UNESP - Universidade Estadual Paulista
“Júlio de Mesquita Filho”
Faculdade de Odontologia de Araraquara

Analú Barros de Oliveira

Potencial da terapia fotodinâmica na modulação da resposta inflamatória e nos fatores de virulência microbianos: avaliação em modelo de co-culturas tridimensionais de fibroblastos gengivais, *Candida albicans* e *Staphylococcus aureus*

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Orientadora: Profª Drª Fernanda L. Brighenti
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Analú Barros de Oliveira

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DADOS CURRICULARES

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"Tudo posso naquele que me fortalece"

(Filipenses 4:13)

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Oliveira AB. Potencial da terapia fotodinâmica na modulação da resposta inflamatória e nos fatores de virulência microbianos: avaliação em modelo de co-culturas tridimensionais de fibroblastos gengivais, Candida albicans e Staphylococcus aureus [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2024.

RESUMO

Este trabalho está dividido em três publicações cujos objetivos foram: a) realizar uma revisão sistemática e metanálise em ensaios clínicos para avaliar a eficácia da terapia fotodinâmica em lesões de mucosite oral (Publicação 1); b) avaliar o potencial *in vitro* de quatro diferentes fotossensibilizadores, sendo dois de origem sintética (Azul de Metileno e Fotoditazina) e dois de origem natural (Curcumina e extrato bruto de *Senna alata*), para terapia fotodinâmica antimicrobiana (TFDa) contra *Candida albicans* e *Staphylococcus aureus* cultivadas em suspensão e biofilme e c) avaliar a efetividade da TFDa mediada pelo extrato bruto de *S. alata* em modelo de cocultura celular tridimensional (3D) contendo fibroblastos gengivais, queratinócitos orais e colágeno tipo I. Publicação 1: A questão de pesquisa e as palavras-chave foram definidas seguindo a estratégia do PICO. A pesquisa do artigo foi realizada em diversas bases de dados, incluindo Embase, Scielo, Scopus, Cochrane Library, Web of Science, Science Direct e Pubmed. Foram selecionados ensaios clínicos randomizados para a revisão. O estudo foi conduzido conforme as normativas do PRISMA para revisão sistemática. Publicação 2: Foram utilizadas as seguintes cepas de referência em suspensão e biofilme: *S. aureus* ATCC 25923 e *C. albicans* ATCC 90028. Foram testados quatro agentes fotossensibilizadores (FS) em duas concentrações diferentes (Photodithazine (PDZ): 0.175 e 0.2 mg/mL; azul de metileno (AM): 0,2 e 2 mg/mL; Curcumina (CUR): 0.014 e 0.029 mg/mL; Extrato vegetal de *S. alata*: 0.05 mg/mL e 0.5 mg/mL. Dois protocolos de TFDa foram utilizados sobre biofilmes cultivados por 24 h e 48 hs: a) em único tratamento e b) em três aplicações sucessivas. As células microbianas em fase planctônica receberam apenas um único tratamento. A eficácia da terapia foi avaliada através da contagem de células viáveis após o tratamento (UFC/mL) e ensaio Live/Dead. O ensaio MTT foi utilizado para avaliar a citotoxicidade. O metabolismo de cada biofilme foi avaliado pelo ensaio XTT. Publicação 3: O modelo de cocultura 3D foi preparado e posteriormente infectado com *S. aureus* ATCC 25923 e *C. albicans* ATCC 90028 por 16 h, seguido pela aplicação da TFDa mediada pelo extrato bruto de *S. alata* nas concentrações de 0,05 e 0,5 mg/mL. Após 24 h do tratamento, foi avaliada a viabilidade microbiana (UFC/mL) e o dano celular pelo ensaio da lactato desidrogenase (LDH). AM nas concentrações de 0,2 e 2 mg/mL foram utilizados para fins de comparação. Os dados das publicações 2 e 3 foram analisados no programa IBM SPSS versão 20.0, com nível de significância de 5%. Dos 727 artigos analisados na publicação 1, foram selecionados 5 artigos para análise qualitativa e 2 para a meta-análise. Os resultados da meta-análise, incluindo nível de evidência e avaliação de risco de viés, demonstraram a eficácia da TFD na redução do tempo de cicatrização em combinação com a terapia a laser de baixa intensidade (TLBI), em comparação com a aplicação isolada da TLBI (p = 0,0005/I2 = 0%).Reduções totais na viabilidade microbiana foram encontradas na publicação 2 para TFDa mediada por curcumina (0.014 e 0.029 mg/mL), TFDa mediada por PDZ (0.175 e 0.2 mg/mL) e a TFDa mediada por *S. alata* (0.05 e 0.5 mg/mL) para ambos os micro-organismos cultivados em suspensão. Biofilmes de *C. albicans* cultivados por 24 horas mostraram redução total na viabilidade microbiana (UFC/mL), para TFDa

mediada por AM (0,2 e 2,0 mg/mL) e TFDa mediada por *S. alata* (0.5 mg/mL) em ambos os protocolos utilizados (aplicação única e aplicações sucessivas). Biofilmes de *C. albicans* cultivados por 48 h apresentaram redução total na viabilidade microbiana para TFDa mediada por AM (2,0 mg/mL). Para *S. aureus*, houve redução significativa na viabilidade microbiana apenas para três aplicações de TFDa (*S. alata*: 0.05 e 0.5 mg/mL; AM: 0,2 e 2,0 mg/mL) em biofilmes cultivados por 24 h e em biofilmes cultivado por 48 h (*S. alata*: 5,0 mg/mL; MB: 0,2 e 2,0 mg/mL). As imagens CLSM para ensaio Live/Dead confirmaram os resultados encontrados para viabilidade microbiana. Todos os fotossensibilizadores apresentaram valores significativamente diminuídos para a atividade metabólica do biofilme de *C. albicans* e *S. aureus* cultivados por 24 horas tratados com uma única aplicação de TFDa. Além disso, as concentrações dos fotossensibilizadores e os parâmetros de irradiação utilizados no presente estudo apresentaram valores aceitáveis (≤70%) de citotoxicidade para ambas as linhagens celulares. Os resultados da publicação 1 destacam que a TFD é eficaz no tratamento da mucosite oral, contribuindo para a cicatrização de feridas, principalmente no tempo necessário para o reparo. Já os resultados da publicação 2 mostraram que a terapia fotodinâmica antimicrobiana (TFDa) mediada por diferentes FSs revelou-se seguro e eficiente para o controle de *C. albicans* e *S. aureus*. Adicionalmente, para a publicação 3, os melhores reduções de *S. aureus* e *C. albicans* foram encontradas para 0,5 mg/mL de *S. alata* e 2 mg/mL de AM, sendo que a TFDa mediada pelo extrato bruto de *S. alata* causam menos danos celulares em comparação com AM ($p>0,05$) e clorexidina 0,12%. Os resultados da publicação 1 destacam que a TFD apresenta resultados promissores para o tratamento da mucosite oral e pode ser uma opção terapêutica eficaz, contribuindo para a cicatrização dos tecidos lesionados principalmente no tempo necessário para o reparo. Já os resultados das publicações 2 e 3 mostraram que a terapia fotodinâmica antimicrobiana (TFDa) mediada pelo extrato bruto de *S. alata* revelou-se seguro e eficiente para o controle de *C. albicans* e *S. aureus*.

Palavras-chave: Fotoquimioterapia. Infecções bacterianas e micoses. Revisão sistemática.

Oliveira AB. Potential of photodynamic therapy in modulating the inflammatory response and microbial virulence factors: evaluation in a three-dimensional co-culture model of gingival fibroblasts, Candida albicans and Staphylococcus aureus [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2024.

ABSTRACT

This study is divided into three publications whose objectives were: a) to conduct a systematic literature review, followed by a meta-analysis of clinical trials to evaluate the efficacy of photodynamic therapy in oral mucositis lesions (Publication 1); b) to evaluate the *in vitro* potential of four different photosensitizers, two of synthetic origin (Methylene Blue and Photodithazine) and two of natural origin (Curcumin and crude extract of *Senna alata*), to be used in antimicrobial photodynamic therapy (aPDT) against *Candida albicans* and *Staphylococcus aureus* cultured in suspension and biofilm; and c) to evaluate the effectiveness of aPDT mediated by the crude extract of *S. alata* in a three-dimensional (3D) cell co-culture model containing gingival fibroblasts, oral keratinocytes, and type I collagen. Publication 1: The research question and keywords were defined following the PICO methodology. The article search was conducted on various databases, including Embase, Scielo, Scopus, Cochrane Library, Web of Science, Science Direct, and PubMed. Randomized clinical trials were selected for the review. The study was conducted following PRISMA guidelines for systematic review. Publication 2: The following reference strains in suspension and biofilm were used: *S. aureus* ATCC 25923 and *C. albicans* ATCC 90028. Four photosensitizing agents (PS) were tested at two different concentrations (Photodithazine (PDZ): 0.175 and 0.2 mg/mL; Methylene Blue (MB): 0.2 and 2 mg/mL; Curcumin (CUR): 0.014 and 0.029 mg/mL; *S. alata* plant extract: 0.05 mg/mL and 0.5 mg/mL). Two aPDT protocols were used on biofilms cultured for 24 h and 48 h: a) single treatment and b) three successive applications. Planktonic microbial cells received only a single treatment. The therapy's efficacy was evaluated through viable cell counting after treatment (CFU/mL) and Live/Dead assay. The MTT assay was used to assess cytotoxicity. Biofilm metabolism was evaluated by the XTT assay. Publication 3: The 3D co-culture model was prepared and subsequently infected with *S. aureus* ATCC 25923 and *C. albicans* ATCC 90028 for 16 h, followed by application of aPDT mediated by the crude extract of *S. alata* at concentrations of 0.05 and 0.5 mg/mL. After 24 h of treatment, microbial viability (CFU/mL) and cell damage were assessed using the lactate dehydrogenase (LDH) assay. MB at concentrations of 0.2 and 2 mg/mL were used for comparison purposes. Data from publications 2 and 3 were analyzed using IBM SPSS version 20.0, with a significance level of 5%. Out of 727 articles analyzed in publication 1, 5 articles were selected for qualitative analysis and 2 for meta-analysis. The meta-analysis results, including the level of evidence and assessment of bias risk, demonstrated the efficacy of aPDT in reducing healing time in combination with low-level laser therapy (LLLT), compared to isolated LLLT application (p = 0.0005/I2 = 0%). Total reductions in microbial viability were found in publication 2 for aPDT mediated by curcumin (0.014 and 0.029 mg/mL), aPDT mediated by PDZ (0.175 and 0.2 mg/mL), and aPDT mediated by *S. alata* (0.05 and 0.5 mg/mL) for both microorganisms cultured in suspension. *C. albicans* biofilms cultured for 24 hours showed total reduction in microbial viability (CFU/mL) for aPDT mediated by MB (0.2 and 2.0 mg/mL) and aPDT mediated by *S. alata* (0.5 mg/mL) in both protocols used (single application and successive applications). *C. albicans* biofilms cultured for 48 h showed total reduction in microbial viability for aPDT mediated by MB (2.0 mg/mL). For *S. aureus*, significant reduction in microbial viability

was observed only for three applications of aPDT (S. alata: 0.05 and 0.5 mg/mL; MB: 0.2 and 2.0 mg/mL) in biofilms cultured for 24 h and in biofilms cultured for 48 h (*S. alata*: 5.0 mg/mL; MB: 0.2 and 2.0 mg/mL). Live/Dead CLSM images confirmed the results found for microbial viability. All photosensitizers showed significantly reduced values for metabolic activity of *C. albicans,* and *S. aureus* biofilms cultured for 24 hours treated with a single application of aPDT. Additionally, the concentrations of photosensitizers and irradiation parameters used in this study showed acceptable levels (≤70%) of cytotoxicity for both cell lines. The results of publication 1 highlight that aPDT is for oral mucositis, contributing to tissue healing mainly in terms of repair time. On the other hand, the results of publications 2 and 3 showed that antimicrobial photodynamic therapy (aPDT) mediated by the crude extract of S. alata proved to be safe and efficient for *C. albicans* and *S. aureus* control.

Keywords: Photochemotherapy. Bacterial infections and mycoses. Systematic review.

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1 INTRODUÇÃO

As doenças infecciosas são distúrbios no hospedeiro causados por micro-organismos como bactérias, vírus, fungos ou parasitas. Em particular, as infecções bacterianas são consideradas uma ameaça mundial devido ao aumento exponencial da resistência aos antibióticos[1]. Estudos estimam que, até o ano de 2050, o aumento da resistência bacteriana causará mais de 300 milhões de mortes, gerando custos de mais de 100 trilhões de dólares na área da saúde. Portanto, o desenvolvimento de novos agentes para controlar o crescimento desses micro-organismos é imprescindível[2]. Czaplewski e coloboradores[3] destacam necessidade do desenvolvimento de novas alternativas tecnológicas antimicrobianas nas quais os micro-organismos não sejam capazes de desenvolver resistência.

Dentro deste contexto, a terapia fotodinâmica antimicrobiana (TFDa) torna-se uma alternativa para o tratamento de processos infecciosos[4]. A TFDa combina mecanismos fotofísicos e fotoquímicos, gerando uma resposta biológica contra micro-organismos por meio de fontes de luz específicas e um agente fotossensibilizador associado ao oxigênio[5]. Essa abordagem oferece vantagens significativas, como ser minimamente invasiva, de aplicação simples e custo acessível, além de causar pouco desconforto ao paciente. Importante destacar que, até o momento, não há relatos de resistência microbiana ao tratamento com TFDa, uma vez que seu mecanismo de ação se baseia na geração de espécies reativas de oxigênio para a rápida eliminação dos micro-organismos[6]..

As superfícies epiteliais do corpo são as primeiras barreiras contra processos infecciosos. A integridade da pele e das mucosas impede a aderência e a penetração de bactérias patogênicas[7]. Porém, se a integridade destas barreiras é afetada, esses micro-organismos penetram nos tecidos, podendo levar ao aparecimento de infecções. Nessas condições, o tratamento é dificultado e a reparação tecidual é retardada, o que ocasiona diversas comorbidades ao paciente e representa um grande desafio para a área médica[8]. Nesse contexto, a TFDa vem se destacando como uma das terapias mais promissoras utilizadas no combate de infecções em lesões ulceradas[9,10].

Na Odontologia, as lesões ulceradas podem ser originadas de processos traumáticos, imunológicos, deficiência nutricional, radioterapia, quimioterapia ou outros processos patológicos, e são uma das queixas mais comuns que envolvem a

mucosa oral e podem causar dores leves a intensas[11]. Em algumas situações, essas úlceras podem ser múltiplas e extensas, podendo acometer toda a mucosa bucal e orofaríngea, ocasionando dor e desconforto, o que compromete a higienização, fonação e alimentação do paciente[11].

Além dos sintomas significativos, as úlceras bucais são propensas à colonização por micro-organismos, resultando em lesões potencialmente mais dolorosas e extensas. Essas lesões podem persistir por mais tempo ou apresentar uma piora no quadro clínico quando há um desequilíbrio na microflora oral e um aumento na proliferação de bactérias prejudiciais[13].

As espécies microbianas potencialmente infectantes, como *Candida* spp., *Staphylococcus* spp. e enterobactérias, são as que apresentam o maior risco para o paciente, pois podem penetrar no tecido e provocar o surgimento de quadros infecciosos sistêmicos[14].

Estudos clínicos prospectivos anteriores observaram que *Candida albicans* e *Staphylococcus aureus* são os micro-organismos potencialmente superinfectantes mais associados às lesões ulceradas da cavidade oral[15,16]. Evidências cientificas demostraram que *C. albicans* aumenta a capacidade de *S. aureus* em colonizar superfícies mucosas [17], enquanto *S. aureus* aumenta a capacidade de invasão de *C. albicans* nas junções epiteliais[18], levando a um aumento da resposta inflamatória da mucosa e alteração da resposta imune do hospedeiro. Esses eventos influenciam a cicatrização das lesões ulceradas e retardam a reepitelização da ferida, desempenhando um papel fundamental na cronicidade da lesão[17,19]. Os produtos tóxicos da parede celular destes patógenos penetram na submucosa, estimulando a liberação de citocinas pró-inflamatórias, resultando em inflamação, dor, infecções secundárias ou influxo sistêmico de micro-organismos e toxinas que, em algumas situações, como pacientes com neutropenia ou imunocomprometidos, aumenta o risco de bacteremia e septicemia[20,21].

Considerando a importância da TFDa no cenário atual mundial e os avanços dessa terapia no tratamento dos processos infecciosos e inflamatórios, diversos estudos foram realizados e evidenciaram que o uso da TFDa induz resposta inflamatória aguda localizada, que leva à ativação do sistema imunológico, migração de células mieloides, monócitos, macrófagos e mastócitos para o local da aplicação, resultando posteriormente na ativação de células TCD8+[22], que contribuem para a eliminação de células e tecidos lesados.

Complementarmente, alguns estudos demonstraram que as lesões ulceradas tratadas com TFDa apresentaram menor infiltrado inflamatório[23], uma vez que esse tratamento promove a redução da carga microbiana[24-26].

No entanto, a maioria dos estudos investigaram o efeito da TFDa apenas nos tecidos da pele; portanto, é necessário explorar essa técnica em tecidos da cavidade oral, uma vez que o uso de TFDa para o controle de micro-organismos bucais é bem documentada, porém o uso da TFDa para desinfecção dos tecidos orais ainda não está bem estabelecido, sendo encontrado poucos estudos que avaliaram a sua eficácia[27,28], além do fato que diferentes tecidos respondem de maneiras diferentes à mesma terapia[29].

A literatura relata a existência de compostos sintéticos e naturais que podem ser utilizados como fotossensibilizadores (FS) para TFDa[30-34]. Embora os compostos sintéticos ofereçam maior estabilidade, os compostos naturais têm sido extensivamente estudados e aceitos, especialmente devido à menor incidência de efeitos colaterais e interações medicamentosas[30,32,35-38]. Nesse sentido, merecem destaque os FS N-metil-D-glucosamina, curcumina e azul de metileno.N-metil-D-glucosamina, comercialmente conhecido como Photodithazine® (PDZ) é um FS de segunda geração, derivado do cloro e6, solúvel em água, com boa estabilidade no armazenamento e tem sido utilizado com sucesso na TFD na inativação de células cancerígenas e micro-organismos, devido ao seu alto rendimento quântico de formação de oxigênio singleto e por possuir altas bandas no espectro eletromagnético (650 a 680 nm), o que o contribui para maior capacidade de penetração nos tecidos biológicos melhorando a ação da TFD[39,40].

A curcumina é um pigmento de cor amarelo-alaranjado obtido do rizoma da planta *Curcuma longa*, amplamente conhecida por ser utilizada como tempero na culinária tradicional asiática e na medicina chinesa. Diversas atividades farmacológicas da curcumina foram relatadas na literatura, tais como anti-inflamatórias, antitumorais, antifúngicas, antibacterianas e anticarcinogênicas[41,42]. Algumas dessas propriedades são potencializadas após a ativação pela luz[42]. A curcumina é ativada pela luz na faixa de comprimento de onda correspondente à luz azul do espectro visível, que varia de 455 a 492 nanômetros. Essa faixa de luz é considerada segura e é amplamente empregada em consultórios odontológicos para a fotopolimerização de compósitos, utilizando aparelhos como LED ou luz halógena.

O Azul de Metileno (AM) é um corante amplamente utilizado na terapia fotodinâmica, pertencente à família das fenotiazinas. Sua faixa de absorção de luz está situada na região vermelha, especificamente entre 600 nm e 660 nm[43]. O AM é aprovado pela FDA para uso oral e intravenoso em humanos em doses elevadas. Além de não apresentar efeitos adversos, o AM possui uma capacidade notável de absorver luz com comprimentos de onda superiores a 620 nanômetros, o que facilita uma penetração mais profunda nos tecidos. Com sua baixa toxicidade, o MB é eficaz na eliminação de vários micro-organismos e células cancerígenas[44].

Recentemente, tem havido um aumento na quantidade de publicações que utilizaram produtos naturais como fonte de substâncias fotoativáveis[33,45-47]. Isso ocorre devido à capacidade dessas substâncias de aderir facilmente ou atravessar a membrana citoplasmática, bem como ao seu potencial de gerar espécies reativas de oxigênio[48]. Assim, a pesquisa em materiais vegetais tem mostrado ser uma fonte promissora na busca por agentes fotossensibilizantes para aplicação em Terapia Fotodinâmica (TFD).

Um estudo anterior do nosso grupo de pesquisa[49] demonstrou que o extrato vegetal de *Senna alata* é capaz de reduzir totalmente a viabilidade microbiana de diversos micro-organismos de interesse médico-odontológico. Além disso, o extrato produziu uma grande quantidade de espécies reativas de oxigênio e apresentou baixa citotoxicidade em culturas de linhagens de células humanas, demonstrando, portanto, potencial para ser utilizado como agente fotossensibilizador.

