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ATIVIDADES BIOLÓGICAS *IN VITRO* DO EXTRATO PIGMENTADO DE *Talaromyces marneffei* ISOLADO DE AMBIENTE MARINHO

IGOR VINÍCIUS PIMENTEL RODRIGUES

São Luís-MA
2019

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Saúde do Adulto e da Criança da Universidade Federal do Maranhão para obtenção do Título de Mestre em Saúde do Adulto.

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“Dedico este trabalho a Deus, a meu pai, minha mãe, minha irmãe aos meus amigos.”

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“Superar o fácil não tem mérito, é obrigação; vencer o difícil é glorificante; ultrapassar o que até então era impossível é esplendoroso.”

(Desconhecido)

RESUMO

Introdução: *Talaromyces marneffei* é um fungo patógeno emergente responsável por causar micose fatal principalmente em indivíduos imunocomprometidos. Os fungos pertencentes ao gênero *Talaromyces* possuem ascomas distintos, geralmente amarelos, brancos, creme, rosados ou avermelhados, nos quais encontram-se ascósporos, que produzem pigmentos variados, dentre eles a pigmentação vermelha utilizada neste trabalho. Estes microrganismos vêm ganhando cada vez mais destaque na indústria farmacêutica devido a sua capacidade de fornecer antibióticos, imunosupressivos, enzimas e drogas anti-câncer. **Objetivo**: Avaliar as atividades antifúngica e antibacteriana *in vitro* do extrato pigmentado vermelho intracelular (IRPE) produzido pelo ascomiceto *Talaromyces marneffei* em *Candida albicans* ATCC 10231, *C. tropicalis* ATCC 1369, *Escherichia coli* ATCC 25922 e *Staphylococcus aureus* ATCC 25923, e atividade citotóxica *in vitro* contra linhagens celulares tumorais (MCF-7) e normais (GM07492). **Metodologia**: Foram realizadas as técnicas de microdiluição em caldo para análise da atividade antimicrobiana. Para análise da atividade citotóxica utilizou-se o ensaio do MTT [Brometo de 3- (4,5-dimetiltiazol-2-il) -2,5-difeniltetrazólio]. **Resultados**: A concentração bactericida mínima (MBC) do IRPE de *T. marneffei* para *E. coli* ATCC 25922 e S. *aureus* ATCC 25923 foi de 1000 µg/mL. As concentrações inibitórias mínimas (MIC) para *E. coli* foi de 250 e para *S. aureus* 500 µg/mL, enquanto para *C. tropicalis* ATCC 1369 foi de 62.5 µg / mL. A metade da concentração inibitória máxima (IC50) para linhagem de células de câncer de mama (MCF-7) foi 45.43 ±1,657 µg/mL, entretanto, não foi observada citotoxicidade na linhagem celular normal testada (GM07492). Portanto, IRPE de *T. marneffei* apresentou atividades antimicrobianas e citotóxica *in vitro* intermediárias para os microrganismos e linhagem celular tumoral testadas, além de não ter apresentado citotoxicidade para a linhagem celular normal testada. Sugere-se a realização do isolamento das substâncias bioativas presentes no extrato a fim de obter-se atividades biológicas mais promissoras.

Palavras-chave: Extrato pigmentado; *Talaromyces marneffei*; Antibacteriano; Citotoxicidade.

ABSTRACT

Introduction: *Talaromyces marneffei* is an emerging pathogenic fungus responsible for causing fatal mycosis mainly in immunocompromised individuals. The fungi belonging to the genus *Talaromyces* have different ascomas, usually yellow, white, cream, rosy or reddish, in which ascospores are found, which produce varied pigments, among them the red pigmentation used in this work. These microorganisms have been gaining increasing prominence in the pharmaceutical industry because of their ability to provide antibiotics, immunosuppressants, enzymes and anti-cancer drugs. **Aims:** To evaluate the *in vitro* antifungal and antibacterial activities of the intracellular red pigmented extract (IRPE) produced by the ascomycetes *Talaromyces marneffei* in *Candida albicans* ATCC 10231, *C. tropicalis* ATCC 1369, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, and cytotoxic activity *in vitro* against strains of tumor cells (MCF-7) and normal cells (GM07492). **Methodology:** Broth microdilution techniques were used to analyze the antimicrobial activity. For analysis of the cytotoxic activity the MTT assay was used [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide]. **Results:** Minimum bactericidal concentration (MBC) of *T. marneffei*'s IRPE for *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 was 1000 μg/ml. The minimum inhibitory concentrations (MIC) for *E. coli* were 250 and for *S. aureus* 500 μg / mL, whereas for *C. tropicalis* ATCC 1369 it was 62.5 μg/mL. Half of the maximal inhibitory concentration (IC50) for breast cancer cell line (MCF-7) was 45.43 ± 1.657 μg / mL, however, no cytotoxicity was observed in the normal cell line tested (GM07492). Therefore, IRPE from *T. marneffei* showed intermediary antimicrobial and cytotoxic activities *in vitro* for the microorganisms and tumor cell line tested, and did not present cytotoxicity to the normal cells tested. It is suggested to carry out the isolation of the bioactive substances present in the extract in order to obtain more promising biological activities.

Key words: Pigmented extract, *Talaromyces marneffei*, antibacterial, cytotoxicity.

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LISTA DE SIGLAS E ABREVIATURAS

ATCC	American Type Culture Collection / Coleção de cultura americana
DMEM	Dulbecco's Modified Eagle Medium / Meio de Eagle modificado da Dulbecco
DMSO	Dimethyl sulfoxide / Dimetilsulfóxido
GM07492	Human fibroblasts cell line / Linhagem de células humanas de fibroblastos
HIV	Human Immunodefficiency Virus / Vírus da Imunodeficiencia adquirida
IC50	Half maximal inhibitory concentration / Metade da concentração inibitória máxima
IRPE	Intracelullar red pigment extract of *T. marneffei* / Extrato pigmentado vermelho intracellular de *T. marneffei*
ITS	Internal transcribed spacer / Espaçador interno transcrito
MCF-7	Human breast cancer cell line / Linhagem cellular de câncer de mama
MRSA	Methicillin-resistent *Staphylococcus aureus* / *Staphylococcus aureus*r esistente à meticilina
PRSA	Penicillin-resistent *Staphylococcus aureus* / *Staphylococcus aureus* resistente à penicilina
RPB1	DNA-directed RNA polymerase II subunit rpb1 / Subunidade da RNA polimerase II dirigida por DNA RPB1

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1 INTRODUÇÃO

O interesse pela utilização de corantes naturais vem aumentando devido à crescente preocupação do consumidor com uma alimentação saudável e balanceada, com a própria saúde física e com a questão ambiental (JOSHI et al., 2003). Os pigmentos ou corantes são compostos químicos que conferem cor aos materiais aos quais se fixam e, por isso, são amplamente utilizados nas indústrias têxteis, alimentícias, cosméticas e farmacêuticas. Eles podem ser de origem natural ou sintética, sendo a utilização dos pigmentos sintéticos desvantajosa, pois estes podem causar efeitos tóxicos à saúde e ao ambiente por possuírem potencial mutagênico, carcinogênico e alergênico (COSTA SOUZA, 2015).

Pigmentos naturais são extraídos principalmente de plantas e microrganismos. Entretanto, o uso de pigmentos derivados de plantas apresenta algumas desvantagens como a indisponibilidade ao longo do ano e a insolubilidade. Além disso, o uso indiscriminado de plantas pode levar à perda de espécies valiosas. Por esses motivos, o processo pode ser considerado inviável (DOWNHAM; COLLINS, 2000). Já os pigmentos fúngicos apresentam várias vantagens em relação aos vegetais: rápido e fácil crescimento em meios de baixo custo, processamento fácil, e a independência do crescimento microbiano em relação às condições ambientais (MANIKPRABHU; LINGAPPA, 2013).

Os metabólitos secundários, dentre eles os pigmentos naturais, são compostos que se formam no começo ou no fim da fase estacionária de crescimento dos microrganismos e não estão diretamente relacionados com o crescimento, desenvolvimento e reprodução dos mesmos. Estão, portanto, relacionados à resistência desses organismos a vários fatores ambientais adversos, como a dissecação, exposição à temperaturas extremas, irradiação e foto-oxidação, ou em interações ecológicas com outros organismos, como esponjas, corais ou outras comunidades microbianas (MARGALITH, 1993). Por essa razão, esses produtos são amplamente utilizados na área da saúde por serem agentes antimicrobianos, antiparasitários, antitumorais, inibidores de enzima e imunosupressivos (DEMAIN, 1999; DEMAIN; FANG, 2000; IMHOFF, 2016).

Os antimicrobianos são substâncias naturais (antibióticos) ou sintéticas (quimioterápicos) que atuam sobre os microrganismos, inibindo o seu crescimento ou causando a sua destruição (SÁEZ-LLORENS et al., 2000). Sabe-se que eles são os fármacos mais comumente utilizados na prática clínica, e que, dentre eles, os antibióticos são os mais usados e prescritos, tanto para uso do hospital quanto para a automedicação (WALSH, 2013).

Recentemente têm-se observado um aumento na resistência dos patógenos fúngicos e bacterianos a esses compostos. Diante disso, há uma demanda crescente por novas substâncias com essas propriedades, o que tem levado cientistas a investigar as atividades antimicrobianas de um número crescente de fungos provenientes de diferentes ambientes, como os fungos endofíticos e os fungos marinhos (XU et al., 2015).

Nesse sentido, os fungos filamentosos, mais especificamente os ascomicetos, são capazes de sintetizar e secretar diversos pigmentos como metabólitos secundários. Além de esses compostos poderem ser utilizados como corantes de alimentos, tecidos e cosméticos, os pigmentos fúngicos também possuem atividades biológicas importantes, como atividade antibacteriana, antifúngica, fitotóxica, antitumoral, antioxidante, entre outras (GEWEELY, 2011).

