UNIVERSIDADE FEDERAL DE MATO GROSSO DO SUL - UFMS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA (PPGBiotec)

CAMILA DE OLIVEIRA GUTIERREZ

PEPAD: UM PEPTÍDEO MULTIFUNCIONAL COM ATIVIDADE ANTIFÚNGICA E ANTICÂNCER

Campo Grande - MS

Dezembro/2024

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Trabalho de defesa de doutorado apresentado ao Programa de Pós-Graduação em Biotecnologia, da Universidade Federal de Mato Grosso do Sul, como requisito parcial para obtenção do grau de doutor.

Orientadora: Dra Maria Lígia Rodrigues Macedo

Coorientadora: Dra Janaina de Cássia Orlandi Sardi

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TERMO DE APROVAÇÃO

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02 de dezembro de 2024

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“Force a si mesmo para ver sempre o lado brilhante que há por trás da nuvem mais escura, e será capaz de encarar negras perspectivas cheio de confiança.”

Lord Baden-Powell

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LISTA DE ABREVIATURAS E SIGLAS

ApTI	Inibidor de Tripsina de *Adenanthera pavonina*
CI	Candidíase Invasiva
CIM	Concentração Inibitória Mínima
CL	Cardiolipina
DAMPS	Padrões Moleculares Associados a Danos
DNA	Ácido Desoxirribonucleico
EROs	Espécies Reativas de Oxigênio
FDA	*Food and Drug Administration*
HAI	*Healthcare-Associated Infections*
IFI	Infecções Fúngicas Invasivas
IFS	Infecções Fúngicas Superficiais
IP	Inibidor de Peptidase Vegetal
LPS	Lipopolissacarídeos
MEC	Matriz Extracelular
PAC	Peptídeo Anticancerígeno
PAM	Peptídeo Antimicrobiano
PC	Fosfatidilserina
PE	Fosfatidiletanolamina
PG	Fosfatidilcolina
PS	Fosfatidilserina
RNA	Ácido Ribonucleico
SM	Esfingomielina
UTI	Unidade de Terapia Intensiva

LISTA DE AMINOÁCIDOS

Aminoácido	Código
Alanina	A
Arginina	R
Asparagina	N
Aspartato	D
Cisteína	C
Fenilalanina	F
Glicina	G
Glutamato	E
Glutamina	Q
Histidina	H
Isoleucina	I
Leucina	L
Lisina	K
Metionina	M
Prolina	P
Serina	S
Tirosina	Y
Treonina	T
Triptofano	W
Valina	V

SUMÁRIO

RESUMO

As infecções fúngicas e o câncer representam duas ameaças distintas à saúde humana, mas que em alguns casos estão relacionadas. As infecções fúngicas recorrentes e as causadas por outros microrganismos específicos podem induzir a mutação de algumas células e consequentemente levar a evolução de diversos tipos de câncer. Por outro lado, pacientes que estão em tratamento contra o câncer se encontram imunocomprometidos, o que favorece infecções por microrganismos. Somando esse vínculo complexo à crescente resistência dos fungos e das células cancerígenas aos medicamentos, faz-se necessário o desenvolvimento de novas substâncias ativas ou fármacos como alternativas terapêuticas. Por meio da técnica de microdiluição em caldo foi verificado que PEPAD apresentou atividade contra 5 espécies de fungos do gênero *Candida*, com CIMs variando entre 2,5 - 5 µM, e apresentou um rápido tempo de ação (60 minutos) para *Candida tropicalis*. Também foi constatado que seu provável mecanismo de ação é por meio da ligação com o ergosterol presente na membrana plasmática. Além disso, PEPAD apresentou ação sinérgica com os antifúngicos anfotericina B e fluconazol, reduzindo consideravelmente as concentrações necessárias de ambas as moléculas. Ensaios de inibição da formação de biofilme e erradicação de biofilme pré-formado também foram realizados e foi constatado que PEPAD foi capaz de inibir e erradicar parte do biofilme de *C. tropicalis*. Para avaliar o potencial anticâncer e a toxicidade do peptídeo, foram realizados ensaios de viabilidade celular pelo método colorimétrico com MTT. O IC_{50} encontrado para células de melanoma murino (B16F10-Nex2) foi de 7,4 µM, já para as células saudáveis de macrófago murino (RAW 264.7) e fibroblasto humano (FN1) até a maior concentração testada (16 µM) não foi possível encontrar o IC_{50}, sugerindo alta seletividade e baixa toxicidade. Após o tratamento com PEPAD em células de melanoma murino, não foram observadas alterações morfológicas na membrana celular, porém foi constatada alterações nas cromatinas do núcleo, que é sugestivo de morte por apoptose. Através de um risco realizado em uma camada de células, foi possível observar que PEPAD retarda a migração celular do melanoma, conferindo um possível potencial antimetastático. Para avaliar o potencial mitocondrial e a morfologia nuclear, foram utilizadas as sondas fluorescentes MitoTracker Deep Red e NucBlue. Foi observado que os núcleos celulares permaneceram intactos; no entanto, foi observado inchaço mitocondrial. A ativação da caspase foi analisada usando o marcador CaspACETM FITC-VAD-FMK, revelando caspases ativas através da ligação do marcador fluorescente a caspases ativadas em células em morte apoptótica. Experimentos de citometria de fluxo também foram conduzidos para confirmar o tipo de morte celular. Os resultados corroboram descobertas anteriores, indicando que o PEPAD induz morte celular por apoptose. Por fim, foram realizados ensaios de ELISA e verificou-se que PEPAD foi capaz de induzir a liberação de calreticulina e HMGB1, que são moléculas conhecidas como Padrões Moleculares Associados a Danos (DAMPS), efeito típico de morte celular imunogênica. Os resultados sugerem o elevado potencial do PEPAD para ser utilizado como protótipo no desenvolvimento de novos agentes antifúngicos e anticancerígenos, dadas as suas características seletivas contra microrganismos e células cancerígenas.

Palavras-chave: resistência, peptídeo antimicrobiano, *Candida*, melanoma, DAMPS.

ABSTRACT

Fungal infections and cancer represent two distinct threats to human health, but in some cases, they are related. Recurrent fungal infections and those caused by other specific microorganisms can induce the mutation of certain cells and consequently lead to the development of various types of cancer. On the other hand, patients undergoing cancer treatment are immunocompromised, which favors infections by microorganisms. Adding this complex link to the growing resistance of fungi and cancer cells to drugs, the development of new active substances or drugs as therapeutic alternatives becomes necessary. Through the broth microdilution technique, it was observed that PEPAD exhibited activity against 5 species of *Candida* fungi, with MICs ranging between 2.5 - 5 µM, and showed a rapid action time (60 minutes) against *Candida tropicalis*. It was also found that its likely mechanism of action is through binding with ergosterol present in the plasma membrane. Furthermore, PEPAD showed synergistic action with the antifungal drugs amphotericin B and fluconazole, significantly reducing the required concentrations of both molecules. Inhibition assays of biofilm formation and eradication of mature biofilm were also conducted, and it was found that PEPAD was able to inhibit and eradicate part of the *C. tropicalis* biofilm. To evaluate the anticancer potential and toxicity of the peptide, cell viability assays were performed using the colorimetric method with MTT. The IC_{50} found for murine melanoma cells (B16F10-Nex2) was 7.4 µM, whereas for healthy murine macrophage (RAW 264.7) and human fibroblast (FN1) cells, it was not possible to find the IC_{50} up to the highest tested concentration (16 µM), suggesting high selectivity and low toxicity. After treatment with PEPAD in murine melanoma cells, no morphological alterations were observed in the cell membrane, but changes in nuclear chromatin were noted, suggestive of apoptosis. Through a scratch assay performed on a cell layer, it was observed that PEPAD delayed the cell migration of melanoma, indicating a potential anti-metastatic effect. To evaluate mitochondrial potential and nuclear morphology, the fluorescent probes MitoTracker Deep Red and NucBlue were used. It was observed that the cell nuclei remained intact; however, mitochondrial swelling was observed. Caspase activation was analyzed using the CaspACETM FITC-VAD-FMK marker, revealing active caspases through the binding of the fluorescent marker to activated caspases in cells undergoing apoptotic death. Flow cytometry experiments were also conducted to confirm the type of cell death. The results support previous findings, indicating that PEPAD induces cell death by apoptosis. Finally, ELISA assays were conducted, and it was found that PEPAD was able to induce the release of calreticulin and HMGB1, which are molecules known as Damage-Associated Molecular Patterns (DAMPs), a typical effect of immunogenic cell death. The results suggest the high potential of PEPAD to be used as a prototype in the development of new antifungal and anticancer agents, given its selective characteristics against microorganisms and cancer cells.

Keywords: resistance, antimicrobial peptide, *Candida*, melanoma, DAMPs.

1. Introdução

1.1. Infecções fúngicas

Embora uma vasta diversidade de fungos esteja presente em todo o mundo, estima-se que em torno de 300 espécies sejam capazes de infectar e causar doenças nos seres humanos, sendo a maioria parte integrante de ambientes naturais e também da microbiota normal de humanos e animais [1]. As infecções fúngicas podem ser categorizadas como superficiais, cutâneas, subcutâneas e invasivas. Isso depende da patogenicidade do fungo, da resposta imunológica do hospedeiro e do local de infecção [2].

As infecções fúngicas superficiais (IFS) geralmente são desencadeadas por espécies como *Malassezia* e *Tinea nigra*, afetando a superfície do corpo, como a pele, cabelos e unhas. No caso das IFS, não há alterações patológicas notáveis e a resposta imunológica celular é limitada. Já as infecções cutâneas, também do tipo superficial, apresentam uma variedade de alterações patológicas. Entre as infecções cutâneas mais comuns estão aquelas causadas por dermatófitos, como *Trichophyton rubrum*, *T. interdigitale*, *T. tonsurans* e *Microsporum canis*, que afetam tecidos ricos em queratina, como a pele. Já as infecções subcutâneas, como por exemplo a cromoblastomicose, afetam camadas mais profundas da derme da pele e do tecido subcutâneo [2].

As infecções fúngicas invasivas (IFIs) têm o potencial de atingir órgãos internos, como os rins, pulmões, fígado, coração, cérebro, entre outras regiões do corpo. Exemplos de infecções sistêmicas frequentes incluem a Candidíase, Aspergilose, Criptococose e Paracoccidioidomicose, sendo causadas respectivamente pelas espécies *Candida* sp., *Aspergillus* sp., *Cryptococcus* sp e *Paracoccidioides* sp [3]. As IFIs são responsáveis por aproximadamente 1,7 milhão de mortes anualmente, sendo que o número de casos continua a aumentar de maneira constante. Dessa forma, as infecções fúngicas estão progressivamente se tornando um desafio para a saúde global, vinculado a índices significativos de morbidade e mortalidade, assim como a consequências socioeconômicas impactantes [4,5].

Uma das principais causadoras de IFIs são os fungos do gênero *Candida*. As espécies de *Candida* são fungos comumente comensais, geralmente presentes no intestino, na pele e em outras superfícies mucosas. Esses fungos podem ocasionar três formas distintas de candidíase: a candidíase comum, a candidíase vaginal e a invasiva (CI). A candidíase invasiva é mais conhecida como candidemia. É uma infecção grave, capaz de afetar órgãos como pulmões, sangue, coração, cérebro, olhos, ossos e outras partes do corpo [3].

Normalmente, o sistema imune do paciente é capaz de combater os fungos que apresentam baixa virulência, porém algumas espécies são patógenas oportunistas, ou seja, quando o paciente está imunocomprometido o fungo se torna virulento [6]. Os indivíduos mais afetados pelas IFIs são os recém-nascidos, os imunocomprometidos, pessoas que receberam transplante de órgãos, pacientes que fazem tratamento quimioterápico e pessoas que fazem uso prolongado de medicamentos imunossupressores, como corticosteroides. Além disso, pacientes que estão internados em unidades de terapia

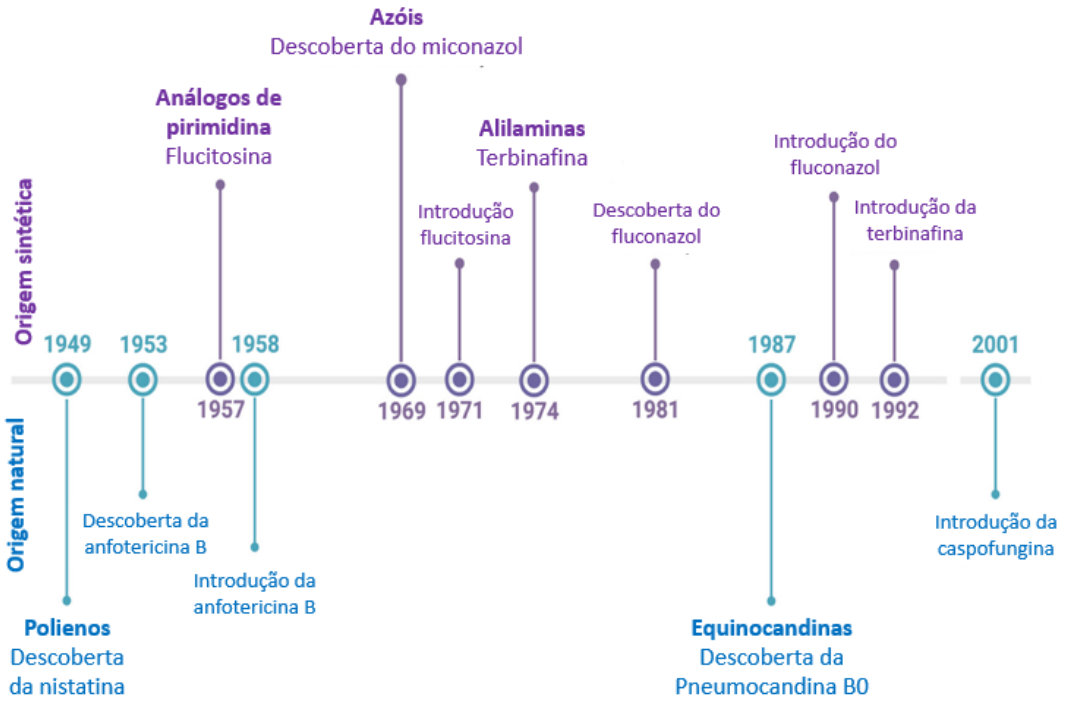
intensiva (UTI) também apresentam maior risco de desenvolver IFIs devido a tratamento com antibióticos [7].

1.2. Medicamentos e resistência aos antifúngicos

A descoberta e desenvolvimento de antimicrobianos foram conquistas fundamentais para a humanidade, proporcionando avanços significativos no tratamento de infecções e melhorando a expectativa de vida da população, permitindo o tratamento eficaz de infecções que anteriormente eram frequentemente fatais. Contudo, a descoberta de novos medicamentos antifúngicos ficou para trás se comparado à descoberta e desenvolvimento dos antibióticos, dessa forma, as infecções fúngicas foram um assunto negligenciado na área da saúde [6].

O primeiro antifúngico descoberto foi a griseofulvina em 1939, que foi isolada pela primeira vez a partir do fungo *Penicillium griseofulvum*. A partir da década de 1950 outras moléculas foram desenvolvidas e atualmente, as opções de tratamento para infecções fúngicas estão limitadas a cinco classes diferentes de antifúngicos: os polienos (anfotericina B e nistatina), análogos de pirimidina (flucitosina), azóis (miconazol, cetoconazol, fluconazol, itraconazol, voriconazol, posaconazol e isavuconazol), alilamina (terbinafina) e equinocandinas (caspofungina, anidulafungina e micafungina) (Figura 1) [5,8].

Figura 1 - Histórico da descoberta dos principais medicamentos antifúngicos. As classes e seus respectivos compostos são divididos de acordo com sua origem, sintética (parte superior) ou natural (parte inferior).



Fonte: Modificado de Vanreppelen, G. *et al*., (2023) [9].

Por serem a classe de antifúngico mais antiga, os polienos marcaram um grande avanço clínico no tratamento das infecções fúngicas. Apesar de ser uma das classes com os antifúngicos mais potentes, os polienos possuem desvantagem por ocasionarem alta nefrotoxicidade, dessa forma são usados como último recurso na clínica médica [9]. Os polienos agem ligando-se aos esteróis da membrana, principalmente ao ergosterol,

formando poros e espécies reativas de oxigênio (EROs), o que induz a apoptose (Figura 2) [10].

A flucitosina é o único membro da classe de análogos de pirimidina. Em 1957 foi inicialmente criada para ser um agente antineoplásico, porém foi descoberto seu potencial antifúngico, então a partir de 1971 começou a ser utilizada para esse fim. Essa molécula compete com a uracila e se liga ao RNA, interrompendo a síntese do RNA e consequentemente prejudicando a síntese de outras proteínas essenciais para a célula fúngica. A flucitosina também afeta a síntese do DNA por inibir o timidilato sintase, que é uma enzima essencial para a biossíntese de timina [11].

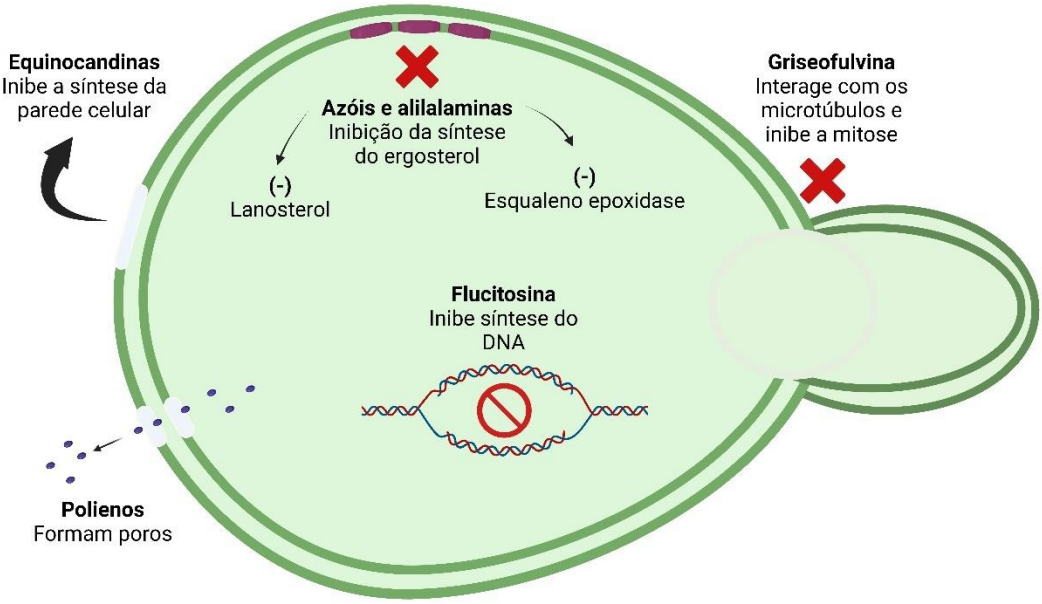
Atualmente os azóis são os antifúngicos mais utilizados devido à sua boa eficácia, menor toxicidade para o paciente e boa farmacocinética. Os azóis interferem com uma enzima crucial chamada lanosterol 14-α-demetilase, que desempenha um papel fundamental na síntese de ergosterol. Dessa forma, a fluidez da membrana é alterada e leva à incapacidade das células fúngicas de formar membranas celulares maduras [12].

