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Dissertação

Bioprospecção de novos compostos monocurcuminoides sintéticos: atividade antioxidante, antimicrobiana e sinérgica e avaliação da citotoxicidade "*in vitro*"

Milena Mattes Cerveira

Pelotas, 2020

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Biopropsecção de novos compostos monocurcuminoides sintéticos: atividade antioxidante, antimicrobiana e sinérgica e avaliação da citotoxicidade "*in vitro*"

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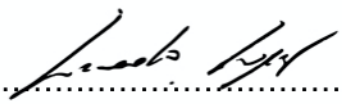

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Dedicatória

Aos meus avós, Lisete Isabel Lehnen Pires Cerveira, Mariza Elaine Brenner Mattes, Ocirio Pires Cerveira e Paulo Guilherme Mattes e à minha mãe, Cristiane Mattes, pelo amor e apoio incondicionais.

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“Nada na vida deve ser temido, somente compreendido.
Agora é hora de compreender mais para temer menos.”
(Marie Curie)

Resumo

CERVEIRA, Milena Mattes. ***Bioprospecção de novos compostos monocurcuminoides sintéticos: atividade antioxidante, antimicrobiana e sinérgica e avaliação da citotoxicidade "in vitro".*** 2020. Dissertação (Mestrado) – Programa de Pós-Graduação em Bioquímica e Bioprospecção, Universidade Federal de Pelotas, Pelotas.

Por consequência da utilização indevida e/ou exacerbada de medicamentos pelo homem, as infecções causadas por micro-organismos, por exemplo, estão emergindo como um grande problema de saúde, uma vez que estratégias convencionais já não são suficientes para o tratamento destas enfermidades. Dessa forma, a busca pelo desenvolvimento e caracterização de novos compostos com atividade biológica tem sido um desafio da área de biopropsecção. Compostos naturais têm surgido como uma alternativa segura e acessível para o estudo de novas moléculas com atividades biológicas, como é o caso da curcumina, extraída do rizoma *Curcuma longa (Zingiberaceae).* Contudo, por consequência do seu teor lipofílico, a biodisponibilidade da curcumina no organismo é baixa, implicando diretamente na eficiência dessa molécula no organismo. Alternativas como a modificação da estrutura química dessa molécula permitem um estudo de novas possibilidades bioativas. Os monocurcuminoides (CNs), derivados da curcumina com apenas uma ponte de carbonila, apresentam-se como grandes candidatos para uma melhor atividade biológica. O estudo foi conduzido com 5 CNs previamente sintetizados (CN, 59, 63, 67 e 77), juntamente com a curcumina (SIGMA) e a cúrcuma em pó (POWDER). A identificação química de SIGMA e POWDER foi realizada por Cromatografia Gasosa acoplado a Espectrofotômetro de Massas (CG-MS) e demonstrou picos em comum, identificando a possível presença de curcumina no tempero obtido comercialmente. A capacidade antioxidante (DPPH e ABTS) foi analisada e os CNs apresentaram valores duas vezes maiores para o ABTS, com valores semelhantes à vitamina C. Os valores de CIM foram avaliados nas diferentes cepas e apresentaram valores entre 4.06 a 150µg/ml. Apenas para *Staphilococcus aureus* a CIM e o CBM foram semelhantes para os CN's 63 e 67, com valores de 150 e 120 µg/ml, respectivamente. O efeito sinérgico dos compostos CN 77 e POWDER foi observado para *Aeromonas hydrophila*, *Candida albicans* e *Pseudomonas aeruginosa* nas concentrações 8.75 e 4.37µg/ml de CN 77 e 9.37 e 4.68µg/ml de POWDER. A terapia fotodinâmica demonstrou ser efetiva na inibição do crescimento microbiológico em 2x CN 59 para *E. faecalis*, com um decréscimo de 4.18 vezes e para *E. coli* o mesmo tratamento quase inibiu totalmente o crescimento. O ensaio de atividade hemolítica demonstrou intervalos seguros para todas as substâncias, com valores de IC_{50} entre 49 e 130 µM. A capacidade anticoagulante dos CNs demonstrou valores dentro dos intervalos normais quando comparados à varfarina mas, quando comparados com heparina, a maioria dos valores estava acima do intervalo recomendado, indicando uma possível atividade anticoagulante na via intrínseca. No geral, os compostos estudados apresentam-se seguros e como uma boa alternativa à curcumina.

Palavras-chave: curcumina, bioprospecção, monocurcuminoides, citotoxicidade, atividade antimicrobiana, resistência antimicrobiana

Abstract

CERVEIRA, Milena Mattes. **Bioprospecction of new synthetic monocurcuminoids: antioxidant, antimicrobial and synergic activities and *in vitro* cytotoxicity**. 2020. Dissertação (Mestrado) – Programa de Pós-Graduação em Bioquímica e Bioprospecção, Universidade Federal de Pelotas, Pelotas.

As a consequence of the improper and/or exarcerbated use of medications, infections such as the ones caused by microorganisms are emerging as a critical health problem considering that conventional strategies are no longer satisfactory for the treatment of these diseases. As a result, the search for the development and characterization of new compounds with biological activity is an obstacle for researchers. Natural compounds are emerging as a safe and accessible alternative for the study bioactive compounds. Curcumin is extracted from the spice turmeric, the power dried rhizome *Curcuma longa* (Zingiberaceae). However due to its lipophilic nature curcumin's bioavailability is low, affecting its efficiency in the organism. Studies regarding structural modifications allow new prospective bioactive molecules. New candidates are presented as monocurcuminoids (CN's) and are based on curcumin's chemical structure with only one methyl bridge. The study was conducted with four previously synthetized CN's (59, 63, 67 and 77) along with curcumin (SIGMA) and turmeric powder (POWDER). Chemical identification of SIGMA and POWDER was performed with GC-MS and showed similar peaks, indicating a possible presence of curcumin in turmeric powder. Antioxidant activity was evaluated by DPPH and ABTS assays. Compounds showed similar values to vitamin c for the ABTS scavenging capacity. MIC values were evaluated in different strains (ATCC and clinical isolates) and presented values between 4.06 and 150µg/ml. Only for *S. aureus* MIC and MBC were equal when treated with CN 63 and 67 with values of 150 and 120µg/ml, respectively. Synergistic effect of CN 77 with POWDER was observed for *A. hydrophila, C. albicans* and *P. aeruginosa,* when treated with 8.75 and 4.37µg/ml of CN 77 and 9.37 and 4.68µg/ml of POWDER. Photodynamic therapy was effective in 2x MIC of CN 59 for *E. faecalis* with growth inhibition by 4.18-fold and on *E. coli* the same treatment almost abolished bacterial growth. Hemolytic cytotoxicity showed safe intervals for all compounds, with IC50 values ranging from 49.65 to 130.9µM. Anticoagulant activity demonstrated values within range for most compounds when compared to warfarin but when compared with heparin most values were above the recommended interval, indicating a possible anticoagulant activity for the intrinsic pathway. Taking together, the results showed that CN's are safe and a promising alternative for curcumin.

Key words: curcumin, bioprospection, monocurcuminoids, cytotoxicity, antimicrobial activity, antimicrobial resistance

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Sumário

1 Introdução

O estudo de novos compostos que apresentam atividade biológica têm sido uma busca constante na área da bioprospecção. O surgimento de vírus de alto risco, aumento das infecções fúngicas no mundo todo, complicações pós-cirúrgicas e resistência aos medicamentos disponíveis no mercado demonstram a necessidade do desenvolvimento de novos métodos para enfrentar essas enfermidades (STROBEL; DAISY, 2003)

As infecções microbianas, por exemplo, estão emergindo como um importante problema de saúde (MEDINA; PIEPER, 2016; PERLIN; RAUTEMAA-RICHARDSON; ALASTRUEY-IZQUIERDO, 2017), devido a tratamentos recorrentes, utilização incorreta de fármacos, cirurgias e/ou sistema imunológico debilitado, não apenas colocando em risco a saúde do homem como também aumentando os custos de tratamento (COMISSION, 2011). Esses patógenos podem causar infecções recorrentes como vaginose bacteriana e candidíase bem como condições com risco de vida como meninginte, pneumonia, asma e tuberculose (COMISSION, 2011; PERLIN; RAUTEMAA-RICHARDSON; ALASTRUEY-IZQUIERDO, 2017). Uma vez que os micro-organismos podem se tornar resistentes aos fármacos utilizadas para os tratamentos, estas se tornam por vezes inviáveis, e o patógeno pode persistir no hospedeiro, aumentando os riscos de saúde e de contaminação de terceiros (WORLD HEALTH ORGANIZATION, 2018).

De acordo com o *website* da ANVISA, estima-se que, a cada ano, em média 700.000 pessoas morrem em decorrência da resistência antimicrobiana, incluindo, HIV, tuberculose e malária. Calcula-se ainda que, até 2050, uma pessoa a cada três segundos morrerá vítima do aumento da resistência dos micro-organismos aos fármacos atuais, representando 10 milhões de óbitos por ano, um número muito maior do que a atual mortalidade relacionada ao câncer, com 8.2 milhões de óbitos por ano (ANVISA, [s.d.]).

Como uma alternativa para a medicina tradicional, o estudo de fitoquímicos tem sido amplamente explorado (MODY; ATHAMNEH; SELEEM, 2020). A curcumina é um polifenol presente na cúrcuma, um tempero conhecido derivado da planta *Curcuma longa,* um rizoma indiano da família do gengibre (Zingiberacea) (FAROOQUI; FAROOQUI, 2019) Essa molécula é utilizada, por conta de seu sabor e coloração, na preparação alimentícia, principalmente na Ásia e Oriente Médio, mas é também um composto largamente utilizado na Medicina Tradicional, como já exposto no *Sushruta's Ayurvedic Compendium,* uma literatura datada em 250 AC, conhecida como o "pai da cirurgia", com mais de 300 procedimentos cirúrgicos, 120 instrumentos e 60 tratamentos para feridas (SINGH, 2017). A curcumina apresenta uma ampla gama de propriedades biológicas reportadas na literatura incluindo a ação sobre diferentes tipos de câncer (SHEHZAD; SHAHZAD; LEE, 2014), anti-inflamatória (GUGLIELMO et al., 2017), antimicrobiana (MODY; ATHAMNEH; SELEEM, 2020) e antiparasitária (CARAPINA DA SILVA et al., 2019b)

Apesar de biologicamente ativa, a curcumina possui baixa biodisponibilidade devido a sua má absorção no organismo. Ainda são encontrados empecilhos para obter um equilíbrio entre alta atividade, eficiência e biodisponibilidade, necessitando de grandes doses diárias para obter uma boa resposta terapêutica e, consequentemente, aumentando os riscos de efeitos adversos como a toxicidade (TRIGO GUTIERREZ et al., 2017).

De forma a ultrapassar essas limitações, diferentes estratégias já foram propostas para melhorar a biodisponibilidade e bioatividade da curcumina, dentre elas a inserção em nanopartículas (KANG et al., 2020), o uso de adjuvantes terapêuticos (BOLAT et al., 2020) e as modificações na estrutura química (CARAPINA DA SILVA et al., 2019). Recentemente, Carapina e colaboradores (2019) sintetizaram diferentes análogos da curcumina e investigaram a sua atividade antiparasitária frente a *T. vaginalis.* Contudo, faz-se necessário um estudo mais aprofundado da atividade biológica dos mesmos.

Tendo em vista as propriedades farmacológicas associadas à curcumina e a dificuldade na biodisponibilidade da mesma, o presente estudo visa bioprospectar monocurcuminoides sintéticos através da avaliação de sua atividade biológica utilizando diferentes bioensaios “*in vitro*” frente a possíveis novas propriedades dessas moléculas.

2 Objetivos

2.1 Objetivo Geral

Avaliar a atividade antioxidante, antimicrobiana, sinérgica e citotoxicidade "*in vitro*" de compostos monocurcuminoides sintéticos.

2.2 Objetivos Específicos

- Caracterizar e analisar os compostos majoritirários da cúrcuma com uma curcumina padrozinada.

- Avaliar a atividade antioxidante das curcuminas.

- Determinar a Concentração Mínima Inibitória (CIM) e avaliar o sinergismo dos compostos frente as bactérias, *A. hydrophila, E. coli, E. faecalis*, *K. pneumoniae*, *P. aeruginosa* e *S. aureus* e a levedura *C. albicans.*

- Determinar a atividade foto dinâmica (aPDT) dos compostos frente às cepas de *A. hydrophila, E. coli, E. faecalis*, *K. pneumoniae*, *P. aeruginosa* e *S. aureus* e a levedura *C. albicans.*

- Analisar a citotoxicidade dos compostos em hemácias.

- Identificar a atividade anticoagulante dos compostos em plasma humano.

