $k=2 \times 10^5 M^{-1}$ for the other two). The binding of H_2TSPP to HSA significantly modifies the photophysics of porphyrin.

On irradiation with light (laser), in the presence of a photosensitizer, DNA undergoes several modifications including chain breaks, DNA-protein cross-links, and basic sites changes coupled with oxidized DNA bases, which have been shown to be able to induce point mutations [27]. The single strand breaks (SSB) are mainly formed through the attack of OH radicals, where the hydrogen bonds between purine bases play an important role. OH radicals react with DNA to remove an H atom, leading to strand rupture. Mitochondria play a central role in the control of apoptosis induced by PDT, which through the mitochondrial permeability transition pore (PTP) lose the integrity of the outer mitochondrial membrane, thus releasing the intermembrane proteins, such as cytochrome *c*, into the cytosol to form the "apoptosome". The apoptosome attacks procaspase-3 and cleaves it to form active caspase-3, which can lead to DNA breakage and nuclear chromatin condensation and cause cell death [28].

The porphyrins are able to induce changes of melting points and viscosity of DNA, which will be fragmented into small chains (Table 1). In this case, two modes of interaction between dye

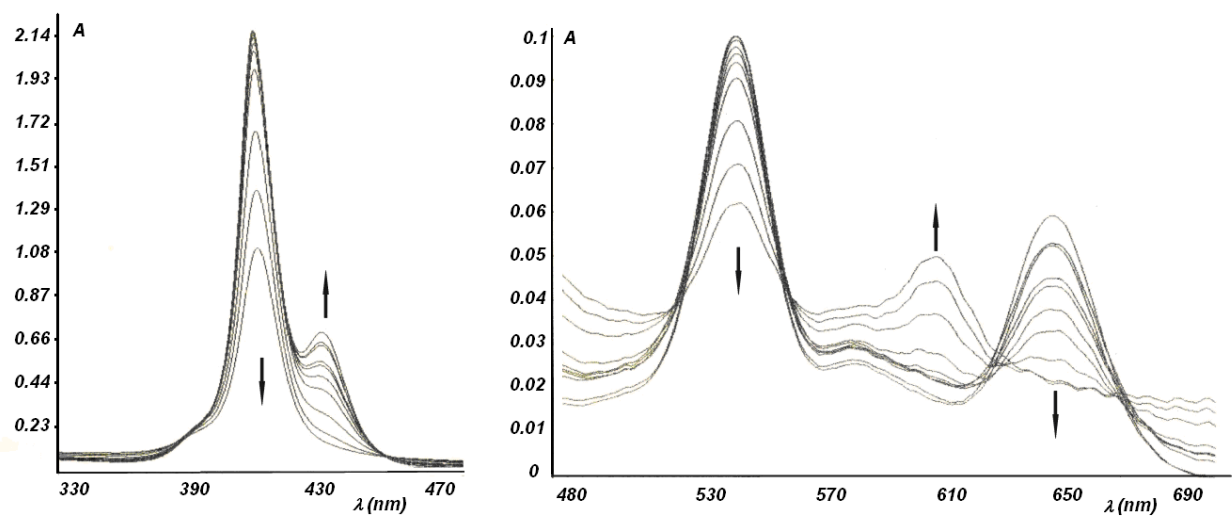


Figure 3. The changes of absorption spectra during the interaction between H₂TSPP and HSA

molecules and DNA are distinguished: a strong binding mode involving about 20-23% of the DNA phosphate groups and a weak binding mode involving the remaining phosphate groups.

DNA (50 µg/ml)	DNA (25 µg/ml)			DNA + TSPP (25 µg/ml + 8 µg/50 ml)		
Sample	1	2	3	4	5	6
Irradiation time (min)	0	0	0	30	45	60
Flow time (min)	49,85	49,38	49,46	48,86	48,61	48,36
η rel	1,009	1	1,002	0,989	0,984	0,979

Table 1. The temperature and viscosity changes of DNA during the photodynamic process

An analysis on DNA degraded by sensitized attack of methylene blue (MB) showed that it had 80% of the guanine residues [29], and similar value for hematoporphyrins and furrocoumarins [30]. Apoptosis was evident in the post-PDT cells through the TdT-mediated dUTP nick end labeling (TUNEL) method and DNA fragmentation assay. Apoptosis was determined by cell morphology with light microscopy and transmission electron microscopy [31].

The two known modes of interaction between dye molecules and DNA are a strong binding mode involving about 20–23% of the DNA phosphate groups and a weak binding mode involving the remaining phosphate groups [32].

Based on the interaction sensitizer-DNA, three groups of porphyrins are known:

- Group I porphyrins, which induce changes characteristic of intercalation in DNA samples with greater 40% GC base composition with an increasing of the linear viscosity of CT DNA,

strongest intercalative binding in GC regions and the localization of AT regions only outside binding.

- Group II porphyrins are believed to be outside binders, with an additional self-stacking features that induce DNA aggregation and a small viscosimetric changes of DNA with greater than 40% GC content.
- Group III porphyrins give results characteristic of outside binding at both AT and GC sites, identified by the absence of an increasing of the solution viscosity for any of the linear or super helical DNA samples; they being able to bind with a preferential orientation.

Cationic porphyrins can interact with DNA either by intercalation between the G–C base-pairs or by outside binding in the minor groove with A–T sites selectively. An exemplification has been checked by personal data for cytosine (C) and guanine(G) by using as sensitizers methylene blue (MB), H₂TSPP and Rhodamine (Rh) (Figure 4).

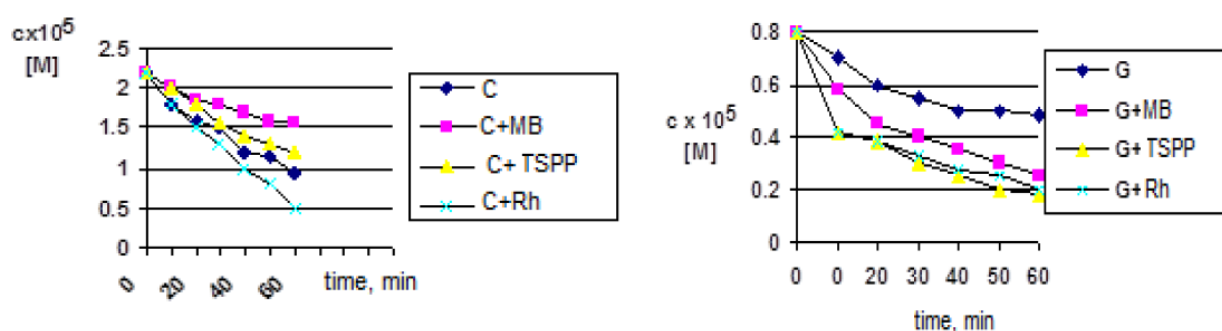


Figure 4. Cytosine (C)- and guanine(G)-sensitized photooxidation by using as sensitizers MB, H₂TSPP and Rh

The weakly bound dye molecules are identified to be attached to the helix exterior by means of electrostatic interaction with the phosphate groups. A red-shifted absorption is due to porphyrin–porphyrin interactions within long-range assemblies on the polymer in exterior. When the porphyrin is intercalated, a strong hypochromicity and a strong bathochromic shift for the absorption bands are observed. As a possibility of external association a small hypochromicity (or even a hyperchromicity) and a small bathochromic shift are observed. If an external association is possible in concert with an aggregation process, only hypochromicity (or hyperchromicity) is important [28]. Some important conclusions had been stated: porphyrins intercalate in DNA with a binding constant of around 10^3 – 10^6 M⁻¹; the porphyrin complex appears to be stabilized by extensive electrostatic interactions, especially in the minor groove; the porphyrin does not form van der Waals stacking contacts with adjacent bases; DNA may require conformational distortion reaching the limit of DNA melting; the resulting severe conformational distortion not resolved upon achievement of the ground-state complex; steric clashing between the DNA backbone and the porphyrin extending the DNA along its helical axis; and de-stacking the interior of the complex.

The aggregation process is very important in this interaction study. If the sensitizer is in a monomer form, the external association with DNA could be favored. The association process

increases its rigidity and changes the distance between the bases creating the adequate distances unfavorable to the dimerization. The Soret band is bathochromically shifted from 412 nm to 433 nm, and the Q-band decreases its intensity. New bands appear at 644 and 707 nm. Also, a new band appears at 490 nm (Figure 5). The band at 490 nm arises from the J-aggregate (edge-to-edge interaction) of porphyrin molecules [33]. Their structures have been identified by atomic force microscopy (AFM) (Figure 6). The porphyrins are externally bound when their planar structure fit into the major or minor groove and interact with DNA electrostatically. Also, hyperchromicity is observed (the greatest absorption value at 260 nm), which is similar with some mono- and di-catenare destructions. It could be presumed that during the laser irradiation support a photooxidation reaction takes place preferably at guanine site. By irradiation with DNA, an increase of viscosity is observed as an effect of a chain-breaks and helix destruction.

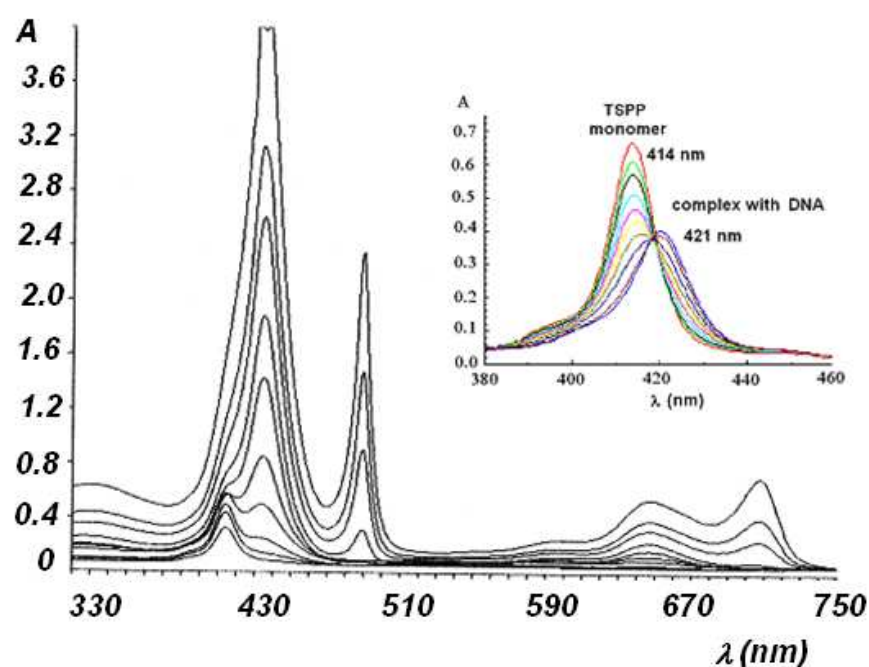


Figure 5. The changes of absorption spectra during the interaction between H_2TSPP and DNA

The fluorescence emission spectra of H_2TSPP , DNA and their complex consist of two bands with maxima little affected by DNA; however, the emission intensity is markedly reduced (Figure 7). For H_2TSPP , in the presence of DNA, the maxima from 605 and 640 nm increase simultaneously, as a proof for the external binding between both compounds. From the amplitude ration I_{604}/I_{640} , it was concluded that the porphyrin/DNA this ratio increases without irradiation and by irradiation with laser beam, this ratio registered a significant increase at the high time of irradiation. A spatial arrangement of porphyrin and DNA takes place on the DNA strand exterior based on electrostatic interaction between phosphate groups of DNA and positive charges of J-aggregated porphyrin. If H_2TSPP has a J-aggregate form, it is possible to cover DNA molecule like two concentric bodies. DNA has a 20 Å diameter, while H_2TSPP -J-aggregate has 0.35–0.40 Å interplanar distances and a 40 Å diameter [34].