As alilaminas foram sintetizados inicialmente para tratar distúrbios no sistema nervoso central, porém em 1992 a terbinafina começou a ser utilizada no tratamento de micoses causadas por fungos dermatófitos e posteriormente como antifúngico oral [13]. Assim como os azóis, as alilaminas inibem a síntese do ergosterol, porém seu alvo é a enzima esqualeno epoxidase. Essa enzima catalisa a conversão de esqualeno para lanosterol, um precursor essencial na síntese do ergosterol. Assim, além de alterar a composição lipídica da membrana, há um acúmulo de esqualeno nas células fúngicas. Esse acúmulo de esqualeno é tóxico para os fungos e interfere na formação adequada dos esteróis necessários para a integridade da membrana celular [14].

As equinocandinas são metabolitos secundários produzidos por fungos filamentosos que consistem em um núcleo hexapeptídico cíclico, acompanhado por uma cadeia lateral lipídica que confere a atividade antifúngica [15]. Essa é a mais nova classe de medicamentos antifúngicos, com a vantagem de possuir um mecanismo de ação totalmente novo. As equinocandinas inibem a enzima β-(1,3)-glucano sintase, que é responsável pela síntese do β-(1,3)-glucano. A integridade estrutural da parede celular do fungo depende da interação glucano + quitina, dessa forma, a interrupção da síntese do glucano afeta essa estrutura causando instabilidade osmótica e consequentemente a lise celular [16].

Os fungos são organismos eucarióticos, o que significa que suas células são biologicamente semelhantes às células humanas. Essa semelhança representa um desafio significativo no desenvolvimento de novos medicamentos antifúngicos, pois encontrar substâncias que sejam eficazes contra os fungos, mas com baixa toxicidade para os hospedeiros (como os humanos), é uma tarefa difícil. Em muitos casos, os medicamentos acabam compartilhando vias metabólicas ou intermediários metabólicos com os hospedeiros, o que torna complicado direcionar especificamente os fungos sem afetar negativamente as células humanas [17].

Figura 2 – Representação esquemática dos mecanismos de ação das classes de antifúngicos.



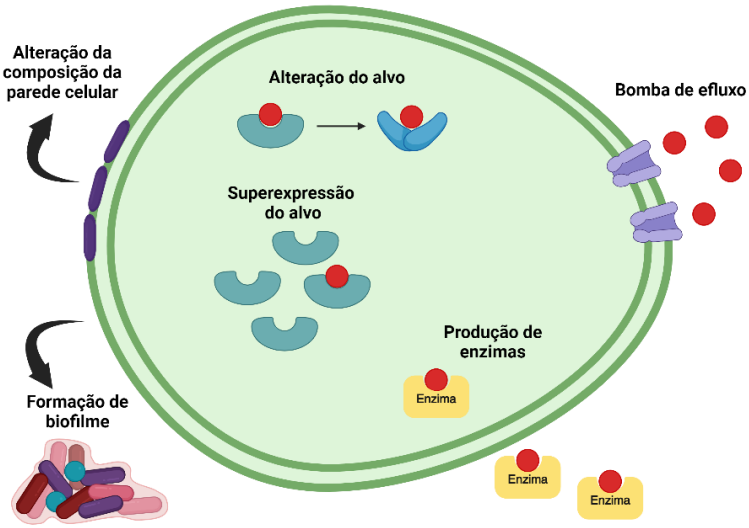
Fonte: Autoria própria. Criado com Biorender.com

Após a aprovação da caspofungina pela *Food and Drug Administration* (FDA) em 2001, o número de antimicrobianos aprovados diminuiu ao mesmo tempo que a resistência fúngica aumentou [18]. A resistência antifúngica é a capacidade dos fungos desenvolverem mecanismos que os tornam menos sensíveis ou totalmente resistentes aos efeitos dos medicamentos antifúngicos. Essa é uma preocupação crescente e destaca a importância do uso responsável de antifúngicos, monitoramento constante e pesquisa contínua para o desenvolvimento de novos agentes antifúngicos [19].

Da mesma forma que as bactérias, os fungos vêm adquirindo resistência aos medicamentos em dosagens terapêuticas. O uso indiscriminado de agentes antifúngicos é um dos principais responsáveis por criar uma pressão seletiva, favorecendo o desenvolvimento de fungos mais resistentes. Isso é especialmente preocupante em ambientes de saúde, onde o uso frequente desses medicamentos é comum [20]. Outros fatores que podem contribuir com a resistência fúngica é a utilização de doses inadequadas, uso prolongado, predisposição genética de alguns fungos para desenvolver resistência e mecanismos de defesa intrínsecos [21].

Os fungos desenvolveram diversos mecanismos de defesa que contribuem para a resistência aos antifúngicos. Dentre esses mecanismos estão a bomba de efluxo, produção de enzimas que inativam os medicamentos, alteração do alvo, formação de biofilmes, superexpressão do alvo do fármaco e alterações na composição da parede celular. Além disso, fungos resistentes frequentemente apresentam uma combinação de vários mecanismos de resistência, tornando-os mais resistentes contra diferentes classes de antifúngicos (Figura 3) [22,23].

Figura 3 – Representação esquemática dos mecanismos de resistência aos antifúngicos desenvolvidos pelos fungos.



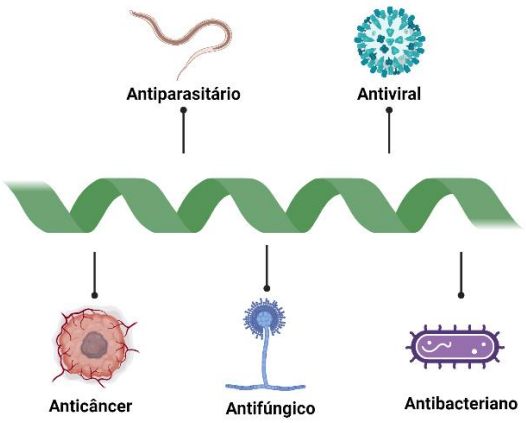
Fonte: Autoria própria. Criado com Biorender.com

Embora a morbidade e mortalidade das infecções fúngicas sejam altas, o seu impacto na saúde pública permanece subestimado. É incontestável que a ameaça decorrente das infecções fúngicas continuará a crescer globalmente, apresentando uma série de desafios que necessitam ser superados. Isso requer uma ação rápida e inovadora em diversos níveis, sendo necessário intensificar a busca por novas opções de tratamento terapêutico [24].

1.3. Peptídeos Antimicrobianos (PAMs)

Um candidato promissor para o desenvolvimento de uma terapia alternativa contra fungos patogênicos são os peptídeos antimicrobianos (PAMs), devido ao seu amplo espectro de ação, rápida ação e à baixa probabilidade de desenvolvimento de resistência [25]. Os PAMs são moléculas com amplo espectro de ação que são produzidas por quase todos os seres vivos como uma forma de mecanismo de defesa do sistema imunológico inato [26]. Eles podem apresentar atividade contra bactérias, fungos, vírus, parasitas e câncer, além de possuir atividade imunomoduladora e anti-inflamatória (Figura 4) [27].

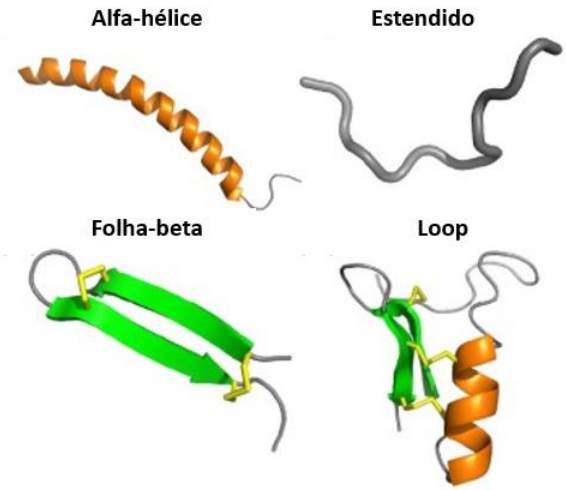
Figura 4 – Desenho ilustrativo do amplo espectro de ação dos peptídeos antimicrobianos.



Fonte: Autoria própria. Criado com Biorender.com

Os PAMs são pequenas moléculas com 5 a 50 aminoácidos, geralmente possuem menos de 10 kDa, com elevada quantidade de resíduos hidrofóbicos e são catiônicos com carga elétrica entre +2 a +9 devido à presença de resíduos de aminoácidos como lisina e arginina [26]. Por serem muito diversos, são classificados em quatro grupos de acordo com sua estrutura secundária, sendo eles: alfa-hélice, folha-beta, estendido e loop (Figura 5) [28].

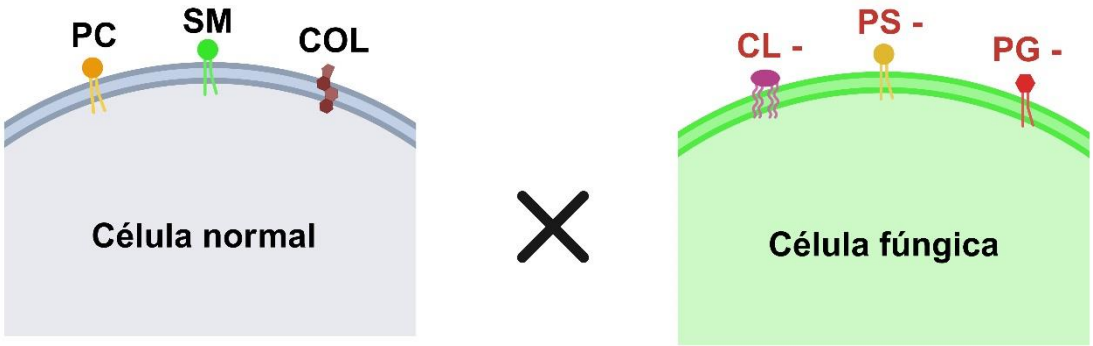
Figura 5 - Estruturas tridimensionais de peptídeos antimicrobianos.



Fonte: Modificado de Koehbach, J. *et* al., (2019) [29].

As membranas dos eucariotos como os fungos possuem carga líquida negativa devido à presença predominante de cardiolipina (CL), fosfatidilserina (PS) e fosfatidilglicerol (PG). Em contrapartida a membrana das células de mamíferos são ricos em fosfatidilcolina (PC), esfingomielina (SM) e colesterol (COL), que são fosfolipídeos zwitteriônicos (com carga líquida neutra) [30]. Dessa forma, a característica catiônica dos PAMs permite que ele seja altamente seletivo contra as células de interesse e não seja tóxico para as células humanas, já que o primeiro passo para a interação peptídeo-célula se dá pela atração eletrostática inicial devido às cargas opostas (Figura 6) [31].

Figura 6 - Diferenças entre as células de mamíferos e fúngicas.



Fonte: Autoria própria. Criado com Biorender.com

Após a atração eletrostática inicial, os peptídeos competem com cátions presentes na membrana que são responsáveis por estabilizar os lipopolissacarídeos (LPS), isso

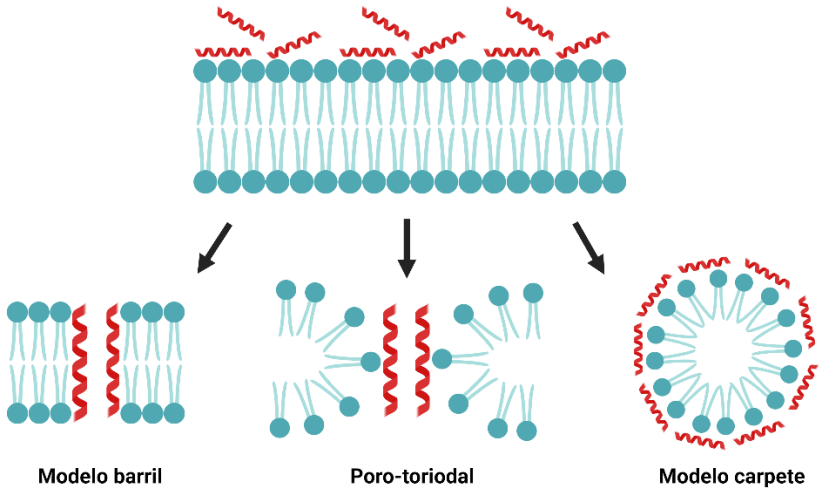
resulta na perturbação da camada externa da membrana e permite a entrada de mais moléculas. Neste momento, os peptídeos que até então estavam em conformação linear adquirem uma estrutura anfipática em alfa-hélice devido ao ambiente hidrofóbico da membrana celular, rompendo essa membrana de diversas formas [32]. A anfipacidade nesses peptídeos é essencial para que ele tenha capacidade lítica, pois isso permite que o peptídeo se incorpore com a membrana de tal forma que os resíduos polares interajam com os fosfatos da bicamada e os resíduos apolares adentrem por meio das cadeias hidrofóbicas dos lipídeos [31].

A hidrofobicidade é outro parâmetro essencial para a ação do peptídeo. Quanto maior a hidrofobicidade melhor a atividade antimicrobiana, porém a toxicidade para células saudáveis também aumenta pois há maior possibilidade de adesão com qualquer membrana. Além disso pode ocorrer uma associação entre os próprios peptídeos ou uma precipitação em água. Por outro lado, a diminuição da hidrofobicidade faz com que o peptídeo não consiga se ligar aos fosfolipídios presentes na membrana, não desempenhando sua função satisfatoriamente. Dessa forma o ideal é o equilíbrio, por isso recomenda-se que a hidrofobicidade esteja entre 30% a 50%, apesar de não ser uma regra limitante [33].

Apesar dos PAM serem moléculas promissoras, existem algumas dificuldades que precisam ser superadas para que eles cheguem a ser usados efetivamente como um medicamento. Dentre os principais desafios temos a baixa estabilidade enzimática e a capacidade de hemólise, mas apesar disso existem mecanismos para superar essas questões, como o nanoencapsulamento e a junção com outras moléculas para garantir maior estabilidade e melhor direcionamento [26].

Os PAM possuem diversos mecanismos de ação, como a inibição da síntese de ácidos nucleicos e de proteínas, inibição da atividade enzimática, inibição do dobramento de proteínas, entre outros [34]. Apesar disso, seu mecanismo inicial e principal se dá pelo dano na membrana plasmática. Há três modelos amplamente aceitos que explica o processo de permeabilização do peptídeo, são eles: o poro-toroidal, modelo barril e o modelo carpete (Figura 7).

Figura 7 - Mecanismos de interação e permeabilização dos peptídeos antimicrobianos em membranas de microrganismos.



Fonte: Autoria própria. Criado com Biorender.com

No modelo barril descrito por Ehrenstein & Lecar, 1977 [35], os peptídeos se posicionam de forma perpendicular em relação à membrana. Após esse primeiro contato ocorre a alteração da fase conformacional do peptídeo, de forma que a parte hidrofóbica do peptídeo se liga à parte interna da membrana plasmática que também é hidrofóbica. Já a porção hidrofílica do peptídeo fica voltada para dentro do canal e sem ficar em contato com a membrana. Dessa forma vários peptídeos se agregam entre si e se inserem na membrana formando poros em forma de "barril", permitindo que substâncias entram e saiam da célula.

No modelo de poro toroidal [34], assim como no modelo anterior, o peptídeo se insere perpendicularmente na membrana. A diferença é que além da parte hidrofóbica ficar em contato com a porção hidrofóbica da membrana, as regiões hidrofílicas do peptídeo e da membrana também interagem. Dessa maneira, esse complexo hidrofílico fica voltado para o centro do poro enquanto a membrana se curva para criar uma cavidade revestida pelos grupos lipídicos e revestida por peptídeos.

Por fim no modelo carpete proposto por Pouny *et al*., 1992 [36], vários peptídeos permanecem de forma paralela na superfície da membrana, formando um "tapete". Isso causa a desestabilização dos fosfolipídeos alterando a fluidez da membrana devido ao deslocamento dos fosfolipídeos pelos peptídeos. No momento que há uma saturação da concentração do peptídeo a membrana sofre uma ruptura devido a energia desfavorável. Diferente dos outros dois modelos, no modelo carpete o peptídeo não se insere na membrana e nem forma poros, pois os peptídeos permanecem em contato apenas com a parte hidrofílica do fosfolipídeos e não com a parte hidrofóbica e interna da membrana.

1.4. Infecções microbianas e o câncer

Além da própria infecção, microrganismos podem induzir diversos tipos de cânceres através de infecções persistentes, resultando em inflamação crônica. Esse processo pode desencadear mutações celulares, especialmente em indivíduos com comprometimento do sistema imunológico. Contudo, mesmo aqueles com sistema imunológico competente não estão isentos desse risco [37].

Apenas alguns microrganismos causam câncer diretamente (como a bactéria *Helicobacter pylori*), mas existem evidências que a inflamação recorrente causada por infecções estimula o potencial metastático das células cancerígenas [38,39]. Calcula-se que, em 2018, aproximadamente 2,2 milhões de casos de câncer atribuíveis a infecções tenham sido diagnosticados globalmente, correspondendo a 13% da incidência total de câncer [40].

A influência bacteriana no câncer foi observada em 1868, enquanto a influência viral foi notada em 1911. Além das 11 bactérias e vírus cancerígenos reconhecidos, há evidências crescentes de que um número maior de bactérias e vírus desempenham um papel na promoção da progressão e metástase do câncer [38]. No entanto, o papel dos fungos no desenvolvimento do câncer tem sido relativamente pouco explorado em comparação, com poucos estudos dedicados a essa investigação [41].

Estudos recentes conduzidos por Dohlman *et al*., 2022 e Vallianou *et al*., 2021 [44,45] revelaram associações entre fungos detectados em tumores, fornecendo

evidências que respaldam o significativo papel do micobioma em cânceres humanos. A presença de fungos do gênero *Candida* foi associada à ativação de vias imunológicas pró-inflamatórias e à ocorrência de metástases em cânceres gastrointestinais [42,44]. Esses fungos podem promover o crescimento do câncer por meio de diversos mecanismos, incluindo a indução de inflamação, ativação de respostas das células T auxiliares, geração de toxinas e substâncias carcinogênicas, danos diretos ao DNA e emprego de mimetismo molecular [45,46]. Além disso, a invasão e colonização dos fungos enfraquece os mecanismos de defesas do hospedeiro, aumentando o risco de desenvolvimento do câncer [47].

