UNIVERSIDADE FEDERAL DE SÃO CARLOS
CENTRO DE CIÊNCIAS EXATAS E DE TECNOLOGIA
Programa de Pós-Graduação em Biotecnologia

STUDY OF DIFFERENT CURCUMINS FOR STAPHYLOCOCCUS AUREUS ANTIMICROBIAL PHOTODYNAMIC THERAPY

Priyankaben Niravkumar Joshi

Dissertação apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade Federal de São Carlos para a obtenção do título de Mestre em Biotecnologia.

Orientador:
Prof. Dr. Vanderlei Salvador Bagnato

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Dedication

I dedicate this work to my loving family-Bhaumik, Truptiben & Rajeshbhai Vyas.

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First and foremost, I want to thank God, for my existence and his blessings, I am grateful to have had the privelege of attending the classes of Ufscar and working at USP. This experience has afforded me the opportunity to work with some of the best and brightest, and the resources for me to achieve great success. Thank you for this opportunity

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Joshi, Priyankaben Niravkumar. **Study of different Curcumins for Staphylococcus Aureus antimicrobial photodynamic therapy** 2022. Dissertação (Programa de Pós-graduação em Biotecnologia) – UFSCar, São Carlos, 2022.

RESUMO

A curcumina é um fotossensibilizador (FS) utilizado para inativação microbiana quando a luz é aplicada. Uma abordagem promissora para o tratamento de infecções bacterianas é a terapia fotodinâmica antimicrobiana (aPDT), que combina um FS e luz em comprimento de onda específico para produzir espécies reativas de oxigênio que promovem a morte dos microrganismos. Este projeto teve como objetivo analisar a eficácia de diferentes formulações de curcumina in vitro para protocolo de aPDT em *Staphylococcus aureus* (S. aureus). Neste estudo comparamos a Curcumina Sintética (Cur-Syn) com a Curcumina Nanoencapsulada (Cur-Nano), assim como as curcuminas modificadas com Cloro(Cl), Selênio(Se) e Iodo(I) (Cur-Cl, Cur-Se, Cur-I). *Staphylococcus aureus* (ATCC 25923) foi incubado com curcumina e irradiado a 450 nm para avaliar os efeitos da aPDT. A taxa de mortalidade microbiana foi medida contando as unidades formadoras de colônias sobreviventes por mililitro (UFC/ml). Parâmetros do FS como a incorporação e o fotobranqueamento foram comparados para as curcuminas de interesse. Nossos resultados in vitro sugerem que a curcumina nanoencapsulada não apresentou atividade significativa como FS quando comparada com Cur-Syn nas mesmas condições. As três modificações realizadas, sendo Cur-Cl, Cur-Se, Cur-I; Cur-Se, apresentaram atividade antimicrobina com a presença de luz principalmente para Cur-Se. Comparando a taxa de internalização do FS pela bacteria e a taxa de fotodegradação de Cur-Syn e Cur-Se foram bastante semelhantes. Nossos resultados sugerem que as modificações na curcumina influencia no efeito da molécula com FS.

Palavras-chave: Terapia fotodinâmica antimicrobiana. Curcumina. Nanoformulação. Modificações da curcumina.

Joshi, Priyankaben Niravkumar. **Study of different Curcumins for Staphylococcus Aureus antimicrobial photodynamic therapy** 2022. Dissertation (Programa de Pós-graduação em Biotecnologia) – UFSCar, São Carlos, 2022.

ABSTRACT

Curcumin is a Photosensitizer (PS) used for microbial inactivation when light is applied. Antimicrobial photodynamic therapy (aPDT) is a promising approach for treatment of bacterial infections, combining a PS + light to produce reactive species of oxygen that kills the microorganisms. This project aims to analyze the effectiveness of different in vitro curcumin formulations for PDT protocol against S.aureus. In this study, we compared Synthetic curcumin (Cur-Syn) with Nanoencapsulated Curcumin (Cur-Nano), as well as curcumins modified with Chlorine (Cl), Selenium (Se), and Iodine(I) (Cur-Cl, Cur-Se, Cur-I). Staphylococcus aureus (ATCC 25923) was incubated with Curcumin and irradiated at 450nm to evaluate the effects of aPDT. M*ortality was measured by counting the surviving colony forming units per milliliter (CFU/ml). The Uptake of photosensitizer (PS) and photobleaching were compared for the curcumins of interest. Our in vitro results suggest that Nanoencapsulated curcumin wasn't able to release curcumin and attack the bacteria when compared with Cur-Syn in the same conditions. Also, of the three modifications of Cur-Cl, Cur-Se, Cur-I; Cur-Se, had detrimental effect on bacteria. However, the rate of uptake and photobleaching capacity of Cur-Syn and Cur-Se were found to be quite similar. Our results suggest that curcumin modifications influence the effect of the molecule on PS.*

Keywords: *Antimicrobial photodynamic therapy. Curcumin. Nanoformulation.* .

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LIST OF ABBREVIATIONS AND ACRONYMS

PS	Photosensitizer
PDT	Photodynamic Therapy
aPDT	Antimicrobial Photodynamic Therapy
PBS	Phosphate Buffered Saline
Cur-Syn	Synthetic Curcumin
Cur-Nano	Nanoencapsulated Curcumin
Cur-Cl	Chlorine modified Curcumin
Cur-Se	Selenium modified Curcumin
Cur-I	Iodine modified Curcumin
S. aureus	*Staphylococcus aureus*

SUMMARY

Chapter 1 – Introduction

1.1. Background and Overview

Bacteria are found everywhere and serve vital functions in our environment. However, only a small fraction of bacteria possess the capacity to provoke infections and diseases. These infections pose substantial challenges to public health, necessitating prompt and accurate diagnosis (LIU et al., 2019). For instance, Staphylococcus aureus, a prominent member of the Staphylococcal bacterial group, which approximately 30% of individuals typically harbor on their skin, nasal passages, or mucous membranes. While it commonly leads to skin and soft tissue infections, it can also rapidly disseminate throughout the body via the bloodstream, potentially causing ailments in various organs such as the lungs, abdomen, heart, and other susceptible sites (LUNA et al., 2010). It has the potential to lead to various conditions like skin infections or abscesses, endocarditis, sepsis, urinary tract infections (UTI), mastitis, meningitis, osteomyelitis, foodborne illnesses, and infections linked with biofilm formation and blood poisoning. Bacterial resistance to antibiotics has been acknowledged since the beginning of the antibiotic era. However, it's only in the last two decades that we've seen a worrying increase in the emergence of dangerous, resistant strains. This rapid evolution of resistance, combined with a decrease in the development of new antibiotics, has led some experts to warn of an impending post-antibiotic era (FAIR; TOR, 2014).

A promising avenue for microbial inactivation is antimicrobial photodynamic therapy (aPDT), as its mechanism of action remains effective against bacteria. aPDT offers several advantages over conventional approaches, including reduced side effects, rapid healing of healthy tissues, and dual selectivity. The photosensitizer (PS) utilized in aPDT can be targeted to specific cells or tissues, where it absorbs light energy to generate reactive oxygen species (ROS). These ROS subsequently damage biomolecules such as proteins, lipids, and nucleic acids. The process of aPDT involves exposing the PS molecule to visible light of a specific wavelength, which elevates it to the excited singlet state (CIEPLIK et al., 2018). This excited state can then undergo intersystem crossing to transition to the slightly lower but longer-lived triplet state, which can react with biomolecules or molecular oxygen, leading to bacterial cell death.

Natural Curcumin (CUR) is a bright yellow compound obtained from the alcoholic extraction of Curcuma longa rhizome in 1842 (HOROSANSKAIA et al., 2020). Photodynamic therapy mediated by curcumin has been used in antibacterial and anticancer applications. It has been observed that when *S. aureus* cells were treated with curcumin

in the presence of blue light, several pit cells were detected, indicating that curcumin-aPDT breaks the structure of bacterial cell membranes and leads to cell death (JIANG et al., 2014; TORTIK; SPAETH; PLAETZER, 2014). Also, the superficial penetration of light needed to activate curcumin composites makes them well-suited for antimicrobial Photodynamic Therapy (aPDT), particularly targeting bacteria and other microorganisms (ARAÚJO et al., 2018).

The mechanism of antimicrobial photodynamic therapy (aPDT) relies on the uptake of a non-toxic dye called photosensitizer (PS) by the target cell and its activation by visible light of a specific wavelength in the presence of oxygen, resulting in the generation of highly damaging reactive oxygen species (ROS). Compared to traditional antimicrobial treatments, aPDT offers several advantages, including (i) the elimination of resistant microorganisms and secreted virulence factors, (ii) localized delivery of PS, and (iii) dual selectivity, exerting deleterious effects only at sites where both PS and light are concurrently applied, leading to an immediate onset of action. Due to the broad and nonspecific nature of cell death induced by ROS and the brief exposure to the PS, the expression of protective factors is impeded, making the development of resistance to aPDT highly unlikely in bacterial cells. While localized application of aPDT minimizes systemic adverse effects, it often necessitates high concentrations of photosensitizers and high-energy light doses to eradicate infectious bacteria, especially in cases of persistent infections. However, this can potentially harm host cells, compromise local immune responses, and even result in tissue necrosis. To address this limitation, aPDT can be combined with other agents or drugs to enhance efficacy, reduce individual concentrations, and mitigate damage to host tissues. In this study, we combined aPDT using Nanoencapsulated curcumin and Curcumins modified by Chlorine, Selenium and Iodine as a modification of PS (curcumin) to assess its potential as a safe alternative to conventional antimicrobials to treat localized infections at low concentrations.

1.2. Objective

1.2.1. General Objectives

The main objective of the work was to analyze *in vitro* effect by modifying the curcumin molecule for photodynamic action in *S. aureus*.

1.2.2. Specific Objectives

The specific objectives were:

- Evaluating the microbial reduction by optimizing the photosensitizer concentration and the light dose for illumination to activate photodynamic therapy
- Evaluating the aPDT effect of Cur-Syn vs Cur-Nano
- Evaluating the aPDT effect by varying the incubation time in Cur-Nano
- Comparing the antimicrobial photodynamic effect for Cur-Cl, Cur-Se and Cur-I with Cur-Syn
- To evaluate the interaction of Cur-Syn and Cur-Se with S. *aureus*;
- To analyze the photodegradation behavior of Cur-Syn and Cur-Se

Chapter 2 – Literature review

2.1 Antimicrobial photodynamic therapy

The mechanism of action of antimicrobial photodynamic therapy (aPDT) is to kill pathogens by using toxic reactive oxygen species (ROS) that are produced when a photoactivatable substance (photosensitizer), oxygen, and light of the suitable wavelength interact. Oskar Raab originally described the antimicrobial effects of photodynamic processes in 1900 when he noticed that the presence of acridine dye reduced the viability of *Paramecium caudatum*. Interestingly, this discovery, like Fleming's accidental discovery of penicillin around 30 years later, was made by accident (GAYNES, 2017; TAN; TATSUMURA, 2015). Since the discovery of antibiotics, photodynamic therapy (PDT) has mostly been used to treat cancer. As a result, research on light-induced disinfection has effectively suppressed the development of alternative antimicrobial therapies. (ANAS et al., 2021; HAMBLIN; HASAN, 2004). As a result of the increase in microbial resistance to antibiotics, aPDT is one of many new antimicrobial techniques that have been developed. aPDT is being thoroughly researched as a potential alternative treatment for localized infections that are also brought on by strains resistant to many drugs (BIYIKLIOGLU et al., 2019; MARTINS ANTUNES DE MELO et al., 2021).

In Jablonski's diagram (**Figure 1**), the processes of light absorption and energy transfer are illustrated, elucidating the mechanism of photodynamic action by delineating the rearrangement of states. Initially in its ground state, the photosensitizer agent (PS) exists as a singlet PS, characterized by two electrons with opposite spins resulting in a total spin of zero, hence denoted as S0 (with the exception of molecular oxygen). Upon absorption of light with the appropriate wavelength (quantum energy), one electron is excited to a higher-energy orbital (I). Due to the inherent instability of the singlet excited-state PS, any surplus energy may dissipate through the fluorescence process or via internal conversion, leading to the generation of heat.

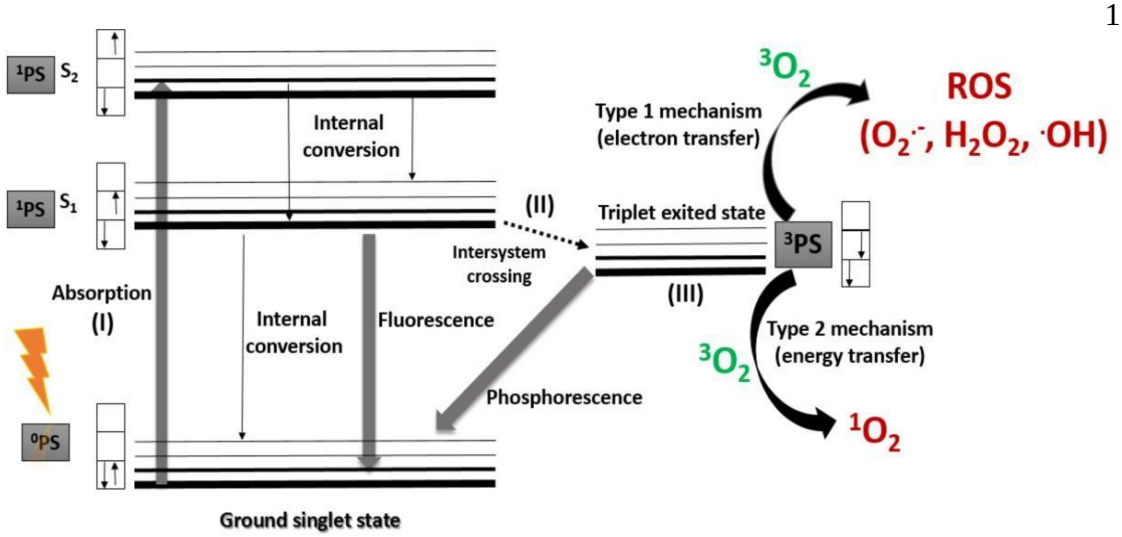


Figure 1: Jablonski diagram for ROS generation by aPDT via type I and type II photodynamic mechanisms. Figure adapted from Lucas D. Dias et al. (DIAS et al., 2020).