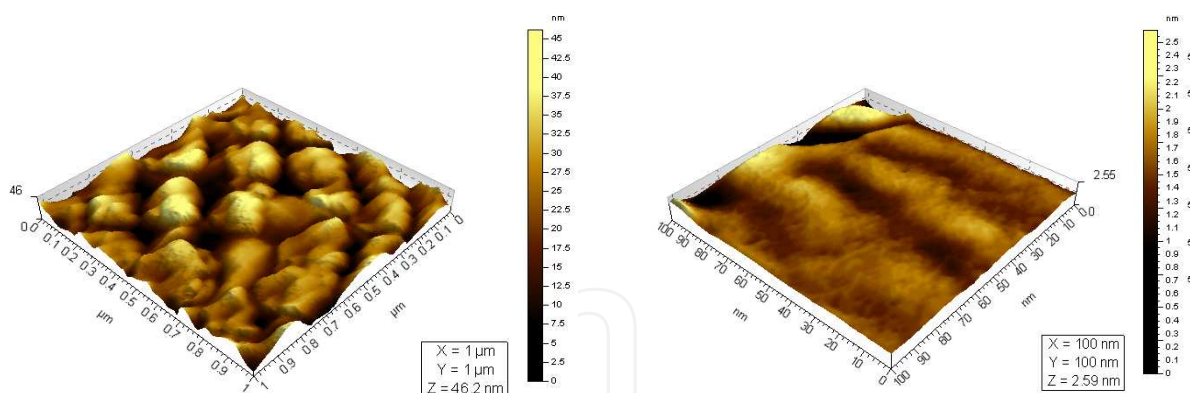


Figure 6. The structure of H₂TSPP in monomer (left) and J-aggregate (right) form

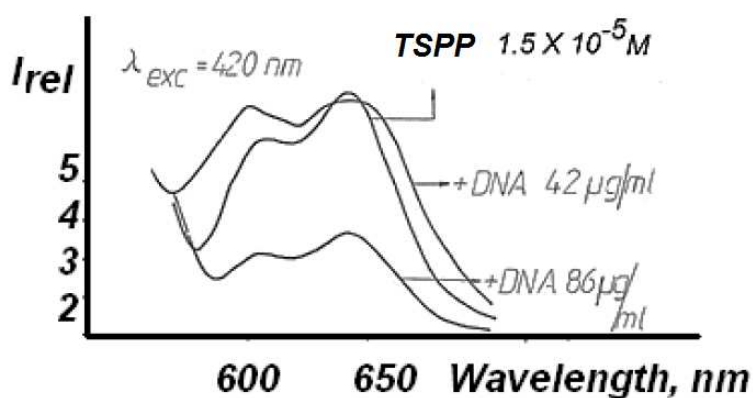


Figure 7. The fluorescence of H₂TSPP and its mixture with DNA at different concentrations.

4. Photosensitizers

4.1. Conventional photosensitizers

The most used and most efficient sensitizers are synthetic compounds, as porphyrins (P) and phthalocyanines (Pc).

Porphyrins constitute a class of the molecules that contain four pyrrole rings linked by the methane carbon bridges. A large group of porphyrins can be chemically modified by introducing the metal at the center of the pyrrole rings or by attaching the peripheral groups to the outer rings of the methane bridges units, respectively. The main characteristic of free-base porphyrin consists in the absorption maxima: a Soret band around 400 nm and four Q-bands in the region of 500–700 nm. Although the porphyrins absorb light poorly in this wavelength region (650 nm, $\epsilon = 30000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [35], as a result of increased transparency of biological tissues at longer wavelength; red light is normally used for PDT. Some exemplification of porphyrins and phthalocyanines are shown in Figure 8.

Phthalocyanines (Pc)/naphthalocyanines (Nc) are molecules composed of four indole units: pyrrole rings linked by nitrogen atoms conjugated with benzene rings characterized by a strong, isolated Q band in the red region of the UV/Vis spectrum, while the less intense B band is found at higher energies (Figure 9). The Q band is characterized by a high molar absorptivity ($\epsilon = 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [36], and it is accompanied by a series of vibrational bands. The B band is broad due to the super-positioning of B1 and B2 [37].

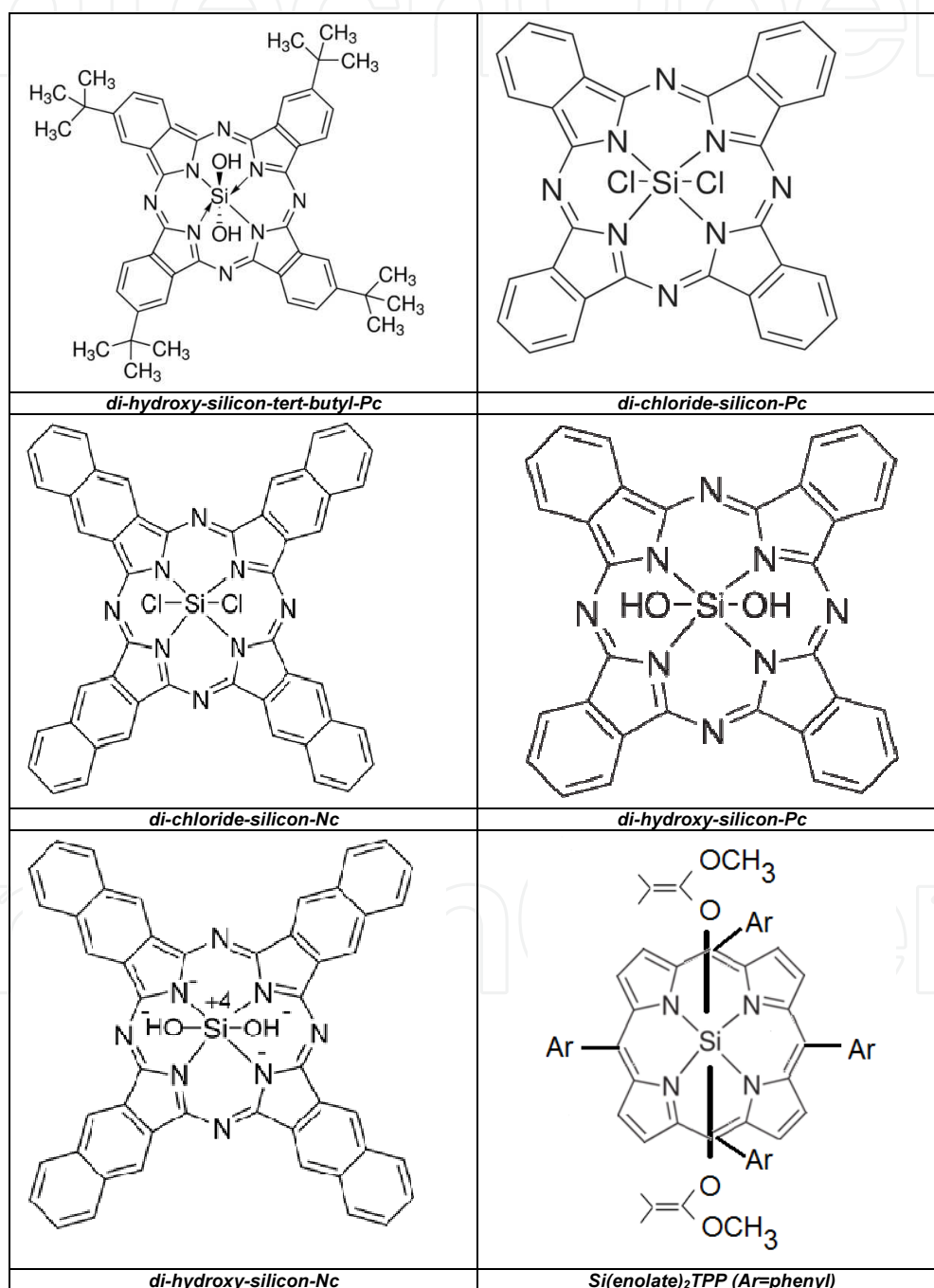


Figure 8. The structure of some SiPc, SiNc and SiTPP

Porphyrins act as free bases and chelated with a variety of metals, the diamagnetic ones enhancing the phototoxicity. Paramagnetic metals shorten the lifetime of the triplet state and as result can make the dyes photoinactive [38]. The photosensitizing activity is quenched by the presence of transition metal ions (as central ions) with a d-electron configuration [39]. An exemplification is Si(enolate)₂ 5, 10, 15, 20-tetra-*p*-phenyl porphyrin (Si(enolate)₂TPP) (Figure 8).

The presence of axial ligands to the centrally coordinated metal ion is often advantageous, since it generates some degree of steric hindrance to intermolecular aggregation, without impairing the photophysical properties of the dye. Several photophysical parameters for tetrasulfonated aluminum porphyrins (Table 2) have been evaluated by means of some in vitro experiments on EL-4 cells [40].