1.5. Câncer - Características, diagnóstico e tratamentos

Câncer é o nome dado ao conjunto de mais de 200 doenças que possuem como característica comum a proliferação descontrolada de células, capacidade de invadir outros órgãos e tecidos (metástase), resistência aos genes supressores tumorais, resistência à morte celular programada, angiogênese, modificações metabólicas, mutações no DNA e resistência ao sistema imune [48]. O câncer é a segunda causa de morte global, dessa forma é essencial assegurar a identificação e o tratamento precoce, visando diminuir a disseminação da doença e sua taxa de mortalidade [49,50].

Apesar de ser um fator geralmente genético, o aumento da incidência e da mortalidade por câncer é favorecido pelo envelhecimento da população, exposição a poluentes e mudanças na dieta. Entre 2023 e 2025 são estimados 704.080 novos casos de câncer no Brasil, com o maior número de casos de câncer de mama, seguido de câncer de próstata [51]. Já no mundo todo é estimado que até 2030 o número de mortes exceda 13 milhões [52].

Inicialmente uma célula se torna cancerígena devido a mutações no material genético, apesar disso, nem todas as células que sofrem mutações se tornam cancerígenas. As mutações genéticas são eventos comuns e naturais que podem ocorrer ao longo da vida de uma célula. Muitas dessas mutações são reparadas pelo próprio sistema de reparo do DNA da célula, e algumas mutações podem não ter efeitos significativos. Caso esse sistema falhe, ainda há células do sistema imune que eliminam essas células defeituosas impedindo-as de se multiplicarem. No entanto, em alguns casos, se as mutações ocorrerem em genes específicos e esses mecanismos de controle falharem, as células podem se tornar cancerígenas e começar a se proliferar descontroladamente [53].

O desenvolvimento do câncer, mais conhecido como carcinogênese é um processo complexo e depende de várias etapas. A carcinogênese é o processo da transformação da célula saudável para célula cancerígena, e é dividida em três etapas. A primeira é a iniciação, que consiste na mutação do material genético ocasionado por diversos fatores, como fatores genéticos, químicos, infecções por patógenos e radiação. Em seguida vem a etapa da promoção, um processo marcado por mutações adicionais e pela multiplicação descontrolada das células. Fatores como hormônios e inflamação podem desempenhar um papel fundamental durante essa fase. E por último tem a progressão, as células que agora são cancerígenas, persistem na divisão celular e adquirem características que as tornam mais agressivas, aumentando sua propensão de se disseminar para outras regiões do corpo e formar tumores malignos [54–56].

A disseminação do câncer para outras partes do corpo, conhecida como metástase, é a principal causa de mortalidade associada ao câncer. Durante o processo de metástase, as células cancerígenas deixam o local de origem e vão para diferentes áreas do organismo, formando novos locais de crescimento e desencadeando disfunção nos órgãos. A primeira fase desse processo é a invasão, em que as células tumorais atravessam a membrana basal circundante e se movem através da matriz extracelular (MEC) no tecido adjacente. Dessa forma, essas células são capazes de atravessar os vasos sanguíneos e se disseminarem por todo o corpo através da corrente sanguínea [57].

O método de tratamento varia de acordo com o estágio do câncer e o estado do paciente, podendo ser um tratamento específico ou uma combinação deles. Os tratamentos utilizados visam eliminar ou diminuir a agressividade do câncer, impedir a progressão da doença e em último caso realizado de forma paliativa para aumentar o tempo de vida do paciente e melhorar a qualidade de vida [58,59]. Os tratamentos geralmente utilizados são:

- Cirurgia oncológica: Consiste na retirada do tumor por meio de cirurgia, também são retirados tecidos circundantes para garantir uma margem de segurança e evitar a recidiva ou disseminação do câncer para outras partes do corpo [60];

- Radioterapia: Essa é uma modalidade que utiliza radiações ionizantes (como raios X ou feixes de elétrons) com o intuito de eliminar ou danificar as células cancerígenas, e pode ser dividida em dois tipos. O primeiro tipo é a radioterapia externa, onde a radiação é administrada por uma fonte externa ao corpo. Já a radioterapia, também conhecida como braquiterapia, a fonte de radiação é colocada dentro ou próximo ao tumor por meio de cateteres, sementes radioativas, entre outros dispositivos [61];

- Imunoterapia: É uma abordagem terapêutica que induz e modula o sistema imunológico do próprio paciente a atacar as células cancerígenas [62];

- Hormonioterapia: Conhecida também como terapia hormonal, é um tratamento que visa alterar os níveis hormonais no corpo para impedir o crescimento ou a disseminação de cânceres hormônio-sensíveis. Este tipo de tratamento é frequentemente utilizado em cânceres de mama e próstata [63];

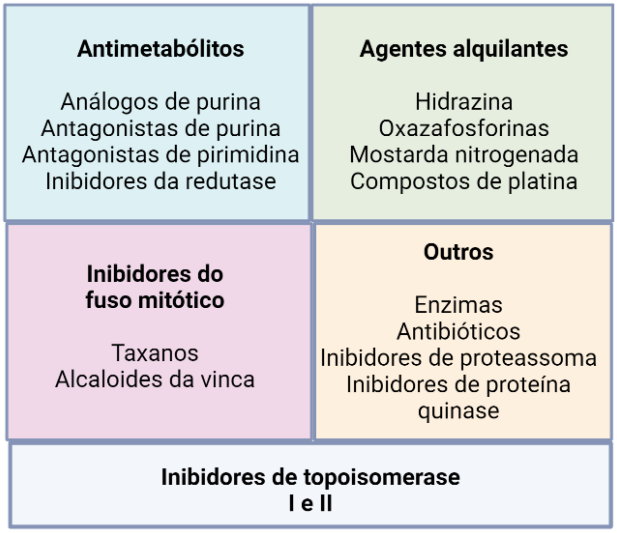
- Quimioterapia: É um tratamento sistêmico que utiliza medicamentos químicos conhecidos como quimioterápicos ou antineoplásicos. Esses medicamentos têm a capacidade de retardar ou interromper a divisão celular, e dessa forma controlar o crescimento e disseminação das células cancerígenas [64].

A radioterapia e a quimioterapia, embora sejam tratamentos amplamente utilizados no combate ao câncer, apresentam desafios significativos devido à falta de seletividade, podendo resultar em efeitos tóxicos nas células saudáveis dos pacientes [65]. Essa questão emerge como um dos principais desafios no tratamento do câncer, impulsionando a necessidade urgente de investigar e desenvolver novas opções antineoplásicas que possuam maior especificidade e menor impacto negativo nas células saudáveis do organismo. Entre os principais efeitos colaterais temos alterações no sistema imunológico, fadiga, tontura, náuseas, inflamação e ulceração das mucosas, dores e inflamações na pele [66–68].

1.6. Medicamentos e resistência aos quimioterápicos

Apesar de existir vários tipos de tratamentos contra o câncer, a quimioterapia continua sendo o método mais utilizado. Eles podem ser classificados de acordo com o mecanismo de ação, divididos em antimetabólitos, agentes alquilantes, inibidores do fuso mitótico, inibidores de topoisomerase I e II, entre outros (Figura 8) [49].

Figura 8 - Classificação dos quimioterápicos de acordo com seu mecanismo de ação.



Fonte: Autoria própria. Criado com Biorender.com

Apesar dos diversos medicamentos utilizados, a resistência a múltiplas drogas apresenta um grande desafio no tratamento do câncer, já que mais de 90% das mortes causadas por câncer se deve à essa resistência [49]. A resistência aos medicamentos pode ser classificada como intrínseca ou adquirida, dependendo do momento em que se desenvolve. A resistência intrínseca ocorre antes do início do tratamento medicamentoso, enquanto que a resistência adquirida surge após a terapia. Ambas as formas ocorrem em aproximadamente 50% dos pacientes com câncer que apresentam resistência aos medicamentos [69].

Dentre os mecanismos de resistência mais comuns, encontra-se o efluxo da droga, alteração do alvo do medicamento, reparo aprimorado ao DNA, fuga da senescência, alterações epigenéticas, supressão da apoptose (morte celular programada), modificações no metabolismo do medicamento [70]. Uma outra forma de resistência é a heterogeneidade genética, onde são encontradas células com genéticas ou tipos celulares diferentes. Essa diversidade aumenta a complexidade e desafios ao tratamento do câncer, tornando praticamente inviável erradicar todas as células cancerígenas com uma única forma de terapia. Dessa forma, essa complexidade estimulou o desenvolvimento de abordagens terapêuticas combinadas e empregadas em diversos tratamentos oncológicos [69].

Em vista de todos os desafios apresentados, existe um interesse crescente na busca por medicamentos anticancerígenos que eliminem com eficiência as células cancerígenas e que não prejudiquem as saudáveis.

1.7. Peptídeos anticancerígenos (PAC)

Diversos estudos com PAMs que eram testados incialmente contra microrganismos patogênicos relataram que o peptídeo também apresentaram atividade

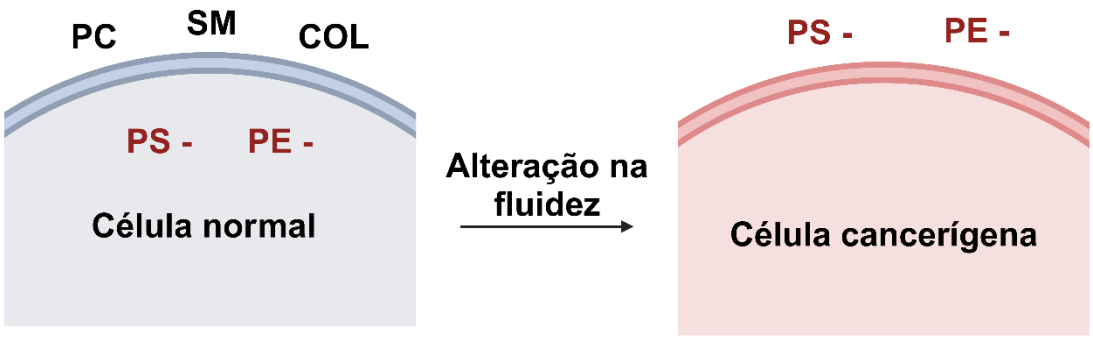
anticancerígena e antitumoral. Vários tipos de células foram analisadas, como as células cancerígenas de próstata, mama, ovário, bexiga, pulmão e pele [71–76]. Embora haja progressos significativos no tratamento do câncer, o desenvolvimento de resistência das células cancerosas aos medicamentos permanece um desafio. Dessa forma, muitas pesquisas estão em andamento para identificar novos agentes anticancerígenos eficazes [70].

Os peptídeos anticâncer (PACs) são pequenos peptídeos com toxicidade direcionada a células cancerígenas, e têm impulsionado o desenvolvimento de medicamentos anticâncer e vacinas baseadas em peptídeos [77]. As aloferonas, que são um dos primeiros inseticidas naturais isolados de insetos, foi classificado como um PAC por demonstrarem atividades estimuladoras nos linfócitos *natural killer* (NK), enquanto experimentos *in vivo* contataram que essas aloferonas induziram supressão tumoral em modelos de câncer em camundongos [78]. Outro PAC que tem potencial como um novo candidato a agente anticancerígeno é o Buforina IIb, seu mecanismo de ação consiste em atravessar a membrana da célula neoplásica sem danificá-la e induzir a apoptose [79,80].

As características dos PAMs, como anfipaticidade, hidrofobicidade e carga positiva, também desempenham papéis importantes na atividade terapêutica dos PACs [81]. Esses peptídeos identificam seu alvo por meio da atração eletrostática inicial [82], já que as células saudáveis e cancerosas são diferentes.

As membranas de células saudáveis apresentam uma distribuição assimétrica com lipídeos zwitteriônicos, como a fosfatidilcolina (PC) e esfingomielina (SM) no folheto externo, enquanto a fosfatidilserina (PS) e fosfatidiletanolamina (PE) que são lipídeos aniônicos ocupam o folheto interno [83]. Essa assimetria no folheto interno é mantida principalmente por enzimas, que transferem esses lipídeos aniônicos da membrana externa para a interna), além do colesterol da parte interna para a externa [83]. Por outro lado, a membrana das células cancerígenas perde essa distribuição assimétrica devido a alterações na fluidez, resultando na exposição da carga negativa da PS e PE na superfície da membrana (Figura 9) [84–86]. Além disso, a quantidade de SM é menor na membrana das células cancerígenas e está associada à tumorigênese [87]. De forma resumida, assim como os fungos essas células apresentam membrana aniônica, e como explicado no tópico anterior, essa característica aumenta a seletividade para as células cancerígenas e diminui a possibilidade de o peptídeo atacar células saudáveis.

Figura 9 - Diferenças entre células saudáveis e cancerígenas.



Fonte: Autoria própria. Criado com Biorender.com

Além do mecanismo de ação extracelular com ação direta na membrana, os PACs podem ter alvos intracelulares. Eles podem atuar regulando a apoptose, inibindo a replicação de DNA, alterando a função das mitocôndrias, danificando a membrana dos lisossomas, inibindo ou ativando o proteossoma e ativando vias imunomoduladora [88]. Esses peptídeos também podem ser utilizados para transportar medicamentos citotóxicos, hormônios, vacinas e radionuclídeos (terapia direcionada) [89].

A terapia com PAMs e PACs apresenta vantagens em relação às proteínas e anticorpos no tratamento de doenças, destacando-se pela alta especificidade e baixa toxicidade [90,91]. Em contrapartida, o uso clínico dos peptídeos encontra desafios, devido à sua instabilidade e meia-vida curta em fluidos corporais, toxicidade para algumas células do hospedeiro e o alto custo de produção, apesar de que esta última está diminuindo com o avanço da tecnologia. Porém, esses desafios podem ser superados substituindo aminoácidos, adicionando moléculas ao peptídeo para interagir com receptores específicos, além do nanoencapsulamento do peptídeo para prolongar sua meia-vida e atingir alvos mais específicos [80,88,92].

Uma perspectiva futura dos PACs é a sua combinação com outros agentes quimioterápicos, visando aprimorar a eficácia na destruição das células tumorais, ao mesmo tempo em que reduz a toxicidade e minimiza o risco de recorrência tumoral [93].

1.8. Desenho *in silico* e bioinformática

Os PAMs e PACs podem ser obtidos tanto de fontes naturais como plantas e animais, como também é possível realizar a síntese química. Esta última é interessante em questão de economia de tempo e pureza, já que a extração de fontes naturais é muito mais demorada. A bioinformática é outra ferramenta utilizada, pois é possível estudar questões biológicas de uma forma tridimensional, possibilitando experimentos *in silico* de um ambiente biológico [28,94].

A bioinformática desempenha um papel crucial no desenvolvimento destas moléculas, pois ajuda a identificar novos peptídeos com atividades medicinais, já que identificar novas moléculas com potencial é um trabalho demorado e custoso [88]. Através de modelagem e dinâmica molecular é possível verificar a eficácia e estabilidade dessas moléculas, além de suas interações com possíveis alvos moleculares. Tudo isso é possível através de algoritmos computacionais que permitem a comparação com outras sequências depositadas em bancos de dados genômicos e proteômicos [95]. Além disso, é possível melhorar peptídeos já existentes, realizar substituições de aminoácidos em regiões estratégicas para favorecer determinadas características físico-químicas desejadas nos PAMs.

Entre 2015 e 2021, foram aprovados pela FDA 258 medicamentos, sendo 27 deles peptídeos antimicrobianos e anticâncer [96–98]. Apesar de parecer pouco no contexto geral, a descoberta de medicamentos peptídicos está crescendo exponencialmente. Estima-se que até 2026, o mercado global de medicamentos com peptídeos irá atingir mais de 60 milhões de dólares. Atualmente é encontrado 197 medicamentos peptídicos disponíveis no mercado e mais 800 em desenvolvimento clínico [88].

1.9. Inibidor de tripsina de *Adenanthera pavonina* (ApTI)

Inibidores de peptidase vegetal (IP) são compostos presentes em plantas que possuem capacidade de inibir a atividade de peptidases. As peptidases, que também conhecidas como proteases ou enzimas proteolíticas, são responsáveis pela clivagem de ligações peptídicas em proteínas, resultando na formação de peptídeos menores. Esses IP também são cruciais na defesa das plantas contra herbívoros e patógenos, pois ocorre a interação do inibidor com as proteases específicas do intestino do inseto, originando um complexo enzima-inibidor. Esse complexo compromete a atividade enzimática habitual, resultando em efeitos adversos como indigestão, problemas na reprodução, diminuição no crescimento, diminuição da ingestão alimentar, e até a morte do inseto. Além disso, alguns desses inibidores possuem propriedades antimicrobianas, contribuindo para a resistência das plantas a patógenos [99]. Dessa forma, os IP são uma estratégia adaptativa das plantas para se protegerem contra predadores e patógenos, conferindo-lhes resistência biológica.

Um IP que tem sido estudado é o Inibidor de Tripsina de *Adenanthera pavonina* (ApTI). Esse inibidor possui uma estrutura de 176 aminoácidos em folha-β composto por duas cadeias polipeptídicas, com 16 kDa e 5 kDa cada, que são ligadas por uma ligação dissulfeto [100]. Esse inibidor é encontrado em sementes da planta *A. pavonina*, que é uma árvore leguminosa de países tropicais e subtropicais, suas sementes possuem coloração vermelha e por esse motivo é conhecida como olho-de-dragão, carolina-tento, tento vermelho ou falso-pau-brasil. Estudos já mostraram que suas sementes possuem atividade anti-inflamatória, antioxidante e antibacteriana [101–103].

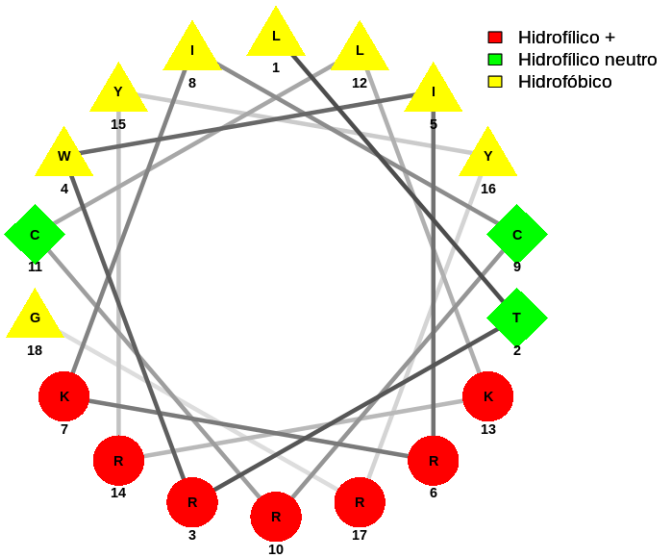
A função primária do ApTI é inibir a tripsina, uma enzima proteolítica encontrada no trato digestivo de muitos animais, incluindo insetos herbívoros, dessa forma ele atua como um mecanismo de defesa da planta [104].