Once the singlet excited-state photosensitizer (PS) is generated, it can undergo an intersystem crossing (IC) process, resulting in the formation of a more stable triplet excited-state with parallel spins. In this state, two unpaired electrons possess a spin angular momentum that collectively adds up to a spin quantum number of 1. Despite the possibility of the triplet excited-state PS decaying back to the ground state by emitting a phosphorescent photon, this process is restricted due to quantum selection rules that deem it a "forbidden process."

With a lifetime of microseconds rather than the singlet excited-state PS's nanoseconds, the triplet state exhibits significantly higher stability compared to the singlet state (DIAS et al., 2020). Consequently, owing to oxygen's distinctive property of existing as a molecular triplet in its ground state, the prolonged lifetime of the triplet state facilitates energy transfer (type II mechanism) to oxygen, thereby generating singlet oxygen ($^1O_2$).

Moreover, the photodynamic process can proceed via a type I mechanism involving electron transfer reactions that yield reactive oxygen species (ROS), including superoxide radical anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\bullet}$) (DIAS et al., 2020). ROS and $^1O_2$ can cause oxidative stress in biomolecules like lipids, nucleic acids, proteins, and others that target harmful microbial species (bacteria, fungus, viruses, and parasites), causing cell damage (**Figure 2**). (MARTINS ANTUNES DE MELO et al., 2021).

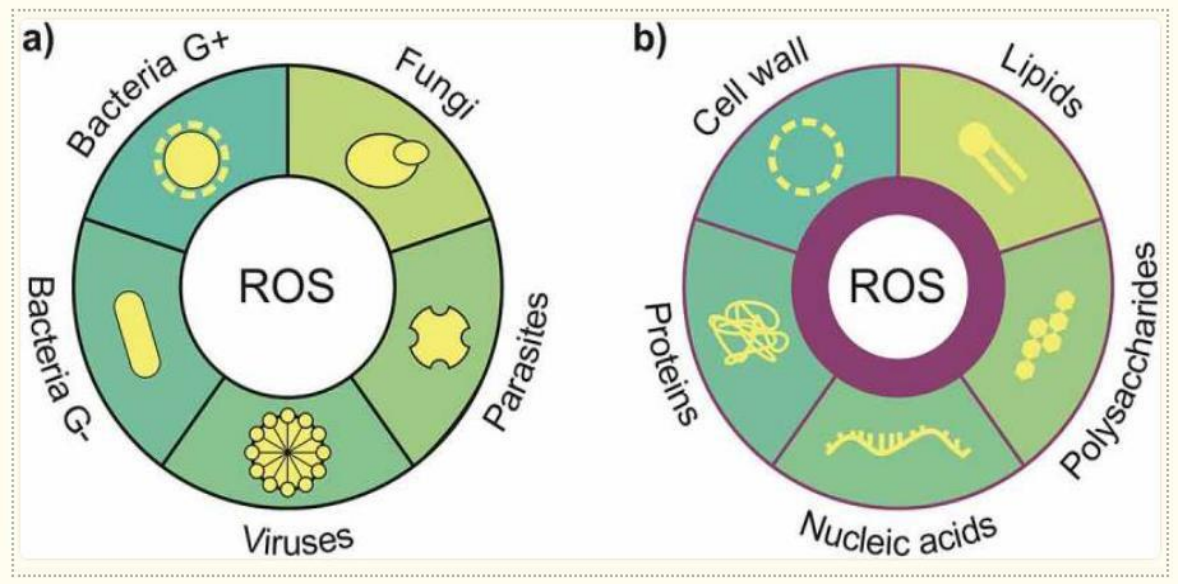


Figure 2: ROS presents (a) a wide therapeutic window that affects different species of microorganisms (bacteria, fungi, viruses and parasites), and (b) it is a nonselective multiple targets oxidizing various biomolecules promoting substantial cell damage. Figure adapted from Wanessa de Cassia Martins Antunes de Melo et al. (MARTINS ANTUNES DE MELO et al., 2021).

2.2 Advantages of PDT

The utilization of PDT, a minimally invasive technique, has been proposed as an alternative strategy for addressing infections caused by various pathogens. Its wide range of effectiveness, ability to deactivate multiple antibiotic-resistant strains, low potential for causing mutations, and absence of selection for photo-resistant microbial cells are among the numerous advantages offered by this therapy (MARTINS ANTUNES DE MELO et al., 2021). Moreover, PDT has substantial advantages over current antibacterial therapy. It appears to be equally effective against multi-drug resistant microorganisms and native bacterial strains. Furthermore, the effect of PDT on bacteria is much faster than that of other antimicrobial agents, and no indication of PDT resistance has been found yet. According to Nicolò Martinelli et al (MARTINELLI et al., 2019), PDT has antimicrobial effects and has been utilized to lower the total and pathogenic microbial load in diabetic ulcers without generating bacterial resistance. It is risk-free and has the potential to improve outcomes. PDT with RLP068 (cationic zinc phthalocyanine derivative) has shown in a clinical experiment to lower the microbial burden of diabetic ulcers in 62 patients. PDT could be useful for antimicrobial therapy of patients who were already receiving multiple medications, for ulcers infected with drug-resistant

bacteria, or in conjunction with other therapies in severe situations.

2.3 Curcuminoids

In many tropical regions of Asia, turmeric, or *Curcuma longa L*., which belongs to the *Zingeberacea* family, is widely farmed for its main polyphenolic pigment, curcumin. Common applications for turmeric include culinary spices in Indian cuisine, skin-care products, and traditional Chinese and Indian medicines. People have used turmeric for thousands of years to treat conditions including the common cold, fever, skin conditions, stomachaches, liver illnesses, open wounds, chronic inflammations, etc. (PRIYADARSINI, 2009). Interest in the medicinal potential of turmeric and the relative simplicity of isolating curcuminoids has led to an extensive investigation of these compounds (NELSON et al., 2017).

Curcumin, isolated in 1815 and described as a pure crystalline compound in 1870, was discovered to be the most active of the curcuminoids present in *C. longa* (GUPTA et al., 2012). Due to its demonstrated safety by regulatory bodies such as the Food and Drug Administration (FDA) of the United States, it is used to treat various ailments, including cancer control and infections caused by a variety of pathogenic bacteria (LU et al., 2020). In addition, curcumin has demonstrated significant potential as a photo-sensitizer (light- sensitive) in photodynamic therapy (PDT) due to its ability to absorb blue light and produce reactive oxygen species (ROS) (DIAS et al., 2020). Curcumin is a diferuloylmethane with the chemical formula $C_{21}H_{20}O_6$, a solid yellow- orange color, a molecular weight of 368.39 g/mol, and a melting point of 183°C. The chemical structure of curcumin has two o-methoxy phenols attached symmetrically through α, β-unsaturated β -diketone linker, which also induces keto-enol tautomerism (**Figure 3**).

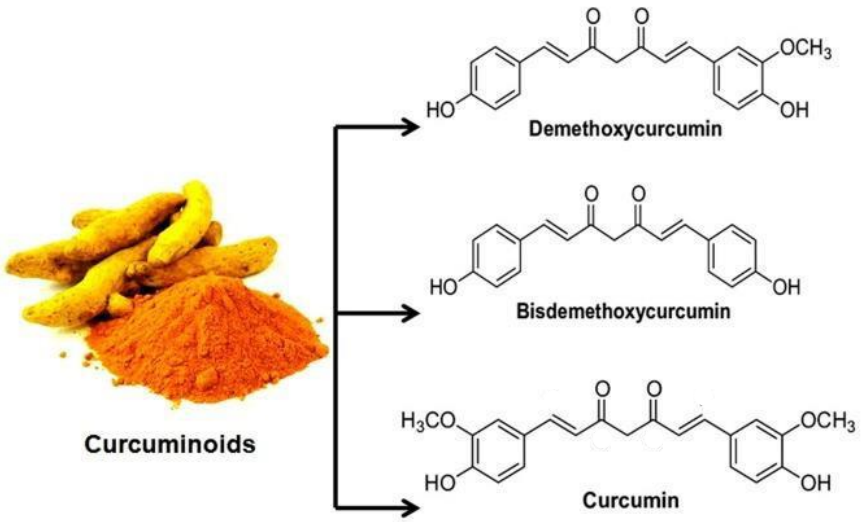


Figure 3: Curcuminoids extracted from the rhizome of Turmeric (*Curcuma longa L.*) and molecular structures of the three major constituents curcumin (CUR) chemically a diferuloylmethane [1,7-bis(4-hydroxy-3-methoxy-phenyl)-hepta-1,6-diene-3,5-dione], demethoxycurcumin (DMC) [4-hydroxycinnamoyl-(4-hydroxy-3-methoxycinnamoyl) methane], and bis(dimethoxy)curcumin (BDMC)) [bis-(4-hydroxy cinnamoyl) methane]. Figure adapted from Kathryn M. Nelson et el. (NELSON et al., 2017).

Curcumin primarily exists in the enol form when it is diluted in ethanol. However, in an aqueous solution, the di-keto form prevails, stabilized by the coordination of a water molecule. These structural attributes impart lipophilic properties to curcumin (Log P=2.5). It behaves as an acid and exhibits high solubility in organic solvents like dimethyl sulfoxide (DMSO), methanol, ethanol, and acetone (SOARES et al., 2020). Due to this, curcumin exhibits many interesting photophysical and photochemical properties. The optimal cut-off point for curcumin stability in an aqueous solution ranges from pH 1-6. In the charged state (pH< 1 or pH > 7), its color changes to red, and exposure to sunlight accelerates its degradation. Diferuloylmethane (60-70%), demethoxycurcumin (20-27%), and bisdemethoxycurcumin (10-15%) are the main components of commercial curcumin and are made by extracting the rhizome of *Curcuma longa* with alcohol. The amount of curcuminoids in *C. longa L.* depends on several factors, including geographic location, climate (temperature and light), etc. (PRIYADARSINI, 2009).

Based on the Lipinski rule of five, it seems that curcumin's molecular weight (MW of 368.38 Da < 500 Da) lets the GI tract absorb it. But many studies have shown that the GI tract doesn't absorb curcumin very well. As curcumin is a lipophilic compound, its lipophilicity plays a key role in its absorption, distribution, metabolism, and elimination (ADME). Still, its lipophilicity favors its uptake by the peripheral tissues, which lowers the amount of free curcumin in the blood (SHARIFI-RAD et al., 2020). Depending on the

molecular target needed, new formulations should be used to increase the amount of free curcumin in the blood or target tissues. To make curcumin more bioavailable, it is essential to use new formulations and select the proper administration route. Different forms of curcumin, such as synthetic curcuminoids, nanoparticles, liposomes, micelles, and phospholipid complexes, have been made to make it more bioavailable. These new ways of making curcumin not only make it more bioavailable, but they also make it last longer, be more permeable, and resist metabolic processes (SHARIFI-RAD et al., 2020).

The nomenclature “curcumin” is sometimes used to describe the pure curcumin or an extract from the turmeric rhizome. Curcuminoids share the same structure with two benzenemethoxy rings, joined by an unsaturated chain. Demethoxycurcumin is a beta-diketone that is curcumin in which one of the methoxy groups is replaced by hydrogen while bisdemethoxycurcumin is a β-diketone that is methane in which two of the hydrogens are substituted by 4-hydroxycinnamoyl groups. Curcuminoids possess three significant functional groups: an aromatic methoxy phenolic group, α,β-unsaturated β-diketo linker, and keto-enol tautomerism. These compounds predominantly exist in the trans-trans keto-enol form. The aromatic groups contribute to their hydrophobic nature, while the linker confers flexibility. Additionally, the tautomeric structures play a role in determining hydrophobicity and polarity. Due to their hydrophobic properties, curcuminoids exhibit poor solubility in water (AMALRAJ et al., 2017).

As a photosensitizer, curcumin has received attention in the management of oral or cutaneous contaminations. Antimicrobial photodynamic treatment can be performed using it because of its ability to absorb blue light and produce ROS. Having its bright yellow color when solid, curcumin absorbs strongly in the UV–visible range, with maximum absorption at a wavelength between 408 and 434 nm (ETEMADI et al., 2021; PRIYADARSINI, 2009).

In aqueous alcohol solutions, curcumin maintains stability under acidic conditions, yet it undergoes hydrolysis and chemical degradation in basic environments. Both in solution and solid state, curcumin exhibits photodegradation. This process yields photoproducts with absorption spectra similar to curcumin's, evident in both aerated and deaerated organic solutions, as well as aqueous micellar solutions. UV photolysis of curcumin leads to the formation of three degradation products, while sunlight exposure in organic solvents produces a greater variety of degradation products compared to UV photolysis. The primary mechanism of degradation involves the cleavage of β-diketone bonds, resulting in the formation of smaller phenolic compounds. Notably, the presence or absence of the phenolic OH group does not influence the degradation process. Photodegradation of curcumin also

yields phenolic compounds such as vanillin and ferulic acid. Furthermore, polymerization of some smaller phenols results in the formation of phenolic polymers. Interestingly, the photodegradation products of non-phenolic curcuminoids, like benzochalcone and flavanone, are formed through this process (PRIYADARSINI, 2009).

2.4 Structural modification of photosensitizer

Curcumin's potential health advantages are hampered by several hurdles. A few researchers have discussed what they call the "evil side of curcumin." Curcumin has been shown to have weaknesses, the most notable of which are its poor pharmacokinetic and pharmacodynamic (PK/PD) qualities, its lack of efficacy in a number of illness models, and its potentially toxic effects under particular testing conditions (NELSON et al., 2017). Curcumin has anti-inflammatory, antioxidant, wound healing, hypoglycemic, and antimicrobial properties, according to evidence. Despite its tremendous biological activities, curcumin has some physical shortcomings, including water insolubility and poor bioavailability (PRASAD et al., 2021). Curcumin is poorly absorbed by intestinal cells and rapidly processed by the liver due to its low solubility and stability in bodily fluids. As a result, curcumin has a poor pharmacokinetic profile overall. A safe oral dose of curcumin (8 g/day) in humans achieves only low circulation quantities (22-41 ng/mL) due to limited absorption. However, excessive doses (>8 g/day) have been linked to negative side effects. Curcumin's limited oral bioavailability is a primary reason for its failure in clinical trials. Many therapeutic uses require higher bioavailability and curcumin levels in circulation to be efficacious (DIAS et al., 2020). To address these concerns, efforts have been made in recent decades to modify its structure, including the synthesis of curcumin analogues (ABD EL-HACK et al., 2021).