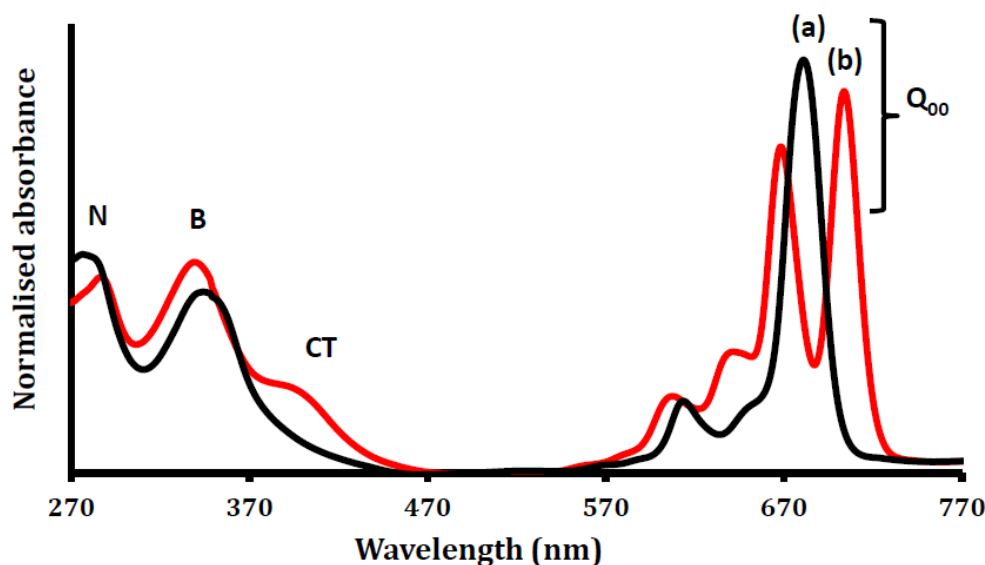


Figure 9. The spectra of Pc as free base (a) and metallic complex (b)

Drug	absorption	excitation	fluorescence ($\lambda_{ex} = 427\text{ nm}$)	phosphorescence ($\lambda_{ex} = 427\text{ nm}$)	Partition coefficient
C ₂ AITSP	215; 230; 263; 266; 432; 570; 603; 645; 827	236; 254; 300; 330; 368; 438; 564; 644	344; 425; 588; 651; 676; 708; 854;	950; 1075	0.23
C ₈ AITSP	212; 222; 264; 428; 564; 606; 645	250; 300; 422; 564; 608	426; 598; 652; 826; 706; 776;	950	0.37
C ₁₂ AITSP	212; 222; 264; 428; 564; 606; 645	442; 644	426; 598; 652; 856	1075	0.32

Table 2. Spectral properties and data for partitioning coefficients of some metallo-porphyrins

The quantum yield for singlet oxygen generation was evaluated in this experiments by using DPBF method [41], and an exemplification is shown in Table 3.

Porphyrin	¹ O ₂ quantum yield	Lifetime for singlet excited states of porphyrins (ps)
C ₂ AITSP	0, 96	5.03
C ₄ AITSP	0.78	40, 8
C ₈ AITSP	0.83	32, 9
C ₁₂ AITSP	0.665	47, 5

Table 3. The singlet oxygen quantum yields and the lifetime values for the first excited states of Al porphyrins

The cellular uptake of different drugs seems to be correlated to their hydrophobicity only when the drugs are very closely related to chemical structure. Aluminum ion has a great influence on the sensitizer hydrophobicity without a corresponding effect on the cellular uptake (Table 4).

Porphyrin	<i>K</i> _{octanol}
TSPP	0.47
C ₂ AITSP	0.23
C ₈ AITSP	0.37
C ₁₂ AITSP	0.32

Table 4. The data for cellular uptake of the aluminum porphyrins

Nevertheless, C₂AITSP is not as well uptaken as H₂TSPP, even in the studied series, porphyrin is the most reactive. The uptake ratio of porphyrins in vitro increases when increasing the lipophilic property of the drug. The uptake of aluminum porphyrins increases linearly with increasing lipophilicity. The inactivation kinetics for EL-4 cells is shown in Figure 10 and their microscopic aspects are shown in Figure 11.

Apoptosis (type I cell death) is different from necrosis (type III cell death) [42, 45]. If apoptosis is a controlled suicide pathway, involving DNA breaks, membrane blebbing, cell shrinkage and phagocytosis, necrosis involves membrane damage, local inflammation and injury, which lead to oncosis. Except of these types, there is type II cell death, which is characterized by an enormous increase of two-membrane autophagic vacuoles in the cytoplasm, which are finally catalyzed by lysosomal hydrolases [43]. Autophagy is a convertible process, which can provoke both survival and death pathways, in contrast to the apoptotic irreversible process leading only to cell death. Apoptotic cell death is the most preferable effect of various anti-cancer therapies, which leads to destruction and elimination of pathological cells. Inflammation does not occur through apoptosis in cancer cells and surroundings tissue [44].

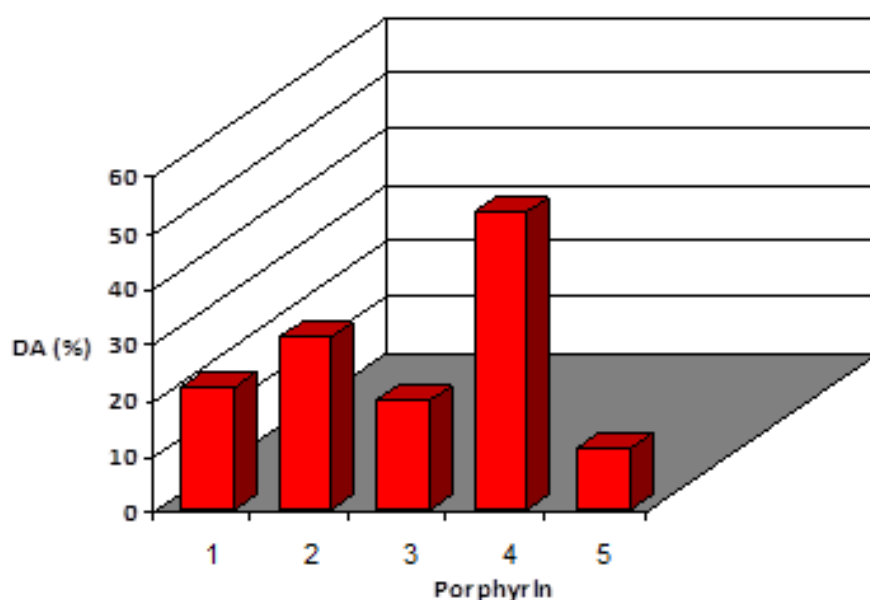


Figure 10. The inactivation diagram for EL-4 cells with aluminum porphyrins (1= $\text{AlC}_{12}\text{TSPP}$; 2= AlC_8TSPP ; 3= Al-i-BuTSPP ; 4= AlC_2TSPP ; 5=Control)

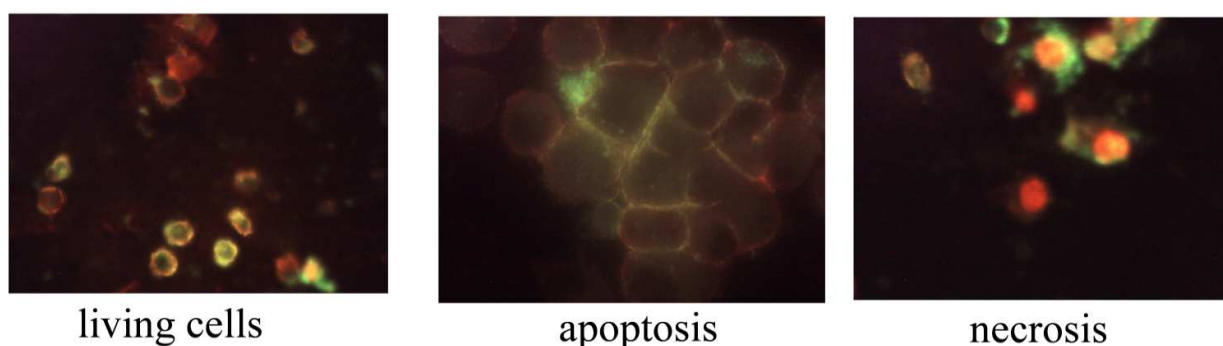


Figure 11. The microscopic aspects of cells before (left) and after PDT (middle and right)

5. Concept of oxidative stress in photodynamic therapy

5.1. Types of Reactive Oxygen Species (ROS)

PDT is a concerted action of a sensitizer, a light source in the presence of oxygen, based on singlet oxygen and ROS production, leading to cell death by different mechanism [46].

Reactive oxygen species (ROS) is a collective term used for a group of oxidants, which are either free radicals or molecular species able to generate free radicals. The most important free radicals occurring in the human body are hydroxyl radical ($\cdot\text{OH}$), singlet oxygen ($^1\text{O}_2$) and superoxide ($\text{O}_2^{\cdot-}$). Except these species, the intracellular generation of ROS mainly comprises nitric oxide ($\text{NO}\cdot$) radicals, which together with $\text{O}_2^{\cdot-}$ radicals, are converted to powerful oxidizing radicals like hydroxyl radical ($\cdot\text{OH}$), alkoxy radicals ($\text{RO}\cdot$), peroxy radicals ($\text{ROO}\cdot$),

singlet oxygen ($^1\text{O}_2$) by complex transformation reactions. Some of the radical species are converted into molecular oxidants like hydrogen peroxide (H_2O_2), peroxyxynitrite (ONOO^-) and hypochlorous acid (HOCl). The oxidative stress damage is targeted mainly at cellular macromolecules, such as lipids, nucleic acids and proteins. Hydrophobic photosensitizers accumulate mainly in cell membranes and they are primarily attacked by free radicals. These oxidizing agents can damage cells by starting chain reactions such as lipid peroxidation, or oxidation of proteins or DNA. Damage to DNA can cause mutations and even major diseases (cancer), while protein oxidation can lead to distortion and degradation. Oxidation or oxidative stress leads to the production of free radicals, for example the hydrogen and oxygen molecules, which are not related to other factors. In their free form these molecules react with other molecules, and contribute to oxidative stress [47-49].

Oxidative stress, arising as a result of an imbalance between free radical production and antioxidant defenses, is associated with damage to a wide range of molecular species including lipids, proteins, and nucleic acids. Oxidative stress is a factor that initiates cell death after photodynamic reaction [50-54]. The formation of reactive oxygen species (ROS) with cytotoxic effects is the key involved in the death of the tumor cells by PDT [55]. PDT may be considered an oxidative stress that induces apoptosis in different types of cancerous cells both in vitro and in vivo [56].