Uma maneira de adquirir novas sequências peptídicas é através da busca por peptídeos criptografados dentro de proteínas. Em outras palavras, é possível encontrar pequenas sequências de aminoácidos com previsões promissoras para atividade antimicrobiana em proteínas maiores [105]. As proteínas podem ter diversas atividades e, assim, esses peptídeos criptografados podem ter diferentes funções biológicas, como antimicrobiana, imunomoduladora, inibidora de enzimas e antioxidante [105,106]. Estudos mostram que vários peptídeos, quando incorporados em proteínas, podem não apresentar atividade. Porém, quando a proteína sofre proteólise, o peptídeo é liberado e demonstra atividade biológica [106]. Alguns trabalhos realizados com peptídeos inspirados em partes da sequência do ApTI já descreveram atividades antibacterianas e antifúngicas, indicando o potencial deste inibidor como um bom modelo para o desenho de novos peptídeos antimicrobianos [28,107].

1.10. Caracterização do peptídeo antimicrobiano PEPAD

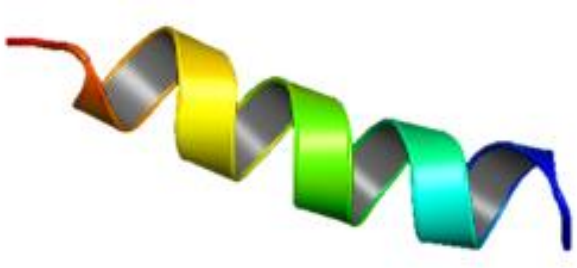
O peptídeo PEPAD foi projetado com base em um peptídeo criptografado do inibidor de peptidase vegetal ApTI. Ele foi projetado com as características físico-químicas necessárias para apresentar atividade antimicrobiana, sendo uma molécula catiônica, anfipática, que possui α-hélice e com resíduos hidrofóbicos (Figuras 10 e 11).

Figura 10 - Representação em roda helicoidal do peptídeo PEPAD ilustrando a distribuição de aminoácidos hidrofóbicos (triângulos amarelos), hidrofílicos neutros (losangos verdes) e hidrofílicos positivos (círculos vermelhos). A disposição alternada desses resíduos ao longo da hélice evidencia a estrutura anfipática, com uma superfície predominantemente hidrofóbica e outra hidrofílica.



Fonte: Imagem gerada pelo software NetWheels.

Figura 11 - Conformação α-hélice do peptídeo PEPAD.



Fonte: Imagem gerada pelo software I-TASSER.

PEPAD foi caracterizado como uma molécula anfipática com 18 aminoácidos LTRWIRKICRCLKRYYRG, com carga líquida +7, hidrofobicidade de 38%, índice de Boman de 3,5 kcal/mol^{-1} e ponto isoelétrico de 10,96. A sequência do PEPAD é protegida por pedido de patente depositado no Instituto Nacional de Propriedade Industrial (INPI) sob número BR 10 2019 008247 0 [108].

Nas previsões *in silico*, PEPAD mostrou melhora na atividade antimicrobiana quando comparado ao seu peptídeo parental. PEPAD foi testado contra uma variedade de bactérias Gram-positivas e Gram-negativas, incluindo *Staphylococcus* resistentes à meticilina. PEPAD apresentou atividade bactericida rápida contra sete bactérias Gram-positivas e seis Gram-negativas, com valores de concentração inibitória mínima (CIM) variando de 4 a 10 μM. Além disso, PEPAD apresenta sinergismo com o antibiótico comercial ciprofloxacina, diminuindo consideravelmente as concentrações necessárias utilizadas dos dois compostos [108].

Além disso foi constatado que ele adota uma conformação α-helicoidal quando em contato com membranas biológicas. Essa característica auxilia no rompimento da membrana e consequente perda do conteúdo do citoplasma, levando à morte celular [109]. Sabendo disso e por meio de outros ensaios foi constatado que o principal mecanismo de ação contra bactérias é por meio de danos à membrana celular.

Por apresentarem um sistema imunológico bem desenvolvido comparável à resposta imune inata dos mamíferos, larvas de *Galleria mellonella* foram utilizadas para verificar a toxicidade do peptídeo [110]. Mesmo em uma concentração muito superior à CIM testada (50 μM), PEPAD não apresentou efeitos tóxicos nas larvas [108].

2. Objetivos

2.1. Objetivo geral

Avaliar o potencial antifúngico e anticâncer *in vitro* de um peptídeo antimicrobiano (PEPAD), que foi desenhado com auxílio de ferramentas computacionais, além de avaliar os possíveis mecanismos de ação.

2.2. Objetivos específicos

- Avaliar a atividade antifúngica do peptídeo frente a espécies de *Candida*;
- Determinar o mecanismo de ação em células de *Candida*;
- Analisar a interferência do peptídeo sobre a formação de biofilme por *Candida*;
- Avaliar a citotoxicidade do peptídeo em linhagens de células cancerígenas e em células normais;
- Verificar os efeitos antiproliferativos *in vitro* e o tipo de morte celular nas células cancerígenas;
- Avaliar a capacidade de indução da morte celular imunogênica *in vitro*.

Capítulo I - Avaliação da atividade antifúngica

Development of new antifungal strategies: Synergistic action of the antimicrobial peptide PEPAD with commercial antifungals against *Candida* species

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Abstract

Fungal infections are the leading cause of death in immunosuppressed individuals. The increasing resistance of fungals to existing antimicrobials, coupled with the absence of selective substances, has become a worldwide public health problem. Therefore, the development of new molecules based on efficient and selective toxicity strategies is necessary. Antimicrobial peptides are an alternative to new molecules against microorganisms due to their broad spectrum and varied mechanisms of action. Therefore, the objective of this study was to evaluate the antifungal potential against *Candida* species, of a synthetic peptide, PEPAD, and to describe its effect and mechanism of action. PEPAD was designed to have specific action against microorganisms such as bacteria and fungi. The results of the broth microdilution assays revealed the antifungal potential of PEPAD against various strains of *Candida*, with minimum inhibitory concentration (MIC) values ranging from 2.5 µM to 20 µM. Subsequently, we chose to continue the assays with the *C. tropicalis* strain, considering its more favorable MIC and clinical relevance. Regarding the characterization of PEPAD's antifungal effect, the death kinetics showed rapid action, resulting in the elimination of treated fungi in just 1 hour. The description of the mechanism of action revealed that the effect of the peptide targets the fungal plasma membrane by binding to ergosterol. Additionally, PEPAD was able to inhibit biofilm formation and showed synergistic effects with the antifungals amphotericin B and fluconazole. Thus, PEPAD proved to be an important antimicrobial peptide with rapid and broad-spectrum action, a relevant inhibitory effect through binding to the fungal membrane, and the ability to inhibit the formation of complex structures such as biofilms. These results support the conduct of complementary assays to use PEPAD for the development of new drugs that can help with the treatment of resistant fungal infections.

Keywords: fungi, ergosterol, drug synergism, biofilm, antifungal agents, synthetic peptide.

1. Introduction

Fungal infections pose a significant threat to global public health, often underestimated in cases of resistance. These infections account for 1.5 billion deaths annually worldwide, and immunosuppressed individuals are the most susceptible [1,2]. Normally, the immune system can combat fungi, in general. However, some species act as opportunistic pathogens, becoming virulent when the patient's defense system is compromised. An example of such a pathogen is the fungus *Candida albicans*, naturally present in the female reproductive system and oral mucosa [3]. *Candida* species are among the four most frequently isolated pathogens in blood cultures, and their origin varies, including through implanted medical devices, through the genitourinary and gastrointestinal tracts, contaminated intravenous solutions, and skin lesions [4].

Candida infection is one of the most common among Healthcare-Associated Infections (HAI) in the hospital environment. There are two types of fungal infection, localized candidiasis and systemic candidemia, the latter being of greater concern, as it is the fourth most common cause of HAI [5]. The most common pathogenic species responsible for more than 95% of the invasive diseases caused by the *Candida* genus are *Candida albicans, Candida glabrata, Candida tropicalis, Candida parapsilosis* and *Candida krusei* [5]. *C. tropicalis* is the second most virulent species and has the ability to develop antifungal resistance during treatment. Patients with *C. tropicalis* candidemia have higher mortality and a worse prognosis compared to other non-albicans *Candida* species. Additionally, *C. tropicalis* has a growing tendency to develop antimicrobial resistance to echinocandins, a class of antifungal drugs [1].

Among fungal resistance mechanisms are cellular efflux pumps, drug target overexpression, metabolic pathway alterations, decreased ergosterol in the membrane, and mutations in various genes, such as ERG11 responsible for ergosterol synthesis and the FKS gene encoding the enzyme targeted by echinocandins [6]. In addition to the mechanisms mentioned above, biofilms represent an important form of resistance for some species. This occurs due to cell protection by an extracellular polymeric matrix, which acts as a protective film. This allows cells to adhere to surfaces, such as medical devices, and prevents drugs from permeating the cell matrix and reaching the cells, making patient treatment more difficult [7].

One of the biggest obstacles to the development of new antifungal drugs is the difficulty in developing selective substances with low toxicity [8]. Fungal infections are

being fought with a limited number of substances, such as polyenes (amphotericin B and nystatin), echinocandins (micafungin, caspofungin and anidulafungin), triazoles, and azoles (itraconazole, posaconazole, miconazole, voriconazole, and fluconazole). Only these three classes of antifungals are used for the treatment of invasive fungal infections. If one of these classes becomes ineffective, the therapeutic options will reduce by 33%, resulting in a significant impact on this scenario [6]. Therefore, it is necessary to find new therapeutic options that are selective, efficient and with minimal toxic effects.

Thus, antimicrobial peptides (AMPs) have emerged as an alternative. These peptides have been studied for the diversity of biological activities that they exhibit, which present antimicrobial, immunomodulatory, antibiofilm, anticancer, and antiviral potential [9,10]. In addition, AMPs have advantages over classical antimicrobials due to their broad spectrum of action, rapid elimination of microorganisms, synergism with classical antimicrobials, and low propensity to develop resistance [11]. The promising therapeutic characteristics of AMPs led us to explore the antifungal potential of the peptide PEPAD, derived from a plant protease inhibitor (ApTI). ApTI is found in the seeds of the plant *Adenanthera pavonina* and has shown insecticidal effects [12–15]. Other studies have also reported two ApTI-derived peptides (adevonin and adepamycin) that showed antimicrobial activity [16,17]. Due to this and the fact that protease inhibitors can have antimicrobial action, ApTI was selected as a template for the rational design of the antimicrobial peptide [18].

The computational design and modeling of PEPAD were performed with the support of bioinformatics tools, providing favorable characteristics to this molecule [19]. PEPAD has a net charge of +7, ideal for initial electrostatic attraction to the microorganism membrane, an alpha-helix structure that provides stability to the molecule when interacting with the membrane, and an amphipathic structure with 38% hydrophobicity so that the molecule can interact with the medium (hydrophilic) and the membrane (hydrophobic) simultaneously. Additionally, this peptide has shown significant results against 13 species of Gram-positive and Gram-negative bacteria, a mechanism of action directed at the cell membrane, synergy with the antibiotic ciprofloxacin, and the absence of toxicity in *in vivo* assays [19]. Considering the proven antibacterial potential of PEPAD, its promising characteristics, and the need for the development of efficient and selective therapeutic alternatives, the objective of this study was to evaluate the antifungal potential and determine the mechanism of action of the PEPAD peptide.

2. Materials and Methods

2.1. Reagents and chemicals

The culture media and reagents used in the assays are from the following brands: Kasvi, Sigma-Merck, Himedia, Synth, and Invitrogen.

2.2. Access to genetic heritage

The access to the genetic heritage of microorganisms has been registered in the National System for Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under the number A297FAA.

2.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The minimum inhibitory concentration (MIC) of the peptide was determined by the broth microdilution technique [20]. The tested concentrations ranged from 0.6 to 20 μM and were evaluated against strains of *C. albicans* ATCC 5314, *C. tropicalis* ATCC 750, *C. guilliermondii* ATCC 6260, *C. krusei* ATCC 6258, and *C. utilis* ATCC 9950. The strains were kept at -20°C in Brain Heart Infusion (BHI) broth supplemented with 20% glycerol. For the assay, the strains were inoculated in BHI broth for 24 hours then subcultured on Sabouraud dextrose agar (SDA) using the streak plate technique and incubated at 37 °C for 24 hours, obtaining isolated colonies for the assays.

To prepare the inoculum, a single colony of the fungus isolated on SDA agar was placed in sterile 0.9% NaCl solution until an optical density of 0.08 to 0.1 at 620 nm, totalling approximately $1.5x10^8$ CFU/mL. Then, 10 μL of this inoculum were transferred to a test tube containing 4990 μL of RPMI 1640 culture medium, reaching a concentration of $3x10^5$ CFU/mL. Subsequently, a microplate was prepared with 100 μL of the serially diluted peptide in RPMI 1640 and 100 μL of the inoculum, totaling $1.5x10^3$ CFU/mL/well. Amphotericin B and fluconazole were used as positive controls, while only 0.9% NaCl was used as a negative control. The microplate was incubated at 37°C for 24 hours and visual reading was performed. The MIC was defined as the lowest concentration of peptide capable of inhibiting visible microbial growth. To determine the

minimum fungicidal concentration (MFC), aliquots of 10 µL taken from the MIC assay plate were cultured on SDA agar and incubated at 37°C for 24 hours. The MFC was determined to be the lowest sample concentration in which no growth was observed in solid medium.

2.4. Kinetics of fungicidal action of PEPAD

To determine the fungicidal action time of PEPAD against *C. tropicalis* ATCC 750, the MFC (5 µM) was used, evaluated at time intervals between 0 and 240 minutes [21]. A volume of 200 µL of the inoculum (prepared in the same way as the previous assays) was added to a microtube previously filled with 200 µL of RPMI 1640 medium and the peptide (the final concentration of the peptide in this solution reached the MFC value). The microtubes were incubated in an oven at 37 °C according to the test time (0, 30, 60, 120, 180, and 240 minutes). After the respective periods, an aliquot of 10 µL was transferred to an agar plate, spread with a Drigalski loop, and incubated for 24 hours at 37 °C. Subsequently, the colony-forming units were counted. Amphotericin B was used as a positive control and 0.9% NaCl was used as a negative control.

2.5. Synergism with Antifungals

The synergistic effect of PEPAD with the antifungals amphotericin B and fluconazole was evaluated using the Checkerboard method [22]. A fungal inoculum of *C. tropicalis* ATCC 750 was prepared as previously described, and combinations of PEPAD with amphotericin B and fluconazole were made from their respective isolated MICs. The fungal density reached a final concentration of $1.5x10^3$ CFU/mL in RPMI-1640 medium and the plates were incubated at 37 °C for 24 hours. The result of the combination assay was interpreted as the lowest concentration of each combination that resulted in complete inhibition of fungal growth. Synergistic was considered when FIC ≤ 0.5, indifferent when FIC > 1 and ≤ 4 and antagonistic when FIC > 4. The fractional inhibitory concentration (FIC) index was calculated using the following equation:

$$\Sigma FIC = FIC\ (PEPAD) + FIC\ (antifungal)$$

$$FIC\ (PEPAD) = CIM\ combination \div CIM\ individual$$

$$FIC\ (antifungal) = CIM\ combination \div CIM\ individual$$

2.6. Membrane Permeability with Fluorescence Microscopy

To assess the ability of PEPAD to damage to the plasma membrane, we used the fluorescent probe Sytox^{TM} Green with fluorescence microscopy. For this, an inoculum of *C. tropicalis* ATCC 750 was treated with PEPAD at 2.5 µM, following the procedures performed in determining the MIC assay, according to Thevissen *et al* methodology [23]. After a 24-hour incubation period, 12 µL of Sytox^{TM} Green (30 µM) was added, followed by incubation for 15 minutes at 37°C. Subsequently, the samples were homogenized and an aliquot was placed between a slide and a coverslip, to be observed under a Leica DM 2000 LED fluorescence microscope, equipped with a Leica DFC 7000 T camera and a set of filters for fluorescein detection (excitation at 450-490 nm and emission at 520 nm). Images were acquired using LAS V4.12 software.

2.7. Mechanism of action on fungal plasma membrane and cell wall

To determine wheter PEPAD could bind to fungal membrane or cell wall sterols, the peptide's MIC was determined again, but for this assay, with and without the addition of exogenous ergosterol and sorbitol to the culture medium. This assay was carried out on a 96-well microplate with *C. tropicalis* ATCC 750, following the same methodology as the assay performed to determine the MIC. Supplementation was performed in RPMI-1640 medium, previously supplemented with ergosterol (400 µg/mL^{-1}) or sorbitol (0.8 M) [24]. RPMI-1640 culture medium without supplementation was used as a negative control, and amphotericin B was used as a positive control. Subsequently, the microplate was incubated at 37 °C for 24 hours and visual reading was performed.

2.8. Inhibition of biofilm formation and eradication of mature biofilms

To evaluate the ability of PEPAD to inhibit biofilm formation or eradicate mature biofilms of *C. tropicalis* ATCC 750, crystal violet dye was used [25]. PEPAD was previously prepared at concentrations of 0.5 X, 1 X and 10 X the MIC. For the inoculum, a portion of *C. tropicalis* ATCC 750 previously isolated on SDA agar was diluted in 0.9% NaCl to reach an optical density (OD) of 0.08 to 0.1 at 620 nm (1.5x10^{8} CFU/mL). Then 500 µL of this inoculum was diluted in 4500 µL of BHI supplemented with 1% glucose

($1.5x10^7$ CFU/mL). To assess inhibition of biofilm formation, 170 µL of BHI broth with 1% glucose + 20 µL of the fungal inoculum prepared previously + 10 µL of the sample were added to a 96-well microplate and then the plate was incubated at 37 °C for 24 hours. To evaluate the eradication of mature biofilm, 180 µL of BHI broth with 1% glucose + 20 µL of the inoculum were added to the microplate, which was then incubated at 37 °C for 24 hours. After incubation, the culture medium was removed and 190 µL of BHI broth with 1% glucose + 10 µL were added to the wells. The plate was again incubated at 37 °C for 24 hours. To perform staining and quantify the biofilm mass, the supernatant was removed from the wells and washed twice with sterile distilled water. Then 125 µL of 0.1% aqueous crystal violet solution was added to the wells and incubated for 10 minutes. The supernatant from the wells was discarded, washed with sterile water, and allowed to dry for 1 hour. The biofilm was diluted in 150 µL of 30% acetic acid solution for 10 minutes, homogenized with a pipette, and an aliquot of 125 µL was transferred to another microplate. OD was determined at 550 nm using a Varioskan Lux microplate reader (Thermo Scientific). Data were expressed as a percentage of inhibition of biofilm formation/eradication compared to the control.