Apesar dos avanços da medicina, o câncer continua sendo uma doença grave e uma das principais causas de morte ao redor do mundo e atualmente não há nenhuma droga eficaz no controle do câncer. Um dos maiores desafios na pesquisa por compostos que apresentem atividade antitumoral é o desenvolvimento de drogas com especificidade para as células cancerígenas e com pouco ou nenhum efeito nas células saudáveis de mamíferos (MARTINS et al., 2014).

A literatura relata que espécies pertencentes aos gêneros *Penicillium* e *Talaromyces* têm sido alvo de investigações na descoberta de compostos bioativos, o que levou a descoberta de novas drogas, como a penicilina (DEMAIN; ELANDER, 1999) e a compactina, que é um agente anticolesterolêmico (CHAKRAVARTI; SAHAI, 2004), drogas antitumorais diversas (NICOLETTI et al., 2008) e micotoxinas que contaminam alimentos (FRISVAD et al., 2004).

A abundante diversidade de espécies e de metabólitos secundários, a utilização de técnicas de melhoramento genético em cepas que produzem micotoxinas e de processos fermentativos relativamente baratos faz dos fungos filamentosos uma promissora fonte de obtenção de produtos naturais bioativos (XU et al., 2015).

Diante disso, este estudo teve como objetivo avaliar as atividades antifúngicas, antibacterianas e antitumoral *in vitro* de um pigmento produzido pelo ascomiceto *Talaromyces marneffei*, espécie isolada do solo do Parque Estadual da Laguna da Jansen, conservada na "Coleção de Fungos da UFMA, Núcleo de Imunologia Básica e Aplicada – NIBA/ UFMA", gentilmente cedida para esta pesquisa, uma vez que se faz necessária caracterizar por biologia molecular os fungos armazenados, para estudar possíveis novos fármacos com utilização na prática clínica em pacientes oncológicos, bem como pacintes acometidos por bactérias e fungos patogênicos.

2 REFERENCIAL TEÓRICO

2.1 Fungos

O Reino Fungi exibe uma enorme diversidade ecológica, fisiológica e morfológica, o que torna difícil uma generalização das características de seus membros. São reconhecidos três grupos distintos nesse reino: fungos filamentosos, leveduras e cogumelos, sendo os dois primeiros microscópicos e o último macroscópico. Para se ter uma ideia da diversidade encontrada nesse reino, no último levantamento realizado estimou-se que existem cerca de 1,5 milhões de espécies fúngicas, sendo que destas foram descritas apenas cerca de 74.000, fazendo dos fungos o segundo grupo de seres vivos mais numerosos, perdendo apenas para os insetos (ESPOSITO; AZEVEDO, 2004; ARCHER; CONNERTON; MACKENZIE, 2008).

Os fungos são organismos eucariotos, isto é, possuem um núcleo cercado por uma membrana que contem vários cromossomos. Possuem também várias organelas citoplasmáticas também cercadas por uma membrana, como: mitocôndria, vacúolos, etc. Outras características compartilhadas pelos demais eucariotos são: fluxo citoplasmático, DNA que contem regiões não-codificantes chamadas íntrons, membranas que contem esteróis, e ribossomos do tipo 80S em contraste com os ribossomos de 70S das bactérias (DEACON, 2006).

Esses organismos possuem parede celular constituída de quitina, com exceção de alguns fungos aquáticos; não realizam fotossíntese, sendo, portanto, heterótrofos, ou seja, precisam de compostos orgânicos pré-formados como fonte de energia e de esqueleto carbônicos para a síntese celular (DEACON, 2006). Reproduzem-se sexuada ou assexuadamente, por esporos. Podem ser uni ou multinucleados. Possuem vida independente e não formam tecidos verdadeiros. Toleram amplas faixas de temperatura, pressão e pH e são capazes de crescer em meios com baixo teor de nutrientes, utilizando-se de vários tipos de carbono para obtenção de energia (ALEXOPOULOS *et al*., 1996; MADIGAN *et al*., 1997).

Os fungos filamentosos crescem como filamentos chamados hifas, que se alongam nas suas pontas, exibindo um crescimento apical. Tais hifas se ramificam repetidamente por detrás de suas pontas, dando origem a uma cadeia denominada micélio. Entretanto, alguns fungos como as leveduras se reproduzem por brotamento, e algumas podem mudar de uma fase leveduriforme para uma fase hifal em resposta às condições ambientais, como *Candida*

albicans, por exemplo. Esses fungos denominados dimórficos geralmente são patógenos sérios de humanos. Geralmente crescem na forma de levedura para proliferação celular nos fluidos corporais, mas se convertem em hifas para a invasão dos tecidos (DEACON, 2006).

2.2 Os gêneros *Penicillium* e *Talaromyces*

O gênero *Penicillium* foi descrito por Link em 1809. É um gênero bastante numeroso e ubíquo, com 354 espécies aceitas (VISAGIE et al., 2014). Os membros desse gênero formam colônias de rápido crescimento, com tempo de maturação por volta do terceiro ou quarto dia. Macroscopicamente, as colônias apresentam inicialmente uma textura algodonosa baixa ou veludosa, de tom branco, logo adquirindo uma coloração amarelo-alaranjada, amarelo-esverdeada, verde, ou azul-esverdeada, sendo esses três últimos tons os mais frequentemente observados. O reverso pode variar do castanho-amarelado ao castanho-avermelhado (PITT; HOCKING, 1997; RAPER; THOMS, 1949; FRISVAD; SAMSON, 2004).

Microscopicamente, observa-se grande número de hifas hialinas septadas, com conidióforos simples ou ramificados, solitários ou agrupados, hialinos ou levemente pigmentados. As células conidiogênicas (fiálides) tem forma de jarro e os conídios são esféricos ou possuem uma forma de limão, podendo ser lisos ou rugosos (Figura 1). A conidiação é do tipo acrópeta, na qual os conídios são produzidos sequencialmente a partir da extremidade do conídio previamente formado (SIDRIM; ROCHA, 2004; KONEMAN *et al.*, 2008).

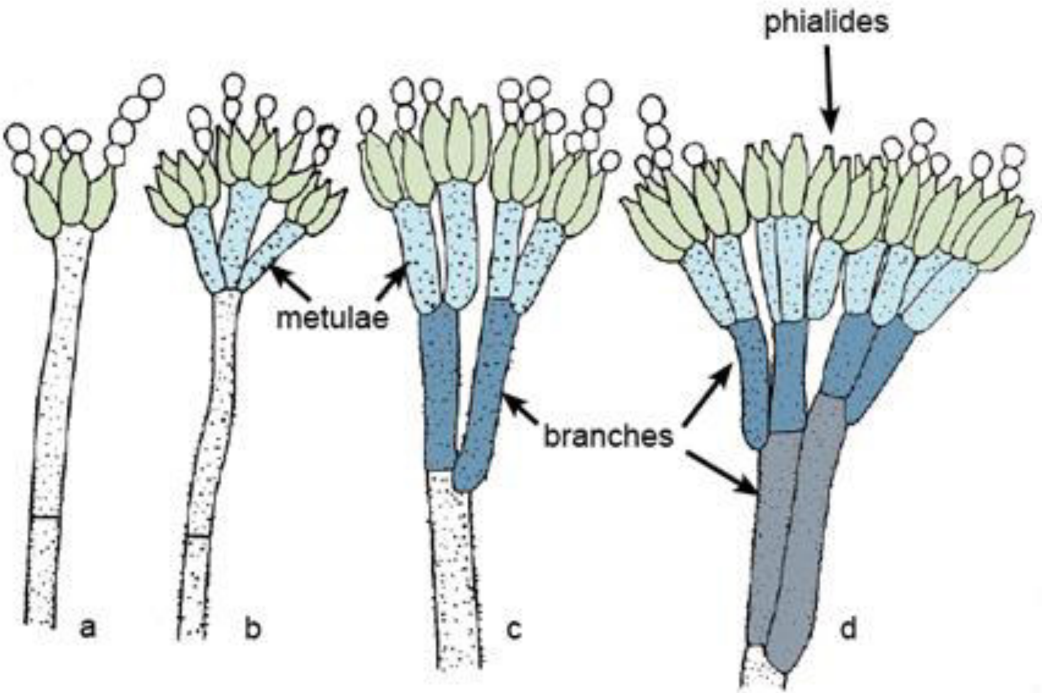

Figura 1. Estruturas microscópicas de frutificação de *Penicillium* spp. A: conidióforo não-ramificado, rugoso e monoverticilado. B: conidióforo biverticilado, rugoso e bisseriado. C: conidióforo terverticilado, rugoso e bisseriado. D: conidióforo quaterverticilado, rugoso e bisseriado. FONTE: VISAGIE et al. (2014).

2.2.1 O gênero *Talaromyces*

O gênero *Talaromyces* foi descrito pela primeira vez por Benjamin em 1955 como sendo o estado sexual de *Penicillium*. Os fungos pertencentes a esse gênero produzem um ascoma de parede mole coberto por hifas entrelaçadas. Estudos filogenéticos reveleram que o subgênero *Biverticillium* de *Penicillium* e *Talaromyces* formam um clado monofilético que é distinto dos outros subgêneros de *Penicillium*. Portanto, de acordo com a recente adoção do conceito "um fungo, um nome", *Penicillium* subgênero *Biverticillium* foi transferido para *Talaromyces* com base no sequenciamento dos locos ITS e RPB1 (SAMSON et al., 2011).

Benjamin (1955) apresentou o gênero *Talaromyces* com a espécie teleomórfica de *Penicillium*, *T. vermiculatus*, como um tipo genérico. O gênero se caracteriza por possuir ascocarpos moles com uma parede cleistotecial formada por hifas entrelaçadas e um ascoma tipicamente amarelado, com ascos ovais a globosos, contendo ascósporos espinhosos, na sua maioria.

Os fungos pertencentes ao gênero *Talaromyces* possuem ascomas distintos, geralmente amarelos, brancos, creme, rosadas ou avermelhadas, nos quais encontram-se ascósporos, que produzem pigmentos variados (STOLK; SAMSON, 1972).