Cells have a highly developed and regulated antioxidant defense system to maintain appropriate intracellular ROS levels and prevent oxidative damage. This system includes antioxidant enzymes such as superoxide dismutase (SOD), catalase and various peroxidases and non-enzymatic systems (GSH, thioredoxin, uric acid, vitamins, coenzyme Q) that effectively remove ROS. Under normal conditions, antioxidant mechanisms scavenge ROS and protect the organism from the damaging effects of ROS. However, under conditions of excessive oxidative stress, cellular antioxidant mechanisms may be unable to prevent the adverse impact of ROS on critical cellular processes. ROS can interact with cellular macromolecules, including DNA, protein and lipids, and interfere with vital cellular functions [35]. Mutations caused by ROS can result in malignant transformation and the development of cancer [57].

To evaluate the presence of oxidative stress in the plasma, we used indirect methods. These quantify the lesions produced by the reactive oxygen species on the organism's biomolecules.

To evaluate the presence of oxidative stress in PDT, some of the following methods could be used [58-64]:

- Detection of malondialdehyde (MDA), the marker used most frequently for lipid peroxidation, using the fluorimetric method with 2-thiobarbituric acid (TBA);
- The carbonylated proteins (CP) with 2, 4-dinitrophenylhydrazine using the Reznick method. To determine the level of proteins from the homogenate samples Bradford method was used.
- The hydrogen-donating capacity (HDA) using the stable free radical: 1, 1 diphenylpicrylhydrazyl (DPPH);

- Detection of the –SH groups with 2, 2-dithiobisnitrobenzoic acid (Ellman's reagent). The zymographic method was used to determine the MMPs activity.

Some synthetic PSs such as porphyrins have the capacity to generate reactive oxygen species with cytotoxic effects leading to the necrosis of tumor cells, and induced a significant oxidative stress response, with peak intensity at 24-hour post-exposure. The most significant responses were the increase of CP, MDA, MMP-2 (matrix metalloproteinase-2) activity and the decrease in HDA levels. The experiment also induced less significant, but present, reduction of the thiol groups.

As an exemplification, PDT with H₂TSPP increases ROS production in plasma and tumor tissue and determines oxidative alterations of biomolecules (lipids, proteins) (Figure 12).

Oxidative stress, responsible for tissues injured in different pathologies, involves the nonequilibrium between the produced radical species and antioxidant defense agents.

There are some priority parameters which should be determined and evaluated:

1. Cell viability test measured by the lactate dehydrogenase release in the supernatant of cell culture as a marker of cell integrity. Viability results were expressed as % of live cells from the suspension subjected to 24 h incubation in the tested agents and/or irradiation.
2. Cell proliferation test measuring the number of viable cells as live cells reduce the kit reagent into a formazan compound that is colorimetric measured at 490 nm. From this point of view the test measures the quantity of viable cells in culture and thus the proliferative capacity of the tested cells.
3. RNA synthesis achieved by tritium-labeled uridine incorporation method, which involves beta-radioactivity measurement for radiolabeled cell cultures.
4. Total cellular RNA offer the concentration of total RNA, measured with SV total RNA isolation system.
5. TUNEL and immunofluorescence essential for apoptosis detection by observing the DNA fragmentation assay and TdT-mediated dUTP nick-end labeling (TUNEL) assay. TUNEL staining was performed to detect internal and end-strand breaks, which often occur in the early stages of apoptosis. TUNEL staining was carried out according to the manufacturer's instructions (Promega, Madison, USA). The procedure is carried out as follows. Biotinylated nucleotide is incorporated at the 3'-OH DNA ends using the terminal deoxynucleotidyl transferase (rTdT) recombinant enzyme. The apoptotic cells were counted under the microscope and photographed.
6. Analysis of genomic DNA fragmentation visualized by staining with ethidium bromide (0.5 µg/ml) and photographed under UV illumination. DNA was separated using standard 1.5% agarose (Bio-Rad) gel electrophoresis at 10 V/cm.
7. Cellular morphology visible by electron microscopy necessary for identifying the apoptotic cells. After PDT treatment, cells are developing morphological features characteristic of apoptosis: chromatin compaction into uniform electron-dense masses with

nuclear margination, nuclear fragmentation, cellular shrinkage, cell membrane vacuolization and blebbing, and the increase in electron-density of the cytosol [63-65].

8. Photodegradation reaction of sensitizers, which are indicators for lifetime of the sensitizers, time efficiency and potential side effects of them (Figure 13).

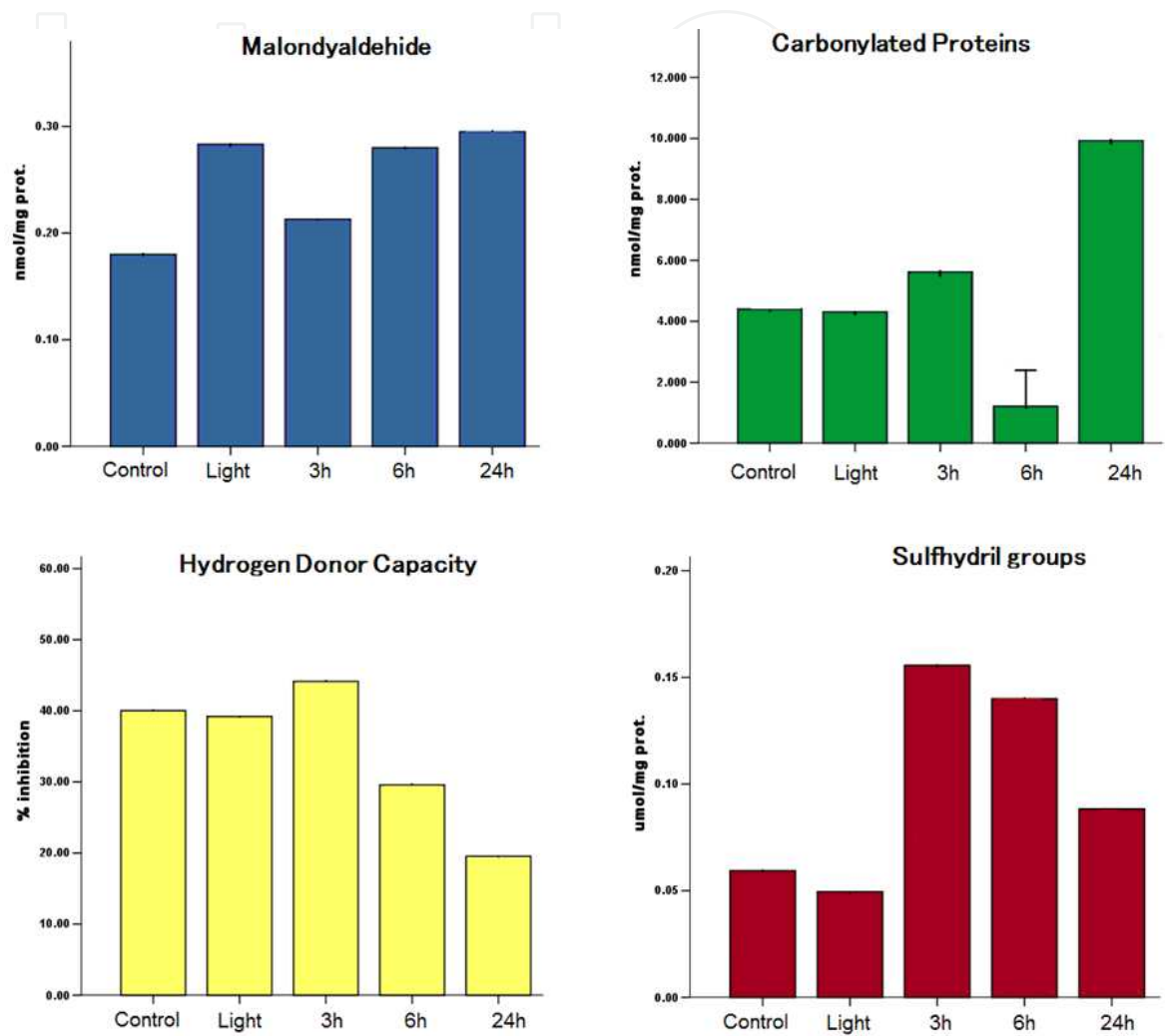


Figure 12. The oxidative stress parameters for PDT with H₂TSPP. Adapted after [55]

Some of these parameters have been calculated for PDT with different sensitizers and for different cells; B16, K562, EL-4, etc. [64–69]. During irradiation, after the first 30 minutes, a strong cellular degradation, especially for TNP-loaded cells, is visible (Figure 14). The results indicate that a free-base porphyrin as 5, 10, 15, 20-tetra-*p*-naphthyl-porphyrin (TNP) induces apoptosis in K562 cells in a time-dependent manner. There were no changes in tail moment of K562 cells in the absence of light, whereas TNP (10 µg/mL) leads to DNA damage at different time incubation (1–4 h), light dose (0, 10 or 25 J/cm²) and time irradiation (5–50 minutes). Less genotoxicity will appear for higher light dose that for higher sensitizer concentration. Unloaded cells subjected to irradiation have a slightly higher LDH release, 56% proliferation

capacity and 13% Urd incorporation compared to control cells. After 48 h of irradiation (Figure 14) cells have a high LDH release compared to control and a reduced proliferative capacity (100 %). After irradiation, the cells were subjected for 24 h, 48 h and 72 h to the LDH, MTS and uridine incorporation tests in order to study the capacity of remaining cells to proliferate after treatment. A significant reduction of RNA has been registered for TNP loaded and non-loaded cells (Figure 15). The viability of the irradiated K562 tumor cells was assessed by the increase of the LDH release and the associated decline of the MTS reduction (Figure 16). We also observed that cells that survive after the PDT procedure are more stable than the irradiated control.