2.9. Statistical Analysis

The experiments were carried out in triplicate in three independent experiments. All graph results were expressed as mean ± standard deviation of the mean. Differences between groups were determined by one-way analysis of variance (ANOVA), followed by Tukey's post-test. The results were analyzed using GraphPad Prism 8.0 software. Data were considered significant when $p < 0.05$.

3. Results

3.1. Antifungal Potential of PEPAD

Tests to determine the antifungal potential revealed a broad spectrum of action of PEPAD against the evaluated strains. MIC and MFC values ranged from 2.5 to 20 μM (Table 1). Overall, the MIC results for PEPAD were higher compared to the reference drug fluconazole, in addition to presenting fungicidal effect, unlike fluconazole, which only has fungistatic effect. Considering the clinical importance and MIC results against *C. tropicalis*, this strain was chosen for subsequent assays.

Table 1
Determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *Candida* yeasts treated with PEPAD, amphotericin B, and fluconazole.

	PEPAD (μM)		**Amphotericin B (μM)**		**Fluconazole (μM)**	
Fungal strains	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC***
C. albicans ATCC 5314	20	>20	0.125	0.5	>52	-
C. guilliermondii ATCC 6260	2.5	5	0.067	0.135	26	-
C. krusei ATCC 6258	10	20	0.25	0.5	>52	-
C. tropicalis ATCC 750	5	5	0.5	0.5	3	-
C. utilis ATCC 9950	2.5	5	0.135	0.135	13	-

*The MFC assay for fluconazole was not conducted because it is a fungistatic drug.

3.2. Rapid fungicidal action of PEPAD

The evaluation of the time-dependent fungicidal potential of PEPAD (Figure 1) represents the time it takes for the peptide to act on the fungal cell. Colony formation units (UFCs) of *C. tropicalis* ATCC 750 were reduced after 30 minutes of incubation with PEPAD in CFM (5 μM). The fungicidal effect of PEPAD was identified at 60 minutes of incubation, with complete eradication of UFC. Compared to amphotericin B, PEPAD exhibited considerably faster activity (4X), as the UFC of *C. tropicalis* when treated with amphotericin B were only eradicated after 240 minutes (Figure 1).

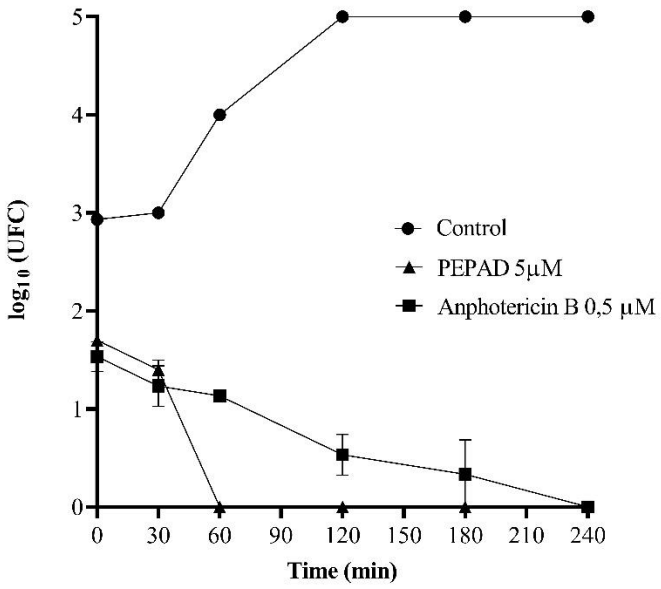

Fig. 1. Time of action of PEPAD and amphotericin B against *C. tropicalis* ATCC 750. The Dunnett's test determined the statistical differences, with $p < 0.0001$.

3.3. Synergism with antifungals

The combinations of PEPAD with the antifungals amphotericin B and fluconazole demonstrated synergy, as Σ FIC for both was less than 0.5 (Table 2). The combination of PEPAD + fluconazole was able to decrease MIC by 8 X and 18 X, respectively, for each compound. Meanwhile, the combination of PEPAD + amphotericin B reduced the MIC of both treatments by 16 X.

Table 2
Synergistic potential of PEPAD. MIC values for *C. tropicalis* ATCC 750 of the peptide alone and in combination with fluconazole and amphotericin B.

Combination	MIC individual (µM)		MIC combination (µM)		Σ FIC	Activity
	a	b	a	b	FIC_A+FIC_B	
PEPAD (a) + Fluconazole (b)	5	3	0.62	0.18	0.2	Sinergism
PEPAD (a) + Anphotericin B (b)	5	0.5	0.31	0.03	0.1	Sinergism

3.4. Alteration in membrane permeability

Sytox™ Green dye is a fluorophore that binds to nucleic acids within cells. It is impermeable to intact cells but penetrates damaged membranes. After treatment of *C. tropicalis* ATCC 750 with PEPAD (2.5 µM), it was possible to observe fluorescence emission from some cells, indicating compromised plasma membrane integrity (Figure 2). Treatment of the fungi at the MIC prevents visualization of intact cells, as it is an

inhibitory concentration, leaving only cellular debris. Therefore, PEPAD treatment for this assay was performed at half the MIC.

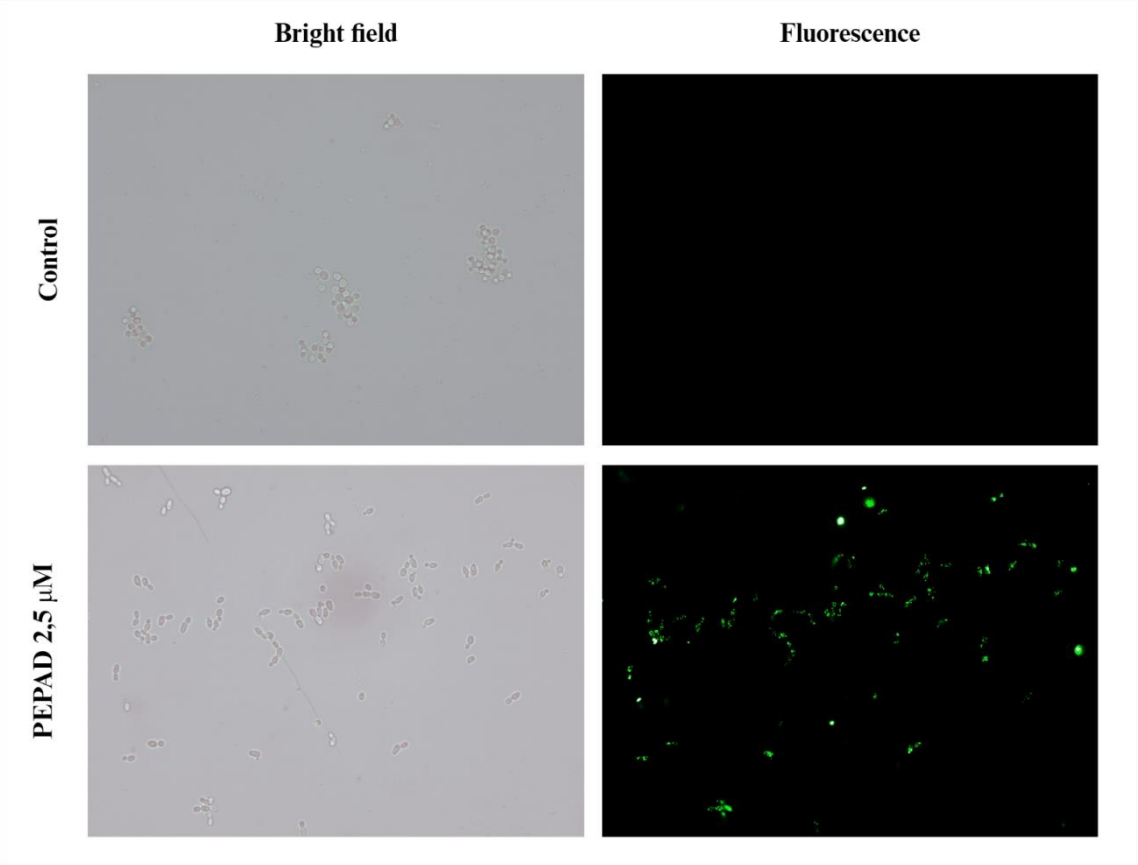

Fig. 2. Permeabilization of the plasma membrane of *C. tropicalis* ATCC 750 cells after treatment with PEPAD at 2.5 µM. Non-viable cells stained green by Sytox™ Green. Objective = 40X.

3.5. Mechanism of action on the fungal plasma membrane and cell wall

To determine the possible mechanism of action of PEPAD on *C. tropicalis* fungal cells, we studied the interaction between the peptide, the ergosterol present in the cell membrane, and the sorbitol, which is an osmotic stabilizer present in the cell wall. The assay showed that the MIC of PEPAD remained at 5 µM in the medium supplemented with exogenous sorbitol but increased to 20 µM when the culture medium was supplemented with exogenous ergosterol (Table 3). The data suggest that one of the mechanisms of action of PEPAD occurs through binding to the ergosterol present in the fungal membrane. This experiment corroborates the previous assay, suggesting that the plasma membrane is one of the targets of the peptide. Amphotericin B was used as a positive control, as it also has the characteristic of binding to ergosterol.

Table 3
Determination of the mechanism of action of PEPAD. Antifungal activity of PEPAD and amphotericin B in the presence of exogenous ergosterol (400 µg/mL) and sorbitol (0.8 M).

	MIC (µM)	MIC in the presence of exogenous ergostero (µM)	MIC in the presence of exogenous sorbitol (µM)
PEPAD	5	20	5
Amphotericin B	0.5	4	0.5

3.6. Inhibition of biofilm formation and eradication of mature biofilm

Several experiments were conducted to assess the ability of PEPAD to inhibit the biofilm formation by *C. tropicalis* ATCC 750. Initially, the determination of biofilm mass inhibition using crystal violet staining revealed that both tested concentrations (1 X and 10 X the MIC) inhibited 41% of biofilm formation, showing a non-dose-dependent behavior. Antifungal fluconazole inhibited 32% in the MIC and 50% in 10 X the MIC (Figure 3A). Subsequently, the data on the reduction of viable cells in the biofilm showed that PEPAD caused a decrease in viable cells by 73% for 1 X MIC and 77% for 10 X MIC. Fluconazole caused a reduction of 73% at 1 X MIC and 79% at 10 X MIC (Figure 3B). Qualitative analysis by fluorescence microscopy, were performed using the Syto 9 probe, which stains viable cells bright green, and propidium iodide (PI), which stains dead cells red (Figure 3C).

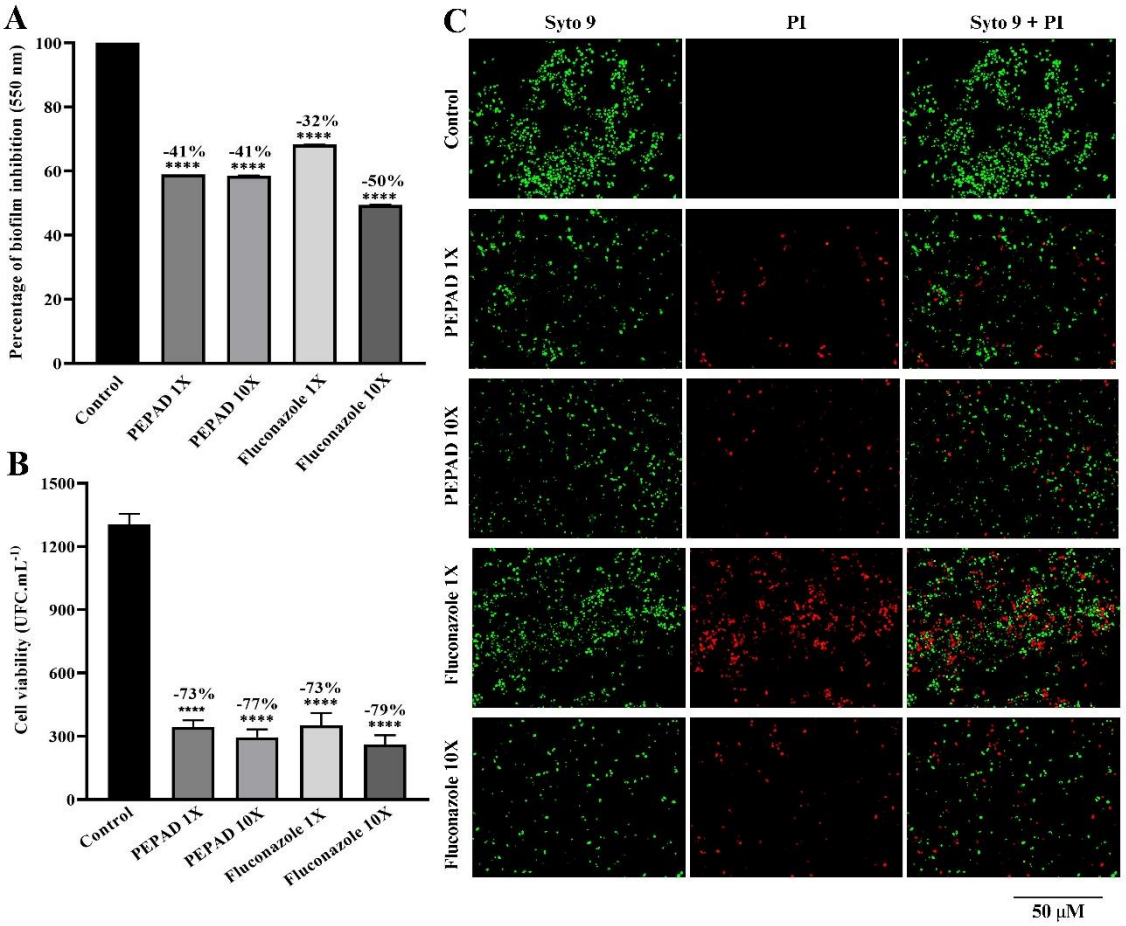

Fig. 3. Effects of PEPAD on inhibition of biofilm formation by *C. tropicalis* ATCC 750. (A) Quantitative analysis of biofilm inhibition; (B) Analysis of cell viability; and (C) Analysis of biofilm inhibition by fluorescence microscopy. The Dunnett's test determined the statistical differences, with $p < 0.0001$.

The analyses of the potential of PEPAD in eradicating mature biofilm revealed that the peptide was able to eradicate 29% (1 X MIC) and 31% (10 X MIC) of the preformed biomass. Antifungal fluconazole was able to eradicate 22% (1 X MIC) and 25% (10 X MIC) (Figure 4A). Regarding viable cells, PEPAD caused a reduction of 40% (1 X MIC) and 53% (10 X MIC), while fluconazole caused a reduction of 78% (1 X MIC) and 95% (10 X MIC) (Figure 4B). Figure 4C shows the fluorescence microscopy images with the same treatments.

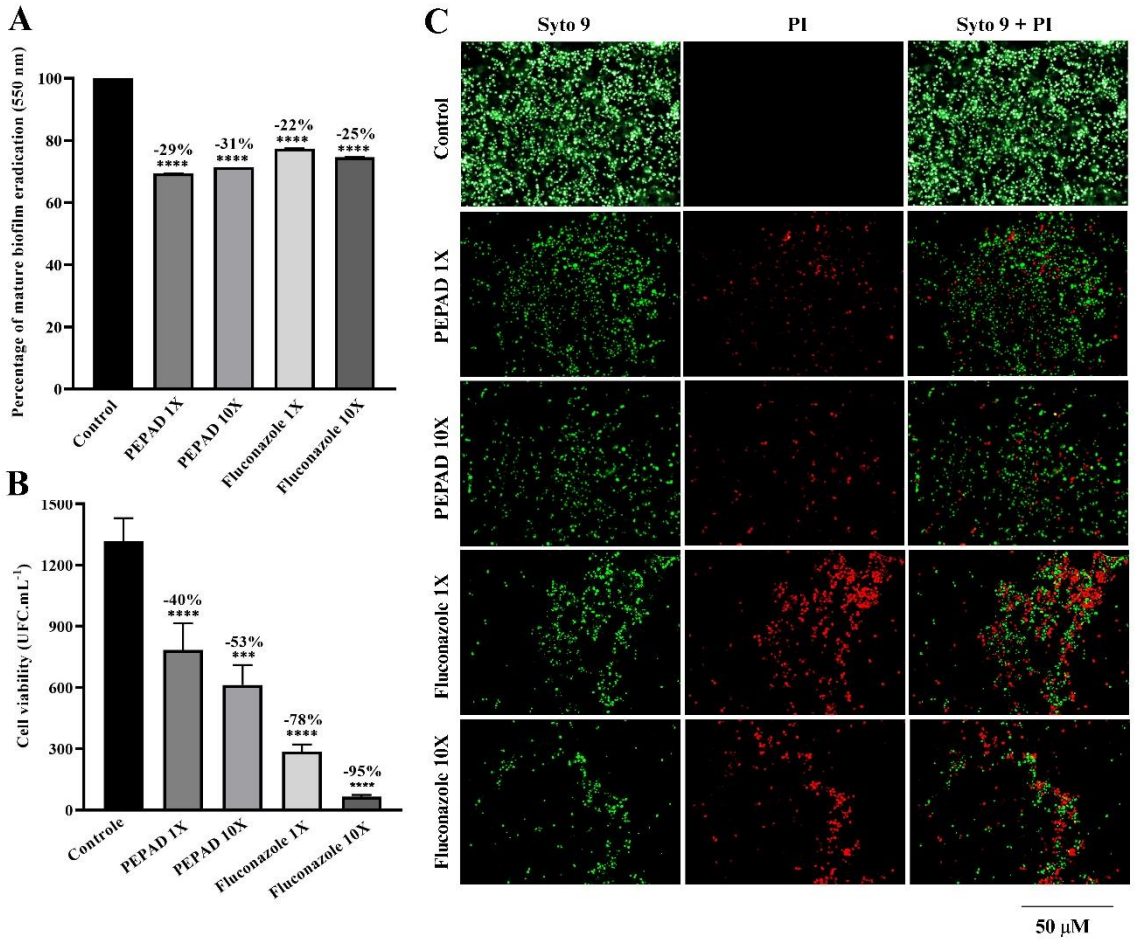

Fig. 4. Effects of PEPAD on the eradication of mature biofilm of *C. tropicalis* ATCC 750. (A) Quantitative analysis on biofilm eradication; (B) cell viability analysis; and (C) biofilm eradication analysis by fluorescence microscopy. The Dunnett's test determined the statistical differences, with $p < 0.0001$.

