UNIVERSIDADE FEDERAL DA GRANDE DOURADOS
FACULDADE DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

PROSPECÇÃO DE NOVOS AGENTES NO COMBATE DE LEVEDURAS DO COMPLEXO *Candida haemulonii* e *Candida auris*

LARISSA RODRIGUES PIMENTEL

Dourados - MS
2024

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Faculdade de Ciências da Saúde da Universidade Federal da Grande Dourados (UFGD), para obtenção do título de Mestre em Ciências da Saúde.
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LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SÍMBOLOS

ADF - Anidulafungina

AIDS - Síndrome da Imunodeficiência Adquirida

ALX -Alexidine

AmB – Anfotericina B

CAS -Caspofungina

CDC - *Centers for Disease Control and Prevention*

CHX - Clorexidina

CIM - Concentração Inibitória Mínima

COVID-19 - Coronavírus

CLSI - *Clinical and Laboratory Standards Institute*

ECM - Matriz extracelular

EUCAST - *European Committee on Antimicrobial Susceptibility Testing*

FICI – *Fractional Inhibitory Concentration Index*

FDA - *Food and Drug Administration*

FLZ – Fluconazol

MCF - Micafungina

MDR – Multirresistentes

MRSA - *Staphylococcus aureus* resistente à meticilina

MIC – Minimal Inhibitory Concentration

NYS - Nistatina

PDR – Pan-resistência

ZnPT – Piritionato de Zinco

TAF – Tafenoquine

UTIN – Unidade de Terapia Intensiva Neonatal

VRC - Voriconazol

PROSPECÇÃO DE NOVOS AGENTES NO COMBATE DE LEVEDURAS DO COMPLEXO *Candida haemulonii* e *Candida auris*

RESUMO PORTUGUÊS

Candida auris e o complexo *Candida haemulonii* são ameaças emergentes devido aos seus perfis multirresistentes a medicamentos (MDR) e às altas taxas de mortalidade causadas. A eficácia limitada das terapias antifúngicas atuais destaca a necessidade urgente de desenvolvimento de novos tratamentos. Neste sentido, o reposicionamento de medicamentos, utilizando compostos com perfis de segurança e farmacológicos conhecidos, apresenta uma via promissora. O piritionato de zinco (ZnPT) e a nistatina (NYS) são reconhecidos por suas propriedades antifúngicas. A tafenoquina (TAF) e a alexidina (ALX) destacam o potencial dos medicamentos reposicionados no enfrentamento de patógenos resistentes. O objetivo deste estudo foi avaliar o potencial antifúngico dos compostos ZnPT, NYS, TAF e ALX para leveduras multirresistentes do complexo Candida haemulonii e Candida auris, buscando contribuir no desenvolvimento de alternativas terapêuticas eficazes.A suscetibilidade antifúngica foi avaliada por meio do método de microdiluição em caldo, para determinar as concentrações inibitórias mínimas (CIMs) e fungicidas mínimas (CFMs) dos compostos frente isolados clínicos de *C. auris* e *C. haemulonii*. A inibição e a erradicação de biofilmes foram testadas. Modelos *ex vivo* de pele suína,permitiram avaliar a inibição de biofilmes por ZnPT + NYS. Estudos dos mecanismos de ação incluíram ensaios de extravasamento de proteínas e nucleotídeos para avaliar a permeabilidade da membrana celular, testes de inibição de bombas de efluxo e ensaios de osmoproteção com sorbitol para investigar os efeitos potenciais dos compostos nas paredes celulares fúngicas. A segurança biológica foi avaliada por meio de ensaios de hemólise para determinar a hemocompatibilidade, testes de toxicidade aguda em larvas de *Tenebrio molitor* e o teste de Ames para avaliar a mutagenicidade. ZnPT + NYS mostraram um efeito sinérgico, inibindo significativamente a formação de biofilmes na pele suína, com erradicação quase completa em tempos curtos de exposição. Estudos de mecanismo de ação revelaram a interrupção seletiva da membrana sem extravasamento significativo de nucleotídeos. Os ensaios de bombas de efluxo confirmaram que a eficácia de ZnPT + NYS não foi afetada por mecanismos de resistência. As avaliações de segurança mostraram que, enquanto o ZnPT sozinho exibiu toxicidade em *T. molitor*, a combinação com NYS mitigou esses efeitos. O teste de Ames confirmou o baixo risco mutagênico do tratamento com ZnPT + NYS. ZnPT e NYS, especialmente quando usados em combinação, demonstraram atividade antifúngica significativa contra *C. auris*, destacando seu potencial como uma poderosa opção de tratamento TAF e ALX demonstraram atividade antifúngica promissora contra *C. haemulonii*. A compatibilidade com agentes reposicionados como TAF e ALX reforça ainda mais sua versatilidade terapêutica, oferecendo uma estratégia promissora para combater infecções fúngicas por *C. haemulonii*. Este estudo inova ao reposicionar medicamentos como piritionato de zinco e nistatina, alexidine, tafenoquine, apresentando uma solução para infecções fúngicas causadas por *Candida auris* e para o

complexo *Candida haemulonii*. A combinação desses compostos mostrou não apenas eficácia antifúngica, incluindo a erradicação de biofilmes, mas também resistência aos mecanismos de defesa fúngica e baixa toxicidade. Esses resultados oferecem uma proposta para o desenvolvimento de novas terapias que utilizem combinações de medicamentos reposicionados. Essa abordagem representa um avanço promissor no enfrentamento dos patógenos estudados, com potencial para impactar positivamente o manejo clínico dessas infecções. Esses resultados destacam a necessidade de ensaios clínicos para confirmar a eficácia desses compostos, abrindo caminho para soluções inovadoras e eficazes no manejo de patógenos fúngicos desafiadores.

Prospection of New Agents to Combat Yeasts from the *Candida haemulonii* and *Candida auris* Complexes

RESUMO INGLÊS

Candida auris and the *Candida haemulonii* complex are emerging threats due to their multidrug-resistant (MDR) profiles and high associated mortality rates. The limited efficacy of current antifungal therapies highlights the urgent need for the development of new treatments. In this context, drug repurposing, leveraging compounds with established safety and pharmacological profiles, presents a promising avenue. Zinc pyrithione (ZnPT) and nystatin (NYS) are recognized for their antifungal properties. Tafenoquine (TAF) and alexidine (ALX) further showcase the potential of repurposed drugs in tackling resistant pathogens. This study aimed to evaluate the antifungal potential of ZnPT, NYS, TAF, and ALX against multidrug-resistant yeasts of the *Candida haemulonii* complex and *Candida auris*, contributing to the development of effective therapeutic alternatives.Antifungal susceptibility was assessed using the broth microdilution method to determine the minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of the compounds against clinical isolates of *C. auris* and *C. haemulonii*. The inhibition and eradication of biofilms were tested. Ex vivo models using porcine skin were employed to evaluate biofilm inhibition by ZnPT + NYS. Mechanism-of-action studies included protein and nucleotide leakage assays to assess cell membrane permeability, efflux pump inhibition tests, and osmoprotection assays with sorbitol to investigate the potential effects of the compounds on fungal cell walls. Biological safety was evaluated through hemolysis assays to determine hemocompatibility, acute toxicity tests in *Tenebrio molitor* larvae, and the Ames test to assess mutagenicity.ZnPT + NYS demonstrated a synergistic effect, significantly inhibiting biofilm formation on porcine skin, with nearly complete eradication achieved in short exposure times. Mechanism-of-action studies revealed selective membrane disruption without significant nucleotide leakage. Efflux pump assays confirmed that the efficacy of ZnPT + NYS was not compromised by resistance mechanisms. Safety evaluations showed that while ZnPT alone exhibited toxicity in *T. molitor*, the combination with NYS mitigated these effects. The Ames test confirmed the low mutagenic risk of ZnPT + NYS treatment. ZnPT and NYS, especially in combination, showed significant antifungal activity against *C. auris*, highlighting their potential as a powerful treatment option. TAF and ALX demonstrated promising antifungal activity against *C. haemulonii*. The compatibility with repurposed agents such as TAF and ALX further reinforces their therapeutic versatility, offering a promising strategy to combat fungal infections caused by *C. haemulonii*.This study innovates by repurposing drugs such as zinc pyrithione, nystatin, alexidine, and tafenoquine, providing a solution for fungal infections caused by *Candida auris* and the *Candida haemulonii* complex. The combination of these compounds demonstrated not only antifungal efficacy, including biofilm eradication, but also resistance to fungal defense mechanisms and low toxicity. These findings provide a proposal for the development of new therapies utilizing combinations of repurposed drugs. This approach represents a promising advancement in addressing the studied pathogens, with potential to positively impact the clinical management of these infections. These results underscore the need for clinical trials to confirm the efficacy of these

compounds, paving the way for innovative and effective solutions in managing challenging fungal pathogens.

SUMÁRIO

1. INTRODUÇÃO

O gênero *Candida* compreende mais de 200 espécies, sendo que 90% das infecções humanas são causadas por *C. albicans*, *Nakaseomyces glabrata*, *C. tropicalis*, *C. parapsilosis* e *Pichia kudriavzevii*. Essas infecções variam de formas superficiais a invasivas, muitas vezes podem resultar em complicações graves, especialmente em pacientes imunocomprometidos (Kaur et al., 2023). A candidíase invasiva apresenta alta mortalidade, frequentemente associada ao diagnóstico tardio e à resistência ao tratamento, sendo *Candida* o quarto principal agente de infecção na corrente sanguínea em hospitais (Riera et al., 2022).

Leveduras raras como *Candida haemulonii* estão ganhando atenção crescente na clínica devido ao potencial de causar infecções invasivas graves e a sua resistência intrínseca a antifúngicos, particularmente ao fluconazol (Ramos et al., 2022). Esses fungos, identificados inicialmente em regiões tropicais, têm relevância clínica global devido à sua capacidade de colonizar superfícies humanas, formando biofilmes resistentes e causando infecções graves, como candidemia, cuja mortalidade associada às infecções varia de 44% a 83%, dependendo do contexto clínico e da disponibilidade de tratamentos específicos (Françoiseid et al., 2023; Pharkjaksu et al., 2021).

Além disso, leveduras emergentes, como *C. auris*, desde sua identificação há pouco mais de uma década, tornou-se um patógeno nosocomial de relevância global. Diferentemente de outras espécies do gênero, *C. auris* coloniza a pele, onde pode persistir por longos períodos, facilitando sua disseminação em instituições de saúde por meio do contato direto ou de dispositivos médicos contaminados (Horton & Nett, 2020; Proctor et al., 2021). Sua resistência a diversas classes de antifúngicos, como azóis, polienos e equinocandinas, complica significativamente o manejo terapêutico (Alanio et al., 2022; Huang et al., 2021; Ostrowsky et al., 2020).

A resistência antifúngica, intrínseca ou adquirida, representa uma preocupação crescente, diminuindo as opções terapêuticas e associando-se a altas taxas de morbimortalidade. Em casos de resistência a múltiplos medicamentos, as infecções podem se tornar intratáveis (Ben-Ami & Kontoyiannis, 2021; Perlin et al., 2017).

Diante desse cenário, estratégias de prevenção e controle são essenciais, assim como o uso adequado de antifúngicos. Além disso, novas abordagens terapêuticas, como tratamentos combinados, estão sendo investigadas para combater a resistência (Ford et al., 2015). A falta de preparo médico no contexto do reposicionamento de medicamentos representa um desafio significativo na prática clínica moderna. Apesar das vantagens dessa estratégia, como a redução de custos e tempo de desenvolvimento, sua implementação clínica enfrenta obstáculos importantes. Entre eles, destaca-se a resistência dos profissionais de saúde, muitas vezes associada à ausência de treinamento específico e ao desconhecimento sobre os novos usos de medicamentos já existentes (Peyclit et al., 2021; Zamami et al., 2021).

O reposicionamento de medicamentos possibilita identificar novas indicações para medicamentos existentes. Um exemplo, é o uso da talidomida para tratar o mieloma múltiplo e o ácido acetilsalicílico (aspirina) em estudos clínicos para prevenir recidivas em tumores sólidos não metastáticos, como no ensaio Add-Aspirin (Serafin et al., 2021). Outro caso de destaque é o reposicionamento do dissulfiram, tradicionalmente usado no tratamento do alcoolismo, para combater glioblastomas ao inibir vias moleculares específicas (Serafin et al., 2021). O reposicionamento de fármacos constitui uma estratégia para acelerar a disponibilidade de tratamentos em áreas críticas.

Assim, este estudo justifica-se pela necessidade urgente de ampliar as opções terapêuticas diante do crescente desafio representado pelas infecções fúngicas resistentes, que têm impacto significativo na saúde pública e no ambiente hospitalar. Nesse cenário, o reposicionamento de fármacos surge como uma abordagem promissora para o desenvolvimento de terapias inovadoras, com potencial para reduzir custos e acelerar a disponibilização de tratamentos eficazes, ao explorar estratégias alternativas, espera-se contribuir para o manejo clínico mais eficaz dessas infecções e para o controle de sua disseminação no contexto nosocomial. Portanto, este estudo visa preencher lacunas críticas no conhecimento e na prática clínica, ajudando a mitigar o impacto das infecções fúngicas resistentes e a melhorar os resultados de saúde pública.

Assim, este estudo justifica-se pela necessidade urgente de ampliar as opções terapêuticas frente às infecções fúngicas resistentes, contribuindo para a melhoria do manejo clínico e para o controle eficaz dessas infecções no contexto nosocomial.

2. REVISÃO BIBLIOGRÁFICA

2.1. Importância de infecções causadas por espécies do gênero *Candida*

Infecções fúngicas causadas por espécies do gênero *Candida* têm ganhado destaque na literatura médica devido ao impacto significativo na saúde pública, especialmente entre pacientes imunocomprometidos e hospitalizados (Pfaller et al., 2011). Essas infecções, conhecidas como candidíases, variam desde manifestações superficiais até formas invasivas graves, como a candidemia, que apresenta altas taxas de mortalidade, o aumento da incidência de candidíase invasiva preocupa particularmente devido ao crescimento da população em Unidades de Terapia Intensiva (UTI), ao uso de antibióticos de amplo espectro e à presença de dispositivos invasivos, como cateteres venosos (Pfaller et al., 2011).

Infecções de corrente sanguínea por *Candida* spp. constituem um problema crescente em hospitais terciários em todo o mundo, impulsionado pelo aumento de pacientes em condições de risco. Estima-se que ocorram aproximadamente 400.000 casos anuais de candidemia no mundo, com taxas de mortalidade entre 27% e 42% (Zheng et al., 2021). No Brasil, essa mortalidade entre pacientes hospitalizados é ainda mais elevada, situando-se entre 50% e 70% (Canela et al., 2018a; de Almeida et al., 2021a), enquanto nos EUA e em países europeus, varia de 30% a 40% (Mazi et al., 2022).

A capacidade de *Candida* de se transformar de um comensal inofensivo para um patógeno oportunista está associada a fatores como plasticidade genética e formação de biofilme (Fisher et al., 2012). A patogênese das espécies de *Candida* envolve múltiplos mecanismos, incluindo evasão do sistema imunológico, multiplicação no hospedeiro e disseminação para outros órgãos (Colombo et al., 2013; Irinyi et al., 2015). Em infecções sistêmicas, a invasão da corrente sanguínea pode resultar em complicações graves, com letalidade chegando a 42% dos casos (Calvo et al., 2016; Prakash et al., 2016).

Embora *C. albicans* seja a espécie mais frequentemente associada a infecções, outras espécies não-*albicans*, como *N. glabrata*, *C. parapsilosis*, *C. tropicalis* e *C. auris*, têm se destacado como patógenos relevantes (Canela et al., 2018b). Em particular, *C.*

auris tornou-se um grande desafio para unidades de saúde globais devido à sua resistência intrínseca a múltiplas classes de antifúngicos e sua persistência em superfícies hospitalares, facilitando surtos de infecção (Centros de Doenças Controle e Prevenção, 2023).

A resistência antifúngica entre espécies de *Candida* não-*albicans* é uma preocupação crescente. *N. glabrata*, por exemplo, mostra resistência elevada aos azóis, especialmente ao fluconazol, com taxas de resistência entre 10% e 15% em ambientes hospitalares, variando conforme a localização geográfica e práticas clínicas locais (Arendrup & Patterson, 2017). Esse quadro aumenta a complexidade do manejo terapêutico, restringindo as opções de tratamento e demandando antifúngicos mais caros e com maior toxicidade, como as equinocandinas (Arendrup & Patterson, 2017).

Diversos fatores predisponentes contribuem para o desenvolvimento de infecções por *Candida*, incluindo uso prolongado de antibióticos, imunossupressão, neutropenia, diabetes mellitus e presença de dispositivos invasivos, como cateteres e próteses, pacientes em UTIs são especialmente vulneráveis, com alta incidência de candidemia, em razão do uso frequente de dispositivos intravasculares, ventilação mecânica e nutrição parenteral, condições que favorecem a colonização e subsequente infecção (Lockhart et al., 2017).

Dada a gravidade das infecções por *Candida* e o aumento da resistência antifúngica, medidas de prevenção e controle são cruciais, a adesão rigorosa às práticas de controle, como higienização das mãos, desinfecção de superfícies e substituição de dispositivos invasivos quando necessário, são essenciais para reduzir a incidência de candidíase em hospitais, o uso criterioso de antibióticos de amplo espectro e a profilaxia antifúngica em populações de alto risco também são estratégias defendidas para limitar o desenvolvimento de resistência (Colombo et al., 2013; de Almeida et al., 2021).

O uso extensivo de antifúngicos, especialmente fluconazol e outros triazólico, em ambientes clínicos, tem contribuído para o aumento da resistência (Borman et al., 2019). *C. auris*, com resistência a múltiplos medicamentos, apresenta desafios significativos, exigindo novos antifúngicos e estratégias de controle (Cortegiani et al., 2019; Lockhart et al., 2017). Portanto, infecções por *Candida* representam grandes desafios clínicos, e o desenvolvimento de novas terapias é crucial para melhorar os desfechos dos pacientes (Borman et al., 2019).

A incidência crescente de infecções por *Candida*, somada ao surgimento de cepas resistentes, destaca a urgência de métodos diagnósticos aprimorados, novas terapias antifúngicas e medidas de controle mais eficazes, uma abordagem multidisciplinar é essencial para enfrentar os desafios impostos por esse gênero em ambientes hospitalares e comunitários. Avanços em diagnósticos moleculares e programas de administração antifúngica representam estratégias promissoras para mitigar a crescente carga de infecções por *Candida* (Fisher et al., 2022; Lockhart et al., 2017).

2.2. *C. auris* e complexo *C. haemulonii*

O complexo de espécies *C. haemulonii* inclui os patógenos emergentes *C. haemulonii sensu stricto*, *C. duobushaemulonii* e *C. haemulonii* var. *vulnera,* este complexo está filogeneticamente relacionado a *C. auris*, (Gade et al., 2020). Esse grupo de leveduras multirresistentes têm sido associado a infecções hospitalares em várias regiões do mundo, incluindo o Brasil, devido à sua resistência a múltiplos antifúngicos e à gravidade das infecções que causa, especialmente em pacientes imunocomprometidos ou hospitalizados por longos períodos (Ramos et al., 2015).

A emergência de *Candida auris* e do complexo *Candida haemulonii* representa um desafio significativo para a saúde pública global, devido ao seu potencial de disseminação rápida em ambientes hospitalares e à sua alta resistência a múltiplos antifúngicos (Jeffery-Smith et al., 2018). Desde sua identificação inicial em 2009 no Japão, *C. auris* demonstrou uma capacidade notável de causar surtos em diversos continentes, incluindo Ásia, África, Europa e Américas, com clados geneticamente distintos identificados nessas regiões (Chow et al., 2020).

Em termos globais, mais de 40 países relataram casos de *C. auris*, com uma incidência crescente em unidades de terapia intensiva (UTIs) devido à sua habilidade de colonizar superfícies hospitalares e persistir em equipamentos médicos, facilitando a transmissão nosocomial (Cortegiani et al., 2018). A taxa de mortalidade associada à candidemia por *C. auris* varia entre 30% e 60%, dependendo da população afetada e das condições clínicas subjacentes (Di Pilato et al., 2021).

Regionalmente, na América do Sul, surtos significativos foram documentados na Venezuela, Colômbia e Brasil, onde *C. auris* foi identificado pela primeira vez em 2020. No Brasil, surtos relacionados à pandemia de COVID-19 evidenciaram a

vulnerabilidade dos pacientes criticamente enfermos à colonização e infecção por *C. auris* (de Almeida et al., 2021). Na Ásia, a disseminação rápida é atribuída à infraestrutura hospitalar densa e ao uso extensivo de antifúngicos, enquanto na Europa, surtos têm sido controlados com rigorosas medidas de controle de infecção (Spivak & Hanson, 2018).

O complexo *Candida haemulonii*, embora menos prevalente que *C. auris*, tem emergido como um patógeno relevante, com casos relatados principalmente na América Latina e na Ásia (Ramos et al., 2022). Estudos indicam que *C. haemulonii* é frequentemente associado a infecções em pacientes com condições subjacentes crônicas e dispositivos invasivos, apresentando taxas de mortalidade semelhantes às de *C. auris* em casos de candidemia (Tsay et al., 2020).

Candida haemulonii foi primeiramente isolada a partir do intestino do peixe *Haemulon scirus*, em 1962 (Uden&Kolipinsky, 1962), sendo caracterizada como uma levedura e denominada inicialmente como *Torulopsis haemulonii*. Apenas em 1984 foi isolada a partir de cultura de sangue de um paciente com insuficiência renal (Lavard et al.,1984). É um patógeno emergente que, embora inicialmente identificado em ambientes marinhos, tornou-se uma preocupação significativa em contextos médicos devido à sua resistência intrínseca a antifúngicos, como anfotericina B e azóis. Estudos recentes destacam sua adaptação genética e plasticidade, características que compartilha com o patógeno relacionado, *C. auris*, sugerindo uma pressão evolutiva comum para resistência antifúngica e adaptação ambiental (Ahearn, 2023).

Além disso, análises genéticas e fenotípicas revelam uma capacidade marcante de formar biofilmes robustos em superfícies médicas, um fator crítico para infecções invasivas associadas a dispositivos médicos (Ramos et al., 2020). Apesar de raramente ser reportado anteriormente, o aumento de infecções por *C. haemulonii*, muitas vezes confundido com outras espécies de *Candida* por métodos fenotípicos convencionais, ressalta a necessidade de diagnósticos moleculares precisos para evitar falhas terapêuticas e conter a disseminação (Cao et al., 2023).

Infecções causadas por *C. haemulonii* e suas espécies relacionadas apresentam altas taxas de mortalidade, muitas vezes superiores a 50% em contextos hospitalares, sendo que no Brasil, a taxa geral de mortalidade por candidemia, incluindo *C. haemulonii*, é consistentemente alta, em torno de 51% a 58%, variando entre regiões e populações específicas (Braga et al., 2018; de Medeiros et al., 2019).

As taxas de mortalidade permanecem altas devido a diagnósticos tardios e limitações nos tratamentos eficazes, dados epidemiológicos recentes também sugerem que espécies emergentes, como *C. haemulonii*, continuam desafiando as estratégias de manejo clínico no país (Rodrigues et al., 2021).

Um estudo realizado no Rio de Janeiro em 2022 revelou que, entre 2011 e 2020, 64% dos isolados clínicos de *C. haemulonii* foram provenientes de amostras de sangue, trazendo sua relevância como agente causador de infecções graves na corrente sanguínea. Essa alta proporção reforça a importância de monitorar essa espécie em ambientes hospitalares, dada sua associação com infecções invasivas e sua resistência intrínseca a antifúngicos frequentemente usados (Pinho et al., 2024). Os isolados apresentaram elevada resistência ao fluconazol, itraconazol e anfotericina B, além de uma significativa capacidade de formação de biofilme, característica que contribui para sua resistência e persistência no ambiente hospitalar (Ramos et al., 2022).

Por outro lado, *C. auris*, é um problema de saúde pública mundial devido à sua resistência a múltiplos medicamentos e à sua rápida disseminação na última década. As razões para o surgimento desse fungo permanecem desconhecidas (Satoh et al., 2009). Contudo, uma análise retrospectiva identificou a presença de *C. auris* na Coreia do Sul já em 1996 (Kwon et al., 2019). Estudos indicam que *C. auris* emergiu de forma simultânea e independente em quatro regiões do mundo (Sul da Ásia, Leste da Ásia, África e América do Sul), formando os clados I, II, III e IV, respectivamente, geneticamente distintos entre si (Jackson et al., 2019). Em 2022 foi identificado um novo clado no Irã (Clado V) (Spruijtenburg et al., 2022), e há indícios de um possível sexto clado descrito na África Oriental (Figura 1), sugerindo uma expansão da diversidade genética de *C. auris* (Suphavilai et al., 2023).

A identificação precisa desses clados é essencial para a vigilância epidemiológica, pois ajudam a monitorar surtos e padrões regionais de disseminação, resistência antifúngica, e impacto clínico (Tabela 1) (Carolus et al., 2021; Kappel et al., 2024).

Tabela 1. Quadro demonstrativo com a localização predominante, características epidemiológicas e referências relacionadas aos cinco principais clados de *C. auris*.

Clado	Região Predominante	Características Principais	Resistência Antifúngica	Referência
Clado I	Sul da Ásia (Índia, Paquistão)	Associado a surtos hospitalares; alta persistência em superfícies, facilitando transmissão em ambientes hospitalares.	Alta resistência ao fluconazol.	(Lockhart et al., 2017)
Clado II	Japão, Coreia do Sul	Menor resistência antifúngica; frequentemente identificado em casos de otite externa, com potencial para infecções invasivas.	Menor resistência antifúngica em comparação com outros clados.	(Ahmadi et al., 2023)
Clado III	África do Sul	Associado a candidemias em pacientes imunocomprometidos; contribui para infecções em UTI.	Alta resistência à anfotericina B.	(van Schalkwyk et al., 2019)
Clado IV	Colômbia, Venezuela	Surtos hospitalares frequentes; resistência significativa ao fluconazol.	Resistência significativa ao fluconazol.	(Escandón et al., 2018)
Clado V	Irã, Oriente Médio	Recente identificação; resistência e dispersão peculiares; dados limitados, mas requer vigilância para prevenir surtos locais.	Resistência variada, com características ainda sendo estudadas.	(Ahmadi et al., 2023)

C. auris é um patógeno de grande preocupação clínica, esse fungo é responsável por infecções invasivas, como otites, infecções de feridas e infecções da corrente sanguínea. No entanto, sua sobrevivência em regiões anaeróbicas, como o intestino e mucosas, é limitada, o que distingue sua capacidade de colonização em comparação com outras espécies de *Candida* (Lockhart et al., 2017). As infecções causadas por *C. auris* têm aumentado globalmente (Osei Sekyere, 2018), sendo considerado endêmico em partes da África e da Ásia (Hinrichs et al., 2022).