Talaromyces contêm espécies de importância médica, como por exemplo *Talaromyces marneffei*, que é um fungo patógeno emergente responsável por causar uma micose fatal principalmente em indivíduos imunocomprometidos provenientes de países do Leste Asiático como China, Taiwan, Tailândia e Vietnã (CHIANG et al., 1998; DENG et al., 1988; HIEN et al., 2001; SUPPARATPINYO et al., 1994), apesar de a literatura relatar infecções em indivíduos HIV negativos (KWAN et al., 1997; SAADIAH; JEFFREY; MOHAMED, 1999).

2.2.2 *Talaromyces marneffei*: história e classificação

A espécie *Talaromyces marneffei* foi isolada pela primeira vez em 1956 a partir de lesões hepáticas de ratos de bambu (*Rhizomys sinensis*) utilizados em infecções experimentais no Instituto da Indochina, Dalat, Vietnã do Sul (CAPPONI; SEGRETAIN; SUREAU, 1956). O fungo foi inicialmente nomeado *Penicillium marneffei* em homenagem a Hubert Marneffe, diretor do Instituto Pasteur da Indochina (SEGRETAIN, 1959a), porém, atualmente todas as espécies aceitas de *Penicillium* subgênero *Biverticilium*, incluindo *Penicillium marneffei*, foram transferidas ao gênero *Talaromyces* com base em uma análise do gene *RPB1* (RNA polymerase II largest subunit, ou subunidade maior da RNA polimerase II) e no sequenciamento da região ITS (incluindo o 5.8S nrDNA) (SAMSON et al., 2011).

A talaromicose marneffei humana foi descrita pela primeira vez como uma infecção laboratorial adquirida quando o pesquisador G. Segretain acidentalmente picou seu próprio dedo com uma agulha infectada com conídios de *P. marneffei* que estava sendo utilizada para inocular hamsters (SEGRETAIN, 1959b). Desenvolveu-se no local da inoculação um pequeno nódulo seguido de uma linfadenopatia axilar. Essa infecção acidental foi curada após intenso tratamento com nistatina oral por 30 dias (CHAN et al., 2016).

Atualmente esta espécie está classificada no: Reino Fungi; Filo: Ascomycota; Classe: Eurotiomycetes; Ordem: Eurotiales; Família: Trichocomaceae; Gênero: *Talaromyces*; Espécie: T. *marneffei* (SEGRETAIN, 1959a). De acordo com Raper e Thoms (1949), está classificado ainda na seção Asymmetrica, subseção Divaricata, que é equivalente ao

subgênero *Biverticillium.* Essa classificação foi confirmada por Frisvad e Filtenborg (1990) baseado na similaridade fisiológica e no perfil de metabólitos secundários.

Esta é a única espécie fúngica dimórfica entre os outros membros do gênero e é um fungo patogênico que pode causar uma micose sistêmica talaromicose fatal em pacientes imunocomprometidos infectados com o vírus da imunodeficiência humana (HIV), sendo essa infecção micótica sistemática a mais prevalente em pacientes infectados com o vírus da imunodeficiência humana (HIV) .Essa doença é endêmica na Ásia tropical, especialmente na Tailândia, nordeste da Índia, China, Hong Kong, Vietnã e Taiwan (VANITTANAKOM et al., 2006) e é, depois da tuberculose e da criptococose, a terceira causa mais comum de infecção em pacientes com AIDS no norte da Tailândia (SUPPARATPINYO et al., 1994).

Outros indivíduos imunocomprometidos, mas acometidos por outras doenças, também são suscetíveis à talaromicose, sendo relatada em pacientes com autoanticorpos contra interferon- γ (LEE et al., 2014). Foi descrito também casos de infecções por *T. marneffei* em pacientes hematológicos recebendo terapias direcionadas (CHAN et al., 2015).

A característica diagnóstica de *T. marneffei* em relação aos outros fungos do gênero é o seu dimorfismo termal, que permite que essa espécie cresça na sua forma micelial a 25ºC e na forma leveduriforme a 37ºC (SEGRETAIN, 1959a). Essa capacidade é o principal fator de virulência desse microrganismo. Sua forma micelial pode ser visualizada na figura abaixo (Figura 2).

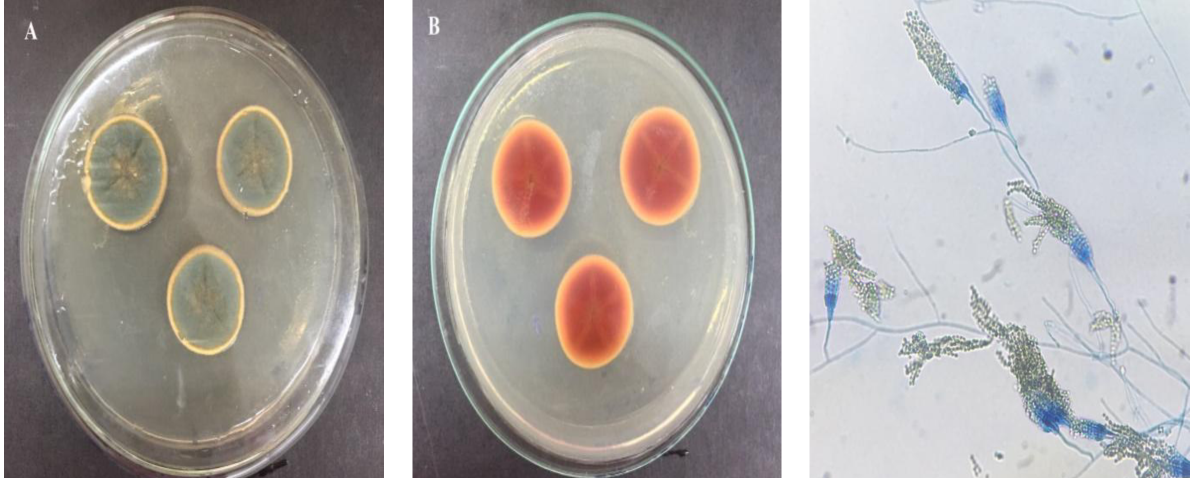

Figura 2. Macroscopia e microscopia de *Talaromyces marneffei*. 2A: Anverso de colônias de *Talaromyces marneffei* isoladas em Ágar Sabouraud-Dextrose. 2B: Reverso de colônias de *T. marneffei* isoladas em Ágar Sabouraud-Dextrose. 2C: Microscopia de *T. marneffei*. (Fonte do autor).

2.3 Identificação fúngica

A identificação taxonômica de fungos filamentosos baseia-se principalmente nas características morfológicas macro e microscópicas. Entretanto, esse método exige tempo e experiência do profissional qualificado para que seja feita uma boa identificação. Atualmente há métodos mais específicos e acurados como as técnicas de biologia molecular que utiliza sequências de DNA ribossomal (rDNA); espectrometria de massas do tipo MALDI-TOF ICMS *Assisted Laser Description/Ionization Time-of-Flight Intact Cell Mass Spectrometry*); SDS-PAGE (Eletroforese em geral de poliacrilamida) para determinar perfis protéicos imunológicos; e espectroscopia no infravermelho por transformada de Fourier (FT-IR) (BUGNI; IRELAND, 2004; HORISAWA; SAKUMA; DOI, 2009; SANTOS et al., 2010; SANTOS et al., 2010a).

3 MICRORGANISMOS-TESTE UTILIZADOS NESSE ESTUDO

3.1 Bactérias

As bactérias são microrganismos relativamente simples e possuem uma única célula (unicelulares). Seu material genético não é envolto por uma membrana nuclear, por isso são chamados de procariotos, que significa pré-núcleo. Apresentam várias formas, entre elas pode-se citar: *bacilos,* isto é, se encontram em forma de bastão; *cocos,* ou seja, são esféricas ou ovoides*; e os espirilos*, possuindo formato saca-rolha ou curvado. Podem formar ainda pares, cadeias, grupos ou outros agrupamentos. Essas características são geralmente de um gênero específico ou até mesmo de uma espécie (TORTORA; FUNKE; CASE, 2017).

O principal componente estrutural da parede celular bacteriana é o peptidioglicano, que é composto por proteínas e carboidratos polimerizados e reticulados, criando uma rede contínua que envolve a célula (ROMANIUK; CEGELSKI, 2015). Nas bactérias gram-positivas os ácidos teicóicos e as proteínas da parede são covalentemente ligados ao peptidoglicano para gerar uma parede celular que protege a célula da pressão do turgor e do estresse externo, e auxilia na adesão às células do hospedeiro durante a infecção (BROWN; SANTA MARIA; WALKER, 2013; NAVARRE; SCHNEEWIND, 1999).

Já o envelope das bactérias Gram-negativas é constituído por uma bicamada lipídica secundária e assimétrica que é composta por glicerofosfolipídios no lado proximal à célula e de lipopolissacarídeos no lado externo da célula. As proteínas são ligadas à membrana citoplasmática, parede celular e membrana externa, de forma que o espaço periplasmático contem proteínas e outras macromoléculas envolvidas na síntese do envelope, além da homeostase, absorção de nutrientes, remoção de resíduos, motilidade, secreção e ligação a superfícies (GUEST; RAIVIO, 2016).