Frente às dificuldades de erradicação de micro-organismos e de controle dos fatores de virulência micro-organismos, particularmente de *C. albicans* e *S. aureus* quando associados à lesões com exposição de tecido conjuntivo, à falta de um protocolo de aplicação da TFDa para esta condição e ao potencial uso de agentes fotossensibilizadores como o PDZ, Curcumina, Azul de metileno e o extrato vegetal de *Senna* spp.- recentemente descoberto pelo nosso grupo de estudo- justifica-se a necessidade de estudos para contornar a infecção e a resistência microbiana em lesões com exposição de tecido conjuntivo de forma efetiva. Desse modo, o presente trabalho está dividido em três estudos. No primeiro estudo, foi realizada uma revisão sistemática e meta-análise em ensaios clínicos para estabelecer a eficácia da terapia fotodinâmica em lesões de mucosite oral. O segundo estudo teve como objetivo avaliar a capacidade *in vitro* de quatro diferentes fotossensibilizadores, dois de origem sintética (Azul de Metileno e Fotoditazina) e dois de origem natural (Curcumina e

extrato bruto de *S. alata*), para uso em terapia fotodinâmica antimicrobiana (TFDa contra *C. albicans* e *S. aureus*. Adicionalmente, foi avaliado os efeitos citotóxicos desses agentes fotossensibilizantes em células orais humanas, além da avaliação da atividade metabólica dos biofilmes pelo ensaio de redução de XTT. O terceiro estudo teve como objetivo avaliar a efetividade do extrato de *Senna alata* como potencial agente fotossensibilizador em TFDa utilizando um modelo celular tridimensional de queratinócitos orais e fibroblastos gengivais humanos infectados com de *C. albicans* e *S. aureus*. Este modelo celular foi utilizado como uma ferramenta representativa para investigação do uso potencial do extrato de *S. alata* em comparação ao fotossensibilizador comercial azul de metileno na TFDa para o controle de de processos infecciosos causados por *C. albicans* e *S. aureus*.

2 PROPOSIÇÃO

O presente trabalho teve como objetivo:

Realizar uma revisão sistemática da literatura e meta-análise com a determinação de avaliar o efeito da terapia fotodinâmica sobre o reparo tecidual de lesões ulceradas de mucosite oral em estudos clínicos randomizados;

Avaliar a relação dose-resposta de diferentes concentrações dos agentes fotossensibilizadores (Curcumina, Azul de metileno, Photodithazine® e extrato vegetal de *S. alata*) utilizando culturas de células em monocamada de fibroblastos e queratinócitos gengivais humanos, bem como sua ação antimicrobiana sob biofilmes simples de *C. albicans* e *S. aureus* e avaliar se a aplicação da TFDa mediada por esses agentes fotossensibilizadores pode alterar a expressão de genes associados à adesão, formação de biofilme e à resposta ao estresse oxidativo de biofilmes de *C. albicans* e *S. aureus* cultivadas *in vitro;*

Avaliar o potencial uso do extrato de *S. alata* como agente fotossensibilizador em TFDa utilizando um modelo celular tridimensional contendo queratinócitos orais, fibroblastos gengivais humanos e componentes microbianos (de *C. albicans* e *S. aureus*), por meio avaliação da viabilidade microbiana e do dano tecidual provocado após a aplicação da TFDa mediada por *S. alata*.

3 PUBLICAÇÕES

O presente estudo está divido em três publicações distintas, sendo:

3.1 Publicação 1*

A reprodução deste material está assegurada pelas políticas de acesso aberto do periódico Photodiagnosis Photodynamic Therapy.

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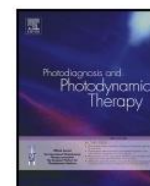

Review

A systematic review and meta-analysis of the effect of photodynamic therapy for the treatment of oral mucositis

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A R T I C L E I N F O

Keywords:
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Photodynamic therapy
Systematic review, meta-analysis review

A B S T R A C T

Background: Oral mucositis is a significant reaction to antineoplastic treatment characterized with pain, nutritional compromise, impact on the quality of life, interruption in cancer therapy and risk for infection. There is no effective standard protocol for the treatment of oral mucositis. This study aims to synthesize the scientific evidence available about the effects of photodynamic therapy on treatment of oral mucositis.
Methods: PubMed, Scopus, Web of Science, Science Direct, Scielo, Embase and Cochrane libraries were searched. Two independent and calibrated researchers (kappa = 0.92) performed all systematic steps according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). To access the risk of bias, RoB 2 and Delphi list criteria for clinical trials were used. Meta-analysis was conducted using the R software with "META" package.
Results: Clinical and randomized clinical trials were included with a total of five articles. Meta-analysis, level of evidence, and risk of bias assessment were performed showing that photodynamic therapy was effective in reducing healing time in association with low-power laser therapy when compared to low-power laser therapy alone ($p = 0.0005$).
Conclusion: Photodynamic therapy presents promising results for the treatment of oral mucositis. It may be an effective therapeutic option, contributing to the healing of injured tissues especially in the time needed for repair.

1. Introduction

Chemotherapy and radiotherapy are non-surgical methods widely used for the treatment of cancer. However, these therapies can cause toxic side-effects, including disorders of the oral mucosa [1–3]. Oral mucositis is one of the most serious and frequent complications in oncological patients undergoing chemotherapy and/or radiotherapy treatment protocols [4].

Oral mucositis is defined as an inflammatory, painful, and debilitating condition that affects oncologic patients. It is characterized by epithelial atrophy, edema, erythema, and the appearance of ulcers, which can be multiple and extensive, affecting the entire oral and oropharyngeal mucosa [5]. The large variability in the prevalence of oral mucositis (33.87 %–100 %) can be explained by the type of oncological therapy used, the type and location of the tumor, and the variation in the treatment protocols, particularly the dosage and frequency [5,6].

On account of pain and discomfort, patients affected by oral mucositis have impairment in oral hygiene, phonation, and feeding [6]. In addition, the severity of some oral mucositis lesions can lead to the hospitalization of patients for dehydration and dysphagia. In more severe situations, the interruption and delay of cancer treatment can occur, which brings negative effects on the prognosis of cancer and on the quality of life of these patients [6].

Currently, there are many scoring systems that classify the severity of oral mucositis. However, the World Health Organization's criteria of 1979 are the most commonly used to date [7]. According to this system, the oral mucositis can be graded into (1) clinical manifestation of

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erythema and patient reporting soreness; (2) clinical manifestation of erythema and ulcers, and the patient can swallow solid food; (3) clinical manifestation of ulcers with extensive erythema, the gums are markedly edematous, the patient cannot swallow solid food and presents risk of infection; and (4) ulcers are more extensive, bleeding gums and infection are observed, the pain is very intense, and the discomfort prevents the patient from ingesting solids and liquids. The incidence of severe mucositis (Grades 3/4) is greater than 97 % in patients with head and neck cancer receiving conventional radiotherapy and greater than 89 % during chemotherapy [4]. Moreover, simultaneous administration of radiotherapy and chemotherapy can result in the harshest and most persistent mucositis [8].

The severity of oral mucositis can also be influenced by inadequate oral hygiene practices, previous periodontal diseases, secretory dysfunction of salivary glands, and inadequate nutritional status in patients over 65 years of age with comorbidities [9]. Thus, oral hygiene protocols, the use of antimicrobial and anti-inflammatory agents and physical therapies (e.g., cryotherapy and laser therapy) are the main options for oral mucositis treatment. However, to date, there is no consensus on the level of evidence presented by previous studies, and there is no agreement on the best therapeutic option for oral mucositis treatment [5,10,11].

In the last decade, great emphasis has been given to low-level light therapy (or photobiomodulation) as an effective modality in preventing oral mucositis and reducing its symptoms [12]. This approach uses a wide range of non-ionizing light sources, such as lasers, light-emitting diodes, and broadband visible light and infrared spectrum at very low non-thermal doses capable of promoting anti-inflammatory, analgesic, and angiogenic action [13,14].

Low-level light therapy activates endogenous chromophores and acts on cytochrome c oxidase causing several biological events that produce a transient increase in reactive oxygen species. Consequently, this process culminates in increased proliferation of epithelial cells and accelerates tissue healing [15]. However, as patients undergoing cancer treatment are generally immunosuppressed, and because the oral cavity is an environment rich in microorganisms, these patients have a greater predisposition to fungal and bacterial infections, especially due to the presence of severe grades of oral mucositis lesions, which can hamper tissue healing and facilitate the occurrence of local or systemic infections [10].

In this context, photodynamic therapy becomes a promising therapeutic option for the treatment of infectious processes of the oral cavity [16]. Photodynamic therapy is a treatment option that combines photophysical and photochemical mechanisms, generating a biological response that is highly selective against microorganisms by using specific light sources and an oxygen-associated photosensitizing agent [16].

The use of photodynamic therapy has the advantage of being safe, easy to applied, cost effective, and capable of causing minimal discomfort to the patient. Additionally, photodynamic therapy can be performed in the hospital environment for patients undergoing antineoplastic therapies [17]. Thus, due to lack of satisfactory scientific evidence and systematic reviews addressing the use of photodynamic therapy in the treatment of oral mucositis—in spite of the importance of photodynamic therapy on the world scenario and the advances of this therapy in the treatment of infections conditions in the oral cavity—the present study aims to conduct a systematic review and meta-analysis in clinical trials to establish the effectiveness of photodynamic therapy on oral mucositis injuries.

2. Methods

2.1. Protocol and registry

The present systematic review and meta-analysis was carried out according to Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines and Enhancing the Quality and Transparency of Health Research (EQUATOR) guidelines [18,19]. The study was registered in the International of Registered Review and Meta-analysis Protocols (INPLASY202130006/DOI: 10.37766/inplasy2021.3.0006).

2.2. Data extraction and question of the study

The "PICO" strategy [20] was used to guide the methodology and selection of the articles and to help to answer this question: "Is photodynamic therapy effective in preventing and/or treating lesions of oral mucositis induced by radio and/or chemotherapy?" The PICO strategy was: P (patients with oral mucositis lesions), I (photodynamic therapy or photodynamic therapy associated with other treatment), C (non--photodynamic therapy applied), O (photodynamic therapy or photodynamic therapy associated with other treatment are able to give more positive results than others for the treatment of oral mucositis lesions).

For the data extraction two independent reviewers (ABO and TMF) gathered the subsequent information of screening articles: authors, publication year, oncologic characteristics (type of cancer, antineoplastic therapy, grade of oral mucositis at baseline, sample size), information of photodynamic therapy (photosensitizer used by the authors, incubation time, irradiation time, number of treatment sessions, wavelength, and intensity), non-photodynamic therapy used by the authors as the control group, and the outcome measurements.

2.3. Eligibility criteria

The inclusion criteria for this systematic review were: clinical trials, studies that evaluated the effectiveness of photodynamic therapy in the treatment of oral mucositis, studies that evaluated the effectiveness of photodynamic therapy combined with other non-photodynamic therapy in the treatment of oral mucositis, and articles written in English. No restrictions were used to the type of photosensitizer or type of light. The exclusion criteria were: other experimental designs of studies such as *in vitro* or animal studies, review articles, letters to the editor, personal opinions, book chapters, or conference abstracts, studies that did not present a control group, articles written in other languages, and articles where full text is not freely available.

2.4. Search strategy

Two independent examiners (ABO and TMF) conducted an electronic search in the PubMed, Scopus, Web of Science, Science Direct, SciELO, Embase and Cochrane library databases using the following search terms: (photodynamic therapy OR photochemotherapy) AND (oral mucositis OR Stomatitis). The kappa calibration was previously performed between the independent examiners at the start of the systematic review. The classification score between the examiners was 0.92 ($p < 0.01$), which can be classified as an "almost perfect" agreement. A manual search was also conducted in specific journals (*Photodiagnosis and Photodynamic Therapy, Journal of Biophotonics, Photochemical e Photobiological Sciences, Journal of Photochemistry and Photobiology, Scientific Reports, Plos One, European Journal of Cancer, Oral Oncology, Critical Reviews in Oncology/Hematology, European Journal of Clinical Investigation, Clinical Oral Investigation* and *Archives of Oral Biology*) with scope in photodynamic therapy and oral mucositis with the intention of finding more articles that could be included in the present systematic review.

Thereafter, the data were extracted from eligible articles. Studies were analyzed and discussed. Any possibility of disagreement during the process was solved before proceeding to the next steps by reaching a consensus, and as appropriate, a third reviewer (CRF) was consulted. An independent researcher (FLB) guided the development of this review. The literature review was carried out from February 1990 to May 2020. The records obtained were exported to Mendeley Reference Manager Software® to detect and exclude duplicates.

2.5. Level of evidence

The included articles in eligibility criteria were classified in levels of evidence based on the guideline of the Centre for Evidence-Based Medicine from the University of Oxford [21].

2.6. Quality of clinical trials assessment

The quality assessment of the clinical trials was performed by two independent reviewers (ABO and TMF) using the Delphi List criteria [22].

2.7. Risk of bias assessment

This stage was carried out by two independent reviewers (ABO and TMF), prioritizing the clear description of the methods and omitting the name of the journals and authors to avoid any conflict of interest or bias for all the eligible studies included. The risk of bias assessment was performed using the RoB 2 tool for clinical trials [23].

2.8. Meta-analysis

The meta-analysis was performed using R software, version 3.6.3 with the "META" package assistant by the RStudio platform, using the random effects model and showing the results in a forest plot. For conducting the meta-analysis, the mean-difference pattern was calculated as an effect measure. The funnel plot was performed to access the publication bias.

3. Results

3.1. Search results

The selection process of the articles can be seen in the flow diagram (Fig. 1). The initial electronic search identified 727 potentially relevant references. A total of 177 duplicated articles were excluded and 550 articles remained in the selection process.

After title and abstract screening, 545 articles were excluded. A total of five articles were eligible for full-text evaluation, data extraction, synthesis of results, quality of clinical trial, risk of bias assessments, and classification of level of evidence. Only two articles met the criteria to be included in the meta-analysis. Information on data extraction and level

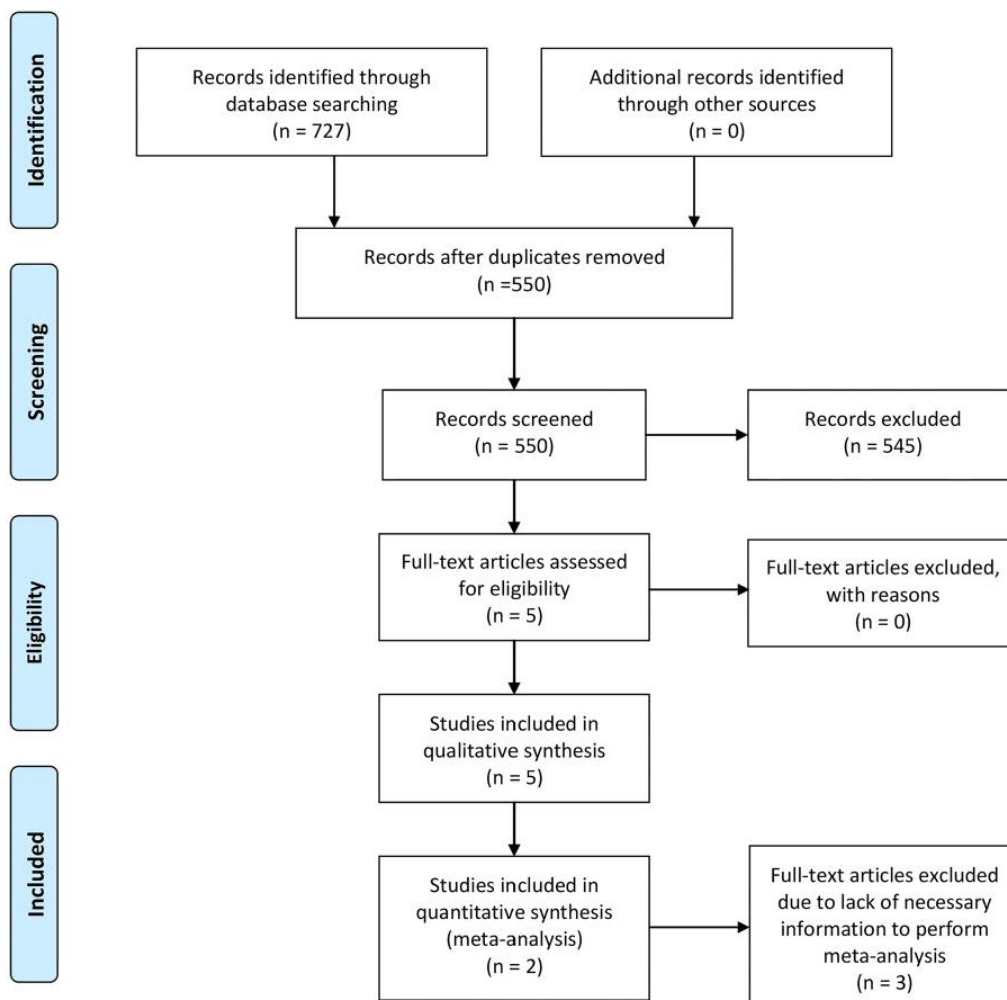

Fig. 1. Flowchart summarizing inclusion and exclusion of the articles for the study.

of evidence of the included articles are summarized in Table 1.

3.2. Synthesis of results

The majority of the articles selected were from Brazil and one was from Iran [24–28]. Among the malignances affected by the studied population, cancer of the lymphatic system (60 %) was the most frequently cited, followed by breast cancer (40 %). The target population of the studies was fairly comprehensive, ranging from pediatric patients (≤ 18 years old) [24,25] to older adults (26–81 years old) [26, 25–28]. Chemotherapy with high doses of methotrexate (≥ 5 g/m^2) was the most widely used antineoplastic treatment. The use of preventive protocols and oral care was reported in only two studies before antineoplastic treatment [27,28].

The majority of studies used the Word Health Organization severity classification system for oral mucositis; Grade III was the most frequent classification reported (100 %) followed by Grades II (60 %) and IV (40 %). The anatomical distribution of mucositis can be influenced by the amount and direction of radiation when the radiotherapy is utilized in patients affected by head and neck cancer. Also, ulcer lesions were observed in the non-keratinized oral tissues (buccal mucosa, lateral tongue, soft palate, floor of the mouth) and upper and lower lips.

Among the photosensitizers used in the included clinical studies, the methylene blue was the most frequent photosensitizer evaluated (60 %) followed by curcumin (40 %) [24,25,27]. The pre-irradiation time of the photosensitizer ranged from 3−10 min [24–27]. Only one study (20 %) [28] did not report the pre-irradiation time used. The light source most often used in the selected studies was red light-emitting diodes (wavelength 660–810 nm) (71 %) [24,25,27] followed by blue light-emitting diodes (wavelength 400–470 nm) (28.57 %) [26,28].

The included studies used two main experimental groups: photodynamic therapy combined with low-level light therapy and photodynamic therapy alone. However, the specifications and details of each treatment protocol employed varied significantly. Irradiation time ranged from 30 s to 10 min. The frequency of application varied from every day (for 8 days) [24], once a week (for 4 weeks) [26,28], an application on the first, third, and fifth day after the appearance of mucositis [27] or until the disappearance of the oral lesion [25].

The effectiveness of photodynamic therapy on the healing process and the reduction in severity of oral mucositis lesions had positive outcomes compared with low-level light therapy [25,27]. In these studies, the authors agreed that photodynamic therapy showed a tissue repair effect similar to low-level light therapy. However, in the presence of infection, photodynamic therapy was more effective. The studies that associated photodynamic therapy and low-level light therapy [24,26, 28] reported that this combination could enhance the healing process of mucositis and reduced lesion remission time when compared with low-level light therapy alone. The level of evidence analysis showed that the majority (60 %) of the studies were classified as level II. Only two articles (40 %) [26,28] were classified as level III. This difference of evidence level is explained by the utilization or non-utilization of randomization procedures in the study's design.

3.3. Quality of clinical trials assessment

The best article evaluated according to Delphi list criteria was Lavaee et al. [27]. This article showed only one negative answer to the question: "Was the outcome assessor-blinded?" The article that received the highest number of negative answers was Pinheiro et al. [26]; it failed to cover randomization and allocation. In addition, Pires Marques et al. [28] had the highest number of answers classified as "Don't know" (Table 2).

3.4. Risk of bias assessment in clinical trials

This step was conducted according to the RoB 2 toll to assess the risk of bias in clinical trials. The article with the best results was Medeiros-Filho et al. [24], followed by Ribeiro da Silva et al. [25], and Lavaee et al. [27]. Interestingly, all the studies that were included in the present systematic review demonstrated bias in the criterion "measurement of the outcome" (Fig. 2).

3.5. Meta-analysis

For the meta-analysis, only the outcome related to healing time in clinical trials could be grouped to perform the statistical analysis. The patients that were treated with photodynamic therapy combined with low-level light therapy achieved the healing of oral mucositis in a period of 4.84 days before the control group, which was formed with patients who received only low-level light therapy as treatment ($p = 0.0005$). Due to the absence of heterogeneity ($I^2 = 0\%$), the fixed effect model and the random effects models showed the same results. However, considering the methodological differences of the studies, the interpretation of the meta-analysis results is more appropriate using the random-effects models. The study of Pires Marques et al. [28] matched 71.9 % of weight in the meta-analysis results, while Pinheiro et al. [26] matched only 28.1 %. Additionally, with the funnel plot analysis, it is evident that there is no publication bias (Fig. 3).