Research has shown that low doses of curcumin can have stronger effects when they are combined with blue light or UVA irradiation. This is because the light energy intake is enhanced in these situations (BERND, 2014; DUJIC et al., 2007). Some photosensitizers such as Rhodamines, when subjected to chemical reactions like alkylation of amino groups or halogenation of rhodamines causes a red shift in the light absorption spectrum (BERG, 2014). In a similar vein, alternate elements like sulphur or selenium can substitute for oxygen. This substitution significantly boosts the quantum yield of singlet oxygen production in photosensitizers such as merocyanine 540 (MC540) and Nile blue. When activated by light, these compounds exhibit antibacterial activity against Gram-positive bacteria (BERG, 2014). Even minor alterations in chemical structure can profoundly impact a compound's photosensitizing efficacy. For instance, the "heavy atom effect"

manifests when a higher atomic number replaces a lower one, thereby increasing the likelihood of transitioning to a triplet state and initiating photosensitization. By brominating rhodamine 123 to create tetrabromorhodamine 123, the singlet oxygen production of the compound is enhanced approximately 50-fold. In a similar fashion, oxygen can be replaced with sulphur or selenium, which results in a significant increase with the quantum yield of singlet oxygen production. This is the case, for example, in merocyanine 540 (MC540) and Nile blue (BERG, 2014).

Figure 4 illustrates the fundamental structure of curcumin and its constituent groups responsible for its biological functions, including anticancer and antioxidant properties. Curcumin consists of a seven-carbon linker and three primary functional groups: an α, β-unsaturated β-diketone moiety, an aromatic O-methoxyphenolic group, and the seven-carbon linker molecule itself (**Figure 4A**). The antioxidant capabilities of curcumin stem from its phenolic group, while the carbon linker molecule contributes to its hydrophobic nature (SALEM; ROHANI; GILLIES, 2014) (**Figure 4B**). Modification of these moieties has yielded curcumin derivatives with increased effectiveness and improved water solubility or stability (TOMEH; HADIANAMREI; ZHAO, 2019).

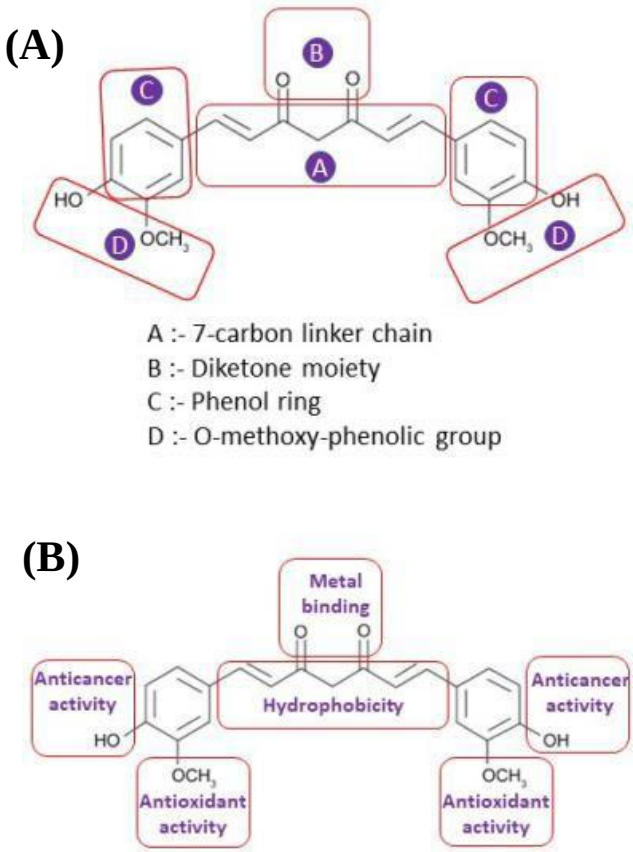


Figure 4: Structure of (A) curcumin. (B) Groups of curcumin responsible for its biological properties. Figure adapted from Sahdeo Prasad et al. (PRASAD et al., 2021).

In recent years, researchers have developed metal complexes of curcumin to enhance its biological activity and address its limitations. These complexes alter curcumin's structure, improving its efficacy. Various halogenated derivatives of curcumin, including fluorine, chlorine, bromine, and iodine compounds, have shown promise in treating Alzheimer's disease. For instance, a chlorinated curcumin analog (Compound 11) has been patented as a potential therapeutic agent for Alzheimer's disease (JOHN R. CASHMANMILAN FIALA, 2009). Additionally, selenium, an essential trace element, plays crucial roles in immune function, and has been explored for its therapeutic potential in various conditions such as cancer, diabetes, and infectious diseases. Selenium also exhibits antioxidant and antimicrobial properties (KIM et al., 2012).

Thus, modifications in the curcumin molecule can potentiate both its photosensitizing and antimicrobial activity. The structure of the modified curcumin prepared by Prof. Dr. Kleber T. de Oliveira at UFSCar is shown below (**Figure 5**) with the modification highlighted in yellow.

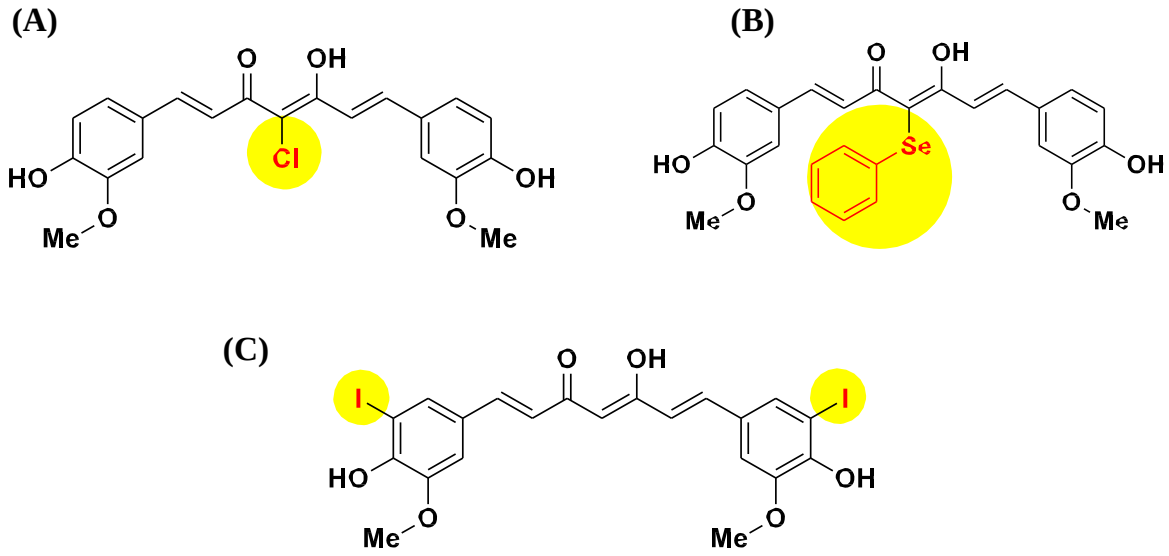


Figure 5: Chemical structure of (A) Chlorine modified curcumin (Cur-Cl), (B) Selenium modified curcumin (Cur-Se) and (C) Iodine modified curcumin (Cur-I). Figure made by Dr. Guilherme M. Martins, Prof. Dr. Kleber Thiago de Oliveira from UFSCar.

From the antimicrobial point of view, these ligands have a property of interest in the inactivation effect. It is known for iodine the antimicrobial activity of bacteria, fungi and viruses damaging proteins (binding to thiol and sulfhydryl groups), nucleotides (blocking hydrogen bonds.) and fatty acids (BRADSHAW, 2011). Chlorine, also a halogen that has antimicrobial properties, but by promoting oxidizing activity and interrupting protein synthesis (GOTTARDI; NAGL, 2005; ODLAUG, 1981) That is, this modification can, in addition to adding the effect of bacterial inactivation, in theory can favor the oxidative

processes characteristic of aPDT. Selenium, despite not being reported antimicrobial effects, only when in nanoparticles, this is an essential micronutrient, since it is the radical of the 21st amino acid, seleno-cysteine, essential for the synthesis of enzymes of antioxidant processes such as glutathione peroxidases (GPx) and thioredoxin reductase (TrxR), that is, its uptake by cells is required (FILIPOVIĆ et al., 2021).

The pharmacokinetics and bioavailability of curcumin can be significantly changed by formulations. Numerous published studies show that curcumin formulations can perform better than the free compound. However, these enhancements varied significantly between various products (JAMWAL, 2018). Encapsulating or integrating (binding) curcumin with other harmless, biocompatible materials has been the most prevalent technique to improving curcumin's limitations. Various carriers such as polymeric nanoparticles, micelles, hydrogels, nanoemulsions, liposomes, solid lipid nanoparticles, polymeric and inorganic nanostructures have garnered significant attention. Nanostructures have shown promise in enhancing therapeutic effects by enhancing the solubility of hydrophobic drugs, reducing potential immunogenicity, and enabling controlled and stimulus-responsive drug release. They offer the potential to modulate the rate and site of drug release effectively (MONTAZERABADI et al., 2019). Nano-formulations have a low size/surface area ratio and provide several benefits, including enhanced transport through the GI mucosa, ensuring prolonged and controlled release, and targeted delivery. The relatively low hydrolytic degradation rate of curcumin contained inside polymeric particles shows that encapsulation, particularly nanoencapsulation (particles 100 nm), is a viable technique for addressing curcumin's high degree of instability and poor bioavailability. Despite its safety and efficacy, curcumin’s limited water solubility and low absorption limit its medicinal application. Curcumin-based nanoformulations with improved stability, bioavailability, and bioactivity have evolved.

Several nanoparticle forms are being investigated for their potential to successfully encapsulate curcumin (BONIFÁCIO et al., 2013). As seen in **Figure 6** (1) Lipophilic particles are integrated into the hydrocarbon bilayer of liposomes, whereas hydrophilic molecules are encapsulated within their aqueous interiors. (2) Polymeric micelles possess both hydrophilic and hydrophobic functional groups. (3) Polymeric nanoparticles have a dense matrix structure that can retain compounds with pharmacological activity. (4) Nanogels-The internal hydrophobic core of a core-shell polystyrene gel layer interacts with pharmaceutically active chemicals. (5) A nanoemulsion is a dispersion of water and oil that has been stabilized using a surfactant and a transfectant and is thermodynamically stable. (6) Solid lipid nanoparticles consist of a solid lipid core matrix supported by surfactants or emulsifiers, offering a means to solubilize lipophilic substances effectively (7) Inclusion complexes involve a mixture of

active medicinal components nestled within the hydrophobic cavity of bulky host molecules like cyclodextrin. (8) Dendrimer Core-shell Many medicinally active compounds are directly tied to stable physical contact or chemical bonding. Typically, nanostructures are assembled layer by layer. (9) Phytosomes are phospholipid complexes composed of pure phospholipids and exhibiting different physicochemical and spectroscopic properties. (10) Curcumin nanoparticles, solely composed of curcumin without any conjugated carrier, are produced by dissolving pure curcumin in ethanol under high pressure and homogenizing it with water containing 0.1% citric acid (BOSE et al., 2015).

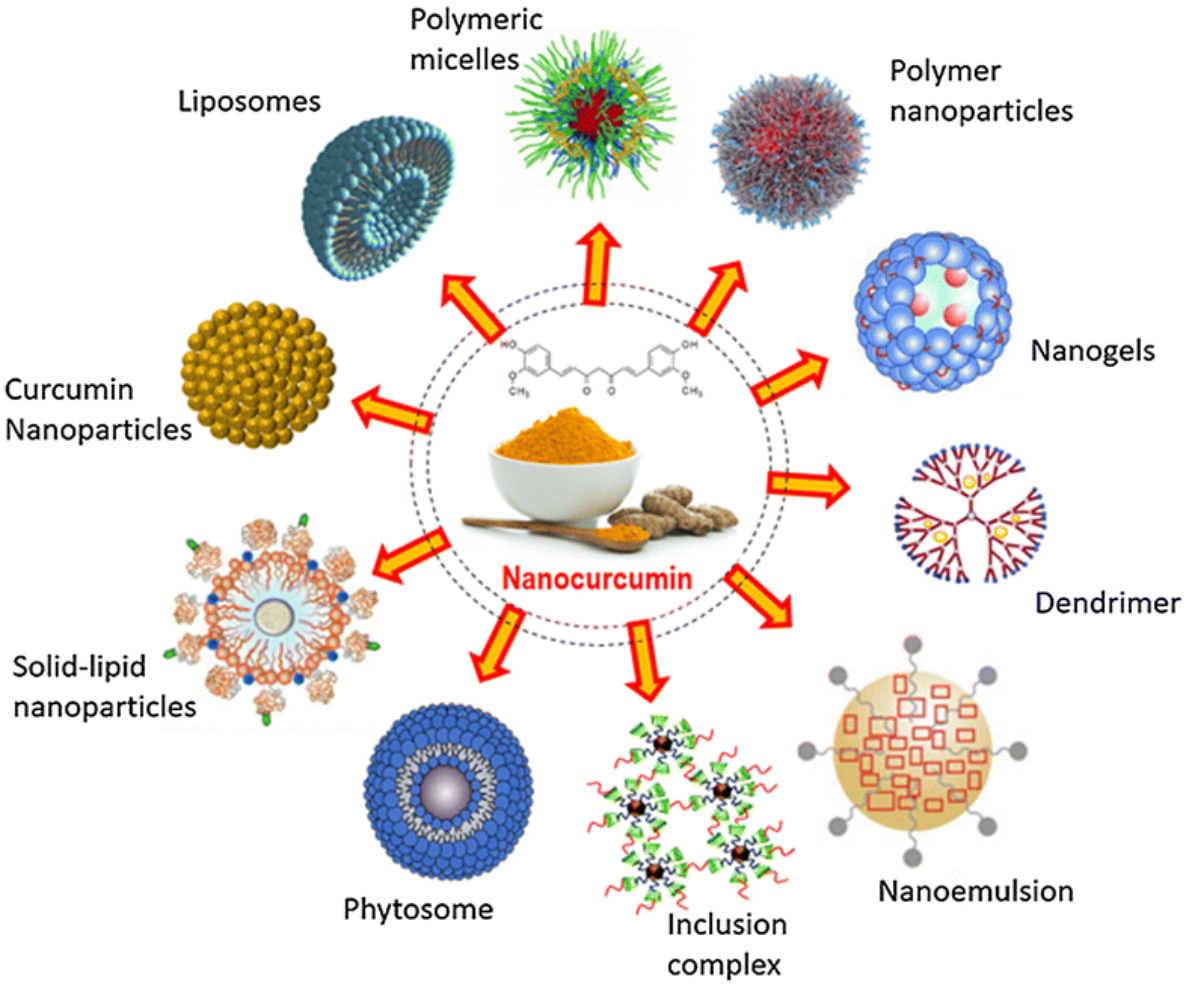


Figure 6: Different forms of Nanocurcumin. Figure adapted from (BOSE et al., 2015).