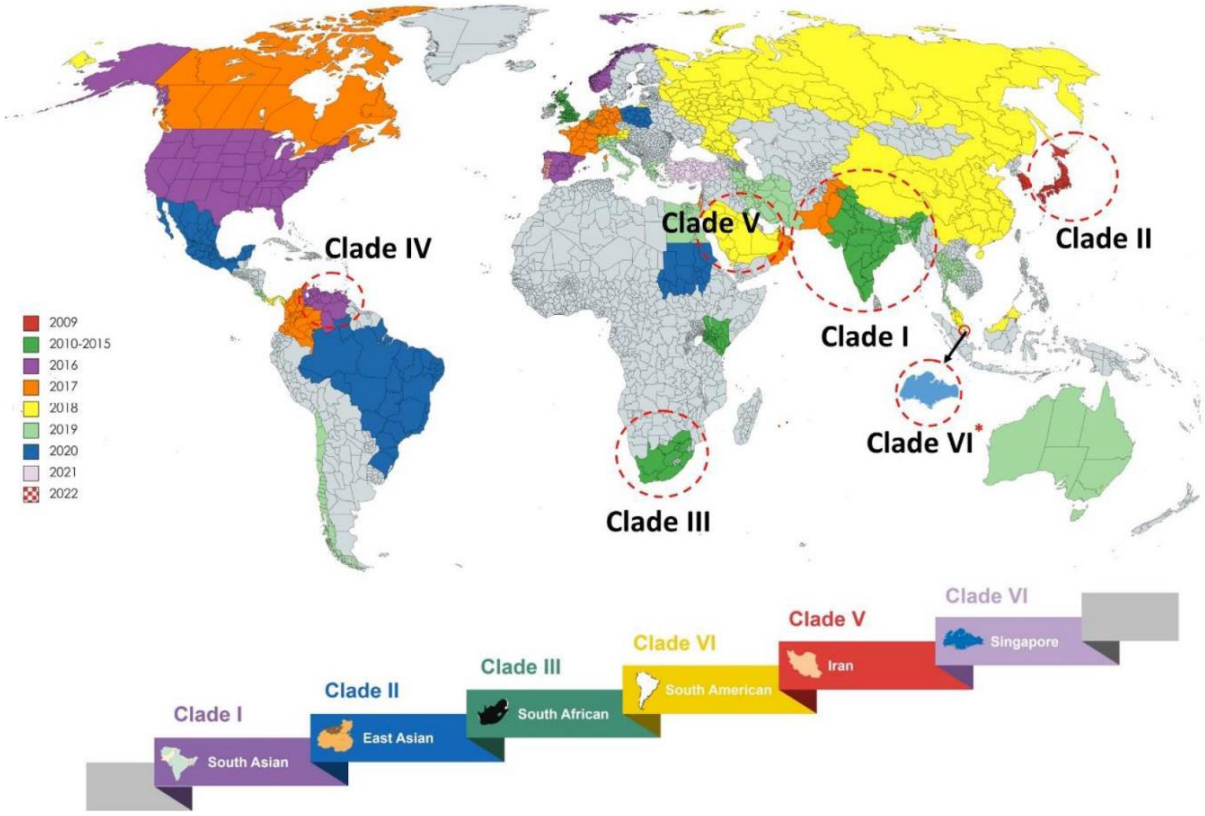

Figura 1. Ilustração do padrão de distribuição de *C. auris* em diferentes regiões geográficas ao redor do mundo. Os dados apresentados aqui são baseados em informações de publicações. Um esquema de cores foi utilizado para representar o ano em que *C. auris* foi relatado. Mais de 40 países reportaram casos positivos de *C. auris* entre 2009 e 2022. Até 2022, cinco clados haviam sido relatados mundialmente; no entanto, dados publicados recentemente indicam a existência de um sexto clado, originado em Singapura. Fonte: (Arumugam et al., 2024).

Surtos de *C. auris* foram relatados em diversos países europeus, incluindo Reino Unido, Espanha e Itália (Di Pilato et al., 2021; Hinrichs et al., 2022; Kohlenberg et al., 2018; Osei Sekyere, 2018). No Brasil, *C. auris* foi identificada pela primeira vez em 2020, quando foi isolada como colonizadora em dois pacientes com COVID-19 em Salvador, Bahia. Um dos casos envolveu amostra de ponta de cateter venoso central, enquanto o outro foi identificado em hemocultura (de Almeida et al., 2021a). A cepa brasileira inicial mostrou-se suscetível a antifúngicos, e a análise genética revelou semelhança com cepas do clado sul-asiático (de Jong et al., 2021). Posteriormente, um surto ocorreu em uma UTI também em Salvador, onde foram identificadas duas cepas clonais de *C. auris* com baixa suscetibilidade a antifúngicos como fluconazol e anfotericina B (de Almeida et al., 2021).

Outro surto expressivo foi registrado entre dezembro de 2021 e março de 2022, sendo o maior documentado no Brasil até o momento. Nesse evento, *C. auris* foi

isolada em 11 pacientes, com a capacidade de formação de biofilmes ressaltada como um fator que contribui para sua resistência e propagação em ambientes hospitalares, dificultando o controle da infecção (de Melo et al., 2023).

Em geral, a candidemia causada por *C. auris* ocorre secundária à colonização; portanto, identificar essa espécie em fases iniciais e compreender os fatores de risco em pacientes colonizados é fundamental para o diagnóstico precoce e a prevenção de infecções invasivas (Briano et al., 2022; Prestel et al., 2021). Em dezembro de 2022, dois anos após o surto em Salvador, *C. auris* foi novamente isolada em um hospital público de Recife, Pernambuco. Esse hospital, um dos maiores da região Nordeste, possui 704 leitos e é referência em várias especialidades, como trauma e cirurgia geral (de Melo et al., 2023).

A resistência a medicamentos como fluconazol e anfotericina B, observada em cepas brasileiras, restringe as opções terapêuticas para o tratamento de infecções invasivas. Além disso, a capacidade de *C. auris* e de espécies pertencentes ao complexo *C. haemulonii* de persistirem em superfícies hospitalares facilita sua disseminação rápida, mesmo em locais onde são seguidas práticas rigorosas de controle de infecções (Figura 2) (Forsberg et al., 2019; Silva et al., 2023).

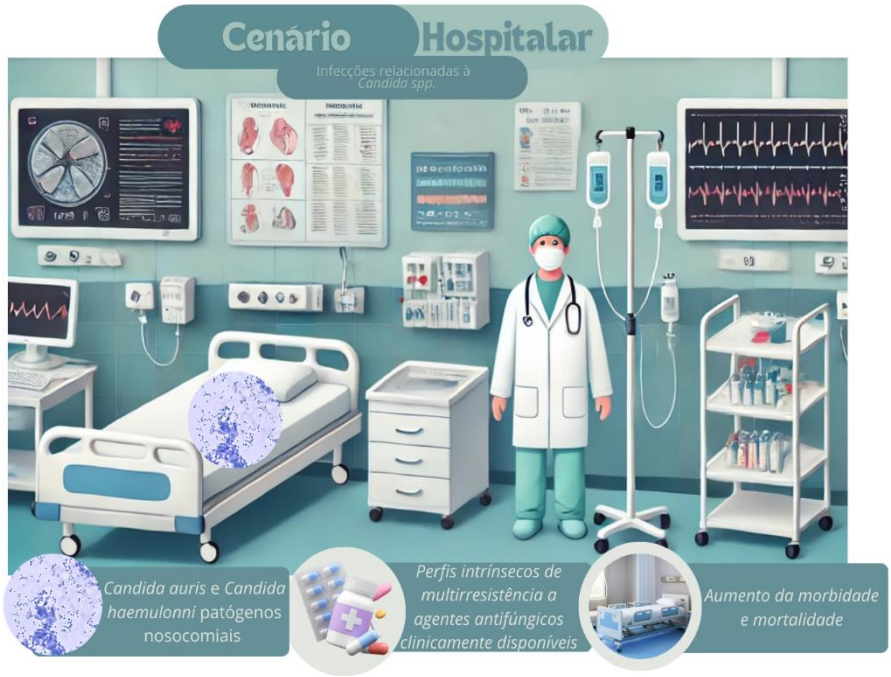

Figura 2. *Cenário hospitalar com infecções por isolados de C. auris e de espécies pertencentes ao complexo C. haemulonii. Fonte: Próprio autor, baseando em (Ben-Ami & Kontoyiannis, 2021; Ramos et al., 2022).*

2.3. Perfil de resistência de *C. auris* e de espécies pertencentes ao complexo *C. haemulonii*

O perfil de resistência a múltiplos antifúngicos em isolados do complexo *C. haemulonii* e de *C. auris*, representa um desafio significativo para o manejo terapêutico das infecções associadas a essas espécies emergentes. Até o momento, não há pontos de corte padronizados para a suscetibilidade antifúngica de *C. auris* estabelecidos pelo *Clinical and Laboratory Standards Institute* (CLSI) (CLSI, 2017).

Com o objetivo de estabelecer parâmetros de corte para os antimicrobianos e orientar possíveis tratamentos contra esses microrganismos, o Centers for Disease Control and Prevention (CDC) propôs critérios específicos com base na relação filogenética de *C. auris* com outras espécies de *Candida*. Entretanto, a relação entre esses pontos de corte microbiológicos e os desfechos clínicos ainda não está totalmente esclarecida. Estudos recentes indicam que aproximadamente 90% dos isolados de *C. auris* são resistentes a pelo menos uma classe de antifúngicos, e aproximadamente 30% apresentam resistência a múltiplas classes, criando um cenário alarmante em relação às opções de tratamento disponíveis (Kainz et al., 2020).

Para *C. auris*, os pontos de corte para antifúngicos, segundo as diretrizes mais recentes do CDC, incluem resistência ao FLZ com CIMs ≥ 32 µg/mL e à AmB com CIMs ≥ 2 µg/mL. A resistência às equinocandinas, geralmente observada com CIMs ≥ 2 µg/mL para CSF, está frequentemente associada a mutações no gene FKS1, com CIMs ≥ 4 µg/mL para MCF e ADF (Tabela 2) (CDC, 2024), reforçando a necessidade de alternativas terapêuticas eficazes (Li et al., 2021).

A resistência a múltiplas drogas em *C. auris* também tem sido observada em outras espécies relacionadas, como aquelas do complexo *C. haemulonii*, nas quais a resistência cruzada está se tornando mais reconhecida. Pesquisas que avaliam a suscetibilidade *in vitro* de diferentes antifúngicos contra isolados do complexo *C. haemulonii* sugerem que equinocandinas, como micafungina (MCF) e anidulafungina (ADF), são mais eficazes do que a caspofungina (CSF) para algumas cepas. No entanto, a capacidade dessas leveduras de se adaptar rapidamente e sobreviver pode favorecer a resistência em contextos clínicos (Muñoz et al., 2020; Ramos et al., 2022).

Isolados do complexo *C. haemulonii* frequentemente apresentam resistência a antifúngicos de primeira linha, como anfotericina B (AmB) mostra valores de CIM

variando de 2 a >16 µg/mL e fluconazol (FLZ) exibem CIMs superiores a 64 µg/mL, com valores de CIM significativamente elevados, ultrapassando os pontos de corte clínicos estabelecidos para outras espécies de *Candida* (Ramos et al., 2020).

Além disso, *C. auris* pode desenvolver resistência às equinocandinas, especialmente à CSF, frequentemente associada a mutações específicas no gene FKS1. Estudos indicam que o aumento da resistência ao FLZ em *C. auris* pode ocorrer devido a duplicações cromossômicas, que intensificam a expressão de genes de resistência, resultando em CIMs elevadas após exposição prolongada ao antifúngico (Narayanan et al., 2021).

Tabela 2. Pontos de corte dos antifúngicos frente a isolados de *C. auris* e de espécies pertencentes ao complexo *C. haemulonii*.

Espécies	Antifúngico	MIC (µg/mL)
C. haemulonii	FLZ	≥ 64
	AmB	2 à ≥ 16
	MCF/ADF	0.25/0.16
C. auris	FLZ	≥ 32
	AmB	≥ 2
	CSF	≥ 2
	MCF	≥ 4
	ADF	≥ 4

Fluconazol (FLZ), Anfotericina B (AmB), Micafungina, (MCF), Anidulafungina (ADF), Caspofungina (CSF). Os pontos de corte de concentração inibitória mínima (CIM) estão expressos em µg/mL. Os dados de resistência foram baseados nas diretrizes do Centers for Disease Control and Prevention (CDC) (CDC, 2024).

2.4. Tratamentos de infecções causadas por *C. auris* e de espécies pertencentes ao complexo *C. haemulonii*

O tratamento atual para *C. auris* são as equinocandinas (como caspofungina e micafungina) consideradas tratamento de primeira linha devido à sua eficácia contra a maioria dos isolados, embora casos de resistência a esta classe também tenham sido relatados, o que justifica o uso alternativo de anfotericina B lipossomal ou isavuconazol em situações específicas (Barantsevich & Barantsevich , 2022) . No caso

do complexo *C. haemulonii*, caracterizado por resistência frequente ao fluconazol e à anfotericina B, as equinocandinas também são o tratamento de escolha, com relatos de sucesso em casos clínicos, no entanto, devido ao aumento de cepas resistentes e à limitação de opções antifúngicas, há um interesse crescente no desenvolvimento de novas terapias, incluindo agentes antifúngicos de nova geração e preparações farmacêuticas, bem como o uso de diagnósticos rápidos para melhoria do manejo clínico (Ben -Ami , 2018).

Isolados multirresistentes (MDR) e pan-resistentes (PDR) de *C. auris* são cada vez mais frequentes em todo o mundo, com a resistência ao fluconazol (FLZ), anfotericina B (AmB) e voriconazol (VRC) (Maphanga et al., 2021). Diante desse cenário, as equinocandinas têm sido amplamente recomendadas para o tratamento de infecções causadas por espécies de *Candida*, devido à sua eficácia comprovada contra a maioria dessas espécies. Aproximadamente 60-80% dos casos de candidemia, especialmente entre pacientes imunocomprometidos, recebem tratamento inicial com equinocandinas, que atuam inibindo a síntese de 1,3-β-glucano, um componente essencial da parede celular fúngica (Worku & Girma, 2020).

Embora as equinocandinas sejam o tratamento preferencial, a resistência a essa classe de medicamentos também tem sido documentada. Assim, as principais opções terapêuticas para infecções por *C. auris* incluem equinocandinas ou AmB, que são as recomendações clínicas mais comuns (Kumar et al., 2016). O ibrexafungerp, derivado da enfumafungina, tem sido introduzido como opção para tratar infecções causadas por espécies de *Candida* resistentes a múltiplos medicamentos, uma vez que inibe a síntese de β-1,3-D-glucano de forma distinta das equinocandinas, oferecendo uma alternativa terapêutica promissora (Jallow & Govender, 2021).

O entendimento dos mecanismos de ação das principais classes dos antifúngicos é fundamental para otimizar o tratamento e contornar desafios relacionados à resistência antifúngica (Perlin et al., 2017). Os polienos, como a anfotericina B, atuam primariamente na membrana celular dos fungos, ligando-se ao ergosterol, um componente essencial da membrana fúngica. Essa interação resulta na formação de poros que desestabilizam a integridade da membrana, levando à perda de íons e metabólitos essenciais, culminando na morte celular (Gintjee et al., 2020).

Os azóis, incluindo fluconazol, voriconazol e posaconazol, inibem a enzima lanosterol 14-α-desmetilase, fundamental para a biossíntese do ergosterol. A inibição

dessa via metabólica resulta na acumulação de esteróis tóxicos e na disfunção da membrana celular, prejudicando o crescimento e a viabilidade fúngica (Berkow & Lockhart, 2017). Já a classe das equinocandinas, como caspofungina, micafungina e anidulafungina, atuam inibindo a β-1,3-D-glucano sintase, enzima crítica para a síntese da parede celular fúngica. A inibição dessa enzima compromete a integridade da parede celular, levando à lise osmótica e morte celular (Szymański et al., 2022).

O tratamento de infecções fúngicas causadas por *C. auris* e pelo complexo *C. haemulonii* enfrenta desafios significativos devido as limitações terapêuticas associadas à toxicidade dos antifúngicos, à eficácia reduzida contra biofilmes e à emergência de resistência antifúngica. Esses fatores contribuem para o aumento da morbidade e mortalidade, especialmente em pacientes imunocomprometidos (Li et al., 2022).

A toxicidade representa uma limitação crítica no uso de antifúngicos, como a anfotericina B, que está associada a nefrotoxicidade significativa, mesmo em formulações lipossomais (Schlottfeldt et al., 2015). Azóis, como o voriconazol, podem causar hepatotoxicidade, alterações visuais e efeitos colaterais neurotóxicos, enquanto as equinocandinas, embora geralmente bem toleradas, podem ocasionar elevações em enzimas hepáticas e reações infusionais (Pappas et al., 2018).

A eficácia reduzida dos antifúngicos em biofilmes formados por *Candida auris* e pelo complexo *Candida haemulonii* representa um dos principais desafios no tratamento clínico das infecções fúngicas. A capacidade dessas espécies de formar biofilmes robustos em superfícies abióticas e em dispositivos médicos invasivos está diretamente associada à persistência das infecções e à resistência aos tratamentos convencionais (Kean et al., 2018; Tsay et al., 2020).

As espécies do complexo *C. haemulonii*, tem demonstrado resistência ao FLZ e AmB, ou ainda a itraconazol, voriconazol, posaconazol, terbinafina e até mesmo às equinocandinas (Ramos et al., 2022). Essas características tornam o tratamento de infecções causadas por essas espécies extremamente desafiador, reforçando a necessidade de alternativas terapêuticas e de uma vigilância contínua da resistência antifúngica.

Identificação precisa e rápida dessa espécie por meio de métodos moleculares é essencial para evitar atrasos no tratamento e melhorar os resultados clínicos. A vigilância contínua e o monitoramento das características de resistência são

fundamentais para lidar com infecções por *C. haemulonii* e outras leveduras emergentes como *C. auris* (Pinho et al., 2024).

Esse cenário ressalta a urgência de desenvolvimento de estratégias terapêuticas inovadoras e do fortalecimento do monitoramento da resistência antifúngica, visando garantir a eficácia dos tratamentos para infecções causadas por essas leveduras emergentes e altamente resistentes.

2.5. Desafios na erradicação de *C. auris* da pele e de dispositivos médicos

A persistência de *C. auris* em dispositivos médicos e sua resistência a tratamentos antifúngicos apresentam desafios consideráveis, cujos mecanismos subjacentes ainda não são totalmente compreendidos. A formação de biofilmes robustos, especialmente em dispositivos médicos, desempenha um papel fundamental nesse processo. A matriz extracelular (ECM) do biofilme de *C. auris* constitui uma rede complexa de polímeros extracelulares, rica em polissacarídeos, proteínas, lipídios e ácidos nucleicos, que confere estrutura, proteção e resistência contra tratamentos antifúngicos e desinfetantes (Mitchell et al., 2015)

Os biofilmes são comunidades microbianas organizadas que se formam em superfícies abióticas e bióticas, envoltas por uma matriz extracelular, este modo de crescimento é o estado predominante para microrganismos em nichos ecológicos naturais (Nobile & Johnson, 2015). No ambiente clínico, os biofilmes podem ser desenvolvidos em tecidos humanos, como camadas mucosas, ou em dispositivos médicos implantados, como cateteres venosos centrais, esses biofilmes frequentemente funcionam como fontes de infecção que podem disseminar microrganismos para outras partes do corpo(Hall-Stoodley et al., 2004).

No caso de *Candida auris*, foi demonstrado que essa espécie é capaz de formar biofilmes em superfícies, embora estes sejam estruturalmente mais frágeis em comparação aos biofilmes de *Candida albicans*, apesar disso, as células em biofilme de *C. auris* apresentam níveis elevados de resistência a agentes antifúngicos, semelhantes aos biofilmes de *C. albicans*, superando significativamente a resistência das células planctônicas (Oh et al., 2010).

A capacidade de formação de biofilmes em *C. auris* varia entre diferentes isolados e clados. Ambas as formas celulares da espécie, agregadas e não agregadas, podem formar biofilmes; entretanto, as células não agregadas demonstram produzir

biofilmes mais robustos (Singh et al., 2019). Estudos de transcrição ao longo do tempo identificaram genes relacionados à adesão, bombas de efluxo e fatores de virulência como sendo regulados positivamente durante o desenvolvimento do biofilme de *C. auris*, embora ainda sejam menos compreendidos em comparação aos biofilmes de outras espécies do gênero *Candida*, os biofilmes de *C. auris* estão claramente associados à sua virulência, resistência antifúngica e capacidade de sobrevivência tanto no ambiente quanto no hospedeiro (Kean et al., 2018).

Estudos indicam que *C. auris* pode sobreviver em diversos materiais, incluindo polietileno e superfícies metálicas, mesmo em condições de baixa umidade (Ledwoch & Maillard, 2018; Lockhart et al., 2017). A presença de dispositivos médicos feitos desses materiais em pacientes pode atuar como um veículo para a transmissão de infecções graves, como candidíase invasiva e candidemia (Horton & Nett, 2020). Comparativamente a *C. albicans*, *C. auris* forma biofilmes mais densos em cateteres, como demonstrado em estudos com modelos murinos. A ECM mais densa de *C. auris* favorece sua adesão e persistência nessas superfícies (Vila et al., 2020).

Enquanto alguns estudos sugerem que os mecanismos de sobrevivência de *C. auris* em dispositivos médicos e na pele humana ou animal ainda não são completamente elucidados (Heaney et al., 2020), outros apontam que essa persistência está relacionada à formação de biofilmes, que protegem o microrganismo contra desinfetantes e tratamentos antifúngicos, permitindo sua sobrevivência prolongada nessas superfícies. A produção de moléculas de adesão e a capacidade de adaptação ao microambiente também são sugeridas como possíveis mecanismos de sobrevivência (Garcia-Bustos et al., 2020). Essa adaptabilidade de *C. auris* ao ambiente favorece sua permanência em superfícies inertes e áreas da pele menos expostas, como dobras cutâneas, axilas, áreas interdigitais e regiões inguinais (Garcia-Bustos et al., 2020).

Em pacientes imunocomprometidos, *C. auris* não é normalmente parte da microbiota, mas pode colonizar a pele ou regiões mucosas como contaminante. Diferente de outras espécies de *Candida*, *C. auris* raramente é isolada da região entérica, embora sua presença em áreas cutâneas possa resultar em infecções graves caso a integridade dessas barreiras esteja comprometida (Garcia-Bustos et al., 2020). O contato direto com superfícies de pele contaminadas ou o uso de dispositivos médicos reutilizáveis, como termômetros e esfigmomanômetros, facilita o

desenvolvimento de biofilmes de *C. auris*, contribuindo para sua transmissão e persistência em ambientes hospitalares (Horton & Nett, 2020).

2.6. Reposicionamento de fármacos para leveduras

Devido às crescentes dificuldades no tratamento de infecções fúngicas e ao tempo prolongado necessário para o desenvolvimento de novos medicamentos, o reposicionamento de fármacos constitui uma alternativa terapêutica promissora para ser explorada (Rudrapal et al., 2020). Essa estratégia busca novas indicações para moléculas que já passaram por todas as fases de pesquisa clínica e aprovação regulatória, aproveitando compostos bioativos cujos perfis de segurança, eficácia e efeitos adversos já são bem conhecidos (Rossato et al., 2021; Rudrapal et al., 2020).

Conforme ilustrado na Figura 3, diferentes fármacos não antifúngicos apresentam mecanismos de ação variados que podem ser explorados contra patógenos resistentes. O reposicionamento de fármacos pode acelerar significativamente o desenvolvimento, reduzindo o tempo de 12-15 anos para apenas 12-18 meses, além de diminuir os custos, que no desenvolvimento completo e testes clínicos variam de 350 milhões a 2 bilhões de dólares por molécula (Trivedi et al., 2020).

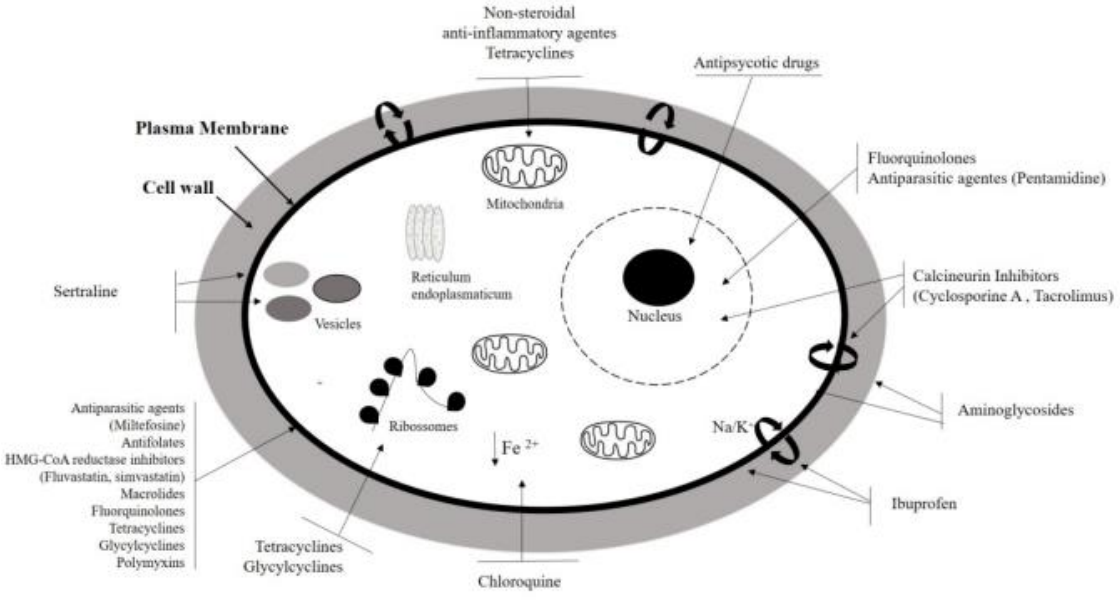

Figura 3.Estrutura celular destacando os alvos de ação de diferentes classes de fármacos. À imagem apresenta uma célula fúngica com várias organelas e partes estruturais, destacando os alvos específicos de diferentes classes farmacológicas. Os alvos incluem a membrana plasmática, parede celular, núcleo, ribossomos, mitocôndria, vesículas, retículo endoplasmático e íons intracelulares como o ferro (Fe^{2+}) e canais de sódio e potássio (Na/K). Representando mecanismo de ação de fármacos não antifúngicos, como antibióticos (tetraciclinas, glicilciclinas, aminoglicosídeos, fluoroquinolonas), agentes antiparasitários (miltefosina, pentamidina), antipsicóticos, anti-inflamatórios não esteroidais (AINEs),

imunossupressores (inibidores da calcineurina) e antidepressivos (sertralina). Fonte: (Rossato et al., 2021).

Casos bem-sucedidos de reposicionamento de fármacos demonstram como medicamentos originalmente desenvolvidos para uma finalidade específica podem ser aplicados em novos contextos terapêuticos. Esses exemplos ilustram a flexibilidade e o potencial dessa estratégia no desenvolvimento de tecnologias inovadoras. Alguns dos casos mais notáveis estão na (Tabela 3).

Tabela 3. A tabela apresenta uma seleção de fármacos que foram reposicionados para novos usos terapêuticos, detalhando sua aplicação original, as indicações atuais e referências relevantes.

Fármaco	Indicação Original	Nova Indicação	Referência	Testes Clínicos
Aspirina	Analgésico/Anti-inflamatório	Prevenção de Eventos Cardiovasculares	(Zhou et al., 2015)(Zhou et al., 2015)	Sim
Atorvastatina	Hiperlipidemia	Redução de Risco em Doenças Inflamatórias	(Elisi et al., 2018)	Sim
Cloroquina e Hidroxicloroquina	Malária	Doenças autoimunes (lúpus, artrite reumatóide)	(Schrezenmeier & Dörner, 2020)	Sim
Metformina	Diabetes Tipo 2	Câncer e Envelhecimento	(Panchapakesan & Pollock, 2018)	Sim
Minoxidil	Hipertensão	Tratamento de Alopecia	(Baker et al., 2018)	Sim
Sildenafil	Hipertensão Pulmonar	Disfunção erétil	(Pushpakom et al., 2018)	Sim
Talidomida	Sedativo/Náuseas na Gravidez	Mieloma múltiplo	(Cha et al., 2018)	Sim
Sertralina	Antidepressivo	Terapia Adjuvante em Câncer	(Gowri et al., 2020)	Sim
Amilorida	Diurético	Fibrose cística	(knowles et al., 1990)	Sim

Além de ser uma via mais rápida, o reposicionamento de fármacos oferece uma opção econômica e segura, pois as cadeias de produção e distribuição já estão estabelecidas, e estudos de fases I e II podem ser dispensados, devido ao conhecimento prévio dos perfis farmacocinéticos e farmacodinâmicos dos compostos (Majumder et al., 2020). Essa abordagem é especialmente relevante no tratamento de infecções fúngicas resistentes, como as causadas por *C. auris*, estudos pré-clínicos sugerem que fármacos desenvolvidos originalmente para outras finalidades, como agentes anticâncer, imunomoduladores e antibióticos, podem apresentar atividade antifúngica, oferecendo novas alternativas para o manejo dessas infecções (Peyclit et al., 2021).

Os antifúngicos convencionais, como os azóis, polienos e equinocandinas, agem principalmente em componentes essenciais da célula fúngica, como a membrana e a parede celular, os azóis, por exemplo, inibem a síntese de ergosterol, um componente vital da membrana celular dos fungos, enquanto os polienos, como a anfotericina B, se ligam ao ergosterol, desestabilizando a membrana e levando ao vazamento de componentes celularess , as equinocandinas, por sua vez, atuam inibindo a síntese de 1,3-β-glucano, um polímero essencial da parede celular fúngica (Johnson, 2021) . No entanto, a eficácia desses antifúngicos é frequentemente limitada devido ao desenvolvimento de resistência, o que torna o reposicionamento de fármacos uma estratégia relevante para expandir os mecanismos de ação disponíveis e superar a resistência (Hua et al., 2022).