4. Discussion

One of the most challenging side-effects that affect patients undergoing antineoplastic therapies is the occurrence and severity of mucositis during and after therapy [29]. Nevertheless, to date, there is no preferred treatment. Thus, new treatment options, as well as the improvement of conventional therapeutic resources and the establishment of robust protocols to treat of oral mucositis, are needed. In this context, the uses of biophotonic approaches are promising, especially when photodynamic therapy acts as an addition to low-level light therapy.

For low-level light therapy, several studies have demonstrated outcomes that can be applied in the treatment of oral mucositis, such as increasing the angiogenic capability of irradiated tissue and anti-inflammatory and analgesic action [30]. However, as the oral cavity is an environment rich in microorganisms, the ulcerated lesions are often associated with infections, which make it necessary to disinfect the area for adequate healing.

Due to the limitations of conventional therapies in oral mucositis lesions decontamination and healing, the search for new therapeutic modalities is necessary [31]. In this way, photodynamic therapy is an alternative treatment for infectious processes present in the oral cavity [24].

The scientific literature has already demonstrated the effectiveness of photodynamic therapy in ulcerated lesions, suggesting that it may be applied as adjuvant therapy for tissue healing and repair [31,32]. Previous studies have shown that photodynamic therapy is able to induce an acute localized inflammatory response, which leads to the activation of the immune system and the migration of myeloid cells, monocytes, macrophages, and mast cells to the injured site. Subsequently, the activation of CD8$^+$ T-cells occurs [33], which contributes to the elimination of injured cells and the repair of damaged tissues. However, some studies have shown that ulcerated lesions treated with photodynamic therapy have less inflammatory infiltrate [34], since this treatment promotes the reduction of microbial load [35–37].

For the success of photodynamic therapy, many factors have to be considered, such as the photosensitizer used and light dosimetry [38]. Among the articles evaluated, the most frequently used photosensitizer was methylene blue. This molecule belongs to the phenothiazine class and shows water and ethanol solubility. The efficiency of methylene blue in photodynamic therapy is related to the intense absorption in the visible region by ultraviolet light, the maximum wavelength of which is 664 nm. This wavelength belongs to the phototherapeutic window,

Table 1
Summary of the characteristics of included studies.

Study		Year	Level of Evidence*	n	Type of cancer	Antioneoplastic treatment	Grade of OM at baseline	Photosensitizer	Incubation time**	Irradiation Time	Therapy	Control group	Number of sessions	Wave-length	Ligth dose
#1	Medeiros-Filho et al. [24]	2017	II	15	Lymphoblastic leucemia	Chemotherapy and radiotherapy	III and IV	Methylene blue	5 min	90 s	PDT + LLLT	LLLT	8	660 nm	100mW
#2	Ribeiro da Silva et al. [25]	2018	II	14	Non-Hodgkin Lymphoma, Osteosarcoma	Chemotherapy	II and III	Methylene blue	3 min	30 s	PDT	LLLT	Daily applications until complete remission of the lesions	660 nm	107 J/ cm^2, 100mW
#3	Pinheiro et al. [26]	2019	III	14	Myeloma, Prostate, Carcinoma, Rectal adenocarcinoma, Lymphoma, Breast, Neuroectodermal, Lung, Testicular and Thyroid	Chemotherapy and radiotherapy	II and III	Mouthwash solution with curcumin	5 min	5 min	PDT + LLLT	LLLT	4	450 nm	142 J/ cm^2, 100mW
#4	Lavaee et al. [27]	2020	II	15	ND	Chemotherapy	I and III	Methylene blue	10 min	10 min	PDT	LLLT	3	660 nm	19,23 J/ cm^2, 25 mW
#5	Pires Marques et al. [28]	2020	III	28	Breast, Head, neck and face carcinoma, Lymphoma, Prostate, Sarcoma, Myeloma, Rectal adenocarcinoma and melanoma	Chemotherapy and radiotherapy	I, II, III and IV	Mouthwash solution with curcumin	N/A	5 min	PDT + LLLT	LLLT	4	450 nm	142 J/ cm^2, 100mW

sec: seconds; min: minutes; Negative: no treatment applied; * Level of evidence according Oxford Centre for Evidence-Based Medicine; n: sample size; ** Pre-irradiation time; RCT: randomized clinical trial; CT: clinical trial; PDT: Photodynamic Therapy; LLLT: Low-level laser therapy; x: versus; +: association of treatments; ND: not documented.

Table 2
Quality assessment of randomized clinical trials included in the systematic review.

Medeiros-Filho 2017 [24]	Ribeiro da Silva 2018 [25]	Pinheiro 2019 [26]	Lavaee 2020 [27]	Pires-Marques 2020 [28]	Delphi List for Quality Assessment of RCTs
Yes	Yes	No	Yes	Don't know	Was a method of randomization performed?
Yes	Yes	No	Yes	Don't know	Was the treatment allocation concealed?
Yes	Yes	Yes	Yes	Yes	Were the groups similar at baseline regarding the most important prognostic indicators?
Yes	Yes	Yes	Yes	Yes	Were the eligibility criteria specified?
Don't know	Don't know	Don't know	No	Don't know	Was the outcome assessor blinded?
No	Don't know	Don't know	No	Don't know	Was the care providor blinded?
Yes	Yes	Don't know	Yes	Don't know	Was the patient blinded?
Yes	Yes	Yes	Yes	Don't know	Were point estimates and measures of variability presented for the primary outcome measures?
Yes	Don't know	Don't know	Yes	Yes	Did the analysis include an intention-to-treat analysis

which is the spectral region ranging from 600 to 1000 nm. This aspect allows deep penetration of light into biological tissues and an expressive quantum yield to form singlet oxygen [39,40].

In addition, the effectiveness of photodynamic therapy mediated by methylene blue in repairing tissue injuries is closely related to the microbial reduction that results from the oxidative stress process caused by photodynamic therapy. This microbial reduction also can be explained by the penetration power of methylene blue in the tissue, allowing more effective action in the lesion [41]. Additionally, there are reports in the literature about the action of photodynamic therapy mediated by methylene blue against bacteria in association with oral ulcerative lesions [42]. The methylene blue has promoted a good response in gram-positive and gram-negative bacteria, since this photosensitizer is positively charged on the molecule and has low molecular mass [39].

The results of the present systematic review have also shown that two other types of photosensitizer (also belonging to the phenothiazine class) can be used in the treatment of oral mucositis. The mouthwash solution containing curcumin was the second most frequently used photosensitizer, followed by indocyanine green. However, further clinical studies with these photosensitizers should be developed to confirm this result.

Considering the pre-irradiation time (i.e., the period in which the photosensitizer remained in contact with the tissue allowing binding to the plasma membrane and/or internalizing the target cells before treatment with light) different times were evaluated. The pre-irradiation time of the included studies ranged from 3–10 min for the different photosensitizers [24–27]. Only one article did not report the time used [28]. This information is essential and should be given by the articles, otherwise the establishment of future treatment protocols of oral mucositis using the photodynamic therapy will be difficult, and the lack of this information will not allow the scientific data to be reproduced [38].

Concerning the effectiveness of photodynamic therapy when compared with low-level light therapy on the healing process of ulcerative lesions of oral mucositis, photodynamic therapy can be safely used in these injuries because this therapy does not affect the tissue repair process and stimulates platelet-derived growth factor BB production and fibroblast growth factor β [43,44]. These inflammatory mediator factors are essential to the healing process. In addition, the data suggest that the healing effect of photodynamic therapy is related to the decreased levels of microbial loads in the target site [43,44]. However, clinical trials should be performed to confirm these results.

The studies of Ribeiro da Silva et al. [25] and Lavaee et al. [27] evaluated the effect of photodynamic therapy and low-level light

Medeiros-Filho et al. 2017	Ribeiro da silva et al. 2018	Pinheiro et al. 2019	Pires Marques et al. 2020	Lavaee et al. 2020	Version 2 of the Cochrane risk-of-bias assessment tool
+	+	+	+	+	Bias arising from the randomization process
+	?	?	+	+	Bias due to deviations from intended interventions
+	+	+	+	+	Bias due to missing outcome data
?	?	?	?	-	Bias in measurement of the outcome
+	+	-	-	+	Bias in selection of the reported result
+	+	?	?	+	Overall risk of bias

Fig. 2. Risk of bias analysis according to the RoB 2 toll for assessment risk of bias to studies included in the systematic review. Red (−) is high risk of bias; Green (+) is low risk of bias, and Yellow (?) is unclear risk of bias.

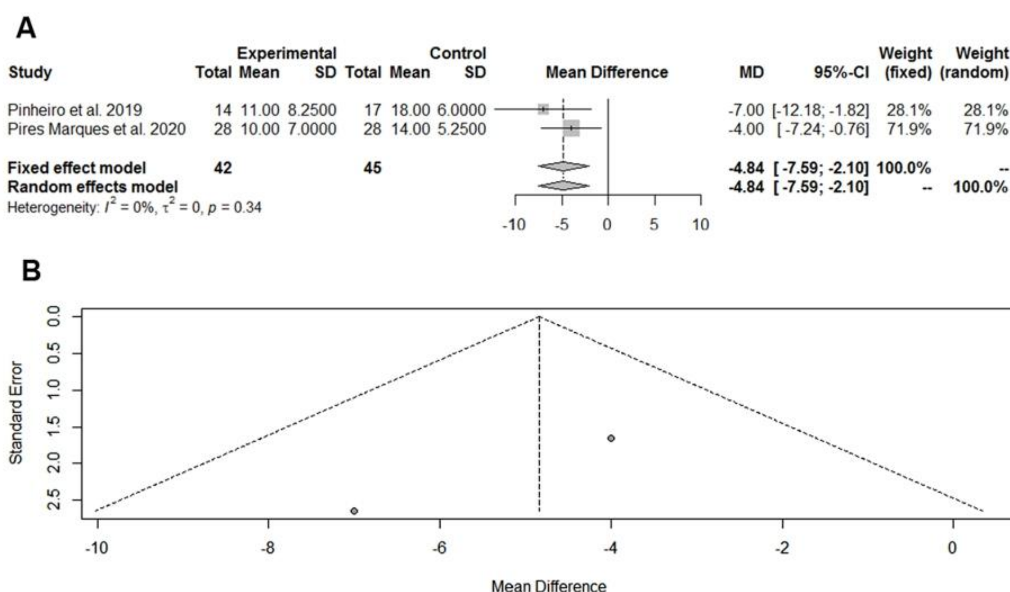

Fig. 3. Quantitative results. A: Meta-analysis results illustrated in a forest plot. B: funnel plot to assess publication bias. Experimental: Low-level laser therapy (LLLT) associated with photodynamic therapy (PDT); Control: LLLT.
CI: confidence interval; MD: mean difference; SD: standard deviation.

therapy in oral mucositis lesions. However, the authors did not find statistically significant differences between the therapies. This finding can be explained by the split-mouth design adopted by both studies. Additionally, the systemic effect of photodynamic therapy on the release of cytokines, interleukins, and other mediators might have caused bias in the interpretation of results [27]. Although the reasons are not clear enough to understand the results of these studies, the time of irradiation and the number of treatment sessions can influence the success rate of photodynamic therapy when compared to low-level light therapy.

Meta-analysis was only conducted for the variable “time to repair”. All other data extracted used different methods to measure the dependent variable, impairing more meta-analyses. Thus, our results highlight that combined treatment with photodynamic therapy and low-level light therapy reduces the need for treatment in approximately 5 days compared to patients who received only low-level light therapy for oral mucositis. Due to publication bias and the lack of heterogeneity, these results are reliable. In addition, photodynamic therapy combined with low-level light therapy may have a synergistic effect in re-epithelization and connective tissue remodeling, which might accelerate healing time [45]. Moreover, photodynamic therapy not only has effects against microorganisms but also can favor the repair process through biomodulation in irradiated tissue and reduction of local inflammation [46]. Nonetheless, more studies should be performed to confirm these findings, since there were only two articles included in the meta-analysis.

The use of blind procedures in scientific studies is one of the best strategies to bring scientific facts closer to reality. The aim of blinding is to eliminate bias related to detection/ascertainment, attrition, performance, co-intervention, and observation. Additionally, the lack of concealment of an intervention—especially those related to participants, patients, and examiners—might increase the effect size estimate. Thus, blind experiments are highly recommended and should be carried out whenever possible [47].

All patients affected by oral mucositis need very strict care, particularly patients receiving chemotherapeutics drugs, such as alkylating agents, antitumor antibiotics, antimetabolites, vinca alkaloids, anthracyclines, taxanes, and others (etoposide, teniposide, and nitrogenated mustards) [48]. In addition, patients receiving a combination of chemotherapy and radiotherapy for head and neck cancer as well as chemotherapy combined with immunosuppressive drugs have increased risk for severe oral mucositis [48].

Considering the difficulty of patients with oral mucositis in keeping their mouths opened for long periods, future treatment protocols should be addressed aiming shorter pre-irradiation and irradiation times in order to reduce patient discomfort. Photosensitizers, such as like methylene blue, curcumin, and indocyanine green, have shown the ability to treat oral mucositis. Thus, as the patients affected by oral mucositis have many oral sites with lesions, the application of mouthwash solutions containing the photosensitizer is an excellent option. In addition, the results of curcumin mouthwashes were strengthened by the meta-analysis results. Nevertheless, photosensitizers with a wavelength between 600 and 1000 nm not only are able to induce microbial reduction but also have more healing properties. Photodynamic therapy can be indicated for Grades 3–4 of oral mucositis, due to the presence of the infection independently of the oral mucositis etiology and the type of cancer; however, special care should be taken in patients affected by oral cancer. In these patients, photodynamic therapy and low-level light therapy should be avoided in the surrounding cancer regions.

Photodynamic therapy has numerous advantages, such as the absence of significant side-effects. It is a non-invasive treatment and can be repeated without cumulative toxicity in the target tissue [16]. From this perspective, many studies indicate that photodynamic therapy is an effective and promising therapy to reduce bacterial viability [49] and to support tissue repair [31].

5. Conclusion

Photodynamic therapy alone and combined with low-level light therapy show promising results for the treatment of oral mucositis, contributing to healing and repairing injured tissue. However, more blind and randomized clinical trials need to be carried out in patients with oral mucositis in order to evaluate different parameters for the use of photodynamic therapy, such as the type of photosensitizer, the concentration, the incubation time of the photosensitizer, and the dose of

light. Finally, evaluation of other factors that surely interfere with the results obtained (i.e., medications used by cancer patients, their nutritional status and oral condition) also need to be taken into consideration for future studies.

Authorship contributions

Design of study and conceptualization: ABO, TMF, CRF, FLB. Data curation and analysis: ABO, TMF, FLB. Original draft construction: ABO, TMF, FLB. Draft review and scientific revisions: ABO, TMF, FGB, CRF, EMAG, FLB. Final draft approval: FGB, CRF, EMAG, FLB.

Compliance with ethics guidelines

This article is based on previously conducted studies and does not contain any studies with human participants or animals performed by any of the authors.

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3.2 Publicação 2*

Senna alata extract as natural photosensitizer in the antimicrobial photodynamic inactivation.

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* O artigo segue as normas do periódico *Oral diseases* para o qual pretende-se submeter.

ABSTRACT

The study evaluated the efficacy of antimicrobial photodynamic therapy (aPDT) using *S. alata* (0.05/0.5 mg/L) against *Candida albicans* and *Staphylococcus aureus* compared to methylene blue (MB: 0.2/2.0 mg/mL), Photodithazine (PDZ: 175/200 mg/mL), and curcumin (CUR: 0.014/0.029 mg/mL) as photosensitizers. The following parameters were analyzed: microbial viability, LIVE/DEAD staining, cytotoxicity in human cells, biofilm metabolic activity, and gene expression (ALS1, CAP1, HPW1, CAT1, SOD1, *fnbA*, *cflA* and *hla*). Different concentrations of each photosensitizer were tested against microbial suspensions and biofilms, with single and multiple aPDT applications. Results showed undetectable microbial viability for CUR, PDZ, and *S. alata*-mediated aPDT in suspensions of both microorganisms. Significant reductions in microbial viability were observed in biofilms after aPDT, particularly with MB and *S. alata*. Biofilm metabolic activity decreased after a single aPDT application. Photosensitizer concentrations and irradiation parameters were non-cytotoxic. Moreover, aPDT significantly reduced the expression of virulence-related genes in both microorganisms. In conclusion, *S. alata*-mediated aPDT effectively controlled biofilms of *S. aureus* and *C. albicans*, showing superior results in reducing virulence factors and maintaining cellular safety compared to other photosensitizers.

1. INTRODUCTION

One of the main challenges facing modern medicine is establishing new effective strategies to deal with infections (McEwen and Collignon; 2018). Microbial biofilms, i.e., an aggregate of microbial cells embedded in a matrix of extracellular polymeric substances (EPS), adhered to each other and/or to a surface (Wingender et al., 1999), are responsible for more than 80% of all bacterial and fungal infections in humans (European Centre for Disease Prevention and Control; 2022). In contrast to cells in suspension, biofilm cells can have different physiological and metabolic aspects (Donlan, 2002), which are essential for microbial survival in challenging and varied environments. In clinical contexts, biofilms foster stubborn infections that are challenging to eliminate, shielding microorganisms from host defense mechanisms, and heightening microbial resistance to numerous drugs (Hall-Stoodley et al., 2004). Moreover, biofilms play a significant role in the development of persistent chronic infectious diseases (Del Pozo, 2018).

Among the microorganisms with potential risks of mortality and morbidity in mankind, *Candida* spp., *Staphylococcus* spp. and enterobacteria are in evidence, especially due to their ability to infiltrate and persist within the host, potentially leading to the spread of infectious diseases (Laheij and Soet, 2014). Additionally, scientific evidence has shown that interkingdom interaction between *Candida albicans* and *Staphylococcus aureus* leads to an elevated inflammatory response in the mucosa, alterations in the host's immune response, and changes in the virulence of these species *C. albicans* increases the colonization ability of *Staphylococcus aureus* on mucosal surfaces (Xu et al., 2014), while *S. aureus* enhances the invasiveness of *C. albicans* at epithelial junctions (Xu et al., 2016).

Toxic products originating from the cell walls of these pathogens can penetrate the submucosa, triggering the release of pro-inflammatory cytokines. As a result, inflammation, pain, risk of secondary infections and, in some situations, a potential systemic influx of microorganisms and toxins may occur. This latter scenario is especially concerning for patients with neutropenia or with compromised immune systems, as these factors increase the risk of bacteremia and septicemia (Sonis, 2004a; Sonis, 2004b). Therefore, it is paramount to search, and advance recourses approaches to combat biofilm infections. Antimicrobial photodynamic therapy (aPDT) has emerged as a viable alternative method to combat infection diseases. aPDT is based on the application a non-toxic photosensitizer (PS) that, in the

presence of visible light at a precise wavelength, can lead cells to death due to production of cytotoxic reactive oxygen species (ROS).

Although synthetic compounds provide greater stability, natural compounds have been extensively researched and embraced, primarily due to their lower likelihood of causing side effects in patients and reduced potential for drug interactions. Additionally, in some situations, natural compounds are also more capable to penetrate microbial cell wall and extracellular matrix (Dovigo et al., 2011; Paschoal et al., 2013; Dovigo et al., 2013; Leite et al., 2014; Rego-Filho et al., 2014; Silva et al., 2015).

Besides, the currently available PS do not meet all the characteristics for an ideal PS. Ideally, a PS should selectively accumulate into the target area, be readily available, possess a high coefficient of absorption in a specific wavelength, ensure that the absorption bands of the PS do not overlap with endogenous dyes such as melanin or hemoglobin, react with the light to produce singlet oxygen or radicals, have few side effects, and exhibit low toxicity while being easily excreted from the body (Kubrak et al., 2022). Therefore, given the lack of a PS with ideal characteristics, studies aimed at discovering new PSs are encouraged.

In a previous study, we assessed the potential of *Senna* spp. extracts as PS for use in aPDT against many types of microorganisms related to human health (de Oliveira et al., 2023). *Senna alata*-mediated aPDT showed the most favorable outcomes against *C. albicans* and *S. aureus* cultured in both suspension and in biofilm following a single application of this treatment (de Oliveira et al., 2023).

Given the difficulties in eradicating and controlling the virulence factors of microorganisms, particularly *C. albicans* and *S. aureus*, the need for studies to effectively overcome infection and microbial resistance is justified. Therefore, the present study aimed to evaluate the *in vitro* potential of *S. alata*-mediated aPDT against *C. albicans* and *S. aureus* cultured in suspension and mono-species biofilm with one or three successive applications of aPDT and to compare the results with three other photosensitizers widely evaluated in scientific literature, two of synthethic origin (Methylene Blue (MB) and Photoditazine (PDZ)) and one of natural source (Curcumin (CUR)). In addition, the cytotoxic effects of these PS on human keratinocyte and fibroblast cells were assessed, along with the evaluation of metabolic activity of biofilms, analysis by confocal laser microscopy and

quantitative expression of virulence factors related to the *C. albicans* and *S. aureus* by real time PCR.