Curcumin's efficacy in various disorders, particularly infectious diseases, may be enhanced by nanoparticulate drug delivery techniques. In various *in vitro* and *in vivo* studies, curcumin nanomaterials outperformed free curcumin in terms of therapeutic efficacy. Curcumin's nanoform improves its water solubility and antimicrobial action. Nanocurcumin is more efficient against Gram-positive bacteria than Gram-negative bacteria and fungi. Recent clinical trials confirm that curcumin in nano form has enhanced absorption, and nanoformulated curcumin can affect inflammation, cancer, infection, and other pathological

states. The effects of toxicity and particle size on the biological activities of nanocurcumin should be studied further (BEYENE et al., 2021; FAROOQI, 2017; KHEZRI et al., 2021; MAHJOOB; STOCHAJ, 2021; MALEKI DIZAJ et al., 2022; WONG et al., 2019; YALLAPU et al., 2015).

Chapter 3 – Materials and Methods

3.1 Microorganisms

The microorganisms used were the Gram-positive bacteria *S. aureus* (ATCC 25923). The microorganism was cultured in a 25 mL Falcon tube containing 1 mL of the frozen bacteria and 9 mL of the culture medium BHI (Brain Heart Infusion, Kasvi®) and incubated at 37 °C and 150 rpm overnight in an incubator shaker. After this, the falcon solution was centrifuged for 15 mins at 3000 rpm, the supernatant was discarded and resuspended in 10 ml phosphate-buffered saline (PBS) and centrifuged for 10 mins at 3000 rpm. This washing for 10 mins at 3000 rpm was repeated again, and the supernatant containing PBS was discarded and replaced with the same volume of PBS. The solution was shaken to disaggregate the bacterial colonies. It was diluted to obtain the inoculum of 10^8 CFU/ml (colony forming units per milliliters), verified by the optical density at 600 nm with Cary UV–VIS 50 Varian (CORRÊA et al., 2019).

3.2 Curcumin

Curcumin (Synthetic and Modified) in powdered form was weighed and crushed, and the absolute alcohol was added to complete the volume. The stock solution for curcumin was prepared by crushing 0.0092 g of curcumin powder using a glass rod followed by adding 5 mL ethyl alcohol (5mM). From this solution, and just before the beginning of the experiment, new decimal dilutions were carried out to obtain concentrations of 3.75μg/mL and 7.5μg/mL in water for aPDT. All curcumin preparations were made immediately before experiments and kept protected from light throughout the experiments. Cur-Syn was obtained from PDTPharma®. *Cur-Cl, Cur-Se, Cur-I; Cur-Se* was synthesized by Prof. Dr. Kleber Thiago de Oliveira from UFSCar.

The Cur-Nano was received as a stock solution of concentration 0.87 mM, from the laboratory of Prof. Gang Zheng, University of Toronto, Canada and was used without further modifications. The experimental solutions were obtained by diluting the stock solution in water to obtain the concentrations of interest (3.75μg/mL and 7.5μg/mL).

Before commencing these experiments, I analyzed measurements with various concentrations, including 3.75μg/mL, 7.5μg/mL, 15μg/mL, and 30μg/mL, as well as different light doses. I selected a specific combination of light dose and concentration because, in these groups, there was no photodynamic effect observed in the absence of light and photosensitizer (PS). This combination was then selected for synthetic curcumin. For better

comparison, the same concentration and light dose were utilized for nano formulations and modified curcumins.

3.3 Illumination

Irradiation was performed with a Biotable®, a light-emitting diode (LED)-based device that consists of 24 emitting centers with the wavelength of 450 nm. The irradiance obtained with this device was 40 mW. LED arrays were arranged so that each LED display was disposed of under a well from the well plate, providing the same average irradiation. The light doses applied were 3.75, 7.5, and 15 J/cm^2 at exposure times of 1 minute 34 seconds, 3 minutes 08 seconds, and 6 minutes 15 seconds, respectively. The light control and aPDT groups were illuminated. The time required for illumination was calculated using Equation 1, where D is the Light Dose (J/Cm^2), I is the Intensity of Biotable used: 40 mW, and T is the time in seconds.

$$D = I.t \quad \text{Eq. 1}$$

3.4 aPDT

The groups were: General control (Bacteria + PBS), light control (Bacteria + PBS + irradiation) and Dark control (Bacteria + PS) and aPDT groups (Bacteria + PS + light) were performed. They were prepared in 24-well plates. Each well contains 200 μL of Bacterial inoculum and 200 μL of PS solution or PBS solution, depending on the group. The final volume of each well was 400 μL. All the groups were incubated at 37 °C for 20 mins in this 24-well plate protected from external light. For this, 100 μL of each sample was transferred into microtubes containing 900 μL of PBS to carry out the serial dilution until 10^{-5} was obtained for all the groups. An aliquot of 10uL of each dilution was plated in the Petri dishes with BHI agar in triplicate. After the plates were dried, they were incubated at 37°C for 24 hours to carry out the counts of CFU. After this time, colonies were counted for each dilution for all the groups. Three independent experiments were performed for each experimental condition.

3.4.1 Uptake of PS

In Eppendorf, 1 ml bacteria and 1 ml PS was taken. Also, the concentrations (3.75 μg/mL and 7.5 μg/mL) were done separately for PS. Each Eppendorf was incubated at 37 °C for 0 min, 20 mins, 40 mins, and 60 mins. Control was 1ml PBS+1ml PS. Then it was centrifuged at 3000 RPM for 5 mins. The supernatant was collected in a cuvette, and simple reads were performed to measure the absorbance in Spectrophotometer UV–vis

(Cary UV-Vis50, Varian) at 422 nm for Cur-Syn and 432 nm for Cur-Se. The percentage of uptake was calculated using Equation 2.

$$Uptake\ (\%) = \left(1 - \frac{Abs_{supernatant}}{Abs_{standard}}\right) \times 100 \qquad \text{Eq. 2}$$

3.4.2 Photobleaching

Photobleaching was investigated using LEDs in the wavelength of 450 nm to illuminate an aqueous solution of curcumin, evaluating its degradation for different exposure times. A stock solution was prepared using curcumin from (PDTPharma®, Cravinhos, Brazil). It was added to absolute alcohol to prepare a concentrated solution. The mother solution was diluted again in water, generating solutions in 20 µM concentrations. Absorption spectroscopy was carried out on a spectrophotometer (VARIAN - CARY 50 BIO UV-Visible Spectrometer), using quartz cuvettes with 1 cm of the optical path. Absorption records were obtained immediately after dilution (at t=0 min). Subsequently, at 1min 34sec, 3 min 08sec and 6 min 15sec in a dark environment at a light dose of 0 J/cm^2, 3.75 J/cm^2, 7.5 J/cm^2, and 15 J/cm^2, respectively and the change in the peak was observed.

3.4.3 Statistical analysis

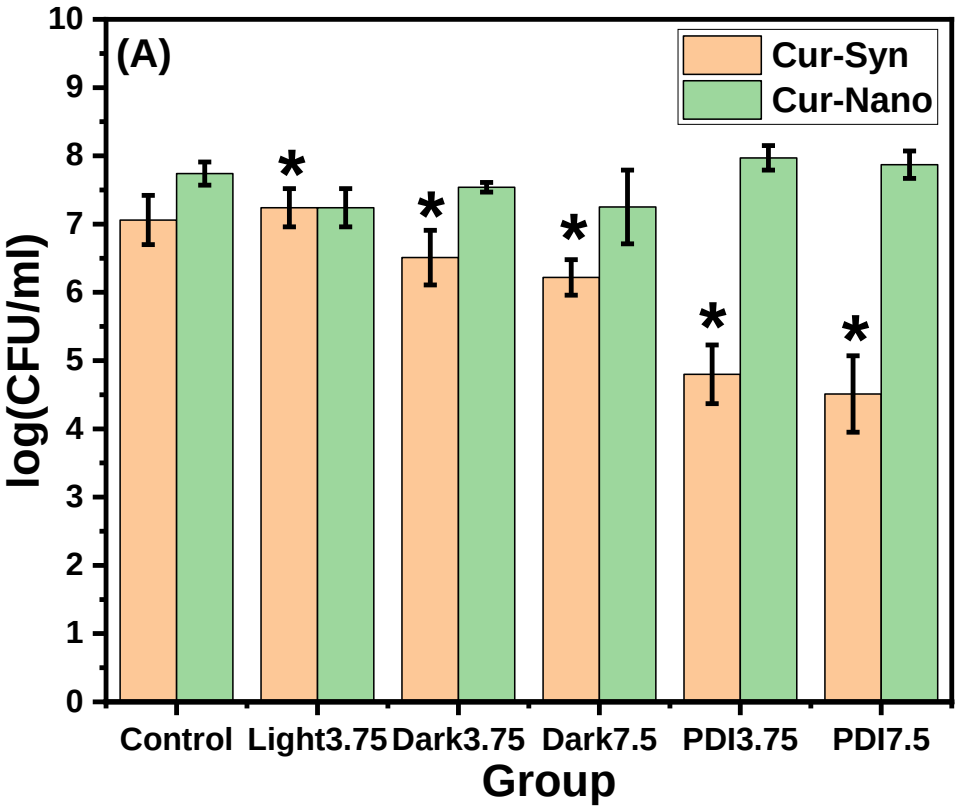
The ANOVA (Analysis of variance) method was used to evaluate the results. The p-value < 0.05 is considered statistically significant. The error bar determined the standard deviation. The experiments were carried out in triplicates on three independent occasions, and the results are presented as mean.

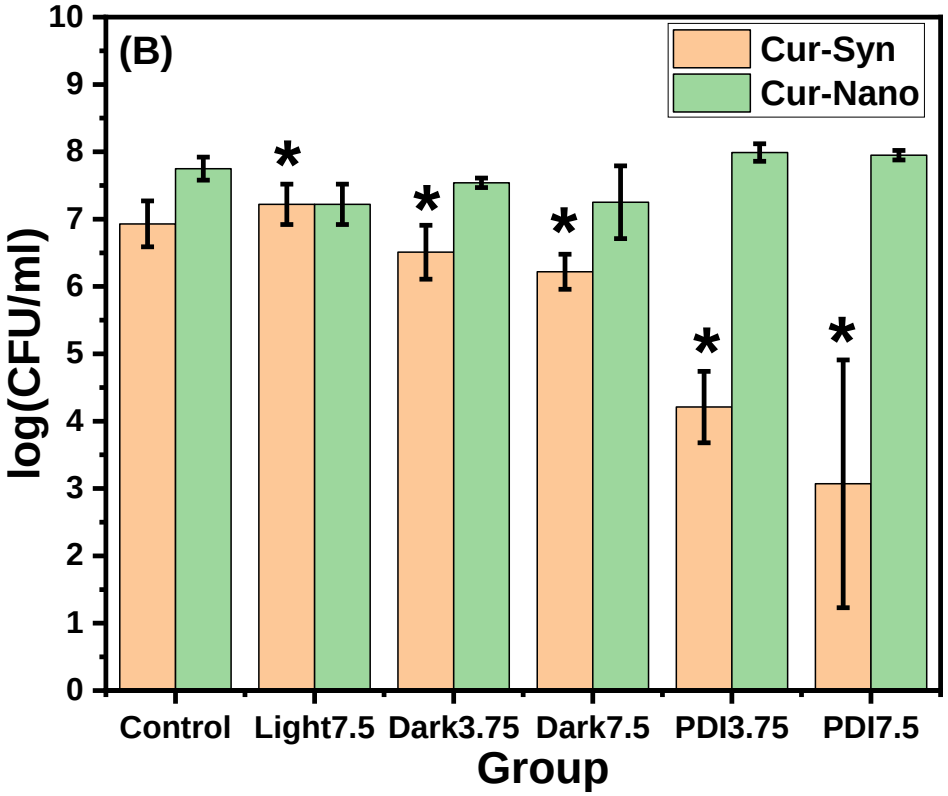
Chapter-4: Results and Discussion

4.1 Nanoformulation

Combination treatment for each photosensitizer

In vitro studies were carried out to evaluate the bacterial reduction using curcumin in different formulations, the Cur-Syn solubilized and the Cur-Nano. Different types of nano-carriers are utilized for curcumin nanoparticle formulation based on their unique properties such as polymeric nanoparticles (controlled release & Enhanced stability), solid-lipid nanoparticles(Improved bioavailability & Targeted delivery), polymeric micelles (Solubility enhancement & improved stability), curcumin nanocrystal Enhanced dissolution rate & Enhanced permeability), nano-emulsions (Improved absorption), nano liposome-encapsulated curcumin (Cellular uptake & Protection from degradation), Cyclodextrin (Increased solubility), curcumin nanosuspension and dendrimers (Enhanced dispersibility &Targeted delivery) (MANCA et al., 2015). Both polymeric nanoparticles and nano liposome-encapsulated curcumin offer advantages in terms of prolonged circulation, improved permeability, and resistance to metabolic processes, making them suitable for enhancing the therapeutic efficacy of curcumin. The Cur-Nano employed in this investigation comprises liposomes, which are spherical vesicles encapsulating curcumin and characterized by the presence of high-density lipoprotein in the membrane. These liposomes closely resemble cellular membranes, providing an effective delivery system for curcumin. The quantity of medication encapsulated within a liposome is influenced by factors such as the number and size of bilayers and the vesicle diameter, all of which play a crucial role in determining the liposome's circulation time (MANCA et al., 2015). We conducted screening experiments to identify the optimal combinations of energy dose and photosensitizer (PS) concentrations. Specifically, we selected two concentrations of each PS paired with an energy dose that did not induce cytotoxic effects in the absence of light (dark group), as well as in groups subjected to irradiation alone (Light group). **Figure 7.** Shows the Microbiological test in S. *aureus* wherein the orange and green bars represent the Photosensitizer: Cur-Syn and Cur-Nano, respectively.