O reposicionamento de fármacos também pode aumentar a eficácia dos tratamentos quando combinado com antifúngicos tradicionais, ajudando a reduzir o risco de desenvolvimento de resistência, essa estratégia já tem sido explorada com sucesso em outras áreas da medicina, além disso, o uso de tecnologias emergentes, como inteligência artificial e modelagem computacional, pode facilitar a descoberta de novas indicações terapêuticas, otimizando o potencial terapêutico das moléculas existentes (Trivedi et al., 2020).

Uma das principais vantagens do reposicionamento é a redução substancial do tempo necessário para que um medicamento chegue ao mercado, enquanto o desenvolvimento de novas moléculas pode levar de 10 a 15 anos, o reposicionamento pode reduzir esse período para 3 a 6 anos, uma vez que muitos testes pré-clínicos e

de segurança já foram realizados (Peyclit et al., 2021). Além disso, os custos associados ao reposicionamento são significativamente menores, variando entre 60% a 70% abaixo dos investimentos necessários para o desenvolvimento de novos compostos (Rossato et al., 2021). Outra vantagem relevante é o aumento da taxa de sucesso clínico, medicamentos reposicionados possuem perfis farmacocinéticos e toxicológicos bem estabelecidos, o que reduz o risco de falhas em fases avançadas de ensaios clínicos. Além disso, essa estratégia permite explorar sinergias terapêuticas quando combinada com antifúngicos convencionais, aumentando a eficácia contra patógenos resistentes (Eldesouky et al., 2020).

Apesar das vantagens, o reposicionamento de fármacos também apresenta limitações, uma delas é a restrição de patentes e direitos de propriedade intelectual, que podem desestimular o investimento por parte da indústria farmacêutica (Hua et al., 2022). O conhecimento prévio do perfil de segurança dos medicamentos reposicionados é um fator crucial para acelerar sua aprovação clínica, fármacos com histórico de uso seguro em humanos permitem uma transição mais rápida para as fases clínicas, com a possibilidade de iniciar diretamente em estudos de fase II, reduzindo o tempo e os recursos necessários (Ioakeim-Skoufa et al., 2023).

2.7. Agentes antifúngicos tópicos para *C. auris*

A erradicação de *C. auris* utilizando compostos tópicos é uma abordagem promissora, especialmente devido à resistência intrínseca desse fungo a diversos antifúngicos convencionais e sua capacidade de persistir em superfícies hospitalares. Compostos tópicos, como soluções à base de clorexidina e peróxido de hidrogênio estabilizado, têm demonstrado eficácia na redução da colonização cutânea e na descontaminação ambiental de *C. auris* em contextos hospitalares, quando utilizados adequadamente (Biswal et al., 2017).

Além disso, óleos essenciais como o de *Thymus vulgaris* e *Lavandula dentata* mostraram propriedades antifúngicas significativas contra biofilmes de *Candida* spp., incluindo *C. auris*, devido à sua capacidade de destruir membranas celulares e inibir a formação de biofilmes (Karpiński et al., 2021). A implementação de agentes tópicos, tanto naturais quanto sintéticos, associados a práticas rigorosas de higiene, é crucial

para prevenir a transmissão e erradicar esse patógeno multirresistente de superfícies e pacientes colonizados(Ji & Ye, 2024).

Na busca por compostos com potencial para combater isolados de *C. auris*, agentes antifúngicos tópicos têm se destacado como uma abordagem promissora, especialmente para infecções cutâneas e superficiais, onde atuam como agentes de descolonização. Aplicados diretamente sobre a pele, esses agentes oferecem concentrações elevadas do fármaco no local da aplicação, o que ajuda a superar as barreiras impostas pela resistência intrínseca de *C. auris* a muitos tratamentos sistêmicos (Hashemi et al., 2018). A eficácia desses tratamentos tópicos é essencial para o manejo de infecções localizadas e para prevenir sua disseminação em ambientes hospitalares (Wasi et al., 2019).

2.7.1. Piritionato de zinco (ZnPT)

O piritionato de zinco (ZnPT), também conhecido como bis(N-oxopiridina-2-tionato) zinco (II), é um complexo com dois ligantes de piritiona quelados a íons de zinco (Figura 4) (Jo et al., 2006). Este composto possui um amplo espectro de atividade antimicrobiana, atuando contra bactérias, algas e fungos, e previne a formação de biofilmes em superfícies expostas ao ambiente (Turley et al., 2005; Yebra et al., 2004). O ZnPT é apresentado como micropartículas para aplicação no epitélio da pele e é amplamente utilizado em shampoos medicinais para o tratamento de caspa e dermatite seborreica, uma inflamação caracterizada por descamação, eritema e prurido (Mangion et al., 2021).

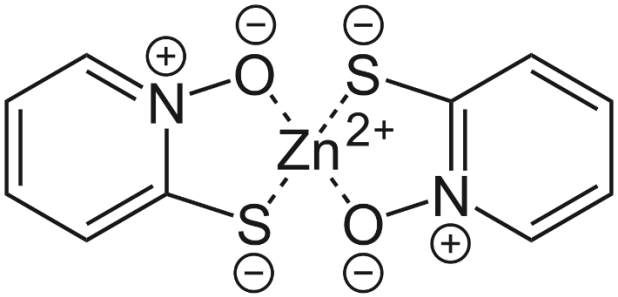

Figura 4. *Estrutura molecular de piritionato de zinco (ZnPT). Fonte: Disponível em: https://commons.wikimedia.org/w/index.php?curid=4548132. (Acesso em: 06 out. 2024).*

Apesar de sua eficácia, o uso contínuo de ZnPT levanta preocupações quanto à toxicidade, tanto para organismos aquáticos quanto para mamíferos. Em experimentos com peixes de água doce, o ZnPT induziu estresse oxidativo,

neurotoxicidade e alterações no comportamento alimentar após exposição crônica, mesmo em concentrações subletais (Falcão et al., 2019). Estudos em *zebrafish* adultos também mostraram que o ZnPT pode causar hepatotoxicidade, com impactos significativos no metabolismo hepático, diferenciando-se entre machos e fêmeas e afetando vias de glicogenólise e metabolismo oxidativo (Zhao et al., 2020).

A eficácia antifúngica do ZnPT tem sido comprovada em diversos contextos. Sua ação está relacionada à interferência no transporte de íons e metabólitos pela membrana celular, o que leva à disfunção metabólica e à inibição do crescimento fúngico. Além disso, o ZnPT provoca um influxo de cobre nas células, inativando proteínas que contêm clusters de ferro-enxofre, essenciais para a sobrevivência celular. Esses mecanismos fazem do ZnPT um candidato promissor para o tratamento de infecções fúngicas resistentes, incluindo aquelas causadas por *C. auris* (Reeder et al., 2011).

Estudos *in vitro* com linfócitos humanos demonstraram que o ZnPT pode causar estresse oxidativo e citotoxicidade em concentrações elevadas, aumentando a produção de espécies reativas de oxigênio (ROS) e impactando a viabilidade celular. No entanto, o ZnPT não apresentou genotoxicidade significativa em concentrações mais baixas, sugerindo que sua toxicidade está associada ao estresse oxidativo em sistemas biológicos (Marcheselli et al., 2011).

Recentemente, novas formulações de ZnPT têm sido investigadas para aprimorar sua eficácia, incluindo sua conjugação com outros compostos para aumentar a penetração no tecido e a afinidade pelas mitocôndrias dos fungos, como demonstrado em estudos de terapia fotodinâmica contra *C. albicans* (Tang et al., 2020). Em um estudo prospectivo, o ZnPT mostrou resultados promissores no tratamento de dermatoses como psoríase, dermatite seborreica e pitiríase versicolor, todas associadas à presença de *Malassezia restricta* e *Malassezia globosa*. O estudo revelou uma redução significativa da colonização fúngica após o uso tópico de ZnPT, com alta taxa de eficácia e ausência de efeitos adversos graves (Samtsov et al., 2023).

2.7.2. Nistatina (NYS)

A nistatina (NYS) é um macrolídeo poliênico ativo de membrana (Figura 5) produzido por cepas de *Streptomyces noursei* e está disponível em várias formas, como suspensão oral, creme tópico e pastilhas orais (Wong et al., 2014). Quando

administrada oralmente, este fármaco não é absorvido pelo trato gastrointestinal (Samaranayake et al., 2009), tornando o uso tópico a via mais comum em odontologia, pois minimiza a exposição sistêmica. Além disso, a nistatina desempenha um papel importante na profilaxia da candidíase oral e sistêmica em recém-nascidos a termo e prematuros, bebês e pacientes imunocomprometidos (por exemplo, pacientes com AIDS, câncer ou transplantados), devido à baixa incidência de interações medicamentosas e ao custo acessível, especialmente em países em desenvolvimento (Fernandez et al., 2012).

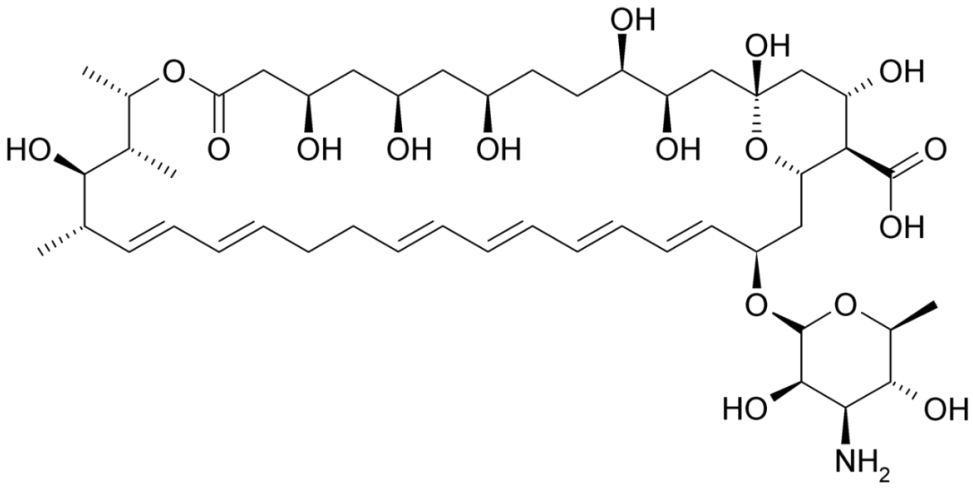

Figura 5. Estrutura molecular de nistatina (NYS). Fonte: (Volpon & Lancelin, 2002).

A nistatina, um antifúngico da classe dos polienos, é amplamente utilizada no tratamento de infecções causadas por *Candida* spp. No entanto, sua toxicidade limita o uso em algumas situações clínicas (AMIR et al., 2022). Estudos recentes têm investigado novas formulações, incluindo derivados de amida e formulações com deoxicolato, para melhorar sua solubilidade e reduzir a toxicidade, um derivado de amida da nistatina demonstrou uma toxicidade 13,5 vezes menor em comparação com a nistatina pura, sendo considerado uma alternativa promissora para futuros desenvolvimentos farmacêuticos (AMIR et al., 2022). Formulações de nistatina em hidrogéis e sistemas micelares também foram desenvolvidas para administração tópica, visando aumentar a eficácia e reduzir efeitos colaterais, demonstrando melhores resultados no tratamento de infecções fúngicas com menor toxicidade em modelos animais, o que sugere uma alternativa segura e eficaz para uso clínico (AbouSamra et al., 2019).

Estudos recentes exploraram novas formulações e combinações de nistatina para ampliar sua eficácia e biodisponibilidade, a combinação de nistatina com

maltodextrina, por exemplo, mostrou-se eficaz na inibição de mais de 80% da formação de biofilmes de *C. albicans*, indicando uma melhoria significativa na atividade antifúngica quando administrada como dispersão sólida (Benavent et al., 2021). Outro estudo destacou o desenvolvimento de sistemas micelares à base de deoxicolato, que aumentaram a penetração da nistatina em tubos endotraqueais e potencializaram sua atividade antifúngica, especialmente contra biofilmes de *C. albicans* (Benavent et al., 2019).

Embora a nistatina seja amplamente utilizada no tratamento da candidíase oral e sistêmica, especialmente em populações vulneráveis como recém-nascidos, bebês e pacientes imunocomprometidos(Marzban et al., 2022). Estudos sobre sua eficácia específica contra *C. auris* ainda são limitados.

2.8. Tafenoquina (TAF) e Alexidine (ALX)

O reposicionamento de agentes farmacológicos surge como uma abordagem promissora para acelerar a disponibilidade de novas opções de tratamento para infecções por *Candida*, especialmente em casos que as terapias convencionais são ineficazes. Essa estratégia não apenas agiliza o desenvolvimento clínico, mas também amplia as alternativas terapêuticas para enfrentar os desafios impostos pela resistência fúngica aos medicamentos (Eldesouky et al., 2020; Yousfi et al., 2019). Pesquisas sobre o potencial de medicamentos existentes no combate a infecções fúngicas revelam que o aproveitamento de seus mecanismos de ação pode resultar em benefícios significativos.

A tafenoquina (TAF) e a alexidina (ALX), antimalárico e antisséptico, respectivamente são descritos como potenciais agentes antifúngicos no tratamento de infecções por *C. haemulonii*, ampliando seu espectro de uso além das indicações originais(Nabeela et al., 2022; Srinivasan et al., 2021). Ambos estão sendo estudados por suas propriedades de reposicionamento terapêutico, oferecendo novas alternativas promissoras diante da crescente resistência observada em tratamentos convencionais (Peyclit et al., 2021).

A TAF é um composto sintético da classe das 8-aminoquinolinas (Figura 6), utilizado historicamente no tratamento e prevenção da malária (Yardley et al., 2010). Sua estrutura química permite a interação com uma variedade de organismos

patogênicos, contribuindo para sua eficácia em diferentes contextos clínicos(Sidrônio et al., 2021).

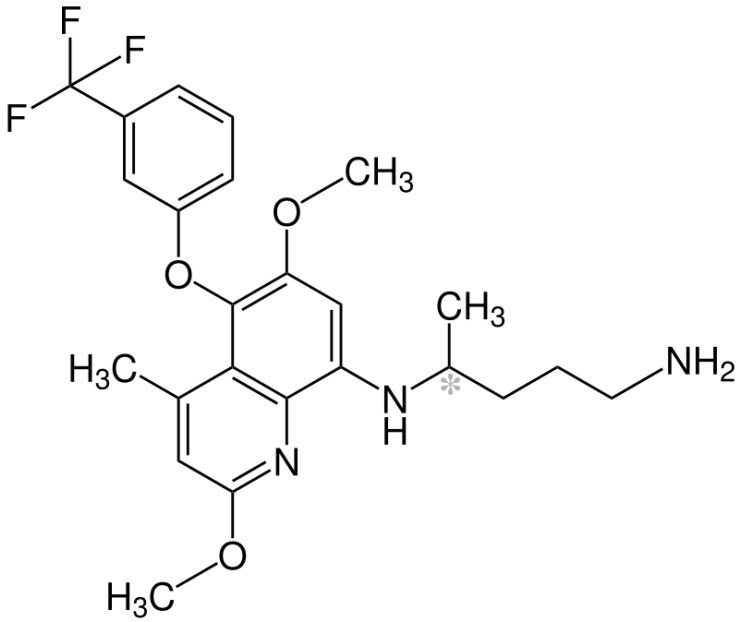

Figura 6. Estrutura molecular de tafenoquina (TAF). Fonte: Disponível em: https://commons.wikimedia.org/w/index.php?curid=64951292. (Acesso em: 06 out. 2024).

Além de sua eficácia antimalárica, a tafenoquina tem demonstrado atividade promissora *in vitro* e *in vivo* contra diversas espécies de Leishmania, incluindo cepas resistentes ao antimônio pentavalente, estudos recentes também sugerem um espectro de atividade antifúngica, embora limitado em dados, o que amplia o potencial do fármaco para usos alternativos (Yardley et al., 2010).

O mecanismo de ação da TAF contra protozoários, como os causadores da malária, envolve a geração de espécies reativas de oxigênio (ROS) que causam estresse oxidativo nas células parasitárias. Em organismos suscetíveis, esse mecanismo é eficaz na destruição dos patógenos. Estudos com derivados de mefloquina, quimicamente relacionados à TAF, indicam que esses compostos também inibem a formação de cápsulas e a melanização em *Cryptococcus neoformans*, sugerindo um mecanismo de ação antifúngica potencialmente similar (Montoya et al., 2020).

Clinicamente, a TAF é amplamente utilizada para tratar e prevenir a malária, sendo eficaz tanto contra a fase sanguínea quanto contra a fase hepática do ciclo do protozoário. Sua versatilidade terapêutica torna-a uma opção promissora para investigações no tratamento de outras infecções, especialmente aquelas para as quais há resistência aos tratamentos convencionais(Nekkab et al., 2021).

Apesar de sua eficácia, o uso da TAF apresenta um risco significativo de toxicidade, especialmente em pacientes com deficiência de glicose-6-fosfato desidrogenase (G6PD). O estresse oxidativo causado pela tafenoquina nas células vermelhas do sangue pode levar à anemia hemolítica, o que torna fundamental a triagem para deficiência de G6PD antes de iniciar o tratamento (Swastika et al., 2019).

Embora os estudos sobre a ação antifúngica da TAF ainda sejam limitados, suas propriedades terapêuticas promissoras contra fungos resistentes a tratamentos convencionais despertam interesse. Pesquisas recentes com derivados de mefloquina, uma classe relacionada à TAF, demonstraram atividade antifúngica de amplo espectro contra leveduras e fungos filamentosos, incluindo isolados clínicos resistentes. Esses compostos se mostraram eficazes na inibição de biofilmes e de estruturas essenciais à sobrevivência dos fungos, indicando o potencial da tafenoquina para reposicionamento como agente antifúngico (Sidrônio et al., 2021).

A alexidine (ALX) é uma bisbiguanida utilizada como antisséptico e desinfetante (Figura 7). Suas propriedades químicas permitem uma ampla aplicação em controle antimicrobiano, sendo especialmente eficaz contra patógenos fúngicos e bacterianos em ambientes clínicos(Nabeela et al., 2022).

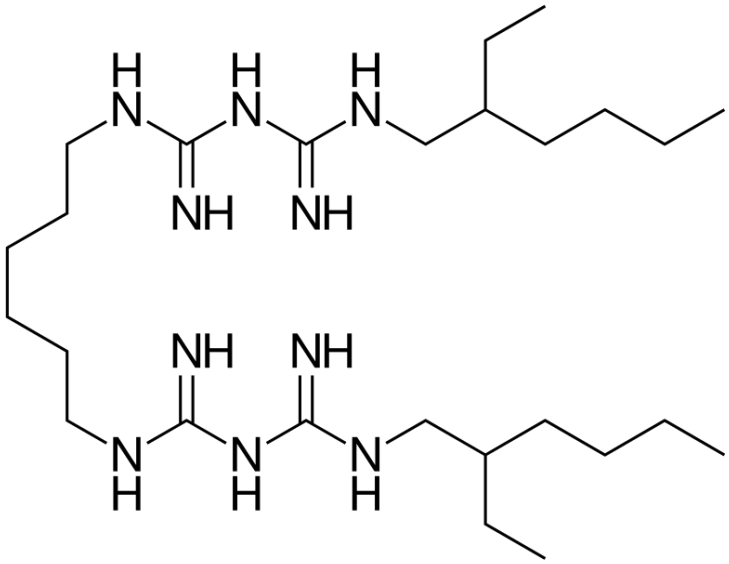

Figura 7. Estrutura molecular de alexidina (ALX). Fonte: Disponível em: https://commons.wikimedia.org/w/index.php?curid=40700421. (Acesso em: 06 out. 2024).

ALX apresenta potentes efeitos inibitórios contra uma ampla gama de patógenos fúngicos, incluindo *C. albicans*. Em estudos comparativos, mostrou-se mais eficaz do que antifúngicos convencionais, como fluconazol (FLZ) e anfotericina B (AmB), especialmente contra espécies de *Candida* que desenvolveram resistência a outros

tratamentos (Ginestra et al., 2023). Sua atividade antimicrobiana ampla, aliada a efeitos anti-inflamatórios e cicatrizantes, oferece um perfil terapêutico vantajoso no combate a infecções fúngicas em ambientes hospitalares (Sherry et al., 2012).

O mecanismo de ação da ALX envolve a ruptura da parede celular e da membrana fúngica, resultando no extravasamento do conteúdo celular e, consequentemente, na morte do patógeno (KAMIGIRI et al., 2004). Esse efeito de desestabilização da membrana torna a ALX particularmente eficaz na inibição de biofilmes fúngicos, estruturas resistentes onde os fungos frequentemente se abrigam, dificultando a ação de tratamentos convencionais (Nabeela et al., 2022).

ALX é amplamente utilizada em produtos de higiene bucal, como enxaguantes, onde é considerada segura em baixas concentrações. Em odontologia, estudos indicam que ALX é comparável ou superior à clorexidina no combate a microrganismos sem formar subprodutos tóxicos, como p-cloroanilina, o que a torna uma alternativa promissora para desinfecção endodôntica e tratamentos bucais (Bukhary & Balto, 2017).

Apesar de sua segurança em baixas concentrações, ALX pode causar irritação e comprometimento da regeneração celular em altas concentrações, especialmente com uso prolongado, o que representa um risco de toxicidade sistêmica (Bukhary & Balto, 2017). Além disso, estudos indicam que, quando combinada com hipoclorito de sódio, a ALX pode apresentar citotoxicidade, o que exige um monitoramento rigoroso em práticas clínicas onde essa combinação é utilizada (Thomas et al., 2019).

A ALX tem se mostrado eficaz contra cepas de *Candida*, especialmente *C. albicans*, um patógeno fúngico comum associado a infecções mucocutâneas e sistêmicas, em estudos comparativos, a ALX demonstrou eficácia antifúngica semelhante ou superior à da clorexidina. Acredita-se que seu mecanismo antifúngico esteja associado à capacidade de desestabilizar a membrana celular fúngica e inibir a formação de biofilmes, reforçando seu potencial como uma alternativa terapêutica para infecções resistentes a tratamentos convencionais (Nabeela et al., 2022).

3. OBJETIVOS

3.1. Objetivo geral

Avaliar o potencial terapêutico de fármacos reposicionados, isolados e em associação com antifúngicos convencionais, contra *Candida auris* e *Candida haemulonii*, incluindo sua atividade antifúngica, inibição de biofilmes, mecanismos de ação e segurança.

3.2. Objetivos específicos

- Avaliar a atividade antifúngica isolada de fármacos de diferentes classes contra isolados clínicos de *Candida auris* e do complexo *Candida haemulonii*;

- Avaliar a eficácia da combinação de fármacos reposicionados com antifúngicos convencionais contra isolados clínicos de *Candida auris* e do complexo *Candida haemulonii;*

- Determinar as propriedades fungicidas ou fungistáticas dos fármacos reposicionados, isoladamente ou em combinação com antifúngicos, contra isolados clínicos de *Candida auris* e do complexo *Candida haemulonii;*

- Investigar a capacidade dos compostos em inibir a formação de biofilmes em isolados clínicos de *Candida auris* e do complexo *Candida haemulonii;*

- Explorar o mecanismo de ação dos compostos, isolados e/ou combinados com antifúngicos, contra isolados clínicos de *Candida auris* e do complexo *Candida haemulonii;*

- Analisar a sobrevida e toxicidade *in vivo* dos fármacos, isolados e/ou em combinação, frente a isolados clínicos de *Candida auris* e do complexo *Candida haemulonii.*

4. ARTIGOS

4.1. Artigo 1

Targeting Multidrug-Resistant *Candida auris*: Synergistic Effects of Zinc Pyrithione and Nystatin

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Abstract

Introduction *Candida auris* has emerged as a multidrug-resistant fungal pathogen, posing a significant global health threat due to its resistance to conventional antifungal therapies. This study aimed to evaluate the antifungal potential of zinc pyrithione (ZnPT) and nystatin (NYS), both individually and in combination, against *C. auris*.

Methods Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) were determined to assess the antifungal potency of ZnPT and NYS. Biofilm inhibition and eradication assays were conducted to evaluate the compounds' effectiveness against *C. auris* biofilm formation, including their impact on biofilm formation on porcine skin. To assess cell membrane integrity and permeability, evaluating the effects of these treatments on *C. auris* cell structure. Additionally, efflux pump inhibition and a sorbitol protection assay were performed to explore potential mechanisms of action. Safety evaluations included hemocompatibility assay, the Ames test to assess mutagenic potential, and acute toxicity on *Tenebrio molitor* larvae.

Results The findings demonstrated that the ZnPT + NYS combination achieved a synergistic effect. Furthermore, the combination was able to inhibit biofilm formation on porcine skin. Protein leakage assays indicated increased cell membrane permeability in *C. auris* following treatment, particularly with the combination. No nucleotide leakage was observed. The Ames test revealed no significant mutagenicity for the ZnPT + NYS combination, suggesting a low mutagenic risk. Acute toxicity assessments on *T. molitor* larvae showed that ZnPT alone exhibited toxicity, whereas the combination remained within safe limits.

Conclusions The combination of ZnPT and NYS shows promising antifungal activity against *C. auris*, with enhanced biofilm inhibition and low toxicity, positioning it as a potential candidate for combating this resistant pathogen. Further studies are warranted to explore clinical applicability and efficacy in treating *C. auris* infections.

Keywords: Zinc pyrithione, Nystatin, Fungal infection, *Candida*.

1. Introduction

Candida auris has emerged as a serious multidrug-resistant fungal pathogen, causing widespread hospital-acquired outbreaks across multiple continents (Kim et al., 2024; Rodrigues

and Nosanchuk, 2020). Since its first identification in 2009 (Satoh et al., 2009), *C. auris* infections have risen sharply worldwide, posing a significant threat to public health (Rossato and Colombo, 2018). Those most at risk include patients who have recently undergone surgery, used invasive medical devices, received prolonged antibiotic treatments, had extended stays in healthcare settings, or have compromised immune systems (Pfaller et al., 2011).

The spread of *C. auris* presents a significant public health challenge due to its ability to persist on surfaces and in diverse environments, contributing to its propagation in healthcare settings (Escandón et al., 2018). Its resistance to common hospital disinfectants further complicates infection control efforts (CDC, 2018). In addition, traditional diagnostic methods often misidentify *C. auris* as other *Candida* species, which can delay appropriate treatment and facilitate its spread (Lockhart et al., 2017).

C. auris causes invasive infections, impacting the bloodstream, wounds, and ears, with mortality rates reported between 28% and 56% (Calvo et al., 2016; Lockhart et al., 2017). Its exceptional persistence within patients and on inanimate surfaces in healthcare environments makes it particularly challenging to control and eradicate (Wasylyshyn and Stoneman, 2024). While early reports in 2009 indicated that *C. auris* was fully susceptible to available antifungals (Du et al., 2020; Satoh et al., 2009), resistance to the azole antifungal fluconazole was observed by 2011 (Tsay et al., 2018). Currently, most *C. auris* isolates show resistance to at least two of the three primary classes of antifungals—polyenes, azoles, and echinocandins—making it critical to explore new therapeutic options (Cortegiani et al., 2019; Retore et al., 2024). One particularly challenging aspect is the pathogen's ability to form biofilms (Sherry et al., 2017). Biofilms provide a protective barrier for the yeast, significantly enhancing its ability to withstand antifungal agents and survive in harsh environments (Butassi et al., 2021). Addressing this challenge, the application of zinc pyrithione (ZnPT) in a topical form offers a direct approach to combating these biofilms. Topical treatments can deliver antifungals directly to the infection site, potentially reducing systemic side effects and increasing drug concentrations at the target location, thereby maximizing the therapeutic effects against biofilms (Kaur and Kakkar, 2010).

The strategy of drug repurposing has gained traction as a rapid approach to identifying effective treatments for emerging infections, targeting a variety of pathogens including viruses, bacteria, parasites, and fungi (Izadi et al., 2022; Sun et al., 2017). This study delves into innovative therapeutic methods to tackle drug-resistant *C. auris* infections, focusing specifically on the *in vitro* and *in vivo* effectiveness of ZnPT as a viable alternative treatment.