3 Revisão Bibliográfica

3.1 Curcumina

3.1.1 Histórico

Amplamente utilizada na medicina tradicional, a curcumina é um composto originário da Índia (NAIR, 2013) extraído do rizoma da *C. longa* (Zingiberaceae). No Brasil, este rizoma é conhecido como Cúrcuma ou açafrão-da-terra, e é amplamente utilizado na culinária como tempero, devido a sua coloração e sabor (NELSON et al., 2017).

Em 1910, a estrutura química da curcumina foi identificada por Milobedzka e Kostanecki e a primeira síntese bem sucedida ocorreu em 1913 pelo mesmo grupo de pesquisadores (LAMPE; MILOBEDZKA, 1913). A primeira utilização da cúrcuma como agente terapêutico foi descrita por Oppenheimer em 1937, no tratamento de doenças humanas biliares.

De acordo com Farooqui e colaboradores (2019), o uso da curcumina foi descrito, primeiramente, *no Sushruta's Ayurvedic Compendium*, em 250 AC, quando foi utilizado para tratar sintomas de intoxicação alimentar. Na Ásia, é utilizado para tratar problemas estomacais e doenças do fígado, como também para o tratamento de feridas e problemas de pele, como catapora (NELSON et al., 2017). Ela também é utilizada em rituais de nascimentos, de forma que uma pasta do rizoma é colocada tanto na barriga da mãe e no cordão umbilical, para evitar maus espíritos para a criança recém-nascida (NAIR, 2013).

3.1.2 Estrutura química

A composição química da *C. longa* consiste em, aproximadamente, 70% carboidratos, 13% umidade, 6% proteínas, 5% gordura, 3% minerais (potássio, cálcio,

fósforo, ferro e sódio) e entre 3-5% curcuminoides (GOEL; KUNNUMAKKARA; AGGARWAL, 2008; PRASAD et al., 2014).

Metabólitos secundários são um grupo de substâncias que possuem atividade biológica, incluindo alcaloides, terpenoides, fenóis e flavonoides. A curcumina é um polifenol extraído da *C. longa* e é o metabólito secundário majoritário (NELSON et al., 2017) responsável pela coloração amarela do rizoma. Dentre os 3-5% de curcuminoides presentes no rizoma, a curcumina apresenta-se em aproximadamente 77% deste valor ((FAROOQUI, 2019; PRASAD; TYAGI; AGGARWAL, 2014).

A curcumina apresenta um peso molecular de 368.37 g/mol, ponto de fusão de 179-183 oC e fórmula molecular $C_{21}H_{20}O_6$ e seu nome pela IUPAC é 1,7-bis(4-hydroxy-3-methoxy-phenyl)-1,7-heptadiene-3,5-dione (1E-64), também conhecida por diferuloilmetano (DFM). É uma molécula simétrica, composta de dois ácidos ferúlicos unidos por uma ponte de metano, e possui dois grupos funcionais majoritários: um grupo a-b insaturado e um grupo *O*-metoxi fenol (SLIKA; PATRA, 2020)**.** O primeiro grupo permite a molécula uma conformação tautomérica, exibindo tanto uma estutura cetônica quando enólica (Figura 1), nas quais fatores como pH e temperatura podem influenciar na sua forma predominante (FAROOQUI, 2019)

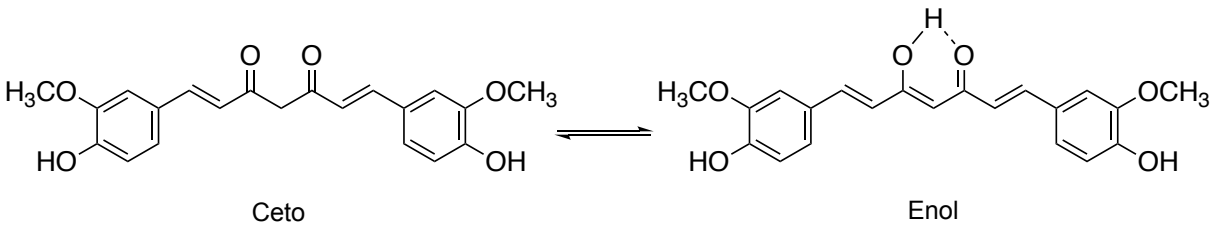

Figura 1: Estrutura e tautomeria ceto-enólica da molécula da curcumina. Fonte: Farooqui, (2019).

3.1.3 Metabolismo e Biodisponibilidade

A curcumina pode interagir com diversas moléculas de sinalização, incluindo moléculas inflamatórias (GUGLIELMO et al., 2017), HIV1-integrase (MAZUMDER et al.,

1995), DNA and RNA (NAFISI et al., 2009) e íons metálicos (MOHAMED et al., 2020; TEDESCO et al., 2020).

Apesar de biologicamente ativa, estudos de outras décadas já demonstraram a instabilidade da curcumina, com até 90% de sua composição em menos de 30 minutos, na presença de tampão fosfato em pH 7.2 (WANG et al., 1997a) e 7.4 (Commandeur apud (WANG et al., 1997). A temperatura também pode ter influência na estabilidade da curcumina. Um estudo realizado por Suresh e colaboradores (2007) analisou diferentes condições de cozimento da cúrcuma, demonstrando que a curcumina apresentou estabilidade até 70ºC por 10 minutos, e que a fervura desta levou a uma perda de 27-32%. O mesmo estudo ainda demonstrou que o cozimento sob pressão (15 psi) por 10 minutos gerou uma perda de 53% de curcumina.

A biodisponibilidade de uma molécula está relacionada com a absorção pelo epitélio do trato gastrointestinal (TGI) para que esta possa entrar na circulação (SANIDAD et al., 2019). Tønnesen e Karlsen (1985) já haviam demonstrado a baixa biodisponibilidade da curcumina por ser um composto hidrofóbico que, em contato com a umidade do GIT, inviabiliza sua absorção. Ainda assim, mesmo se a curcumina fosse absorvida pelo GIT, esta não seria transportada para a circulação, mas sim para que fosse excretada (SANIDAD et al., 2019).

Vareed e colaboradores (2008) também demonstraram a baixa biodisponibilidade em plasma humano, após a ingestão de 10 a 12 g de cúrcuma, indicando a necessidade de uma dose maior. Cheng et al (2019) também estudou a presença, em plasma humano, após a ingestão de 4g de cúrcuma nos intervalos de 0, 0,5, 0,75, 1, 2, 4, 6, 8 e 12 horas utilizando HPLC-ITMS/MS/MS. A presença de curcumina não foi detectada, apenas o metabólito principal desta, a curcumina-glucoronida, no intervalo de 0,5 horas, com pico máximo em 2,7 horas, sugerindo um rápido metabolismo da curcumina pelo organismo e consequente baixa biodisponibilidade.

A curcumina é também fotossensível. O armazenamento da curcumina em um frasco transparente durante o preparo de amostras demonstrou um decréscimo de 5% na absorbância (COPPER, 1994 apud WANG et al., 1997). Concomitantemente, a exposição de uma solução alcoólica de curcumina por 4 horas em comprimentos de onda entre 400 e 510 nm demonstrou a perda de dois hidrogênios da estrutura, através da análise por técnicas espectrométricas, como a Ressonância Magnética Nuclear e Espectrometria de Massas, por um m/z 366 (TØNNESEN; KARLSEN, 1985).

Nos últimos anos tem sido desenvolvidas estratégias para melhorar a absorção de curcumina pelo organismo e a consequente melhor suas atividades biológicas. Como uma das alternativas tem-se a modificação de sua estrutura química (PRASAD et al., 2014).

3.1.4 Atividade Antimicrobiana e aPDT

A primeira referência científica sobre a atividade antimicrobiana da curcumina foi publicada em 1949 na revista *Nature*, atuando sobre a inibição do crescimento de *S. aureus (*(SCHRAUFSTÄTTER; BERNT, 1949). Diversos estudos confirmam a atividade antimicrobiana da curcumina, desde a aplicação direta em micro-organismos (ABDULRAHMAN et al., 2020; ADAMCZAK; OŻAROWSKI; KARPIŃSKI, 2020; DE OLIVEIRA et al., 2018; JORDÃO et al., 2020) como também na preparação de embalagens para alimentos (ROY; RHIM, 2020).

De acordo com Zheng e colaboradores (2020), a curcumina é um composto fotosensitivo e fototóxico que, quando excitado à luz azul, apresentando efeito bactericida em diferentes cepas de micro-organismos a partir da influência sobre o sistema *quorum sensing,* impedindo, por exemplo, a formação de biofilmes.

O potencial da aPDT foi descoberto no início do século XX, quando Moan e Peng (2003) avaliaram o efeito da laranja de acridina na presença de luz sob a morte de *Paramecium.* Desde então, é uma técnica emergente no combate a micro-organismos

resistentes, a qual ocorre em um determinado comprimento de onda sob a presença de moléculas fotossensibilizadoras (FS), como é o caso da curcumina (CIEPLIK et al., 2014).

Essa estratégia é indolor, seletiva e menos invasiva para o ser humano, uma vez que é possível a focalização nas células alvo sem que haja a produção de células fotorresistentes (SANTIN et al., 2014).

Os mecanismos da reação fotodinâmica dependem das moléculas de oxigênio dentro das células, e podem atuar por duas vias distintas (Figura 2). A primeira etapa é semelhante para ambos: a entrada da FS na célula e a irradiação de uma luz dentro de uma faixa de comprimento de onda específica. Esse processo gera a forma terapêutica da FS – um estado tripleto excitado (T1).

No primeiro mecanismo, o T1 pode transferir parte da sua energia para as moléculas vizinhas, resultando na emissão de espécies reativas de oxigênio (EROs) e consequente interação com moléculas de oxigênio, e gerando um desequilíbrio com a formação adicional de EROs como ânion superóxido ($O2^{-\cdot}$), radical hidroxila ($HO^{\cdot}$) e peróxido de hidrogênio (H_2O_2). Em decorrência do estresse oxidativo, as células são destruídas. Já no segundo mecanismo, a energia de T1 é transferida diretamente para o oxigênio presente no citoplasma, sem a necessidade de biomoléculas intermediárias. Essa transferência direta entre T1 e oxigênio molecular gera a formação de oxigênio singleto, o qual apresenta alta atividade oxidante. Por não interagirem diretamente com as moléculas orgânicas presentes, não causam danos nas estruturas celulares. A ocorrência prevalente de um dos mecanismos depende de diversos fatores, como o pH do local da infecção, concentração de oxigênio e estrutura do FS (CASTANO; DEMIDOVA; HAMBLIN, 2004; FONSECA et al., 2006; JUZENIENE; MOAN, 2007; LUKSIENE, 2003)

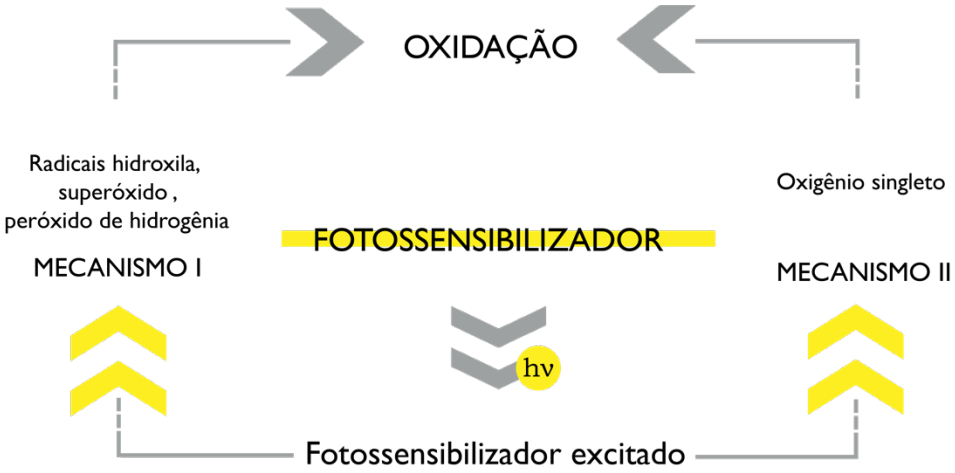

Figura 2: Mecanismos da terapia fotodinâmica (aPDT).

Diferentes estudos têm demonstrado a efetividade dos efeitos fotodinâmicos através da produção de ROS na formação de biofilmes de *P. aeruginosa*, com a redução de mais de 6 vezes em espessura (de > 30 μm para < 5 μm) (ABDULRAHMAN et al., 2020). Outros pesquisadores, também utilizando as técnicas de aPDT, mostram a eficácia da curcumina em diferentes micro-organismos, como é o caso da inativação de *E. coli* através da combinação da curcumina com luz UV-A na prevenção da contaminação cruzada entre água contaminada e lavagem de saladas, como espinafre e tomate (DE OLIVEIRA et al., 2018); aPDT com curcumina também já foi testada frente a levedura *C. albicans*, através a diminuição de genes relacionados à formação de biofilmes e adesão superficial (JORDÃO et al., 2020).