(A)
Cur-Syn
Cur-Nano
log(CFU/ml)
0
1
2
3
4
5
6
7
8
9
10
Control
Light3.75
Dark3.75
Dark7.5
PDI3.75
PDI7.5
Group


(B)
Cur-Syn
Cur-Nano
log(CFU/ml)
0
1
2
3
4
5
6
7
8
9
10
Control
Light7.5
Dark3.75
Dark7.5
PDI3.75
PDI7.5
Group

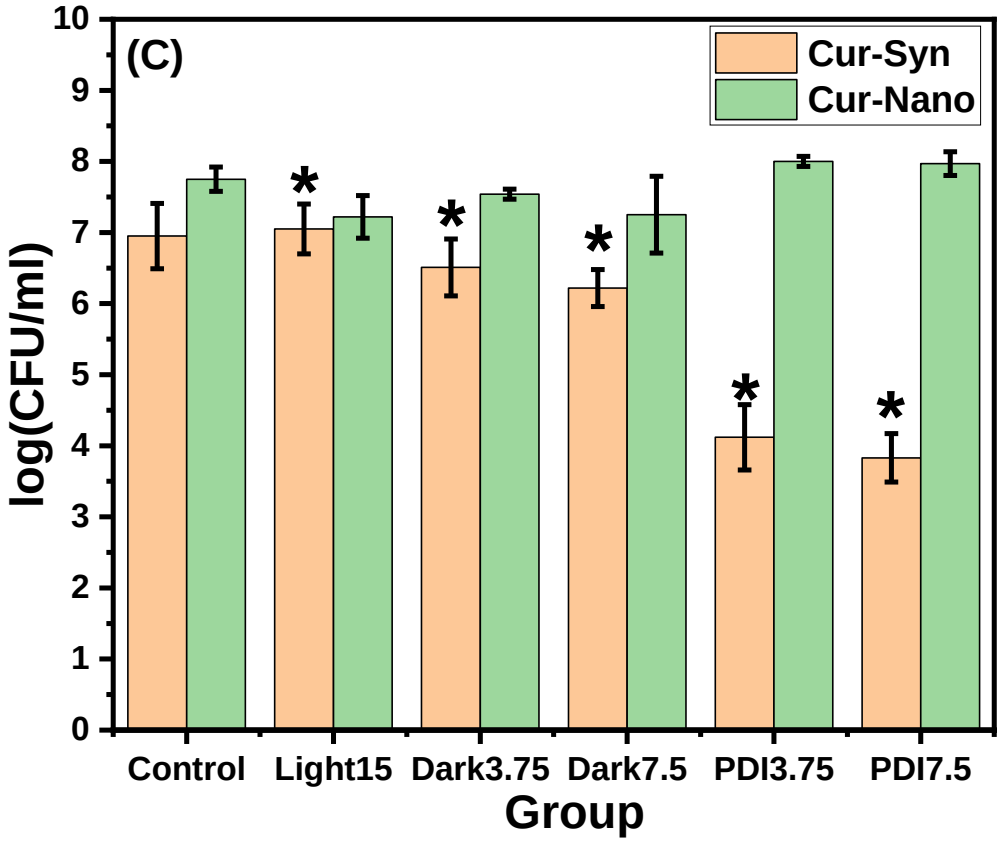


Figure 7. Comparison of aPDT of Synthetic Curcumin (Cur-Syn) vs. Nano Curcumin (Cur-Nano) against S. aureus at two different concentrations, PDI 3.75 and PDI 7.5 at Light Dose (A) 3.75 J/cm^2 (B) 7.5 J/cm^2 and (C) 15 J/cm^2, * indicates statistic difference for $p<0.05$.

For each experiment, the groups taken are as shown in the **Table 1**, Control (C) which is Bacteria + PBS, Light Control (LC) which is Bacteria + PBS + Light, Dark Control (DC) which is Bacteria + PS, and PDI groups, which has Bacteria + PS + Light, were performed. Control groups were performed in each experiment to ensure the observed effects were due to PDT.

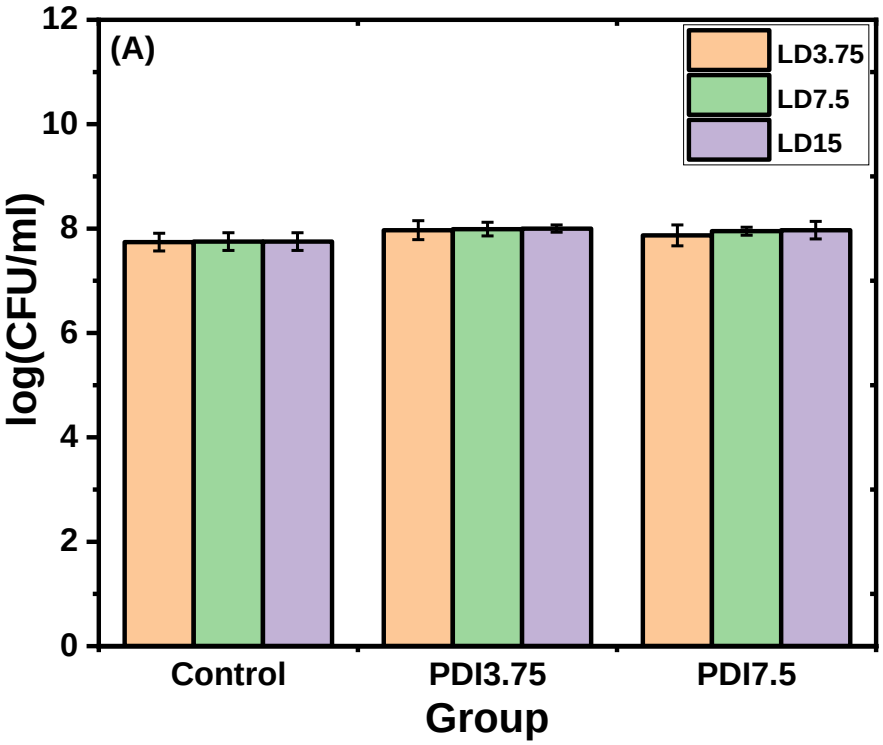
Control groups were performed in each experiment to ensure the observed effects were due to PDT. The results of samples were plotted in a graph of log CFU/ml vs. different groups performed during the experiment. For Light dose 3.75 J/cm^2 **(Figure 7A)**, the bar graph suggests that only Cur-Syn presented a statistical significant effect compared to Cur-Nano. The same pattern was observed for Light dose 7.5 J/cm^2 **(Figure 7B)** and Light dose 15 J/cm^2 **(Figure 7C).**

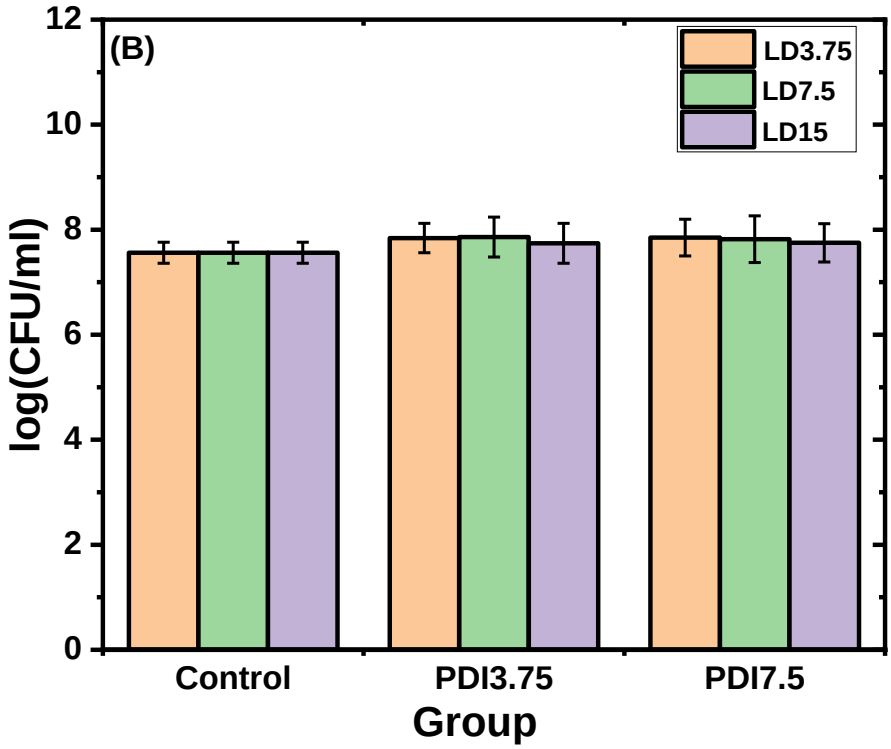
Table 1: Details about each group and their respective contents

Groups	**Contents**
Control (C)	Bacteria + PBS
Light Control (LC)	Bacteria + PBS + Light
Dark Control (DC)	Bacteria + PS
PDI groups	Bacteria + PS + Light

Cur-Syn showed significant reductions of about 2-4 log CFU/ml using low concentrations of PS. Of which the best reduction (about 4 log CFU/ml) was observed for Cur-Syn using Light dose 7.5 J/cm^2 for PDI 7.5, these groups were statistically different from the control group with Tukey test with p-value < 0.05. Cur-Nano didn't show reductions under any concentration of PS, which was not statistically significant considering that the log CFU/ml remained similar for both the concentrations and the three light doses. Since the nanoformulation involves curcumin, which is not readily available for interaction with the bacterial cell, its release is required. Also, the cellular microenvironment plays a significant role in the degradation of nanoparticles and curcumin release from the nanoformulations (YALLAPU et al., 2015). Additionally, the type of nanoparticle and the production methods impact release at desired site (GERA et al., 2017). In contrast, Cur-Syn is comparatively able to interact with bacteria in this environment.

To understand whether incubation time could improve the photodynamic inactivation response, further studies were performed by increasing the incubation time **(Figure 8)**. Microbiological experiments were performed for Cur-Nano using the same concentration (Concentration: PS 3.75 and 7.5) and Light dose (LD3.75, 7.5, 15) as used for Cur-Syn for better comparison. The results were plotted in the graph of log CFU/ml vs. groups, where the orange bar indicates a light dose of 3.75 J/cm^2; the green bar indicated light dose of 7.5 J/cm^2 and the purple bar shows a light dose of 15 J/cm^2 performed during the experiment for 20 mins **(Figure 8A)**, 40mins **(Figure 8B)** and 60mins **(Figure 8C).** The graphs revealed no significant differences in the evaluated incubation times for the Nanoformulation, being these groups not statistically significant (p > 0.05).