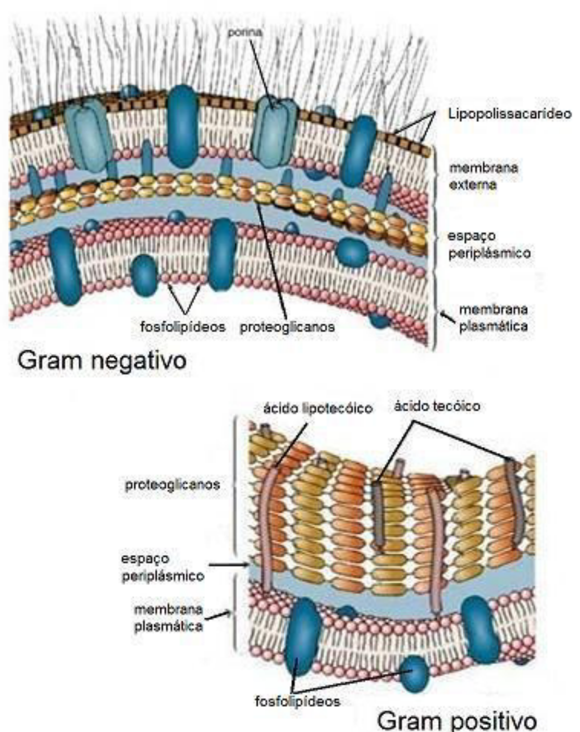

Figura 3. Composição química das paredes celulares de bactérias Gram-negativas (acima) e bactérias Gram-positivas (abaixo). FONTE: Cardoso, et al., (2011).

3.1.1 *Escherichia coli*

E. coli é a espécie-tipo (espécie que fixa o nome de um determinado gênero) pertencente ao gênero *Escherichia*, que é composto principalmente por bacilos Gram-negativos móveis inseridos dentro da família *Enterobacteriaceae.* É uma bactéria anaeróbia facultativa e se encontra predominantemente na microbiota humana. Esse microrganismo coloniza o trato gastrointestional da criança nas primeiras horas de vida e, a partir daí, *E. coli* e o hospedeiro mantêm uma relação mutuamente benéfica por décadas. Essas cepas de *E. coli* comensais raramente causam doença, exceto em hospedeiros imunocomprometidos ou naqueles onde as barreiras gastrintestinais normais são rompidas - como na peritonite, por exemplo (KAPER; NATARO; MOBLEY, 2004).

A possível transmissão de cepas de *E. coli* virulentas e/ou resistentes entre animais e humanos através de várias vias, como por exemplo, contato direto, contato com excrementos de animais ou por meio da cadeia alimentar é alvo de grande preocupação. Essa bactéria também possui vários genes de resistência que dificultam o tratamento em humanos e

animais, o que acaba se constituindo em um problema a nível mundial e precisa ser considerado como uma preocupação real para a saúde pública (POIREL et al., 2018).

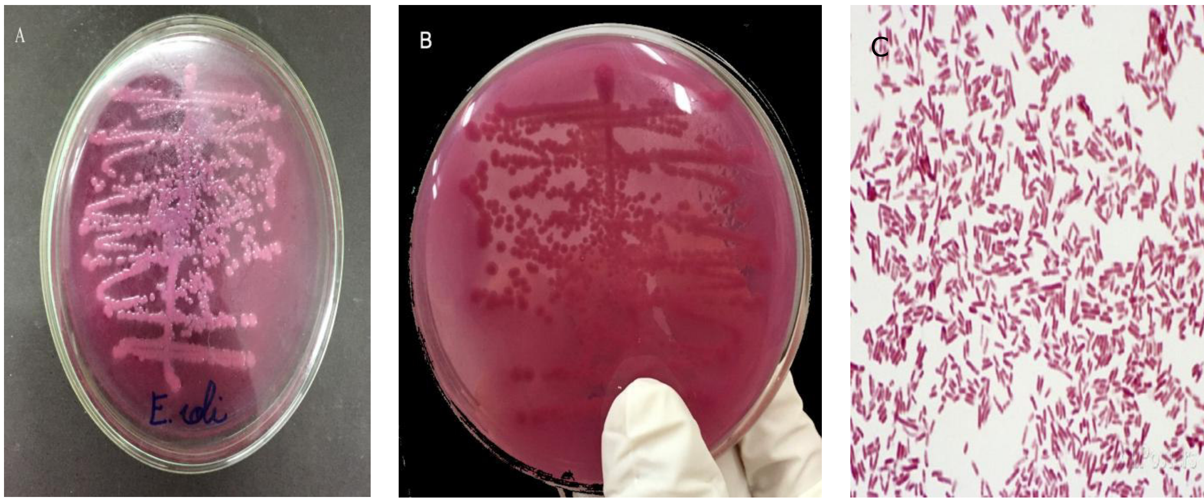

Figura 4. Macroscopia e microscopia de *E. coli*. 4A. Anverso de colônias de *E. coli* em Ágar MacConkey. 4B. Reverso de colônias de *E. coli* em Ágar MacConkey. Fonte do autor. 4C. Microscopia de *E. coli.* FONTE: Cangussu (2016).

3.1.2 Staphylococcus aureus

Staphylococcus aureus é uma das principais causas de infecções nosocomiais e adquiridas na comunidade e consiste em um grande problema para os sistemas de saúde de todo o mundo. *S. aureus* é uma bactéria Gram-positiva que coloniza persistentemente as narinas de aproximadamente20-25% da população de adultos saudáveis, enquanto que em torno de 60% são colonizados intermitentemente (ELLIS et al., 2014).

Em meados do século 20, antibióticos como a penicilina e a meticilina eram eficazes contra *S. aureu*s. Entretanto, essa bactéria logo adquiriu resistência a esses antibióticos, tornando as infecções causadas por *S. aureus* resistente a penicilina (PRSA) e *S. aureus* resistente a meticilina (MRSA) difíceis de serem tratadas. Mesmo com progressos recentes no combate a essa infecção, MRSA continua sendo uma ameaça a saúde a nível global (MCGUINNESS; MALACHOWA; DELEO, 2017).

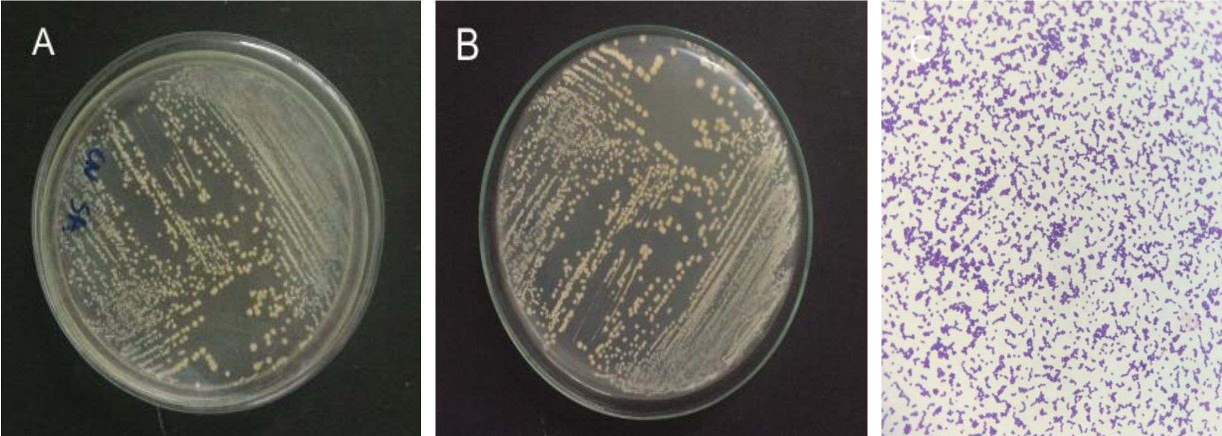

Figura 5. Macroscopia e microscopia de *S. aureus.* 5A. Anverso de colônias de *S. aureus* em Ágar Mueller-Hinton. 5B. Reverso de colônias de *S. aureus* em Ágar Mueller-Hinton. 5C. Microscopia de *S. aureus*. Fonte do autor.

3.2 Leveduras

3.2.1 *Candida albicans*

C. albicans tem se tornado um problema de saúde pública durante as últimas duas décadas. Essa espécie pode causar um amplo espectro de doenças, que varia desde infecções vaginais, que acomete até 75% das mulheres pelo menos uma vez em suas vidas, até graves infecções em pacientes hospitalizados, que podem levar à altas taxas de morbidade e mortalidade (POULAIN, 2015).

Essa levedura normalmente coloniza a mucosa digestiva e vaginal do ser humano, sendo que o benefício dessa colonização ainda é desconhecido. Quando a mãe é colonizada, a transmissão desse microrganismo se dá no momento do nascimento, logo ocorrendo uma estimulação no sistema imune do bebê (WILLINGER et al., 1994). Entretanto, como já supracitado, a colonização por *Candida* também tem seu lado negativo, sendo que a candidíase orofaringeal ocorre em 90% dos pacientes infectados com HIV (DE REPENTIGNY; LEWANDOWSKI, JOLICOEUR (2004).

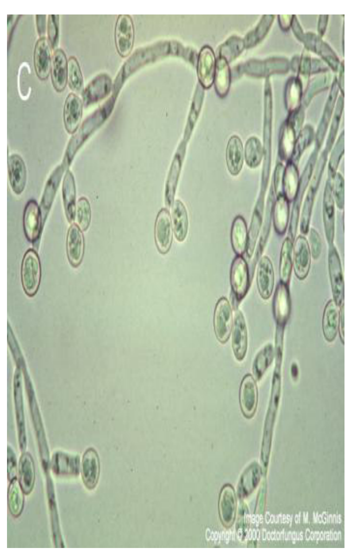

Figura 6. 6A. Anverso de colônias de *C. albicans* em Ágar Sabouraud-Dextrose 6B. Reverso de colônias de *C. albicans* em Ágar Sabouraud-Dextrose. FONTE: Rodrigues, I.V.P. (2018). 6C. Microscopia de *C. albicans*. Fonte: Zimmerman, (2012).

3.2.2 *Candida tropicalis*

Nas duas últimas décadas, tem-se observado um aumento na incidência de infecções causadas por espécies de *Candida* não-*albicans* (NCAC) (KAUFFMAN et al., 2000; RUAN; HSUEH, 2009). Esse crescente envolvimento dessas espécies pode estar parcialmente relacionado a melhorias nas técnicas de diagnóstico laboratorial, como o uso de meios cromogênios que permitem a diferenciação de espécies de *Candida*, bem como a introdução de técnicas moleculares que são utilizadas para a identificação rotineira desses patógenos (LIGUORI et al., 2009).