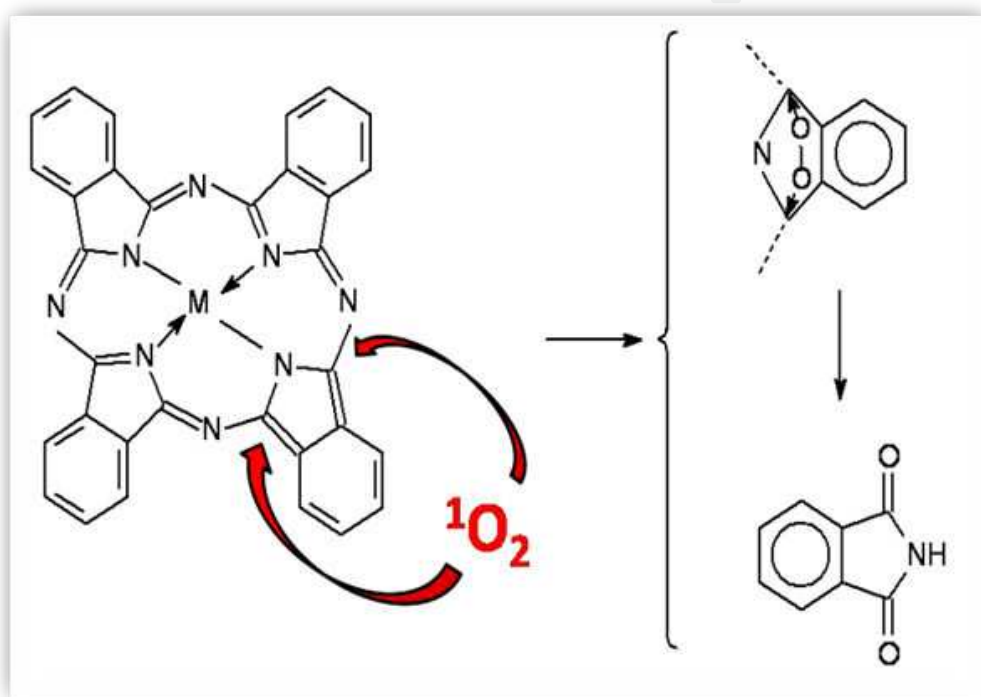


Figure 13. The photo degradation scheme of a sensitizer

After irradiation, the K562 cell viability is strongly affected both for TNP-loaded cells and for those unloaded. TNP activated increases the cell mortality by comparison with control cells (Mi) (Figure 15).

At different time post-irradiation, TNP intracellular loaded and activated shows a homogeneous effect on the cells' capacity to deliver LDH (Figure 16).

RNA isolation from K562 cells has been achieved in the first 4 hours after irradiation. A 35% decrease of RNA content has been registered in the case of H₂TSPP (Figure 17) [72], Also, there is a good correlation between uridine incorporation and number of live cells in 24 h post-irradiation in the presence of the same sensitizer, both of them decreasing (Figure 18). Also, the amount of total cellular RNA isolated from the K562 cells drastically decreased after irradiation (Figure 19).

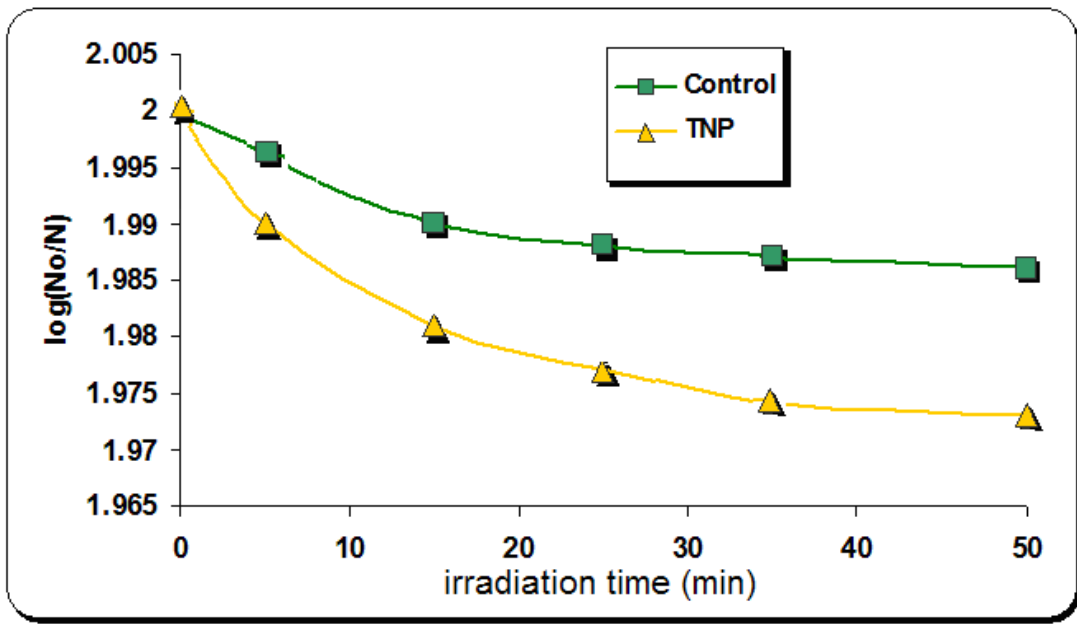


Figure 14. Time evolution of the cellular density for TNP-loaded K562 cells (10 µg/ml TNP)

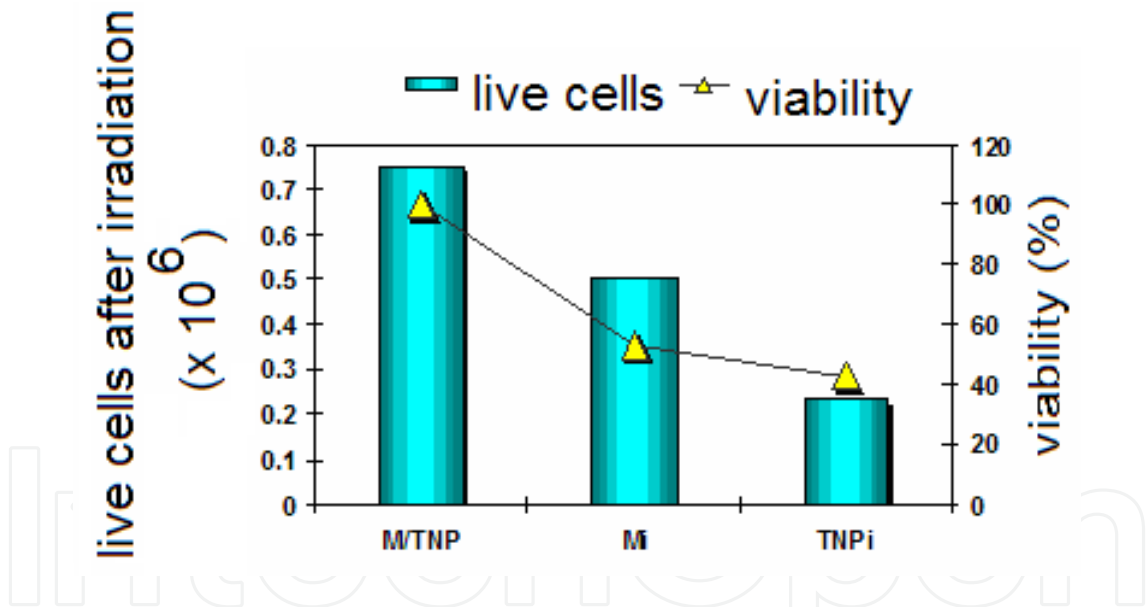


Figure 15. The viability of K562 loaded with TNP after the first hours after irradiation

At different time intervals after treatment, the cells were analyzed for caspase-3. At 4 h post-PDT, 100% of the cells displayed protease activities. After PDT, 30.6% of the cells showed total caspase activity. Thereafter, the fraction of cells with caspase activity increased to 48.6%.

When present in the cytoplasm, a number of caspases have been activated following PDT and responsible for the cleavage of multiple cellular proteins, DNA fragmentation, and cell death. Activation of procaspase-3 after PDT has been demonstrated in multiple experimental settings. The morphological manifestation of apoptosis (“execution” phase) can be ascribed as degra-

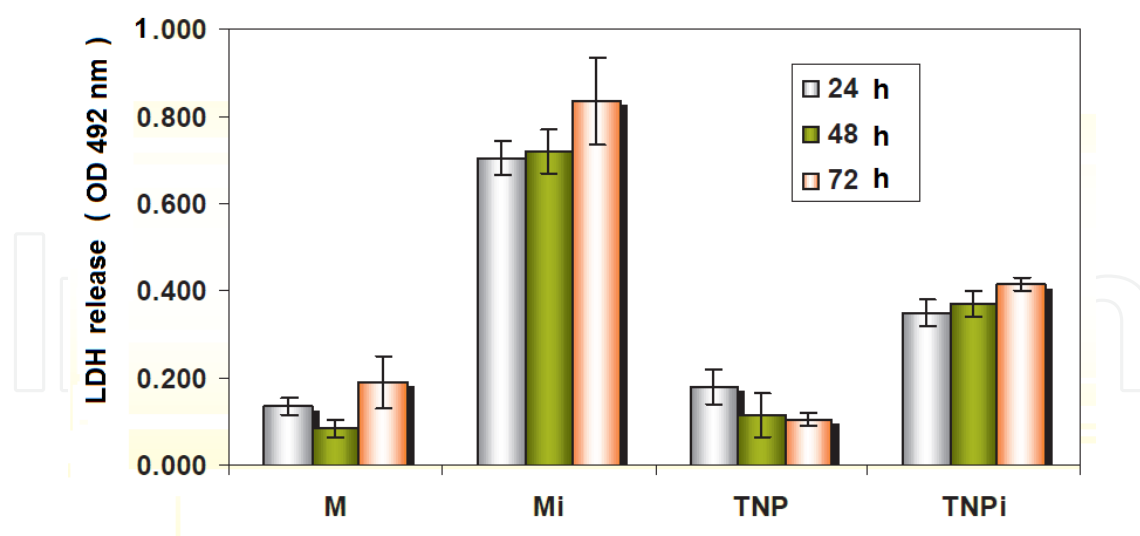


Figure 16. Time evolution of K562 cells viability after laser activation in the presence of TNP

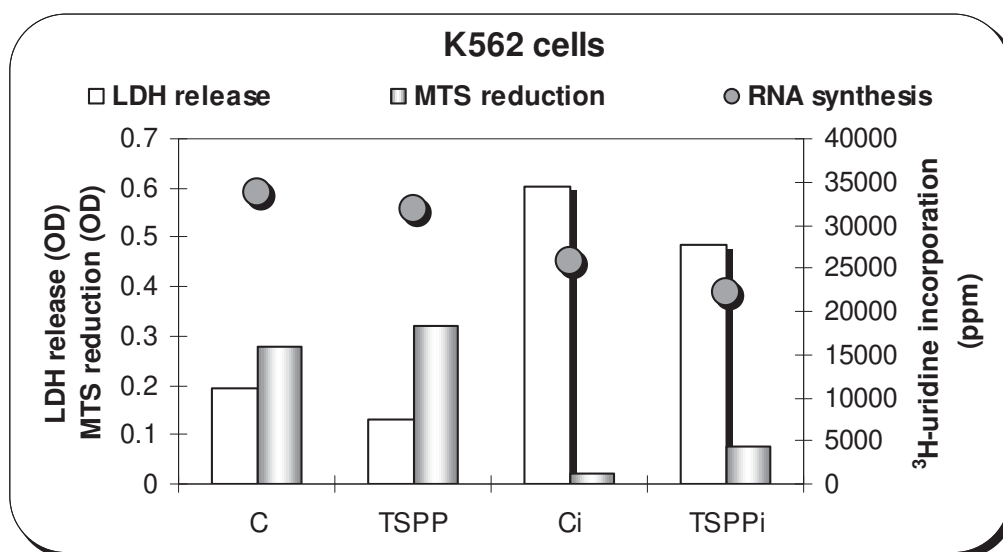


Figure 17. The viability and multiplication rate of K562 tumor cells at 24h post-irradiation. C = nonirradiated unloaded cells; TSPP = nonirradiated loaded cells; Ci = irradiated unloaded cells; TSPPi = irradiated loaded cells.

dation of various structural proteins and DNA. After loading K562 cells with TNP, and irradiation, a strong decrease has been obtained for caspase-3 activity (Figure 20).