2. Material and methods

2.1. Strain and culture conditions

The fungal strain used in this study was obtained from the Mycotheque of the Núcleo de Estudos em Micologia Médica Aplicada (NEMMA) at the Health Sciences Research Laboratory (LPCS), Federal University of Grande Dourados (UFGD). We utilized an isolate of the *C. auris* (150/23), previously identified (Calvo et al., 2016), was preserved in 20% glycerol at –80°C until use. For testing, the strain was transferred onto freshly prepared Sabouraud Dextrose Agar (SDA) and incubated for 24 hours at 37ºC before being used to inoculate the testing media.

2.2. Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentrations (MICs) of nystatin (NYS) and ZnPT were determined using the broth microdilution method, in accordance with the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, 2017). Sensitivity profiles for ZnPT and NYS were assessed using dilution ranges of 0.5 to 256 µg/mL and 0.12 to 64 µg/mL, respectively. Fungal suspensions were prepared, diluted in RPMI-1640 medium buffered with MOPS, and added to the wells of 96-well plates at a density of 1–2.5 × 10^3 CFU/mL. The plates were then incubated at 37°C for 24 hours. MIC was defined as the lowest concentration that visually inhibited 100% of the strain's growth. To determine the Minimum Fungicidal Concentrations (MFC), 10 µL from each well of the MIC plates was transferred to SDA plates and incubated at 37°C for 24 hours. MFC was defined as the lowest concentration at which no fungal growth was observed. Additionally, these experiments included the *C. auris* CBS reference strain as quality control.

2.3. Checkboard assay

The combination of ZnPT and NYS was evaluated using the checkerboard microdilution method (Odds, 2003), which allows assessment across all concentration combinations within the desired range. ZnPT concentrations from 0.5 to 256 µg/mL and NYS concentrations from 2 to 64 µg/mL were used. In setting up the plates, 50 µL of NYS was added horizontally across the wells, while 50 µL of ZnPT was added vertically in a flat-bottom 96-well plate. Then, 100 µL of inoculum (10^3 cells/mL) was added to

each well. Eight wells containing only RPMI-1640 medium with MOPS buffer served as the sterility control. Subsequently, 100 µL of inoculum was added to each well containing the compounds and eight wells containing only RPMI-1640 medium with MOPS buffer (positive control). The plates were incubated at 37°C for 24 hours. Following incubation, each plate was visually assessed for fungal growth, and the lowest concentration that inhibited 100% growth was recorded as the MIC for that strain.

The interaction between ZnPT and NYS was quantified using the Fractional Inhibitory Concentration Index (FICI), calculated as follows:

$$\mathrm{FICI} = \frac{\text{MIC of Drug 1 in combination}}{\text{MIC of Drug 1 alone}} + \frac{\text{MIC of Drug 2 in combination}}{\text{MIC of Drug 2 alone}}$$

Each FICI value was determined for all concentration combinations, with interactions classified according to (Odds, 2003): FICI ≤ 0.5 indicated synergistic interaction, 0.5 < FICI ≤ 4 indicated indifference, and FICI > 4 indicated antagonism.

2.4. Inhibition growth assay

Cells were standardized to 0.5 on the McFarland scale, then diluted 1:100, followed by a 1:20 dilution, resulting in a final concentration of 1–2.5 × 10^3 cells/mL. This suspension was mixed with ZnPT (at MIC, 64 µg/mL), NYS (at MIC, 32 µg/mL), ZnPT (at FIC, 1 µg/mL) + NYS (at FIC, 2 µg/mL), AMB (0.5 µg/mL as a negative control), and RPMI-1640 medium with MOPS buffer (as a positive control). The tubes were then incubated at 37°C. Absorbance was measured at 595 nm at 0, 2, 4, 6, 8, 12, and 24 hours. Turbidity versus incubation time graphs were plotted, and growth rate curves were analyzed to assess any fungicidal effects of the compounds (Retore et al., 2024). Disinfectant controls such as chlorhexidine (CHX), benzalkonium chloride (BZK), and didecyldimethylammonium chloride (DDA) were included.

2.5. Antibiofilm activity

Antibiofilm activity was determined according to the method described by (Sushmitha et al., 2023), with minor modifications. Briefly, a standardized inoculum of *C. auris* (1–2.5 × 10^3 cells/mL) in RPMI-1640 medium with MOPS buffer was added to the wells of a 96-well flat-bottom plate. The plate was then incubated at 37°C for 24 hours to promote fungal growth and biofilm formation. After the adhesion phase, planktonic cells

were removed, and the adherent biomass was washed three times with distilled water. The biomass was then stained with a 0.1% crystal violet solution for 20 minutes. Excess dye was removed, and the stained cells were then resuspended in 70% ethanol for quantification at 595 nm using a microplate absorbance reader (iMark™ Microplate, Bio-Rad, São Paulo, SP, Brazil) at 595 nm.

The degree of biofilm inhibition was calculated relative to biofilm growth in the absence of compounds (defined as 100% biofilm formation) and the sterility control with medium alone (defined as 0% biofilm formation). The percentage of biofilm inhibition for each compound, alone and in combination, was calculated using the following formula (Wang et al., 2021):

$$\%\ \text{Biofilm Inhibition} = \left(\frac{\text{OD of the control} - \text{OD of the treatment}}{\text{OD of the control}}\right) \text{x } 100$$

2.6. Effect of ZnPT and NYS on the inhibition of C. auris biofilm adhesion to porcine skin

The capacity of ZnPT combined with NYS to inhibit biofilm formation was assessed in an *ex vivo* porcine skin model, following the methodology described by Horton et al. (Horton et al., 2020), with modifications. Porcine skin samples, sourced from a local supplier, were cut into 12 mm discs using a biopsy punch and decontaminated by immersion in an antibiotic solution (streptomycin 1,000 µg/mL and penicillin 1,000 units/mL; Sigma-Aldrich) for 18 hours. The samples were then rinsed with Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma-Aldrich) and transferred to 12-well plates containing semisolid Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich), supplemented with 10% fetal bovine serum (Sigma-Aldrich).

C. auris was cultured overnight in Sabouraud broth at 37°C, and the cell concentration was adjusted to 1–2.5 × 10^3 cells/mL. Ten microliters of the cell suspension were applied to the surface of each porcine skin sample, followed by 10 µL of ZnPT combined with NYS, both at a concentration of 2 µg/mL, or 10 µL of CHX at 1% as a comparison treatment. The samples were incubated at 37°C for 0, 15 minutes, 45 minutes, 4 hours, and 24 hours.

After incubation, each skin sample was transferred to a tube containing 10 mL of NaCl solution and vortexed for 10 minutes to dislodge the cells. Serial dilutions were performed, and the suspensions were plated on SDA. Colony-forming units (CFU) were

counted after 24 hours of incubation at 37°C. Results were expressed as a percentage of inhibition using the formula:

$$\%\ \text{inhibition} = \left(\frac{\text{CFU/mL of positive control} - \text{CFU/mL of treatment}}{\text{CFU/mL of positive control}}\right) \text{x } 100$$

2.7. Biofilm eradication assay

C. auris cells were cultured on SDA for 24 hours, then resuspended in peptone water (HiMedia) at a concentration of 10^3 CFU/mL. Hospital devices, specifically double-lumen central venous catheters, were immersed in this yeast suspension and incubated for 24 hours at 37ºC to promote biofilm formation. A parallel control group was prepared using catheters exposed solely to peptone water without yeast, serving as the sterility control. After the incubation period, the catheters were rinsed three times with sterile distilled water to remove non-adherent cells. The catheters with adhered biofilm were then treated with 64 µg/mL of ZnPT, 32 µg/mL of NYS, 1 µg/mL of ZnPT + 2 µg/mL of NYS, and peptone water (untreated control) for 15 and 45 minutes.

Biofilm was collected from the catheters using physical agitation. After plating the samples on SDA, they were incubated at 37ºC, and the CFU count was determined. Biofilm eradication percentage was calculated with the formula (Pippi et al., 2018):

$$\%\ \text{Biofilm Eradication} = \left(\frac{\text{CFU of Untreated Biofilm} - \text{CFU of Treated Biofilm}}{\text{CFU of Untreated Biofilm}}\right) \text{x } 100$$

2.8. Sorbitol protection assay

We used a previously described methodology to evaluate the osmoprotective effect of sorbitol (Escalante et al., 2008). Serial microdilutions were prepared in a sterile 96-well microplate containing RPMI-1640 medium with MOPS buffer and enriched with 0.8 M sorbitol. The ZnPT stock solution was diluted to concentrations ranging from 0.5 to 256 µg/mL, and NYS concentrations ranged from 0.12 to 64 µg/mL, with micafungin (MCF) serving as a positive control. MIC values were determined after incubation at 37°C for 24 and 48 hours.

2.9. Efflux pump inhibition assay

The overexpression of drug efflux pumps in the plasma membrane is a well-recognized mechanism by which fungi evade antifungal drugs (Castelo-Branco et al., 2013). To assess the effects of ZnPT and NYS on efflux pump inhibition, a phenotypic susceptibility assay was conducted using promethazine, an established inhibitor of plasma membrane efflux pumps (Castelo-Branco et al., 2013). A combined phenotypic susceptibility assay was performed to assess the effect of ZnPT (0.5 to 256 µg/mL) and NYS (0.12 to 64 µg/mL) on efflux pump inhibition by incorporating sub-inhibitory concentrations of promethazine (128 µg/mL) into the final fungal inoculum.

2.10. Alteration of cell membrane permeability

Cell membrane permeability changes were evaluated using the Pierce™ BCA Protein Assay Kit. *C. auris* cells were prepared in sterile distilled water and adjusted to a concentration of 1–2.5 × 10^3 cells/mL. This cell suspension was combined with ZnPT (at MIC 64 µg/mL), NYS (at MIC 32 µg/mL), and a mixture of ZnPT (at MIC 1 µg/mL) + NYS (at MIC 2 µg/mL), then incubated at 37°C for intervals of 0, 1, 2, 3, and 4 hours. After incubation, the samples were centrifuged at 908 g for 5 minutes at 4°C. Following this, 25 µL of the supernatant was transferred to a flat-bottom 96-well plate, and 200 µL of BCA working reagent was added to each well. The plate was shaken for 30 seconds and incubated at 37°C for 30 minutes. Absorbance readings were taken at 595 nm after incubation.

The absorbance average from the control wells was subtracted from the absorbance readings of each treatment. A blank control, containing only the treatment and BCA working reagent, was also prepared for each treatment, and its absorbance value was subtracted from the results. The protein concentration (µg/mL) was then calculated using a linear equation from the kit's calibration curve, allowing correlation between absorbance and the quantity of protein released by the yeast cells (Retore et al., 2024).

2.11. Nucleotide leakage

Nucleotide leakage analysis was conducted following the method outlined by Campos et al. (Campos et al., 2023). The *C. auris* strain was first cultured on SDA at 37°C for 24 hours. *C. auris* cells were then suspended in 0.9% saline solution to achieve a final concentration of 1–2.5 × 10^3 cells/mL. These suspensions were treated with ZnPT (64 µg/mL), NYS (32 µg/mL), or a combination of ZnPT (at MIC 1 µg/mL) + NYS at MIC 2 µg/mL) for different incubation times

(samples collected at 0, 1, 2, and 4 hours). Following incubation, the samples were centrifuged at 1300 g for 15 minutes, and the supernatants were analyzed for nucleotide leakage at 260 nm.

2.12. Hemolysis assay

Hemolysis was determined acording to priviosly described (Lin et al., 2022). Fresh mouse blood was diluted 25 times with sterile PBS, and then 250 µL of the diluted blood was mixed with ZnPT (at MIC 64 µg/mL), NYS (at MIC 32 µg/mL), and ZnPT (at MIC 1 µg/mL) + NYS (at MIC 2 µg/mL). PBS and Triton (0.1%, v/v) were used as negative and positive controls, respectively. The samples were incubated at 37°C for 1 hour and subsequently centrifuged at 700 g for 5 min. The supernatant from each sample (100 µL) was transferred into each well of a 96-well flat-bottom plate. Absorbance was measured at 490 nm with a microplate reader to calculate the hemolysis ratio (%) using the following equation:

$$\left(\frac{\mathrm{ODs}-\mathrm{ODnc}}{\mathrm{ODpc}-\mathrm{ODnc}}\right)\times 100$$

ODs: OD490 values for samples, ODnc: OD490 values for negative controls, ODpc: OD490 values for positive controls.

2.13. Ames test

The mutagenic potential of the compound ZnPT in combination with NYS was evaluated using the Ames test (Kado et al., 1983), with the *Salmonella* Typhimurium strains TA98 and TA100, provided by the Toxicology and Genotoxicity department of the São Paulo State Environmental Company (CETESB). The bacterial suspension, at a concentration of 1-2 × 10^9 cells/mL, was added to tubes containing different concentrations of the test compound (2.0 to 150 µg/mL) in the presence of phosphate buffer or S9 fraction (MOLTOX® - Molecular Toxicology, USA). The mutagens 2-aminoanthracene and 4-nitro-o-phenylenediamine were used as positive controls in the presence and absence of metabolic activation, respectively. Dimethyl sulfoxide (DMSO) was used as the negative control. The tubes were pre-incubated at 37ºC for 90 minutes.

After pre-incubation, 2 mL of top agar was added to each tube, and the mixture was poured onto glucose minimal agar plates. The plates were incubated for 48-66 hours at 37°C, and the revertant colonies were counted. The mutagenicity index (MI) was calculated using the formula: MI = number of induced revertants / number of spontaneous revertants. The compound was

considered to have mutagenic potential when MI ≥ 2 in at least one of the tested concentrations (Valente-Campos et al., 2009). Concentrations with an MI ≤ 0.7 were considered cytotoxic (Kummrow et al., 2006). The results were analyzed using the Salanal statistical software (U.S. Environmental Protection Agency, Monitoring Systems Laboratory, USA, version 1.0, by the Research Triangle Institute, RTP, USA).

2.14. Acute toxicity tests using the Tenebrio molitor model

Tenebrio molitor larvae, weighing between 0.110 and 0.200 g, were divided into four groups, each consisting of 15 larvae. These groups were then incubated at 37°C for 24 hours without food. Following incubation, 5 µL of the respective treatments was injected directly into the larval hemocoel, between the third and fourth abdominal segments, using a Hamilton syringe (Hamilton, USA). The experimental groups were as follows: Group 1: PBS only (negative control); Group 2: ZnPT (at MIC 64 µg/mL); Group 3: NYS (at MIC 32 µg/mL); Group 4: Combination of ZnPT (at MIC 1 µg/mL) + NYS (at MIC 2 µg/mL). All *T. molitor* larvae were maintained at 37°C, and the number of live larvae (responsive to touch) was recorded every 12 hours for a total of 72 hours.

2.15. Statistical analysis

Tukey test was used to compare the results from the hemolysis assay, and Kaplan–Meier survival curves were generated for *T. molitor*, with statistical significance evaluated through the log-rank test. All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, CA, USA), with a significance threshold set at $P < 0.05$. All tests were performed in duplicate.

3. Results

3.1. Antifungal susceptibility testing

ZnPT showed an MIC of 64 µg/mL, while NYS had an MIC of 32 µg/mL for the *C. auris* strain (Table 1). Fungicidal activity was confirmed at these same concentrations: 64 µg/mL for ZnPT and 32 µg/mL for NYS (Table 1). MIC and MFC values for the *C. auris* CBS strain, used as a control, are also provided in Table 1. The combination of ZnPT and NYS demonstrated a synergistic effect against the *C. auris* strain. The

combination resulted in a 64-fold reduction in the concentration of ZnPT and a 16-fold reduction for NYS, yielding a FICI value of 0.07 (Table 1). This synergistic efficacy was confirmed using Synergy Finder Software, with ZIP synergy scores above 10 indicating synergism (Figure 1) (Ianevski et al., 2022).

Table 1. Minimum Inhibitory Concentrations (MICs) and Minimum Fungicidal Concentrations (MFCs) of ZnPT and NYS against *C. auris* strains.

	MIC (µg/mL)				MFC (µg/mL)		Checkboard	
Drug	**ZnPT (i)**	**ZnPT (c)**	**NYS (i)**	**NYS (c)**	**ZnPT**	**NYS**	**FICI**	**Interaction**
C. auris (150/23)	64	1	32	2	64	32	0.07	SYN
C. auris CBS	32	-	8	-	32	8	-	-

MIC: minimum inhibitory concentration, MFC: minimum fungicidal concentration. ZnPT: zinc pyrithione, NYS: nystatin. (i): isolate. (c): combinate. FICI (fractional inhibitory concentration index) is used to measure the interaction between the tested combinations. FICI interpretation corresponded to the following definitions: synergism (SYN), FICI ≤ 0.5; additivity (ADD), FICI > 0.5 and ≤1; and indifference (IND), FICI > 1 and ≤4; antagonism (ANT), FICI ≥ 4.

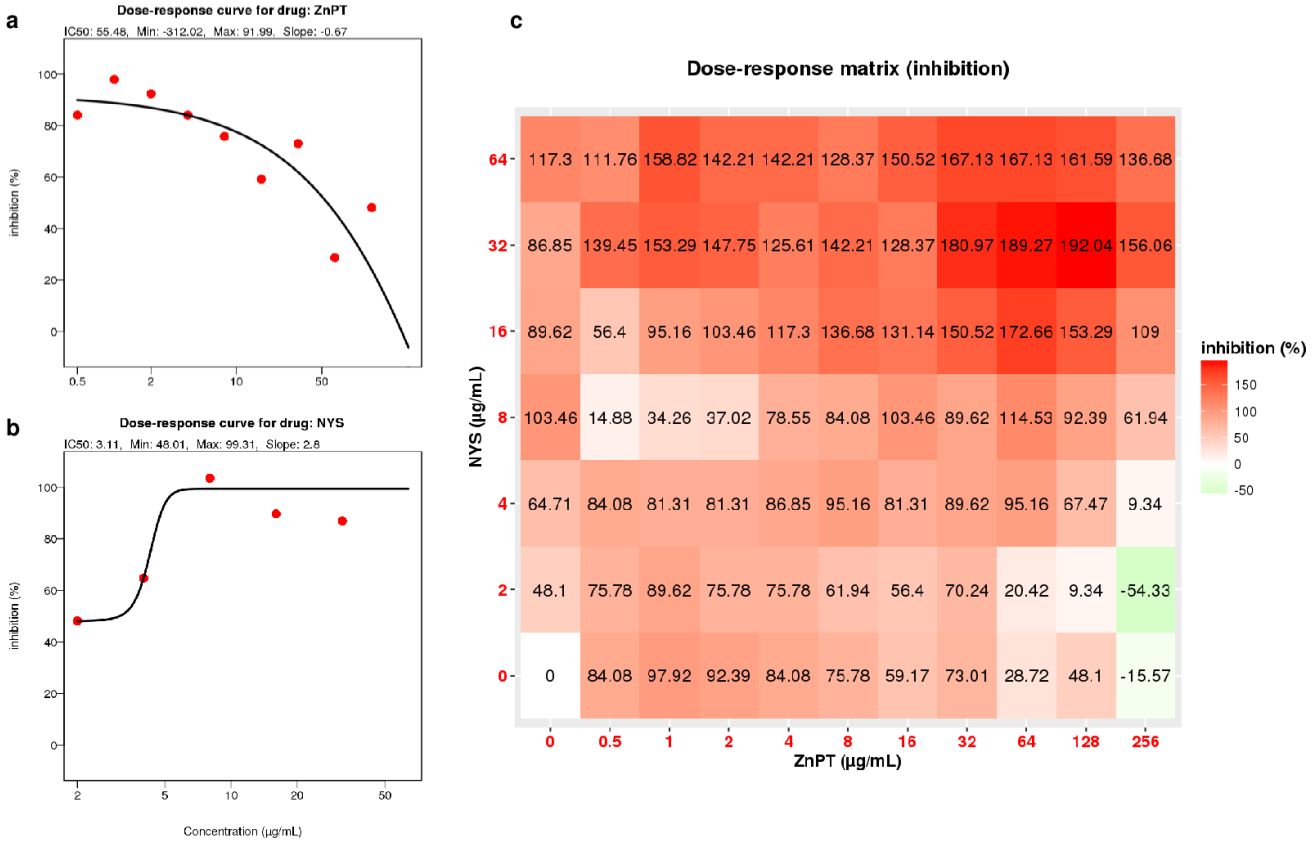

Figure 1. Synergistic activity of ZnPT in combination with NYS against *C. auris*. **a.** The dose-response curve of ZnPT alone (when the dose of NYS was 0). **b.** The dose-response curve of NYS alone (when the dose of ZnPT was 0). **c.** Synergy scores were calculated using the SynergyFinder software. ZIP Synergy scores >10 indicate synergism (red regions), and scores < - 10 indicate antagonism (green regions). The corresponding X- and Y-axes for rectangular highlights indicate the concentrations encompassing the region of greatest synergy. ZnPT: zinc pyrithione. NYS: nystatin.

3.2. Fungal inhibition assay

The efficacy of ZnPT, both as a standalone treatment and in combination with NYS, in inhibiting *C. auris* growth revealed a reduction in fungal growth after 12 hours of incubation compared to the positive control. No statistically significant differences were observed among the treatments, except for a significant difference between the positive control (*C. auris* alone) and BZK (Figure 2). The other treatments did not exhibit significant differences, either when compared to one another or to the positive control.

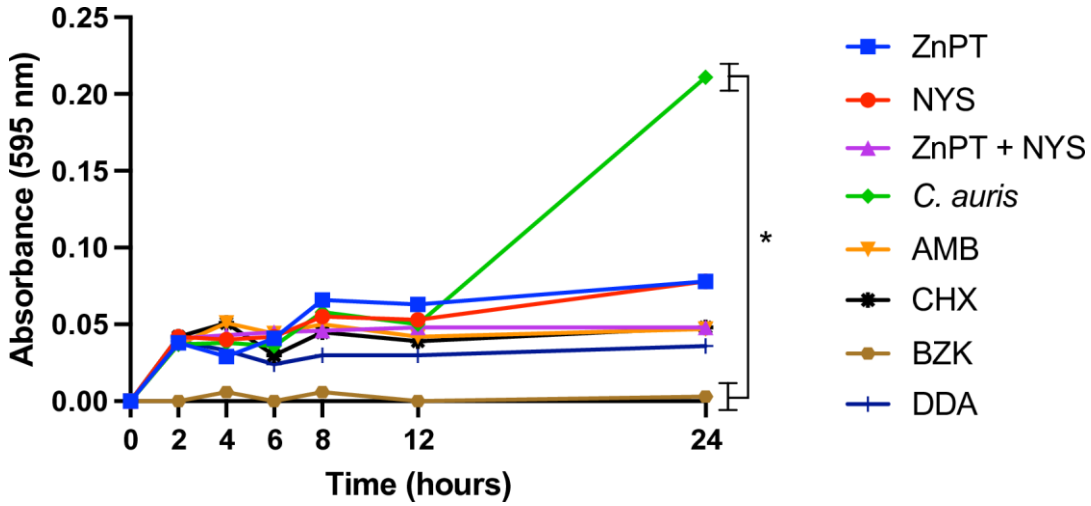

Figure 2. Growth curve of *C. auris* in the presence and absence of ZnPT. The test was conducted at concentrations of ZnPT (at MIC 64 µg/mL), NYS (at MIC 32 µg/mL), ZnPT (at FIC 1 µg/mL) + NYS (at FIC 2 µg/mL). ZnPT: zinc pyrithione, NYS: nystatin, AMB: amphotericin B, CHX: chlorhexidine, BZK: benzalkonium chloride, DDA: didecyldimethylammonium chloride. * $p < 0.05$.

3.3. Antibiofilm activity

Antibiofilm activity assay was conducted on *C. auris* using ZnPT at 64 µg/mL, NYS at 32 µg/mL, and a combination of ZnPT (1 µg/mL) + NYS (2 µg/mL). The combination of ZnPT + NYS demonstrated superior efficacy, achieving an antibiofilm activity rate of 50.39%. ZnPT alone exhibited an activity rate of 31.70%, while nystatin showed 20.45%. These results highlight that the ZnPT + NYS combination is significantly more

effective in inhibiting biofilm formation compared to either compound used individually, as shown in Figure 3.

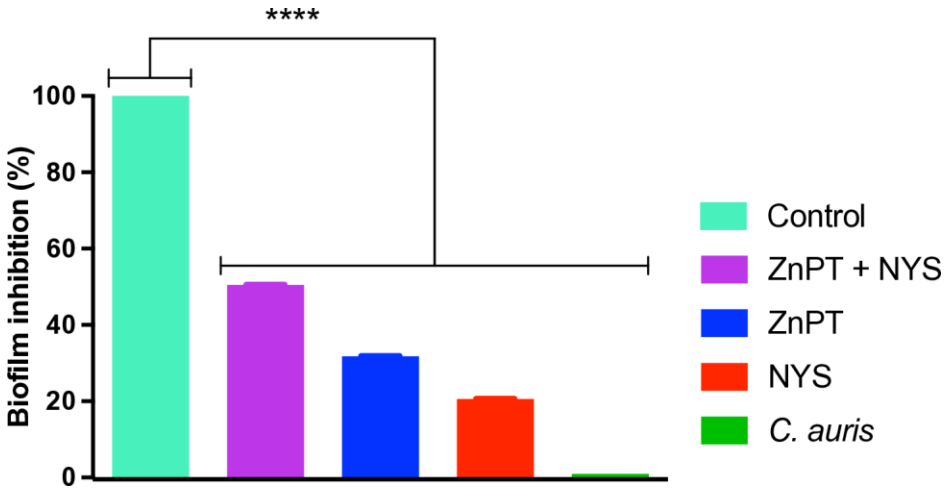

Figure 3. Antibiofilm activity. The antibiofilm activity test was conducted at concentrations of ZnPT (at MIC 64 µg/mL), NYS (at MIC 32 µg/mL), ZnPT (at FIC 1 µg/mL) + NYS (at 2 µg/mL). The control indicates the absence of yeast, implying no biofilm formation. ZnPT: zinc pyrithione, NYS: nystatin. **** $p < 0.0001$.

3.4. Effect of ZnPT and NYS on the inhibiting C. auris biofilm adhesion to porcine skin

The combination of ZnPT + NYS was able to inhibit *C. auris* biofilm formation on porcine skin over time, with inhibition rates nearing 100%. At exposure times of 15 and 45 minutes, the ZnPT + NYS treatment achieved inhibition rates of approximately 96% and 98.2%, respectively. At subsequent times of 4 hours and 24 hours, the inhibition rate reached over 99%. Statistical analysis using the Mann-Whitney test revealed no statistically significant difference between treatments with ZnPT + NYS and CHX ($p = 0.5397$) (Figure 4).

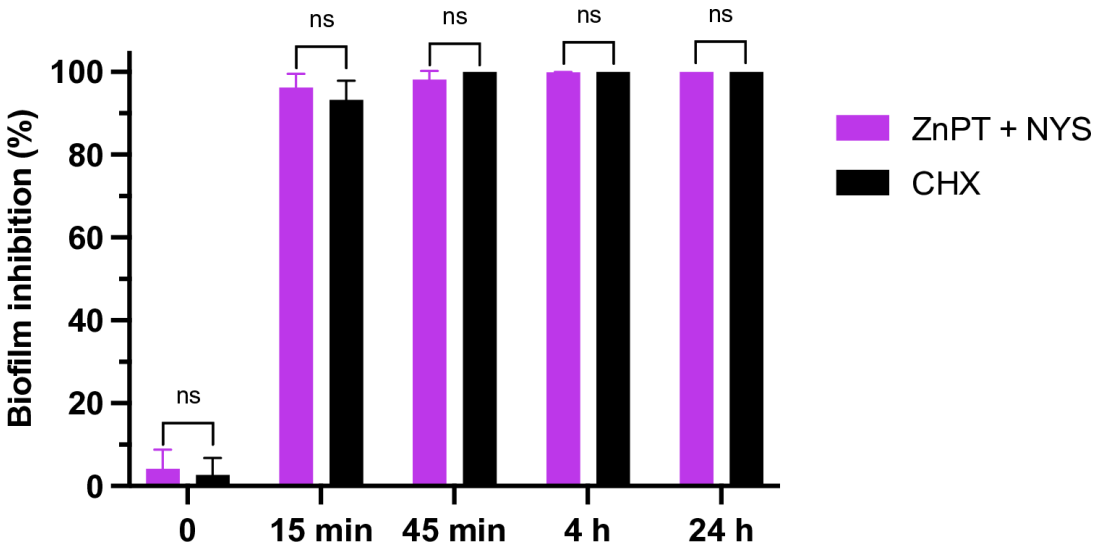

Figure 4. Effects of the ZnPT + NYS combination on biofilm formation of a resistant *C. auris* isolate. CHX: Chlorhexidine, ZnPT: zinc pyrithione, NYS: nystatin. ns: not significant. Error bars represent standard deviation (SD), Shapiro-Wilk test, Mann-Whitney test.