Ainda, a alta prevalência das NCAC em pacientes pode também ser devido ao aumento da resistência dessas leveduras a algumas drogas antifúngicas (GONZÁLEZ; ELIZONDO; AYALLA, 2008), quando comparado com *C. albicans*, uma vez que isso promove a persistência delas e possivelmente ao detrimento de *C. albicans*, em infecções mistas tratadas com antifúngicos tradicionais (SILVA *et al.*, 2011).

Enquanto que *Candida albicans* é uma espécie polimórfica verdadeira, devido à sua capacidade de formar hifa e/ou pseudo-hifa e tubo germinativo, sendo esta última uma característica diagnóstica, *C. tropicalis*, produz blastóporos ovais, pseudo-hifas e eventualmente hifas verdadeiras.

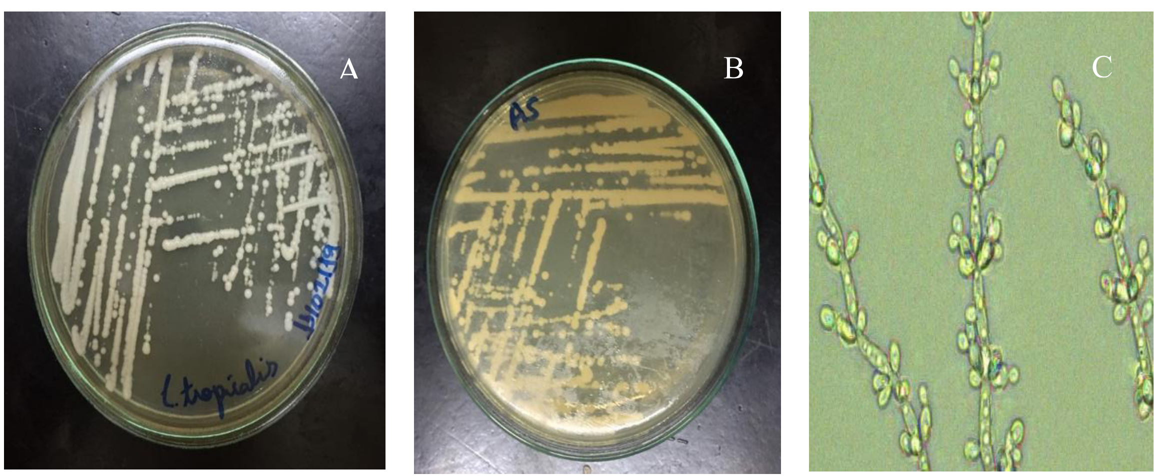

Figura 7. 7A. Anverso de colônias de *C. tropicalis* em Ágar Sabouraud-Dextrose 7B. Reverso de colônias de *C. tropicalis* em Ágar Sabouraud-Dextrose. **Fonte**: Rodrigues, (2018). 7C. Microscopia de *C. tropicalis*. **Fonte**: Alchetron (2018).

4. O USO DE FUNGOS FILAMENTOSOS NAS INDÚSTRIAS ALIMENTARES E FARMACÊUTICAS

Os microrganismos estão sendo extensivamente utilizados como fontes de agentes bioativos, dado o destaque cada vez maior que os produtos naturais vêm recebendo na obtenção de fármacos para o tratamento de doenças humanas. Os fungos, particularmente, são importantes fontes de novos metabólitos com atividade antibacteriana, antifúngica, antitumoral e antiviral (COSTA SOUZA et al., 2015).

Dentre os fungos microscópicos, alguns gêneros como *Aspergillus e Penicillium,* conhecidos por serem ubíquos e sintetizarem esporos abundantemente, têm sido utilizados na busca de compostos com atividade biológica. De aproximadamente 6.500 metabólitos bioativos de fungos microscópicos, mais de 30% foram obtidos destes dois gêneros (LUCAS; DE CASTRO; TAKAHASHI, 2007; DE SILVA et al., 2009; SURYANARAYANAN et al., 2009).

Uma grande vantagem da prospecção de metabólitos fúngicos em relação às outras fontes reside no fato de que os fungos podem ser cultivados em larga escala em fermentadores, sendo, portanto, ecologicamente benéficos, pois essa metodologia não necessita da retirada de plantas e algas dos seus habitats naturais, nem enfrenta problemas éticos como os que podem surgir da prospecção de metabólitos bioativos de insetos, anfíbios e outros animais (LUCAS; DE CASTRO; TAKAHASHI, 2007).

Dentre os metabólitos secundários produzidos por fungos filamentosos podemos citar os pigmentos, que podem pertencer a diferentes classes, como os carotenóides, melaninas, flavinas, fenazinas, quinonas, monascinas, violaceína e índigo (DUFOSSÉ et al., 2014). Como exemplo de um fungo produtor de um pigmento bastante conhecido na Ásia Oriental, podemos citar Mo*nascus purpureus* (ou fungo vermelho do arroz), que é um microrganismo amplamente utilizado nas indústrias alimentar e farmacêutica desde os tempos antigos para a produção de arroz fermentado vermelho também conhecido como "koji" no Japão, que significa "grão ou feijão coberto com uma cultura de bolor" (SHURTLEFF; AOYAGI, 2012).

As espécies de *Monascus* são capazes de produzir pigmentos amarelos (ankaflavina, monascina), laranjas (rubropuncatina, monascorubina) e roxos (rubropuncatimina, monascorubramina) comumente consumidos em alimentos orientais, especialmente no sul da China, no Japão e no Sudeste Asiático (DUFOSSE et al., 2005). Além disso, os pigmentos de

Monascus spp. apresentam atividades antimicrobianas, anticancerígenas, anti-mutagênicas e anti-obesidade (FENG; SHAO; CHEN, 2012).

Entretanto, sabe-se que alguns fungos filamentosos produzem também micotoxinas quando são submetidos à fermentação, que muitas das vezes são nocivas à saúde do ser humano (LI et al., 2006). Portanto, para que o consumo desses pigmentos naturais seja bem-sucedido deve-se haver uma boa aceitabilidade pelos consumidores, aprovação regulatória e um bom investimento de capital para que o produto seja comercializado (DUFOSSE et al., 2014). Dessa forma, para evitar a produção de micotoxinas, alguns métodos foram desenvolvidos:

a) A cepa não deve ser patogênica: algumas micotoxinas são produzidas somente em algumas cepas, indicando que a produção de toxina é específica de cada cepa.

b) A biossíntese do metabólito deve ser controlada: a produção de toxina pode ser controlada através do processo de biossíntese. Para que isso seja feito, a via metabólica deve estar bem elucidada.

c) Seleção do meio de cultura: já foi demonstrado por estudos anteriores (DUFOSSE et al., 2005; HAJJAJ et al., 2000; CHEN et al., 2017), que a adição ou remoção de íons metálicos, fontes de carbono e nitrogênio podem afetar a produção de toxinas. Portanto, deve-se selecionar os meios de cultura adequados para que haja um melhor controle da produção de toxinas.

Além do problema das micotoxinas, a literatura relata ainda que as vias biossintéticas principais da maioria das cepas de microrganismos, incluindo os fungos filamentosos, estão silenciadas e, portanto, impossibilitadas de produzir metabolitos secundários sob condições laboratoriais convencionais. Portanto, várias estratégias foram desenvolvidas para acordar essas vias biossintéticas silenciadas para ter acesso a esses metabolitos secundários (BRAKHAGE; SCHROECKH, 2011).

Entre estas, a estratégia de uma única estirpe de muitos compostos (OSMAC) (BODE et al., 2002), engenharia de ribossomos e a epigenética são também aplicadas por químicos de produtos naturais devido a seus procedimentos experimentais práticos. Ainda, os pesquisadores da área podem introduzir genes de resistência a antibióticos em microrganismos, induzindo-os a produzir compostos bioativos. Um desses mecanismos pode ser a mutagênese aleatória induzida pelo sulfato de dietilo, por exemplo (FANG et al., 2012).

Os fungos filamentosos também sintetizam uma ampla gama de compostos bioativos de baixo peso molecular. Dentre eles destacam-se os metabólitos com atividade farmacológica, como as β-lactamas (penicilina e cefalosporina), estatinas (lovastatina,

mevinolina, compactina, pravastatina e atrovastina) e imunossupressores (ciclosporina e ergotamina) (BÉRDY, 2005; MISIEK; HOFFMEISTER, 2007; FOX; HOWLETT, 2008).

4.1 Resistência de bactérias a antibióticos

A era dos antibióticos começou em 1929 com a descoberta da penicilina a partir da espécie fúngica *Penicillium notatum* e, a partir da década de 40, sua produção comercial prosperous (DEMAIN; FANG, 2000). Desde então, vários antibióticos foram sendo obtidos a partir de fungos e actinomicetos na busca por propriedades farmacológicas eficientes no combate a patógenos emergentes. Pesquisas continuadas na área levaram à descoberta de vários antibióticos, como a cefalosporina, tetraciclina, macrólidos, ansamacrolídeos, aminoglicosídeos, cloranfenicol, glicopéptidos, inibidores de peptídeos e antraciclinas (HASSAN et al., 2012). Esses compostos inibem várias vias, como a da síntese de ácidos nucléicos, síntese proteica, formação celular e via de transporte de elétrons (DEMAIN, 2007).

Antibióticos são compostos químicos específicos derivados de ou produzidos por organismos vivos que podem inibir outros organismos em pequenas quantidades. Em um sentindo mais amplo, o termo “antibiótico” frequentemente designa substâncias que inibem microrganismos que são patogênicos ao homem, outros animais superiores ou plantas. Esses microrganismos incluem bactérias, fungos, amebas e mais raramente vírus (APSIMON, 2009).