(A)
LD3.75
LD7.5
LD15
log(CFU/ml)
0
2
4
6
8
10
12
Control
PDI3.75
PDI7.5
Group


(B)
LD3.75
LD7.5
LD15
log(CFU/ml)
0
2
4
6
8
10
12
Control
PDI3.75
PDI7.5
Group

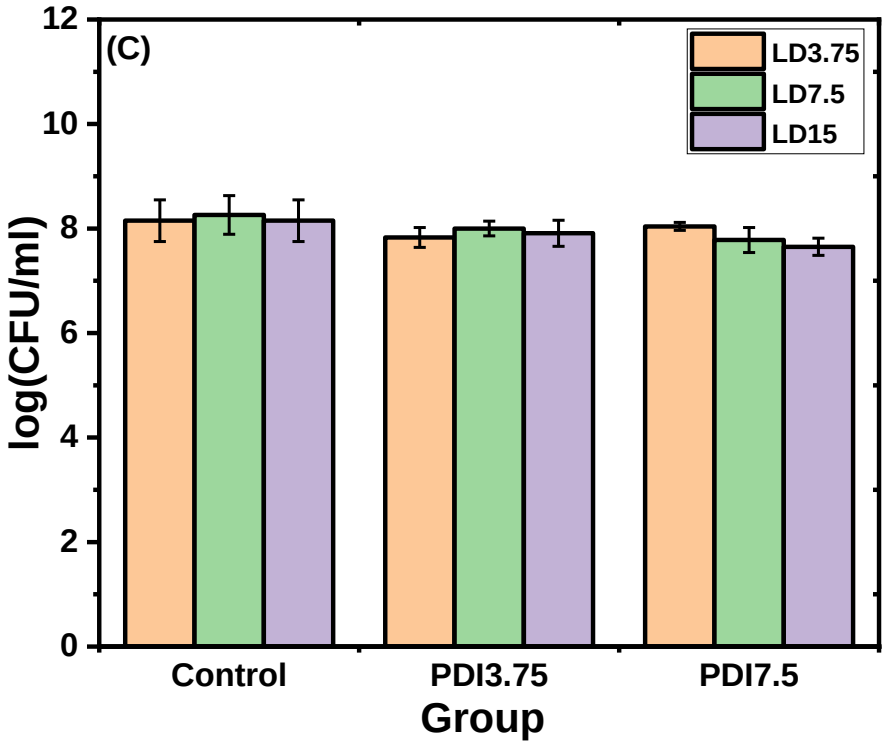


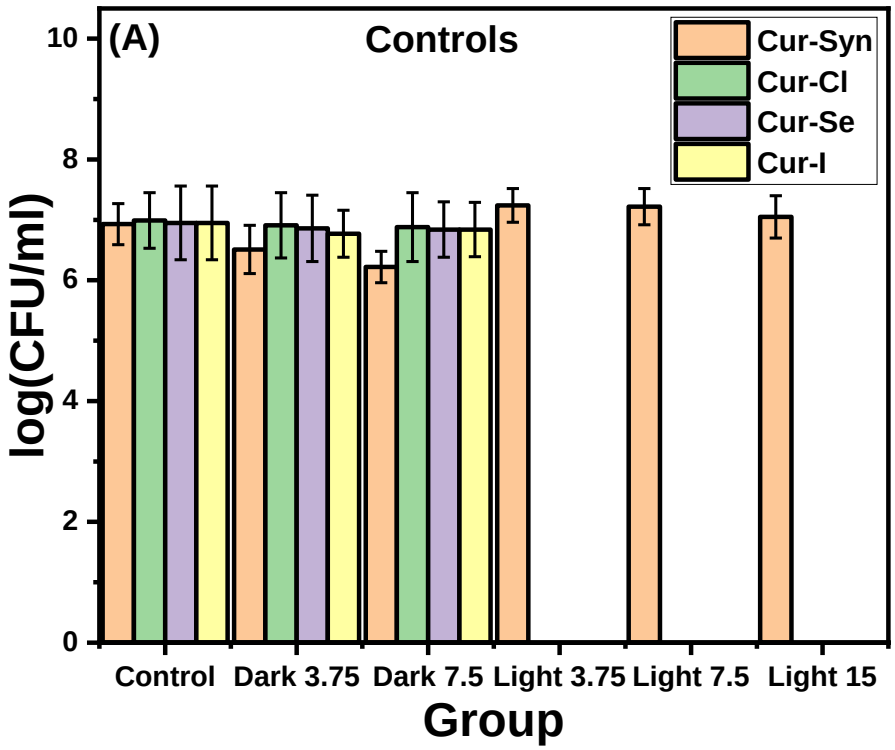
Figure 8. Comparison of aPDT of Cur-Nano against S. aureus at two different concentrations, PDI3.75 μM and PDI7.5 μM at Light Dose 3.75 J/cm^2, 7.5 J/cm^2, and 15 J/cm^2 by varying the incubation time (A) 20 mins (B) 40 mins and (C) 60 mins

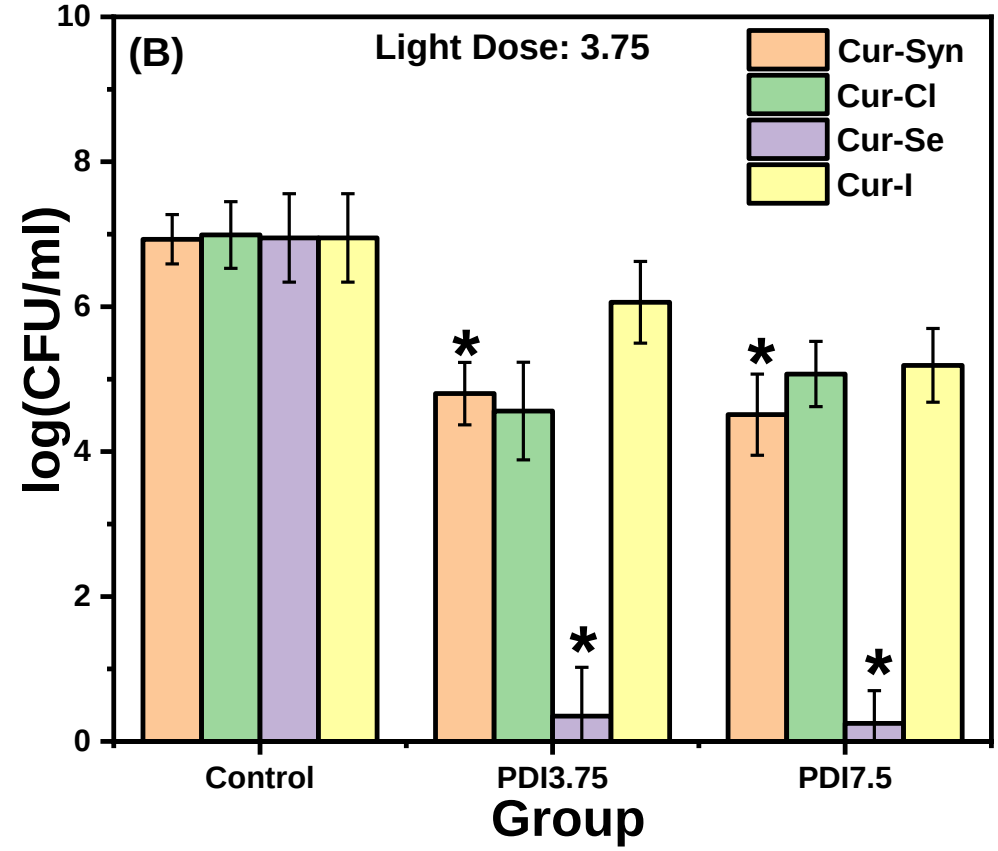
Therefore, the results indicate that Cur-Syn is the most suitable for aPDT. In spite of this, nanoformulation is a good strategy for application in PDT protocols, mainly if it is considered for pharmacokinetic studies. This is because the study of encapsulating photosensitizers aims to protect the molecule, make it more soluble in water, and deliver it directly to the tissue of interest. This increases bioavailability and permits targeting and transport across barriers which accelerates treatment. Also, much research has been done on the development and *in vitro* testing of Cur-Nano, which is designed to deliver curcumin to a specific site and has high permeability, longer circulation, and increased biodistribution, all of which lead to significant responses in terms of effectiveness (GERA et al., 2017). There is, however, a dearth of evidence-based studies that specifically investigate the therapeutic roles of the nanocarrier-based delivery systems in improving antibacterial properties. Therefore, there is much still to be explored. (DAI et al., 2022).

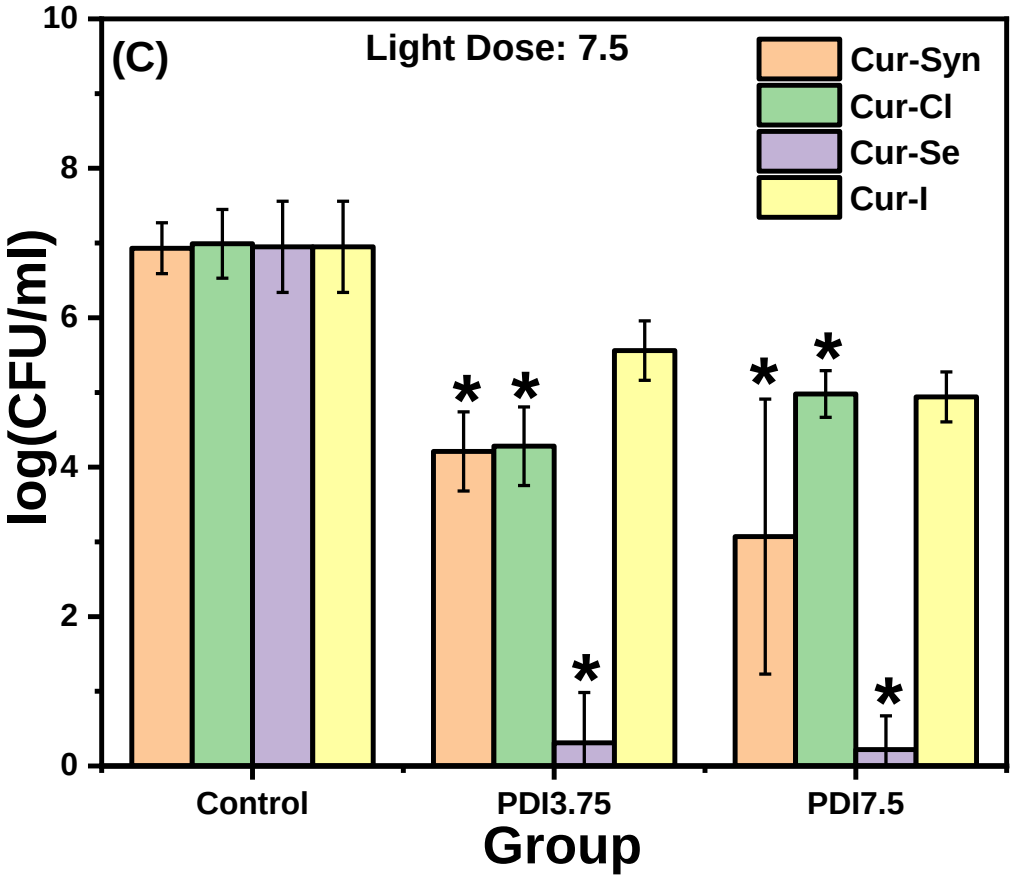
4.2 Curcumin modification

Several studies have shown that curcumin works better against bacteria when it is combined with traditional antibacterial drugs (like polymyxins, meropenem, oxacillin,

tetracycline, ciprofloxacin, ampicillin, and norfloxacin), natural products (like epigallocatechin gallate and berberine), or metals (like Cu^{2+}, Zn^{2+}, and Fe^{3+}) (DAI et al., 2022). Many metals have been used because they have been around for a long time. Their potential molecular mechanism is involved in oxidative stress, protein dysfunction, or membrane damage in bacterial cells (DAI et al., 2022). Modifications in the curcumin molecule **(Figure 5)** were performed by Professor Kleber Thiago de Oliveira from UFSCar and were provided for studying aPDT activity. These modifications consist of bonding atoms of Chlorine, Selenium, and Iodine. Based on the results obtained for Cur-Syn (**Figure 3**), the same concentration and light dose (Concentration: PS 3.75 and 7.5, Light Dose: LD3.75, 7.5, 15) were used for the three modified curcumins.




(B)
Light Dose: 3.75
Cur-Syn
Cur-Cl
Cur-Se
Cur-I
log(CFU/ml)
10
8
6
4
2
0
*
*
*
Control
PDI3.75
PDI7.5
Group


(C)
Light Dose: 7.5
Cur-Syn
Cur-Cl
Cur-Se
Cur-I
log(CFU/ml)
10
8
6
4
2
0
*
*
*
*
*
*
Control
PDI3.75
PDI7.5
Group

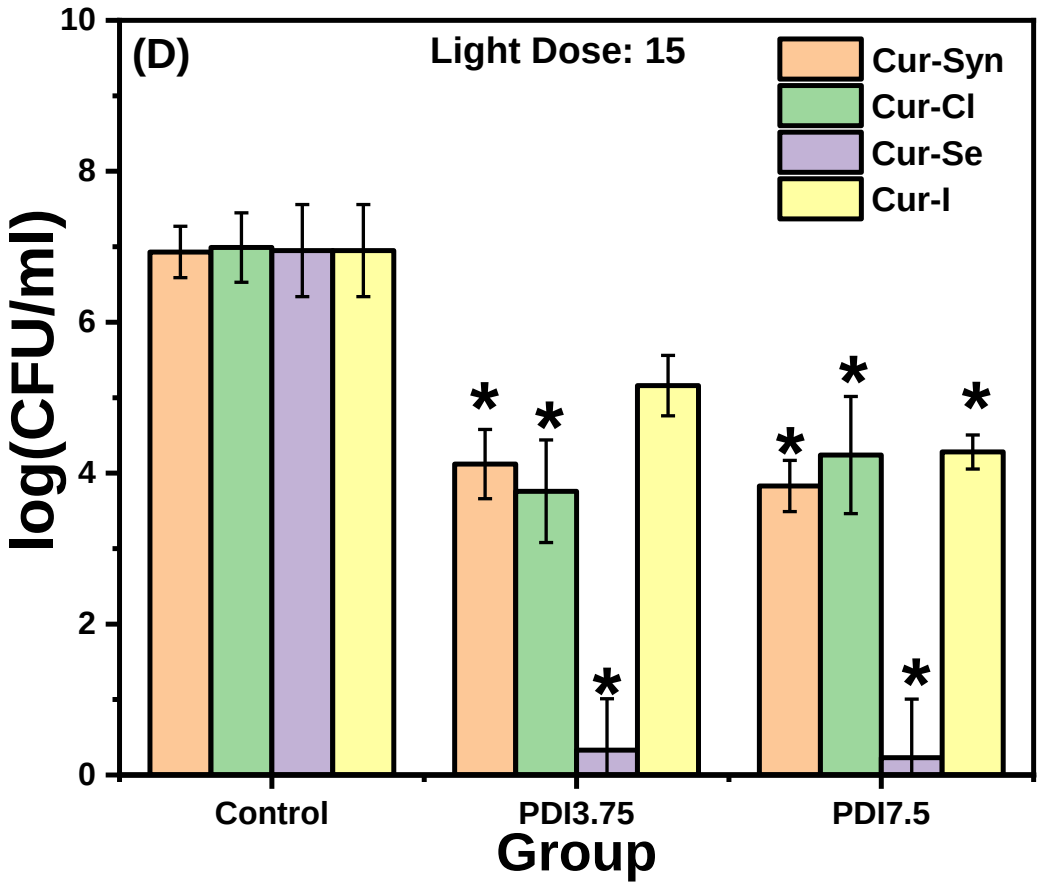


Figure 9. Comparison for (A) controls, aPDT for Cur-Syn, Cur-Cl, Cur-Se, and Cur-I against S. aureus at two different concentrations PDI 3.75μM and PDI 7.5μM at Light Dose (B) 3.75 J/cm^2, (C) 7.5 J/cm^2 and (D) 15 J/cm^2. * indicates statistic difference for $p<0.05$

The bar plot of log CFU/ml vs. PDI groups for different light doses LD3.75J/cm^2 **(Figure 9B)**, LD7.5J/cm^2 **(Figure 9C),** LD15J/cm^2 **(Figure 9D)** shows the reduction in viability of *S. aureus* after the combined treatment of photosensitizer and blue light irradiation where each color indicates PS used: orange color: Cur-Syn, green color: Cur-Cl, purple color: Cur-Se and yellow color: Cur-I. **Figure 9A** shows no significant reduction for dark controls and light controls, and statistically, the values are similar to control, with the Tukey test with p-value >0.05 since there is no photodynamic action and ROS production in the absence of light and PS. When the groups were illuminated with a light Dose of 3.75 J/cm^2 **(Figure 9B)**, Cur-Se showed significant results of 100% reduction in both the concentrations in comparison with the control, being this reduction important ($p<0.05$) followed by Cur-Syn reduced by 2.5 log for PDI 7.5 due to aPDT action being these groups statistically different between them ($p<0.05$). Cur-I didn't reduce significantly for this light dose in any concentration.