3.5. Biofilm eradication assay

A biofilm eradication assay was performed on *C. auris* using ZnPT at 64 µg/mL, NYS at 32 µg/mL, and a combination of ZnPT (1 µg/mL) + NYS (2 µg/mL), with CHX, BZK, and DDA included as comparative disinfectants. The ZnPT + NYS combination achieved biofilm eradication rates of 85.21% at 15 minutes and 85.96% at 45 minutes. Interestingly, ZnPT showed a biofilm eradication rate of 9.67% at 15 minutes but did not demonstrate any biofilm eradication at 45 minutes. In contrast, nystatin exhibited a biofilm eradication rate of 41.70% at 15 minutes and 59.14% at 45 minutes. BZK and CHX showed eradication rates of 70.27% and 59.21%, respectively, at both 15 minutes (Figure 5).

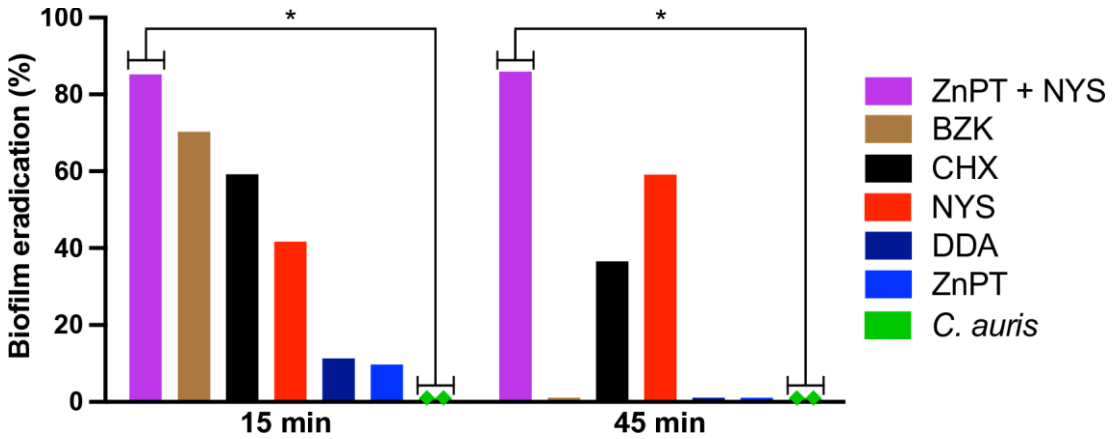

Figure 5. Biofilm eradication. The biofilm eradication test was conducted at concentrations of ZnPT (at MIC 64 µg/mL), NYS (at MIC 32 µg/mL), ZnPT (at FIC 1 µg/mL) + NYS (at 2 µg/mL). ZnPT: zinc pyrithione, NYS: nystatin, CHX: chlorhexidine, BZK: benzalkonium chloride, DDA: didecyldimethylammonium chloride. * $p < 0.05$.

3.6. Sorbitol protection assay

The impact of ZnPT, NYS, and their combination on the cell wall integrity of *C. auris* suggests that they compromise the cell wall structure via a sorbitol-dependent pathway, as indicated by an increase in MIC in the presence of sorbitol. According to established criteria, cell wall damage is implied when the MIC of a compound rises in the presence of sorbitol compared to its absence. These findings, detailed in Table 2, confirm that the antifungal action of these compounds involves direct disruption of cell wall integrity.

Table 2. Minimum Inhibitory Concentration of ZnPT and NYS in the presence or absence of sorbitol.

Treatment	**ZnPT**								**NYS**								**MCF**			
	24 hours				**48 hours**				**24 hours**				**48 hours**				**24 hours**		**48 hours**	
	MICi		**MICc**		**MICi**		**MICc**		**MICi**		**MICc**		**MICi**		**MICc**		**MICi**		**MICi**	
Strain	**S-**	**S+**	**S-**	**S+**	**S-**	**S+**	**S-**	**S+**	**S-**	**S+**	**S-**	**S+**	**S-**	**S+**	**S-**	**S+**	**S-**	**S+**	**S-**	**S+**
C. auris	64	≥256	1	2	64	≥256	1	16	32	16	2	2	32	32	2	16	≤0,01	0,06	0,5	≥8

S-: Absence of sorbitol, S+: Presence of sorbitol, MICi: Minimum Inhibitory Concentration (isolated), MICc: Minimum Inhibitory Concentration (combined), ZnPT: zinc pyrithione, NYS: nystatin, MCF: micafungin.

3.7. Efflux pump inhibition assay

The study also included tests to assess whether ZnPT, NYS, and their combination could inhibit efflux pumps, a common mechanism contributing to resistance. For the efflux pump assay, *C. auris* cells were exposed to treatments at concentrations of 64 µg/mL of ZnPT, 32 µg/mL of NYS, and 1 µg/mL of ZnPT + 2 µg/mL of NYS, with and without the addition of promethazine (128 µg/mL), an established efflux pump inhibitor. The efflux pump inhibition assay showed that the presence of promethazine led to a decrease in the MIC values for ZnPT and NYS, suggesting that the reduction in MIC in the presence of promethazine indicates interference with the functioning of efflux pumps, preventing the antifungal from being expelled

by the yeast. Promethazine, as an efflux pump inhibitor, appears to impair efflux activity, allowing greater intracellular retention of ZnPT and NYS (Table 3).

Table 3. Minimum Inhibitory Concentration of ZnPT and NYS in the presence or absence of promethazine.

Treatment	**ZnPT**								**NYS**							
	24 hours				**48 hours**				**24 hours**				**48 hours**			
	MICi		**MICc**		**MICi**		**MICc**		**MICi**		**MICc**		**MICi**		**MICc**	
Strain	**P-**	**P+**	**P-**	**P+**	**P-**	**P+**	**P-**	**P+**	**P-**	**P+**	**P-**	**P+**	**P-**	**P+**	**P-**	**P+**
C. auris	64	0.06	1	0.01	64	0.06	1	1	32	1	2	0.01	32	8	2	1

P-: Absence of promethazine, P+: Presence of promethazine, MICi: Minimum Inhibitory Concentration (isolated), MICc: Minimum Inhibitory Concentration (combined), ZnPT: zinc pyrithione, NYS: nystatin.

3.8. Alteration of cell membrane permeability

Protein leakage increased after treatments with ZnPT and NYS, both individually and in combination, compared to AMB. However, statistical analyses revealed no significant differences between the treatments and the positive control (*C. auris* alone). The results indicated that ZnPT alone showed minimal protein leakage after 2 hours compared to the combination treatment, which caused greater disruption to the fungal cell membrane, leading to higher protein concentrations in the supernatant over time. After 4 hours, treatments with ZnPT and NYS alone resulted in protein leakage levels of 38.33 µg/mL and 70.83 µg/mL, respectively, while the combination showed a level of 28.33 µg/mL (Figure 6).

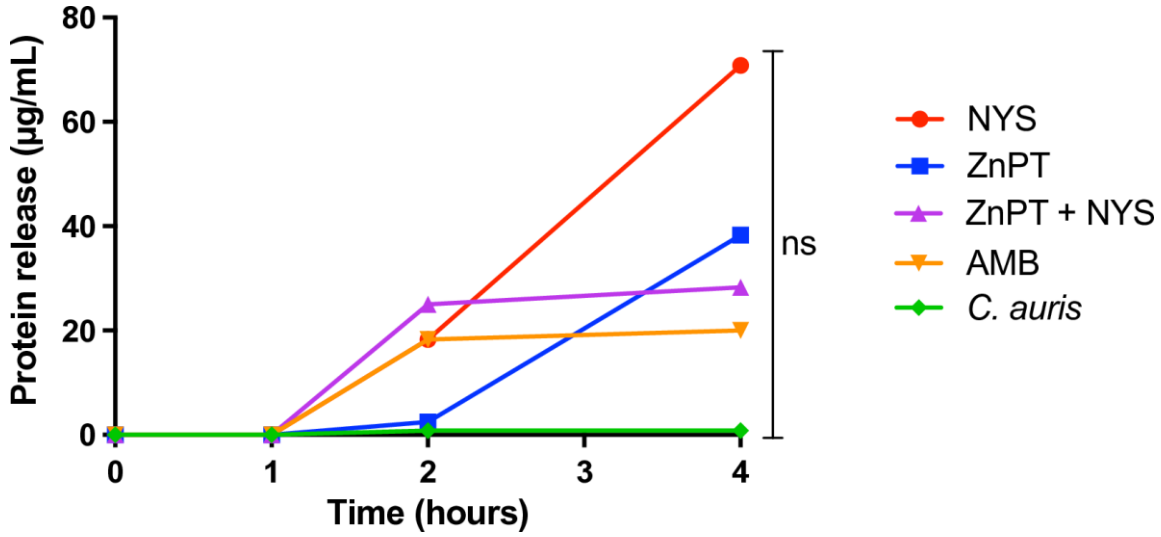

Figure 6. Cell membrane permeability assay for isolated and combined treatments of ZnPT and NYS. Treatments included ZnPT (64 µg/mL), NYS (32 µg/mL), and ZnPT (1 µg/mL) + NYS (2 µg/mL), AMB as the negative control, and *C. auris* alone as the positive control. ZnPT: zinc pyrithione, NYS: nystatin, AMB: amphotericin B, ns: not significant.

3.9. Nucleotide leakage

Nucleotide leakage showed no significant differences between the compounds and the positive control, AMB. The results indicated that ZnPT alone reached peak activity at 2 hours, with a level of 0.04 µg/mL, while NYS alone showed no measurable nucleotide presence at 2 hours. After 4 hours, the ZnPT + NYS combination resulted in an increase in nucleotide levels in the supernatant, reaching 0.75 µg/mL, compared to 0.95 µg/mL in the positive control (Figure 7).

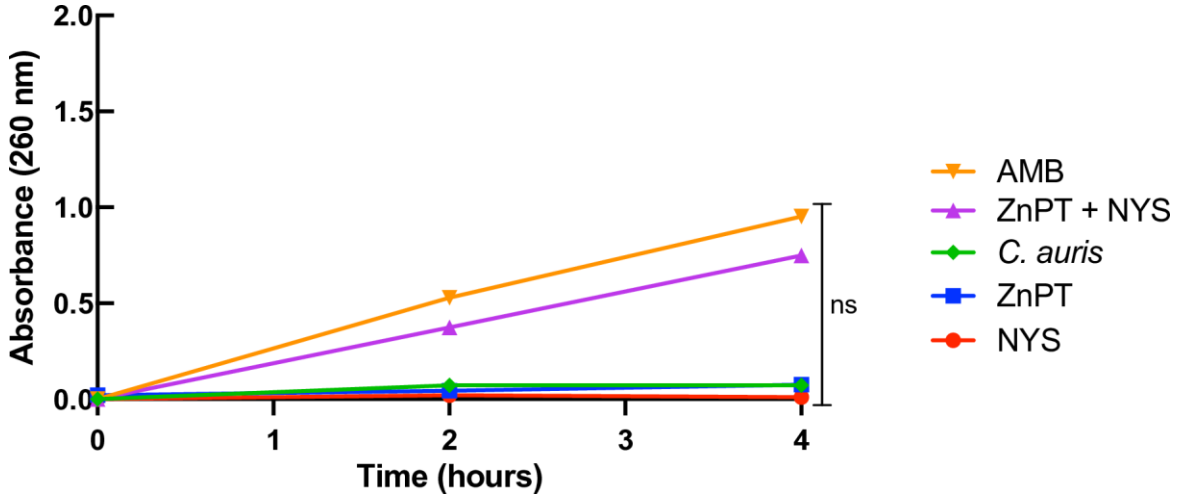

Figure 7. Nucleotide leakage from *C. auris* following isolated and combined treatments with ZnPT and NYS. Treatments included ZnPT (64 µg/mL), NYS (32 µg/mL), the combination of ZnPT (1 µg/mL) + NYS

(2 µg/mL), AMB as the negative control, and *C. auris* as the positive control. ZnPT: zinc pyrithione, NYS: nystatin, AMB: amphotericin B, ns: not significant.

3.10. Hemolysis Assay

In this investigation, hemolysis assay was conducted to assess the hemocompatibility of ZnPT and NYS, both individually and in combination. At isolated concentrations of 64 µg/mL for ZnPT and 32 µg/mL for NYS, neither compound showed evidence of inducing hemolysis, nor did the combination of ZnPT (1 µg/mL) + NYS (2 µg/mL). These results indicate that both ZnPT and NYS are hemocompatible and non-hemolytic (Figure 8).

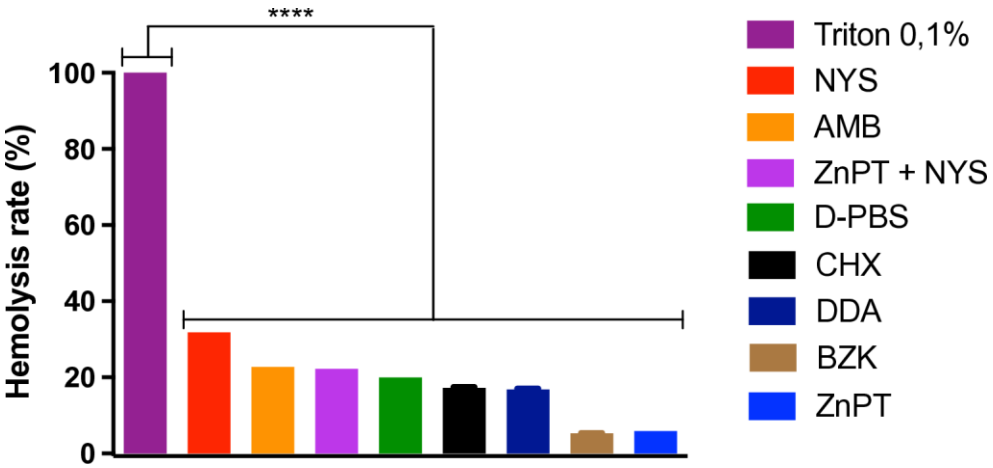

Figure 8. Hemolysis test for isolated and combined treatments of ZnPT and NYS. Treatments included ZnPT (64 µg/mL), NYS (32 µg/mL), and the combination (1 µg/mL ZnPT + 2 µg/mL NYS). D-PBS was used as the negative control, and 0.1% Triton X-100 served as the positive control. ZnPT: zinc pyrithione, NYS: nystatin, AMB: amphotericin B, CHX: chlorhexidine, BZK: benzalkonium chloride, DDA: didecyldimethylammonium chloride. **** $p < 0.0001$.

3.11. Ames test

Results were analyzed using Salanal software, which indicated no significant mutagenicity for ZnPT + NYS at the tested concentrations, supporting a low risk of mutagenic effects (Table 4).

Table 4. Mutagenic activity of ZnPT combined with NYS, expressed as the mean number of revertant colonies/plate ± standard deviation and the mutagenicity index against *S.* Typhimurium strains TA98 and TA100 in the absence (-S9) and presence (+S9) of metabolic activation.

µg/placa	TA98		TA100	
	-S9	+S9	-S9	+S9
0[a]	35 ± 2	33 ± 4	155 ± 7	102 ± 5
2	33 ± 1 (1.0)	29 ± 3 (0.9)	143 ± 5 (0.9)	128 ± 2** (1.2)
5	35 ± 6 (1.0)	27 ± 2 (0.8)	131 ± 5 (0.8)	129 ± 4** (1.3)
15	35 ± 6 (1.0)	28 ± 2 (0.8)	131 ± 3 (0.8)	139 ± 2** (1.4)
50	32 ± 2 (0.9)	30 ± 2 (0.9)	130 ± 5 (0.8)	137 ± 2** (1.3)
150	41 ± 2 (1.2)	30 ± 2 (0.9)	139 ± 6 (0.9)	140 ± 9* (1.4)
C+	260 ± 9[b]	293 ± 7[c]	677 ± 9[b]	708 ± 7[c]

Negative control: [a]DMSO, Positive control (C+): [b]4-nitrophenylenediamine (10 µg/plate) and [c]2AA-aminoanthracene (1.5 µg/plate). Significant difference (ANOVA): * $p < 0.05$, ** $p < 0.01$.

3.12. Acute toxicity tests using the Tenebrio molitor model

Acute toxicity assays on *T. molitor* revealed significant differences in survival among the treatments. Comparisons were conducted using ZnPT at 64 µg/mL, NYS at 32 µg/mL, and their combination of ZnPT at 1 µg/mL with NYS at 2 µg/mL, based on the concentrations determined from the MIC tests. Statistically, only ZnPT alone showed a significant difference when compared to the negative control (PBS), suggesting that this treatment may be considered toxic in the *T. molitor* model (Figure 9).

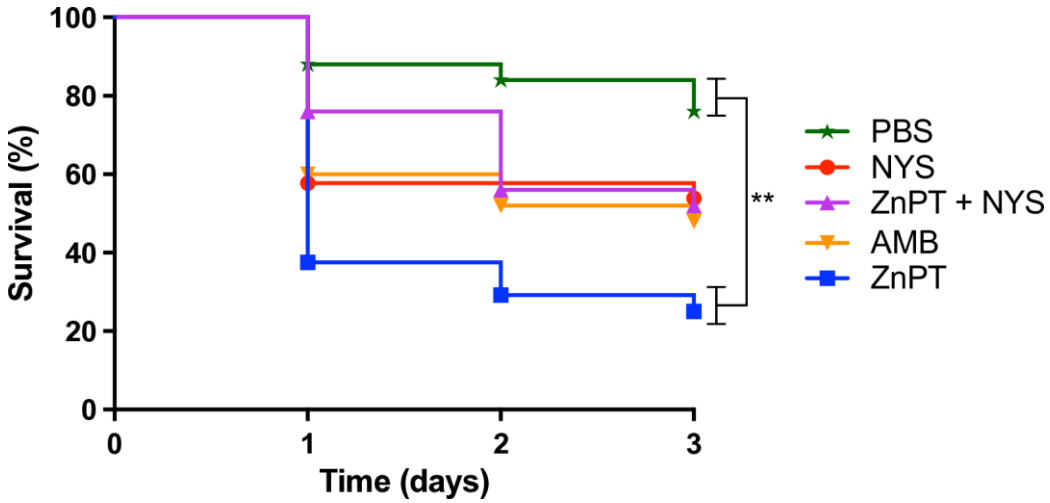

Figure 9. *In vivo* acute toxicity assays for isolated and combined treatments of ZnPT and NYS were conducted. Treatments included ZnPT (64 µg/mL), NYS (32 µg/mL), and the combination (1 µg/mL ZnPT + 2 µg/mL NYS). ZnPT: zinc pyrithione, NYS: nystatin, AMB: amphotericin B. ** $p < 0.01$.

4. Discussion

Since its discovery over a decade ago, *C. auris* has emerged as a significant nosocomial pathogen with unique skin tropism, enabling persistent colonization and widespread hospital outbreaks. This challenges current treatment strategies and underscores the urgent need for new antifungal solutions (Alanio et al., 2022; Huang et al., 2021; Ostrowsky et al., 2020). This study explored the antifungal potential of ZnPT and NYS, both individually and in combination with *C. auris*, through a series of *in vitro* and *in vivo* assays to evaluate their efficacy. ZnPT is a zinc coordination complex approved by the United States Food and Drug Administration (FDA) as an additive for treating seborrheic dermatitis and dandruff, owing to its well-documented antimicrobial and antifungal properties (Mangion et al., 2021). NYS, on the other hand, is an established antifungal medication indicated for the treatment of candidiasis in the digestive tract. Beyond evaluating the individual effects of these compounds, our study explored their combination, which exhibited synergistic action. This combination not only demonstrated the ability to inhibit the growth of resistant *C. auris* but also significantly reduced the minimum inhibitory concentrations (MICs) of both compounds after just two hours of exposure. Moreover, the combined treatment effectively inhibited biofilm formation, further highlighting its potential as a robust therapeutic strategy against resistant yeast strains.

In our study, treatment with ZnPT, both alone and in combination with NYS, did not show significant differences compared to the controls. However, there is a noticeable decrease in absorbance relative to the control containing only yeast and medium, suggesting that ZnPT and NYS exhibit fungicidal activity. After 24 hours of combined treatment, fungal proliferation does not resume. ZnPT has been shown to inhibit fungal growth by increasing cellular levels of copper, which damages iron-sulfur clusters essential for fungal metabolism (Reeder et al., 2011). Nystatin exerts its antifungal effect by binding to ergosterol in the fungal cell membrane, leading to pore formation and cell death (de Castro et al., 2015).

Our biofilm inhibition tests, including those conducted on porcine skin, demonstrated that the combination of ZnPT and NYS significantly reduced *C. auris* biofilm formation. This finding is particularly significant for *C. auris*, as its biofilm formation is a major factor contributing to antifungal resistance, complicating treatment and infection control

efforts, especially in healthcare settings (Rudramurthy et al., 2017). The ability of ZnPT + NYS to inhibit biofilm formation on a model simulating human skin underscores its potential as an effective topical treatment against *C. auris*, particularly in preventing skin colonization in healthcare environments. Studies exploring the use of NYS and ZnPT for treating *C. auris* infections in porcine skin models are limited. However, porcine skin models have been used to compare fungal colonization across different *Candida* species, biofilm formation, and the effectiveness of various treatments (Eix et al., 2022; Horton et al., 2020).

ZnPT + NYS proved superior to individual treatments in eradicating *C. auris* biofilm, reaching almost 100% eradication rates, particularly within shorter exposure times. This eradication capacity emphasizes the combination's strength in breaking down established biofilms, a critical step in managing persistent infections (de Melo et al., 2023). Compared to standard agents like chlorhexidine, ZnPT + NYS demonstrated similar or greater efficacy, positioning it as a promising candidate for biofilm management. Sorbitol plays an osmoprotective role and is crucial for fungal growth. Fungal cells with damaged cell walls lose their ability to regulate osmotic pressure and cannot grow in the absence of sorbitol. However, when sorbitol is added to the culture medium, it restores osmotic balance, enabling growth despite cell wall damage. This characteristic allows the identification of fungal cell wall inhibitors, as their MIC values increase in the presence of sorbitol. The addition of sorbitol stabilizes the cell's osmotic environment, permitting growth even in the presence of drugs targeting the cell wall (FROST et al., 1995). The MIC values of ZnPT and NYS for *C. auris* increased when the assay was performed in the presence of 0.8M sorbitol in the culture medium. This result indicates a possible action of the ZnPT and NYS on the fungal cell wall inhibition.

Efflux pump inhibition assays demonstrated that efflux mechanisms, which contribute to the expulsion of ZnPT and NYS from fungal cells, are being inhibited in the presence of promethazine, as evidenced by the reduction in MIC values. This inhibition allows for greater intracellular retention of ZnPT and NYS, enhancing their antifungal efficacy. Despite the involvement of efflux pumps under normal conditions, ZnPT and NYS maintain significant antifungal activity due to mechanisms of action that are not solely reliant on intracellular retention. NYS acts by binding to ergosterol in the fungal cell membrane, forming pores that lead to cell death, a mechanism not countered by efflux pumps (Sanglard et al., 2009). While ZnPT increases intracellular zinc levels, disrupting

iron-sulfur proteins critical for fungal cell functionality. This mechanism is not effectively countered by efflux pumps, as ZnPT's action involves intracellular processes that efflux mechanisms cannot mitigate (Reeder et al., 2011). This combination of mechanisms supports the overall effectiveness against *C. auris*, even in the presence of efflux pump activation, reinforcing the potential of ZnPT + NYS as a promising therapeutic alternative against resistant strains.

We investigated the potential mechanisms of action of ZnPT and NYS, both compounds demonstrated measurable protein leakage, supporting their membrane-permeability properties. ZnPT, a membrane-active compound, likely compromises fungal cell integrity by disrupting the phospholipid head structure in the outer membrane, leading to the loss of essential cellular components and impaired cell function (Reeder et al., 2011). NYS, on the other hand, exerts its antifungal action by binding to sterols within the fungal cell membrane, altering membrane permeability and inducing leakage of cytoplasmic contents (dos Santos et al., 2017). Notably, when ZnPT and NYS are combined, the degree of protein extravasation surpasses that observed with amphotericin B, a well-established membrane- permeability antifungal (MORAES, 2023). This enhanced disruption suggests that the synergistic effects of ZnPT and NYS may amplify their ability to destabilize fungal membranes.

Nucleotide measurements revealed no significant nucleotide release, suggesting that ZnPT + NYS affects membrane integrity selectively, avoiding extensive cytoplasmic leakage. A study on the ethanolic extract of *Centrosema coriaceum* leaves, using nystatin as a reference, demonstrated a gradual increase in absorbance at 260 nm over a 5-hour period, indicating nucleotide leakage (LEMOS et al., 2022). The mechanism of action of nystatin is well-established, involving binding to membrane sterols, which alters cell membrane permeability and leads to the leakage of cytoplasmic content, as observed in *C. glabrata* (LEMOS et al., 2022).

Additionally, our study demonstrated no hemolytic activity for either compound, with the combination of ZnPT and NYS showing an even lower hemolysis rate compared to the individual agents. The hemolytic activity of NYS in some formulations was evaluated and found to be influenced by its aggregation state, with reduced hemolytic activity under optimized conditions, indicating compatibility with red blood cells (Rodighiero et al., 2004). However, nystatin is known to exhibit toxicity at higher concentrations, particularly when administered systemically, due to its mechanism of action that

involves binding to sterols in cell membranes, which can also affect mammalian cells (Larson, 2000). Therefore, its use is typically limited to topical and localized applications to minimize systemic toxicity. In contrast, ZnPT can cause increased hemolysis at higher concentrations, emphasizing the importance of carefully assessing its concentration and application conditions to ensure safety in human use (Lamore and Wondrak, 2011). These findings highlight the strong safety profile of ZnPT and NYS, particularly when used together in controlled concentrations, making them promising candidates for topical antifungal therapies.

Ames test results indicated no significant mutagenicity for ZnPT + NYS, supporting a low mutagenic risk. This finding aligns with safety requirements for potential clinical use, further confirming the suitability of ZnPT + NYS for therapeutic applications without a substantial risk of genetic mutations. A review of the toxicological characteristics of ZnPT highlights its environmental behavior and potential risks in different conditions, though not specifically addressing mutagenicity in clinical settings (Soon et al., 2019). Similarly, a study evaluated the reproductive and developmental toxicity of Nyotran, a liposomal formulation of nystatin, and reported no significant genotoxic or mutagenic effects in animal models, further supporting its safety for therapeutic use (Larson, 2000).

The acute toxicity tests using the *T. molitor* model have demonstrated that while ZnPT exhibits some toxic effects, the combination of ZnPT and NYS maintains an acceptable safety profile at the concentrations used. This model is particularly valuable for studying fungal infections, including the action of antifungal compounds, due to its immune system, which is conserved with vertebrates (Canteri de Souza et al., 2018). This highlights the potential for utilizing this combination in topical treatments against *C. auris* infections. However, the study's limitation lies in its narrow focus on a limited number of isolates, which might not fully represent the genetic diversity and varying resistance profiles of *C. auris* globally. Future studies should expand on these findings by including a wider array of isolates from different clades and geographic regions. Additionally, exploring the long-term effects of this treatment on host tissues and its efficacy in a clinical setting are crucial steps forward. These efforts will help in understanding the broader implications of using ZnPT and NYS in the treatment of persistent fungal infections and pave the way for more targeted and effective therapeutic strategies.

5. Conclusions

This study underscores the synergistic effect of ZnPT and NYS in treating superficial infections caused by *C. auris*, advocating for their potential as a combined therapeutic strategy. The antifungal efficacy and acceptable safety profile highlighted here pave the way for clinical trials to validate and refine the use of this combination therapy. Further research should focus on the clinical applications and effectiveness of ZnPT and NYS, particularly for topical treatments, to combat drug-resistant *C. auris* infections.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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4.2. Artigo 2

Repurposing Tafenoquine as a Potent Antifungal Agent Against *Candida haemulonii sensu stricto*

Short title: Tafenoquine: A Potent Antifungal Against *C. haemulonii*

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Abstract

Background: The rise in fungal infections caused by multidrug-resistant (MDR) pathogens like *Candida haemulonii sensu stricto* presents a significant global health challenge. The common resistance to current treatments underscores the urgency to explore alternative therapeutic strategies, including drug repurposing.