Induced apoptosis of K562 cells loaded with 10 $\mu\text{g/ml}$ TNP and activated with laser (TUNEL) has been shown in Figure 21, as results of TUNEL test. Many apoptotic cells characterized with brown nuclei can be seen in the TNP-PDT groups.

The PDT efficacy relies on the concerted action of sensitizer and light, with none of them alone inducing apoptosis.

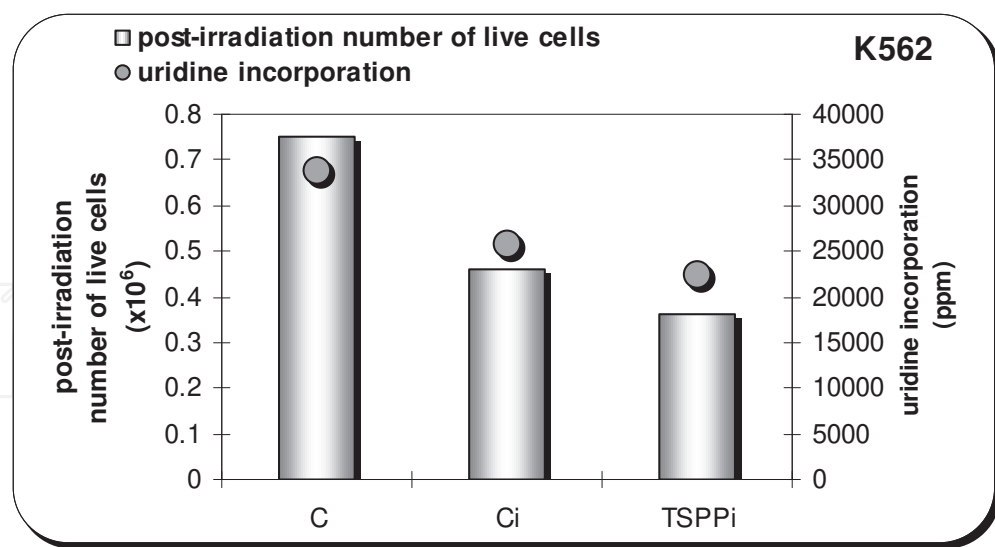


Figure 18. Correlation between uridine incorporation and number of live cells in 24 h post-irradiation. C = non-irradiated unloaded cells; Ci = irradiated unloaded cells; H_2TSPP_i = irradiated loaded cells.

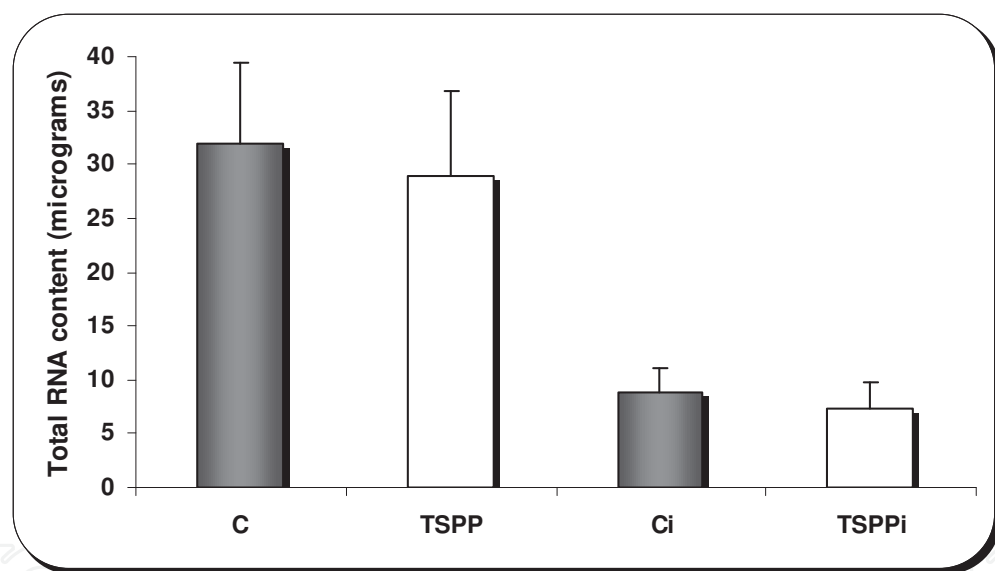


Figure 19. The amount of total cellular RNA isolated from the K562 cells. C = unloaded non-irradiated cells; Ci = irradiated unloaded cells; TSPP = non-irradiated loaded cells; TSPPi = irradiated loaded cells.

6. Natural products as antioxidants

An antioxidant is a molecule stable able to donate an electron to a free radical, neutralizing it, thus reducing its capacity to damage. The antioxidants delay or inhibit cellular damage mainly through their free radical scavenging property. A variety of dietary plants including grams, legumes, fruits, vegetables, tea, wine etc. contain antioxidants. The most important antioxidants seem to be nonenzymatic antioxidants derived from plant sources including vitamins (vitamin A, C, E, K), flavonoids (quercetin, catechin, epigallocatechin gallate, hesperidin,

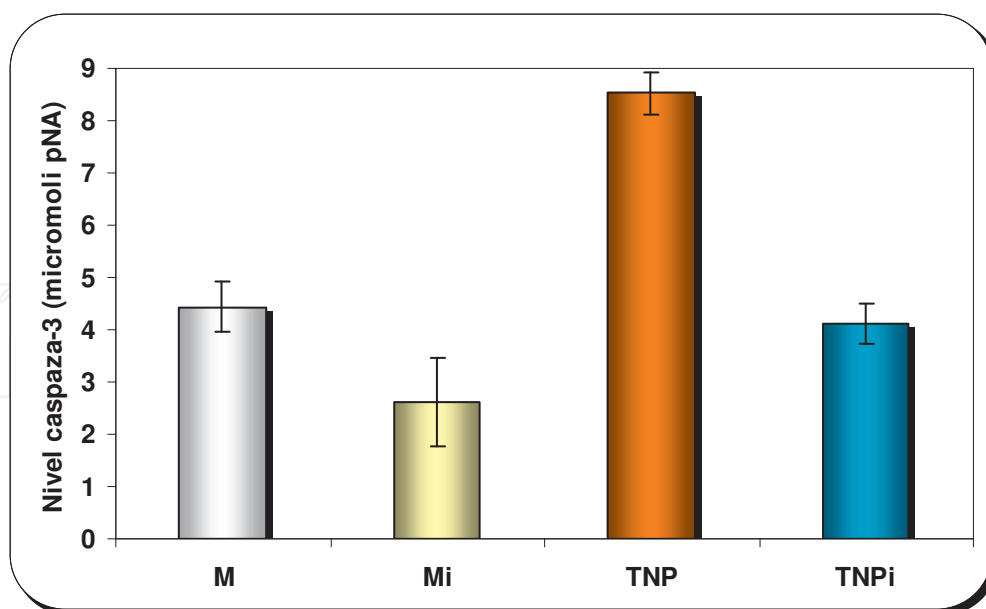


Figure 20. Caspase 3 activity in K562 cells loaded with 10 µg/ml TNP and activation by irradiation.

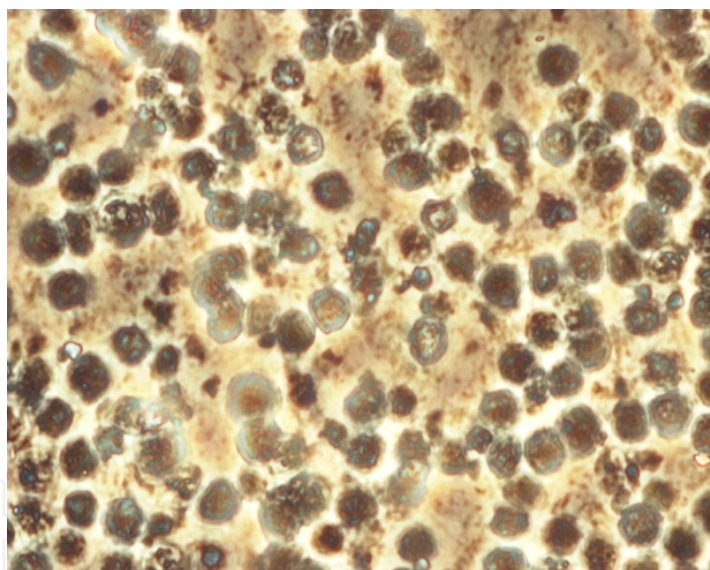


Figure 21. Induced apoptosis of K562 cells loaded with 10 µg/ml TNP and activated with laser (TUNEL). Results of TUNEL. Many apoptotic cells characterized with brown nuclei can be seen in the TNP-PDT groups.

hesperetin, diosmin, and many others), phenolic acids (cinnamic acid derivatives, curcumin, caffeine, catechins, gallic acid derivatives, salicylic acid derivatives, chlorogenic acid, resveratrol, folate, anthocyanins and tannins) [74–80].

Carotenoids, known as naturally fat-soluble pigments and synthesized by vegetal organisms, are sources of different colors [81]. They could be classified into carotenes (beta-carotene and lycopene) and xanthophylls (lutein and zeaxanthin). Their structure is shown in Figure 22. Beta-carotene and lycopene are widely regarded as being effective antioxidants, with small sizes

(nm), specific absorption and fluorescence spectra, and are easy to detect (Figures 23–25). The synergistic antioxidant effect of the mixture lycopene-beta-carotene-vitamin E on some cellular systems (in vitro and in vivo), has been reported [82–84]. Some antioxidants with health-protective effects (lycopene, beta-carotene, vitamin C, quercetin-glycosides, naringenin-chalcone, chlorogenic acid) are seen in tomato plants.