Os antibióticos podem ser classificados segundo a sua estrutura química, rota de biossíntese, fonte, modo de ação, via de administração e seu alcance efetivo. As tetracicilinas, por exemplo, são as mais estudadas devido ao seu amplo-espectro de ação e são utilizadas no tratamento de doenças causadas por uma ampla gama de microrganismos, incluindo bactérias gram positivas e negativas, protozoários parasitas, clamídias, ricketsia e micoplasma (CHOPRA; ROBERTS, 2001).

Para que um antibiótico seja útil terapeuticamente este fármaco deve ter toxicidade seletiva, isto é, deve ter pouca ou nenhuma toxicidade ao paciente devendo inibir ou matar o patógeno em questão. Além disso, deve ser capaz de ser absorvido por via oral (exceto quando a aplicação tópica é mandatória) e deve permanecer em níveis adequados na corrente sanguínea por um período de tempo razoável. Deve também possuir ampla distribuição pelos vários tecidos do corpo do hospedeiro (APSIMON, 2009).

Devido a essas limitações, poucos são os compostos orgânicos que podem ser agentes quimioterápicos. Os que podem ser classificados nessa categoria são, em sua maioria, de origem microbiana, apesar de alguns poderem ser derivados de formas de vida superiores. Alguns são fabricados sinteticamente, e a maioria na verdade são híbridos de sínteses bioquímicas e laboratoriais. Estes são modificações feitas em laboratório de antibióticos pré-existentes e tais compostos modificados estão se tornando cada vez mais necessários, visto que muitas cepas de microrganismos vêm criando resistência aos antimicrobianos que são mais utilizados na prática clínica (APSIMON, 2009).

A resistência que microrganismos patógenos a seres humanos vêm adquirindo nos últimos anos já é considerada como um problema de saúde global. O surgimento de bactérias resistentes a várias drogas (MDRs) pode ser atribuído ao uso indiscriminado de antibióticos de amplo-espectro. Entretanto, um componente que muita das vezes não é levado em consideração são os sistemas genéticos que as bactérias adquirem e que regulam uma gama de mecanismos de resistência a antibióticos (BASSEGODA et al., 2018).

Um dos mecanismos de aquisição de resistência a antibióticos desenvolvidos pelas bactérias é a transferência horizontal de genes, o que acaba favorecendo a ocorrência desse fenômeno entre diferentes espécies e cepas bacterianas. A produção de β-lactamases, isto é, enzimas que degradam os antibióticos betalactâmicos, é um mecanismo de resistência comumente desenvolvido pelas bactérias Gram-negativas. A propagação dessas enzimas entre esse grupo de bactérias tem comprometido o uso clínico de várias clássicas de antibióticos betalactâmicos, e com a disseminação de genes como o bla_{KPC}, o uso dos carbapenemos pode também ser prejudicado no futuro (HAWKEY; JONES, 2009).

Também tem sido extensivamente reportada na literatura a resistência a antibióticos entre bactérias Gram-positivas, como o surgimento da cepa de *Staphylococcus aureus* resistente à meticilina (MRSA) associada à comunidade, o que tem dificultado a distinção entre cepas adquiridas na comunidade e no hospital (HAWKEY; JONES, 2009).

4.2 Resistência a antifúngicos

Antifúngicos são medicamentos utilizados no combate às infecções fúngicas. Atualmente existem cinco classes de antifúngicos utilizados na prática clínica: os azóis, equinocandinas, polienos, alilaminas e análogos da pirimidina. Entretanto, além da resistência

fúngica à maioria dessas classes de antifúngicos, o tratamento das micoses ainda possui várias limitações: propriedades farmacocinéticas, efeitos colaterais indesejáveis, espectro de atividade restrita e pequeno número de alvos (PFALLER, 2012; PFALLER et al., 2013; PIANALTO; ALSPAUGH, 2016). Além disso, nao há introdução de uma nova classe de antifúngicos no mercado desde 2006, quando a Agência Europeia de Medicamentos e a Administracao de Comidas e Drogas (FDA) aprovaram a anidulafungina (CAMPOY; ADRIO, 2017).

Apesar de os antifúngicos pertencentes à classe dos azóis serem os mais utilizados nas terapias antifúngicas, casos de resistência já têm sido documentados na literatura (PFALLER, 2012). Existem quatro mecanismos principais de resistência a essaes medicamentos: a) aumento no efluxo das drogas; b) mutação-alvo; c) desregulação na expressão do alvo; d) alteração na via biossintética do ergosterol (TOBUDIC; KRATZER; PRESTERL, 2012).

Quando ocorre a ativação das bombas de efluxo associadas à membrana, a droga é expelida da célula, diminuindo a sua concentração intracelular, resultando em uma diminuição da sua concentração dentro da célula (PFALLER, 2012). Nos fungos, dois sistemas de efluxo da droga estão envolvidos na retirada dos azóis de dentro do citoplasma: Superfamília dos Cassetes Ligantes ao ATP e a Superfamília Facilitadora Principal (TOBUDIC; KRATZER; PRESTERL, 2012). A resistencia ao azói acontece entao quando há uma super expressão dos genes que codificam para essas proteínas de transporte (TOBUDIC; KRATZER; PRESTERL, 2012).

O gene *ERG11* codifica a produção da enzima lanosterol 14α-desmetilase nas leveduras, que é uma enzima-alvo para os azóis. Portanto, mutações pontuais nesse gene resulta na diminuicao ou inabilidade dessa enzima de se ligar à droga devido à uma alteração no sítio de ligação ao azol (FUENTEFRIA et al., 2018).

Na presença de um azol, algumas espécies de *Candida* podem desenvolver um mecanismo de *feedback* em resposta à destruição do ergosterol a partir da super expressão do gene *ERG11*, resultando em um aumento na síntese da lanosterol 14α-desmetilase e tal fenômeno requer um aumento na quantidade da droga antifúngica, o que contribui para um aumento na resistência fúngica (VANDEPUTTE; FERRARI; COSTE, 2012).

Além da mutação no gene que codifica para a enzima lanosterol 14α-desmetilase, tal resistência também altera outras enzimas da mesma via biossintética (PFALLER, 2012). A exposição aos azóis resulta em uma depleção do ergosterol presente na membrana citoplasmática fúngica e também no acúmulo do inibidor do crescimento fúngico 14α-metil-

3,6-diol. Mutações no gene *ERG3* impedem a formação da 14α-metil-3,6-diol a partir do 14α-metil-fecosterol e acumula os precussores que podem substituir o ergosterol celular. Esses dois fatores restituem a membrana fúngica ao mesmo tempo que contribui ao desenvolvimento da resistência das espécies de *Candida* (TOBUDIC; KRATZER; PRESTERL, 2012).

As mutações no gene ERG3 estao associadas com resistência cruzada aos polienos, provavelmente causada pela depleção do ergosterol. Ademais, mutações em outros genes da via biossintética do ergosterol, como *ERG2*, *ERG6* e *ERG24* podem estar ligados a uma redução na susceptibilidade aos azóis (SCORZONI et al., 2017).

Outro fator que contribui para o aumento da resistência a antifúngicos é o biofilme, que é uma comunidade de células microbianas protegida por uma matriz polimérica, que facilita a adesão entre as células e dessas com superfícies e interfaces. Os biofilmes fúngicos colonizam superfícies abióticas e contribuem para infecções relacionadas à dispositivos médicos, por exemplo, cateteres, marca-passos, lentes de contato e dispositivos prostéticos. Além disso, os biofilmes podem ser compostos por muitas espécies e podem mudar com o tempo, captando microrganismos “imigrantes” e, consequentemente, alterando a estrutura e função daquela comunidade microbiana (SARDI et al., 2014).

Os biofilmes tornam aquela comunidade microbiana menos suscetível aos mecanismos de defesas do hospedeiro e, fundalmente, levam à resistência à maioria dos antifúngicos tradicionais, dificultando o tratamento e contribuindo para altas taxas de mortalidade e morbidade. Por isso, é de suma importância desvendar os mecanismos moleculares envolvidos no desenvolvimento dos biofilmes fúngicos para que sejam realizadas novas estratégias para combater essas infecções (KUMAR; MISHRA; SINGH, 2018).

4.3 Câncer e terapia anticâncer baseada em produtos naturais

O câncer é uma das principais causas de mortalidade ao redor do mundo. Estimativas preveem um aumento para 19.3 milhões de novos casos de câncer por ano para 2025 (FERLAY et al., 2015). Mais da metade dos casos e mortalidade do câncer ocorrem em países de baixa e média renda, e espera-se que esses números aumentem até 2025. As células cancerígenas se instalam e crescem em vários tecidos e órgãos do corpo humano, sendo que

os cinco sítios preferenciais do câncer em homens são os pulmões, próstata, cólon-reto, estômago e fígado, e entre as mulheres são as mamas, cólon-reto, pulmões, colo do útero e estômago (TORRES et al., 2015).

O tratamento para o câncer inclui cirurgia, radioterapia e tratamentos sistêmicos que compreendem quimioterapia citotóxica, terapia hormonal, imunoterapia e terapias direcionadas (PALUMBO et al., 2013). As maiores desvantagens da quimioterapia são a recorrência do câncer associada à resistência aos medicamentos utilizados, e ainda os diversos efeitos colaterais que restringem o uso de algumas drogas anticâncer e que acabam prejudicando a qualidade de vida dos pacientes. Apesar disso, a quimioterapia continua sendo um dos tratamentos mais utilizados para todos os tipos de câncer e em todos os estágios de progressão da doença. Como resultado, o câncer continua sendo um desafio para o tratamento clínico, por isso, a busca por terapias alternativas e efetivas tem sido contínua (RAYAN; RAIYN; FALAH, 2017).