2. MATERIALS AND METHODS

2.1 *Photosensitizing Agents*

To carry out this study, Photodithazine® (PDZ) (Veta-grand Co., Moscow, Russia), methylene blue (MB) (Sigma-Aldrich Co. LLC, St Louis, USA), CUR (Sigma-Aldrich Co. LLC, St Louis, USA) and crude extract of *Senna alata* were used as photosensitizing agents. The choice of these photosensitizing agents is based on results from previous studies, which demonstrated their ability to promote a significant reduction in the of biofilms' viability of *C. albicans* and *S. aureus* in PDT (de Oliveira et al., 2023; Dovigo et al., 2013; Carmello et al., 2015; Carmello et al., 2016).

PDZ was initially supplied at 5,000 mg/L by the Biophotonics Laboratory of the São Carlos Physics Institute – USP (IFSC). Subsequently, PDZ was diluted in 0.85% NaCl to achieve concentrations of 0.175 and 0.2 mg/mL (Quishida et al., 2014). A stock solution of MB (10 mg/mL; Sigma Aldrich Co. LLC, St Louis, MO, USA) was prepared in distilled water. Working concentrations of 0.2 mg/mL and 2 mg/mL were further prepared (Abo-Neima et al., 2023). CUR was diluted in 1% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) to obtain working concentrations of 0.014 and 0.029 mg/mL. All PS were stored sealed and protected from light at room temperature.

S. alata extract was prepared as previously described (de Oliveira et al., 2023). Briefly, the plant material was dehydrated at 40 °C for 36 h, followed by grinding extraction in an ultrasonic bath at a ratio of 100 mg of powder to 3 mL of methanol. The resulting extract was filtered and concentrated under vacuum in a rotary evaporator (Bueno et al., 2015). The plant extract was suspended in 1% DMSO and subsequently diluted in culture medium to obtain a final concentration of 0.05 and 0.5 mg/mL.

2.2 *Light Sources*

Two different irradiation systems were used (IrradLED® – Biopdi, São Carlos, SP, Brazil), one consisting of blue LEDs and the other of red LEDs, which allow a homogeneous distribution of light in each well of the plate. The photosensitizing agents *S. alata* plant extract and Curcumin were illuminated at 460 nm and the photosensitizing agents PDZ and methylene blue were illuminated at 660 nm.

2.3 *Assessment of activity in aPDT and cytotoxicity in relation to the concentration of photosensitizing agents and the dose of light*

2.3.1 Microbial strains and growth conditions

The reference strains used were *Candida albicans* (ATCC® SC5314™) and *Staphylococcus aureus* (ATCC® 25923™), sourced from the National Institute for Health Quality Control (INCQS) of the Oswaldo Cruz Foundation (FIOCRUZ - Manguinhos, RJ, Brazil). Prior to use, the strains were individually cultured on Petri dishes (TPP Techno Plastic Products AG, Switzerland) containing Sabouraud Dextrose Agar (SDA – Acumedia Manufactures Inc., Baltimore, MD, USA) for *C. albicans* or Brain Infusion Agar and Heart (BHI) for *S. aureus*. The Petri dishes were then incubated at 37ºC for 48 hours (Bacteriological greenhouse, Marconi Equipamentos Laboratoriais Ltda, Piracicaba, SP, Brazil).

For the *S. aureus* pre-inoculum, seven colonies from newly cultured plates were transferred to 10 mL of Tryptic Soy Broth culture medium (TSB-Acumedia Manufactures Inc., Baltimore, MD, USA) and incubated in an oven at 37 ºC for 18 hours. The resulting inoculum was then diluted at a ratio of 1:20 (1 mL of pre-inoculum in 19 mL of TSB medium). To prepare the *C. albicans* pre-inoculum, 10 freshly cultured colonies were transferred to 10 mL of Yeast Nitrogen Base broth culture medium (YNB-Difco, Becton Dickinson Sparks, MD, USA) supplemented with 100 mM glucose and incubated in an oven at 37 ºC for 16 hours. The inoculum was diluted at a ratio of 1:10 (1 mL of pre-inoculum in 9 mL of sterile liquid culture medium).

Suspensions of *C. albicans* and *S. aureus* were standardized using a spectrophotometer at an optical density of 540 nm and an optical density at 600 nm of 0.7 ± 0.01, which corresponds to the middle of the exponential phase and represents a microbial concentration of 10^7 CFU/mL of each microorganism.

2.3.2 *aPDT with microorganisms cultured in suspension.*

To provide the dose-response relationship between the concentration of photosensitizing agents and the dose of light, the following parameters were used (Table 1).

Table 1. aPDT experimental parameters

Photosensitizing agent	Concentration (mg/mL)	Dose of light (J/cm²)	Pre-irradiation time (min)	Irradiation time (seg)	References
Photodithazine®	0.175 and 0.2	50	20	746	Quishida et al., 2015
Methylene blue	0.2 and 2	50	20	746	Su et al., 2021
Curcumin	0.014 and 0.029*	50	20	322	Jordão et al., 2020
Senna alata	0.05 and 0.5	50	20	322	de Oliveira et al., 2023

*Corresponding to 40 μM and 80 μM, respectively

aPDT was conducted by administering each photosensitizing agent at the concentrations specified in table 1 and exposing them to 50 J/cm^2 of LED light (P+L+). Besides, samples were treated with only the photosensitizing agents (P+ L−) or only LED light (P−L+). Untreated control samples did not receive exposure to light or photosensitizing agents (P-L-). After preparing and adjusting the inoculum of each microorganism, 50 μL of the prepared inoculum, as described in item 2.3.1, were transferred to wells of 96-well plates. Then, 50 μL of each photosensitizing agent were added to the wells designated for the P+L+ and P+L− groups, resulting in a final volume of 100 μL per well and a 50% dilution of the solutions and inoculum. For the P−L+ and P−L− groups, 50 μL of Phosphate-buffered saline (PBS) (pH 6.8) were added. Subsequently, the plates were incubated in darkness for 20 minutes (pre-irradiation time). Next, samples from groups P+L+ and P−L+ were exposed to LED light and illuminated for either 322 or 746 seconds (50 J/cm^2), while groups P+L− and P−L− remained in darkness for the same duration.

After irradiation of the samples, the microbial suspensions from each well were diluted and seeded using the agar drop method (de Freitas et al., 2018). The microbial colonies formed were counted after 24-48 h of incubation under the conditions described in item 2.3.1 at 37 °C.

2.3.3 aPDT with microorganisms cultured in biofilm.

100 μL aliquots of the prepared *C. albicans* and *S. aureus* inoculum, as described in item 2.3.1, were transferred to 96-well plates containing 100 μL of specific culture

medium for each microorganism. These plates were then incubated in an orbital shaker (AP 56, Phoenix Ind with Scientific Equipment Ltd., Araraquara, SP, Brazil) at 75 rpm for 1.5 hours (adhesion phase) (Panariello et al., 2018). Following this, to remove non-adherent cells, the suspensions were gently aspirated and washed with 150 µL of PBS (pH 6.8). Biofilms were then allowed to develop for 24 and 48 hours in RPMI buffered with HEPES (pH 7.0) for *C. albicans* and TSB for *S. aureus*, all on an orbital shaker. Once mature biofilms had formed, the culture media were removed by washing the wells twice with PBS (pH 6.8) (Panariello et al., 2018).

aPDT with microorganisms in biofilm was performed with one or three successive applications, mediated by the four photosensitizing agents under the conditions described in table 1. Each experimental condition was conducted on three separate occasions in quadruplicate (n=12 per group).

For single-application therapy tests, following biofilm formation, the wells were washed twice with PBS (pH 6.8). Depending on the experimental groups, 150 µL of photosensitizing agents were added to the corresponding wells for the P+ L+ and P+L− groups. For the P−L+ and P−L− groups, 150 µL of PBS (pH 6.8) was added instead. The plates were then incubated in darkness for 20 minutes (pre-irradiation time). Then, samples from the P+L+ and P-L+ groups were exposed to LED light and irradiated according to the times specified in table 1, while the P+L− and P−L− groups remained in darkness for the same duration as the irradiation period for each photosensitizer.

For the three successive applications of the treatment, immediately after the first aPDT treatment (i.e., illuminating the samples [P−L+, P+L+] or incubating them in the dark [P−L−, P+L−]), the incubation and irradiation procedures were repeated two more times. For each successive application, were washed with PBS. Next, the treatment solution (PS or PBS) was inserted, the incubation period was observed, and light was applied as describe above.

After treatments, each well was washed twice with PBS (pH 6.8), and biofilms were detached by scraping for 30 seconds. Next, the biofilm dispersions were diluted and seeded using the agar drop method (de Freitas et al., 2018). The microbial colonies formed were counted after incubation for 24 h (*S. aureus*) or 48 h (*C. albicans*).

2.3.4 *Metabolic activity of biofilms (XTT assay)*

A new set of biofilms was grown and treated as described in item 2.3.3. Following the treatments, the metabolic activity of the biofilms was assessed using the XTT assay (XTT, Sigma-Aldrich). To conduct the assay, a mixture of 158 µL of PBS (pH 6.8) with 200 mM glucose, 40 µL of XTT, and 2 µL of 0.4 mM menadione (Sigma Aldrich) was homogenized and inserted to each well. Subsequently, the plates were incubated in darkness for 3 hours at 37 °C.

After the incubation period, 100 µL of the XTT saline solution from each well was transferred to a new 96-well plate, and the metabolic activity was evaluated by assessing colorimetric changes and measuring light absorbance using a microtiter plate reader (Thermo Plate - TP Reader) at 492 nm.

2.3.5 *Confocal laser scanning microscopy (CLSM)*

Cultures of *C. albicans* and *S. aureus* biofilms were cultivated in 96-well plates (SensoPlate™ - Greiner Bio-One Brazil). The experimental groups were treated in duplicate (n=2) for confocal fluorescence microscopy analysis (CARLS ZEISS LSM 800 Airyscan with GaAsp detector, Germany). Following treatment, 200 µL of 0.89% NaCl was used to wash the wells and then, 100 µL of a 1:1000 dilution of Syto 9 and Propidium Iodide (PI) solution in 1X PBS from the LIVE/DEAD ®BacLight™ kit (Invitrogen; Carslab, California, USA) was added. Syto 9 marks the nucleic content of bacterial DNA (green), while PI marks dead cells (red) (Foglia et al., 2017). 30 minutes was the time establish to incubate the plate. After, the wells were washed again with 100 µL of 0.89% NaCl. For image acquisition, at 10× magnification, 100 µL of 0.89% NaCl was added in the well plates. The configuration settings were as follows: Syto 9 - excitation: 488 nm; detection: 520 nm / propidium iodide: excitation: 561 nm; detection: 620nm). Image analysis was performed using Zen Blue LITE software (version 2.3).

2.3.6 *Cytotoxicity assessment*

Oral keratinocyte (NOK-SI) and oral fibroblast (FGH) cells were cultured separately in 75 cm2 cell culture flasks and maintained in a CO2 incubator at 37°C with 5% CO2 using the MCO-17AC model from Sanyo Electric Co., Ltd., Osaka, Japan. The cells were cultured in DMEM culture medium (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil), with medium renewal every 48 hours.

Negative control experiments utilized Triton X-100, while DMEM supplemented with 10% FBS served as the growth control. Upon reaching 80% confluency, the cells were trypsinized. A cell suspension containing 1.7×10^5 cells/mL of each cell line was then seeded into 96-well plates and incubated for 24 hours in 5% CO_2 (Kathirvel and Ravi, 2012).

Subsequently, the cells were subjected to treatment with photosensitizing agents and controls under the same experimental conditions with three successive applications, following the protocol outlined in item 2.3.3. Cell viability was assessed using the MTT assay (Mosmann, 1983) by adding 100 µL of MTT solution (3 mg/mL, Sigma Aldrich, San Luis, MO, USA) to each plate. After three hours, the absorbance was measured at 562 nm, as instructed by the manufacturer (Datasheet Sigma Aldrich, catalog no. M2128). Experiments were conducted in triplicate. The same concentrations of photosensitizing agents and light doses (J/cm^2) used in the biofilm evaluation were employed with three successive applications (see table 1).

2.3.7 *RT-qPCR Analysis*

The RT-qPCR test was conducted to surviving *C. albicans* and *S. aureus* cells recovered from monospecies biofilms after three successive applications of aPDT.

The RNA extraction utilized the phenol-chloroform with ethanol precipitation method (Cury et al., 2008), associated with a mechanical cell rupture by employing glass beads (Yin et al., 2001; Alonso et al., 2018; Jordão et al., 2020; Jordão et al., 2021). The resulting pellet was resuspended in 100 µL of H_2O and kept on ice for 1 hour for hydration. Subsequently, RNA purification was conducted with DNase on column (RNeasy Micro Kit, Qiagen, Hilden, Germany) and solution (Turbo DNase; Ambion, Austin, TX, USA) (Cury et al. 2008).

RNA integrity was evaluated using agarose gel electrophoresis (1% agarose containing 0.3 µg/mL ethidium bromide), and images were captured using a gel-doc system (Bio-Rad, Laboratories, Hercules, CA, USA). The RNA yield and purity were assessed using a nanospectrophotometer (OD260nm and OD260/280 ratio) (DS-11, DeNovix, Wilmington, DE, USA).

For cDNA synthesis, 1 µg of RNA was utilized for reverse transcription with the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Alfred Nobel Drive, Hercules, CA, USA) following the manufacturer's instructions. The presence of DNA contamination

was assessed using negative controls. Post-procedure, all samples were stored at -20°C. RT-qPCR analysis was conducted for the genes outlined in table 2.

Table 2. Characteristics of virulence genes used to analyze gene expression in *C. albicans* and *S. aureus*

Microorganisms	Gene	Gene description	*Primers*	References
C. albicans	ALS1	Agglutinin-Like Sequence 1 (Agglutinin), encodes cell wall proteins required for adhesion during the early phase of biofilm formation.	F – CATCATTGACTCAGTTGT R – CAGTGGAAGTAGATTGTG	Alonso et al., 2018
	CAP1	CAP1 (Adenylate cyclase-associated protein), is accountable for triggering oxidative stress genes in *C. albicans*. Once activated, it stimulates the transcription of numerous antioxidant genes.	F – GACTGCTTACATTCAAAC R – AACTTACCAAATCTTCTCA	Jordão et al., 2021
	HPW1	The hyphal wall protein plays a role in *C. albicans'* adhesion to epithelial cells, acting as a substrate for transglutaminase. It mediates the attachment of *C. albicans* to epithelial cells and holds significance in the pathogenesis process.	F– CTCCAAAATCATCAGCTC R– CACTAGCCAAAACAGAAG	Jordão et al., 2021
	CAT1	Catalase, which acts as an oxidative stress gene, is one of the transcription factors that significantly contribute to the virulence of *C. albicans.*	F – GACTGCTTACATTCAAAC R – AACTTACCAAATCTTCTCA	Alonso et al., 2018
	SOD 1	Superoxide dismutase 1, contributes to the virulence of *C. albicans* by expressing proteins against oxidative stress.	F – TTGAACAAGAATCCGAATCC R – AGCCAATGACACCACAAGCAG	Zhu et al., 2011
S. aureus	fnbA	The fibronectin-binding protein A encodes a protein located in the *S. aureus* cell wall and responsible for binding to host ligands present in extracellular matrix of tissues. The protein is essential for microbial cell adhesion	F – GATACAAACCCAGGTGGTGG R – TGTGCTTGACCATGCTCTTC	Montanaro et al., 1999
	clfA	Adhesin clumping factor (cflA) encodes a surface adhesin protein responsible to bind to host tissue	F – GATTCTGACCCAGGTTCAGA R– CTGTATCTGGTAATGGTTCTTT	Acosta et al., 2018
	hla	α-hemolysin, a pore-forming toxin, is identified as a key virulence factor in *S. aureus*. This toxin could lyse macrophages, red blood cells, and lymphocytes, leading to their death. Its role is crucial in promoting and advancing infections.	F–CTGATTACTATCCAAGAAATTCGATTG R– CTTTCCAGCCTACTTTTTTATCAGT	Jarraud et al., 2002

2.4 Statistical analysis

The data were analyzed using the IBM SPSS version 20.0 program. Firstly, a descriptive analysis of the all data was carried out followed by evaluation of normal distribution and homoscedasticity using the Shapiro-Wilk test ($p>0.05$) and Levene test ($p>0.05$). Outliers were not detected. Then, based on the number of independent factors, either a three or two-factor Analysis of Variance test was performed. In the presence of values equal to zero in a group, these groups were not included in the parametric inferential analysis. However, these groups were added to the figures.

For the analysis of the effect of aPDT against *C. albicans* and *S. aureus* cultured in suspension, as well as for XTT assay, cytotoxicity assessment and qRT-PCR, two-factor Analysis of Variance (two-way ANOVA) was conducted. The presence and absence of light and the type of photosensitizer used were the independent factors.

For the analysis of the effect of aPDT against *C. albicans* and *S. aureus* cultured in biofilm, three-factor Analysis of Variance (three-way ANOVA) was conducted. In this scenario, the type of photosensitizing agent, the concentration of the photosensitizing agent and the number of applications (one or three successive applications) were the independent factors evaluated. Since the exposure to the light was significantly in suspension assays and therefore confirming the interaction between the photosensitizer and the light, for analysis of biofilm, we did not include presence and absence of light as independent factors. As consequence, the group P+L- was not included in the analysis. The comparative analysis was carried out by means estimation with a 95% confidence interval and for the results from qRT-PCR Tukey’s test for multiple comparison was conducted. The results from CLSM were descriptive.

3. RESULTS

3.1 aPDT against C. albicans and S. aureus grown in suspension.

Statistically significant differences were observed for the interaction between all independent factors ($p<0.0001$). The results for the application of aPDT mediated by different photosensitizing agents in suspension phase provided total reduction in microbial cell viability (5.30 log CFU/mL for *C. albicans*

and 6.60 log CFU/mL for *S. aureus*) at the two concentrations evaluated (Figure 1). Both concentrations of each photosensitizing agents showed no statistically significant difference between them when exposed to light. Furthermore, the concentrations of photosensitizing agents in the absence of light also did not show antimicrobial activity (Figure 1).

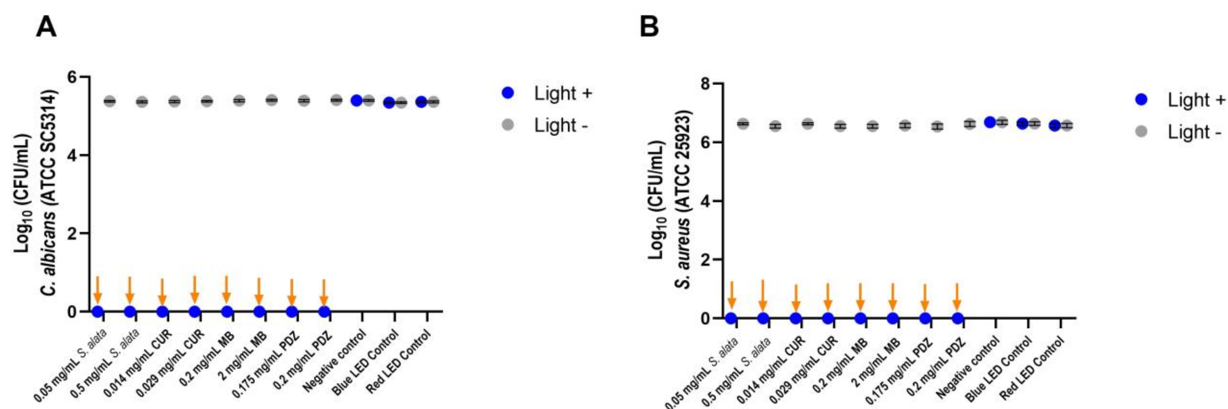

Figure 1. Effect of Photodynamic Therapy on suspensions of *C. albicans* (Figure 1A) and *S. aureus* (Figure 1B). Two-way ANOVA and mean ± confidence interval (95%) for multiple comparations (n=12). Statistically significant difference for association between independent factors (light + photosensitizing agent) $p<0.0001$. Orange arrows demonstrate total microbial reduction.

3.2 aPDT against C. albicans and S. aureus grown in biofilm.

For aPDT against *C. albicans* and *S. aureus* grown in biofilm, statistically significant differences were observed for the interaction between all independent factors ($p<0.0001$).