From ancient times, plants have been used intuitively for medicinal purposes. A large number of plants have been investigated and various species have been reported to exhibit antioxidant activity, including Marigold flower (*Calendula officinalis*), belonging to the Asteraceae family, which is a medicinal plant which contains oleanolic acid and other compounds, which present considerable potential health benefits, protective effects against the development of cancer, adverse effects of chemotherapy and radiation therapy, inhibition of existing tumor cells, anti-inflammatory activity, antioxidant activity, protective cardiovascular and antiviral effects [85]. The medicinal plants contain many ingredients with antioxidant capacity, as the pigments: antocyanins, chlorophyll, carotenoids, flavonoids, and so on [86]. Reactive oxygen species (ROS) comprise singlet oxygen (1O_2) and a range of oxidizing free radicals. The interaction of carotenoids with such species is important for the understanding of many important aspects of life such as photosynthesis, vision, various medical treatments from dermatology to cancer, as well as understanding possible deleterious reactions affecting man and also for commercial reasons, such as, investigations into the stability of carotenoids used as food dyes [87–90].

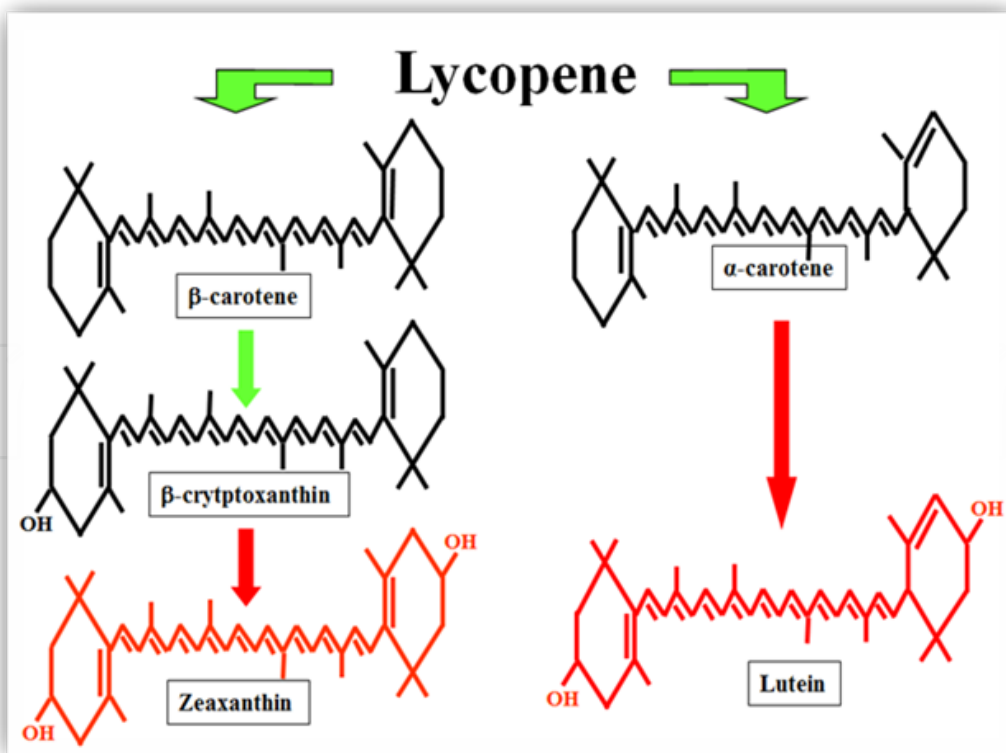


Figure 22. The transformation diagram of lycopene

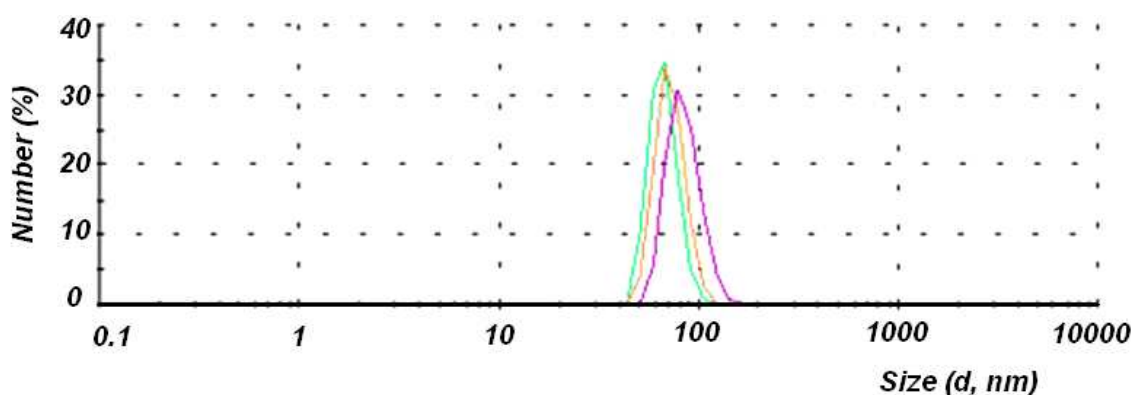


Figure 23. The DLS measurement of lycopene

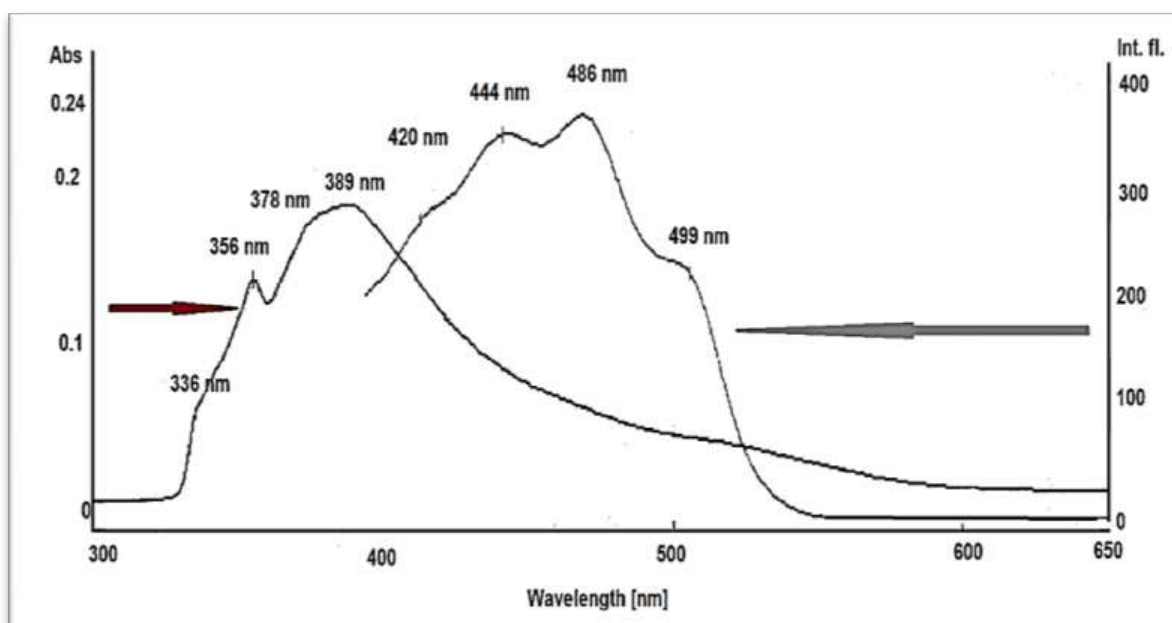


Figure 24. The absorption (left) and fluorescence (right) spectra of lycopene

Many antioxidants could be identified in tea leaves and fruits. For example, green tea, produced from the leaves of the plant (*Camellia sinensis*) contains polyphenols, which are potent antioxidants and, based on studies in preclinical models, have several photoprotective properties. The most active constituents are polyphenolic catechins, of which epigallocatechin-3-gallate is the most potent. Tea polyphenols have been shown to inhibit carcinogenesis in many animal models, and the significance of catechins, the main constituents of green tea, has been increasingly recognized to play a role in cancer prevention. Green tea contains some catechins, such as (–)-epigallocatechin-3-gallate (EGCG), epicatechin (EC), epicatechin -3-gallate (ECG), and epigallocatechin (EGC) (Figure 26), the first one being recognized as an efficient anticancer agent. Except the catechins, tea polyphenols exhibit carcinogenesis effect [91–101].

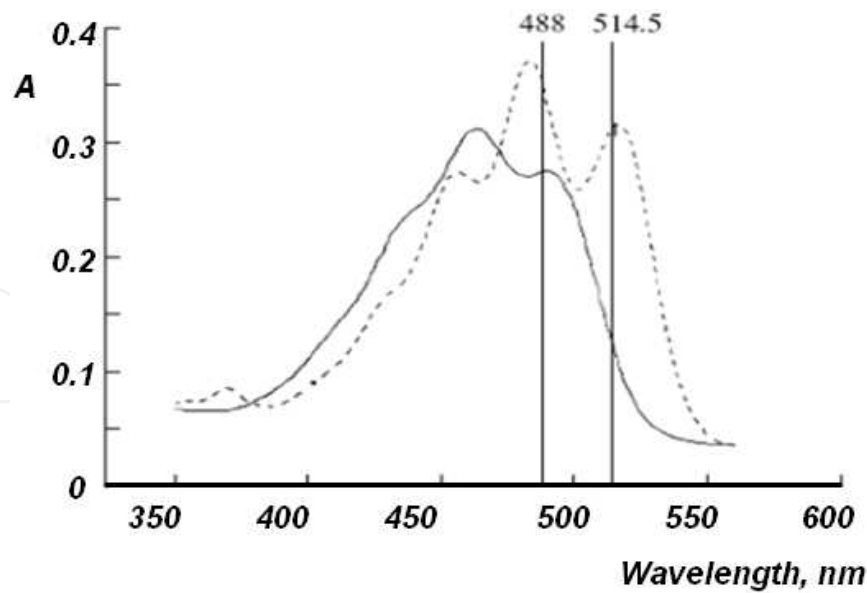


Figure 25. The absorption spectra of lycopene (---) and carotene (—)