Objectives: To assess the potential of repurposing tafenoquine, an antimalarial agent, for antifungal use against *C. haemulonii sensu stricto*.

Methods: The efficacy of tafenoquine was tested using *in vitro* assays for minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), biofilm inhibition, cell damage, cell membrane integrity, nucleotide leakage, sorbitol protection assay, and efflux pump inhibition. The compound's cytotoxicity was assessed through a hemolysis assay, and *in vivo* safety and efficacy were tested using *Tenebrio molitor* larvae.

Results: Tafenoquine exhibited potent fungicidal activity against *C. haemulonii sensu stricto* with an MIC of 4 mg/L and significantly inhibited biofilm formation by 60.63%. Tafenoquine also impaired mitochondrial functionality, leading to compromised cellular respiration. Despite these effects, tafenoquine did not cause significant protein leakage, indicating a distinct mechanism from membrane-targeting agents. *In vivo* study confirmed tafenoquine's non-toxic profile with no observed hemolysis or acute toxicity in the *T. molitor* model. During antifungal treatment with tafenoquine, a survival rate of approximately 60% was observed after 3 days.

Conclusions: The findings of this study highlight tafenoquine's potential as a promising candidate for antifungal drug repurposing, especially against *C. haemulonii sensu stricto*. Its effectiveness in inhibiting fungal growth and biofilm formation underscores its viability for further clinical development as a novel antifungal therapy.

Keywords: Tafenoquine, *C. haemulonii sensu stricto*, fungal infection, drug repurposing.

Background

Candidemia is one of the most significant causes of morbidity and mortality in healthcare environments, particularly in intensive care units and among immunocompromised patients, such as those undergoing chemotherapy, organ transplants, or with complex medical conditions.[1–3] This invasive fungal infection not only increases hospital stays but also

contributes to high healthcare costs due to the complexity of its management and the need for prolonged treatments.[4]

In recent years, there has been a notable rise in the incidence of invasive fungal infections (IFIs) caused by cryptic *Candida* species, which are often more difficult to identify and treat due to their diverse virulence factors and resistance mechanisms.[5,6] Among these, the *Candida haemulonii* species complex has gained particular attention. In 2012, this group was redefined to include *Candida haemulonii sensu stricto*, *Candida duobushaemulonii*, and *Candida haemulonii* var. *vulnera*.[3]

The identification and treatment of infections caused by this complex are made more challenging by the organisms' multidrug-resistant (MDR) characteristics.[7] These yeasts often exhibit high minimum inhibitory concentrations (MICs) for fluconazole,[8] a first-line antifungal therapy in many regions, particularly in low-income countries where alternative treatments may be limited. They also show reduced susceptibility to amphotericin B,[9] a potent antifungal for severe infections. While echinocandins are generally effective, emerging echinocandin-resistant strains are a growing concern.[10]

The challenges of MDR fungal infections and limitations of existing antifungals make drug repurposing a promising strategy for new treatments.[11] This strategy allows for the rapid identification of new therapeutic uses for existing drugs, bypassing the lengthy process of drug discovery and benefiting from known safety profiles.[11–13] Recent studies have highlighted the potential of repurposing antimalarial drugs as antifungal agents,[14] with tafenoquine—originally developed to treat malaria—emerging as a particularly strong candidate.[15,16] This study explores tafenoquine's potential as a novel antifungal against *C. haemulonii sensu stricto*, using both *in vitro* and *in vivo* approaches.

Material and methods

Strain and culture conditions

C. haemulonii sensu stricto strain (132/23) used in this study was sourced from the library of the Center for Studies in Applied Medical Mycology (CSAMM) at the Health Sciences Research Laboratory (HSRL) of the Universidade Federal of Grande Dourados (UFGD). This strain was previously characterized for biofilm production and identified by sequencing (ITS) region of rDNA.[17] The isolate, obtained from a blood culture, demonstrated resistance to amphotericin B

and fluconazole.[18] The strain was maintained in 20% glycerol at –80°C until use. For the experiments, it was cultured in Sabouraud agar (SDA), and incubated at 37°C for 48 h.

Minimum Inhibitory Concentration (MIC)

The MIC was determined by broth microdilution following EUCAST guidelines.[19] The sensitivity profile of the compound tafenoquine was assessed using dilutions ranging from 0.25-128 mg/L. Fungal suspensions were diluted in sterile distilled water and plated in 96-well plates at 2.5 x 10^5 cells/mL. The plates were incubated at 37°C for 48 h. Antifungal activity was determined spectrophotometrically at 530 nm and the MIC corresponded to the smallest compound concentrations able to inhibit 90% of growth. To determine the Minimum Fungicidal Concentrations (MFC), 10 µL of each well from the MIC plates was transferred to SDA plates and incubated at 37°C for 48 h. Amphotericin B concentrations ranging from 0.03-16 mg/L were used as a resistance control. The procedure was conducted in duplicate.

Checkboard assay

Tafenoquine was evaluated combined with fluconazole, amphotericin B, and micafungin using the checkerboard microdilution method.[20] Tafenoquine concentrations ranged from 0.25 to 128 mg/L, fluconazole from 0.125 to 64 mg/L, amphotericin B from 0.031 to 16 mg/L, and micafungin to 0.015-8 mg/L. For plate setup, 50 µL of tafenoquine was placed horizontally, and 50 µL of antifungal was placed vertically in a 96-well plate with a flat bottom. Subsequently, 100 µL of inoculum (10^5 cells/mL) was added to each well containing the compounds and eight wells containing only 2 × RPMI medium supplemented with 2% glucose and 165 mM MOPS (pH 7.0) (positive control). Sterility control was also performed, with eight wells containing only 2 × RPMI medium supplemented with 2% glucose and 165 mM MOPS (pH 7.0). The properly capped plates were incubated for 48 h at 37°C. The procedure was conducted in duplicate.

The interaction between the compounds was quantified by the Fractional Inhibitory Concentration Index (FICI): FICI = [MIC tafenoquine in combination]/[MIC isolated tafenoquine] + [MIC drug in combination]/[MIC isolated drug]. Each plate had its FICI calculated at all interaction concentrations and was classified according to,[21] being synergistic when FICI ≤0.5; indifference if 0.5 < FICI ≤4; and antagonism if FICI > 4.

Inhibition growth assay

For the growth curves, an inoculum of 2.5 × 10^5 cells/mL was prepared. This suspension was mixed with tafenoquine (MIC 4 mg/L), 2 × RPMI medium supplemented with 2% glucose and 165 mM MOPS (pH 7.0) as a positive control. The negative control consisted of the medium without yeast, and amphotericin B was used as a resistance control. The plates were then incubated at 37°C for 48 h. Absorbance was measured at 530 nm at 0, 12, 24, 36, and 48 h. Growth rate curves were analyzed to assess signs of fungicidal effects of tafenoquine.[15] The procedure was conducted in duplicate.

Antibiofilm activity

Inhibition of biofilm formation was assessed using a previously described method,[22] with some modifications. Briefly, a standardized inoculum of *C. haemulonii sensu stricto* (2.5 × 10^5 cells/mL) in 2 x RPMI medium supplemented with 2% glucose and 165 mM MOPS (pH 7.0) was added to wells containing the selected compound (tafenoquine: 0.25-128 mg/L). This suspension was added to the wells of 96-well polystyrene microtiter plates, which were then subjected to static incubation at 37°C for 48 h to facilitate fungal growth and biofilm formation.

Adhered biomass was washed, stained with 0.1% crystal violet for 20 minutes, and resuspended in 70% ethanol. Fungal growth was measured at 530 nm using an absorbance reader. *C. haemulonii sensu stricto* in 2 × RPMI medium with glucose and MOPS (pH 7.0) was the positive control; medium without yeast was the negative control. Tests were done in duplicate.

The extent of biofilm inhibition was calculated relative to the biofilm growth observed in the absence of the compound and the sterility control containing only the medium. Biofilm formation inhibition was calculated with the formula:[23]

$$\%\ Biofilm\ Inhibition = \left(\frac{OD\ of\ the\ control - OD\ of\ the\ treatment}{OD\ of\ the\ control}\right) x\ 100$$

Cell damage

To evaluate and quantify the cellular damage induced by tafenoquine, we conducted the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), followed established methodology.[24,25] This assay is crucial as it helps determine the extent of cellular impairment caused by the compound, which is key to understanding its cytotoxic effects on fungal cells.

After incubating for 24 h, the plates were centrifuged at 40 g for 10 min at room temperature, and the supernatant was removed. The pelletized cells were then incubated for an additional 3 h at 37°C with 200 µL of aqueous MTT solution (0.05 mg/mL). Following centrifugation, formazan crystals were solubilized using 150 µL of isopropyl alcohol. Subsequently, 100 µL aliquots from each well were transferred to clean wells for absorbance measurements at 595 (A530) nm and 655 (A655) nm. Cellular damage was quantified and presented graphically as a bar chart. All tests were performed in duplicate. The formula used to calculate cellular damage is:

$$Cell\ damage\ (\%) = \left[1 - \frac{(A595 - A655\ of\ treated\ cells)}{(A595 - A655\ of\ positive\ control\ cells)}\right] x\ 100$$

Sorbitol protection assay

We utilized a methodology previously described to evaluate the osmoprotection provided by sorbitol.[26,27] Serial microdilution was conducted in a sterile 96-well microplate containing 2 × RPMI medium supplemented with 2% glucose and 165 mM MOPS (pH 7.0) enriched with 0.8 M sorbitol. Micafungin serving as a positive control. MIC values were determined after incubation at 37°C for 48 and 72 h. All tests were performed in duplicate.

Efflux pump inhibition assay

The overexpression of drug efflux pumps located at the plasma membrane is recognized as a mechanism by which fungi evade the effects of antifungal drugs [28]. Therefore, to evaluate the impact of the compound tafenoquine on the inhibition of these efflux pumps, a phenotypic susceptibility assay was conducted using promethazine, an inhibitor of plasma membrane efflux pumps.[28] A combined phenotypic susceptibility assay was conducted to evaluate the impact of tafenoquine (0.25-128 mg/L) on the inhibition of efflux pumps by incorporating sub-inhibitory concentrations of promethazine (128 mg/L). All tests were performed in duplicate.

Alteration of cell membrane permeability

Modulation of cell membrane permeability was assessed using the Pierce™ BCA Protein Assay Kit. Cells of the *C. haemulonii sensu stricto* strain were suspended in sterile distilled water and adjusted to 2.5 × 10^5 cells/mL. The inoculum was mixed with tafenoquine (MIC 4 mg/L) and incubated at 37°C for 0, 12, 24, 36, and 48 h. After incubation, the samples were

centrifuged at 908 g for 5 min at 4°C. Subsequently, 25 µL of the supernatant was transferred to a 96-well plate, and 200 µL of BCA working reagent was added to each well. The plate was shaken for 30 s and then incubated at 37°C for 30 min. Absorbance at 530 nm was measured post-incubation. Protein concentration (mg/L) was calculated from the kit's calibration curve.[29] All tests were performed in duplicate.

Nucleotide leakage

The methodology followed the procedure previously described.[30] *C. haemulonii sensu stricto* was incubated in SDA at 37°C for 48 h. Subsequently, the cells were suspended in 0.9% saline to achieve concentrations of 2.5 × 10^5 cells/mL. The microorganism was then incubated with tafenoquine (MIC 4 mg/L) for durations of 0, 12, 24, 36, and 48 h. Cells incubated with 0.9% saline served as a negative control, and amphotericin B was used as a resistance control. Supernatants from the suspensions were centrifuged at 1300 g for 15 min and analyzed at 260 nm. All tests were performed in duplicate.

Hemolysis assay

Hemolysis was determined acording to priviosly described.[31] Fresh mouse blood was diluted 25 times with sterile PBS, and then 250 µL of the diluted blood was mixed with tafenoquine (MIC 4 mg/L). PBS and Triton (0.1%, v/v) were used as negative and positive controls, respectively. The samples were incubated at 37°C for 1 h and subsequently centrifuged at 700 g for 5 min. The supernatant from each sample (100 µL) was transferred into each well of a 96-well flat-bottom plate. All tests were performed in duplicate. Absorbance was measured at 490 nm with a microplate reader to calculate the hemolysis ratio (%) using the following equation:

$$\left(\frac{ODs - ODnc}{ODpc - ODnc}\right) \times 100$$

ODs: OD490 values for samples, ODnc: OD490 values for negative controls, ODpc: OD490 values for positive controls.

Acute toxicity tests in the Tenebrio molitor model

T. molitor larvae, weighing between 0.110 and 0.200 g, were divided into four groups, each consisting of 25 larvae per group. These groups were incubated for 24 h at 37°C without food. Subsequently, 5 µL of the treatments were injected directly into the larval

hemocoel between the third and fourth abdominal sternites using a Hamilton syringe (Hamilton, USA). The groups were as follows: Group 1: only PBS (negative control); Group 2: only tafenoquine (4 mg/L); Group 3: only amphotericin B (4 mg/L); and Group 4: tafenoquine with 2× MIC (8 mg/L), to evaluate toxicity. The *T. molitor* larvae were then incubated at 37°C, and the number of live larvae was determined every 24 h for 72 h. All tests were performed in duplicate.

In vivo survival assay and antifungal treatment

To evaluate the ability of tafenoquine to treat *C. haemulonii sensu stricto* infection, we utilized a technique described by.[29] Following the same groups and procedures as described previously for the toxicity assay, *C. haemulonii sensu stricto* cells cultured for 48 h in SDA at 37°C were suspended in PBS to a density of 2.5×10^5 cells/mL. A 5-µL aliquot of the cell suspension was injected into the larval hemocoel between the third and fourth abdominal segments using a Hamilton syringe, along with 5 µL of the treatment. *T. molitor* larvae were incubated at 37°C, with live larvae counted every 24 h for 72 h. All tests were duplicated.

Statistical analysis

The Tukey test compared hemolysis assay results, and Kaplan–Meier survival curves for *T. molitor* were analyzed with the log-rank test. All analyses used GraphPad Prism 8, with significance set at $P < 0.05$.

Results

Minimum inhibitory concentration (MIC)

The MIC of tafenoquine against *C. haemulonii sensu stricto* was determined to be 4 mg/L. The MICs for amphotericin B, fluconazole, and micafungin were 4 mg/L, 32 mg/L, and 0.25 mg/L, respectively. Tafenoquine demonstrated fungicidal activity against the *C. haemulonii sensu stricto* strain (4 mg/L) (Figure 1). Furthermore, the combination of tafenoquine with amphotericin B, fluconazole, or micafungin displayed an indifferent effect against the *C. haemulonii* isolate, as indicated in Table 1. An indifferent effect in this context means that the combined use of the drugs did not

significantly enhance or reduce their efficacy compared to when they are used separately. This outcome suggests that there is no synergistic interaction.

Inhibition growth assay

The efficacy of tafenoquine in inhibiting the growth of *C. haemulonii sensu stricto* showed that after 12 h of incubation, a reduction in fungal growth was observed when compared to the positive control. However, when comparing the tafenoquine treatment directly to the controls, the differences were not statistically significant. The only statistically significant reduction in fungal growth occurred when comparing the positive control, which consisted solely of *C. haemulonii sensu stricto*, with the treatment using micafungin, as shown in Figure 2.

Antibiofilm activity

Tafenoquine exhibited a significant inhibitory effect on biofilm formation, which achieved a 60.63% inhibition of biofilm formation, surpassing amphotericin B, which demonstrated an inhibition rate of 39.90% (Figure 3). Based on the statistical analyses conducted among the experimental groups, it is evident that the differences in inhibition rates between these compounds are statistically significant (Figure 3).

Cell damage

The effect of tafenoquine on mitochondrial functionality in *C. haemulonii sensu stricto* cells was investigated. Statistical analysis showed significant differences between the tafenoquine-treated group and both the positive and negative controls. Notably, tafenoquine induced substantial mitochondrial impairment, affecting 95.49% of the fungal cells, which led to significant disruption in their cellular respiration (Figure 4). This highlights tafenoquine's potent impact on the cellular mechanisms of *C. haemulonii sensu stricto*.

Sorbitol protection assay

The effect of tafenoquine on the integrity of the cell wall of *C. haemulonii sensu stricto* indicated that tafenoquine did not cause damage to the cell wall via the sorbitol pathway. A cell wall is considered damaged if the MIC of a compound in the presence of sorbitol is higher than the MIC without sorbitol. The detailed results are presented in Table 2.

Efflux pump inhibition assay

In the efflux pump assay, *C. haemulonii sensu stricto* was treated with tafenoquine at concentration of 4 mg/L, in the presence and absence of the drug promethazine, an efflux pump inhibitor, at concentration of 128 mg/L. Our results demonstrated that the activity of the tafenoquine compound remained unchanged after 48 and 72 h, indicating that efflux pumps were not activated (Table 3).

Alteration of cell membrane permeability

In our study, we evaluated the integrity of the fungal cell membrane by quantifying protein leakage from the cells, which serves as a critical indicator of cellular disruption caused by antifungal agents. Our findings revealed that tafenoquine-treated cells did not show any protein leakage, suggesting that the compound does not compromise the cell membrane integrity. Statistical analysis further supported these observations, indicating no significant differences in protein leakage between the tafenoquine-treated groups and the control groups.

Nucleotide leakage

To deepen our understanding of tafenoquine's impact on cell membrane integrity and its potential cytotoxic effects, we measured nucleotide leakage from the cells. Nucleotide leakage is a critical marker that can indicate disruption of cellular barriers and potential cellular damage. Throughout the 48-hour test period, our analysis demonstrated that cultures treated with tafenoquine did not exhibit any nucleotide leakage, suggesting that the integrity of the cell membrane was maintained under treatment. Furthermore, statistical comparisons revealed no significant differences in nucleotide leakage between the tafenoquine-treated samples and the control groups. This outcome indicates that tafenoquine's mechanism of action might not involve compromising the cell membrane, aligning with our observations of its non-disruptive effects on cellular structures.

Hemolysis assay

To assess the hemocompatibility of tafenoquine, we conducted hemolysis assays at the MIC of 4 mg/L. In our tests, no hemolysis was observed in samples treated with

tafenoquine, indicating that the compound did not disrupt the integrity of red blood cell membranes. The absence of hemolysis at this concentration suggests that tafenoquine is hemocompatible and does not induce hemolytic activity (Figure 5).

Acute toxicity tests in the T. molitor model

To evaluate the safety profile of tafenoquine, we performed acute toxicity assays using *T. molitor* larvae as a model organism. In our study, larvae were exposed to two concentrations of tafenoquine: the MIC of 4 mg/L and a higher dose of 8 mg/L. The results revealed that survival rates of the larvae decreased in a dose-dependent manner when treated with tafenoquine compared to the untreated controls. Specifically, larvae exposed to the higher concentration of 8 mg/L exhibited lower survival rates than those treated with the MIC of 4 mg/L, indicating increased toxicity with higher doses of the drug. These findings, depicted in Figure 6, underscore the importance of dose management in minimizing the toxicological impact of tafenoquine while maintaining its therapeutic efficacy.

In vivo survival assay and antifungal treatment

To understand the relative efficacy and safety of tafenoquine compared to established antifungals, we conducted an analysis of survival curves over time using *T. molitor* larvae. During the course of the experiment, we monitored and compared survival rates across different treatment groups. By the third day of treatment, notable differences emerged: the survival rate in the group treated with amphotericin B significantly decreased to 40%. In contrast, the group treated with tafenoquine exhibited a more moderate decline in survival, maintaining 60% survival by the same time point. This result, illustrated in Figure 7, suggests that tafenoquine may have a milder impact on the health of the larvae compared to amphotericin B, potentially indicating a better safety profile which could be advantageous in clinical settings where reducing drug-related toxicity is critical.

Discussion

Candida infections is increasing due to rising antimicrobial resistance and the limited availability of therapeutic alternatives. Drug repurposing has emerged as a promising alternative approach.[32]

The findings of this study demonstrate the potential of repurposing tafenoquine as an effective antifungal agent against *C. haemulonii sensu stricto*, an amphotericin B-resistant strain that poses significant clinical challenges.[18] The results indicate that tafenoquine shows fungicidal activity against *C. haemulonii sensu stricto*.

Tafenoquine treatment effectively inhibits the growth of *C. haemulonii sensu stricto* after 12 h, reinforcing its potential as a potent antifungal agent. Prior studies reported mean MICs of tafenoquine against panels of yeast and filamentous/dimorphic fungi at 4.9 and 8.3 mg/L.[15] This panel includes clinically relevant species like *C. parapsilosis*, *C. albicans*, *C. auris*, *C. guilliermondii*, *Fusarium*, and *Aspergillus*, with varied resistance profiles; notably, *C. auris* shows high resistance to conventional antifungals. Additionally, tafenoquine reduced lung fungal burden in a dose-dependent manner against a susceptible *Rhizopus* strain (MIC 4 mg/L) in a lung infection model.[15] Tafenoquine has also shown antibacterial activity against methicillin-resistant *Staphylococcus aureus*.[16]

Tafenoquine exhibited a notable reduction in biofilm formation, a critical factor contributing to antifungal resistance and persistent infections by offering a protective haven for fungal cells.[33] The compound decreased biofilm formation by 60.63%, showcasing its ability to disrupt these protective structures and negatively affect mitochondrial function, which in turn impacts cellular respiration.

Recent studies have underscored the broad-spectrum antifungal activity of tafenoquine against a range of *Candida* species, including *C. auris*, highlighting its potential as a repurposed antifungal agent.[15] Similar to our findings, these studies have shown that tafenoquine not only inhibits fungal growth but also biofilm formation, which is crucial for treating infections caused by biofilm-forming pathogens like *C. haemulonii sensu stricto*. The ability of tafenoquine to disrupt biofilm structures, as reported in our results, aligns with observations that tafenoquine decreases fungal burden in lung models of invasive fungal infections.[15] This alignment emphasizes the clinical relevance of our results, suggesting that tafenoquine could be effectively integrated into treatment regimens for severe fungal infections.

The observed impairment in mitochondrial activity is likely due to tafenoquine's interference with mitochondrial processes, leading to a decrease in energy production. Similar effects have been observed in studies with *Leishmania*, where tafenoquine was found to disrupt mitochondrial functionality, reduce ATP levels, and impair respiratory processes by targeting cytochrome c reductase (complex III).[34] Our observations regarding the mitochondrial impairment caused by tafenoquine provide a novel insight into its antifungal mechanism. This mechanistic action mirrors the findings from other studies where tafenoquine has been shown

to impair mitochondrial function in protozoan parasites, leading to inhibited respiration and ATP production.[34] By comparing these effects across different pathogens, our study not only extends the known pharmacological impacts of tafenoquine but also supports its potential utility in a broader antimicrobial context. Understanding this mitochondrial disruption is crucial, as it offers a therapeutic target that is distinct from those of many current antifungals, which primarily focus on cell wall synthesis and integrity.

We investigated tafenoquine's mechanism by examining cell membrane integrity and efflux pump activity. Minimal protein leakage suggests tafenoquine does not disrupt the fungal cell membrane, indicating a non-membrane-targeting mechanism. The lack of efflux pump activation suggests tafenoquine's antifungal effects are unaffected by common resistance mechanisms, indicating its potential in combination therapies to overcome resistance and enhance treatment outcomes. The observed nucleotide leakage suggests tafenoquine may compromise cell membrane integrity through alternative mechanisms. For example, caspofungin inhibits glucan synthesis, leading to osmotic instability and fungal cell death,[35] while fluconazole disrupts ergosterol synthesis, compromising the cell membrane and causing cell lysis.[36]

In vivo studies with the *T. molitor* model showed tafenoquine is well-tolerated, non-hemolytic, and lacks acute toxicity in the concentrations used, confirming a favorable safety profile and supporting its potential for antifungal repurposing.[15] Tafenoquine exhibited no significant toxicity at the tested concentrations and conditions within the *Galleria mellonella* model, supporting its potential as a therapeutic agent against fungal infections.[37] Additionally, tafenoquine is safe and effective for chemoprophylaxis of malaria in humans[38] and prevents malaria in mice at a dose of 5 mg/kg per day.[39] This same dosage increased survival and decreased fungal burden in Rhizopus-infected mice, highlighting tafenoquine's potential as a pharmacological candidate against fungal lung infections in humans.

Tafenoquine showed antifungal activity in the *T. molitor* infection model, with a survival rate of about 60% after three days in the treated group. This result suggests that tafenoquine exhibits therapeutic potential for treating *C. haemulonii sensu stricto* infections. The moderate survival rate suggests that while tafenoquine is effective, its clinical use may require dosage optimization or combination with other antifungals. A zebrafish infection study showed *C. haemulonii* caused about 80% mortality, highlighting its virulence and the need for potent treatments.[40]

Our findings highlight tafenoquine as a strong candidate for drug repurposing, showing promise as an antifungal against *C. haemulonii sensu stricto* by inhibiting growth and biofilm

formation. Further research is needed to enhance efficacy, explore synergy, and understand its molecular action to address potential resistance mechanisms.

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Transparency declarations

None to declare.

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Table 1. Minimum Inhibitory Concentration of tafenoquine and antifungal agents against *C. haemulonii sensu stricto*, alone and in combination

	MIC (mg/L)		Combined use MIC (mg/L)			
Strain	**Tafenoquine**	**Amphotericin B**	**Tafenoquine**	**Amphotericin B**	**FICI**	**I**
C. haemulonii sensu stricto	4	4	8	0.5	2.12	IND
	Tafenoquine	**Fluconazole**	**Tafenoquine**	**Fluconazole**	**FICI**	**I**
C. haemulonii sensu stricto	4	32	4	2	1.06	IND
	Tafenoquine	**Micafungin**	**Tafenoquine**	**Micafungin**	**FICI**	**I**
C. haemulonii sensu stricto	4	0.25	8	0.25	3.00	IND

MIC: Minimum Inhibitory Concentration. FICI (fractional inhibitory concentration index). FICI interpretation corresponded to the following definitions: synergism (SYN), FICI ≤0.5; indifference (IND), FICI >0.5-≤4; antagonism (ANT), FICI >4. I: interpretation.

Table 2. Minimum Inhibitory Concentration (MIC) of tafenoquine in the presence or absence of sorbitol

Treatment	**Incubation time**			
	48h MIC (mg/L)		**72h** MIC (mg/L)	
	S-	**S+**	**S-**	**S+**
Tafenoquine	4	4	4	4
Micafungin	1	≥8	1	≥8

S-: Sorbitol absence, S+: Presence sorbitol.

Table 3. Minimum Inhibitory Concentration (MIC) of tafenoquine in the presence or absence of promethazine

Treatment	Incubation time			
	48 h MIC (mg/L)		72 h MIC (mg/L)	
	P-	P+	P-	P+
Tafenoquine	4	128	4	128

P-: Absence of promethazine, P+: Presence of promethazine.

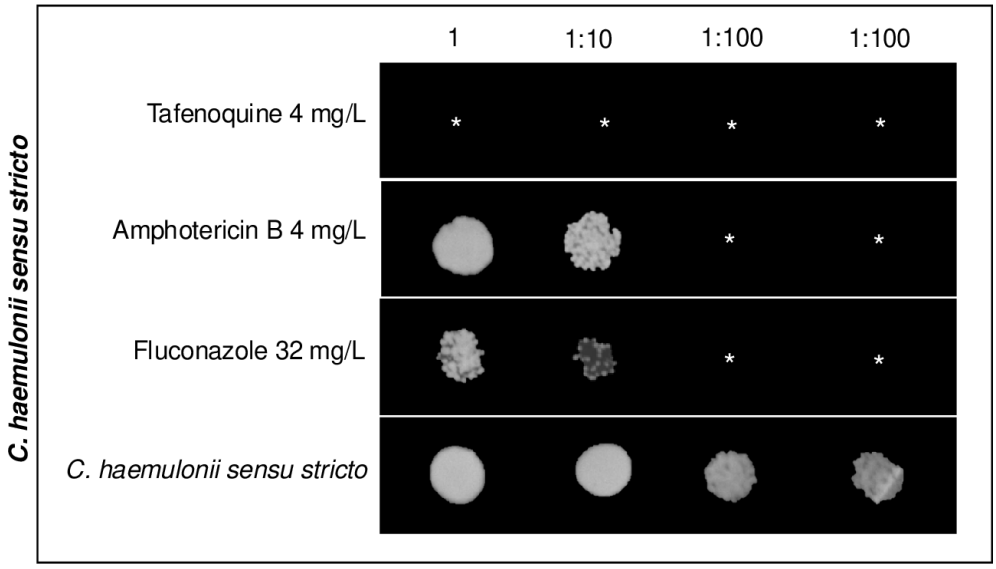

Figure 1. Minimum fungicidal concentration (MFC) determination for tafenoquine. Tafenoquine resulted in fungicidal activity.