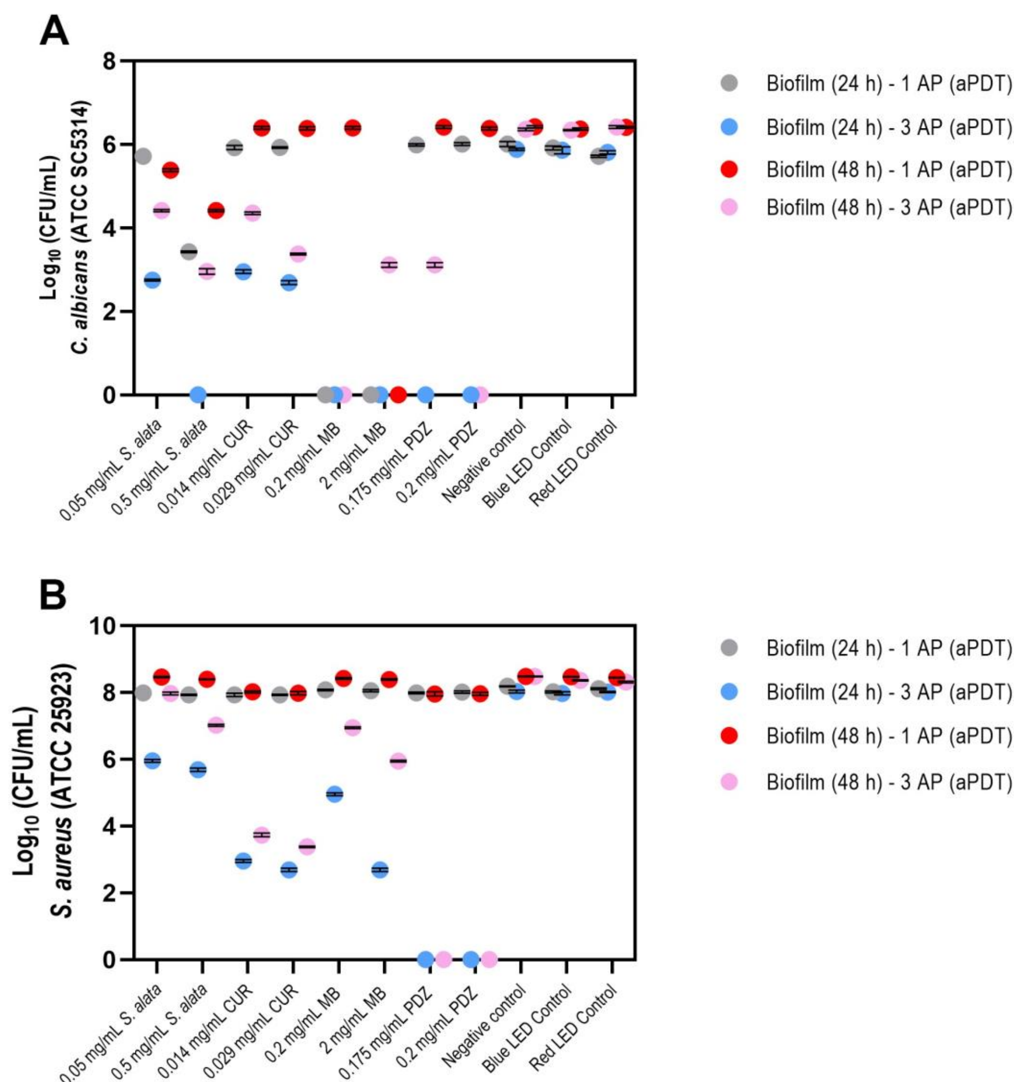

Figura 2. Effect of antimicrobial photodynamic therapy on 24 and 48-h biofilms of *C. albicans* (A) and *S. aureus* (B). Three-way ANOVA and mean ± confidence interval (95%) for multiple comparations (n=12). Statistically significant difference for association between independent factors (photosensitizing agent, photosensitizing agent concentration and number of therapy applications) $p<0.0001$. 1AP: one application; 3AP: three successive applications.

The results obtained with one application of aPDT mediated by different photosensitizing agents after 24 and 48 h of biofilm formation demonstrated no reduction in cell viability (Figure 2), except for *C. albicans* biofilm after 24 hours of formation. Under this growth condition, therapy with 0.5 mg/mL *S. alata* resulted in a reduction of 3.6 log CFU/mL compared to the negative control.

However, with three successive applications, a significant reduction in microbial viability for both microorganisms evaluated and for all photosensitizing

agents applied was noted. The total reduction in microbial viability was particularly notable for the highest concentrations of each photosensitizing agent.

3.3 Confocal laser scanning microscopy.

Figures 3 and 4 display composite images overlaying green fluorescence (SYTO 9) with red fluorescence (PI) across all experimental conditions for *C. albicans* and *S. aureus* biofilms, respectively.

In Figures 3 and 4, for the control groups without treatment (A and A') and light-only control (F and F'), all colonies remained green, indicating that the cells for both biofilms were viable during the experimental period, with either one or three light applications. When evaluating the groups with one and three applications of aPDT, the groups with three successive applications showed a greater number of colonies stained red or orange, especially with the higher concentration of photosensitizing agents (Figures 3 and 4).

After one application of aPDT for *C. albicans* (Figure 3), a visual reduction of biomass was observed for 0.2 and 2.0 mg/mL MB-mediated aPDT. In contrast, there was no reduction of the biomass with only sparse dead cells observed for 80 mM CUR and 5 mg/mL *S. alata*-mediated aPDT. After three successive applications of aPDT, there was a reduction in biomass with the presence of dead cells for CUR (40 and 80 mM), *S. alata* (5 mg/mL), MB (0.2 and 2 mg/mL) and PDZ (0.175 and 0.2 mg/mL)-mediated aPDT.

After one application of aPDT, for *S. aureus* (Figure 4), there was a biomass reduction for MB (0.2 and 2 mg/mL) and PDZ (0.175 and 0.2 mg/mL)-mediated aPDT. After the successive applications of aPDT, there was a reduction in biomass observed for *S. alata* (0.2 and 2 mg/mL)-mediated aPDT. Additionally, a biomass reduction with presence of microbial dead cells was noted for CUR (0.014 and 0.029 mg/mL), MB (0.2 and 2 mg/mL) and PDZ (0.175 and 0.2 mg/mL)-mediated aPDT).

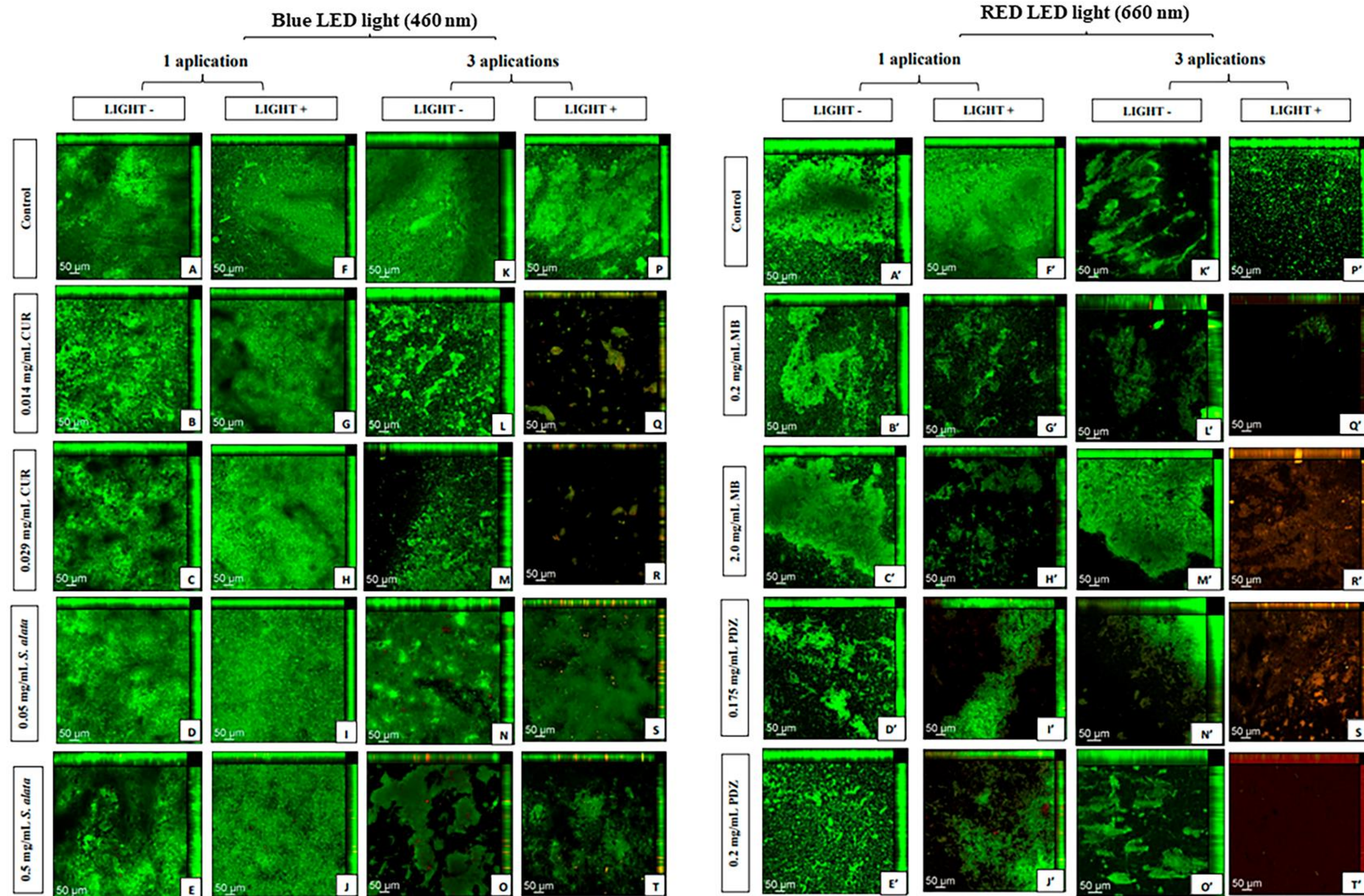

Figure 3. Overview of *C. albicans* biofilm. A-J and A'- J': Groups with 1 application without (A-E and A'-E') or with (F-J and F'-J') light irradiation. A and A': control without treatment; B and G: 0.014 mg/mL curcumin; C and H: 0.029 mg/mL curcumin; D and I: 0.05 mg/mL *S. alata* extract; E and J: 0.5 mg/mL *S. alata* extract; B' and G': 0.2 mg/mL Methylene blue; C' and H': 2 mg/mL Methylene blue; D' and I': 0.175mg/mL PDZ; E' and J': 0.2 mg/mL PDZ. F and F': group exposed to light only; K-T and K'-T': Groups with 3 applications without (K-O and K'-O') or with (P-T and P'-T') light irradiation. K and K': control without treatment; L and O: 0.014 mg/mL curcumin; L' and O' : Methylene blue 0.2 mg/mL; M and R: 0.0.29 mg/mL curcumin; M' and R': Methylene blue 2 mg/mL; N and S: 0.05 mg/mL *S. alata* extract; N' and S': 0.175 mg/mL PDZ; O and T: 0.5 mg/mL *S. alata* extract; O' and T': 0.2 mg/mL PDZ. The predominance of green fluorescence of SYTO 9 indicates viable fungi cells with 1 application of therapy. In images with 3 applications, the presence of red fluorescence indicates dead cells. Original magnification: ×10.

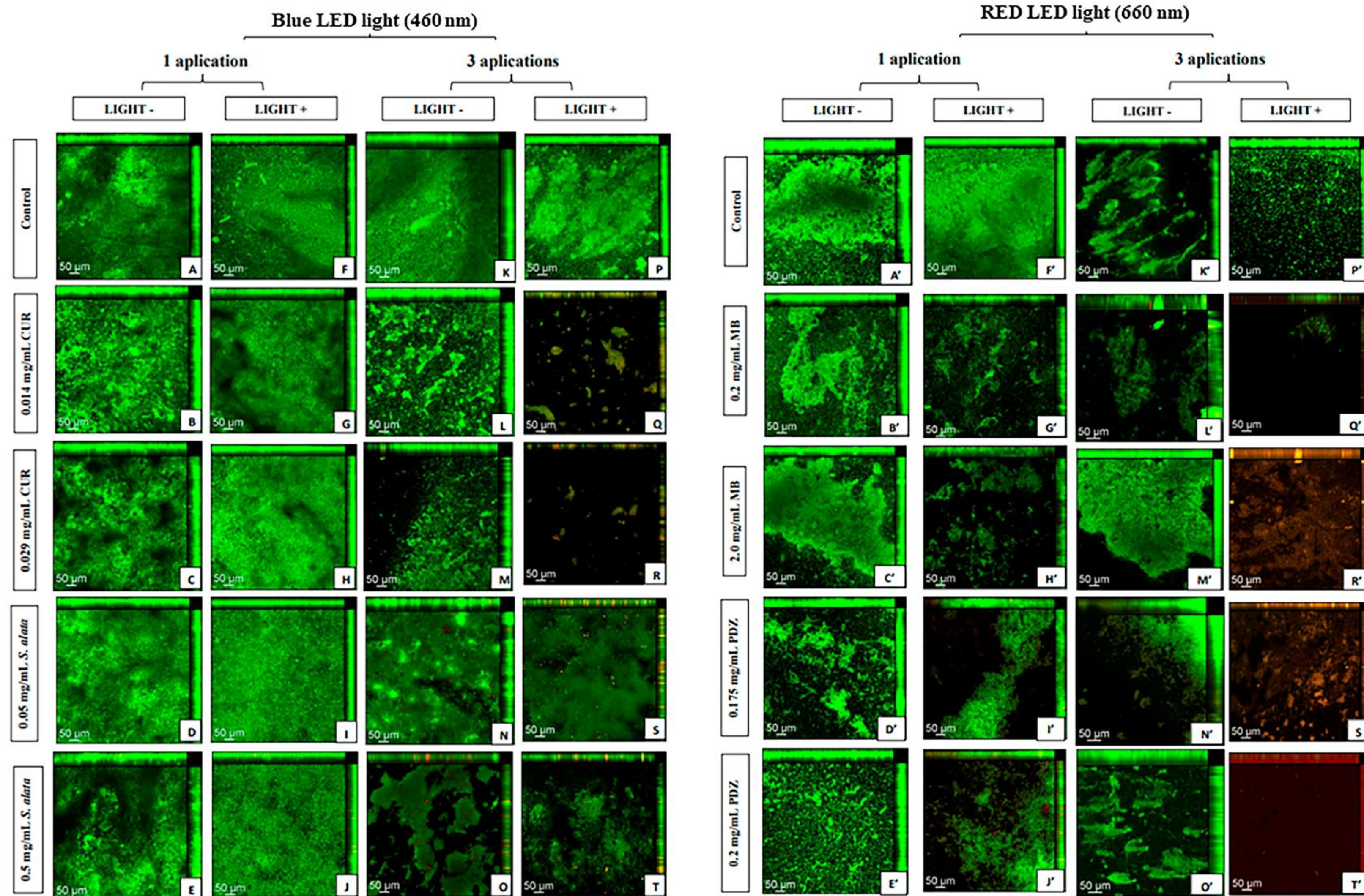

Figure 4. Overview of *S. aureus* biofilm. A-J and A'- J': Groups with 1 application without (A-E and A'-E') or with (F-J and F'-J') light irradiation. A and A': control without treatment; B and G: 0.014 mg/mL curcumin; C and H: 0.029 mg/mL curcumin; D and I: 0.05 mg/mL *S. alata* extract; E and J: 0.5 mg/mL *S. alata* extract; B' and G': 0.2 mg/mL Methylene blue; C' and H': 2 mg/mL Methylene blue; D' and I': 0.175mg/mL PDZ; E' and J': 0.2 mg/mL PDZ. F and F': group exposed to light only; K-T and K'-T': Groups with 3 applications without (K-O and K'-O') or with (P-T and P'-T') light irradiation. K and K': control without treatment; L and O: 0.014 mg/mL curcumin; L' and O' : Methylene blue 0.2 mg/mL; M and R: 0.0.29 mg/mL curcumin; M' and R': Methylene blue 2 mg/mL; N and S: 0.05 mg/mL *S. alata* extract; N' and S': 0.175 mg/mL PDZ; O and T: 0.5 mg/mL *S. alata* extract; O' and T': 0.2 mg/mL PDZ. The predominance of green fluorescence of SYTO 9 indicates viable bacterial cells with 1 application of therapy. In images with 3 applications, the presence of red fluorescence indicates dead cells. Original magnification: ×10.

3.4 Metabolic activity of biofilms (XTT assay)

Figure 5 depicts the metabolic activity values of *S. aureus* (Figure 5A) and *C. albicans* (Figure 5B) biofilms after 24 hours of cultivation. Compared to the negative control group (no treatment), the four photosensitizing agents showed a decrease in the absorbance values of *S. aureus* and *C. albicans* when exposed to light (aPDT), with the reduction depending on the concentration of the photosensitizing agent. The untreated groups with 100% metabolic activity (Figure 5) showed a significant decrease in metabolism for *C. albicans* biofilms subjected to aPDT, ranging from 63.54% to 91.18% depending on the photosensitizing agent concentration. Similarly, there were notable reductions in the viability of *S. aureus* biofilms subjected to aPDT (11.72% – 87.46%), with greater reductions observed at higher concentrations of photosensitizing agents.

Importantly, *S. aureus* biofilms tend to have their metabolism less affected by aPDT compared to *C. albicans* biofilms under all treatment conditions. For both biofilms, the two tested concentrations of the photosensitive agent methylene blue had the potential to provoke a significant decrease in the cellular metabolism of both microbial species compared to the untreated group. The highest concentration of curcumin decreased biofilm metabolism by values greater than 75% for both *S. aureus* and *C. albicans* biofilms (Figure 5).

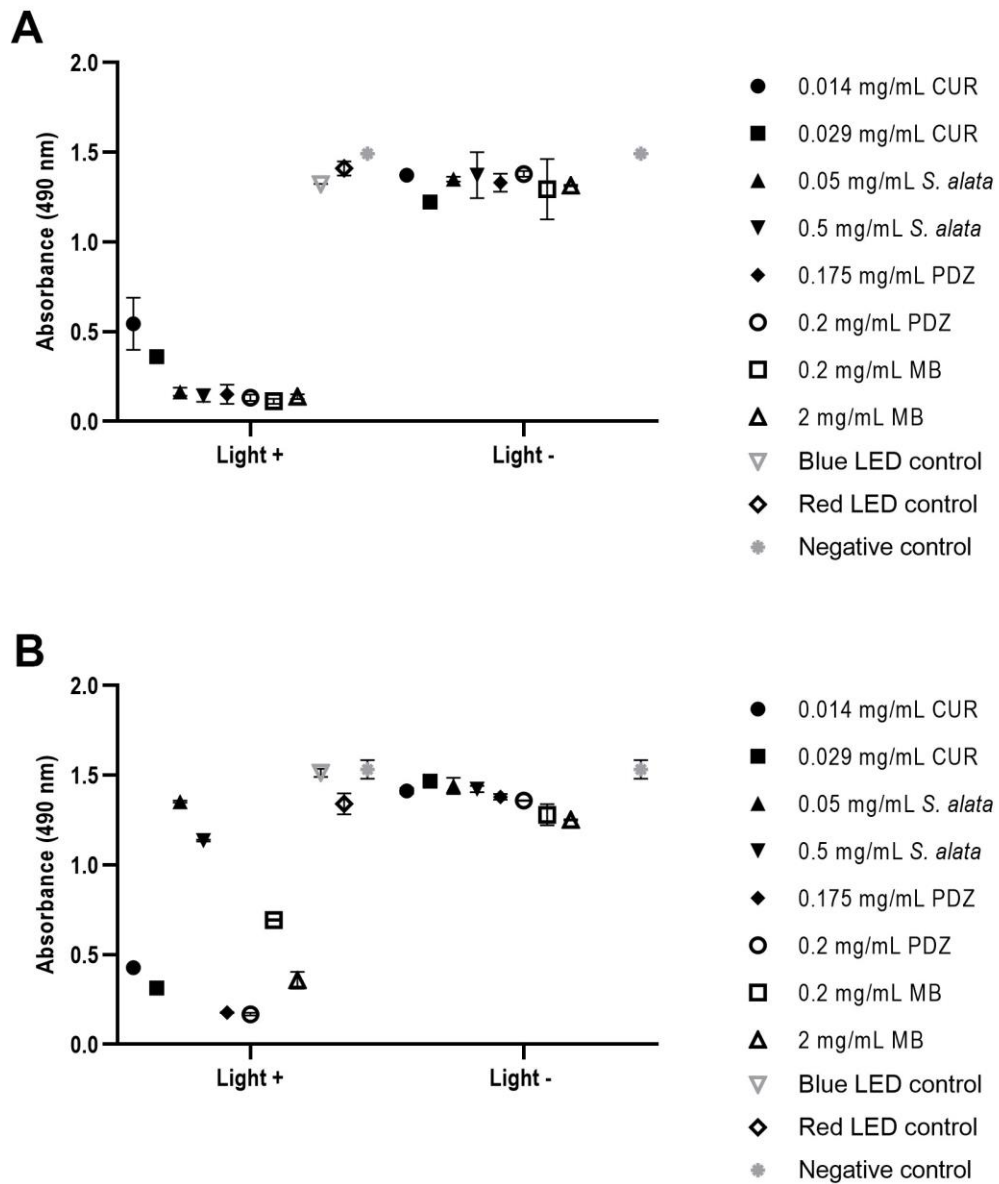

Figure 5. Results of the metabolic activity test (XTT reduction assay) for *S. aureus* (A) and *C. albicans* (B) biofilms cultured in 24 h. Statistically significant difference for association between independent factors $p<0.0001$ (presence and absence of light and photosensitizing agents) (ANOVA for two independent factors; N=12). Mean graph ± confidence interval (95%). Light + = application of light; Light- = absence of light application.