Izui e colaboradores (2016) demonstraram a inibição da formação de biofilmes e consequente redução do número de bactérias em doenças periodontais. Packiavathy e colaboradores mostraram que biofilmes do trato urinário de *E. coli* e *P. aeruginosa* PA01 tratados com curcumina reduziram em espessura e apresentaram deterioração nas estruturas dos biofilmes.

3.1.5 Modificações Estruturais

O órgão americano *Food and Drug Adminstration* (FDA) avalia a utilização desta como “geralmente considerada segura” e a dose aceita pelo “*Joint Food and Agriculture Organization of the United Nations/World Health Organization Expert Committee on Food Additives”* é de 0,1 a 3mg/kg de peso corporal. Dessa forma, um indivíduo de 80kg poderia ingerir, por dia, no máximo 240mg. Contudo, um estudo conduzido por Vareed e colaboradores (2008) demonstrou a baixa detecção da curcumina no plasma sanguíneo humano em uma dosagem de 10 a 12g/dia, dose cerca de cinquenta vezes maior que a recomendada.

Tønnesen e Karlsen demonstraram em 1985, que a curcumina apresenta empecilhos na absorção via oral e que não entraria na corrente sanguínea mesmo que fosse absorvida pelo GIT, uma vez que é uma molécula hidrofóbica (SANIDAD et al., 2019).

De acordo com Prasad e colaboradores (2014) diversos estudos já foram publicados na tentativa de melhorar a biodisponibilidade da curcumina, dentre elas formulações em hidrogéis, ácido hialurônico e diversas formas de nanopartículas (ouro, quitosana, sílica). Além disso, diferentes grupos de pesquisa têm procurado por novos compostos com propriedades similares e/ou superiores à curcumina, como é o caso de análogos monocarbonílicos (MACs), também conhecidos por monocurcuminoides (SHETTY et al., 2015).

Modificações estruturais na molécula da curcumina implicam diretamente na solubilidade da mesma; a remoção e/ou troca dos substituintes da porção aromática da molécula, bem como a exclusão de uma das pontes de carbonila da porção α,ß-insaturada, evitam a rápida decomposição das moléculas sintetizadas e melhoram as propriedades farmacológicas destas (BUKHARI et al., 2013; LIANG et al., 2009).

Um estudo conduzido por Rani e colaboradores (2010) demonstrou a atividade antibacteriana para diferentes MACs e chalconas e identificaram que o composto 1a (**Figura 2**) demonstrou uma melhora na atividade antimicrobiana frente a *K. pneumoniae* e *S. aureus*, por consequência da estrutura simétrica (como na curcumina) ao redor da carbonila, com valores de MIC de 500µg/ml. Em outro estudo, outros compostos similares identificados por 1a, 2a e 3a (Figura 3) apresentaram também uma leve atividade antibacteriana frente a cepas de outras bactérias, como é o caso da *E. faecalis*, com valores de MICs em > 400mM (VIEIRA et al., 2018).

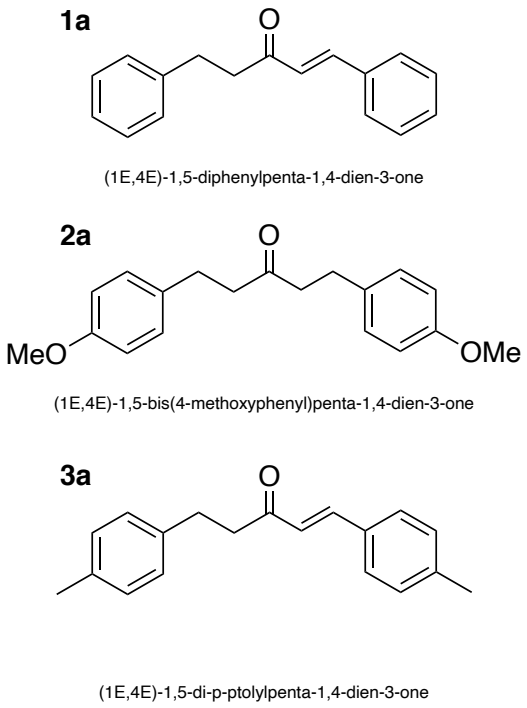

Figura 3: Estrutura dos monocurcuminoides com atividade antibacteriana.

3.2 Atividade antioxidante

EROs são espécies quimicamente reativas que possuem um elétron desparelhado (FAROOQUI, 2019). O corpo humano possui defesas endógenas que incluem enzimas como superóxido desmutase (SOD), catalase (CAT) e glutationa (GSH). A liberação de ROS implica, em baixas concentrações, em um metabolismo fisiológico; contudo, a superprodução de ROS pode causar um desequilíbrio entre moléculas pró-

oxidantes e antioxidantes e induzir a oxidação de compostos celulares importantes, não permitindo ao organismo que se detoxifique sozinho (FAROOQUI; FAROOQUI, 2019).

A atividade antioxidante da curcumina já foi comparada, há muitos anos, com as vitaminas C e E, de acordo com Toda e colaboradores (1985). Essa atividade pode ser explicada através da contribuição, pela sua estrutura, da doação de prótons/elétrons livres dos hidrogênios para as ROS. Duas décadas atrás também já se demonstrou um estudo *in vitro* utilizando células endoteliais bovinas, no aumento da enzima heme-oxigenase-1 (HO-1), responsável pela quebra da heme e prevenção de inflamações vasculares (MOTTERLINI et al., 2000). Neste mesmo estudo também se observou um aumento resistência celular ao estresse oxidativo quando as células foram encubadas por 18h com curcumina.

Cheng e colaboradores (2019) demonstraram, a partir da ingestão de 4g diárias de curcumina em doze voluntários saudáveis, que ocorreu um aumento na expressão de mRNA de genes antioxidantes como o fator nuclear eritroide 2 (NFR2) e heme oxigenase 1 (HO-1). A curcumina também demonstrou capacidade na restauração da atividade mitocondrial pela modulação do poro de transição de permeabilidade mitocondrial, através da supressão da produção de ROS. (MOUZAOUI; BANERJEE; DJERDJOURI, 2020). Neste mesmo estudo, também se observou a diminuição da inflamação intestinal pela inibição de moléculas pro-oxidantes e restauração da atividade da catalase em mitocôndrias extraídas do cólon de camundongos NMRI com a administração de 5uM de curcumina.

Um estudo clínico randomizado com 67 pacientes avaliou a ingestão de 1500mg/dia de curcumina no estresse oxidativo em mulheres portadoras da Síndrome de Ovário Policístico (SOP). Os autores demonstraram o aumento de níveis de PGC-1a depois de três meses de suplementação, e um consequente aumento da enzima glutationa peroxidase. (HESHMATI et al., 2020)

Além disso, a curcumina pode reduzir a toxicidade do estresse oxidativo através da formação de complexos metálicos com metais pesados, tais como cádmio (Cd^{+2}) (TEDESCO et al., 2020), cromo (Cr^{+3}) (MOHAMED et al., 2020) e chumbo (Pb^{+2}) (MAILAFIYA et al., 2020)

3.3 Resistência Antimicrobiana

Um dos principais problemas de saúde do século XXI é a resistência antimicrobiana (AMR) (SINJARI et al., 2019). A AMR é um fenômeno natural (COMISSION, 2011): bactérias, por exemplo, desenvolveram mecanismos para defender e sobreviver em seus nichos, capazes de evitar (KUMAR; SCHWEIZER, 2005), expulsar (NIKAIDO, 1994) e destruir (QUEENAN; BUSH, 2007) compostos que foram denominados, pelo homem, de antibióticos (DENYER et al., 2004).

À medida que a resistência antimicrobiana cresce, os medicamentos utilizados para tratar essas infecções começam a perder efeito gradativamente. A baixa na efetividade não apenas prejudica a habilidade de tratar infecções normalmente, como também pode levar a complicações em pacientes com o sistema imune debilitado. Sem a efetividade de antibióticos, por exemplo, procedimentos e doenças, como transplante de órgãos e diabetes, se tornariam mais agravantes (CENTER FOR DISEASE AND CONTROL, 2019).

Há quase 20 anos, a WHO já trabalhava em soluções para tentar reduzir a velocidade do aumento da resistência antimicrobiana (WORLD HEALTH ORGANIZATION, 2001). Em 2012, a mesma organização publicou um documento em que promovia intervenções como uma melhora nos sistemas de saúde, monitoramento dos casos de resistência antimicrobiana, maior controle na utilização de fármacos e apoio à pesquisa de novas drogas e vacinas (WORLD HEALTH ORGANIZATION, 2012).

3.3.1 Resistência Antibacteriana

A descoberta dos antibióticos é, até hoje, um dos avanços mais significativos da medicina moderna. Os antibióticos não só tratam infecções (e salvam milhares de vidas) como também atuam na prevenção das mesmas em humanos com o sistema imune comprometido, como é o caso de indivíduos sob quimioterapia ou após um transplante de órgãos (CENTER FOR DISEASE AND CONTROL, 2013; PENDLETON; GORMAN; GILMORE, 2013).

Um estudo de 2018 listou as 200 drogas mais prescritas nos Estados Unidos, dentre elas diversos antibióticos, como amoxicilina, azitromicina e sulfametoxazol + trimetoprima (FUENTES; PINEDA; VENKATA, 2018). Contudo, por consequência do uso incorreto e/ou excessivo de medicamentos (WORLD HEALTH ORGANIZATION, 2012), e utilização de antibióticos (muitas vezes desnecessários) na indústria animal (COMISSION, 2011), o aumento da resistência antibacteriana tem emergido como um risco à saúde pública.

Antibióticos são fármacos com um grande poder para combater infecções bacterianas; porém, em alguns casos, podem causar efeitos colaterais, como alergias, diarreias e interferências na efetividade de outros medicamentos contínuos (CENTER FOR DISEASE AND CONTROL, 2013). Concomitantemente, bactérias benéficas ao organismo são destruídas, deixando o organismo susceptível a infecções. Além disso, de acordo com o relatório do CDC publicado em 2019, mais de 140.000 emergências (4 em 5 casos) nos Estados Unidos são registradas por consequência de reações alérgicas (CENTER FOR DISEASE AND CONTROL, 2019).

Com a emergente evolução da resistência antibacteriana aos antibióticos convencionais, alternativas como o uso de fitoquímicos estão sendo exploradas, uma vez que a utilização de plantas no tratamento de doenças humanas pode ser encontrada na medicina tradicional (MODY; ATHAMNEH; SELEEM, 2020). A cúrcuma, por exemplo, apresenta propriedades medicinais já confirmadas na literatura em diversas doenças,

incluindo câncer (SHEHZAD; SHAHZAD; LEE, 2014), atividade antimicrobiana (MODY; ATHAMNEH; SELEEM, 2020) e antiparasitária (CARAPINA DA SILVA et al., 2019).

Até o presente momento, não foram encontradas referências que sugerem a resistência antimicrobiana frente a compostos fitoquímicos mas sim, a utilização dos mesmos como terapia alternativa.

3.3.2 Resistência Antifúngica

O iodeto de potássio (KI) foi a primeira substância a ser utilizada no tratamento de infecções antifúngicas, em 1903. Atualmente, a *C. Albicans* é a levedura de maior patogenicidade, uma vez que é o fungo de maior invasão na corrente sanguínea. (TRABULSI; ALTERTHUM, 2015).

A utilização de alguns medicamentos, como os antibióticos, gera um desequilíbrio na homeostase do organismo e permitem a proliferação desta levedura, a qual pode entrar em contato com o sistema urogenital e causar, por exemplo, infecções vaginais (JAMES G. CAPPUCCINO, 2019).

A candidíase sistêmica é considerada muito grave, uma vez que atinge órgãos como cérebro, rins e coração (FRITZ H. KAYSER et al., 2004). É uma doença de difícil diagnóstico por consequência do polimorfismo das lesões e o isolamento da *C. albicans* do sangue nem sempre é efetivo. (TRABULSI; ALTERTHUM, 2015).

O fluconazol foi introduzido no final dos anos 1980 como um excelente fármaco para o tratamento de candidíase mucosa; porém, já em 1992 os primeiros casos de resistência a esta droga foram registrados, decorrentes do tratamento a longo prazo para evitar relapsos em pacientes com HIV (SMAC, 1998).

Apesar de diversas pesquisas de novos medicamentos, atualmente no mercado encontram-se apenas quatro classes de moléculas capazes de atuar em três diferentes

vias fúngicas: análogos da fluoropirimidina, polienos, azois e ecquinocandinas. Outras classes são utilizadas apenas topicamente por apresentarem baixa eficácia quando administradas sistemicamente (VANDEPUTTE; FERRARI; COSTE, 2012). Desta forma, os fármacos atuais apresentam limitações quanto à efetividade uma vez que, por apresentar poucas alternativas, viabilizam o desenvolvimento da resistência dos micro-organismos.