A compreensão dos mecanismos moleculares envolvidos na progressão do câncer levou ao desenvolvimento de várias drogas antitumorais, entretanto o uso de muitas drogas sintetizadas quimicamente tem causado danos aos pacientes, geralmente na forma de supressão do sistema imune. Por isso, a descoberta de novas drogas baseadas em produtos naturais têm sido o foco de várias pesquisas (WRIGHT, 2017; YAO et al., 2017). Alcalóides, terpenóides, polissacarídeos, saponinas e outras substâncias têm sido relatadas como produtos naturais bioativos com atividade anticâncer. A maioria das drogas anticâncer (>60%) que fazem parte da rotina clínica e que têm demonstrado uma eficácia significativa no combate ao câncer origina-se de produtos naturais derivados de plantas, organismos marinhos e microrganismos (SEELINGER et al., 2012).

5 OBJETIVOS

5.1. Geral

Caracterizar quimicamente o corante obtido de *Talaromyces marneffei* e avaliar suas atividades biológicas *in vitro*.

5.2 Específicos

- Obter o pigmento intracelular (contido no micélio do fungo) produzido por *T. marneffei* em meio de cultura líquido;
- Identificar molecularmente por PCR convencional o *T. marneffei* isolado de ambiente marinho (Parque Estadual da Laguna da Jansen) utilizado neste trabalho;
- Caracterizar quimicamente o extrato pigmentado vermelho intracelular através de HPLC-DAD (Cromatografia líquida de alta precisão com detector Diode Array) comparando-os com substâncias similares já descritas na literatura;
- Avaliar as atividades antifúngicas do extrato pigmentado vermelho intracelular de *T. marneffei* sobre *Candida albicans* ATCC 10231e *Candida tropicalis ATCC 1369;* antibacterianas sobre *Escherichia coli ATCC 25922* e *Staphylococcus aureus* ATCC 25923; e citotóxicas sobre a linhagem celular de câncer de mama (MCF-7) e fibroblastos de pulmão humano (GM07492).

6 ARTIGO: Biological activities of an intracellular pigmented extract from *Talaromyces marneffei.*

Biological activities of an intracellular pigmented extract from *Talaromyces marneffei*.
O manuscrito foi submetido na revista Marine Drugs (Qualis A2 para Medicina II).

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Running headline: Biological activities of fungal pigment.

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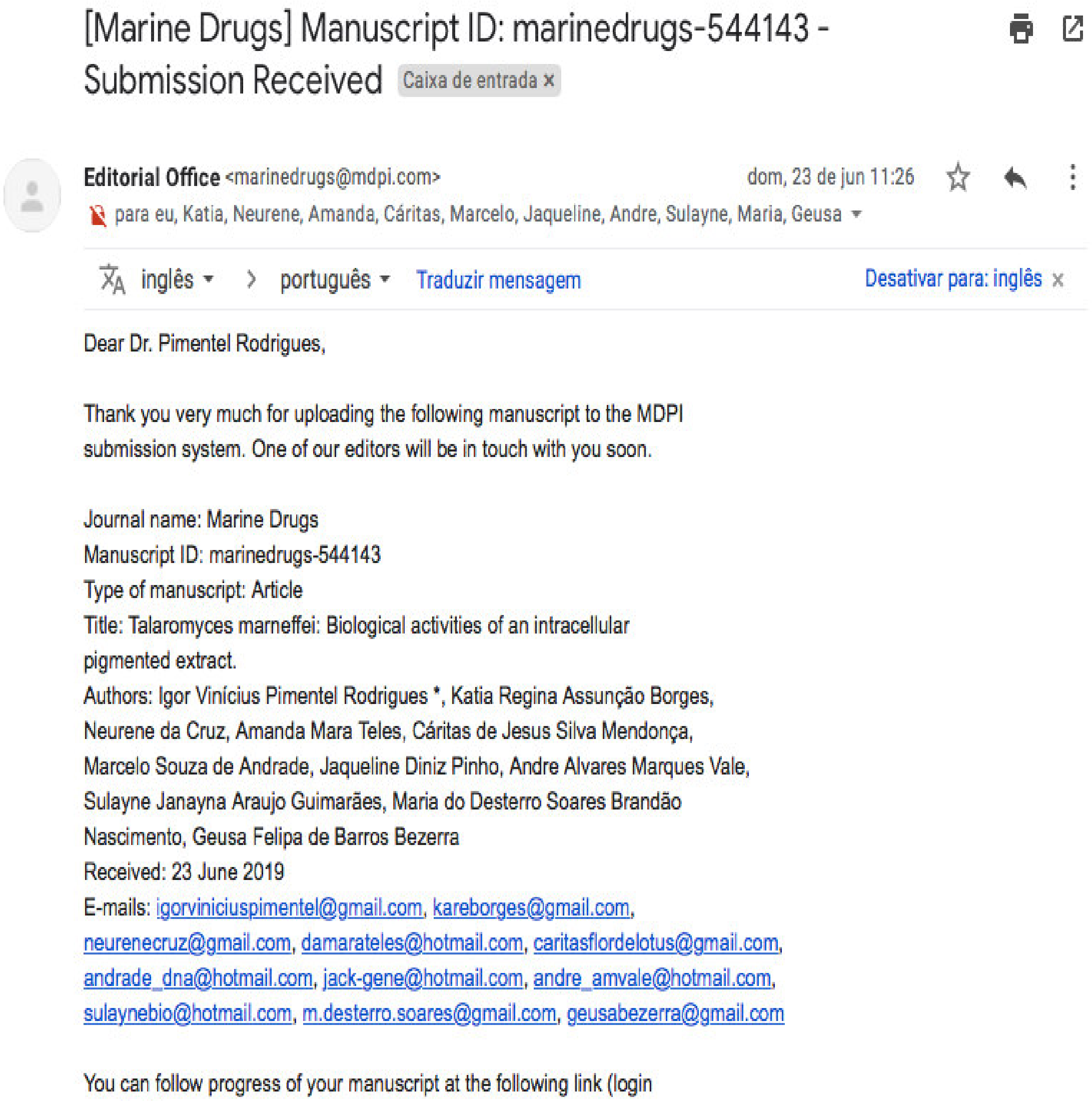

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Authors: Igor Vinícius Pimentel Rodrigues *, Katia Regina Assunção Borges, Neurene da Cruz, Amanda Mara Teles, Cáritas de Jesus Silva Mendonça, Marcelo Souza de Andrade, Jaqueline Diniz Pinho, Andre Alvares Marques Vale, Sulayne Janayna Araujo Guimarães, Maria do Desterro Soares Brandão Nascimento, Geusa Felipa de Barros Bezerra
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ABSTRACT

Introduction: *Talaromyces marneffei* is an emerging pathogenic fungus responsible for causing fatal mycosis mainly in immunocompromised individuals. The fungi belonging to the genus *Talaromyces* often produce varied pigments, among them the red pigmentation used in this work. These microorganisms have been gaining increasing prominence in the pharmaceutical industry because of their ability to provide antibiotics, immunosuppressants, enzymes and anti-cancer drugs. **Aims:** To evaluate the *in vitro* antifungal and antibacterial activities of the intracellular red pigmented extract (IRPE) produced by the ascomycetes *Talaromyces marneffei* in *Candida albicans* ATCC 10231, *C. tropicalis* ATCC 1369, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, and cytotoxic *in vitro* against strains tumor cells (MCF-7) and normal (GM07492). **Methodology:** Broth microdilution techniques were used to analyze the antimicrobial activity. For analysis of the cytotoxic activity the MTT assay was used [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide]. **Results:** Minimum bactericidal concentration (MBC) of *T. marneffei*'s IRPE for *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 was 1000 µg/ml. The minimum inhibitory concentrations (MIC) for *E. coli* were 250 and for *S. aureus* 500 µg / mL, whereas for *C. tropicalis* ATCC 1369 was 62.5 µg/mL. Half of the maximal inhibitory concentration (IC50) for breast cancer cell line (MCF-7) was 45.43 ± 1.657 µg / mL, however, no cytotoxicity was observed in the normal cell line tested (GM07492). Considering these results, it is suggested that more studies be done extending the use of this pigmented extract in the textile, cosmetic, food, and other industries.

Key words: Pigment; *Talaromyces marneffei*; antibacterial; cytotoxicity; natural product.

1 Introduction

Secondary metabolites, among them fungal pigments, are related to the resistance of these organisms to various adverse environmental factors, such as dissection, exposure to extreme temperatures, irradiation and photo-oxidation, or in ecological interactions with other organisms such as sponges, corals or other microbial communities[1]. Because they are substances with unique chemical structures, these substances are widely used in the health

industry because they are antimicrobial, antiparasitic and antitumor agents, enzyme inhibitors and immunosuppressants[2].

Biosynthetically, many extrolites produced by filamentous fungi are polyketides, and several papers report that polyketides seem to dominate natural products of marine-derived fungi[3]. Polyketides represent an array of often structurally complex natural products including the classes of anthraquinones, hydroxyanthraquinones, naphthalenes, naphthoquinones, flavonoids, macrolides, polyenes, tetracyclines, and tropolones.

Extract yield of a secondary metabolite produced by fungi may vary according to the solvent used, the time and temperature of extraction and, especially, its chemical composition. Thus, the choice of solvents to be used in the extraction process depends on the class of the desired substance. In this study, ethyl acetate was used because it is able to extract both polar and non-polar substances, so it is possible to obtain a wide range of compounds that can be tested for their biological activities[4]. Furthermore, many of the compounds extracted by these solvents are reported to have potent antimicrobial and anti-neoplastic activities[5-7].

Species belonging to *Penicillium* and *Talaromyces* genres have been the subject of investigations in the discovery of bioactive compounds, which has led to the discovery of drugs, such as penicillin[8] and compactin, which is an anticholesterolemic agent[9], diverse antitumoral drugs[10] and mycotoxins that contaminates food[11]. Many studies point that microorganisms that are exposed to stressful conditions, like soil pollution, very high or low temperatures and/or salinity, are more likely to produce bioactive compounds as they are related to the survival of these organisms under such harsh conditions[12-14].