3.5 Evaluation of the cytotoxicity of irradiation parameters and concentration of photosensitizing agents in cell cultures of gingival fibroblasts in monolayer.

After the cytotoxicity assay (MTT) we observed a significant difference among the experimental groups and both live and dead control groups (Figure 6). In addition, the experimental groups showed a viability rate above 70% for the two cell lines tested (NOK-si and FGH).

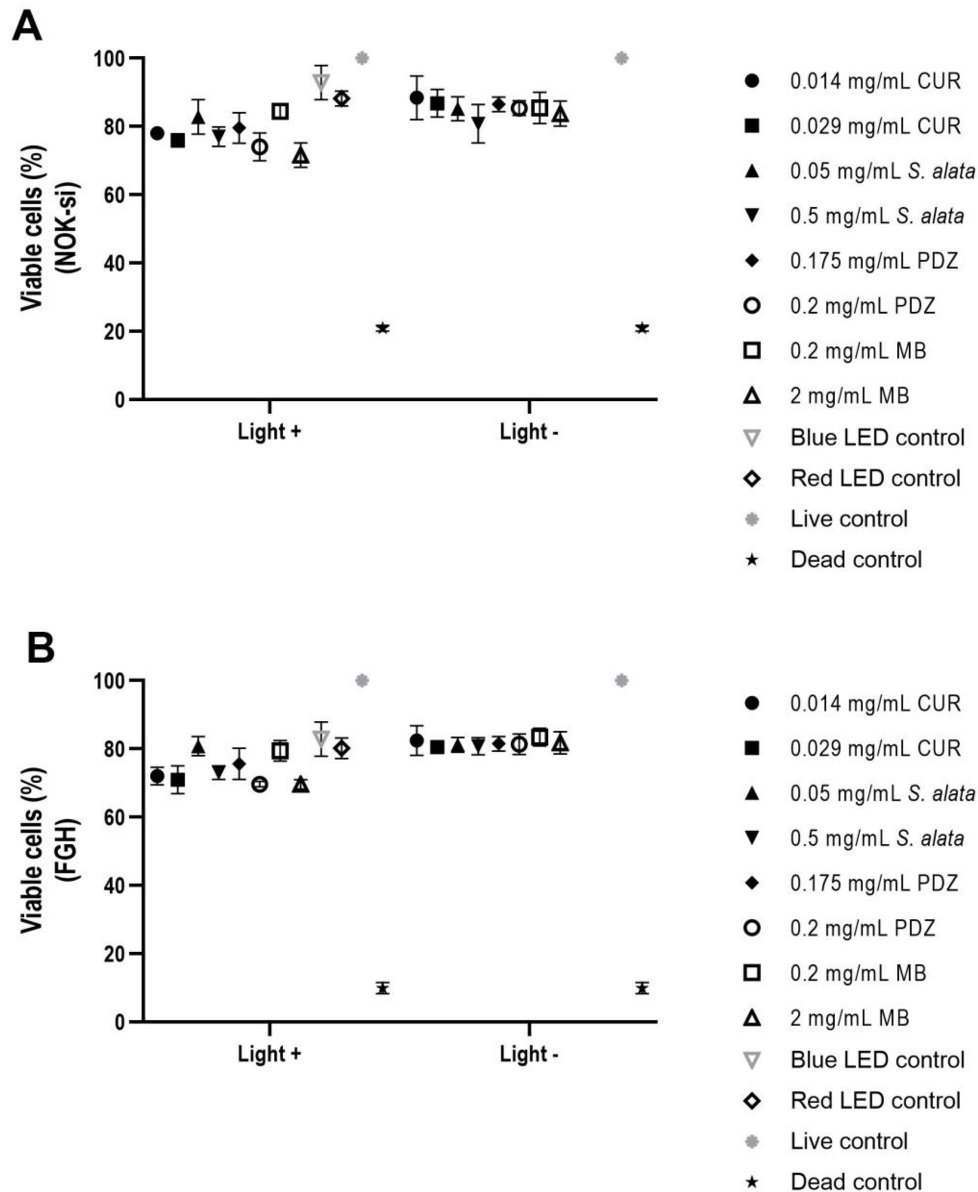

Figure 6. Results of cellular cytotoxicity test (MTT assay) for NOK-si (A) and FHG (B). Statistically significant difference for association between independent factors $p<0.0001$ (presence and absence of light and photosensitizing agents) (ANOVA with two independent factors; n=12). Mean graph ± confidence interval (95%). L+ = application of light; L- = absence of light application.

3.6 RT-qPCR Analysis

Due to total reduction of *C. albicans* in both concentrations of PDZ and MB-mediated aPDT and 0.5 mg/mL *S. alata*-mediated aPDT and for *S. aureus* cells after PDZ-mediated aPDT, only viable cells treated with other groups were collected, and gene expression were quantified. Interactions between independent factors (presence and absence of light) were significant in all variance analysis performed ($p<0.0001$) and at least one photosensitizer reduced genes expression level.

For *C. albicans,* significant reductions in *ALS1*, *CAP1*, *HWP1* and *CAT1* were noted after the treatment with 0.5 mg/mL *S. alata*-mediated aPDT compared to the negative control and blue LED control. In addition, SOD1 was only significantly reduced by 0.5 mg/mL *S. alata*-mediated aPDT. CUR-mediated aPDT significantly reduced the levels of *ALS1* (40 and 80 mM), *CAP1* (80 mM), *HWP1* (40 and 80 mM) and CAT1 (40 and 80 mM) compared to the negative control and blue LED control. Gene expression reductions were significantly more pronounced in 80 mM CUR-mediated aPDT than in 0.05 and 0.5 mg/mL *S. alata*-mediated aPDT for *ALS1* and *CAT1*. On the other hand, for *SOD1* and *CAP1*, the gene expression reductions were more significant in 0.5 mg/mL *S. alata-mediated* aPDT than 40- and 80-mM CUR-mediated aPDT. For *HWP1* gene, both photosensitizers reached the same reductions levels when exposed to light (Figure 7).

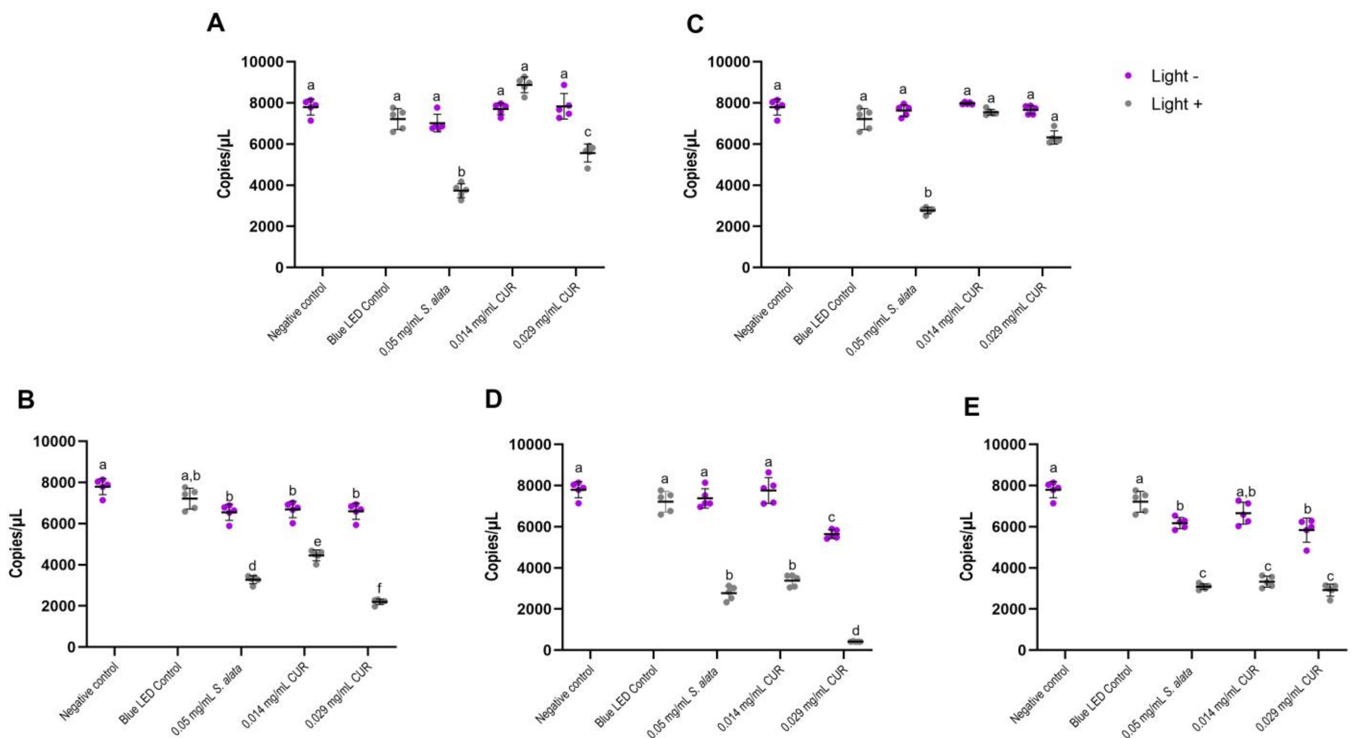

Figure 7. Results from *C. albicans* gene expression in different groups in presence (Light +) or absence of light (Light -). **A**: *CAP1* levels; **B**: *CAT1* levels; **C**: *SOD1* levels; **D**: *ALS1* levels; **E**: *HWP1* levels. Two-way analysis of variance with Tukey`s test for multiple comparisons. Different letters mean statistical significance (n=5).

For *S. aureus,* all photosensitizers evaluated (CUR, *S. alata*, and MB) exhibited significant reductions in gene expressions. The most effective results for *fnbA* and *clfA* were observed with 2.0 mg/mL MB-mediated aPDT, followed by 0.5 mg/mL *S. alata*-mediated aPDT and 80 mM CUR-mediated aPDT. Additionally, 0.2 mg/mL MB-mediated aPDT also demonstrated a significant reduction for *clfA* compared to the negative control and blue and red LED control. For *HLA* gene, the lowest expressions were similarly reached with 2 mg/mL MB-mediated aPDT and 0.5 mg/mL *S. alata*-mediated aPDT (Figure 8).

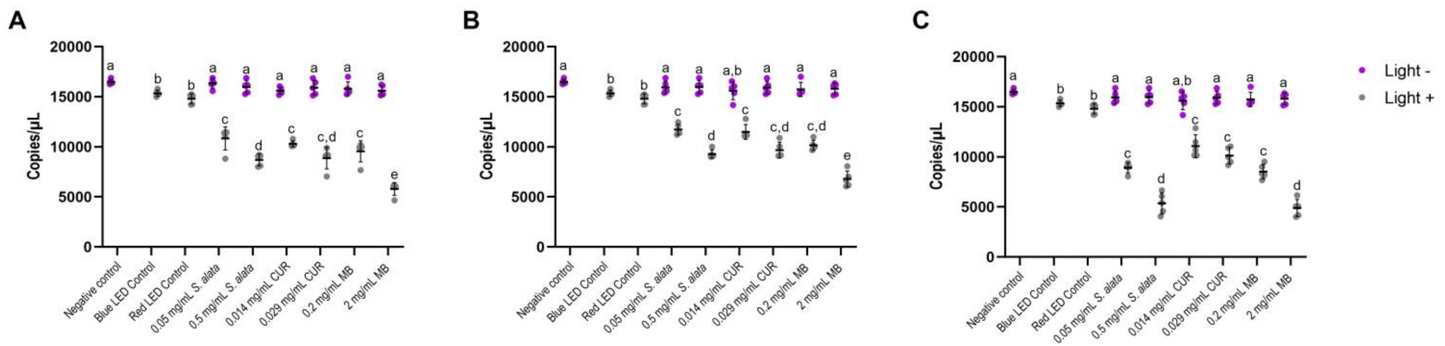

Figure 8. Results from *S. aureus* gene expression in different groups in presence (Light +) and absence of light (Light -). **A**: *fnbA* levels; **B**: *cflA* levels; **C**: *hla* levels. Two-way analysis of variance with Tukey`s test for multiple comparisons. Different letters mean statistical significance of confidence interval (n=5).

4. DISCUSSION

S. aureus and *C. albicans* are opportunistic pathogens that can cause severe and invasive infections in humans. While traditional antibiotic and antifungal treatments can be effective against these microbes, the rise of drug-resistant strains has led to treatment failures and increased mortality rates (McEwen and Collignon, 2018). Developing new antibiotics is a complex and costly process (Brad Spellber, 2014). aPDT has emerged as a potential therapeutic option for diseases caused by microorganisms, aiming to reduce the side effects associated with conventional therapies (Kashef and Hamblin, 2017). To the extent of our knowledge, this is the first *in vitro* study to assess multiple applications of aPDT with a lethal dose mediated by *S. alata* in the planktonic phase and monospecies biofilms of *C. albicans* and *S. aureus*.

The results of the present study demonstrate that both the marketed photosensitizers and the experimental photosensitizer from *S. alata* extract have achieved a desirable property of photosensitizer agents: they exhibit biological activity only when irradiated by light. Moreover, aPDT promoted a total reduction for all microorganisms cultured in suspension. In biofilms, the majority of photosensitizing agents in aPDT promoted a microbial reduction greater than 3 log CFU/mL for both

microorganisms. These results are positive and provide valuable insights for the design of further *in vivo* studies and clinical trials.

48-h biofilms were less susceptible to aPDT than 24-h biofilms (Figure 2), regardless of the microbial species, type of photosensitizing agent and number of therapy applications. Similar results were found comparing phenothiazinium dyes-mediated aPDT with toluidine blue, rose Bengal and MB-mediated aPDT against *C. albicans* biofilms (Bapat et al., 2021). However, corresponding findings regarding *S. aureus* biofilm have not yet been reported in the literature. 24 h-biofilms are in the maturation phase (Machado et al., 2023), which brings the greatest architectural stability. This phase is characterized by EPS production, which enhances resistance against antimicrobial agents (de Barros et al., 2017, Warrier et al., 2021; Songca and Adjei, 2022). Additionally, it creates a proper environment for genetic exchange, retention, and protection of exoenzymes and stabilization of environment DNA (Flemming et al., 2023). These features of biofilms explain the increased susceptibility of *C. albicans* and *S. aureus* in suspension to oxidative damage (Figure 1). Comparable findings have been reported previously (Dovigo et al., 2011; de Oliveira et al., 2023; Chan et al., 2023). Thus, biofilms in early stages possess fragile structures, high metabolism rates, and early adhered microorganisms that are more susceptible to antimicrobial therapies (Fu et al., 2021). Therefore, timely intervention in biofilms can lead to improved prognoses.

The results from microorganisms cultured in biofilm demonstrated that three applications of aPDT resulted in a greater reduction of microbial viability (Figure 2) and cellular metabolism (Figure 5), which agrees with the study of Quishida et al. (2015). The higher efficiency found after three successive aPDT applications is related to weakening biofilm surface, making it more susceptible to treatment (Figueiredo et al., 2017). However, Quishida et al. (2015) did not achieve the same level of antimicrobial inactivation reported in the present study. These conflicting findings may be related to different substrates used for biofilm growth and to the fact that Quishida et al. (2015) used a multispecies biofilm, which is known for its increased resistance (Prabhukhot et al., 2023). Our results can guide clinical applications in infections, reinforcing the use successive applications of aPDT to reach greater reduction of microbial load.

Despite the decrease in *S. aureus* viability following aPDT, this reduction was less pronounced than that found for *C. albicans* (Figure 2). A possible explanation for this outcome is that *S. aureus* tends to aggregate during the initial exponential growth phase in suspension cultures. By the time they reach the stationary phase, more than 90% of the population becomes attached to aggregates (Kragh et al., 2023). These aggregates can have a profound impact on growth rate and on biofilm formation, consequently affecting susceptibility to treatments in in vitro experiments (Kragh et al., 2023).

XTT assay results (Figure 5) showed that, regardless of the number of applications, aPDT-groups demonstrated reduced cellular metabolism compared to the control groups. These results are in line with those found in the literature (Quishida et al., 2015; Dovigo et al., 2011; Dovigo et al., 2013; Chan et al., 2023). This suggests a possible reduction in functions, mechanical stability and in the production of macromolecules metabolites, potentially resulting in a reduction in biofilm virulence factors and increased susceptibility to antimicrobial treatments (Flemming et al., 2022). While metabolic activity results agreed with viability assay findings, it is important to note that the XTT assay may present restrictions, particularly in mature biofilms, as its absorption is limited to the outer layer of the biofilm (Xie et al., 2011), which might not always correlate with other tests (Brighenti et al., 2014). This reinforces the importance of employing a wide variety of assays to ensure reliable results, especially when evaluating antimicrobial action of new photosensitizers (da Collina et al., 2022), such as *S. alata*.

The CLSM analysis contributed to a better understanding of the mechanisms underlying the PS evaluated in the present study, particularly distinguishing in presence and absence of dead cells and biomass reduction (Figure 3 and Figure 4). The images confirm the antimicrobial activity already demonstrated by the metabolic and viability assays, indicating the efficacy of this in disrupting the microbial membrane and leading microbial death. The findings reported in the literature about CLSM outcomes in aPDT are controversial. While Teixeira et al., (2020) observed similar results for CUR-mediated aPDT, Wang et al., (2022) found that *C. albicans* biofilms treated with 2,6-diiodo-1,3,5,7-tetramethyl BODIPY-mediated aPDT resulted in a greater number of dead cells without a reduction in biomass (Wang et al., 2022).

In cytotoxicity tests (Figure 6), the aPDT groups showed viability rates above 70% for the two cell lines tested, which agrees with Kashef et al., (2012) who observed similar results with MB and toluidine blue O-mediated aPDT. However, it is important to mention that oxidative damage can persist within the cell, leading to delayed cell death (Kamiloglu et al., 2020). Hence, it is possible that our viability rates may decrease after 24 h of aPDT treatment (Kamiloglu et al., 2020). In addition, caution must be taken when analyzing these results, as the toxic effects of PSs can also vary not only when exposed to the light (P+L+), but also according to PS concentration (P+L-), solvent (vehicle control), light application (P-L+) cell type used to access cytotoxicity effects and exposure time of the treatment (Pavani et al., 2009). and not necessary only after aPDT treatment. Moreover, there are a natural and *in situ* release of antioxidants agents like peroxidases and catalases by the target cells after the oxidative stress. These agents facilitate the enzymatic breakdown of highly reactive and unstable molecules, preventing excessive tissue damage (Esposito et al., 2003). In the present study, the cytotoxicity assay was conducted only immediately after the aPDT treatment, requiring further investigation into the delayed effects of aPDT mediated by the evaluated PSs with the defined application protocols.

Despite favorable results of the cytotoxicity assay, which offer valuable insights in pre-clinical models, caution is advised in their interpretation as cell monolayer studies are limited for predicting human outcomes (Delben et al., 2016; Svobodová et al., 2021). As an example of this limitation, studies have demonstrated that 0.12% chlorhexidine, commonly used in dentistry without serious toxicity reports, exhibits cytotoxicity when evaluated using this model (Albuquerque et al., 2018).

C. albicans oxidative stress genes were differentially downregulated depending on PS used. Notably, *S. alata*-mediated aPDT reduced either *SOD1* or *CAP1* and *CAT1* levels, depending on the concentration used. While literature presents conflicting views on the expression of these genes following PDZ-mediated aPDT (Jordão et al., 2020; Jordão et al., 2021), the findings of the present study highlight an important mechanism of action of *S. alata*-mediated aPDT against oxidative stress-induced cell tolerance. This suggests that surviving *C. albicans* cells after *S. alata*-mediated aPDT may lack such tolerance phenotypes and will not activate this cell protection mechanism (Dantas et al., 2015).

The importance of *S. alata*-mediated aPDT in reducing SOD1 levels in *C. albicans'* after treatment is strengthened by the fact that *C. albicans* double mutant Δgrx2/sod1 is highly susceptible to neutrophil killing and fails to form hyphae, greatly decreasing the yeast's capability to tissue invasion (Chaves et al., 2012). In other words, despite *S. alata*-mediated aPDT did not completely eliminate all *C. albicans* cells cultured in biofilm (Figure 2), the surviving cells exhibit lower virulence factors, thereby increasing *C. albicans* susceptibility to the host's immune system. The downregulation of SOD1 levels found in the present study highlights as an advantage of *S. alata*-mediated aPDT over the other PS evaluated.