Atualmente não há avanços consideráveis em medicamentos antifúngicos, e estudos estão sendo voltados na testagem de compostos de origem natural, como plantas e outros micro-organismos.

3.4 Citotoxicidade

O estudo da segurança de um novo composto ou fórmula é essencial antes da inserção dos mesmos no mercado. As análises *in vivo* são amplamente utilizadas para tais avaliações; contudo, devido a um movimento ético dentro da pesquisa científica, há uma tendência em tentar adereçar os estudos de toxicidade através de metodologias sem a utilização de animais, principalmente na área de cosméticos, a qual a União Europeia já adereçou a proibição de testes em animais, como também em São Paulo. (VINKEN; BLAAUBOER, 2017).

A utilização de estudos *in vitro* promove, além de um menor custo, a análise da citotoxicidade de diferentes compostos simultaneamente frente a diferentes células humanas antes da testagem em animais. A maioria dos estudos de citotoxicidade utiliza o ensaio de MTT, primeiramente publicado por Mosmann (1983). O ensaio de MTT utiliza-se de um corante de tetrazólio, insolúvel em água que, em contato com células metabolicamente ativas, converte o sal de tetrazólio (amarelo) em formazan (roxo).

Seo e colaboradores (2016) demonstraram que, em células mononucleares do sangue periférico o tratamento com 15uM de curcumina por 24h não afetou a viabilidade celular destas, mas nas células tumorais de ovário MDAH2774, SKOV3 e PA1

apresentou um comportamento de concentração e tempo-dependentes, com valores de IC_{50} de 29.7, 3.5 e 18uM no tratamento de 24h. Sun e colaboradores (2020) também demonstraram que a curcumina não apresenta nenhum efeito antiproliferativo frente a PBMCs, mas que em células Raji (linfoma não Hodgkin) a viabilidade celular também mostrou ser concentração e tempo-dependentes.

O ensaio da atividade hemolítica também é utilizado para estudar os efeitos tóxicos de um composto, através do grau de liberação de hemoglobina pela ruptura dos eritrócitos, a qual é de extrema importância quando contida no interior da célula, desempenhando um papel fundamental no transporte de oxigênio, porém é prejudicial à saúde quando livre no plasma (SCHAER et al., 2013). A curcumina apresentou uma baixa citotoxicidade nos eritrócitos, possivelmente pelo uso de etanol para a solubilização da mesma, mas a viabilidade celular permaneceu acima de 90% durante as 5h do experimento (MARTAKOV; SHEVCHENKO, 2020)

4 Manuscrito

A metodologia e os resultados obtidos nesta dissertação serão apresentados na forma de manuscrito e representam, na íntegra, o estudo. O presente manuscrito está formatado de acordo com as normas da revista a qual foi submetido: Biomedicine & Pharmacotheraphy (Fator de impacto: 4.545; ISSN: 0753-3322)

Bioprospection of novel synthetic monocurcuminoids: Antioxidant, antimicrobial, and *in vitro* cytotoxic activities

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ABSTRACT

The irrational use of medications has increased the incidence of microbial infections, which are a major threat to public health. Moreover, conventional therapeutic strategies are starting to become ineffective to treat these infections. Hence, there is a need to develop and characterize novel antimicrobial compounds. Phytochemicals are emerging as a safe and accessible alternative to conventional therapeutics for treating infectious diseases. Curcumin is extracted from the dried rhizome of the spice turmeric (*Curcuma longa* (Zingiberaceae)). However, the bioavailability of curcumin is low owing to its lipophilic property and thus has a low therapeutic efficacy in the host. A previous study synthesized structural variants of curcumin, which are called monocurcuminoids (CNs). CNs are synthesized based on the chemical structure of curcumin with only one methyl bridge. The biological activities of four previously synthesized CNs (CN59, CN63, CN67, and CN77), curcumin, and turmeric powder were examined in this study. Gas chromatography-tandem mass spectrometry analysis of curcumin and turmeric powder revealed similar peaks, which indicated the presence of curcumin in turmeric powder. The antioxidant activity of the test compounds was evaluated using the 2,2-diphenyl-1-picrylhydrazyl and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) assays. The ABTS radical scavenging activities of the test compounds were similar to those of vitamin C. The minimum inhibitory concentration (MIC) values of the test compounds against seven microbial strains were in the range of 4.06–150 µg/mL. The MIC value was equal to minimum bactericidal concentration value for CN63 (150 µg/mL) and CN67 (120 µg/mL) against *Staphylococcus aureus*. The treatment combination of CN77 (8.75 or 4.37 µg/mL) and turmeric powder (9.37 or 4.68 µg/mL) exerted synergistic growth-inhibiting effects on *Aeromonas hydrophila*, *Candida albicans*, and *Pseudomonas aeruginosa*. Photodynamic therapy using 2X MIC of CN59 decreased the growth of *Enterococcus faecalis* by 4.18-fold compared to the control group and completely inhibited the growth of *Escherichia coli*. The results of the hemolytic assay revealed that the test compounds were not cytotoxic with half-maximal inhibitory concentration values ranging from 49.65 to 130.9 µM. The anticoagulant activity of most compounds was comparable to that of warfarin but higher than that of heparin. This indicated that these compounds target the intrinsic coagulation pathway. These results demonstrated that these CNs are a safe and promising alternative for curcumin.

Keywords: curcumin, bioprospection, monocurcuminoids, cytotoxicity, antimicrobial activity, antimicrobial resistance

1. Introduction

There are ongoing efforts to identify novel molecules with therapeutic activity that can be used to treat various diseases. In particular, there is a need to devise novel therapeutic strategies to address the increased incidences of viral and fungal infections worldwide, post-surgery infections, and microbial drug resistance.

Microbial infections are a serious threat to public health [1,2]. The need for recurrent treatment, irrational use of antibiotics, and infections caused due to surgeries and/or compromised immune system are not only a threat to human life but also increase the treatment costs [3]. These pathogens can cause recurrent infections, such as bacterial vaginosis and candidiasis, and life-threatening conditions, including meningitis, pneumonia, asthma, and tuberculosis [1,4]. Additionally, the pathogens can persist in the host as they develop resistance to the antibiotics, which contributes to aggravating the disease and spreading of infection [5].

Some studies have suggested that phytochemicals can be a potential alternative to conventional therapeutic agents [6]. Curcumin is a polyphenol that is found in the rhizome of the spice turmeric (*Curcuma longa* (Zingiberaceae)) [7]. *C. longa*, which is used as a spice and food colorant due to its flavor and color, has been used to treat different ailments. The therapeutic activity of *C. longa* was reported in 250 BC in *Ayurvedic Compendium of Sushruta*, who is known as the “father of surgery” [8]. Curcumin is reported to exhibit various biological activities, including anti-cancer [9], anti-inflammatory [10], and antimicrobial [11] activities. However, the bioavailability of curcumin is low due to its lipophilic nature. Hence, a high daily dose of curcumin must be administered for a satisfactory therapeutic response, which may increase the side effects [12].

To improve the therapeutic efficacy of curcumin, different strategies, such as nanoparticle preparation [13], use of therapeutic adjuvants [14] and chemical modifications [15] (for example, the synthesis of monocarbonyl curcumin mimics) [16] have been proposed. Recently, Carapina et al. [15] synthesized different curcumin analogs and investigated their growth-inhibiting activity against *Trichomonas vaginalis*. However, further studies are needed to examine the biological activities of these curcumin

analogs. This study aimed to characterize the antioxidant, antimicrobial, and *in vitro* cytotoxic activities of the synthetic monocurcuminoids reported by Carapina et al. [15].

2. Material and Methods

2.1 Chemicals and reagents

Monocurcuminoids (CN58, CN59, CN63, CN67, and CN77) were synthesized as reported previously by Carapina et al. [15]. Curcumin (Sigma Aldrich®) was a kind gift from the same research group. Defibrinated sheep blood and phosphate-buffered saline (PBS) were purchased from Laborclin®. Citrated plasma was obtained from the Charity's Hospital of Santa Maria. The triphenyl tetrazolium chloride (TTC) was purchased from INLAB. Histopaque®-1077, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich®. The commercial kits to examine activated partial thromboplastin time (aPTT) and prothrombin time (PT) were obtained from BioTecnica®. Rosewell Park Memorial Institute-1640 medium was purchased from Gibco®. Warfarin and heparin were purchased from FQM® and Cristalia®, respectively. Turmeric powder was obtained from a local supermarket.

2.2 Preparation of stock solutions

The stock solutions (1000 µM) of all test compounds (CN58, CN59, CN63, CN67, CN77, curcumin, and turmeric powder) were prepared in 0.5% dimethyl sulfoxide (DMSO) and stored at −20°C until use to minimize volatilization. The structures of the compounds are shown in **Figure 1**. For the preparation of turmeric powder stock solution, the molecular weight of curcumin was considered for the calculations. CN58 was not included in further analysis as it did not solubilize in 0.5% DMSO.

2.3 Chemical identification

The monocarbonyl analogs were synthesized and identified based on melting point, infrared spectra, mass spectra, and nuclear magnetic resonance by Carapina et al. [15]. In this study, curcumin (Sigma®) and turmeric powder were subjected to gas chromatography-tandem mass spectrometry (GC-MS) using the GC-MS QP2020 (Shimadzu®) system equipped with an AOC-20i automatic injector. The chromatography conditions were as follows: capillary column, RTx-5MS (30 m × 0.25 mm × 0.25 µm);

carrier gas flow rate, 1.20 mL/min; carrier gas, helium; column temperature, initially maintained at 100°C (1 min hold) and gradually increased to 300°C (4 min hold) in 1°C/min steps; column temperature, 100°C; injection temperature, 260°C. The mass spectra were recorded at a scan range of 40–500 m/z. The analytes were identified based on GC retention time and by comparing the mass spectra of the test compound with those available in the mass spectrum library.

2.4 Antioxidant activity

2.4.1 DPPH assay

Antioxidant activity of the test compounds was assessed using the DPPH assay, following the protocols of Choi et al. [17]. The compounds were serially diluted two-fold in 2.5 mL of absolute ethanol into six concentrations starting from 1000 µM. To each concentration of the test compounds, 1 mL of 0.3 mM DPPH solution was added and the mixture was vortexed for complete solubilization. The samples were allowed to react for 30 min in the dark at room temperature. The blank group comprised 1 mL of ethanol and 2.5 mL of two-fold serially diluted test compounds. The positive control (0.0975 mg/ml of vitamin C) was prepared in 0.3 mM DPPH solution and ethanol, whereas the negative control comprised only ethanol. The DPPH scavenging activity was calculated using the following equation:

[% scavenging activity = [100 – (Abs_{sample} – Abs_{blank})/Abs_{ctrl}] × 100]

where Abs_{sample} was the absorbance from the compound solution, ABS_{blank} was the absorbance for the negative control and Abs_{ctrl} was the absorbance for the positive control:

2.4.2 ABTS assay

The ABTS assay was performed following the protocols of Re et al. [18] with modifications. $ABTS^{\bullet+}$ solution (7 mM) was prepared in 2.45 mM sodium sulfate solution in PBS (pH 7.4) and stored in the dark at room temperature for 16 h. $ABTS^{\bullet+}$ solution was diluted in PBS (pH 7.4) until the absorbance at 734 nm was 0.700 ± 0.02 at 30 °C. The test compounds were serially diluted two-fold in PBS. To 3 mL of the serially diluted test compound, 1 mL of $ABTS^{\bullet+}$ solution was added. The absorbance of the mixture was recorded at 734 nm for up to 6 min. The negative control comprised only PBS, whereas

the positive control (0.0975 mg/ml of vitamin C) was prepared in 1 mL of $ABTS_{\bullet}^{+}$ in 3 mL of PBS. The $ABTS_{\bullet}^{+}$ scavenging activity of the test compounds was calculated using the following equation:

$$[\% \text{ scavenging activity} = [100 - (Abs_{sample} - Abs_{blank})/Abs_{ctrl}] \times 100)]$$

where Abs_{sample} was the absorbance from the copound solution, ABS_{blank} was the absorbance for the negative control and Abs_{ctrl} was the absorbance for the positive control.

2.5 Antimicrobial activity

2.5.1 Strains

In this study, seven microbial strains selected from the available laboratory stock were used to examine the antimicrobial activities of the test compounds. The following six strains cataloged in the American Type Culture Collection (ATCC) and one clinical isolate were used: *Aeromonas hydrophila* (clinical isolate), *Escherichia coli* (ATCC 8733), *Enterococcus faecalis* (ATCC 29212), *Klebsiella pneumoniae* (ATCC 1705), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 6538), and *Candida albicans* (ATCC 24433). The strains were cultured in a selective agar (MacConkey, nutrient, or Sabouraud agar) at 37 °C for 24 h. All strains were then inoculated at the McFarland turbidity scale of 0.5 (1.5×10^8 CFU/ mL) in a 0.9% saline solution and refrigerated until use. The microdilution method was used to analyze minimum inhibitory concentration (MIC) and perform the checkerboard synergy assay and antimicrobial photodynamic therapy (aPDT).