4. Discussion

Annually, approximately 150 million people are infected with invasive fungi, making them responsible for 1.5 million deaths worldwide [26]. Fungal resistance can further complicate the treatment of these infections and increase associated morbidity and mortality. Fungal resistance has long been neglected as a threat to public health and was not included in antimicrobial resistance (AMR) programs [27]. However, this scenario is now beginning to receive the attention it deserves. In 2019, the fungi *Candida auris* and *Aspergillus fumigatus* were included in the urgent threat list of AMR published by the Centers for Disease Control and Prevention (CDC) [28]. Improper use of antifungals is one of the main factors contributing to the emergence of fungal resistance, including incomplete treatments, overuse of medications, and inadequate doses. This creates a selective pressure on fungi, promoting the growth of those resistant to antimicrobials [27].

There is a limited number of antifungals available for the treatment of infections, and the increasing prevalence of AMR makes this scenario concerning. Thus, the discovery of new molecules capable of combating these infections is paramount. The development of new peptide-based antimicrobials has been a promising strategy in the search for new drugs to combat antimicrobial resistance [29]. The peptide-based antimicrobial peptide (PAM) known as PEPAD [19] has demonstrated significant antibacterial activity against various species of bacteria. Based on its antimicrobial potential, we sought to evaluate its antifungal potential, especially against strains of *C. tropicalis*, as well as to describe its mechanism of action, synergistic potential, and antibiofilm properties.

To determine the antifungal potential of PEPAD, a screening was performed against five *Candida* species. The results for MIC ranged from 2.5 µM to 20 µM, and for MFC, they were between 5 µM and >20 µM. Interestingly, fluconazole, which is a widely used fungistatic antifungal drug in clinics, showed MIC results higher than PEPAD (3 µM to >52 µM). On the other hand, the results for amphotericin B ranged from lower MIC values (0.067 µM to 0.5 µM). Despite its lower values, it is a drug with high nephrotoxicity due to its affinity for cholesterol, an essential component of healthy cell membranes [30], which limits its use in clinics. PEPAD showed superior results compared to other antimicrobial peptides in clinical trials for Food and Drug Administration (FDA) approval, such as Omiganan, a fungicidal cationic peptide that is being developed in topical gel form for the prevention of catheter-associated infections. MIC values for Omiganan ranged from 8 µM to 130 µM against *C. albicans, C. glabrata, C. krusei, C. parapsilosis*, and *C. tropicalis* [31,32]. PEPAD has also outperformed other peptides in development, such as TA4 and C10:0-A2, which have MIC values starting from 40 µM and 163 µM, respectively, against *C. albicans* and *C. tropicalis* [31].

In addition to describing the spectrum of antifungal action of PEPAD, it is also important to describe the time required to achieve this effect, and the kinetic assay revealed the rapid action of the peptide against *C. tropicalis*, which occurred after 1 hour of treatment, when no UFC growth was observed. In comparison, amphotericin B showed a linear decrease in UFC count over time, but the fungicidal effect was only observed after 240 minutes of incubation. A similar result to PEPAD was found for the Cecropin peptide, which during its evaluation against *C. albicans*, showed a fungicidal effect up to 40 minutes into treatment [33]. Regarding the *C. tropicalis* strain, the available literature

reports times of 12 hours and 15 hours for the peptides CGA-N9 [34] and CGA-N12 [35], respectively.

The combination of PAMs with other drugs has become an interesting approach to combating fungal resistance because, like standard antifungals, PAMs are also susceptible to fungal resistance [36]. Furthermore, combined therapy allows the use of lower concentrations of both treatments, reducing the toxicity often associated with therapy [37]. Synergy occurs when two drugs exhibit better activity together than when they are isolated. In the combinations between PEPAD and fluconazole and PEPAD and amphotericin B, synergy against *C. tropicalis* was observed for both associations. It was observed that the drugs together with PEPAD significantly reduced MICs. These results may be associated with the fact that one of the mechanisms of fungal resistance occurs through blocking the entry of therapeutic agents through the plasma membrane. Thus, the combination of two compounds with different mechanisms of action can be considered a therapeutic advantage. By interfering with the permeability of the plasma membrane, the peptide not only facilitates the access of another drug to its target, but also causes ruptures and perforations in the cell membrane [37,38].

It is known that PAMs can have more than one type of mechanism of action against the same microorganism, making resistance emergence more difficult [39]. Despite the diversity of targets, the focus of action of PAMs is through the cell membrane of microorganisms. This effect occurs because of the initial electrostatic attraction of the peptide, which has a cationic characteristic, with the anionic membrane of the pathogen. In addition, the amphipathicity of the peptide allows it to insert into the lipid bilayer of the cell, causing disruption in the membrane integrity and consequent leakage of intracellular content and cell death [40,41]. Therefore, the mechanism of action of PEPAD was investigated to determine whether it involves attacking the fungus membrane. This hypothesis was confirmed by using the Sytox^{TM} Green fluorescent probe, which permeabilizes the plasma membrane when compromised. The determination of this effect was carried out using quantitative methods, using microplate reading, and qualitative methods, through fluorescence microscopy. The obtained data confirmed that the mechanism of action of PEPAD occurs through the permeabilization of the plasma membrane, as indicated by the fluorescent probe Sytox^{TM} Green. Additionally, the data obtained in the ergosterol assay confirmed the mechanism of action of PEPAD, which acts directly on the fungus membrane by binding to one of the main sterols of the fungal membrane. This conclusion was reached because exogenously supplemented ergosterol

in the culture medium prevented binding of ergosterol to the fungal membrane with PEPAD, indicating a higher affinity of the peptide for the exogenous portion of ergosterol than for the constituent portion of the membrane. Thus, it can be confirmed that the plasma membrane is at least in part one of the targets of PEPAD, as evidenced by the increase in the MIC against this yeast. The specificity of PEPAD in its interaction with ergosterol supports the results that it is active against the fungal membrane. The presence of ergosterol in fungal membranes and its absence in eukaryotic membranes make it a useful target for antifungal drugs, as it provides greater selectivity, reducing the toxicity often associated with antifungals [42]. Binding to ergosterol is part of the mechanism of action of several antifungals, such as amphotericin B and nystatin, as well as the peptides KWI18, VG16KRKP, and Valinomycin [29,43–45].

Cells within mature biofilms are more difficult to target as a result of the polysaccharide matrix, which is more well-structured compared to biofilms still in formation. Studies indicate that the best method to combat biofilms is combined therapy with various drugs [39]. Biofilms are one of the main mechanisms of microbial resistance and are responsible for about 80% of microbial infections in the human body and 65% of chronic infections. For this reason, several researchers are committed to developing efficient therapeutic alternatives that can help combat both planktonic microorganisms and biofilms [46]. In the inhibition assay of the formation of *C. tropicalis* biofilms, PEPAD reduced biomass by 41% at both concentrations tested, while cell viability was reduced by 73% (1 X MIC) and 77% (10 X MIC). Despite the described effect, the data obtained with the peptide did not show significant differences compared to fluconazole. In the mature biofilm eradication assay, PEPAD was more efficient in eradicating preformed biomass than fluconazole, with reductions of 29% (1 X MIC) and 31% (10 X MIC) compared to 22% (1 X MIC) and 25% (10 X MIC) for fluconazole. Regarding the viability of cells in the mature biofilm, fluconazole showed better results, making 78% and 95% of nonviable cells compared to 40% and 53% for PEPAD. These results were confirmed through fluorescence microscopy imaging, which evidenced the results obtained with PEPAD.

5. Conclusion

On the basis of the results obtained, PEPAD exhibited antifungal activity at low concentrations over a short period, as well as having a synergistic effect with the

antifungals fluconazole and amphotericin B. The potential of the peptide may be associated with the mechanism of action that involves damage to the microorganism's membrane, as evidenced by fluorescence and culture medium supplementation assays. Furthermore, PEPAD was able to reduce the biomass and cell viability of fungal cells in both mature and forming biofilms. Together, the already proven favorable characteristics of PEPAD, combined with the activities demonstrated here, provides support for further trials in the development of an antifungal that may assist in the treatment of resistant infections, especially those caused by *C. tropicalis*.

Declaration of interest

The authors did not report potential conflicts of interest.

Author contributions

Camila de Oliveira Gutierrez: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, visualization, writing – original draft. **Luís Henrique de Oliveira Almeida:** Conceptualization, investigation, methodology. **Claudiane Vilharroel Almeida:** Conceptualization, investigation, methodology. **Suellen Rodrigues Ramalho:** Conceptualization, investigation. **Tamaeh Monteiro-Alfredo:** Visualization, writing – review & editing. **Janaína de Cássia Orlandi Sardi:** Conceptualization, methodology, resources. **Maria Lígia Rodrigues Macedo:** Funding acquisition, resources, supervision.

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Capítulo II - Avaliação da atividade anticancerígena de PEPAD

PEPAD: A promising therapeutic approach to the treatment of murine melanoma (B16F10-Nex2)

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Abstract

Cancer claims millions of lives yearly due to limited therapeutic options, with skin cancer being notably prevalent and deadly in Brazil. Despite advancements, the need remains for anticancer drugs with novel mechanisms that are affordable, effective, and selective, ensuring fewer side effects and broader accessibility. This study evaluated the cytotoxic potential of the peptide PEPAD, developed through computational tools. Using the MTT assay, PEPAD's IC50 was 7.4 µM in murine melanoma cells (B16F10-Nex 2) and 18.2 µM in human melanoma cells (Sk-mel-28), while breast cancer (MCF-7) and cervical cancer (HeLa) cells exhibited IC50 values of 65.2 µM and 59.7 µM, respectively. Healthy cells (RAW 264.7 and FN1) showed low toxicity with IC50 values of 56 µM and 64 µM, indicating high selectivity. Morphological analysis revealed chromatin condensation, hinting at apoptosis, while scratch tests demonstrated delayed cell migration, suggesting antimetastatic potential. Using MitoTracker Deep Red and NucBlue probes, intact nuclei and mitochondrial swelling were observed. Caspase activation, confirmed with the CaspACETM marker, supported apoptosis, and flow cytometry confirmed apoptotic cell death. PEPAD also promoted immunogenic cell death, indicated by the release of DAMPs (calreticulin and HMGB1) after brief incubation. These results highlight PEPAD's selective toxicity towards melanoma cells, its antimetastatic properties, and its potential in anticancer therapy, marking it as a promising drug candidate.

Keywords: Cancer, apoptosis, cell migration, selectivity, immunogenic cell death, *Adenanthera pavonina*.

1. Introduction

Cancer is a group of diseases characterized by the abnormal and uncontrolled growth of cells. Cancer initially arises from DNA mutations, which can occur due to genetic predisposition, microbial infections, or exposure to carcinogenic agents [1,2]. There are various types of cancer, depending on the cell or tissue of origin. Among these types, melanoma originates in melanocytes, the cells responsible for producing melanin [3].

There are two types of skin cancer: non-melanoma skin cancer and melanoma. Non-melanoma skin cancer is the most common type, it has a low mortality rate. It arises in basal or squamous cells and is more common in sun-exposed areas of the body, such as the forearms, neck, ears, and face. Prolonged exposure to the sun is thus a major risk factor [4]. Melanoma, although less common, is the most aggressive and leading cause of skin cancer death (80%) and represents 1.7% of global cancer diagnoses [5]. Melanoma presents as a spot on the skin or a mole in brown or black shades, and can exhibit changes in size, shape, and color [6]. It affects melanin-producing cells (melanocytes) and mainly affects people with fair skin. The main risk factors include exposure to UV rays from sunlight or indoor tanning, genetic predisposition, immunosuppressed patients, and even individuals with little exposure to UVA and UVB, which reduces cell immunosurveillance [5]. The diagnosis is generally made by biopsy in most cases, and when discovered early, melanoma has a good survival rate [7]. The survival rate and prognosis become poor when melanoma is diagnosed late, as it has the highest incidence rate of metastasis among malignant neoplasms, the lymphatic system being the main route of cell dissemination [8].

Melanoma treatment involves various approaches, such as surgical excision, radiation therapy, and chemotherapy, which constitute conventional therapy and are employed in most cases [7]. The therapeutic strategy is defined according to the disease and patient conditions, such as the stage of the melanoma, the health of the patient, and the genetic characteristics of the tumor. Based on these properties, the type of approach and therapeutic modalities adopted are defined, which may involve individualized or combined therapy, with the aim of achieving more favourable outcomes [9]. For five decades, cytotoxic chemotherapy has been used for the treatment of metastatic melanoma. The first drug approved by the Food and Drug Administration (FDA) for chemotherapy treatment of cancer was Dacarbazine in 1974, followed by others such as Interferon α-2b (1995), Interleukin-2 (1998) and Ontak (1999). Subsequently, in 2011, Perginterferon α-2b, Vemurafenib, and Ipilimumab were introduced, followed by Dabrafenib and Trametinib in 2013, Nivolumab, Pembrolizumab, and Dabrafenib + Trametinib in 2014, and finally, Talimogene laherparepvec and Cobimetinib + Vemurafenib in 2015 [9]. In addition to conventional therapy, there are also new therapeutic alternatives, including immunotherapy and adoptive cell therapy, specific targeted therapy for BRAF and MEK mutations, Toll-like receptor-9 agonists, gene therapy, among others [10,11].

Antineoplastics act through various mechanisms and are represented by a wide variety of techniques. Some can act directly on cancer cells, while others, in particular, may induce immunogenic cell death (ICD), which constitutes a part of immunotherapy. Immunotherapeutic agents are capable of restoring the immunological surveillance functions of cells, a condition that is naturally disturbed by cancer cells and have significant therapeutic efficacy [12]. In ICD, dying cells release signals that alert the

immune system to the presence of dying cells. These signals are known as damage-associated molecular patterns (DAMPs) and comprise molecules expressed on the cell membrane or released by cells, notably calreticulin, ATP release (adenosine triphosphate), and intracellular proteins such as High Mobility Group Box 1 (HMGB1). Recognition of these molecules by immune system cells, especially dendritic cells (DCs), stimulates an adaptive immune response that induces phagocytosis of apoptotic cells and an increase in DC maturation markers. These cells present specific antigens to T lymphocytes, promoting an antitumor response with immunological memory. As a result, therapeutic agents capable of inducing DAMP-mediated DC promote lasting therapeutic responses [13,14].

Although the techniques and medications used for melanoma treatment have advanced, there are still challenges to overcome. Some of these issues involve drug resistance, chemotherapy toxicity that affects healthy cells, tumor genetic complexity that causes each subtype to react differently to treatment, propensity for metastasis, patient immunosuppression, and high therapy costs [15,16]. Therefore, the discovery of new molecules capable of combating melanoma with greater selectivity and different mechanisms of action, such as DAMP-mediated immunotherapy, is necessary. The successes achieved through this therapeutic alternative encourage the development and advancement of new research to identify new agents and mechanisms that act through immunological pathways. In recent years, studies with antimicrobial peptides (AMPs) have been developed to explore their anticancer potential. They are part of the innate immune system of many organisms and can modulate various biological responses. Interest in the use of AMPs in the treatment of melanoma arises from their ability to interact with cell membranes and induce apoptosis, with low toxicity and better selectivity [17,18].

AMPs with anticancer activity are small molecules composed of amino acids and are usually hydrophobic, amphipathic, and cationic. This latter characteristic is essential for the initial electrostatic attraction to cancer cell membranes, which have membranes with negative net charge (anionic) due to abnormal exposure of negatively charged phospholipids, such as phosphatidylserine [17]. An interesting way to select potential candidates for the development of antineoplastic agents is to evaluate peptides that already have reported biological activities. In this regard, the peptide PEPAD, recognized for its significant impact against pathogenic bacteria, which, like cancer cells, also have anionic membranes, was selected for this study [19]. PEPAD is an antimicrobial peptide designed with the aid of computational tools from a fragment of the plant peptidase inhibitor (ApTI) found in seeds of *Adenanthera pavonina*. The peptide was designed to have the necessary characteristics to promote interaction with negative membranes, ensuring selectivity and safe use. Thus, the aim of this work was to evaluate and characterize the cytotoxic effects of the PEPAD peptide on murine melanoma cells *in vitro*.

2. Materials and Methods

2.1. Reagents and chemicals

The salts and reagents used in the assays were purchased from Sigma-Aldrich/Merck, Gibco, Fischer Scientific, Bioassay Technology Laboratory, Cytiva, and Invitrogen.

2.2. Cell culture

In this study, we used murine melanoma (B16F10-Nex2), human melanoma (Sk-mel-28), human breast cancer (MCF-7), cervical cancer (Hela), murine macrophage (RAW 264.7) and human fibroblast (FN1) cell. All cell lines were stored in liquid nitrogen cryopreservation at a temperature of approximately −196 ◦C at the LPPFB, Universidade Federal de Mato Grosso do Sul (UFMS: Campo Grande, MS, Brazil). Cells line were cultured in Roswell Park Memorial Institute Medium (RPMI 1640, Sigma, United States), supplemented with 10% fetal bovine serum (FBS), 100 U.mL−1 penicillin, and 100 µg.mL−1 streptomycin (Gibco: Waltham, MA, USA) at 37 ◦C in an incubator at 5% CO2.

2.3. Cell viability assays

Cell viability was assessed by determining metabolic activity using a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) [20].B16F10-Nex2), Sk-mel-28 MCF-7, Hela, RAW 264.7 and FN1 cell lines were seeded in 96-well microplates at densities of $5x10^4$, $1X10^4$, $1x10^5$, $1X10^4$, $3\ X10^4$ and $5X10^5$mL.well^{-1}, respectively. At 80% confluence, cells were treated with different concentrations of the peptide (1 – 128 µM – diluted in sterile water) and incubated in their respective culture medium at 37 °C and 5% CO_2. After 24 hours of incubation, the supernatant was discarded and 100 µL of MTT solution (1 mg/mL was added , followed by another 4 hours of incubation). Subsequently, MTT was removed and 100 µL of dimethylsulfoxide (DMSO) was added to solubilize formazan crystals. Absorbances were then measured at 630 nm using a Varioskan Lux microplate reader (Thermo Scientific) and cell viability was calculated using the SkanIt 6.0 software (Thermo Scientific). Results were expressed as mean ± standard deviation of the mean. The 50% inhibitory concentration (IC_{50}) values were determined using GraphPad Prism 8.0 software.

2.4. Analysis of cellular morphological changes

Cell morphological analysis assay allows the evaluation of changes that cells may undergo during treatment to determine membrane integrity and early signs of potential cell death [21]. For this assay, B16F10-Nex2 cells were seeded in 24-well microplates ($5x10^4$ cells.well^{-1}) and, upon reaching 90% confluence was reached, treated with PEPAD at IC_{50} concentration (7.4 µM). The plate was incubated in a CO_2 incubator at 37 °C for 48 hours. Cells were monitored by capturing images every 5 minutes using the ZenCELL owl 24-channel microscope.