Since aPDT is based of oxidative damage, it is crucial to search photosensitizers able to reduce the level of *SOD1*, *CAP1* and *CAT1*. In the present study, *S. alata*-mediated aPDT was the only PS that significantly reduces the level of these three genes. This suggests a remarkable mechanism of action of *S. alata*-mediated aPDT which downregulate three different pathways related to resistance of oxidative stress in *C. albicans* (Zhu et al., 2011; Alonso et al., 2018; Jordão et al., 2021). However, for the other PS evaluated in the present study, opposite results were found for expression of *CAT1* after PDZ-mediated aPDT (Jordão et al., 2021) and similar results was found *SOD1* and *CAP1* genes after PDZ-mediated aPDT (Jordão et al., 2020; Jordão et al., 2021). Differences in photosensitizer`s concentrations and study design can explain the opposite results.

In addition, *S. alata* and CUR-mediated aPDT significantly reduces *ALS1* and *HWP1* genes expression in *C. albicans* surviving cells. These results indicate a potential reduction in initial adhesion in the substrate and, consequently, in biofilm development (Nobile et al., 2008; Finkel, 2011 and Nailis et al., 2010). Moreover, as HWP1 and ALS1 can have their expression directly correlated, in most situations these genes can show similar expression profile (Nobile et al., 2008). This phenomenon occurred because HWP1 can induce the expression of ALS1 (Nobile et al., 2008). Thus, the reduction of these genes may indicate that surviving cells after aPDT treatment in *C. albicans* biofilm will have reduced capability to adhere to substrates and to form biofilms, thereby hindering the establishment and formation of future biofilms. Similar results were found in the literature using the same light doses and CUR concentration (Jordão et al., 2021), which strengthen the findings of aPDT in

modulation genes expression of *C. albicans* related to fungal adhesion to surfaces and biofilm development.

To the best of our knowledge this is the first scientific report evaluating the modulation capabilities of aPDT in expression of virulent genes of *S. aureus.* In this context, significant downregulations of *fnbA* and *cflA* genes can indicate a decreased capability of *S. aureus* to adhere on tissues (Que et al., 2001; Casillas-Ituarte et al., 2017). Therefore, achieving such downregulation is an important result to antimicrobial approach. In the present study, with exception of PDZ, all other PS showed, included *S. alata* showed downregulation in *fnbA* and *cflA* gene expression. Previous studies using antibacterial compound, such as a novel oxazolidinone or the association of peptide with methicillin can significantly reduce the expression of either *fnbA* or *cflA* in *S. aureus* (Wang et al., 2023; Belguesmia et al., 2021).

S. alata-mediated aPDT was the only natural PS that downregulates *hla* gene expression in the present study. This gene is considered the major virulence factor in *S. aureus* and is responsible for promoting infection and host tissue invasion (Bhakdi et al., 1991; Valeva et al., 1997). This reduction may indicate improved resolution of *S. aureus* infections, especially methicillin- and vancomycin-resistant *S. aureus*, which can express higher levels of *hla* (Verdú-Expósito et al., 2020; Abd El-Hamid et al., 2024). Thus, *S. alata*-mediated aPDT can further be tested in pre-clinical studies such as more robust cell culture experimental models and animal models. Subsequently, it can be considered for clinical trials as a potential treatment against *S. aureus* infections. Treatment options for *S. aureus* are of high priority, as emphasized by the Word Health Organization (OMS) (Tacconelli et al., 2018). MB-mediated aPDT also showed significant results in downregulating *hla* gene expression. Similar outcomes were found with the treatment of *S. aureus* using andrographolide, a terpenoid compound that showed to be capable to reduce the levels of *hla, fnbA* and *cflA* genes (Wang et al., 2022).

S. alata showed promising results against *C. albicans* and *S. aureus* cultured in both suspension and monospecies biofilm. It exhibited a good biocompatibility, reduced biofilm metabolic activity, and biomass, and significantly reduced genes expression related to virulence factors of *C. albicans* and *S. aureus*. *S. alata* photodynamic mechanism is probably associated with the presence of aphthopyrone and anthraquinones and its mechanisms of action is characterized by the high amount

$_1OH$ radical that can act on carbohydrates, lipids, protein, and nucleic acids causing cells death (de Oliveira et al., 2023). This characteristic differentiates *S. alata* from other PS such as MB, PDZ and CUR, which produce high concentration of singlet oxygen (Abrahamse & Hamblin, 2016; Dias et al., 2020; Sampaio et al., 2020). The $_1OH$ radical is the most harmful and damaging reactive oxygen species because organisms exposed to it cannot develop mechanisms to inhibit its action, primarily due to its extremely short half-life (Drzeżdżon et al., 2018; Gligorovski et al., 2018).

As *S. alata* is a crude extract with a complex composition, it is hard to identify all the chemical components present in its constitution, as well as to identify the specific photoactive molecule responsible for the photodynamic action evaluated here. These complexities represent the disadvantages of using *S alata* in aPDT. In addition, the challenges related to *S. alala* obtaining *S. alata*, as well as extract preparation and possible changes in the composition cross different batches need to be consider as potential disadvantages. Our research group is currently conducting a study aiming at obtaining the enriched fraction of *S. alata* and identifying the molecules responsible for the observed aPDT effects. In addition, it is essential to assess heat release during oxidative damage caused by aPDT to confirm *S. alata* as a genuine photosensitizer agent.

In summary, 0.5 mg/mL *S.alata*-mediated aPDT showed promising results for reduction of microbial viability, decreasing microbial metabolism and downregulating essential genes related to the virulence of both *C. albicans* and *S. aureus*. Therefore, clinical trials evaluating the efficacy of *S. alata*-mediated aPDT in conditions where these microorganisms play a central role should be designed.

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3.3 Publicação 3*

Photodynamic therapy mediated by *Senna alata* plant in a three-dimensional oral mucosa model infected.

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* O artigo segue as normas do periódico *Oral diseases* para o qual pretende-se submeter.

ABSTRACT

This study proposed to evaluate the antimicrobial Photodynamic Therapy (aPDT) mediated by *Senna alata* plant extract in a type I collagen three-dimensional (3D) cell coculture model. The 3D coculture model was prepared and subsequently infected with *Candida albicans* and *Staphylococcus aureus* for 16 h, followed by the application of 0.05 and 0.5 mg/mL *S. alata*-mediated aPDT. After 24 h, microbial viability (CFU/mL) and cellular damage by lactate dehydrogenase assay (LDH) were performed. 0.2 and 2 mg/mL methylene blue (MB) and chlorhexidine 0.12% were used for comparison purposes. Data were analyzed with two-way analysis of variance (α=0.05). The best reductions of *S. aureus* and *C. albicans* were found for 0.5 mg/mL *S. alata* (2.8 and 3.1 log CFU/mL, respectively) and 2 mg/mL MB (3.5 and 4.6 log CFU/mL, respectively). Both concentrations of *S. alata* mediated-aPDT causes less cellular damage compared to 2 mg/mL MB-mediated aPDT ($p>0.05$) and chlorhexidine 0.12%. *S. alata*-mediated aPDT demonstrated to be effective for eliminating *S. aureus* and *C. albicans* and safe for oral cells in a (3D) cell coculture model.

1 INTRODUCTION

By the year 2050, it is estimated that microbial resistance will cause more than 300 million deaths and lead to healthcare systems overload by over 100 trillion dollars (de Kraker et al., 2016). Therefore, improving alternative therapies to control the growth and virulence factors of microorganisms is of utmost importance (Kashef et al., 2017). In this context, prioritizing therapies in which microorganisms are unlikely to develop microbial resistance is the most favorable horizon instead of investing substantial sums in developing new antimicrobial agents that might soon become obsolete (Czaplewski et al. 2016).

Opportunistic and pathogenic microbial infections have been described as important causes of morbidity and mortality (Silva-Rocha, 2017). Among these microorganisms, *Candida albicans* and *Staphylococcus aureus* stand out as highly relevant species capable of infecting humans (Hong et al., 2019; Negrini et al., 2019). *Candida albicans* infections are facilitated by relevant virulence factors, including the production of proteinases and phospholipases, which that play an important role in the colonization and invasion of host tissues (Ferreira et al., 2010). *S. aureus* is currently classified as high priority microorganism to search for new antibiotics and antimicrobial agents due to antimicrobial resistance concerns (Tacconelli et al., 2018). This bacterium is known for causing infectious diseases and inflammation through the production of a wide range of exotoxins and proteases (Oliveira et al., 2018). In addition, both microorganisms can form biofilms, which contributes to the expression of virulence factors and to the protection of microbial species (Ramage et al., 2012).

Oral ulcers are constantly susceptible to infections, which worsen their clinical conditions and regression. *S. aureus* and *C. albicans* are among of the microorganisms with the highest ability to colonize oral ulcers (Laheij and Soet, 2014; Hong et al., 2019; Negrini et al., 2019). Besides their virulence factors, these species display symbiotic behavior, which increases the tissue invasion ability of both microorganisms, leading to an inflammatory response and changes in the host immune system (Belkaid et al., 2014).

Antimicrobial photodynamic therapy (aPDT) has emerged as an alternative for the treatment of infectious conditions. Unlike traditional antimicrobial treatments, aPDT does not lead to microbial resistance because it causes unspecific damage caused by oxidative stress (Hamblin and Hasan, 2004; Medeiros et al., 2017). While the use of aPDT for controlling oral microorganisms is well-documented, its application for

disinfecting oral tissues remains relatively unestablished. Up to now, few studies have evaluated the effectiveness of aPDT in this field (Deyhimi et al., 2011; Maya et al., 2020; Ferrisse et al., 2022). In addition, the modulation of inflammatory response after aPDT in infected tissues is currently an open gap (Nie et al., 2024). At the same time, there has been an increase in publications using natural products as a source of photoactivatable substances. These substances have shown greater capability to generate oxidative stress and easily adherence to the cytoplasmic membrane, facilitating their penetration into the cell target (Xiao et al., 2018; Kubrak et al., 2022; Afrasiabi et al., 2022).

With the advances of PDT, several photosensitizers (PS) have been investigated. While certain photosensitizers (PSs) have been commercialized and extensively employed in experimental clinical investigations, they come with drawbacks, including limited water solubility, inadequate selectivity for pathogenic tissues, challenges in purifying molecules, and potential induction of skin sensitivity (Dobson et al., 2018; Imberti et al., 2020). These limitations stimulated research searching for new photosensitizing agents. In this context, Oliveira et al., (2023) observed that the plant extract of *Senna alata* completely reduced the viability of several microbial species, including *S. aureus* and *C. albicans.* In addition, the extract also produced a large amount of reactive oxygen species and showed low cytotoxicity in monolayer cultures of human cells, demonstrating the potential to be used as a photosensitizing agent.

Given the need to overcome infection and microbial resistance in oral lesions, the present investigation evaluated the aPDT mediated by *Senna alata* plant extract in a three-dimensional (3D) cell coculture model of fibroblasts and keratinocytes. For this purpose, we evaluated the cell damage and the antimicrobial activity against *C. albicans* and *S. aureus*. The development of this study is motivated by several concerns: i) the challenges in eradicating microorganisms and controlling their virulence factors, particularly *C. albicans* and *S. aureus* when associated with lesions with connective tissue exposure; ii) the lack of a protocol for applying aPDT for this condition and iii) the potential use of *S. alata* plant extract, which has been recently discovered. The present study may contribute to the treatment of oral lesions by proposing an alternative topical treatment aimed at controlling the microbial load without the need for systemic antibiotics. This approach has the potential to reduce

costs, avoid microbial selection and enhance the effectiveness of the healing process for these injuries.

2 MATERIALS AND METHODS

2.1 *Photosensitizers agents*

The extraction and production of *Senna alata* plant extract was conducted according to the method described by de Oliveira et al. (2023). In summary, the plant materials were dehydrated at 40 °C for 36 hours, ground, and then subjected to extraction in an ultrasonic bath at a ratio of 100 mg of powder to 3 mL of methanol. Subsequently, the extracts were filtered and concentrated under vacuum using a rotary evaporator, following the procedure outlined by Bueno et al. (2015). For biological testing purposes, the plant extracts were initially diluted in dimethyl sulfoxide (DMSO; Labsynth, Brazil) to a concentration of 5 mg/mL, and further diluted in culture medium to achieve final concentrations of either 0.05 mg/mL or 0.50 mg/mL.

Additionally, 0.2 mg/mL and 2 mg/mL methylene blue (MB) (Sigma-Aldrich, St. Louis, MO) was used for comparison purposes. This PS was selected for comparison purposes and because MB exhibits intense absorption in the red spectrum (600-660 nm), making it favorable for PDT as it falls within the “photo-therapeutic window”, known for its efficient light penetration into biological tissues (Hu et al., 2018). Moreover, MB is cost-effective and produces reactive oxygen species.

All PS’ solutions were freshly prepared in light protected environment.

2.2. *Light sources*

PDT was carried out using IrradLED® - Biopdi (São Carlos, SP, Brazil), which is equipped with 48 LEDs and a cooling system to prevent overheating. Pilot studies were carried out to set the best parameters (light dose, intensity, and concentration of material plants; data not shown). The best microbial reduction was achieved with a light dose of 50 J/cm^2 at an intensity of 155.2 mW/cm^2 for the blue LED light source (460 nm) and an intensity of 67.32 mW/cm^2 for the red LED light source (660 nm) when and at fractionated mode every 30 s.

2.3 *Expansion of the cell population*

The cell lines used in this study were the Human gingival fibroblast (FGH) (obtained from the Rio de Janeiro Cell Bank with the code: 0089) and oral keratinocyte (NOK-si

cells) (provided by Professor Carlos Rossa Jr., from the Cellular and Molecular Biology Laboratory, Department of Periodontics, School of Dentistry, São Paulo State University—UNESP). NOK-si and FGH cells were cultured in high-glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM; Gibco, NY, USA) supplemented with L-glutamine (2 mM/L; Lonza, Basel, Switzerland), fetal bovine serum (FBS; 10% vol/vol; Gibco), and 1% vol/vol antibiotic/antimycotic solution (penicillin G—10,000 µg/mL, streptomycin—10,000 µg/mL, and amphotericin B—25 µg/mL) (Sigma-Aldrich, MO, USA) as described by Castilho et al. (2010). The cells were maintained at 37°C in a 5% CO_2 atmosphere.

2.4 *Three-Dimensional Cell Coculture Model*

To establish the 3D cell coculture model, cells were cultured until they reached 90% confluence. Subsequently, the cells (FGH and NOK-si) were rinsed with sterile PBS (pH 7.2), detached using trypsin solution (0.05% v/v EDTA; 0.53 mM/L) (Sigma-Aldrich), and centrifuged at 400×g for 5 minutes. The pellet resulted was resuspended in fresh cell culture medium, stained with trypan blue (in a 1:1 ratio), and counted using the countess II FL cell counter (Life Technologies, Carlsbad, CA, USA). The methodology followed was adapted from Pimentel et al. (2022). In brief, a mixture was prepared consisting of 2.3 mL of DMEM medium, 450 µL of fetal bovine serum (FBS), 1.5 mL of rat tail collagen (First Link, Wolverhampton, United Kingdom), 200 µL of NaOH (1 M), and 250 µL of the FGH cell suspension (at a concentration of 6×10^6 cells/mL). The FGH cell suspension was thoroughly mixed and plated (500 µL) in 24-well plates. The plates were then incubated at 37°C with 5% CO_2 until the collagen polymerized, which typically took around 4 hours. After polymerization, 75 µL of the NOK-si cell suspension (at a concentration of 2×10^6 cells/mL) was added to the wells. Following NOK-si seeding, the tissues were cultured until the epithelial cells reached confluence, which usually occurred within approximately 24 hours.

2.5 *Three-Dimensional Coculture Model Infection with C. albicans and S. aureus Biofilm*

2.5.1 *Microbial strains, growth conditions, and tissue infection*

C. albicans SC5314 and *S. aureus* ATCC25923 microorganisms were used to create dual-species cultures following the procedures outlined by Peters et al. (2012) and Zago et al. (2015). Before use, C. albicans was stored at -80°C in Yeast Peptone

Glucose medium (YEPD: 1% yeast extract, 2% Bacto peptone, 2% D-glucose, and 2% agar). To reactivate cultures of *C. albicans*, *C. albicans* samples was seemed in a petri dish containing Sabouraud Dextrose Agar plates (SDA-Acumedia Manufactures Inc., Baltimore, MD, USA) supplemented with chloramphenicol (0.05 g/L) and then incubated at 37°C for 48 hours. 5-10 colonies were transferred to a falcon tube containing Yeast Nitrogen Base broth (YNB—Difco, Becton Dickinson Sparks, MD, USA) supplemented with 100 mM glucose and incubated at 37°C overnight (16 hours). This overnight culture was then diluted 1:10 into fresh YNB supplemented with 100 mM glucose and allowed to grow at 37°C until reaching mid-log phase (approximately 8 hours, OD_{540nm} 0.55 ± 0.1), which corresponds to a final concentration of $1x10^4$ cells/mL.

S. aureus was kept in Tryptic Soy Broth medium (TSB - Acumedia Manufactures Inc., Baltimore, MD, USA) and stored at -80 °C was seemed in petri dishes containing Tryptic Soy Agar (TSA - Acumedia Manufactures Inc., Baltimore, MD, USA) medium, and around 10 colonies were transferred to TSB liquid medium and allowed to grow overnight (18 hours) at 37 °C. A 1:20 dilution of the overnight culture was then made into fresh TSB, and it was left to grow at 37 °C until reaching the mid-log phase (approximately 4 hours, OD_{600nm} 0.7±0.01), resulting in a final concentration of $1x10^6$ cells/mL.

After growth, both microorganisms underwent two washes in phosphate-buffered saline (PBS) through centrifugation at 5,000 ×g for 5 minutes and were re-suspended in RPMI 1640 buffered with HEPES (25 mM) and supplemented with 2 mM L-glutamine and 2 g/L sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA) (Dias et al., 2016).

The suspensions of microorganisms were added onto the reconstituted *in vitro* oral mucosa tissue in DMEM medium without antibiotics (Mohiti-Asli et al., 2014; Mailänder-Sánchez et al., 2017). The plates were then incubated at 37°C and 5% CO2 for 16 hours.

2.6 *Antimicrobial Photodynamic Therapy (aPDT) on the Infected 3D Coculture Model*

The experiments were done in quadruplicate on two separate occasions (n = 8). To perform the comparison among groups, the mean was calculated from the duplicate experiments.

aPDT was performed by administering MB (0.2 or 2.0 mg/mL) or *S. alata* extract (0.05 or 0.5 mg/mL) and exposure to 50 J/cm^2 of LED light (P+L+). Additional samples were treated with photosensitizing agents only (P+L−) or LED light alone (P−L+). Untreated control samples were not exposed to light or photosensitizing agents (P-L-). The parameters and experimental groups used are described in Table 1.

Table 1. Experimental parameters of the study

Experimental group	Ps Concentration (mg/mL)	Dose of light (J/cm²)	Intensity (mW /cm^2)	Pre-irradiation time (min)	Irradiation time in fractionated mode (s)
Negative control (P-L-)	N/A	0	0	0	0
Blue Light (P-L+)	N/A	50	155.00	N/A	322
Red Light (P-L+)	N/A	50	67.32	N/A	746
MB (P+L-)	0.20 and 2.0	0	0	N/A	N/A
S. alata (P+L-)	0.05 and 0.50	0	0	N/A	N/A
PDT 1 (MB) (P+L+)	0.20 and 2.0	50	67.32	20	746
PDT 2 (*S. alata*) (P+L+)	0.05 and 0.50	50	155.2	20	322

N/A: Not applicable. Triton X-100: Death control. CHX: chlorhexidine digluconate 0.12%. MB: methylene blue. P-L-: negative control. P-L+: Light control. P+L-: application of only the PS without light. P+L+: aPDT

According to the experimental groups, 150 µL of photosensitizing agents were added to the wells corresponding to the P+ L+ and P+L− groups. For the P−L+ and P−L− groups, 150 µL of PBS (pH 6.8) was added. The plates were then incubated in the dark for 20 minutes as a pre-irradiation step. After this, samples from the P+L+ and P-L+ groups were exposed to LED light according to the parameters outlined in table 1, while groups P+L− and P−L− remained in the dark for the same duration as the irradiated samples. Three successive applications of aPDT were performed. After the first light application (irradiated samples; P−L+, P+L+) or incubation in the dark (non-irradiated samples; P−L−, P+L−), the samples were washed once with PBS solution (pH 6.8). Subsequently, 150 µL of fresh solutions with photosensitizing agents (P+L−, P+L+) or physiological solution (P−L−, P−L+) were added. The samples were incubated again in the dark for 20 minutes and then either illuminated (P−L+, P+L+) or kept in the dark for the same duration as the irradiated samples (P−L−, P+L−). Immediately after this second application, the samples were washed again with PBS solution (pH 6.8), and the same procedures were repeated.