2.5.2 MIC and minimum bactericidal/fungicidal concentration (MBC/MFC)

MIC was determined using the microdilution technique according to the Clinical and Laboratory Standard Institute method M7–A6 [19]. The assay was performed in a 96-well plate containing 100 µL of Mueller-Hinton (MH) broth. The MH broth was supplemented with serial concentrations of the drugs (final volume: 100 µL) from rows 1 to 12 in duplicate columns for each test compound. Next, 10 µL strain suspension (0.5 McFarland) was added to each well and incubated at 37°C for 24 h. MIC was defined as the lowest concentration of the compound at which there was no visible growth after centrifugation for 5 min at 3000 *g*. MIC values were interpreted according to their bioactivity, as follows: no bioactivity: MIC > 1000 µg/ml; mild: MIC = 501 - 1000 µg/ml; moderate: MIC = 126 to

500 µg/ml; good: MIC = 26 - 125 µg/ml; strong: MIC = 10 - 25 µg/ml; very strong: MIC < 10 µg/ml. MBC/MFC were determined after subculturing 1 µL of the culture treated with 1x MIC. 0.5x MIC and 2x MIC values of the test compounds in MH agar plates and incubated at 37°C for 24 h. Moreover, bactericidal/fungicidal effect were calculated by the division of MBC/MFC value by MIC value, and were interpreted as possessing bactericidal/fungicidal effect if the division was ≤ 1 and bacteriostatic/fungistatic if the result was > 1. The test was performed in duplicate.

2.5.3 Checkboard assay

To examine synergistic antimicrobial effects of the test compounds, the following three strains were selected for the assay: *A. hydrophila* (gram-negative bacterium), *C. albicans* (yeast), and *P. aeruginosa* (gram-negative bacterium). Synergism was determined according to the method of Chin et al. [20] with modifications. Of the six compounds, three (CN67, CN77, and turmeric powder) were selected based on the hemolysis assay results (groups exhibiting ≥ 60% cell viability). These three agents were serially diluted two-fold starting from 125 µM. The test agents were prepared as follows: 200 µL of MH broth was added to all wells of a 96-well plate, followed by the addition of serial two-fold dilution of 200 µL of compound A and 200 µL of compound B. The final volume of the reaction mixture in each well was 200 µL. Next, 20 µL of 0.5 McFarland inoculum (1.5×10^8 CFU/mL) was added. The microplates were incubated for 24 h at 37 °C. The antimicrobial activity was recorded based on the lack of visible growth after centrifugation for 5 min at 3000 *g*. The tests were performed in duplicate. The fractional inhibitory concentration (FIC) index was calculated using the following equation:

$$C/MIC_A + C/MIC_A = FIC_A + FIC_B = FIC$$

where C is the MIC from the combination of drugs A and B; MIC_A and MIC_B are the MICs of drugs A and B, respectively; FIC_A and FIC_B are the FICs of the drugs A and B, respectively.

The FIC values were interpreted as follows: ≤ 0.5, synergy; > 0.5 to 1.0, addition; >1.0 to 4.0, indifference; ≥ 4 antagonism.

2.5.4 aPDT

aPDT was performed using the microdilution technique. The test compounds were added to 100 µL of MHB at the concentrations of 2X, 1X, and 0.5X MIC, followed by the

addition of 10 µL of the microbial suspension (1.5×10^8 CFU/mL). The samples were incubated at room temperature for 30 min in the dark. Next, the samples were exposed to UV-light (< 300 nm) (T+L+) for 30 min. To evaluate the compounds alone, a plate replicate was maintained in the dark for 60 min (T+L−) corresponding to both times (dark and light). The positive control comprised 10 µL of the microbial suspension in 100 µL of MH broth in the presence (C+L+) or absence (C+L−) of light. The effect of light alone was tested by replacing the test compounds with the same volume of saline solution (0.9%) (C−L− and C−L+). The plates were subsequently incubated at 37 °C for 24 h. Further, 50 µL of TTC (5 mg/mL) was added to each well and incubated at 37 °C for 1 h. The absorbance of the red color mixture was evaluated at 540 nm relative to the bacterial growth. The test was performed in duplicate.

2.6 Cytotoxicity assays

2.6.1 Detection of hemolytic activity

Hemolysis assay was performed according to the methods of Vaucher et al. [21] with modifications. The defibrinated sheep red blood cells were used for the assay. Erythrocytes were resuspended in PBS at a final concentration of 4% (v/v). The cells were incubated with two-fold serially diluted test compounds (diluted from stock solutions) in a water bath at 37 °C for 1 h. Next, the cells were centrifuged for 10 min at 800 *g*. The supernatant was transferred to a 96-well plate. Saline solution (0.9%) and TritonX-100 were used as negative and positive controls, respectively. Hemolytic activity was considered safe in the groups exhibiting cell viability higher than 60%. The absorbance of the mixture at 409 nm was assessed using a microplate reader (Thermoplate, China). The percentage of hemolysis was calculated using the following formula:

$$[Abs_{sample} – Abs_{blank})/(Abs_{ctrl} – Abs_{blank}) \times 100)]$$

where Abs_{sample} was the absorbance from the compound solution, ABS_{blank} was the absorbance for the negative control and Abs_{ctrl} was the absorbance for the positive control.

2.7 Anticoagulant activity

Anticoagulant activity was determined based on aPTT and PT, which indicate the intrinsic and extrinsic coagulation pathways, respectively, in citrated plasma. The heparin and warfarin calibration curves were generated for determining aPTT and PT,

respectively. The calibration curve of heparin was generated at concentrations of 0.0075, 0.0449, 0.1789, and 0.4014 UI, while that of warfarin was generated at concentrations of 0.00238, 0.00909, 0.0333, 0.0692, and 0.0225 mg (data not shown). To examine the effect of the test compounds, the compounds were serially diluted two-fold from the stock solutions. Plasma was incubated with heparin, warfarin, or test compounds at 37°C for 1 h with shaking (140 rpm). The samples were then centrifuged at 800 *g* for 10 min. Saline solution (0.9%) was used as a negative control. aPTT and PT were determined using a mono channel coagulometer (Clotimer, Brazil) with the commercial kits (Biotecnica, Brazil), following the manufacturer's instructions.

2.8 Statistical analysis

The data are expressed as mean ± standard deviation from duplicate or triplicates. The means were compared using two-way analysis of variance test, followed by Dunnett's multiple comparison test. All statistical analyses were performed using GraphPad Prism 8.0 software.

3. Results

3.1 Chemical identification

In this study, curcumin and turmeric powder were subjected to GC-MS analysis. The major chemical constituents of curcumin and turmeric powder are shown **Table 1**, while their retention times are shown in **Figure 2**. Six and ten compounds were identified in curcumin and turmeric powder, respectively. The major constituents of curcumin were 3-methoxy-4-hydroxybenzalacetone, representing a peak area (w/w) of 57.81% and 2-methoxy-4-vinylphenol (23.34%), while those of turmeric powder were aR-turmerone (29.95%), squalene (24.08%), 1-methyl-6-(4-methylenecyclohex-2-en-1-yl) (17.48%), and tumerone (13.21%).

3.2 Antioxidant activity

To evaluate the free radical scavenging activity of the test compounds, the DPPH and ABTS assays were performed. Treatment with 15.625 and 500 μM of curcumin decreased the DPPH radical level by 67.45% and up to 100%, respectively, compared to the control group. The DPPH radical scavenging activities of CN59 and CN67 did not

exceed 50%. CN63 (500 μM), CN77 (500 μM), and turmeric power (15.623, 62.5, 250, and 500 μM) exhibited DPPH scavenging activities higher than 50% (**Figure 3**).

All samples, except curcumin, exhibited ABTS scavenging activities higher than 50% (**Figure 3**). The ABTS scavenging activity of the test compounds was not concentration-dependent. Interestingly, the ABTS scavenging activity of curcumin was inversely proportional to its concentration.

In the DPPH and ABTS assays, the minimum concentrations for Vitamin C at which more than 50% scavenging activity were 0.04875 (58.5%) and 0.00609 (84.68%) mg/mL, respectively, were used as positive controls.

3.3 Antimicrobial activity

3.3.1 Determination of MIC and MBC/MFC

The MIC and MBC results are shown in **Table 2**. Both positive and negative controls were used to validate the tests. The MIC values varied depending on the test compounds and/or strains. All test compounds presented antimicrobial activity. Some compounds showed better efficacy than curcumin, as seen for CN59 that exhibited a very strong bioactivity for *C. albicans* whereas curcumin only a strong activity. CN59 and turmeric powder displayed a very strong bioactivity for *P. aeruginosa* whilst curcumin only showed a good bioactivity. CN 63 showed a very strong bioactivity for *C. albicans* and *E. coli*. CN 67 exhibited a very strong bioactivity only for *A. hydrophila*. The other test compounds presented a lower or similar bioactivity than curcumin. MBC/MFC was determined based on the MIC values to assess the bactericidal/fungicidal effect, and all compounds presented bacteriostatic/fungistatic effect, with the exception of CN63 and CN67 at high concentrations on *S. aureus* (150 and 120 μg/mL, respectively), that exerted bactericidal effect.

3.3.2 Synergistic antimicrobial activity

The growth-inhibiting activity of a combination of two selected test compounds and turmeric powder against three selected microbial strains (*A. hydrophila*, *P. aeruginosa*, and *C. albicans*) was tested using the checkboard method. Three agents were examined in this assay based on the 60% cell viability rule: CN67, CN77, and turmeric power. The FIC values are represented as isobolograms (**Figure 4**). The individual MIC values were used to draw the effect line. Additionally, one data point in the graph of growth-inhibiting

activity of the combination of CN77 and turmeric power against *P. aeruginosa* is represented above the line because the turmeric powder FIC value was the same as its MIC. The concentrations below the line were considered synergistic (FIC ≤ 0.5) or additive (0.5 < FIC < 1.0), while those above the line represented an indifferent (1.0 < FIC < 4.0) or an antagonist (FIC ≥ 4.0) effect. The combination of CN77 and turmeric powder exerted a synergistic effect on all three strains at the two lowest concentrations. The combination of CN67 and CN77 yielded two extra synergistic points at the lowest concentrations. Interestingly, the combination of CN67 and CN77 yielded FIC values that exerted indifferent or antagonist effect on *A. hydrophila* but did not yield visible growth in the agar plate.

3.3.4 aPDT

Figure 5 shows the results for aPDT against different microbial strains. The results were considered effective if the treatment group exhibited at least 2-fold lower growth than the control groups. Compared with that in the T−L− group, the growth of *C. albicans*, *E. faecalis*, *and S. aureus* decreased by 2.5-, 1.77-, and 1.7-fold, respectively, in the T−L+ group (data not shown). The growth of *E. faecalis* in the group treated with 2X MIC value of CN59 decreased by 4.16-fold when compared with that in the positive control. Treatment with 2X MIC of CN59 and CN77 almost completely inhibited the growth of *E. coli.* The growth of *S. aureus* decreased by 2.7-fold compared to positive control upon treatment with 2X MIC of CN63. Interestingly, the growth of *A. hydrophila*, *E. faecalis*, and *S. aureus* in the T+L+ group increased by 3.35-, 1.5-, and 1.27-fold compared to the control group upon treatment with 1X MIC of curcumin, respectively. Moreover, treatments with 2X, 1X, and 0.5X MICs of CN63 increased the growth of *C. albicans* by 2.19-, 2,04-, and 2.01-fold compared to the control group. The growth of *A. hydrophila* increased by 2.58-fold upon treatment with 1X MIC of curcumin, while that of *P. aeruginosa* increased by 1.6-fold, compared to the control group.

3.4 Evaluation of cytotoxicity

3.4.1 Hemolytic activity

In this study, we examined the cytotoxicity of test compounds against red blood cells. As shown in **Figure 6**, the test compounds exhibited concentration-dependent hemolytic activity. CN77, curcumin, and turmeric powder were safe at concentrations of

125, 62.5, 31.25, and 15.625 μM, respectively. The viability was lesser than 60% upon treatment with CN59, CN63, or CN67. Treatment with turmeric powder at the lowest concentration (15.625 μM) did not affect the cell viability (100%). The half-maximal inhibitory concentration (IC_{50}) values of all test compounds, which were calculated based on cell viability, ranged from 49.65 to 130.9 μM.

3.5 Anticoagulant activity

The commercial kits used in this study considered 26–38 s as the safe range for PTT and 10–14 s as the safe range for PT. As shown in **Table 3**, the PT of the test compounds was safe, except for the highest concentration of all compounds in which no clot was observed after 4 min.