2.5. Effects on melanoma cell migration

Melanoma cells have a high capacity for metastasis. Therefore, an assay was conducted to assess the inhibitory effect of PEPAD on cell migration using the Scratch assay [22]. B16F10-Nex2 melanoma cells were seeded in 24-well plates at a density of $5x10^4$ cells.well^{-1} and incubated for 24 hours at 37 °C and 5% CO_2 Upon reaching 90% confluence, a vertical scratch was made in each of the wells using a sterile 200 µL pipette tip. Cells were washed once with PBS to remove detached cells and then incubated with PEPAD (IC_{50}). Culture medium were used as negative controls. Cells were incubated at 37 °C in a CO_2 incubator and photos were captured at 0, 24 and 48 hours intervals, at 4x magnification using the EVOS XL Core inverted phase contrast microscope (Thermo Fisher). The area of the scratch in each well was calculated using ImageJ software.

2.6. Mictochondrial and nuclear changes

The experiment to evaluate nuclear and mitochondrial morphological alterations in B16F10-Nex2 cells was adapted from the protocol of Wodlej et al. [23]. The cells were seeded ($5x10^4$ cells.well^{-1} with confluency >80%) in a 24-well microplate previously prepared with circular coverslips. The cells were treated with 500 µL of the peptide at the IC_{50} concentration diluted in RPMI 1640 and incubated for 30 min, 1, 2, 6, 12, 24, and 48 hours. After incubation, the cells were washed with PBS and fixed with 1% paraformaldehyde for 15 minutes. To stain the mitochondria, 500 µL of RPMI and 20 µL of MitoTracker Deep Red (Molecular Probes Inc., Eugene, OR, USA) (excitation wavelength 650 nm; emission wavelength 668 nm) at 50 µM were added to each well and incubated for 10 minutes. Subsequently, to stain the nucleus, a drop (~5 µL) of NucBlue Live ReadyProbes Reagent (Molecular Probes Inc., Eugene, OR, USA) (excitation wavelength 359 nm; emission wavelength 461 nm) was added to each well and incubated for 5 minutes. The coverslip was removed and observed with glycerol under a Leica DM 2000 LED microscope equipped with a Leica DFC 7000 T camera.

2.7. Detection of active caspases in cells

The B16F10-Nex2 cells were cultured ($5x10^4$ cells.well^{-1}) in a 12-well plate containing RPMI 1640 without FBS and incubated for 24 hours. After reaching confluency (≥ 80%), the medium was replaced with treatment using the peptide PEPAD at its IC_{50} concentration for an additional 24 hours. As a negative control, only RPMI 1640 medium was used. After the incubation period, the medium was discarded, and the cells were washed with PBS. Then, 500 µL of CaspACETM FITC-VAD-FMK In Situ Marker diluted in RPMI 1640 were added to the wells at a concentration of 10 µM, followed by 20 minutes of incubation in a light-protected environment. The medium was then removed, and the cells were washed twice with PBS. After the final wash, the cells were homogenized and resuspended in PBS, and then placed in microtubes for centrifugation at 7000 rpm for 1 minute. The supernatant was removed, and the pellet was resuspended in 100 µL of PBS. Subsequently, an aliquot was placed between a slide and a coverslip. Fluorescence analysis was performed using a Leica DM 2000 LED microscope equipped with a Leica DFC 7000 T camera.

2.8. Cell Death Profile

The cell death profile was determined using the method described by Paredes-Pesarinia et al. (2018) [24] with a few modifications. B16F10-Nex2 cells were plated in 6-well plates ($1x10^6$ cells.well^{-1}) and cultured in RPMI 1640 with 10% FBS for 24 h. After reaching confluency (≥ 80%), the medium was replaced with treatment using the peptide PEPAD at its IC_{50} concentration for an additional 24 hours. After this period, the cells were washed with phosphate-buffered saline (PBS), detached, and resuspended in buffer solution (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl2). The suspension was labeled with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (BD Pharmingen ™) according to the manufacturer's instructions. The cells were incubated for 15 min at room temperature, and subsequently, 50,000 events per sample were collected and analyzed in the flow cytometer.

2.9. Release of DAMPs (Calreticulin and HMGB1)

Induction of DAMP release and consequent stimulation of immunogenic cell death can be determined by ELISA assays, which quantify the release of calreticulin and HMGB1 by B16F10-Nex2 cells in process of death. For this, cells were seeded at a density of $5x10^5$ cells.well^{-1} in a 6-well microplate and treated with PEPAD at the IC_{50} concentration. The ELISA kits used for the assay were the Mouse Calreticulin ELISA Kit and Mouse High Mobility Group Protein B1 (HMGB-1) ELISA Kit (Bioassay Technology Laboratory). The assays were performed according to the manufacturer's instructions.

2.7. Statistical analysis

All results were expressed as mean ± standard deviation of the mean, performed in triplicate in three independent experiments. Differences between groups were determined by one-way analysis of variance (ANOVA), followed by a Student-Newman-Keuls posttest. The results were analyzed using the GraphPad Prism 8.0 software. Data were considered significant when $p < 0.05$.

3. Results

3.1. Cytotoxic profile of PEPAD

To evaluate the cytotoxic capacity of PEPAD, cell lines B16F10-Nex 2, Sk-mel-28, MCF-7 and Hela were tested. In addition to healthy cell RAW 264.7 and FN1. The IC50 value obtained for B16F10-Nex2 cells was 7.4 μM, for Sk-mel-28 it was 18.2 μM, for MCF-7 it was 65.2 μM, and for Hela it was 59.7 μM. While for normal cells like RAW 264.7 it was 56 μM and FN1 was 63 μM (Table 1).

The selectivity index (SI), calculated as the ratio of the IC_{50} of normal cells (FN1) to the IC_{50} of tumor cells, showed significant selectivity of PEPAD for B16F10-Nex2 cells, with an SI of 8.5. For Sk-mell-28 cells, the SI was 3.5; for MCF-7, the SI was 0.97; and for HeLa, the SI was 1.1. These data indicate that PEPAD exhibits selective cytotoxic

activity, being more effective against B16F10-Nex2 cells compared to normal FN1 cells and other tumor cell lines.

Table 1. Cell viability of different cell lines treated with PEPAD.

Cell type	Cell lineage	PEPAD (µM)	IS*
Murine melanoma	B16F10-Nex 2	7.4	8.5
Human melanoma	Sk-mell-28	18.2	3.5
Human breast cancer	MCF-7	65.2	0.9
Cervical cancer	Hela	59.7	1.1
Murine macrophage	RAW 264.7	56	-
Human fibroblast	FN1	63	-

* selectivity index

3.2. Analysis of cellular morphological changes

Cellular morphological changes were monitored over a period of 48 hours. This assay allows observation of potential alterations that murine melanoma B16F10-Nex2 cells can undergo when subjected to PEPAD treatment. Cells remained adhered and did not show membrane alterations; however, in terms of chromatin characteristics, they exhibited increased condensation, a suggestive condition of apoptosis (Figure 1). This effect was evidenced by more intense nuclear staining, becoming more pronounced after 48 hours of treatment."

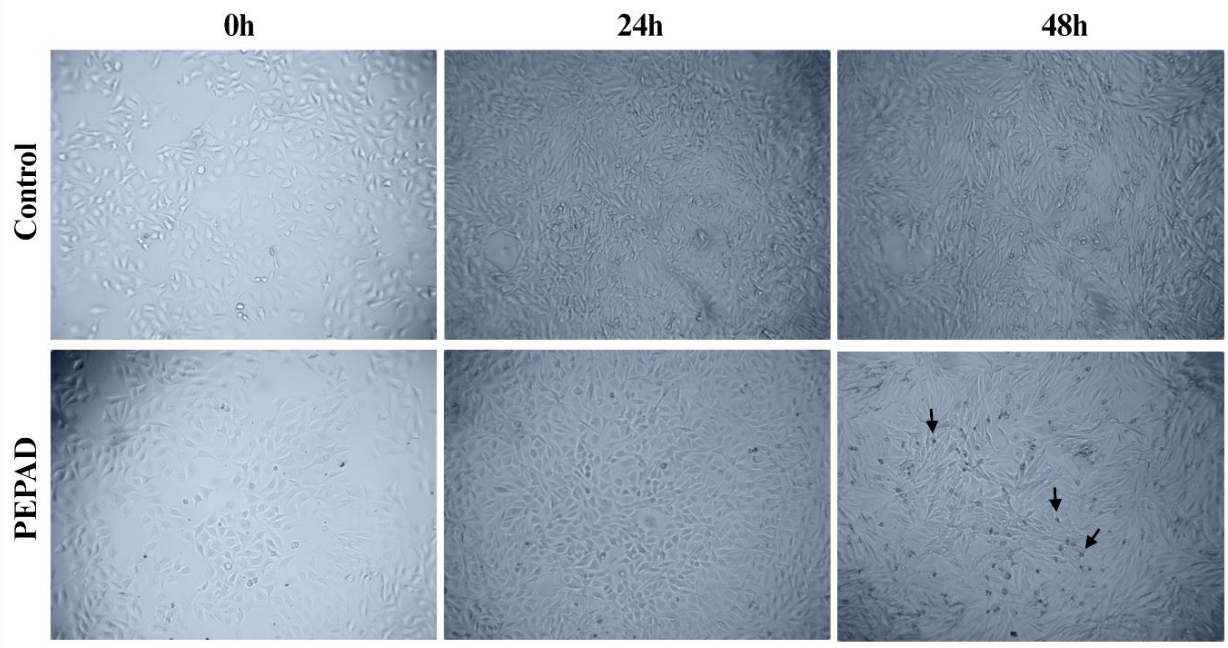

Fig. 1. Morphological and changes in B16F10-Nex2 cells treated and untreated with PEPAD at 7.4 µM, at 0h, 24h, and 48 h.

3.3. Effects on melanoma cell migration

To determine the effect of PEPAD on the metastatic activity of murine melanoma B16F10-Nex2 cells, we investigated cell migration using the scratch assay. As shown in the representative images in Figure 2A, after 48 hours, treatment with the PEPAD peptide was able to reduce cell migration. This effect intensified after 48 hours of treatment, where it was practically no longer possible to observe the scratch made in the control

group. Quantification of the open wound area showed that control cells migrated and filled the gap in less time compared to cells treated with PEPAD (Figure 2B). This shows that PEPAD decreased the migration of murine melanoma cells and possibly the metastatic activity of these cells.

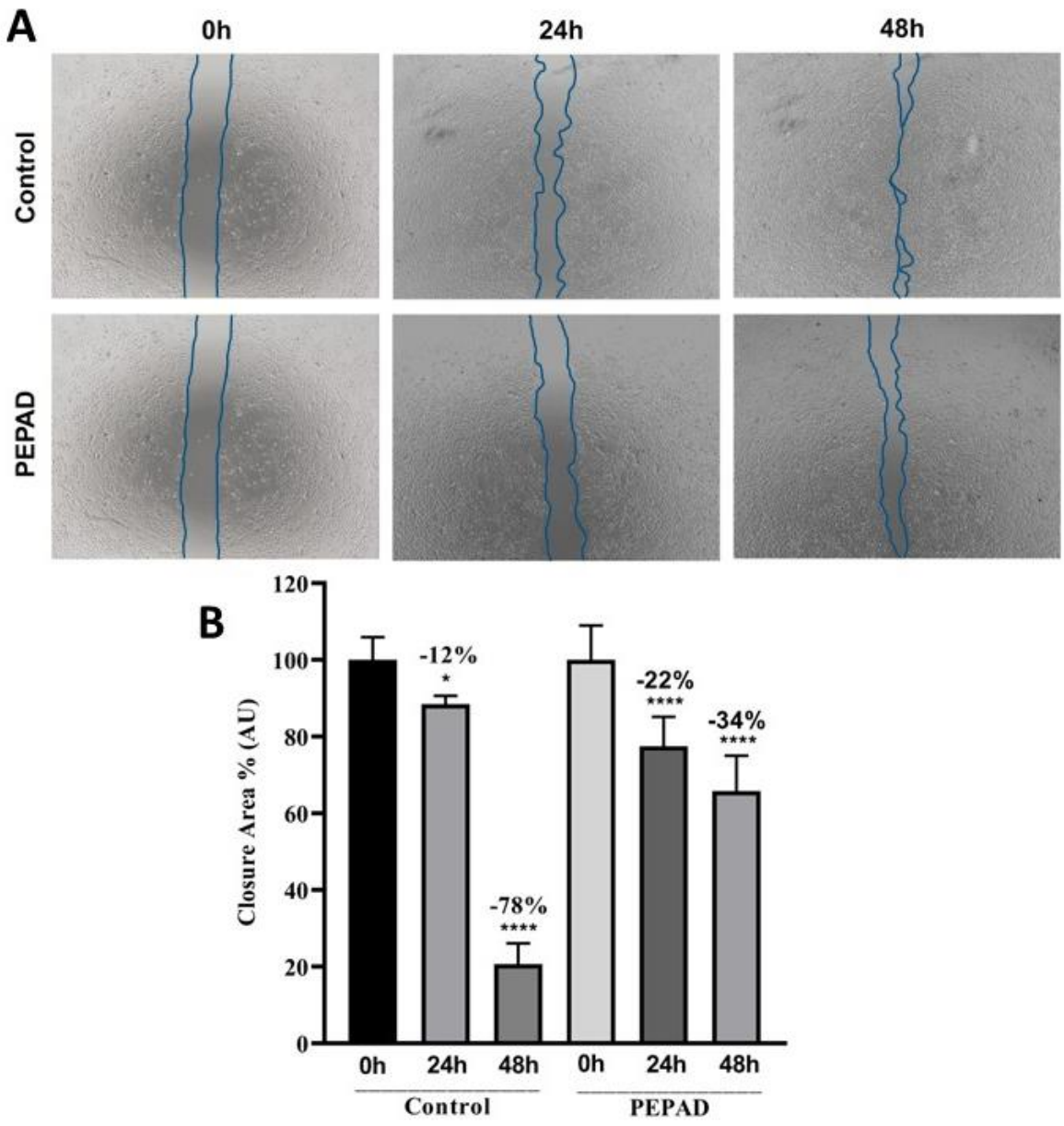

Fig. 2. Effects of PEPAD (7.4 µM) on the migration of murine melanoma B16F10-Nex2 cells. (A) Images of the scratch area. (B) Quantitative analysis of the "wound" area. The Tukey test determined the statistical differences, with $p < 0.0001$.

3.4. Mictochondrial and nuclear changes

During cell death, certain changes occur within the cell. To assess mitochondrial and nuclear alterations following treatment with PEPAD, B16F10-Nex2 cells were treated with two dyes that allowed these changes to be observed through fluorescence microscopy. Figure 3 shows the cells at different treatment times, where mitochondria are

stained with MitoTracker (red) and nuclei are stained with NucBlue (blue). The progression of treatment over time reveals mitochondrial and nuclear morphological changes indicative of apoptosis.

In the control, well-distributed and intensely stained mitochondria are visible, with clearly defined nuclei. After 30 minutes, some cells show mitochondrial swelling (indicated by arrows) as well as a reduction in the number of mitochondria, which can be attributed to mitochondrial membrane potential loss. From 1 hour onward, increased nuclear fluorescence intensity is also visible, indicating cell death, as NucBlue binds to DNA, and the condensed chromatin facilitates dye binding. Additionally, as cell and nuclear membranes become more permeable in the final stages of apoptosis, this may allow NucBlue to enter cells more easily, further increasing fluorescence intensity. After 48 hours, the absence of mitochondrial fluorescence indicates that mitochondria have lost their membrane potential, suggesting that the cells have completed the apoptotic process.

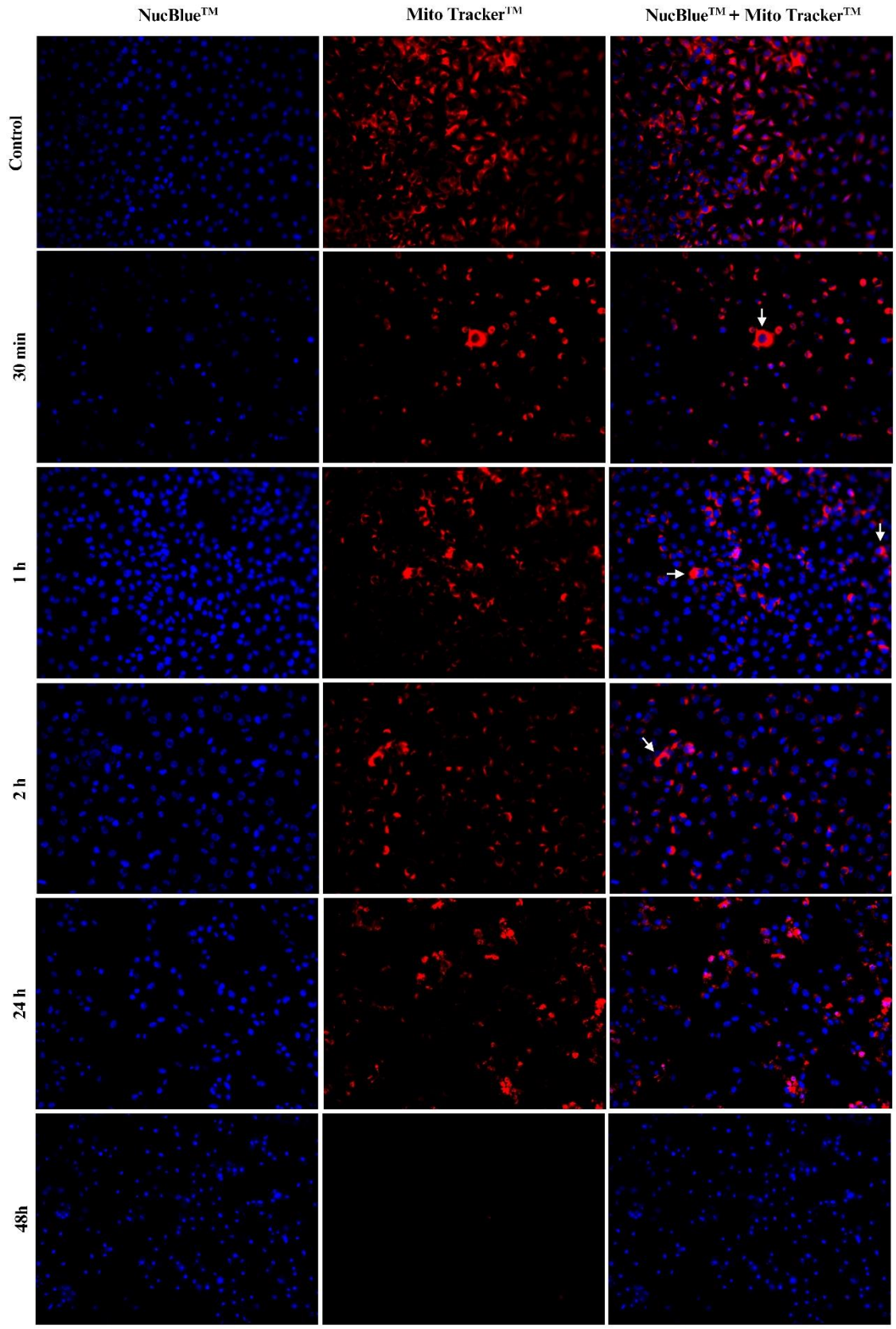

Fig. 3. Analysis of the nuclear and mitochondrial morphology of cells treated with PEPAD (7.4 µM) over time, with mitochondria stained with MitoTracker™ (red) and nuclei stained with NucBlue™ (blue).