After treatments, samples were promptly washed with PBS and then incubated again in antibiotic-free DMEM medium for an additional 24 hours. To assess the antimicrobial efficacy of photodynamic therapy mediated by Senna alata extract, the 3D coculture model was retrieved using sterile forceps and subjected to vortexing with 1 mL of PBS for 5 minutes. Subsequently, 500 µl of the supernatant was diluted at a ratio of 1:1,000 v/v in PBS. Following this, 200 µL of each diluted solution was plated onto Sabouraud Dextrose Agar (SDA) supplemented with chloramphenicol (0.1 g/L) for *C. albicans* and Brain Heart Infusion (BHI) agar supplemented with amphotericin B (0.025 g/L) for *S. aureus*. The plates were then incubated at 37 °C for 24 hours, after which the number of colonies was enumerated. The results were quantified and expressed in log CFU/mL.

2.7 *Lactate dehydrogenase assay*

Lactate dehydrogenase (LDH) release from tissues into the surrounding medium served as a marker for epithelial and fibroblast cell damage assessment. The measurement of LDH release into the culture media from cultures containing infected epithelial cells was conducted following a 16-hour incubation period. LDH activity was determined by monitoring the fluorescence rate corresponding to the disappearance of NADH at excitation and emission wavelengths of 540 nm and 590 nm, respectively. This was achieved through the LDH-mediated conversion of pyruvate to lactate using the CytoTox-ONE kit (Promega, G7890) as per the manufacturer’s instructions, employing FluoroskanAscent FL (Thermo labsystems) for analysis. A standard drug control using 0.12% Chlorhexidine digluconate (CHX 0.12% – Periogard®) and a death cell control with Triton X-100 (0.1% vol/vol) were included for comparison. The treatment duration and number of applications for these groups were consistent with those used for aPDT. Each experiment was conducted in quadruplicate on two separate occasions (n = 8).

2.8 *Statistical analyses*

The present data was considered normal (p>0.05) and homoscedastic (p>0.05). In addition, no outliers were detected. Based on this, two-way ANOVA were used as inferential test. For microbial viability as well as for LDH assay, the groups and exposure or not to the light were the independent factors. For multiple comparisons among the groups, mean estimate with 95% of confidence interval was used for

evaluation of CFU/mL and LDH levels. For all statistical approaches, the significance level of 5% was applied. For each *in vitro*, experiments were conducted in quadruplicate in two independent occasions (n=8).

3 RESULTS

3.1 Efficiency of aPDT in 3D Coculture Model against dual-species infection of C. albicans and S. aureus

After three successive applications of *S. alata* and MB-mediated aPDT in the 3D model infected by *C. albicans* and *S. aureus*, the microorganism cells were recovered, and bacterial viability was assessed using specific media.

For both PSs used, the best results were achieved using the highest concentration. aPDT mediated by 0.5 mg/mL *S. alata* reached a reduction of 2.75 log CFU/mL of *S. aureus* compared to negative control while 2 mg/mL MB-mediated aPDT achieved a reduction of 3.5 log CFU/mL of *S. aureus*. This different between PSs was considered significant. The lowest concentration of each PS used (0.05 mg/mL *S. alata* and 0.2 mg/mL MB) showed, respectively, 1.50 and 2.23 log CFU/mL for *S. aureus* microbial viability. This difference in microbial viability between the lowest PS concentration was also considered significant (Figure1).

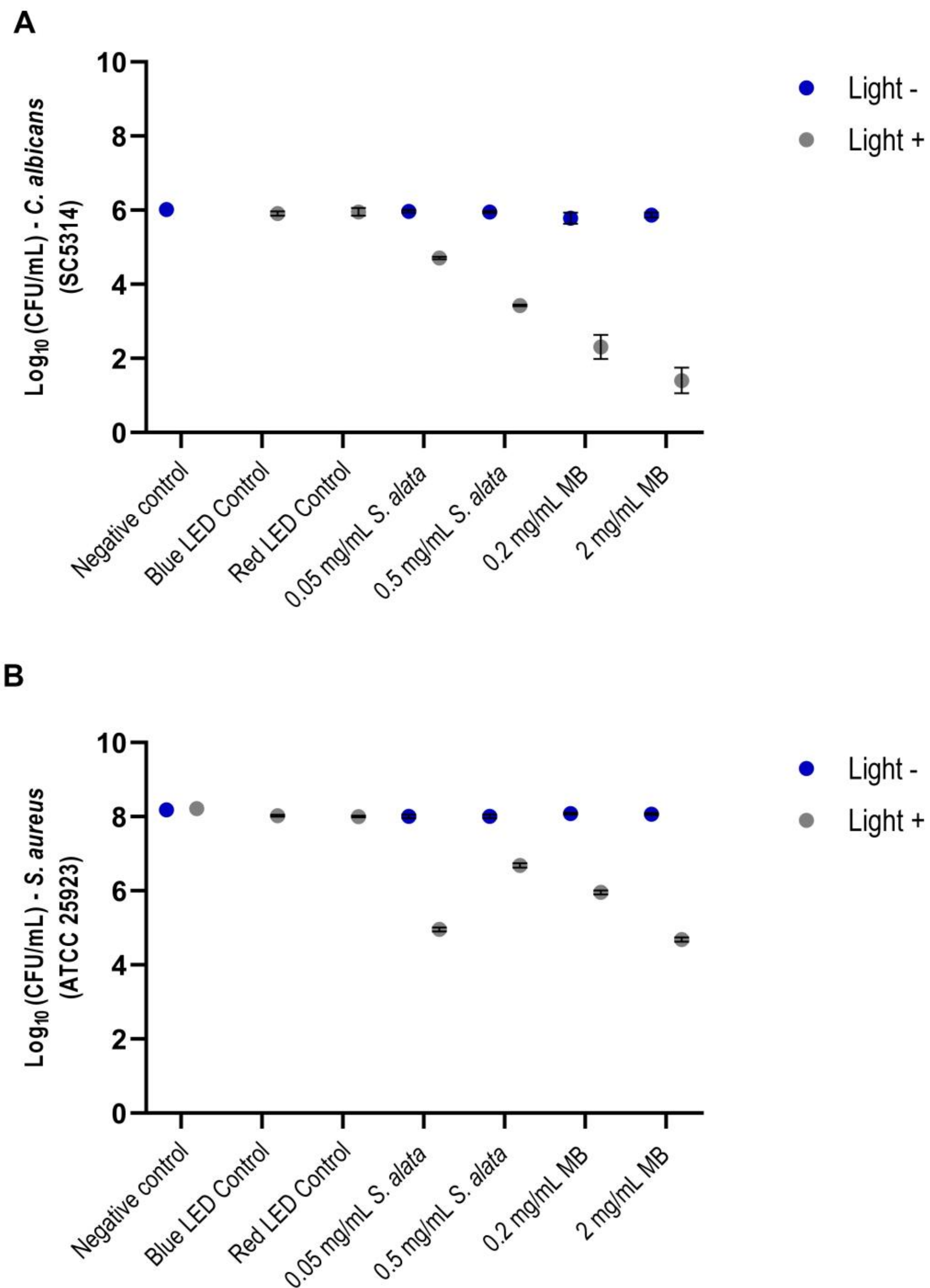

Figure 2. Microbial viability after *Senna alata* and MB-mediated aPDT against *C. albicans* (A) and *S. aureus* (B). Two-way Anova and mean estimated with 95% of confidence interval. Independent factors: presence/absence of light exposure and groups. Light +: light exposure. Light -: absence of light

Similar results were also found for *C. albicans* microbial load, in which the best results were obtained with the highest PS concentration used (0.5 mg/mL S. *alata* and 2 mg/mL MB). Thus, *S. alata*-mediated aPDT reduced 3.06 log CFU/mL and MB-

mediated aPDT reached a reduction of 4.6 log CFU/mL (negative control = 6.02 log CFU/mL). The lowest concentration of *S. alata* (0.05 mg/mL) and MB (0.02 mg/mL) reduced, respectively 1.30 log CFU/mL and 3.7 log CFU/mL of *Candida albicans*. For both microbial species, all P+L+ groups were significantly different from each other and from the light control groups (red and blue LED control). Moreover, the light control groups were not significantly different compared to negative control (inoculum control) (Figure 2).

3.2 Epithelial and fibroblast cell damage by aPDT

2 mg/mL MB-mediated aPDT showed the highest level of cell damage among the experimental groups exposed to light and this difference was considered significant. *S. alata*-mediated aPDT showed the same levels of cell damage compared to 0.2 mg/mL MB-mediated aPDT. Notably, for *S. alata*, the difference in concentration used did not influence the levels of damaged cells. The blue LED control showed the same LDH levels compared to the infected control and all PSs groups that were not exposed to light. The red LED control induced a higher cell damage compared to the blue LED control, with similar effects observed for *S. alata*-mediated aPDT in both concentrations and 0.2 mg/mL MB-mediated aPDT. The death control showed the highest level of cell damage, followed by CHX 0.12%. In addition, the levels of cell damage were significantly higher to all PSs used and exposed to the light (Figure 3).

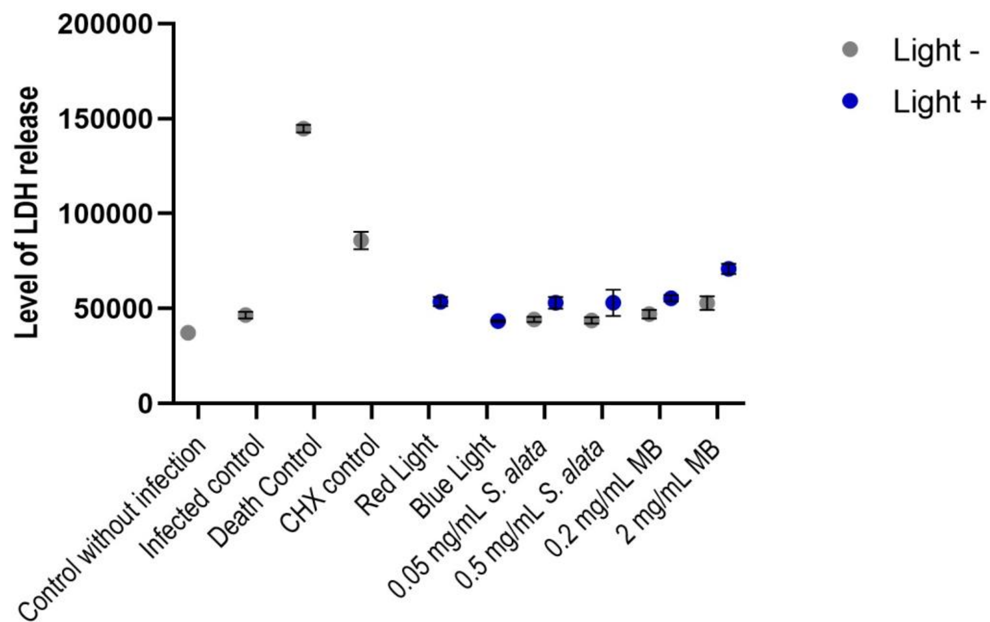

Figure 2. Cell damage assessed by LDH concentration in culture media after *S. alata* and MB-mediated aPDT in a 3D-coculture model. Two-way Anova and mean estimated with 95% of confidence interval. Independent factors: presence/absence of light exposure and groups. Light +: light exposure. Light -: absence of light.

4. DISCUSSION

In the present study, we evaluated the antimicrobial efficacy of *S. alata*-mediated aPDT in a more complex system using tissue engineering technology. This technology added an extra layer to the protection against antimicrobial therapy for *S. aureus* and *C. albicans*, as 3D coculture using oral keratinocytes and fibroblast embedded in a collagen matrix (scaffold-free technology). In addition, the evaluation of cell damage induced by aPDT was also possible.

This work expands previous findings from our research group, which demonstrated that the crude extract of *S. alata* can be used in aPDT against *C. albicans* and *S. aureus* (Oliveira et al., 2023). However, Oliveira et al., (2023) used a simple model of mono-species biofilms to screen the most active extracts, which do not reproduce the challenges employed in the present study, namely, intra- and inter-species interaction organized in a complex 3D-cellular model that mimics the oral mucosa in clinical infections.

To our knowledge, this is the first time that a three-dimensional cellular model containing FGH, collagen type 1 and NOK-si cells and infected with *C. albicans* and *S. aureus* dual-species biofilm has been used to compare the effectiveness of different PS (PS) in antimicrobial photodynamic therapy (aPDT). The clinical importance of evaluating new therapeutic strategies in more robust models, with the interaction of different cell types, is widely indicated due to predict capability for clinical studies (Joshi et al., 2023).

Our results showed that photoactivation of *S. alata* crude extract at different concentrations (0.05 and 0.5 mg/mL) significantly reduced the viability of *C. albicans* and *S. aureus*. In addition, the reduction in *S. aureus* viability using *S. alata*-mediated aPDT was similar to that achieved with the marketed photosensitizer MB, which was tested in two different concentrations in the present study. The similar results between a natural extract (*S. alata*) and an isolated compound (MB) strengthens the present findings, suggesting possible synergistic effects among different compounds found in the plant extract, as described previously (Koo et al., 2003; Brighenti et al., 2012). This observation highlights the significance of exploring natural sources for their therapeutic potential and for understanding the complexities of their chemical compositions in biomedical research.

Three applications of the treatment significantly reduced microbial viability of the two species evaluated in comparison to the control group, regardless of the photosensitizing agent used. The microbial reduction achieved in the present study for *S. alata*-mediated aPDT was lower for *C. albicans* and higher for *S. aureus* in comparison to our prior study (Oliveira et al., 2023). There are some reasons that can explain these findings. First, Oliveira et al., (2023) evaluated the effectiveness of *S. alata*-mediated aPDT using mono-species biofilms with a single application of light.

The limitations of using mono-species biofilms include the lack of intra-species interactions, as well as the presence of a thinner cytoplasmic membrane and a more porous peptidoglycan and lipoteichoic acid layer. These structural characteristics enable penetration of substances into the inner layers of biofilm and heighten microbial susceptibility to treatments compared to their mixed-species counterparts (Moons et al., 2009; Rendueles & Chigo, 2012; Kaya et al., 2024). In addition, simple models of biofilm formation based on monospecies provide information regarding to cells and matrix of the biofilm. That explain the reason of this type of model is largely used to test new techniques of treatment. However, eventually, more complex biofilms are

required to better evaluated a biological system in a more realist scenario (Sutherland, 2001).

In this context, the relationship between *C. albicans* and *S. aureus* can play a role in favoring *C. albicans* survival while impairing that of *S. aureus* (Gould et al., 2023). *S. aureus* can bind to the *C. albicans* hypha cell wall (Hernandes-Cuellar et al., 2022) possibly enhancing the surface mobility of this bacterium (Ren et al., 2022). Therefore, we can hypothesize that during an oral tissue infection, *S. aureus* can invade the tissue by binding to *C. albicans'* hyphae, thus serving as a physical barrier, which decreases the PS uptake in *C. albicans* cells, hampering the efficacy of aPDT against *C. albicans.* As consequence, *S. aureus* uptakes a great amount of PS, becoming more susceptible to aPDT.

Further investigation to understand the mechanism of these interactions and their implications in oral infections should be carried out in the future. This will not only deepen our understanding of microbial pathogenesis but also aid in the development of effective therapeutic approaches against *S. aureus* and *C. albicans* infections, paving the way for more targeted and effective interventions in clinical practice.

Lastly, we can mention the greater resistance photosensitizing agents encounter in penetrating deeper tissue layers and reaching the target microorganisms. This resistance arises from diffusion challenges due to cellular and collagen density present in the experimental model. Other factors such as pH changes, hypoxia and different cell proliferation rates contribute to this greater resistance (Li and Chang., 2008).

In addition, during the dual-species biofilm infection, *C. albicans* can damage the host cell favoring the penetration of *S. aureus* into the epithetical area. As the infection progresses, these microorganisms can invade the tissue deeply, reaching the subepithelial collagen matrix and leaving an irregular and altered epithelial surface above the infection loci (de Carvalho Dias et al., 2018). This fact can also decrease the uptake of the PS by the microorganism during the incubation period, which explains the challenge of eliminating microorganisms in 3D cell coculture model after aPDT compared to well plastic plates.

In the present study, all PSs exposed to light showed significant lower levels of cell damage (LDH concentration) compared to 0.12% CHX. This can be considered a promising result, since 0.12% CHX is a highly effective commercial antiseptic in microbiological control in the prevention of infectious diseases.

Furthermore, LDH levels in samples treated with *S. alata*-mediated aPDT at both concentrations (0.05 and 0.5 mg/mL) were significantly lower compared to 2 mg/mL MB-mediated aPDT. These findings associated with the similar microbial load reduction archived between *S. alata* and MB-mediated aPDT emphasizes the potential of *S. alata* as a new photosensitizer. For this reason, the results of *S. alata*-mediated aPDT should be tested in future pre-clinical studies and clinical trials. Different from *Senna-alata* mediated aPDT, MB-mediated aPDT showed high levels of LDH. This fact can be explained by the increased number of inflammatory cells after aPDT treatment found in subcutaneous tissue of rats (Cintra et al., 2024). However, the correlation between LDH levels and inflammatory cells should be further investigated.

LDH is an enzyme that aids the conversion of sugar into energy for cells. It is present in various organs and tissues and can be released when exposed to a damage or injury offering energy to cells to recover itself. The low levels of LDH observed in the *S. alata*-mediated aPDT group may indicate that a cellular homeostasis was not hampering, what leads to the production of lactic acid instead of the formation of ATP and CO_2. (Khajah et al., 2024).

Senna alata have antioxidant and anti-inflammatory properties (Aung et al., 2023). In addition, this plant can also contain a huge proportion of anthraquinone in its composition, and this fact is reported to protect hepatic injury and increases the synthesis of type II collagen (Fatmawati et al., 2020). Therefore, is possible that *Senna alata* can protect the tissue against the aPDT application using antioxidant and anti-inflammatory properties which confers lower levels of LDH when compared to MB-mediated aPDT at highest concentration.

As *S. alata* is a crude extract with a complex composition, identifying all the chemical constituents and the specific photoactive molecule responsible for the photodynamic action under evaluation is challenging. These complexities show the drawbacks associated with employing *S. alata* in aPDT. Furthermore, the obstacles related to acquiring *S. alata*, preparing its extract, and potential compositional variations across different batches warrant consideration as potential disadvantages. Current research, conducted by our group seeks to obtain an enriched fraction of *S. alata* while identifying the molecules accountable for the aPDT effects observed in this study. Moreover, evaluating the heat release during oxidative damage induced by aPDT is necessary to validate *S. alata* as a genuine photosensitizer agent.

As in vitro research, the present study has some limitations because it was conducted under controlled laboratory conditions, which cannot be directly extrapolated to the clinical context. As an example, there was no simulation of other protection mechanisms, such as presence of inflammatory cells and the multispecies interaction of biofilms. On the other hand, as a primary study, it provides data in a controlled manner, aligned with the objective of the study, thereby preventing other variables from interfering with the results. However, it is important to highlight that *S. alata* mediated aPDT have a potential to be used as a new photosensitizer since it have showed similar microbial load reduction for *C. albicans* and *S. aureus* as a commercially available PS (MB) and lower capability to produce LDH. Moreover, in our previous study, *S. alata* mediated aPDT showed significant reduction in microbial load for *Escherichia coli, Streptococcus mutans* and *Cutibacterium acnes* cultured in planktonic and in biofilm (Oliveira et al., 2023). In this context, further studies are recommended to compare the susceptibility of these microorganisms while also correlating other factors, such as the participation of inflammatory and immunological cells. This is important for the correct indication of the crude extract *of S. alata* as a photosensitizing agent.

5. CONCLUSION

Due to the great microbial load reduction associated with lower levels of LDH found in *Senna alata*-mediated aPDT, this natural PS is safe and promising and therefore their efficacy should be evaluated in further pre-clinical studies and clinical trials.

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4 CONCLUSÃO

A terapia fotodinâmica tem se mostrado uma modalidade promissora para o tratamento de doenças causadas por micro-organismos, principalmente na área da Odontologia. O desenvolvimento de novos fotossensibilizadores e o aprimoramento de protocolos de aplicação da terapia é importante para aumentar a quantidade de recursos terapêuticos disponíveis para o tratamento de doenças infecciosas. Neste contexto, a TFD associada a laserterapia é capaz de reduzir significativamente o tempo de cicatrização de lesões de mucosite oral infectada por micro-organismos. E ainda, TFD mediada por *S.alata* na maior concentração (0.5 mg/mL) apresentou resultados promissores na redução microbiana de *C. albicans* e *S. aureus* cultivados em suspensão e em biofilme simples e misto, principalmente após 3 aplicações sucessivas. TFDa mediada por *S. alata* também apresentou resultados significativos para redução dos fatores de virulência dos micro-organismos acima citados, em especial para a redução de SOD1 além de apresentar baixos níveis de LDH. Em resumo, essas descobertas fornecem uma nova opção de agente fotossensibilizante de origem natural, com boa ação antimicrobiana e sem comprometer a viabilidade das células humanas. Como perspectiva futura, o extrato de *S. alata* deve ser considerado para aplicação contra uma ampla gama de micro-organismos patogênicos, como micro-organismos resistentes, e submetido a avaliação em protocolos em larga escala.

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* De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-atualizado.pdf

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