Similarly, when the groups were illuminated with a light dose of 7.5 J/cm^2 **(Figure 9C),** Cur-Se showed significant results of 100% reduction in both the concentrations compared with the control, followed by Cur-Syn (about 4-log reduction) for PDI 7.5. and Cur-Cl (about 3 log reduction) for PDI 3.75, being this reduction statistically significant ($p<0.05$). Cur-I didn't reduce significantly for this light dose in any concentration. Moreover, when the groups were illuminated with a light Dose of 15 J/cm^2 **(Figure 9D),** Cur-Se showed significant results of 100% reduction in both the concentrations in comparison with the control, being this reduction important ($p<0.05$) followed by Cur-Syn (about 3-log reduction) for PDI 7.5. and Cur-Cl (about 3 log reduction) for PDI 3.75, being this reduction statistically significant ($p<0.05$). Whereas for Cur-I, below 3logs of reduction was observed for PDI7.5.

In summary, with Cur-Syn, initially 4-logs of reduction was observed for PDI 7.5μM at LD7.5 J/cm², which reached 3-logs of reduction at higher light doses (LD15 J/cm²), which may often be related to aggregation of the molecule (BUZZÁ et al., 2019). While for Cur-Cl, about 50% reduction was achieved at the highest light dose of 15 J/cm² for PDI 3.75 μM. **(Figure 9D)**. Finally, In the case of Cur-Se, even at the lowest light dose and concentration 100% of the cell population was killed. On the other hand, for Cur-I below 3-log CFU/ml of reduction was observed for the highest light dose of 15 J/cm² for PDI 7.5 μM **(Figure 9D)** where the p-value < 0.05 and considered statistically significant.

It is worth mentioning that Cur-Se had a deleterious impact on the viability of cells even at the lowest concentration and lowest energy dose. Selenium is a micronutrient necessary for the maintenance of cellular processes such as DNA synthesis and significant functions of the thyroid and male reproduction. It also possesses an excellent antibacterial action (FILIPOVIĆ et al., 2021; MOJADADI et al., 2021). Selenium prevents bacterial colonization on biomaterial surfaces and *S. aureus* biofilm formation (ARIBI et al., 2015).

While, the low reductions in Cur-Cl seem to improve with increasing the light dose, as evident from the graph of PDI3.75 in **Figure 9A**, which reached 3 logs CFU/ml of reduction when the highest light dose is used **(Figure 9C)**. While in the case of Cur-I, an increase in the dose does not correspond to an increase in the photodynamic effect. Under all the conditions, there were no notable reduction for Cur-I. This may often be related to the state of aggregation of the molecule. Because of its high molecular weight, Cur-I may not generate singlet oxygen effectively. Jianhua Zou et al. studied the heavy atom effect on boron dipyrromethene (BODIPY) derivatives to understand the singlet oxygen ($^1O_2$)

quantum yield (QY), which is one of the critical factors in determining the photosensitizer's performance. They concluded that not all iodine atoms in BBDPI are effective for the generation of singlet oxygen. Instead, with more iodine atoms, the dark toxicity of the photosensitizer increases significantly. They also mentioned that the relationship between the heavy atoms and structural configuration on the effect of $^1O_2$ QYs of the photosensitizers is unclear (ZOU et al., 2017).

Since Selenium compounds alter the expression and activities of several cell cycle regulatory proteins, signaling molecules, mitochondrial associated factors, and others, depending on the form, these selenium compounds can target separate pathways (SINHA; EL-BAYOUMY, 2005). The modification of curcumin with Selenium atom may likely have been favored for this reason compared to Cur-Cl and Cur- I. These marked synergistic effects may be related to the improvement of curcumin or intracellular uptake of curcumin (DAI et al., 2022). Also, at high concentrations, selenium is a prooxidant. Indeed, both selenite and methylselenol can generate superoxide anions in the presence of reduced glutathione. Both superoxide anions and depletion of intracellular pools of reduced glutathione contribute to selenium-induced oxidative stress (TANGUY et al., 2012).

4.3 PS uptake and photobleaching

Based on previous studies **(Figure 9)**, Se-modified curcumins had the highest aPDT activity, so we chose that to compare with Cur-Syn. Both the interaction of PS with the bacteria and its photophysical activity were investigated by measuring the uptake rates and photodegradation, respectively. The results of these investigations are presented here. **Figure 10** illustrates the PS rate of uptake (%) vs. Incubation time (mins) for Cur-Syn and Cur-Se. The graph demonstrates that after 20 minutes of incorporation, bacteria has become saturated by approximately 67.87% for Cur-Syn **(Figure 10A)** and 64.45% for Cur-Se **(Figure 10B).** These results show that the aPDT experiments were carried out with the incubation times being carefully considered and optimized. When both PS are compared, their uptake is similar. Maximum absorbance for Cur-Syn is at 422 nm, whereas for Cur-Se is at 432nm. They are both saturated, but Cur-Syn is saturating a little above the selenium with no significant difference (<10%) after 20 mins, so increasing the incubation time will probably not make a difference.

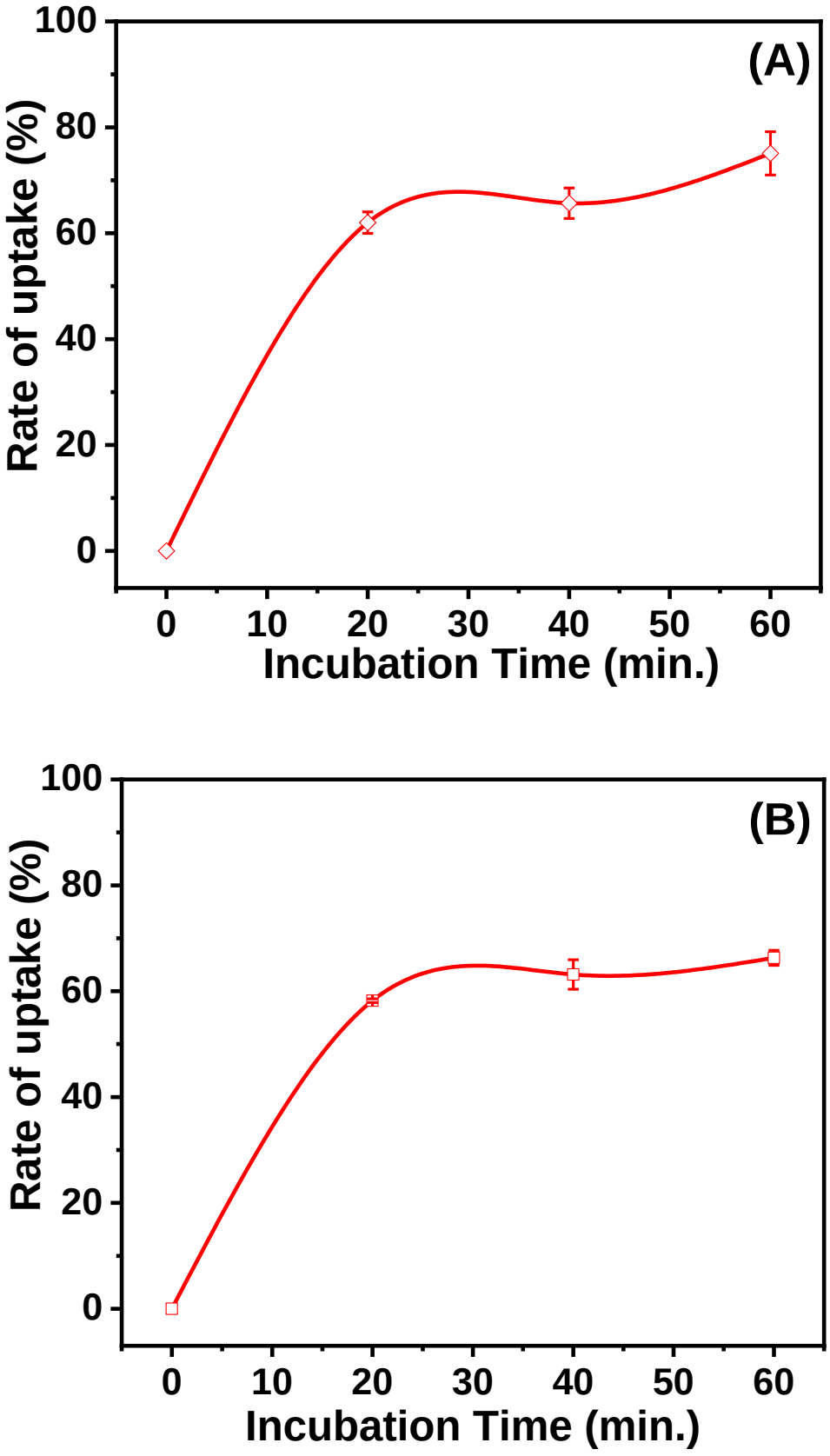
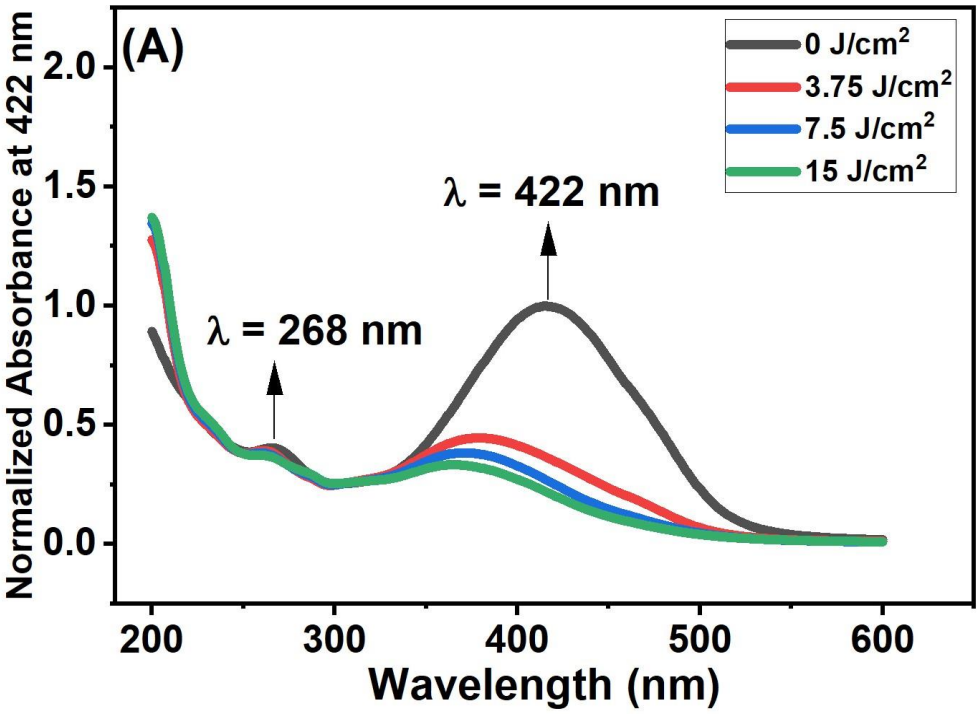
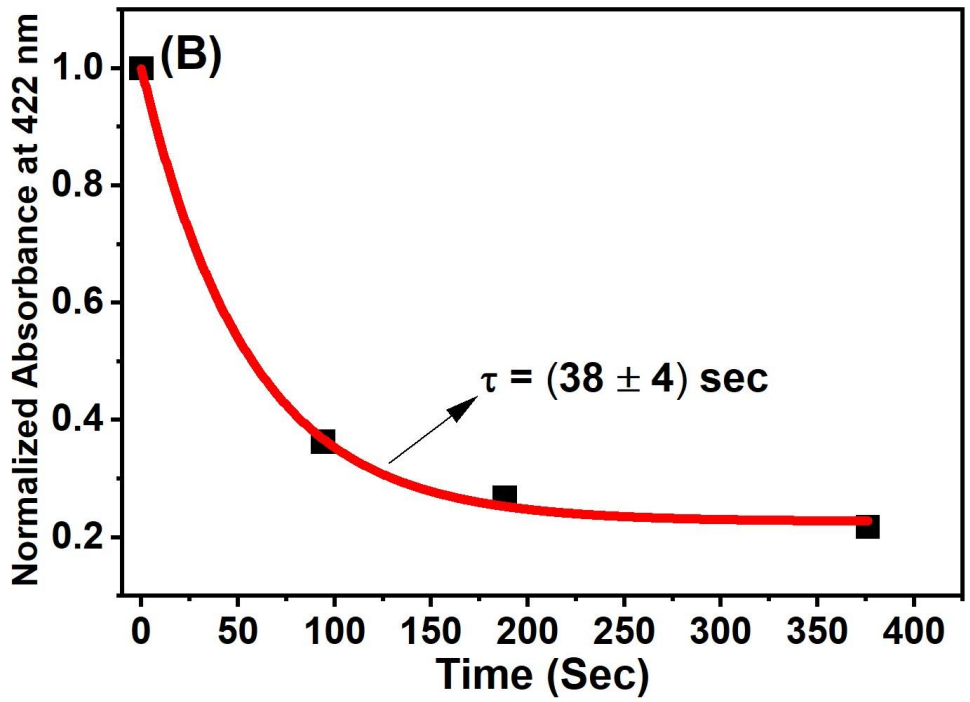


Figure 10: Rate of uptake vs. incubation time for (A) Cur-Syn and (B) Cur-Se

Photobleaching is a phenomenon that takes place when oxidative chemical species are formed because of the highly efficient optical excitation of a photosensitizing molecule. This destroys the molecules immediately adjacent to the photosensitizing molecule, which may be of the same type as the molecule that is absorbing light. This fact arises as a consequence of the brief lifetime of the produced reactive species. The fact that they could show the survival of curcuminoids in aqueous solutions makes absorption an important topic to discuss in this context. This process results in a relative shift that occurs over time in the absorption peaks (REGO-FILHO et al., 2014). Photobleaching experiments for curcumin reveal a clear relationship between its degradation profile and singlet oxygen production. **Figure 11** shows the spectra of Cur-Syn and Cur-Se solutions of 20 µM vs. wavelength at 0 min (0 J/cm^2, black curve), 1 min 34 sec (3.75 J/cm^2, red curve), 3 min 08 sec (7.5 J/cm^2, blue curve), and 6 min 15 sec (15 J/cm^2, green curve), of blue light irradiation. Changes in absorbance for curcumin derivatives relative to the illumination time were monitored. For Cur-Syn at $t_{0,}$ the peak is observed at 422 nm with an absorbance value of 0.99. Upon illumination at time-1 min 34 sec, the peak shifts to 378 nm with an absorbance value of 0.44, which further shifts to 369 nm with an absorbance value of 0.38 and lastly at 363 nm with an absorbance value of 0.33. Similarly, For Cur-Se at $t_{0,}$ the peak is observed at 432 nm with an absorbance value of 0.64. Upon illumination at time-1 min 34 sec, the peak shifts to 390 with an absorbance value of 0.45, which further shifts to 364 with an absorbance value of 0.35, and lastly at 350nm with an absorbance value of 0.33.