The aPTT results did not follow a linear pattern. The aPTT values of CN59 and CN63 at concentrations of 125 μM and 63.5 μM were within the acceptable range. However, the PTT values of only 62.5 and 15.625 μM of CN77 were within the acceptable range. The PTT values of turmeric powder at concentrations of 500 and 15.625 μM were within the safe range, while those of curcumin at a concentration of 125 μM were within the safe range. The PTT values of CN67 were not within the safe range at all tested concentrations.

The IC_{50} values of test compounds obtained from the hemolytic assay were also tested in the anticoagulant assay. The PT values of all test compounds, except CN59 (8.4 s), curcumin (9.15 s), and turmeric powder (7.6 s), were within the acceptable range. CN63 (40.35 s), CN67 (21.95 s), and curcumin (21.35 s) exhibited divergent PTT values.

4. Discussion

The biological activities of five monocurcuminoids synthesized by Carapina et al. [15], curcumin, and turmeric powder were examined in this study.

Turmeric powder, which was obtained from a local supermarket, was chemically characterized using GC-MS. As shown in **Table 1**, only two peaks (aR-turmerone and tumerone) were similar between curcumin and turmeric powder. This indicated the presence of curcumin in turmeric powder. Curcumin is one of the major curcuminoids in turmeric powder [7]. However, the GC-MS analysis did not reveal the presence of curcumin in turmeric powder in this study. A previous GC-MS analysis [22] also revealed that curcumin was not detected in the mass spectrum of turmeric and that curcumene

and zingiberene were the main constituents of the rhizome. Similarly, Kawai et al. [23] did not detect curcumin through GC-MS analysis but reported *p*-cymene, 1,8-cineol, β-elemene, and β-caryophyllene as the major components of turmeric powder. The results of this study were similar to those of Singh et al. [24] who reported that the major constituent in the fresh and dried *C. longa* rhizomes was aR-turmerone (24.4% and 21.4%, respectively) through GC-MS analysis. Govindarajan [25] reported that the ratio of aR-turmerone to tumerone was 2.5:1. In this study, the ratio of aR-turmerone to tumerone was 2.14:1 and 2.26:1 in curcumin and turmeric powder, respectively. Moreover, the differences in the GC-MS results could be due to contamination and variability in the composition of turmeric powder from different geographical regions and/or hybridization with other *Curcuma* species [26].

Curcumin is reported to exhibit a potent antioxidant activity [27,28]. The DPPH and ABTS radical scavenging activities of all test compounds were examined. DPPH is a stable free radical that becomes stable after accepting an electron or hydrogen radical. The DPPH radical absorbs light at 518 nm. The reduction by an antioxidant agent decreases the absorption of DPPH radical, which increases the non-radical forms of DPPH [17]. ABTS radical scavenging protocol is based on the reduction of a pre-formed radical cation of ABTS.+ via an electron-transfer process that can be observed with a spectrometric assay at 734 nm [18].

In this study, curcumin concentration-dependently scavenged the DPPH radicals. Ak and Gülçin [29] had also reported that curcumin concentration-dependently scavenged both DPPH and ABTS radicals. However, high concentrations of curcumin exhibited decreased ABTS scavenging activity as curcumin may function as a pro-oxidant at these concentrations [30]. The test compounds exhibited a potent ABTS scavenging activity, which may be due to the high sensitivity of the ABTS assay resulting from its rapid kinetics [31].

Next, the antimicrobial activity of the test compounds was examined. The MIC values of most compounds were lower (45 µg/mL) than those reported in the literature for curcumin. To the best of our knowledge, this is the only study to comparatively examine the antimicrobial effect of these CNs to curcumin using six ATCC strains. Previous studies have examined the antimicrobial effect of curcumin using *S. epidermis (*ATCC 12228), *S.*

*aureus (*ATCC 25923), *K. pneumoniae* (ATCC 10031), and *E. coli* (ATCC 25922) with MIC values between 4000 and 16000 µg/mL [32]. The MIC value of curcumin against *C. albicans* (ATCC 18804) was reported to be 64 µg/mL [33]. Gunes et al. [34] demonstrated that the MIC values of curcumin against *S. aureus*, *E. faecalis*, *P. aeruginosa*, and *E. coli* were in the range of 175–293 µg/mL. These findings indicated that the MICs for the test compounds reported in this study were lower than those reported in previous studies.

The three agents selected for the checkerboard assay (CN67, CN77, and turmeric powder) individually exhibited a low MIC value. However, the combination of CN 77 and curcumin powder exerted a synergistic growth-inhibiting effect on *A. hydrophila*, *C. albicans*, and *P. aeruginosa* in laboratory culture. This synergistic combination enables the development of a rapid and low-dose therapy against gram-negative bacteria and/or fungus.

aPDT was performed to determine the effect of test compounds on the growth of bacteria/fungus exposed to UV-light. Light, photosensitizing agent, and oxygen are harmless when treated individually. However, the combination of these agents can exert a potent cytotoxic effect on selective cells [35] by promoting the production of reactive oxygen species, which are harmful at high concentrations. Zheng et al. [36] reported that curcumin is a photosensitive and phototoxic compound that exerts bactericidal effect upon exposure to blue light by interfering with the quorum sensing system. To the best of our knowledge, this is only study to perform aPDT using the test compounds.

One study analyzed the effect of curcumin in the prevention of *E. coli* cross-contamination in spinach and tomato during washing with contaminated water [37]. The authors demonstrated that the treatment combination of low concentrations of curcumin (1–10 mg/L) and UV-A light decreased the *E. coli* count by 5 log CFU/mL. Additionally, the authors reported that this treatment combination downregulates the expression of genes related to biofilm formation and superficial adhesion in *C. albicans*. Compounds that increase bacterial/fungal growth can act as a barrier for UV-light to enter the cells, which may be due to their antioxidant properties, as shown in the ABTS assay in the same study.

These results indicated that aPDT may be effective for selective microorganisms and that it selectively inactivates gram-negative bacteria but protects the yeast against light-induced damages.

In vitro cytotoxicity of test compounds was evaluated using the hemolysis assay, which is a routine procedure used in hospitals, clinical laboratories, and research laboratories to rapidly analyze the effect of test agents on red blood cells. The hemolytic activity indicates the ability of the test compound to damage the erythrocyte membrane [26].

Several cellular components, including proteins and carbohydrates, are involved in hemolysis. Spectrophotometric analysis revealed that treatment with Triton X-100 resulted in 100% hemolysis. As the same sample of whole blood was used for all tests, we can conclude that the 'foreign' components did not contribute to hemolysis. These results suggest that all compounds were considered safe at concentrations of 62.5, 31.25, and 15.625 μM, except curcumin (31.25 μM), CN58 (62.5 μM), and CN59 (62.5 μM). Carapina et al. [11] performed an MTT assay to evaluate the effect of CN67 on the viability of VERO cells. CN67 exerted a potent cytotoxic effect on the VERO cells (viability: 2.8%) after 24 h at the tested MIC (80 μM) and a moderate cytotoxic effect at the IC_{50} value (50 μM). These findings may be explained by the long exposure rather than the 1-h incubation. Additionally, the cell type may also contribute to the cytotoxicity of CN67 as VERO cells are sensitive.

One study reported that the presence of flavonoids and polyphenols in various plant extracts, including *Artemisia absinthium*, *Lippia* spp., and *Cymbopogon citratus* extracts, protected the osmotic stability of the erythrocyte membrane due to their antioxidant properties [39]. In this study, treatment with low concentrations of test compounds enhanced cell viability, which can be attributed to their antioxidant potential. In contrast, treatment with high concentrations (250 and 500 μM) of the test compounds decreased the cell viability, which may be attributed to their pro-oxidant activity. This is consistent with the properties of other known antioxidant molecules, such as ascorbic acid [40].

The IC_{50} values of all test compounds, which were determined using the hemolytic assay, varied from 49.65 to 130.9 μM. This indicated that the dose response was in the micromolar range. The IC_{50} value of CN67 (122.8 μM) in this study was lower than that

reported by Carapina et al. [15] (50 μM), which was calculated from the MIC assay. This may be because Carapina et al. [15] performed the anti-parasite assay and not an *in vitro* cytotoxicity assay.

There is a need to identify therapeutic agents that can modify the coagulation pathways to prevent and treat thromboembolic disorders, such as venous thromboembolism (VTE). VTE, which is considered the third most common cause of death among patients with cardiovascular diseases [41], is defined as the formation of an occlusive blood clot that prevents blood flow in the venous system [30].

The test compounds did not affect PT, which indicated that they did not affect the extrinsic coagulation cascade. aPTT of some concentrations of the test compounds was above the accepted range. This indicated that these compounds can activate the intrinsic coagulation pathway and that they are potential candidates for developing an anticoagulant drug. However, one study [43] reported that curcumin exhibited concentration-dependent anticoagulant activity. The PT and aPTT values of 10 μM curcumin were 27.5 ± 0.5 and 77.5 ± 2.1 s, respectively, while those of 50 μM curcumin were 35.2 ± 0.4 and 119.8 ± 0.9 s, respectively. Future studies must verify the anticoagulant efficacy at the obtained levels.

5. Conclusions

The results of the cytotoxicity assay suggest that the synthetic monocurcuminoids used in this study are safe at low concentrations. The synthetic monocurcuminoids are potential antimicrobial candidates, especially for gram-negative bacteria, with low MIC values and effective activity in aPDT. Some concentrations of the test compounds exhibited intrinsic anticoagulant activity as they can delay the coagulation cascade. The chemical modification of curcumin could be a novel strategy for the development of new antimicrobial drugs.

Competing interests

The authors declare no competing financial interests.

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Table 1: Chemical composition of curcumin and turmeric powder examined using GC-MS.

Compound	% Peak Area Content				
	Curcumin (%)*	RT[a] (min)	Powder (%)	RT (min)	Peak #
2-Methoxyphenol	7.1	5.552	-	-	1a[b]
2-Methoxy-4-vinylphenol	23.34	7.7532	-	-	2a
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	5.04	10.115	-	-	3a
aR-Turmerone	4.58	10.693	29.95	9.655	4a, 4b[c]
Tumerone	2.14	10.724	13.21	9.703	5a, 5b
3-Methoxy-4-hydroxybenzalacetone	57.81	11.828	-	-	6a
(undentified compound)	-	-	0.87	8.673	1b
Tricyclo[4.3.1.1(3,8)]undecan-1-amine	-	-	1.08	9.067	2b
Tridecane	-	-	1.98	9.213	3b
2-Methyl-6-(4-methylenecyclohex-2-en-1-yl)	-	-	17.48	10.074	6b
(6R,7R)-Bisabolone	-	-	1.79	10.564	7b
(E)-Atlantone	-	-	3.14	10.86	8b
n-Hexadecanoic acid	-	-	6.43	12.363	9b
Squalene	-	-	24.08	19.751	10b
Total Area	100%		100%		

* Percentage of w/w; [a]RT: Retention Time; [b]a: curcumin; [c]b: turmeric powder

Table 2: Bactericidal and bacteriostatic activities of monocurcuminoids (µg/mL) on selected microorganisms.

	CN 59		CN 63		CN 67		CN 77		SIGMA		POWDER	
Microorganism	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
A. hydrophila	-*	na+	-	na	3.75	15	35	140	11.25	45	37.5	150
C. albicans	4.06	16.25	4.69	18.75	60	240	140	na	22.5	90	37.5	75
E. coli	130	Na	4.69	18.75	30	120	130	na	11.25	45	37.5	150
E. faecalis	130	na	75	300	30	120	35	140	22.5	90	18.75	75
K. pneumoniae	65	260	18.75	75	120	na	140	na	90	360	150	na
P. aeruginosa	4.06	16.25	75	300	30	120	17.5	70	45	180	9.375	37.5
S. aureus	16.25	65	150	150	120	120	35	140	45	180	150	na

* MIC value was not within the tested concentration ranged; + na: MBC value was not within the tested concentration range.. MIC values were interpreted according to their bioactivity: no bioactivity: MIC > 1000 µg/ml; mild: MIC = 501 - 1000 µg/ml; moderate: MIC = 126 to 500 µg/ml; good: MIC = 26 - 125 µg/ml; strong: MIC = 10 - 25 µg/ml; very strong: MIC < 10 µg/ml. All tests were performed in duplicates

Table 3: Anticoagulant activity of test compounds for intrinsic and extrinsic pathways.