3.5. Detection of active caspases in cells

To investigate the mechanism of action of the PEPAD peptide, murine melanoma B16F10-Nex2 cells were treated at the IC_{50} and evaluated using the FITC-VAD-FMK caspase detection kit. This permeable and non-toxic fluorescent marker irreversibly binds to activated caspases in cells undergoing apoptosis. Fluorescence intensity was observed (Figure 4). Cells treated with PEPAD for 24 hours emitted intense fluorescence, indicating that caspase activation is involved in the peptide's mechanism of action, leading to apoptosis. In contrast, control cells did not emit fluorescence, indicating their integrity and viability. These results reinforce the hypothesis that PEPAD induces apoptosis in B16F10-Nex2 cells as one of its potential mechanisms of action.

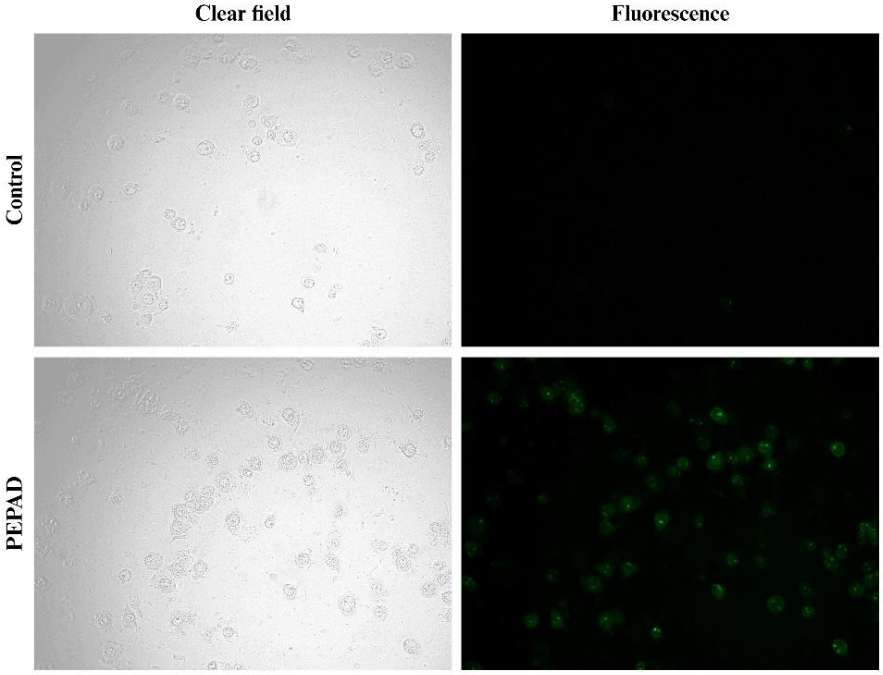

Fig. 4. Detection of active caspases in B16F10-Nex2 cells treated with PEPAD at a concentration of 7.4 µM. The cells that appear green indicate the presence of active caspase in the cells.

3.6. Flow cytometry

Flow cytometry was used to analyzed B16F10-Nex2 cells treated with the PEPAD peptide at 7.4 µM, using Annexin V-FITC and propidium iodide (PI) as markers to detect apoptosis and necrosis, respectively. Data analysis showed that a portion of the cells was located in quadrant Q4, indicating early apoptosis and suggesting that the PEPAD peptide induces apoptosis in murine melanoma cells. Furthermore, a significant population of cells was found in quadrant Q2, indicating progression to late apoptosis.

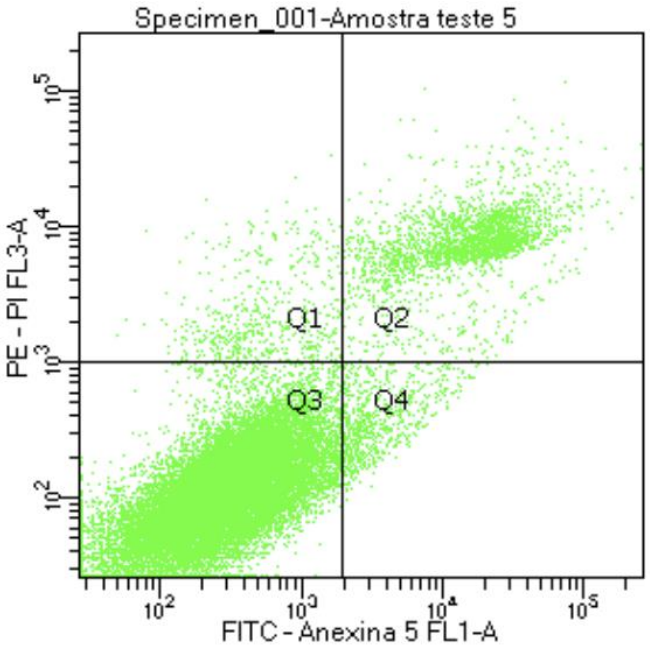

Fig. 5. Flow cytometry analysis of B16F10-Nex2 cells treated with PEPAD at 7.4 µM. The quadrants (Q) represent different cellular states: Q1 - necrotic cells [PI (+) / Annexin V (-)], Q2 - late apoptotic cells [PI (+) / Annexin V (+)], Q3 - healthy cells [PI (-) / Annexin V (-)], and Q4 - apoptotic cells [PI (-) / Annexin V (+)]. Most cells were found in Q2 (late apoptosis) and Q3 (healthy).

3.7. Release of DAMPs (Calreticulin and HMGB1)

Evaluation of the release of DAMPs, Calreticulin and HMGB1 induced by the PEPAD peptide revealed that after 30 minutes of treatment, a significant release of Calreticulin molecules (10.4 mol.mL^{-1}) and HMGB1 (224 mol.mL^{-1}) was observed (Figure 6A and 6B, respectively). The purpose of this assay was to verify if PEPAD is capable of inducing DAMPs release in murine melanoma B16F10-Nex2 cells, because these molecules are commonly externalized from the membrane during immunogenic cell death.

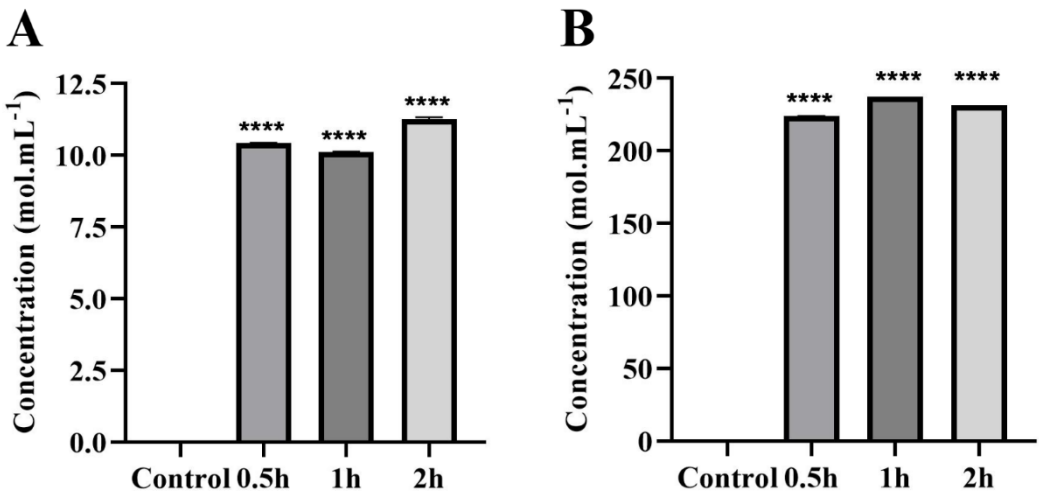

Fig. 6. Extracellular concentrations of calreticulin (A) and HMGB1 (B) in murine melanoma cells (B16F10-Nex2) treated with PEPAD at 7.4 µM. The Tukey test determined the statistical differences, with $p < 0.0001$.

4. Discussion

The limited efficacy of anticancer drugs and the development of drug resistance by cancer cells have made cancer a serious global public health problem. The need to generate new drugs capable of overcoming resistance and being more selective [18] and having fewer toxic and side effects [25] has led peptides to enter the pharmaceutical industry as a promising new class of antineoplastics, known as anticancer peptides (ACP) [18]. So, in this study we sought to evaluate the anticancer potential of a synthetic peptide that has already shown positive results against pathogenic bacteria, called PEPAD [19]. It was designed to present specific characteristics, with the aim of ensuring efficient biological activity. One of the main characteristics is its electrical charge (+7), which grants it the capacity to perform initial electrostatic attraction with the anionic membrane of the microorganism [26].

PEPAD has a hydrophobicity of 38%, giving it the ability to interact effectively with lipid membranes. This hydrophobicity requires a balance of up to 50% hydrophobic amino acids, since too high hydrophobicity can cause it to lose selectivity and interact with any membrane, while low hydrophobicity compromises the ability of the peptide to interact with the membrane [27]. Another crucial characteristic of the activity of PEPAD is its amphipathicity and α-helix structure, where hydrophobic residues cluster in the core of the helix, while hydrophilic residues orient outward, interacting with the surrounding aqueous solvent. Thus, the amphipathicity of the peptide allows the hydrophobic part to insert into the lipid bilayer of the membrane, while the hydrophilic part interacts with the hydrophilic components of the membrane. This results in membrane destabilization, causing intracellular material leakage and, consequently, cell death [27,28].

PEPAD has been characterized as an antimicrobial peptide because of its effectiveness in eliminating a wide range of microorganisms. It was effective in eliminating 13 Gram-positive and Gram-negative bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA). PEPAD acts rapidly against these microorganisms, and its main mechanism of action involves binding to the plasma membrane. It also showed synergy when combined with the antibiotic ciprofloxacin. Furthermore, no toxicity was observed when PEPAD was administered to *Galleria mellonella* larvae [19]. Similar to how antimicrobial peptides (AMPs) interact with the phospholipids of microbial membranes, anticancer peptides (ACPs) interact with cancer cells through the presence of phosphatidylserine, which is an anionic phospholipid. Phosphatidylserine is externalized to the plasma membrane when the cell becomes cancerous, allowing the initial electrostatic attraction with the cationic peptide. This feature facilitates the interaction of peptide and cancer cells while ensuring selectivity by preserving healthy cells with zwitterionic membranes [29].

This potential was confirmed through cell viability assays, where PEPAD exhibited high cytotoxicity against cells B16F10-Nex2, Sk-mell-28, MCF-7 and HeLa. In contrast, it did not show significant cytotoxicity against healthy murine macrophage (RAW 264.7) and human fibroblast (FN1) cells, demonstrating a high selectivity index, particularly for B16F10-Nex2 (Table 1). The selectivity feature is a crucial condition for the development of future anticancer drug candidates. And the selective cytotoxic capacity against cancer cells presented by PEPAD is a significant property that characterizes it as a promising agent in the prospecting of new therapeutic alternatives [30]. Other ACPs with activity against B16F10 have been reported in the literature, such

as Brevinin-1RL1 derived from the skin secretion of the frog *Rana limnocharis* with an IC_{50} of 6.65 µM [31], LVTX-9 derived from the spider *Lycosa vittata venom gland* with an IC_{50} of 59.2 µM [32], and Figainin 1 derived from the skin secretion of the frog *Boana raniceps* that showed an IC_{50} of 10.7 µM [33]. In addition to these, we have Gomesin derived from the hemocytes of the spider *Acanthoscurria gomesiana*, which showed an IC_{50} of 3.58 µM for murine melanoma cells and 5.3 µM for healthy HUVEC cells, with this concentration very close compared to the amount needed to kill melanoma cells [34].

Treatment with anticancer drugs can cause various cellular alteration, including changes in cell morphology. These changes vary depending on the treatment and its mechanism of action. Common alterations include cell shrinkage, apoptotic body formation, vacuolated cytoplasm, cytoplasmic swelling, and changes in the cell membrane [35–37]. In some cases, nuclear changes also occur, as observed in Figure 1. Notably, PEPAD treatment caused chromatin condensation in B16F10-Nex2 cells. Since cancer cells can evade the immune system of the body, leading to suppression and prevention of apoptosis [38], it is possible to suggest that PEPAD is capable of inducing apoptosis in melanoma cells.

Cellular morphology is closely linked to metastatic processes, making its evaluation critical for understanding the anticancer potential of a treatment [39]. Tumor metastasis requires several cellular mechanisms. Initially, epithelial cells undergo a transition to mesenchymal-like characteristics.. These mesenchymal cells, which are elongated and mobile, lose adhesion to neighboring cells and the extracellular matrix, enabling them to invade tissues and spread through the bloodstream Chemotaxis plays a role in this movement, with cells either attracted by or repelled from surrounding tissues. Cell migration is the final and crucial step in metastasis. [40]. Cell migration is the final and crucial step in metastasis. Consequentlt, therapeutic strategies aim to inhibit migration to limit cancer dissemination. In this study, we evaluated the ability of PEPAD to inhibit cell migration. As shown in Figure 2A, PEPAD significantly delayed scratch closure compared to the control group (Figure 2B).

Other qualitative assays reported here supportthe hypothesis thatPEPAD induces apoptosis. Figure 3 shows progressive mitochondrial changes, including fragmentation, swelling, and loss of membrane potential, along with initially preserved nuclear integrity. These changes, followed by signs of cell death over time, align with characteristics of apoptotic cell death [23,41]. The detection of active caspases, illustredin Figure 4, further reinforces this finding, caspases are proteases responsible for cleavage specific proteins, Leading to the controlled disintegration of the cancer cell [41].

To further assess cell viability and the apoptose state, flow cytometry was performed(Figure 5). The graph shows a significant distribution of cells in quadrant Q4, indicating that a substantial number of cells are in early apoptosis. This suggests that the treatment effectivelyinduce apoptotic without immediately compromising membrane integrity. Additionally, the presence of a population in Q2 indicates that some cells have progressed to late apoptosis, confirming the continuous apoptotic stimulus [42].

Chemotherapeutic agents can induce cell death via necrosis or apoptosis, though apoptose is oftem the preferred mechanism. In apoptosis, cells are fragmented into apoptotic bodies, which are encapsulated by a plasma membrane and marked with externalized phosphatidylserine, signaling phagocytes for their removal. This process prevents the release of cellular contents and avoids triggering an inflammatory response.

In contrast, necrosis is a more chaotic form of cell death, releasing cellular material into the environment and potentially causing inflammation [43,44].

Another promising approach to combat cancer cells is the activation of immunogenic cell death (ICD). For ICD to occur, the chemotherapeutic agent must stimulate the release of DAMPs through a series of events [45]. Firsty, the agent induces the externalization of DAMPs on the cancer cell membrane. These signals are then recognized by antigen-presenting cells (APCs), wich phagocytose the dying cells. Following this, the APCs activate T cells, leading to immune response that targets and destroys cancer cells [12,46].

PEPAD demonstrated the ability to induce the release oflarge amounts of DAMPs in melanoma cells, suggesting its potential immunotherapeutic efficacy through ICD activation. Specifically, treatment with PEPAD led to the rapid release of Calreticulin and HMGB1- key DAMP molecules - within the first 30 minutes of treatment, a response that persisted throughout the observation period. This process not only leads to cancer cell death but also suggests the possibility of developing therapeutic cancer vaccines to prevent recurrences by priming the immune system to recognize and attack cancer cells in the future. Other chemotherapeutic agents, such as oxaliplatin, ciproflavin, and doxorubicin, are also known to stimulate ICD. However, these drugs are associated with significant toxicity, which limits their therapeutic window. In contrast, PEPAD's ability to induce ICD with potentially lower toxicity highlights its promise as an immunotherapeutic agent.

5. Conclusions

In this study, we demonstrated that PEPAD has significant antiproliferative effects, reducing the viability of murine melanoma cells (B16F10-Nex2) without affecting healthy cells, such as murine macrophages RAW 264.7 and human fibroblasts FN1. In addition to its antiproliferative properties, PEPAD inhibited cancer cell migration and induced nuclear in malignant cells, such as condensation of chromatin, which is indicative of apoptosis. Moreover, PEPAD showed antimetastatic potential and promoted the release of DAMPs in melanoma cells, suggesting its immunogenic cell death-inducing capability. These findings highlight PEPAD as a promising anticancer agent with both efficiency and selectivity. However, further studies are needed to investigate the molecular mechanisms underlying its antimetastatic effects and to evaluate its therapeutic potential in vivo, with the goal of developing a new strategy for cancer treatment.

Declaration of interest

The authors did not report any potential conflicts of interest.

Authors' contributions

Camila de Oliveira Gutierrez: conceptualization, formal analysis, investigation, methodology, project administration, visualization, writing – original draft. **Luís Henrique de Oliveira Almeida:** conceptualization, investigation, and methodology. **Claudiane Vilharroel Almeida:** investigation, methodology. **Caio Fernando Ramalho**

de Oliveira: conceptualization, formal analysis, investigation, methodology, resources. **Ana Cristina Jacobowski:** investigation. **Rodrigo Juliano Oliveira:** formal analysis, investigation. **Thais de Andrade Farias Rodrigues:** formal analysis, investigation. **Tamaeh Monteiro Alfredo:** conceptualization, formal analysis, investigation, methodology, validation, writing – review & editing. **Ana Paula de Araújo Boleti:** conceptualization, formal analysis, investigation, validation, writing – review & editing. **Maria Lígia Rodrigues Macedo:** acquisition, acquisition of funding resources, supervision.

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3. Conclusão

Por meio dos resultados aqui relatados, PEPAD apresentou uma rápida e potente atividade contra espécies de *Candida* em concentrações micromolares. Apresentou efeito sinérgico com fluconazol e anfotericina B, reduzindo a CIM dos dois compostos. Também foi possível determinar que um dos prováveis mecanismos de ação seja por meio de danos a membrana pela ligação com o ergosterol. PEPAD foi capaz de inibir parte do biofilme, porém não foi capaz de erradicar o biofilme maduro. O peptídeo apresentou alta seletividade contra células de melanoma murino e melanoma humano, não afetando significativamente as células saudáveis testadas. Também foi possível constatar que o peptídeo retarda a migração celular de células cancerígenas, e induz a liberação de DAMPS característicos de morte celular imunogênica. Além disso, foi constatado que o tipo de morte causado por PEPAD em células cancerígenas seja por apoptose, indicada pela condensação das cromatinas, detecção de caspase ativa, avaliação do potencial mitocondrial e confirmada pela citometria de fluxo.

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