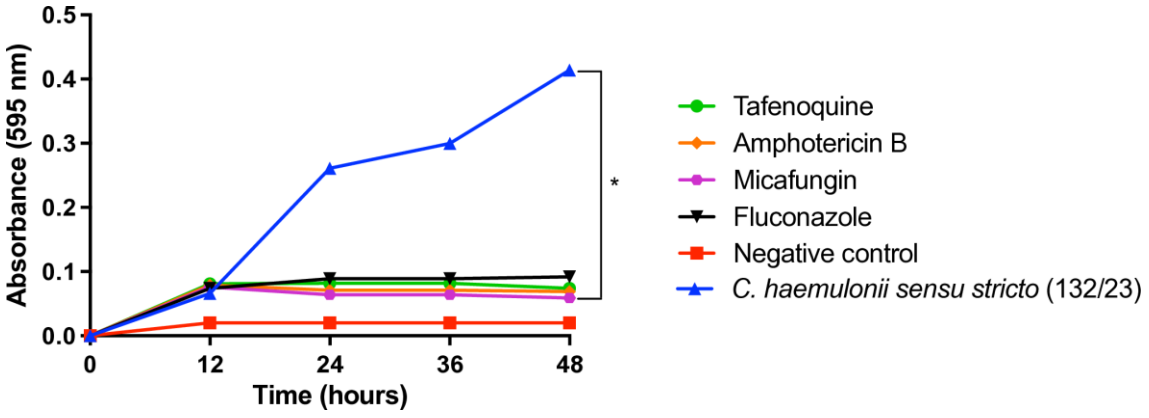

Figure 2. Growth curve of *C. haemulonii sensu stricto* in the presence and absence of tafenoquine. The test was conducted at concentration of tafenoquine (at MIC 4 mg/L), amphotericin B (at MIC 4 mg/L), micafungin (at MIC 0.25 mg/L), and fluconazole (at MIC 32 mg/L). * $p < 0.05$: micafungin and *C. haemulonii sensu stricto*.

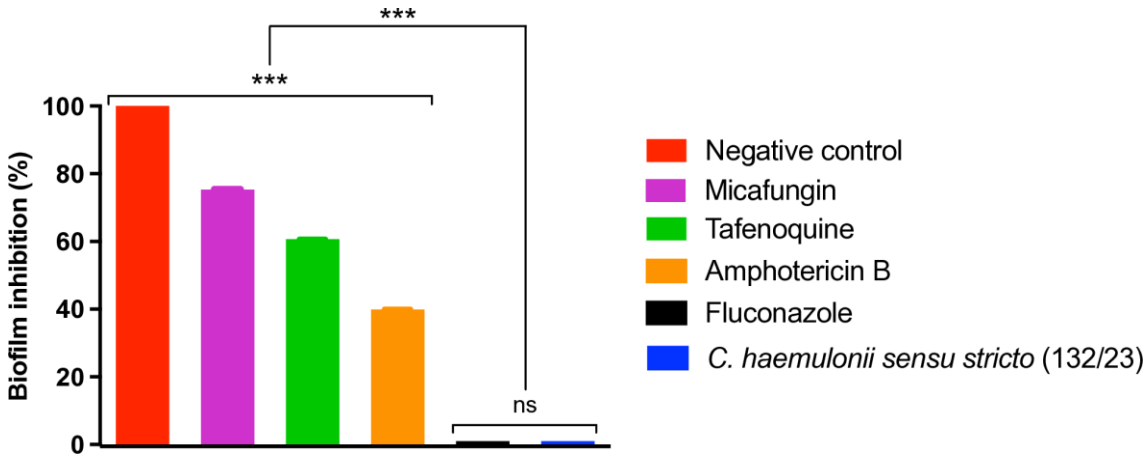

Figure 3. Antibiofilm activity of tafenoquine against *C. haemulonii sensu stricto*. The test was conducted at concentration of tafenoquine (at MIC 4 mg/L), amphotericin B (at MIC 4 mg/L), micafungin (at MIC 0.25 mg/L), and fluconazole (at MIC 32 mg/L). ns: not significant. *** $p < 0.001$.

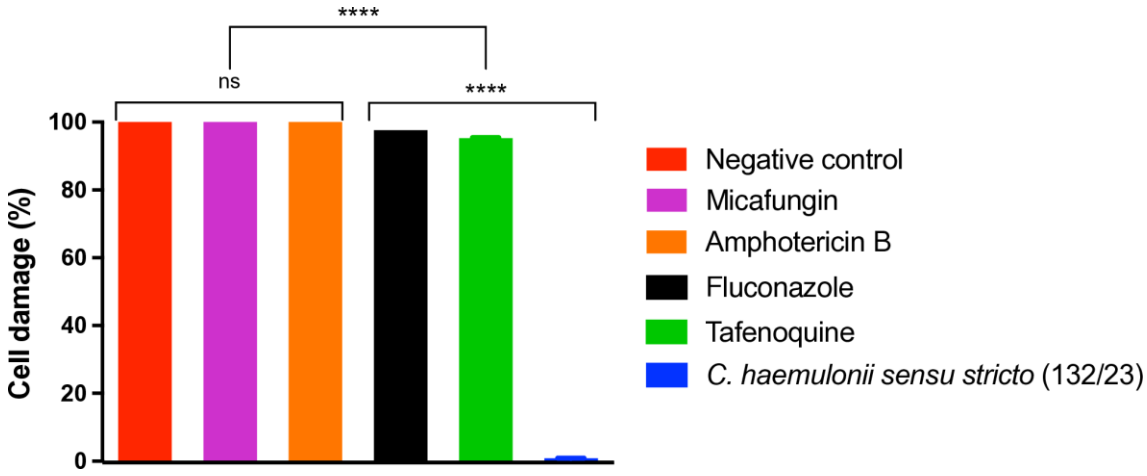

Figure 4. Damage to mitochondria of *C. haemulonii sensu stricto* cells in the presence and absence of tafenoquine (at MIC 4 mg/L). The test was conducted at concentration of amphotericin B (at MIC 4 mg/L), micafungin (at MIC 0.25 mg/L), and fluconazole (at MIC 32 mg/L). ns: not significant. **** $p < 0.0001$.

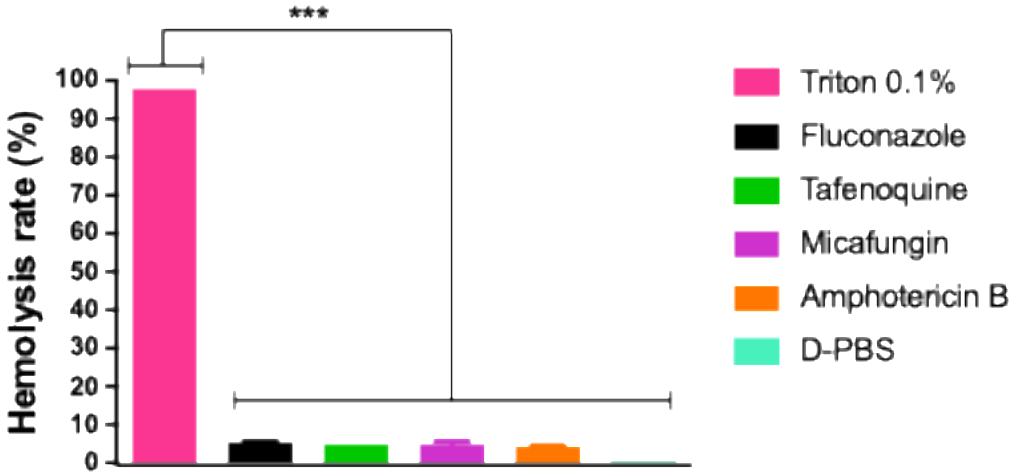

Figure 5. Hemolysis assay. The relative hemolysis rate in mouse red blood cells after incubation with tafenoquine (at MIC 4 mg/L), amphotericin B (at MIC 4 mg/L), micafungin (at MIC 0.25 mg/L), and fluconazole (at MIC 32 mg/L). *** $p < 0.001$.

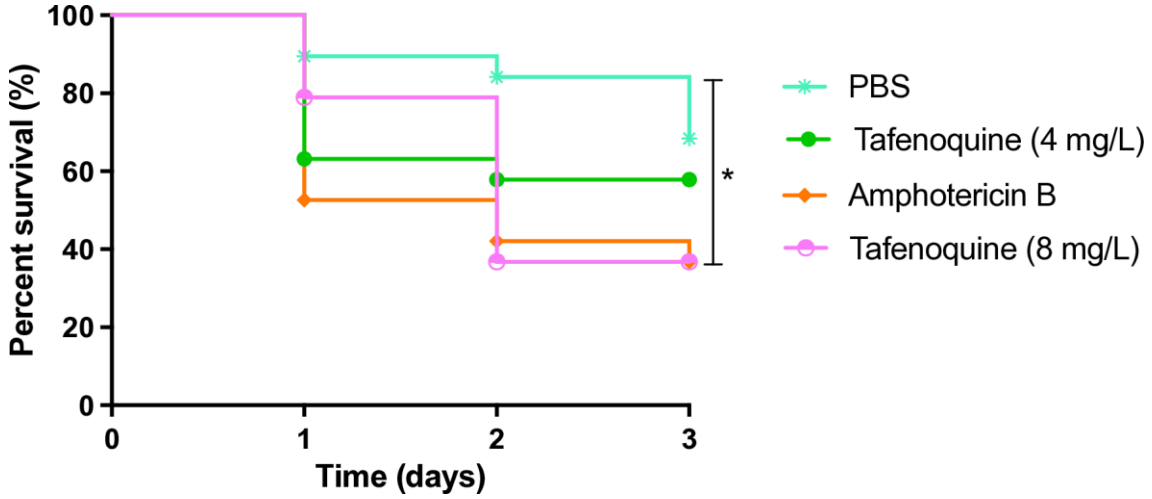

Figure 6. Acute toxicity of tafenoquine, consisting of 25 larvae per group. PBS was used as a negative control. The test was conducted at concentration of amphotericin B (at MIC 4 mg/L). *$p < 0.05$, determined by the log-rank test.

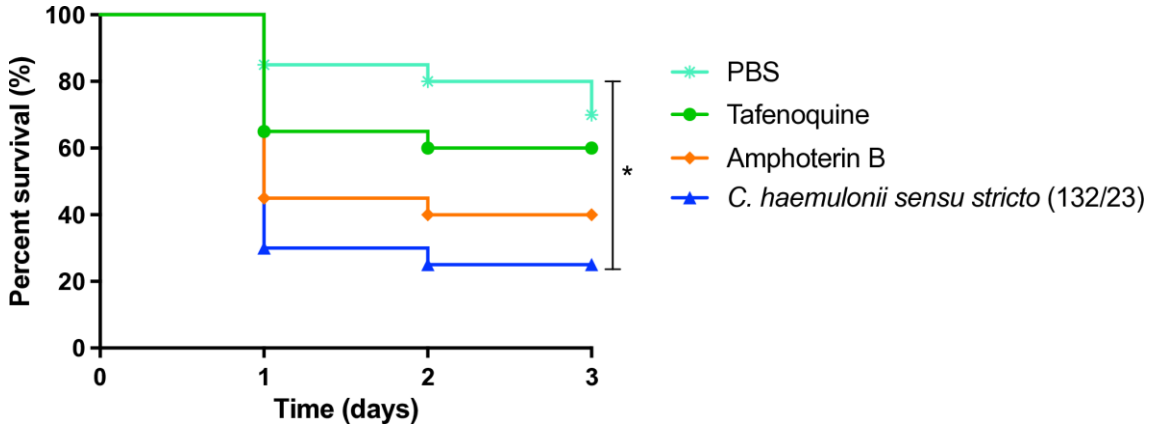

Figure 7. Survival of *T. molitor* larvae infected with *C. haemulonii sensu stricto*, consisting of 25 larvae per group. Tafenoquine (at MIC 4 mg/L); amphotericin B (at MIC 4 mg/L); PBS as a negative control. * $p < 0.05$, determined by the log-rank test.

4.3. Artigo 3

Alexidine as a Potent Antifungal Agent Against *Candida haemulonii sensu stricto*

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ABSTRACT: The increasing prevalence of infections by *Candida haemulonii sensu stricto*, particularly due to its resistance to standard antifungal therapies, represents a significant healthcare challenge. Traditional treatments often fail, emphasizing the need to explore alternative therapeutic strategies. Drug repurposing, which re-evaluates existing drugs for new applications, offers a promising path. This study examines the potential of repurposing alexidine dihydrochloride as an antifungal agent against *C. haemulonii sensu stricto*. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values were established using broth microdilution methods. To further assess antifungal activity, different assays were conducted, including growth inhibition, biofilm inhibition, biofilm eradication, and cell damage. Checkerboard assays were employed to study the compound's fungicidal potential and interactions with other antifungals. Additional tests, sorbitol protection assay, efflux pump inhibition, cell membrane permeability assays, and nucleotide leakage were performed. *In vivo* efficacy and safety were evaluated in *Tenebrio molitor* larvae. Alexidine demonstrated fungicidal activity against *C. haemulonii sensu stricto*, with an MIC of 0.5 µg/mL. Biofilm formation was significantly inhibited, with a reduction of 78.69%. Mechanistic studies revealed nucleotide leakage, indicating membrane impact, but no significant protein leakage was detected. *In vivo*, alexidine displayed a favorable safety profile, with no evidence of hemolysis or acute toxicity in the *T. molitor* model. These findings support alexidine as a strong candidate for antifungal drug repurposing, especially for treating *C. haemulonii sensu stricto* infections. Its efficacy in inhibiting growth and biofilm formation, combined with a positive safety profile, underscores its potential for clinical development as an antifungal therapy.

KEYWORDS: Alexidine, biofilm, drug repurposing, virulence.

INTRODUCTION

The rise of infections caused by species in the *Candida haemulonii* complex, including *C. haemulonii sensu stricto*, *C. haemulonii var. vulnera*, and *C. duobushaemulonii*, presents a growing challenge in healthcare settings, particularly among immunocompromised patients. These yeasts have been increasingly identified in tropical regions,[1,2] and exhibit concerning traits, such as multidrug resistance,[3,4] and a strong ability to adhere to prosthetic materials.[4,5]

Although *C. haemulonii* complex infections have been rarely reported since their discovery.[6] *C. haemulonii* complex species often demonstrate high Minimum Inhibitory Concentrations (MICs) for fluconazole, a primary antifungal agent used in treating candidemia and other *Candida* infections,[7,8] as well as reduced susceptibility to amphotericin B, commonly used in severe or refractory cases. Although echinocandins remain effective for most isolates, cases of resistance have also been documented.[9]

Consequently, there is an urgent need to explore alternative therapeutic strategies to effectively combat these infections. Drug repurposing, which entails re-evaluating existing pharmaceuticals for novel therapeutic uses, has emerged as a promising strategy to address this escalating challenge.[10] This approach can expedite the identification of effective treatments by leveraging known safety profiles and pharmacodynamics, offering a faster path to clinical application.[11,12]

Alexidine dihydrochloride, a bis-biguanide compound, is a well-established antibacterial agent also recognized for its anti-inflammatory and anticancer properties. It induces apoptosis by inhibiting the mitochondrial tyrosine phosphatase PTPM1.[13] Currently, alexidine is used in mouthwash as an antiplaque agent and is applied in endodontic treatments to effectively remove biofilms.[14] Recent studies have shown that alexidine exhibits antifungal activity against some species, including *C. albicans*, *Trichophyton mentagrophytes*, *C. auris*, and *Aspergillus fumigatus*. Notably, *Trichophyton* spp. have displayed acquired resistance to terbinafine and itraconazole, which are traditionally the drugs of choice for treating dermatophyte infections. *C. auris*, a multidrug-resistant pathogen, is among the most invasive human pathogens, alongside *C. albicans* and *A. fumigatus* both of which also show significant drug resistance.[15–17] This suggests that alexidine could be a promising candidate for treating candidiasis, as it may inhibit yeast adhesion, biofilm formation, and other pathogenic traits crucial for managing fungal infections.[18]

This study aims to evaluate the potential of AXD as a repositioned antifungal agent for developing effective treatments against *C. haemulonii sensu stricto* infections.

RESULTS

Minimum inhibitory concentration (MIC)

The MIC of alexidine against *C. haemulonii sensu stricto* was found to be 0.5 µg/mL. The MIC values for amphotericin B, fluconazole, and micafungin were determined as 4 µg/mL, 16 µg/mL, and 0.25 µg/mL, respectively. At its MIC of 0.5 µg/mL, alexidine displayed fungicidal activity against *C. haemulonii sensu stricto*. However, when alexidine was combined with

amphotericin B, fluconazole, or micafungin, no synergistic effects were observed against the *C. haemulonii sensu stricto* isolate (Table 1).

Table 1. Minimum Inhibitory Concentration of alexidine and antifungal agents against *C. haemulonii sensu stricto*, alone and in combination

	MIC (µg/mL)					
	Alexidine		Test Agent			
Test Agent	Alone	Combined	Alone	Combined	ΣFICI[a]	Interaction
Amphotericin B	0.5	1	4	0.5	2.12	IND
Fluconazole	0.5	0.5	16	2	1.12	IND
Micafungin	0.5	0.5	0.25	0.25	2.00	IND

MIC: Minimum Inhibitory Concentration. [a]ΣFICI (fractional inhibitory concentration index) is used to measure the interaction between the tested combinations. ΣFICI interpretation corresponded to the following definitions: synergism (SYN), ΣFICI ≤ 0.5; additivity (ADD), ΣFICI > 0.5 and ≤1; and indifference (IND), ΣFICI > 1 and ≤4; antagonism (ANT), ΣFICI ≥ 4

Inhibition growth assay

The efficacy of alexidine in inhibiting the growth of *C. haemulonii sensu stricto* revealed a reduction in fungal growth after 12 hours of incubation compared to the positive control, indicating a statistically significant difference between alexidine and the positive control, further underscoring the inhibitory effect of this agent (Figure 1). Additionally, significant differences were observed when comparing the positive control with both micafungin and amphotericin B (Figure 1).

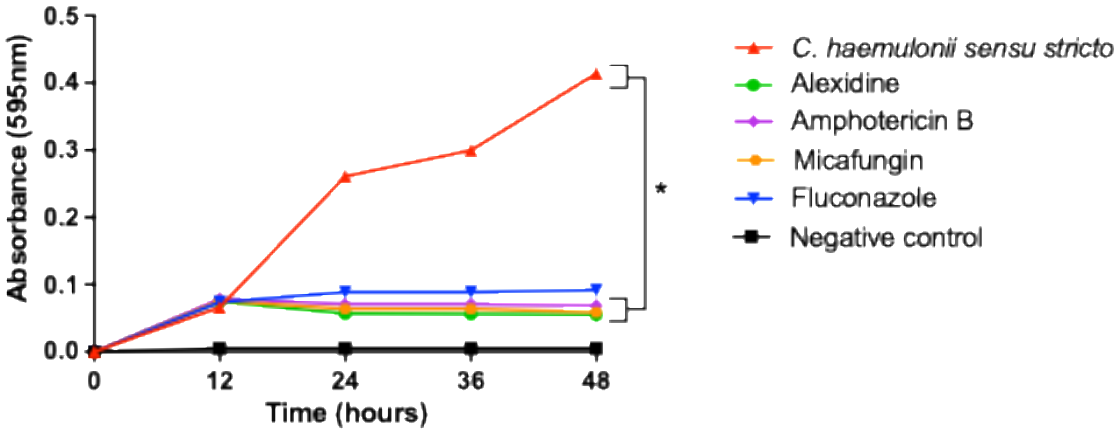

Figure 1. Growth curve of *C. haemulonii sensu stricto* in the presence and absence of alexidine. The test was conducted at concentration of alexidine (MIC 0.5 µg/mL). The negative control represents the absence of yeast. * $p < 0.05$.

Alexidine inhibited biofilm formation

Alexidine demonstrated a notable inhibitory effect compared to fluconazole, achieving a biofilm formation inhibition rate of 78.69%. In comparison, micafungin, chlorhexidine, and amphotericin B showed inhibition rates of 75.27%, 72.93%, and 39.9%, respectively. Among all treatments tested, alexidine exhibited the highest effectiveness in inhibiting biofilm formation (Figure 2).

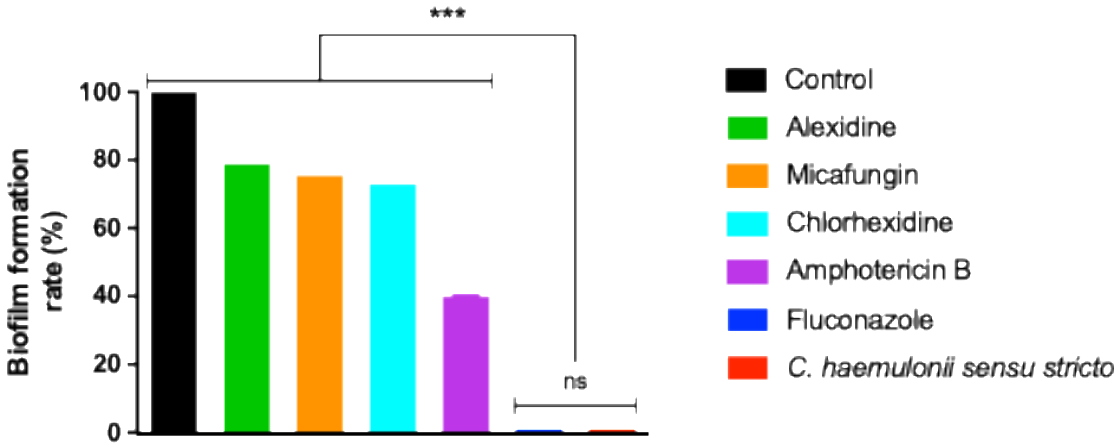

Figure 2. Biofilm formation inhibition test. The test was conducted at concentration of alexidine (MIC 0.5 µg/mL). The control represents the absence of yeast. Not significant (ns). *** $p < 0.001$

Biofilm eradication assay

The biofilm eradication assay was performed on *C. haemulonii sensu stricto* using alexidine at a concentration of 0.5 µg/mL, with chlorhexidine (0.12%) included as a comparative disinfectant. Alexidine achieved a biofilm eradication rate of 71.42% at 15 minutes and 58.53% at 45 minutes, whereas chlorhexidine showed eradication rates of 100.00% at 15 and 45 minutes (Figure 3).

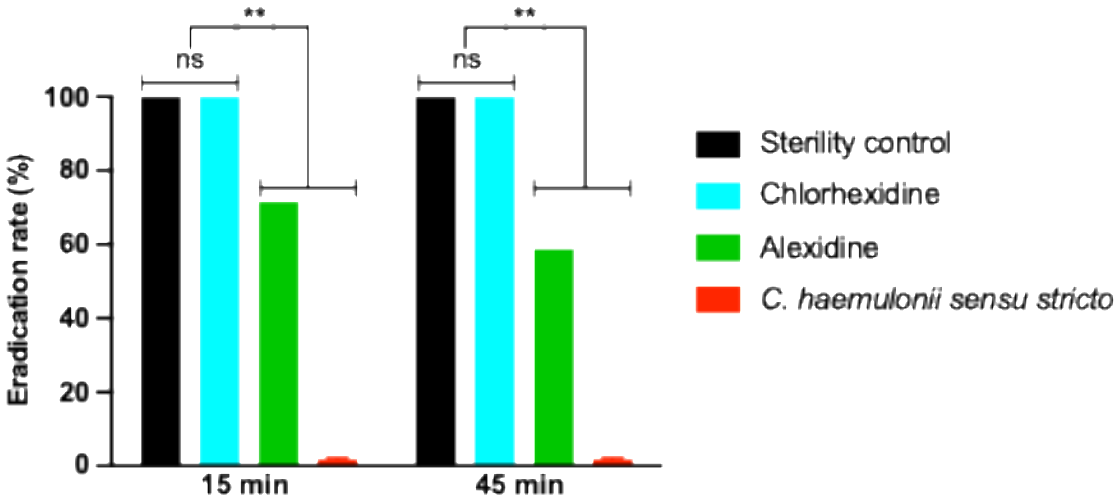

Figure 3. Biofilm eradication. The biofilm eradication test was conducted using alexidine (0.5 µg/mL), chlorhexidine (0.12%), sterility control (only peptone water), and *C. haemulonii sensu stricto*. Not significant (ns). ** $p < 0.01$.

Cell damage

The results indicate that while amphotericin B and micafungin demonstrated superior efficacy compared to alexidine, the use of alexidine still resulted in a significant impact on mitochondrial functionality. Specifically, alexidine induced mitochondrial dysfunction in 48.73% of *C. haemulonii sensu stricto* cells, leading to notable disruption in cellular respiration. These findings emphasize the potential of alexidine as a valuable option in targeting mitochondrial integrity, even when its effectiveness is slightly lower than that of amphotericin B and micafungin (Figure 4).

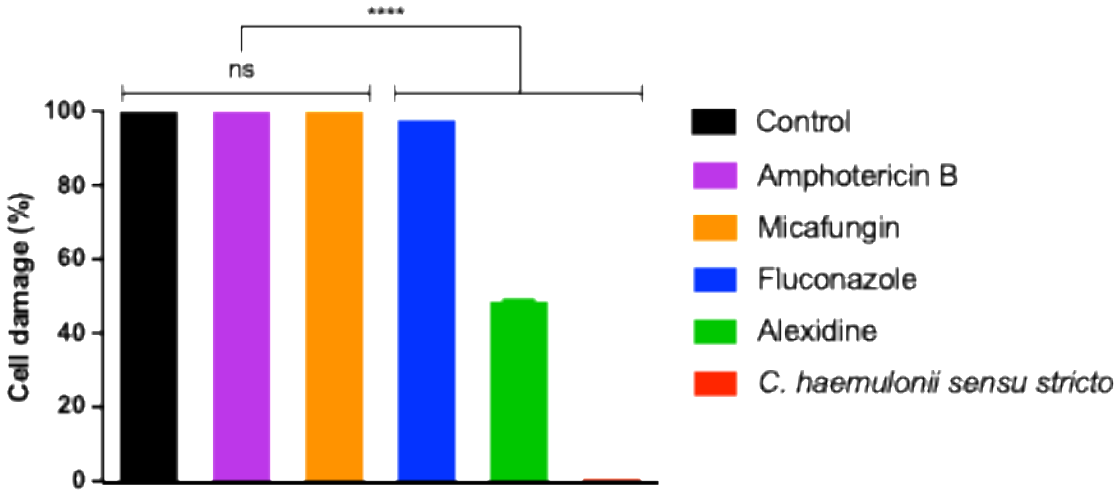

Figure 4. Damage to mitochondria of *C. haemulonii* sensu stricto cells in the presence and absence of alexidine. The test was performed at MIC concentration (0.5 µg/mL). The control represents the absence of yeast. Not significant (ns). **** $p < 0.0001$.

Sorbitol protection assay

The impact of alexidine on the cell wall integrity of *C. haemulonii sensu stricto* showed that alexidine did not compromise the cell wall structure via the sorbitol-dependent pathway, as no increase in MIC was observed when sorbitol was present. According to established criteria, cell wall damage is indicated when the MIC of a compound rises in the presence of sorbitol compared to its absence. These findings are detailed in Table 2, confirming that alexidine's antifungal action does not involve direct disruption of cell wall integrity.