		IC_{50}	15.625 µM	31.25 µM	62.5 µM	125 µM	250 µM	500 µM
CN59	**PT**	8.4 ± 0.14	13.6 ± 0.99	12.75 ± 0.21	12.4±0.42	11.8 ± 1.68	12.6 ± 0.57	-*
	aPTT	26.75 ± 0.07	60.75 ± 21**	133.65 ± 24****	34.25 ± 0.78	26.25 ± 8.8	24.65 ± 78	-
CN63	**PT**	11.2 ± 5.94	12.45 ± 0.35	12.7 ± 0.85	12.7 ± 0.71	13.5 ± 0.1	13.65 ± 0.5	-
	aPTT	40.35 ± 0.49	35.9 ± 6.08	-	28.8 ± 4.81	13.5 ± 0,1	13.65 ± 0.5	-
CN67	**PT**	12.6 ± 0.57	11.9 ± 0.42	12.1 ± 0.57	12.4 ± 0.42	12.65 ± 0.5	13.45 ± 0.21	-
	aPTT	21.95 ± 2.75	60.6 ± 6.22**	41.65 ± 1.91	69.4 ± 2.97****	38.6 ± 5.8	62.5 ± 3.11**	-
CN77	**PT**	12.45 ± 0.78	10.8 ± 1.7*	11.85 ± 0.07	11.85 ± 0.07	14 ± 0.14	12.95 ± 0.7	-
	aPTT	27.6 ± 1.84	36 ± 6.08	65.5 ± 0.92***	23.85 ± 0.21	58.7 ± 11.03**	64.4 ± 6.22***	-
Sigma	**PT**	9.15 ± 1.63	12.85 ± 0.35	13.05 ± 0.21	12.25 ± 0.21	12.15 ± 0.5	12.65 ± 0.07	-
	aPTT	21.35 ± 3.6	39 ± 6.51	51.1 ± 7.21	46.1 ± 0.42	26.25 ± 2.33	38.85 ± 14.1	-
Powder	**PT**	7.6 ± 0.57	12.1 ± 0.92	12.25 ± 0.92	12.05 ± 0.78	12.25 ± 0.35	12.5 ± 0.71	-
	aPTT	32.2 ± 2.4	41.95 ± 1.34	41.95 ± 1.34	42.2 ± 10.32	51.5 ± 15.7	33.7 ± 2.55	-

*- no clot was observed, visually, after 4 mins; +IC_{50} values for the compounds CN59 (49.68 µM), CN63 (84.75 µM), CN67 (122.8 µM), CN77 (130.9 µM), Sigma (74.73 µM), Powder (74.73 µM). All tests were performed in duplicates. Data is shown as mean ± SD. Normal range for PT was 10-14 s and for aPTT was 26-38 s. * p<0.05, ** p<0.01, **** p<0.001, **** p<0.0001 compared with positive control (warfarin or heparin).

Figures

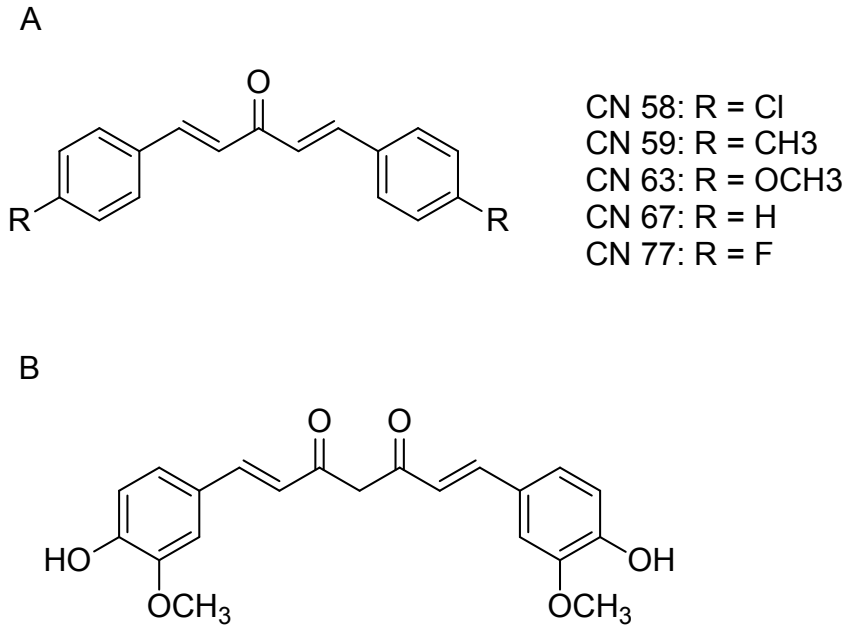


Figure 1: Chemical composition of (a) synthetic monocurcuminoids and (b) curcumin (adapted from Carapina et al. [15]).

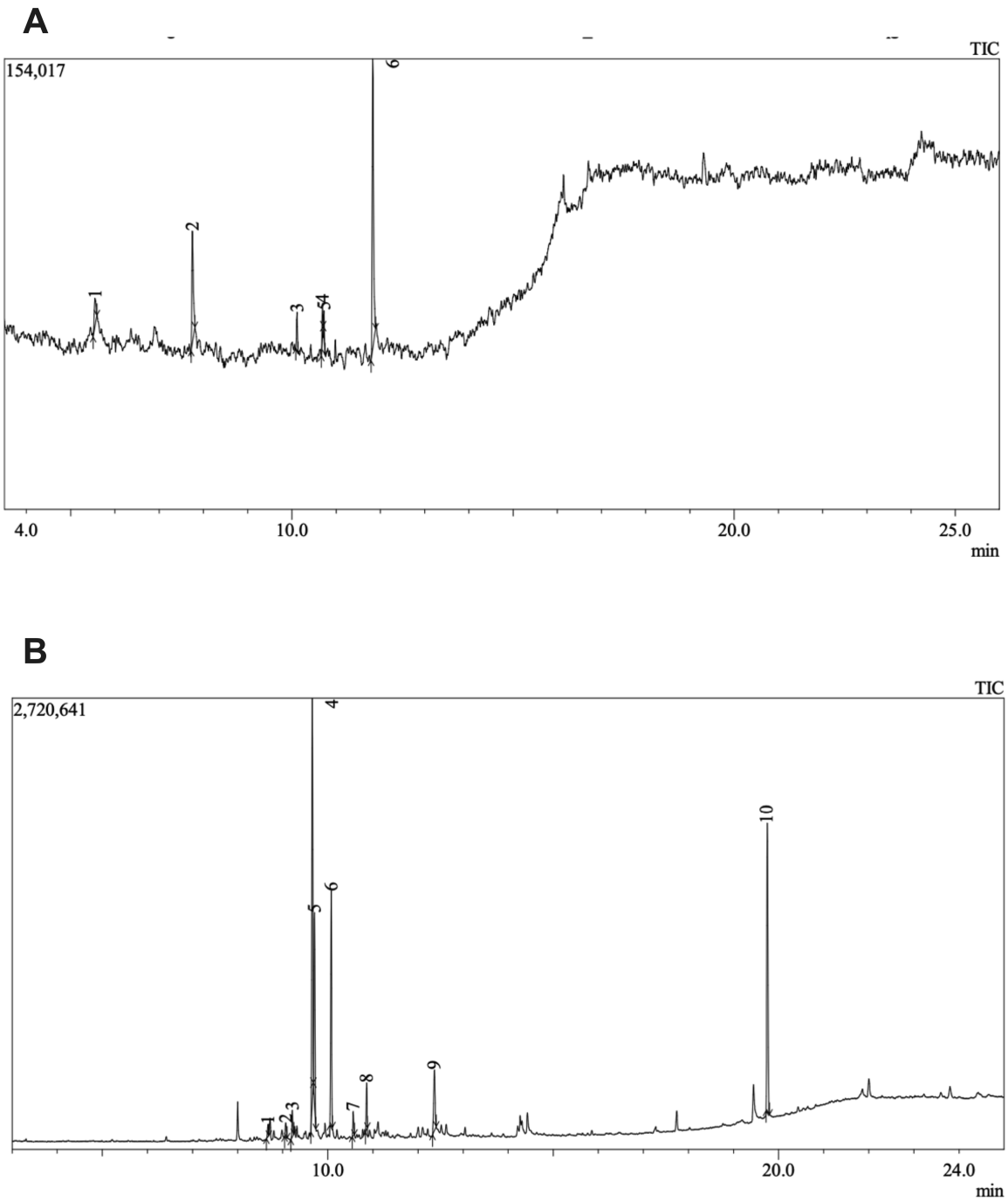
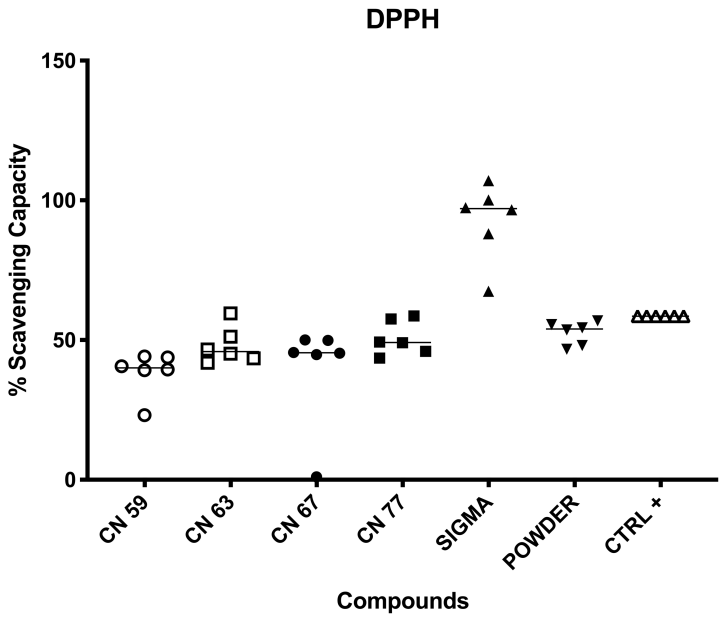


Figure 2: Gas chromatography-tandem mass spectrometry (GC-MS) chromatogram of (a) curcumin (SIGMA) and (b) turmeric powder (POWDER).

a)



b)

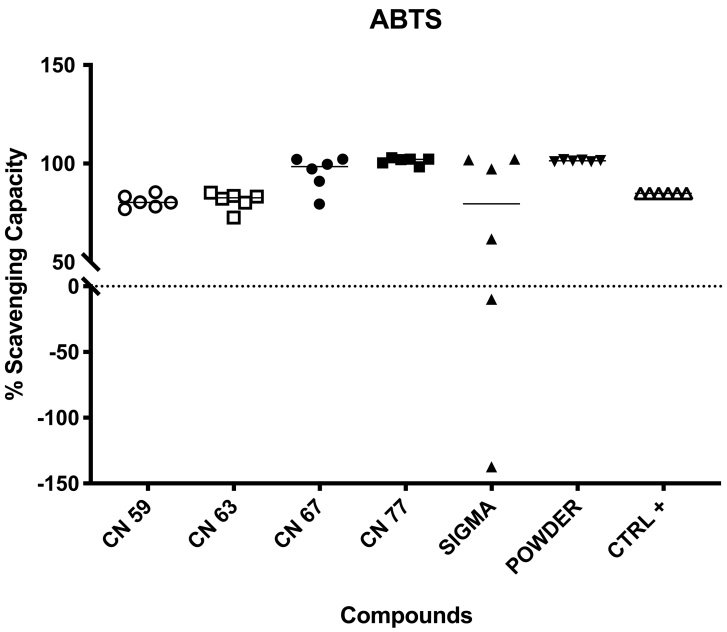


Figure 3: Scavenging of (a) 2,2-diphenyl-1-picrylhydrazyl (DPPH) and (b) 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radicals. The absorbance at 518 nm (DPPH) or 734 nm (ABTS) was examined using a spectrophotometer. Data are represented as mean ± standard deviation from triplicate readings. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared with positive control (CTRL +). Errors bars may not be visible due to their small size.