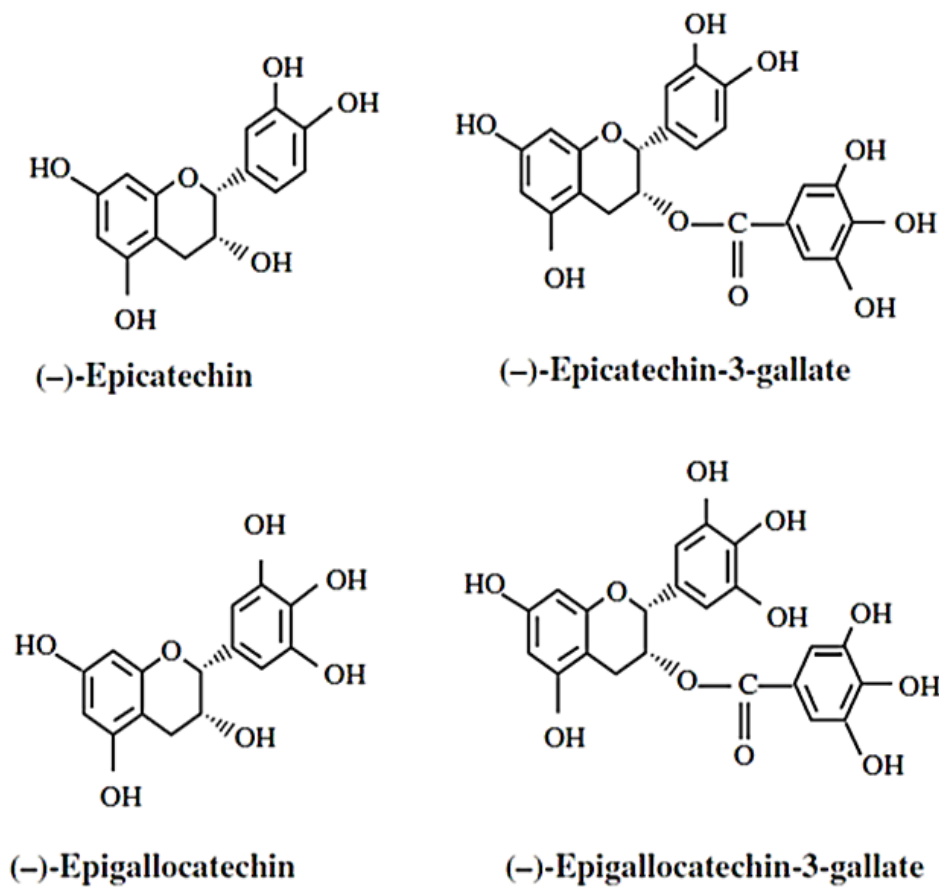


Figure 26. The chemical structure of the main epicatechin derivatives/polyphenols from green tea

The antioxidant activity (AA%) of the studied samples and their inhibitory effect against free radicals was evaluated using the DPPH method, by used the following formula:

$$AA\% = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100$$

where: A_{control} is the absorbance of a DPPH solution without sample, A_{sample} is the absorbance of the sample mixed with DPPH solution.

Some citrus extracts are very important for their antioxidant activity such as hesperidin, hesperitin and diosmin (Figure 27). Among the flavonoids used in oral administration in chronic leg ulcer, hesperidin is a glucoside that is abundant in citrus fruits. Recently, formulating hesperidin in nanocrystals, has provided its dermatological application, assessing its antioxidant effect. In vitro studies have shown its clear antioxidant properties, and using them as nutrients has shown its vaso-protective action.

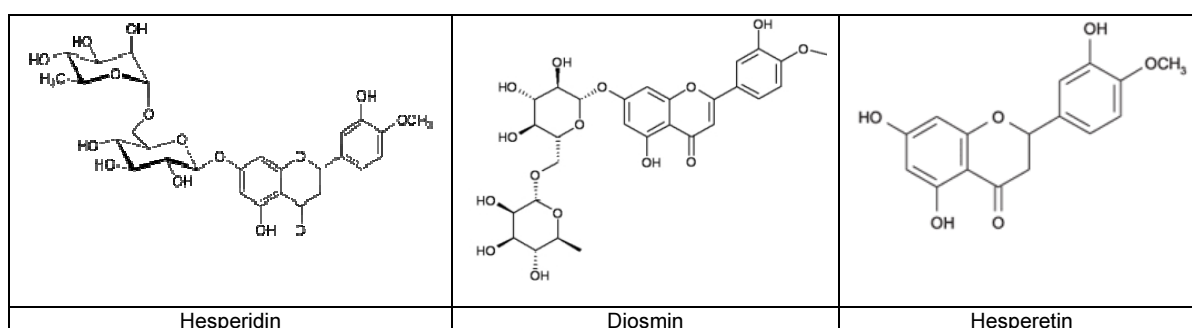


Figure 27. The structure of hesperidin, diosmin and hesperetin

Among mostly exploited flavonoids in chronic venous condition, hesperidin ($C_{28}H_{34}O_{15}$) [+/-3, 5, 7-trihydroxy-4'-methoxyflavanone 7-rhamnoglucoside] is a flavanone glycoside group of flavonoids found in large amounts in citrus fruits, grapefruit peels, lemon, oranges, blond grapefruit (*Citrus paradisi*), pummelo-blond grapefruit hybrid (*Citrus paradisi* var Jaffa Sweetie), or Chinese herbal medicine, with the highest concentration being found in the peel and the white parts of the fruit. Once formulated as nanocrystals, hesperidin also becomes dermally available and its antioxidant effect could be measured, a topic that is the subject of protected patents. In in vitro studies, this compound has a clear antioxidant action. In human nutrition, it contributes to maintenance of the integrity of blood vessels. Hesperidin has similar structure and properties with naringin, which is difficult to be separated from citrics [102]. In the same manner, diosmin is a hesperidin derivative, from the flavonoid family. As a synthetic drug, it is used for venous disease and for hemoroidal diseases [102]. Hesperetin, as a flavonoid from oranges and grapfruits, is a good protector of heart disease. It has antioxidant, anti-inflammatory, antiallergic and anticancer properties [103]. In spite of their structure differences, all of them show similar absorption spectra (Figure 28).

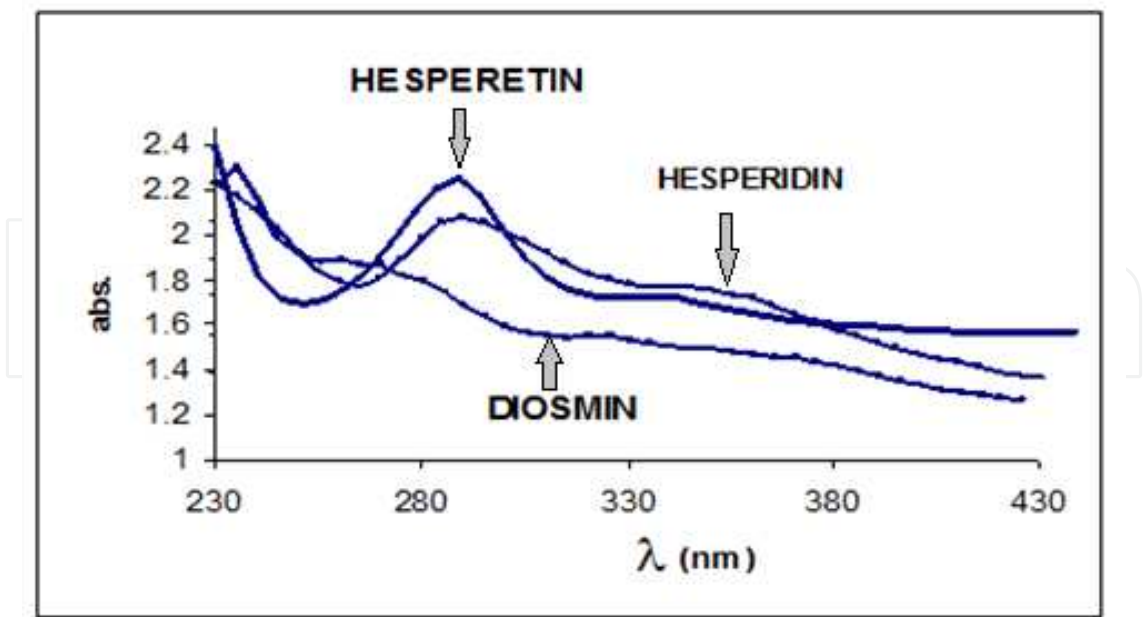


Figure 28. The absorption spectra of hesperidin, hesperetin and diosmin

For all of them, total flavonoids content (TFC), total poliphenols content (TPC) and antioxidant activity (AA%) have been calculated following literature methods (Table 5) [103–105].

Sample	TFC (mg CE/L)	TPC (mg GAE/L)	AA (%)
Diosmin etalon in MeOH	85.53	521.186	55.79
Hesperidin etalon in MeOH	43.1	466	59.31
Hesperetin etalon in MeOH	169.16	1814.406	73.34

Table 5. TFC, TPC and AA content

All the above measured parameters show very close values to the literature reports [106].

7. Conclusions

In spite of numerous advantages, the photodynamic therapy has a number of limitations. The light source should be close to the appropriate site in order to be effective before the diagnostic to be established. Persistent skin photosensitivity is observed some weeks after the treatment, which is considered as the main side effect. Also, this method is not possible without a light source and therefore entails high costs for the whole treatment. PDT acts both on ill cells and in a small manner on healthy cells. The intracellular Ca^{2+} from the cells induce low levels of shear stress on them, without any morphological changes [112]. The reactive oxygen species are localized to the cancer cells selectively destroying them and not the surrounding normal

tissue. The presence of the lymphoid cells in tumor immunity has been demonstrated during PDT with various photosensitizers. The immunologic effects include the production of interleukin 1-beta, interleukin 2, tumor necrosis factor-alpha, and granulocyte colony-stimulating factor. PDT acts to induce oxidative stress by the generation of free radicals to damage DNA and proteins, and eventually cell death, by necrosis and apoptosis. The reactive oxygen species lead to the state called oxidative stress. The antioxidants offer protection against lipid oxidation, react and interfere with free radicals, reduce oxidative stress, and stop low-density lipoproteins from being oxidized. Also, they protect healthy tissues and lower the incidence of treatment-related side effects. In the context where many clinical studies with respect to the application of antioxidants as sensitizers are lacking, this chapter showed a systematic review by showing evidence of the antioxidant action in photochemotherapy and their comparison with synthetic sensitizers (porphyrins and phthalocyanines). Under such circumstances, PDT is extremely important for the treatment of different diseases: lung, bladder, and skin cancers (precancerous and even melanoma).

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