Table 2. Minimum Inhibitory Concentration of alexidine in the presence or absence of sorbitol

Treatment	Incubation time			
	48h MIC (µg/mL)		72h MIC (µg/mL)	
	S-	S+	S-	S+
Alexidine	0.5	0.5	0.5	0.5
Micafungin	1	≥8	1	≥8

S-: Sorbitol absence, S+: Presence sorbitol, MIC: Minimum Inhibitory Concentration

Efflux pump inhibition assay

The study also included tests to assess whether the alexidine could inhibit efflux pumps, a common mechanism contributing to resistance. For the efflux pump assay, *C. haemulonii sensu stricto* cells were exposed to alexidine at a concentration of 0.5 µg/mL, both with and without the addition of promethazine (128 µg/mL), an established efflux pump inhibitor. Results showed that the antifungal activity of alexidine remained consistent at both 48 and 72 hours, suggesting that efflux pumps were not activated under these conditions. Specifically, the presence of promethazine did not influence the activity of alexidine, indicating no involvement of efflux pumps in mediating resistance (Table 3).

Table 3. Minimum Inhibitory Concentration of alexidine in the presence or absence of promethazine

Treatment	Incubation time			
	48 h MIC (µg/mL)		72 h MIC (µg/mL)	
	P-	P+	P-	P+
Alexidine	0.5	64	0.5	64

P-: Sorbitol absence, P+: Presence sorbitol, MIC: Minimum Inhibitory Concentration

Alteration of cell membrane permeability

To evaluate the integrity of the fungal cell membrane, protein leakage was measured as an indicator of potential damage or disruption caused by alexidine treatment. Protein leakage is a commonly used marker to assess whether compounds compromise the cellular structure, as it reflects the ability of the membrane to retain essential macromolecules. In this study, cells treated with alexidine showed no detectable protein leakage, indicating that the membrane remained intact and functional. This suggests that, under the tested conditions, alexidine did not cause significant structural damage to the fungal cell membrane, preserving its ability to maintain cellular homeostasis.

Nucleotide leakage

To further investigate cell membrane integrity and assess the potential cytotoxic effects of alexidine, nucleotide leakage was measured. Results indicated that cultures treated with alexidine exhibited nucleotide leakage, with peak levels observed at 12 hours. Statistical analysis revealed significant differences between the alexidine treatment and control groups, highlighting its impact on membrane integrity (Figure 5).

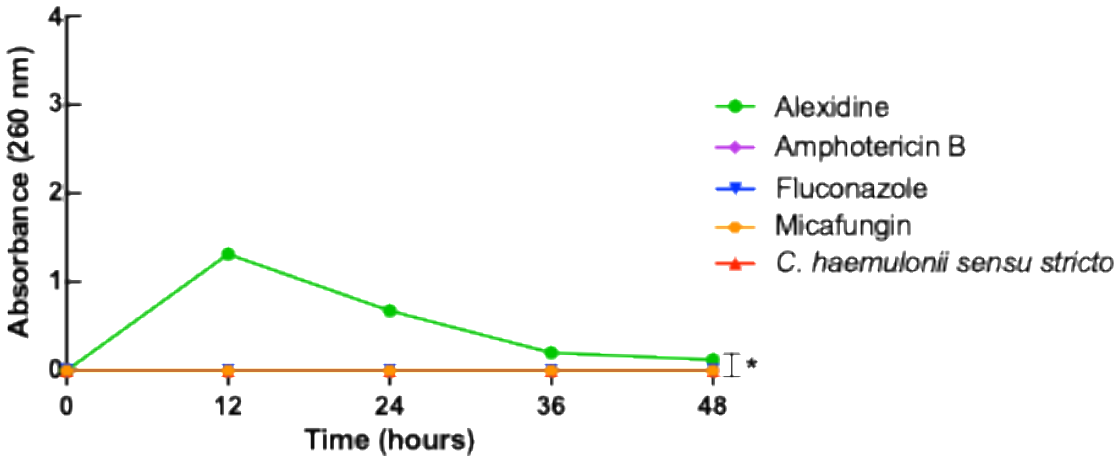

Figure 5. Nucleotides extravasated from *C. haemulonii sensu stricto* after treatment with alexidine. The test was conducted at MIC concentration (0.5 µg/mL). * $p < 0.05$.

Hemolysis assay

Hemolysis assays were performed to assess the hemocompatibility of alexidine. At a concentration of 0.5 µg/mL, alexidine showed no evidence of inducing hemolysis. These results indicate that alexidine is both hemocompatible and non-hemolytic (Figure 6).

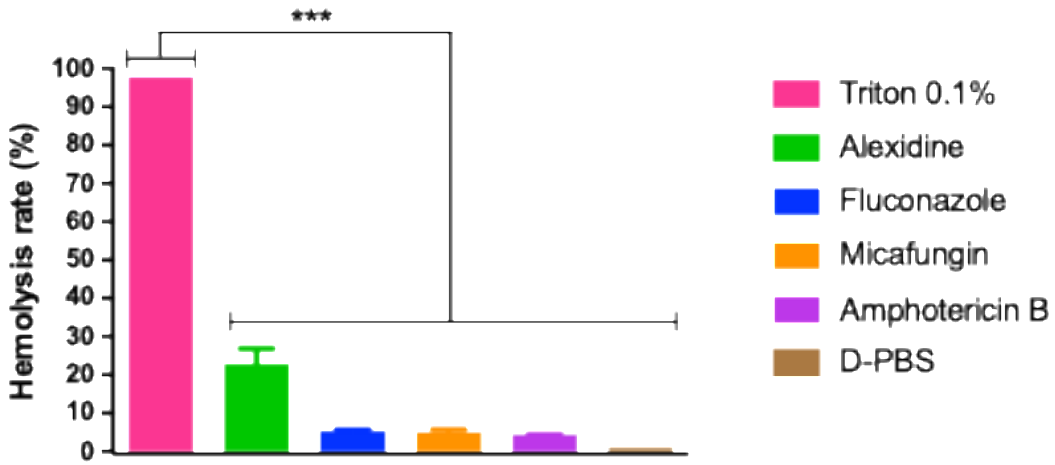

Figure 6. Hemolysis assay. The relative hemolysis rate in commercially sourced defibrinated sheep blood after incubation with alexidine (MIC 0.5 µg/mL), D-PBS (negative control), and 0.1 % Triton (positive control). *** $p < 0.001$.

In vivo survival assay and antifungal treatment

Survival curves were generated and analyzed to assess differences in survival rates among the treatment groups over time. The PBS control group displayed a steady

decrease in survival over the 3-day observation period. In contrast, the amphotericin B-treated group showed a more pronounced decline, with survival rates dropping to approximately 40% by day 3. The group treated with alexidine exhibited a slower decline in survival, maintaining approximately 61% at the end of the 3-day period (Figure 7).

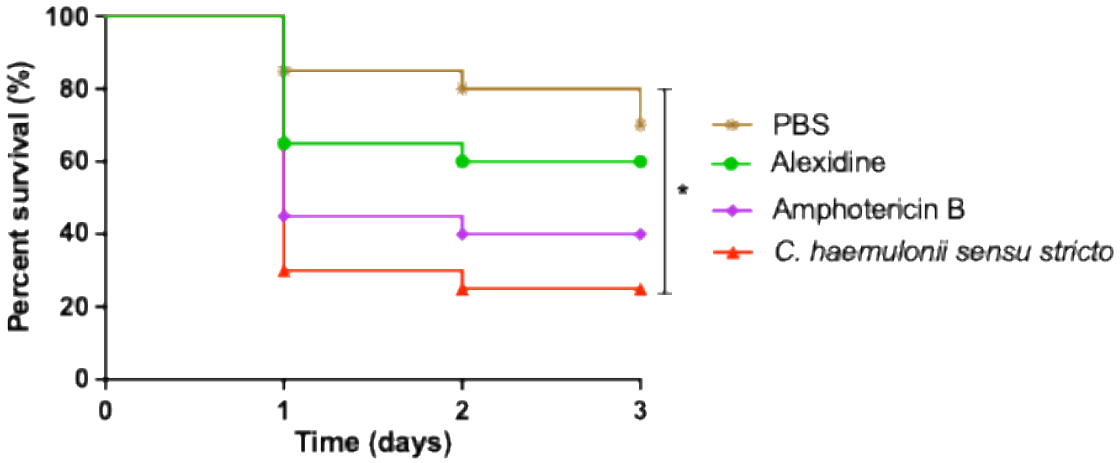

Figure 7. Survival of *T. molitor* larvae infected with *C. haemulonii sensu stricto*, consisting of 25 larvae per group. Alexidine (MIC 0.5 µg/mL); PBS: negative control. * $p < 0.05$, determined by the log-rank test.

DISCUSSION

The severity of *Candida* infections is escalating due to rising antimicrobial resistance and a scarcity of effective treatment options. Drug repurposing offers a viable approach to address these challenges, offering the potential to expedite the discovery of new therapeutic uses for existing drugs. This study explores the antifungal potential of alexidine against the *C. haemulonii sensu stricto* strain. Specifically, the investigation assessed the impact of alexidine on various growth and virulence-related factors, including biofilm formation, membrane integrity, and mitochondrial function.

Alexidine is primarily used for its antimicrobial properties in applications such as contact lens solutions and mouthwashes.[37] In this study, however, alexidine effectively inhibited the growth of *C. haemulonii sensu stricto*. Supporting these findings, Mamouei et al.[14] conducted a screening of the New Prestwick Chemical Library, revealing that alexidine suppresses growth by at least 50% in several *Candida* species (including *C. albicans* and *C. auris*), as well as *Cryptococcus neoformans* and *A. fumigatus*. Furthermore, alexidine's efficacy against various filamentous fungi, including *Fusarium solani* and *F. oxysporum* was documented.[38] Together

with previous studies, our results further underscore the broad-spectrum antifungal potential of alexidine, extending its efficacy to *C. haemulonii sensu stricto*.

Biofilms exhibit unique developmental characteristics, making them significantly more challenging to treat than planktonic cells.[39] This complexity is especially relevant given the widespread issue of antimicrobial resistance, which emphasizes the critical need to evaluate both existing and novel antifungal agents for efficacy against biofilm-associated cells.[27] In this context, our study's findings on alexidine are particularly compelling, as it demonstrated a significant reduction in biofilm formation. Building on previous research that highlighted the anti-biofilm potential of alexidine in species such as *A. fumigatus*, *C. neoformans*, and multiple *Candida* species, and noting its effectiveness against preformed *C. albicans* biofilms in both *in vitro* and *in vivo* catheter models.[17,40] The effect of alexidine on *C. haemulonii sensu stricto* biofilms had not been previously evaluated; however, our data demonstrated that alexidine was the most effective agent against biofilms of this species when compared to other tested drugs.

The mechanism of action of alexidine in fungi remains little explored. In bacterial cells, the positively charged alexidine is attracted to the negatively charged bacterial cell wall, where it induces lipid-phase separation and the formation of lipid rafts within bacterial membranes.[37,40] Given that the fungal cell wall is also negatively charged, a similar interaction with fungal membranes may be inferred.

Furthermore, studies have shown that in certain cancer cell lines, alexidine affects membrane permeability and induces mitochondrial damage by targeting the mitochondrial phosphatase PTPMT1, suggesting that this mechanism might extend to other eukaryotic cells, including fungi.[41,42] In *C. haemulonii sensu stricto*, alexidine led to the leakage of ions or nucleotides without causing membrane destabilization. The overexpression of drug efflux pumps in the plasma membrane is a well-known mechanism by which fungi resist the effects of antifungal agents,[32] but alexidine's mechanism appears to bypass this resistance.

The cytotoxicity of alexidine has been previously documented in the literature.[40,43] Mamouei et al.[17] reported that alexidine caused damage to HUVEC (an endothelial cell line) and A549 cells at a concentration of 14.7 µg/mL. However, only concentrations exceeding the MICs required for antifungal activity are likely to harm host cells. Although our study produced favorable results in hemolysis assays, further research is necessary to fully assess the safety profile of alexidine.

The study of Nabeela et al.[16] demonstrated that the topical application of alexidine achieves both clinical and mycological clearance of fungal infections, specifically dermatophytosis. By day 7 post-treatment, lesions were completely healed, showing a substantial reduction in

infection following topical administration. While untreated, infected mice displayed areas of affected skin and positive cultures, those treated with alexidine showed significantly reduced erythema (83%–91% efficacy; $p < 0.0001$) and a complete absence of fungal growth in culture, indicating 100% mycological clearance post-treatment. These findings align with our results, where a survival rate of 61% was observed in *T. molitor* treated with alexidine, outperforming other control groups and underscoring its therapeutic potential.

There is an urgent need to identify and characterize new agents with efficacy against *C. haemulonii sensu stricto*. Our findings highlight alexidine as a promising candidate for addressing *C. haemulonii sensu stricto* infections. Its existing clinical approval offers a valuable advantage, potentially accelerating the repurposing process and enabling a more rapid integration into antifungal treatment strategies.

In conclusion, this study identifies alexidine as a promising antifungal agent against *C. haemulonii sensu stricto*, particularly for its effectiveness in inhibiting and eradicating biofilms. These findings are significant given the strong biofilm-mediated resistance exhibited by *C. haemulonii sensu stricto*, which poses challenges for treatment and infection control in healthcare settings. Our findings also reveal alexidine's mechanism of action, marked by nucleotide leakage without compromising cell wall integrity, and demonstrate its efficacy *in vivo*. This approach provides a swift pathway for developing effective antifungal therapies and may support new strategies for managing complex hospital infections involving resistant pathogens, as demonstrated by alexidine's ability to inhibit biofilm formation and disrupt other critical cellular processes in *C. haemulonii sensu stricto*.

METHODS

Strain and culture conditions

The *C. haemulonii sensu stricto* (132/23) strain utilized in this study was obtained from the strain library of the Center for Studies in Applied Medical Mycology (CSAMM) at the Health Sciences Research Laboratory (HSRL), Universidade Federal de Grande Dourados (UFGD). This strain had been previously characterized for its biofilm production and identified through sequencing of the Internal Transcribed Spacer (ITS) region of rDNA.[19,20] To assess the interaction between tafenoquine and antifungal agents, we selected this strain, an isolate obtained from a blood culture, which demonstrated resistance to amphotericin B.[7] The strain was stored in 20% glycerol at -80°C until required for experimentation. For the experimental

procedures, the strain was cultured on Sabouraud Dextrose Agar (SDA), and incubated at 37°C for 48 hours.

Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the drug was determined using the broth microdilution method, following the guidelines set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 7.4).[44] The sensitivity of alexidine dihydrochloride was evaluated across a concentration range of 0.25 to 128 µg/mL. Fungal suspensions were prepared, diluted in sterile distilled water, and inoculated into 96-well plates at a final density of 2.5×10^5 cells/mL. The plates were incubated at 37°C for 48 hours. Antifungal activity was assessed spectrophotometrically at 530 nm, with the MIC defined as the lowest concentration of the compound that inhibited 90% of fungal growth. To determine the Minimum Fungicidal Concentration (MFC), 10 µL samples from each MIC well were transferred to Sabouraud agar plates and incubated at 37°C for 48 hours. The MFC was defined as the lowest concentration at which no visible fungal growth occurred. Amphotericin B, at concentrations ranging from 0.03 to 16 µg/mL, served as a resistance control.

Checkboard assay

Checkerboard microdilution method was employed to assess the interaction of alexidine with fluconazole, amphotericin B, and micafungin.[22] The concentration ranges tested were 0.25 to 128 µg/mL for alexidine dihydrochloride, 0.125 to 64 µg/mL for fluconazole, 0.031 to 16 µg/mL for amphotericin B, and 0.015 to 8 µg/mL for micafungin. For each plate setup, 50 µL of alexidine dihydrochloride was added horizontally, and 50 µL of the antifungal agent was added vertically in a 96-well flat-bottom plate. Then, 100 µL of inoculum (final concentration of 10^5 cells/mL) was added to each well containing the compounds. Positive controls were included in eight wells containing only 2× RPMI medium supplemented with 2% glucose and 165 mM MOPS (pH 7.0), while sterility controls were performed in eight wells containing only the medium. The plates were incubated for 48 hours at 37°C.

The interaction between the compounds was quantified using the Fractional Inhibitory Concentration Index (FICI), calculated as follows: FICI = [MIC of alexidine dihydrochloride in combination]/[MIC of alexidine dihydrochloride alone] + [MIC of drug in combination]/[MIC of drug alone]. Each plate's FICI was calculated for all interaction concentrations, and classified

according to the criteria described in,[23] synergy (FICI ≤ 0.5), indifference (0.5 < FICI ≤ 4), or antagonism (FICI > 4).

Inhibition growth assay

For the growth curve analysis, an inoculum of 2.5 × 10^5 cells/mL was prepared. This suspension was combined with alexidine dihydrochloride (MIC 0.5 µg/mL) and 2× RPMI medium supplemented with 2% glucose and 165 mM MOPS (pH 7.0), which served as the positive control. The negative control contained only the medium without yeast, and amphotericin B was included as the resistance control. The plates were incubated at 37°C, and absorbance was measured at 530 nm at 0, 12, 24, 36, and 48 hours. Turbidity was plotted against incubation time, and growth rate curves were analyzed to evaluate the fungicidal effects of alexidine dihydrochloride.[24] All experiments were performed in triplicate.

Antibiofilm activity

Biofilm inhibition was evaluated following a previously established method,[25] with modifications. In brief, a standardized inoculum of *C. haemulonii sensu stricto* (2.5 × 10^5 cells/mL) in 2× RPMI medium, enriched with 2% glucose and buffered with 165 mM MOPS at pH 7.0, was combined with varying concentrations of the selected compound (alexidine dihydrochloride: 0.25–128 µg/mL). This suspension was added to the wells of 96-well polystyrene microtiter plates, which were then incubated without agitation at 37°C for 48 hours to promote fungal growth and biofilm formation.

After incubation, planktonic cells were carefully removed, and the biomass adhered to the plate was washed three times with distilled water. The remaining biofilm was stained with 0.1% crystal violet for 20 minutes. Excess dye was discarded, and the stained biomass was resuspended in 70% ethanol. Fungal growth was quantified by measuring absorbance at 595 nm using a microplate reader (iMarkTM Microplate, Bio-Rad, São Paulo, SP, Brazil). The controls consisted of *C. haemulonii sensu stricto* cells in 2× RPMI medium containing 2% glucose and 165 mM MOPS (pH 7.0), as well as the medium without yeast.

The percentage of biofilm inhibition was calculated relative to the untreated biofilm (considered 100% biofilm formation) and the sterility control containing only medium (considered 0% biofilm). Biofilm inhibition was determined according to the formula provided:[26]

$$\%\ Biofilm\ Inhibition = \left(\frac{OD\ of\ the\ control - OD\ of\ the\ treatment}{OD\ of\ the\ control}\right) x\ 100$$

Biofilm eradication assay

Candida haemulonii sensu stricto cells were cultured on SDA for 48 hours, then resuspended in peptone water (HiMedia) at a concentration of 10^5 CFU/mL. Hospital devices, specifically endotracheal tubes, were immersed in this yeast suspension and incubated for 48 hours at 37ºC to promote biofilm formation. A parallel control group was prepared using endotracheal tubes that were exposed solely to peptone water without yeast, serving as the sterility control. After the incubation period, the endotracheal tubes were rinsed three times with sterile distilled water to remove non-adherent cells. The endotracheal tubes with adhered biofilm were then treated with 4 µg/mL of tafenoquine, 0.12% chlorhexidine, and peptone water (untreated control) for 15 and 45 minutes.

Biofilm was collected from the endotracheal tubes using physical agitation. After plating the samples on SDA, they were incubated at 37ºC, and the CFU count was determined. Biofilm eradication percentage was calculated with the formula: % biofilm eradication = [(CFU of Untreated Biofilm – CFU of Treated Biofilm) / CFU of Untreated Biofilm] × 100, comparing CFU counts of treated samples to the untreated control.[27]

Cell damage

To assess and quantify cellular damage caused by alexidine dihydrochloride, we performed an MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) following established protocols.[28,29] After incubating for 24 hours, the plates were centrifuged at 40 g for 10 minutes at room temperature, and the supernatant was carefully removed. The resulting cell pellets were then exposed to 200 µL of an aqueous MTT solution (0.05 mg/mL) and incubated for an additional 3 hours at 37°C. Post-incubation, the plates were centrifuged again, and the formazan crystals were dissolved using 150 µL of isopropyl alcohol. For absorbance measurements, 100 µL from each well was transferred to fresh wells, where absorbance was read at both 595 nm (A595) and 655 nm (A655). The extent of cellular damage was calculated and presented graphically in a bar chart. Each experiment was conducted in triplicate. The formula used to determine cellular damage is as follows:

$$Cell\ damage\ (\%) = \left[1 - \frac{(A595 - A655\ of\ treated\ cells)}{(A595 - A655\ of\ positive\ control\ cells)}\right] x\ 100$$

Sorbitol protection assay

Following a previously established methodology, we assessed the osmoprotective effect of sorbitol.[30,31] A serial microdilution was performed in a sterile 96-well microplate containing 2× RPMI medium with 2% glucose and 165 mM MOPS (pH 7.0), supplemented with 0.8 M sorbitol. The alexidine dihydrochloride stock solution was prepared at concentrations ranging from 0.25 to 128 µg/mL, with micafungin included as a positive control. MIC values were assessed after incubation at 37°C for 48 and 72 hours. Each test was conducted in duplicate.

Efflux pump inhibition assay

To assess whether alexidine dihydrochloride can inhibit these efflux pumps, we conducted a phenotypic susceptibility assay utilizing promethazine, a known inhibitor of plasma membrane efflux pumps.[32] This assay was performed with alexidine dihydrochloride at concentrations ranging from 0.25 to 128 µg/mL, while incorporating sub-inhibitory concentrations of promethazine (128 µg/mL) into the fungal inoculum to observe any potential synergistic effect on efflux pump inhibition.

Alteration of cell membrane permeability

Cell membrane permeability was evaluated using the Pierce™ BCA Protein Assay Kit. *C. haemulonii sensu stricto* cells were suspended in sterile distilled water at a concentration of 2.5 × 10^5 cells/mL. The inoculum was then combined with alexidine dihydrochloride (MIC: 0.5 µg/mL) and incubated at 37°C for 0, 12, 24, 36, and 48 hours. Following each incubation period, samples were centrifuged at 908 g for 5 minutes at 4°C. After centrifugation, 25 µL of the supernatant was transferred to a flat-bottom 96-well plate, where 200 µL of the BCA working reagent was added to each well. The plate was shaken for 30 seconds and incubated at 37°C for 30 minutes. Absorbance was then measured at 595 nm.

To account for background absorbance, the average absorbance of the control wells was subtracted from that of the treatment wells. For each treatment, a blank containing only the treatment and the BCA working reagent was also prepared, and this blank value was subtracted from the treatment results. The protein concentration (µg/mL) was determined using a linear

formula derived from the kit's calibration curve, allowing for a direct correlation between absorbance and the amount of protein released by the yeast cells.[33]

Nucleotide leakage

The methodology adhered to a previously established procedure.[34] *C. haemulonii sensu stricto* cultures were grown on SDA at 37°C for 48 hours. After incubation, cells were suspended in 0.9% saline to reach a concentration of 2.5 × 10^5 cells/mL. The microorganism was then exposed to alexidine dihydrochloride at its MIC (0.5 µg/mL) for intervals of 0, 12, 24, 36, and 48 hours. Cells incubated solely with 0.9% saline served as a negative control, while amphotericin B was included as a resistance control. The supernatants from these suspensions were centrifuged at 1300 g for 15 minutes, and absorbance was measured at 260 nm to assess cellular response. Each procedure was conducted in triplicate to ensure reproducibility.

Hemolysis assay

Hemolysis was evaluated to determine the hemocompatibility of alexidine dihydrochloride, focusing on its potential application as a antifungal agent. Following a previously described method,[35] commercially sourced defibrinated sheep blood was diluted 1:25 in sterile PBS, and 250 µL of this diluted blood was incubated with alexidine dihydrochloride at its MIC (0.5 µg/mL). PBS was used as the negative control, ensuring baseline compatibility, while 0.1% (v/v) Triton served as the positive control to induce complete hemolysis. The samples were incubated at 37°C for 1 hour, then centrifuged at 700 g for 5 minutes to separate plasma from intact red blood cells. After centrifugation, 100 µL of the supernatant from each sample was transferred to a 96-well flat-bottom plate, and absorbance was measured at 490 nm using a microplate reader. The hemolysis ratio (%) was calculated using the following formula:

$$\left(\frac{ODs - ODnc}{ODpc - ODnc}\right) \times 100$$

ODs: OD490 values for samples, ODnc: OD490 values for negative controls, ODpc: OD490 values for positive controls.

In vivo survival assay and antifungal treatment in *T. molitor* model

To assess alexidine dihydrochloride's efficacy in treating *C. haemulonii sensu stricto* infection, we applied a modified version of a previously described method.[33,36] The experimental groups were structured as follows: Group 1 received only PBS (negative control); Group 2 was treated with alexidine dihydrochloride at MIC (0.5 µg/mL); and Group 3 received amphotericin B (4 µg/mL). *C. haemulonii sensu stricto* cells, cultured on SDA at 37°C for 48 hours, were suspended in PBS to reach a density of 2.5×10^5 cells/mL. A 5-µL aliquot of this cell suspension was injected into the larval hemocoel using a Hamilton syringe (Hamilton, USA), targeting the area between the third and fourth abdominal sternites. Simultaneously, 5 µL of the assigned treatment was injected. The *Tenebrio molitor* larvae were incubated at 37°C, and survival was monitored by counting larvae responsive to touch every 24 hours for a total of 72 hours.

Statistical analysis

The Tukey test was employed to compare the outcomes of the hemolysis assay, while Kaplan–Meier survival curves were generated for *T. molitor*, with statistical significance evaluated using the log-rank test. All statistical analyses were performed in GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, CA, USA), with significance defined at P values <0.05.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY

Data will be made available on request.

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5. CONCLUSÃO

Este estudo avaliou o potencial antifúngico do ZnPT, NYS, TAF e ALX contra *C. auris* e *C. haemulonii sensu stricto* MDR. Esta pesquisa ressalta o potencial de compostos reposicionados no combate a essas infecções resistentes.

ZnPT e NYS, especialmente em combinação, demonstraram atividade antifúngica frente *C. auris*, reduzindo as MICs de ambos os compostos, aumentando atividade fungicida e mitigando a toxicidade observada com ZnPT isolado. As drogas combinadas também inibiram a formação de biofilmes em pele suína e erradicação em dispositivo médico hospitalar. A combinação interrompeu a seletividade da membrana

fúngica sem causar extravasamento significativo de nucleotídeos ou ativação de bombas de efluxo

Por outro lado, a TAF e ALX isoladamente apresentaram atividade antifúngica contra *C. haemulonii sensu stricto*, inibiram a formação e erradicação de biofilme e não apresentaram toxicidade nos testes realizados. Os compostos apresentam ação na mitocôndria e aumentaram a sobrevivência das larvas de *T. molitor.*

Este estudo destaca o potencial de ZnPT + NYS, TAF e ALX, como alternativas viáveis para desenvolvimento de terapias antifúngicas. A capacidade desses compostos de inibir a formação de biofilmes, contornar mecanismos de resistência e manter um perfil de segurança favorável posiciona-os como ferramentas inovadoras no enfrentamento de infecções fúngicas MDR.

As perspectivas e possibilidades inovadoras deste estudo abrem caminhos promissores para o avanço no combate às infecções fúngicas MDR. Com base nos resultados obtidos, os próximos passos devem considerar a transição para estudos clínicos, avaliando rigorosamente a eficácia e a segurança dos compostos em modelos mais próximos de condições humanas. Critérios como a toxicidade em tecidos humanos, a estabilidade das formulações, a biodisponibilidade, e a capacidade de atingir concentrações terapêuticas nos locais de infecção serão fundamentais para orientar essa etapa. Além disso, é necessário explorar o potencial de formulações específicas que maximizem a sinergia observada, especialmente no caso da combinação ZnPT + NYS, bem como definir dosagens otimizadas que mantenham a eficácia enquanto minimizam os efeitos adversos. Essas iniciativas poderão não apenas validar as descobertas, mas também estabelecer diretrizes para o desenvolvimento de tratamentos clínicos, ampliando as opções terapêuticas contra infecções resistentes em ambientes hospitalares e comunitários.

Pesquisas futuras devem se concentrar em ensaios clínicos para validar esses achados e otimizar as estratégias de dosagem, especialmente para terapias combinadas. Além disso, estudos moleculares são necessários para elucidar melhor os mecanismos de ação e o potencial de resistência desses compostos. Este estudo fornece uma base crítica para o desenvolvimento de tratamentos antifúngicos eficazes e seguros, oferecendo esperança no combate ao desafio global representado pelos patógenos fúngicos MDR.

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