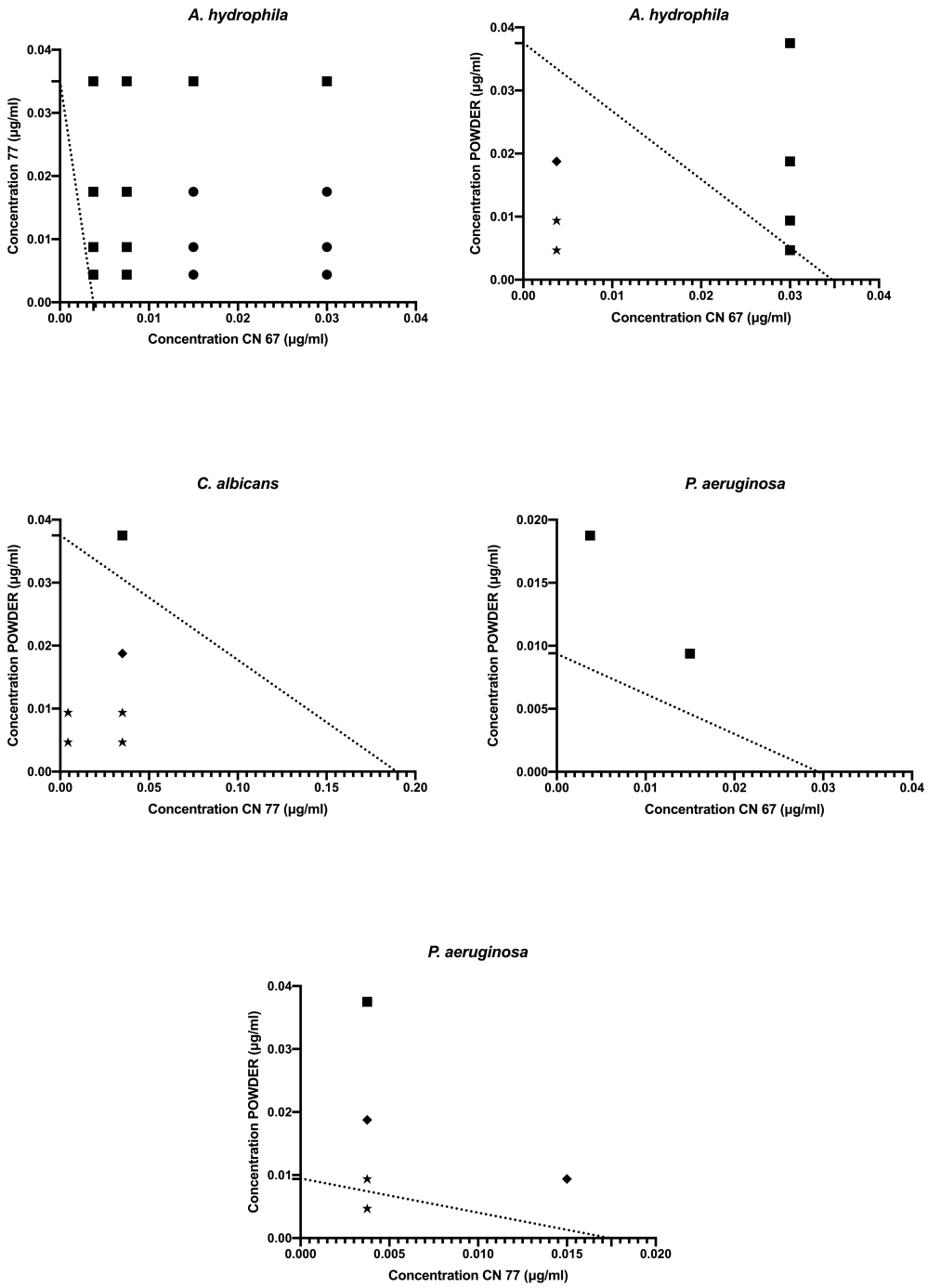


Figure 4: Isobologram analysis of monocurcuminoid combination. The individual minimum inhibitory concentrations (MICs) are used to draw the effect line. Except for one data point in the graph of the growth-inhibiting activity of the combination of CN77 and turmeric powder against Pseudomonas aeruginosa, the synergistic effect of other combinations (fractional inhibitory concentration (FIC) ≤ 0.5) is shown below the effect line (★) along with the addictive (0.5 < FIC <1) effect (✦). Points above the effect line represent FIC values between 1 and 4, which considered as indifferent (●) while the FIC values above 4 are considered antagonist (■).

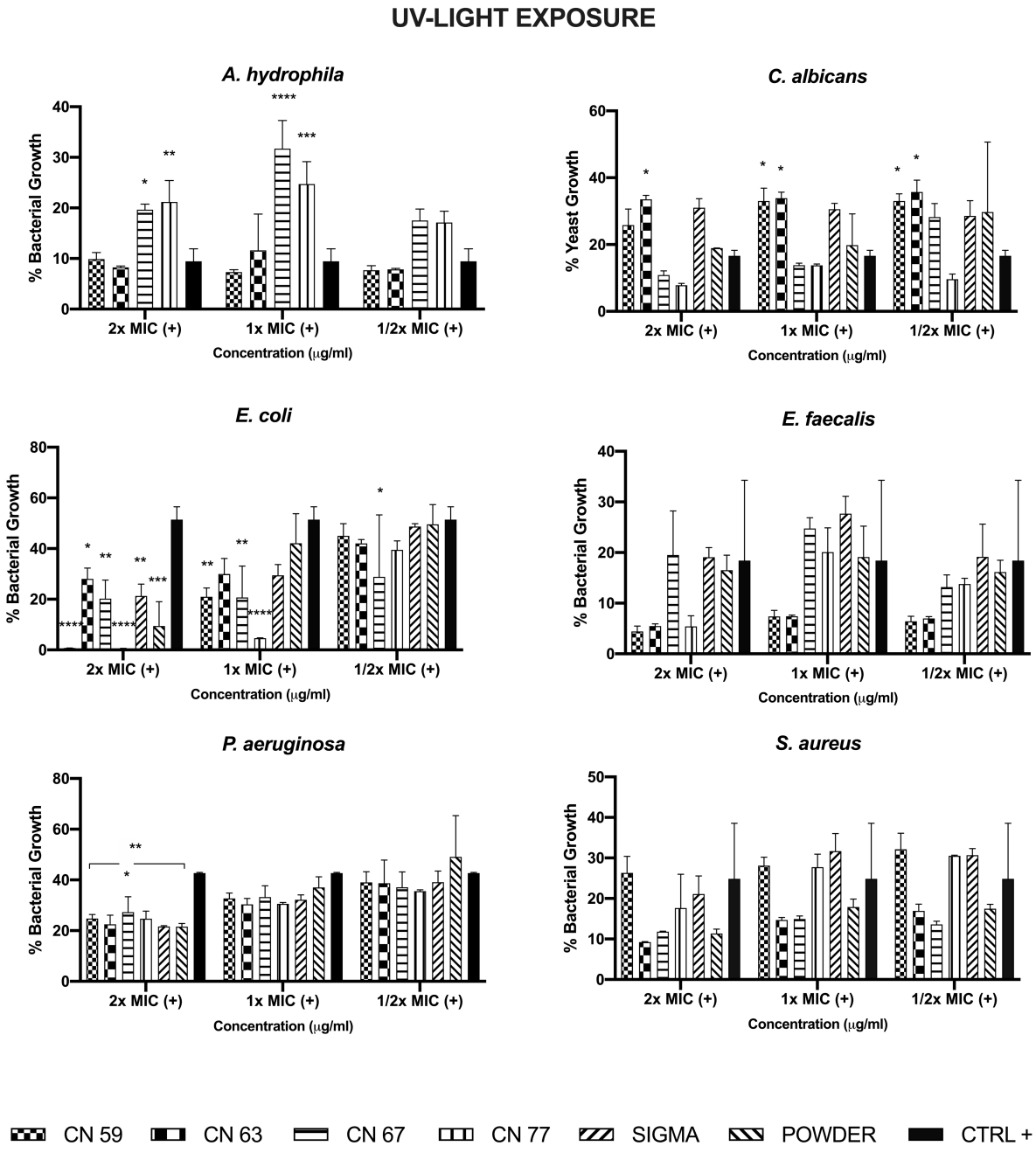


Figure 5: Photodynamic therapy using UV-light exposure**.**Data are represented as mean ± standard deviation from duplicate experiments. * p<0.05, ** p<0.01,*** p<0.001, **** p<0.0001 compared with positive control (CTRL +). Errors bars may not be visible due to their small size.

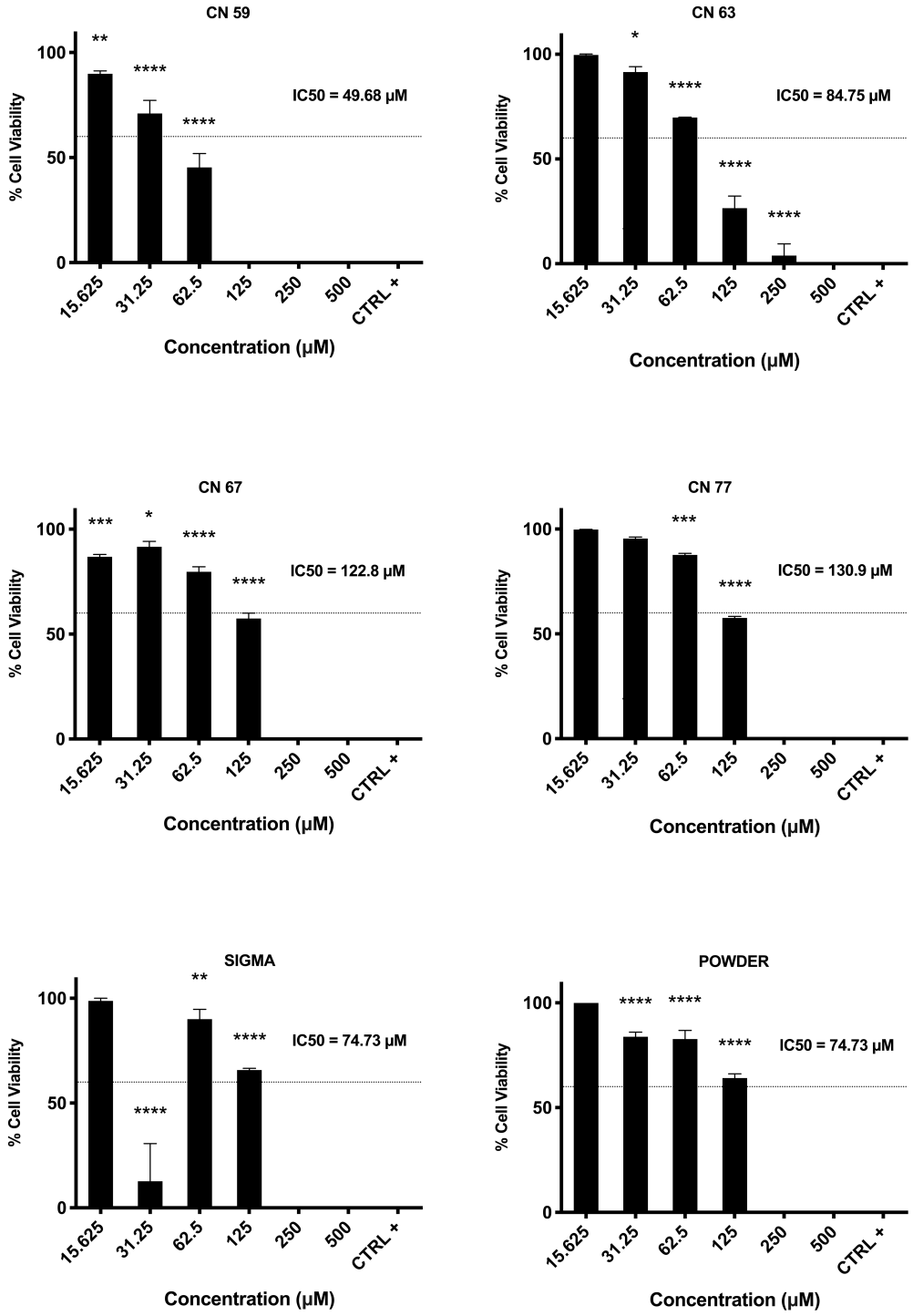


Figure 6: Hemolytic activity of test compounds. Data were represented as percentage of viable cells. TritonX-100 was used as positive control (CTRL +). Data are represented as mean ± standard deviation from duplicate experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared with positive control (CTRL +). The half-maximal inhibitory concentration (IC_{50}) values are shown for each compound.

5 Conclusões

- A identificação da presença de curcumina na cúrcuma foi verificada, podendo haver diferenças na composição quando comparadas a outros estudos por consequência da geolocalização do rizoma;

- A citotoxicidade dos compostos foi estudada e apresentou intervalos seguros para todos os compostos nos ensaios de atividade hemolítica e anticoagulante;

- Alguns compostos apresentaram uma atividade anticoagulante superior à varfarina e à heparina, indiciando uma segunda possível aplicação para os monocurcuminoides;

- As atividades antioxidantes frente aos ensaios de DPPH e ABTS apresentaram-se satisfatórias, visto que no método mais sensível (ABTS) todos os compostos apresentaram atividade semelhante à vitamina C

- Os testes microbiológicos demonstraram uma boa atividade dos monocurcuminoides frente a diferentes micro-organismos, indicando um amplo espectro de ação

- A atividade fotodinâmica apresentou-se muito satisfatória para algunas cepas, principalmente para *E.coli* em 2x MIC, em que o crescimento quase foi impedido totalmente.

- Estudos futuros são necessários para a correta aplicabilidade dos monocurcuminoides, incluindo estudos *in vivo* em Zebrafish *(Danio rerio)* e avaliação da expressão gênica em macrófagos.

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Anexo

Anexo A – Comprovante de submissão do manuscrito à Revista *Biomedicine & Pharmacotherapy*

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