(A)
0 J/cm2
3.75 J/cm2
7.5 J/cm2
15 J/cm2
λ = 422 nm
λ = 268 nm
Normalized Absorbance at 422 nm
Wavelength (nm)
2.0
1.5
1.0
0.5
0.0
200
300
400
500
600


(B)
τ = (38 ± 4) sec
Normalized Absorbance at 422 nm
Time (Sec)
1.0
0.8
0.6
0.4
0.2
0
50
100
150
200
250
300
350
400

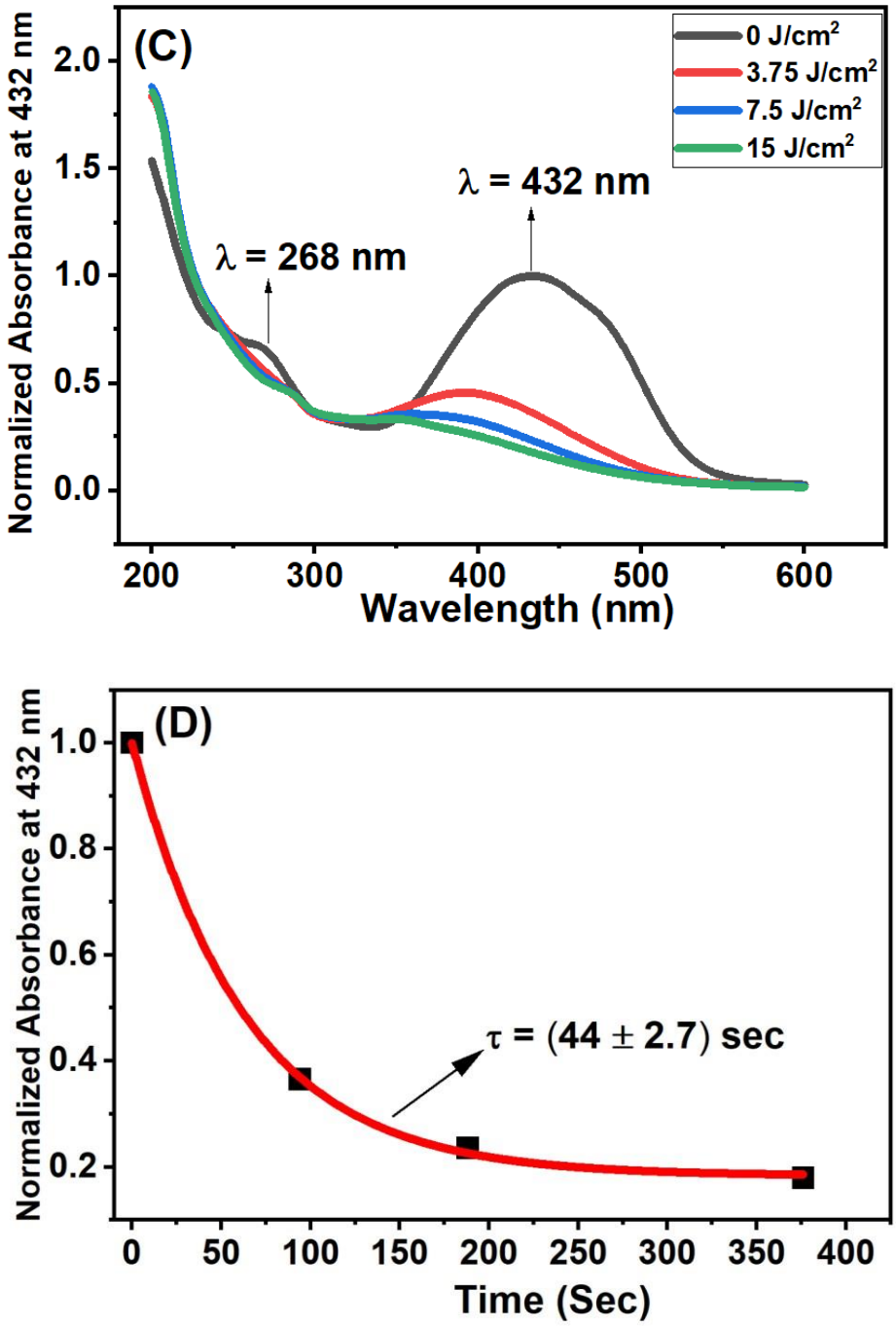


Figure 11: Absorption spectra irradiated at 0, 3.75, 7.5 and 15 J/cm^2 for (A) Cur-Syn and Cur-Se. The arrows show the spectral changes upon irradiation and photoproduct formation at 268 nm. Absorption spectra decrease in (B) Cur-Syn (422 nm) and (D) Cur-Se (432 nm) in the function of irradiation time. The decay time is represented by τ.

Looking closely at the graph in **Figure 11 (A and C),** it can be observed that the peak decreases. This may indicate the natural instability of curcumin-based compounds in the presence of water due to solvation (MUKERJEE et al., 2010; REGO-

FILHO et al., 2014) and a spectral change from blue to UV-A region by the end of light irradiation (6 min 15 sec). The spectrophotometric analysis also highlighted new absorption bands (a small hump) at 268 nm for Cur-Se and Cur-Syn, indicating photoproducts generation. Also, Cur- Se showed the presence of photoproducts at t=0, absorbance 0.43. After illumination (t=3.75,7.5,15), it showed the presence of another photoproduct at absorbance 0.45, which could mean that different photoproducts are generated upon illumination. Both the curcumins presented the same photodegradation behavior, completely photobleached after 6 min 15 sec of light exposure. The characteristic exponential decay τ represents the decay time of each PS. The concentrations were associated with the amplitude of absorption and fitted with an exponential time-dependent Equation 3.

$$C(t) = C_0\, e^{-t/\tau}$$

Eq. 3

It can be seen from **Figures 11 (B and D)** that both decrease exponentially and no significant difference in the decay rate is observed. When Cur-Syn and Cur-Se are exposed to the same amount of light, i.e., the same number of photons, the absorption of synthetic and selenium are not different. Even though decaying equally, Selenium makes more efficient energy transfer to form free radicals. Cur-Syn decays at 38 sec, which is faster than Cur-Se, with a decay time of 44 sec. Cur-Se is relatively more available for action and indeed modifies the bleaching mechanism and promotes a higher efficacy against the bacteria *S. aureus.*

The mechanism of binding of the PS to the cells and its specific cellular localization is not yet fully understood. The site of binding will be where the first damage to the photodynamic effect will occur due to the *in-situ* generation of $^1O_2$ and free radicals (ALVES et al., 2014). Light-activated curcumin significantly increased the intracellular ROS in *S. Aureus* (JIANG et al., 2014). One hypothesis for these results could be that when Cur-Syn and Cur-Se are exposed to the same amount of light, an increased amount of ROS is produced and bleaching the molecule as the lifetime of ROS is short (DIAS et al., 2020; JIANG et al., 2014), the only target of the ROS produced is the photosensitizer itself. Also, we found that this mechanism doesn't seem to be different for both the PS. On the other hand, if the target is present, as bacteria, it seems selenium is more efficient, wherein Cur-Se, when exposed to light, generates ROS. The generated oxygen likely has more affinity for bacterial molecules than for the Cur-Se molecule itself, so these free radicals reach better to the target bacteria following the intrinsic mechanism compared to the situation when there is no target. PS may bind to the cells, loosely or

tightly, being located more externally or internally by diffusion, depending on the interaction mode with cellular components; or it attaches to the cells and is taken up (actively transported into the cell), or translocated through the wall to the plasma membrane thereby destroying the bacterial defense system (ALVES et al., 2014). This is probably because either oxygen is produced farther, and the cross-section is much bigger, even with the same amount. Lastly, the severe damage PDT causes to bacteria makes it difficult for them to transmit their self- adaptivity to the next generation in a short time (REN et al., 2020). Also, The PS binding and its internalization into cells are affected by several factors such as the PS structure and the degree of hydrophobicity, the number of positive charges, and the spatial distribution of substituents seem to play important roles in these relationships.

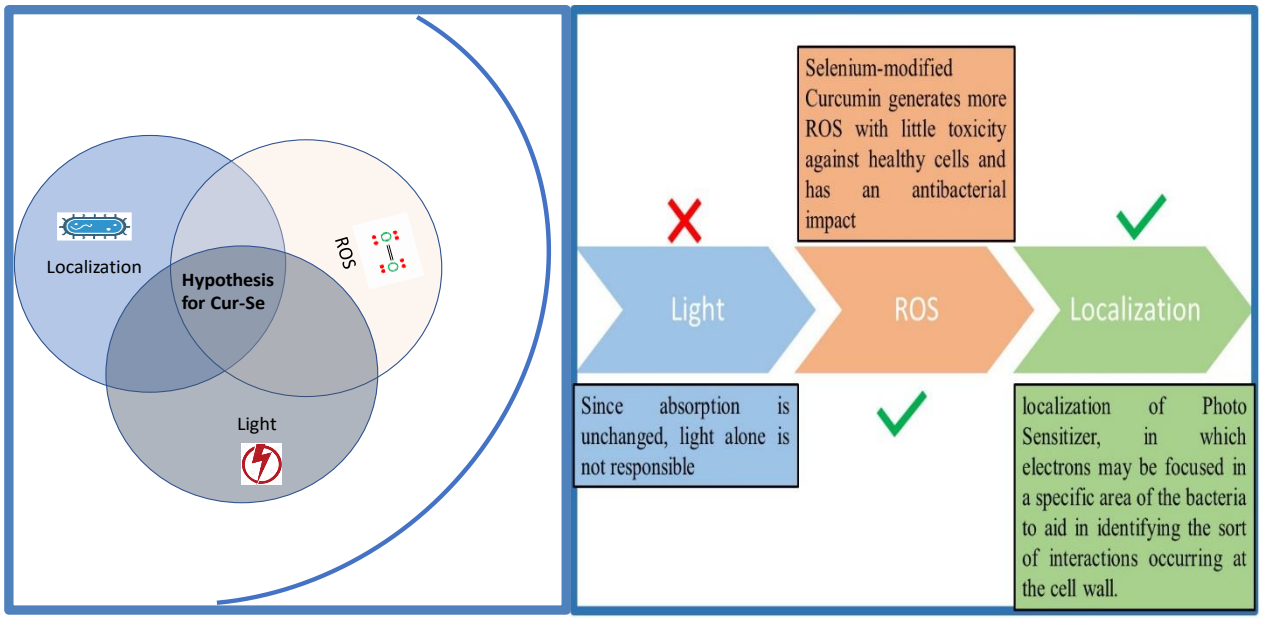


Figure 12: Hypothesis for the mechanism of action of Cur-Se. Figure created by the author.

Figure 12 indicates the three factors could be responsible for this Light, the generation of ROS and the localization of PS (KHORSANDI et al., 2022). Since absorption is unchanged, light alone is not responsible (MALLIDI et al., 2016). An alternative hypothesis could be that Selenium-modified Curcumin generates more ROS with little toxicity against healthy cells and has an antibacterial impact, hence it would be important to measure singlet oxygen. Another factor is the localization of photosensitizer, in which electrons may be focused in a specific area of the bacteria to aid in identifying the sort of interactions occurring at the cell wall (DOLMANS; FUKUMURA; JAIN, 2003; GUNAYDIN; GEDIK; AYAN, 2021).

Chapter 5 – Conclusion

The main conclusions of this project are as below:

- This work demonstrated the comparison of Cur-Syn vs. Cur-Nano. The results show that Cur-Syn has better effects concerning log reduction than Cur-Nano.
- Curcumin Modification - This work compared Cur-Syn with modified curcumins. (Cur-Cl, Cur-Se, Cur-I). The results show that Cur-Se prepared by Prof. Dr. Kleber T. de Oliveira at UFSCar, Sao Carlos, Brazil has the best results in both conditions using the lowest light dose and concentration. Cur-I didn't reduce significantly for any light dose and concentration used.
- Rate of Uptake: The uptake of Cur-Syn and Cur-Se by bacteria is similar as both saturate after 20 minutes.
- Photobleaching: The decay rate for Synthetic curcumin is lower than 38 ± 4 sec, while for Cur-Se is 44 ± 2.7 sec. Although both have similar decay profiles, Cur-Se is degrading a little later than synthetic, which means it is more available for bacteria.

Chapter 6 – Perspective for future studies

- More investigations are needed to explore the aPDT action of the Cur-Se and Cur-Cl.
- Additionally, it would be interesting to see the aPDT of nanoparticles prepared with Cur-Se.
- In vivo applicability of Cur-Se would be another subject of further investigations. evaluate the effects of modified curcumins upon biofilms and in G - planktonic bacteria.
- Test the cytotoxicity in human fibroblasts.
- Quantify ROS production and cellular localization with confocal microscopy.

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