The species *Talaromyces marneffei* was first isolated in 1956 from hepatic lesions of bamboo rats (*Rhizomys sinensis*) used in experimental infections at the Indochina Institute, Dalat, South Vietnam[15]. The fungus was initially named *Penicillium marneffei* in honor of Hubert Marneffe, director of the Pasteur Institute of Indochina[16], but currently all accepted species of *Penicillium* subgenus *Biverticilium*, including *P. marneffei*, were transferred to the genus *Talaromyces* based on an analysis of the *RPB1* (RNA polymerase II largest subunit) and on the sequencing of ITS region (including 5.8S nrDNA)[17][33].

Due to the increasing resistance acquired by microorganisms to antibiotic and antifungal drugs commonly used in the clinical setting, antimicrobial agents of natural origin can be used to treat microbial infections, thus saving millions of lives[18]. Furthermore, the use of many chemically synthesized anti-cancer drugs has been causing harm to patients, usually in the form of immune system suppression. Therefore, the discovery of new drugs based on natural products has been the focus of several researches[19,20]. Most of the anticancer drugs

(>60%) that are part of the clinical routine and that have demonstrated significant effectiveness in the fight against cancer originate from natural products derived from plants, marine organisms and microorganisms[21].

In the present study, we investigated the *in* vitro antifungal, antibacterial and cytotoxic activities of a intracellular red pigment extract from *Talaromyces marneffei* against *Candida albicans ATCC 10231*, *Candida tropicalis ATCC 1369*, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, respectively, and against breast cancer cell line (MCF-7) and human lung fibroblasts cell line (GM07492). To our knowledge, there are no previous studies in the scientific literature that reports such *in vitro* biological activities of the red pigment of this fungal species.

2 Results and discussion

2.1. Yield of the extract

From a total of 1.5 liters of Potato-Dextrose Broth where the fungus was cultivated, we obtained a total of 6.660 grams of fungal biomass and 0.857 grams of intracellular pigment, which was contained in the mycelium of the fungus.

2.2 Chemical composition of the extract

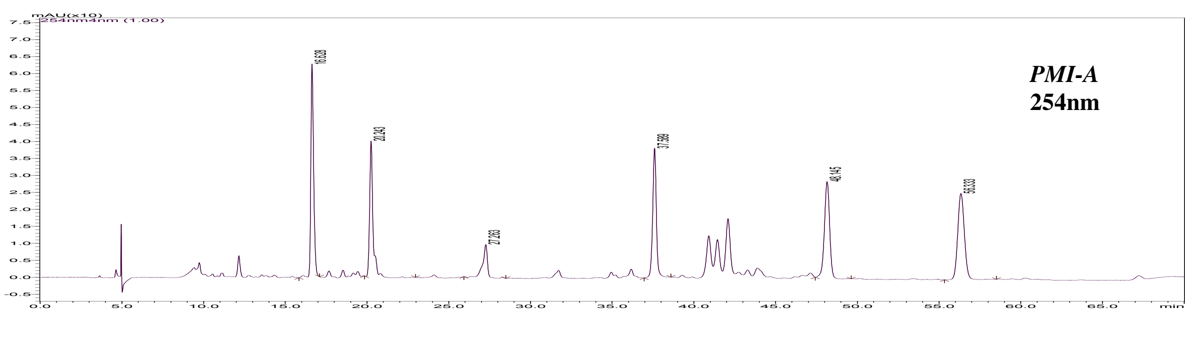
The chemical profile showed a large metabolic variety, with chromatographic bands varying from medium to high polarity. Good chromatographic resolution were visualized in various wavelengths (Figure 1).

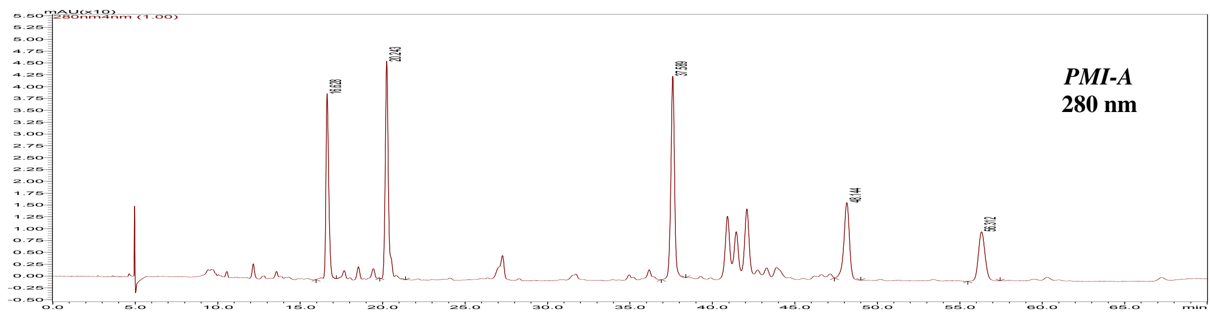
The intracellular pigment extract (PMI) showed the major peaks at the retention times of 16.628, 20.243 and 37.589 min. The latter peak showed a higher intensity compared to the others, mainly at the wavelength of 280 nm.

It was verified a maximum absorption (λmax) of chromophores, which suggests the presence of compounds of the class of anthraquinones, triterpenes and phenolic acids. Literature reports that high intensity bands in which in the UV spectrum are visualized at a λmax of 222 to 276 nm, indicates the presence of chromophores belonging to the class of anthraquinones[22].

PMI showed a high intensity band with tR of 48.145 min (Figure 1). Literature reports that λmax of chromophores ranging from 250 to 470 nm, suggests the presence of

triterpenes[23] and bands with tR of 56.33 min with λmax of 250 nm, suggest the presence of phenolic acids[24].





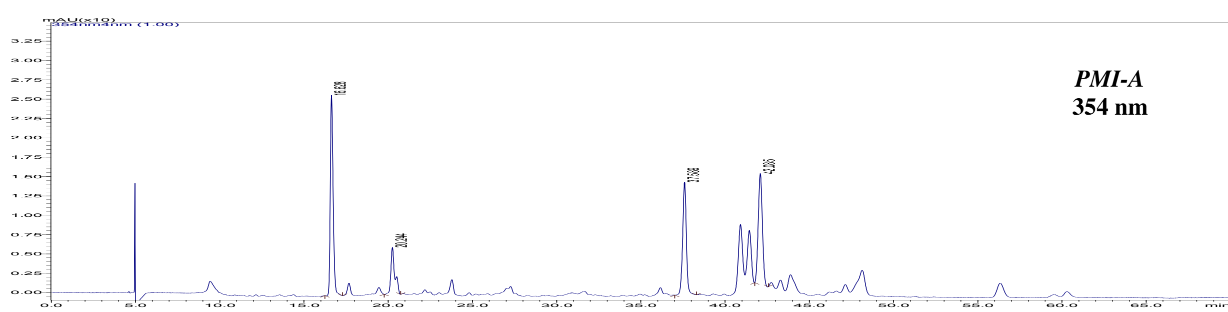


Figure 1: Exploratory gradient chromatographic profile of pigmented extract of PMI-A at different wavelengths (254-354 nm).

2.3 Molecular identification

The size of the ITS1-5.8S-ITS2 rDNA region defined by the pair of primers TM1was 391 base pairs (bp) (Figure 2). The amplified sequence had 100% of identify with

Talaromyces marneffei strains deposited on GenBank (number of accession of the reference sequence: MH707052.1).

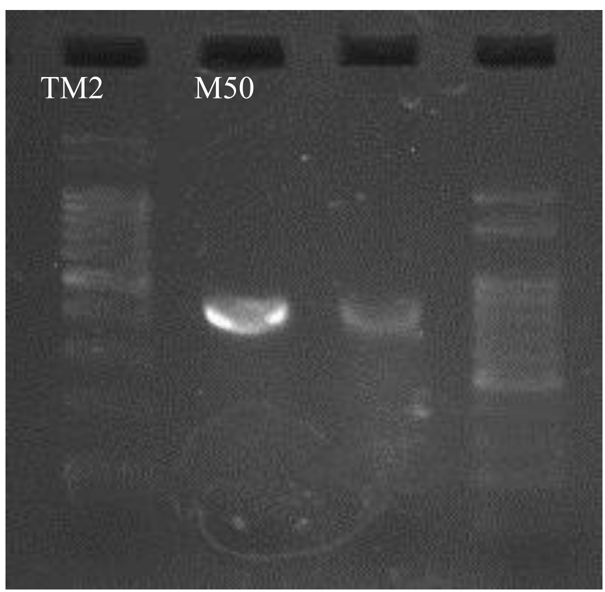


Figure 2. PCR amplification of DNA from *T. marneffei* with specific primers TM1 and TM2. M100: Size marker of 100-bp-ladder standard DNA. M50: size marker of 50-bp-ladder standard DNA. TM1: DNA extracted with DNEasy Plant Mini Kit from Qiagen. TM2: DNA extracted with a non-specific kit (Genomic DNA Extraction Biopur).

2.4 Broth microdilution

The intracellular red pigment extract (IRPE) from *T. marneffei* was bactericidal against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 at the concentration of 1000µg/mL and bacteriostatic against both microorganisms at the concentration of 500 µg/mL. MICs (Minimum Inhibitory Concentrations) against these bacteria were 250 ($p = 0.0005$) and 500 µg/mL ($p= 0.0078$), respectively, since the log of colony units forming (CFUs) showed a significant statistical difference compared to the negative control (Figures 3A and 3B). There was no growth of colonies in the positive controls.

MIC value for *C. tropicalis* ATCC 1369 was 62.5µg/mL ($p = 0.0363$) (Figure 3C). Despite the fact that our tested substance was not active against *C. albicans* ATCC 1023 since there was no significantly statistical difference between any of the concentrations and the negative control, there was a significantly statistical difference ($p<0.05$) between the concentrations of 125 and 500 µg/mL, 7.8 and 500 µg/mL and 125 and 250 µg/mL, indicating that the concentrations of 125 and 7.8 µg/mL were fungistatic against *C. albicans* ATCC 1023

compared to these other concentrations. There was no growth of the colonies in the positive controls.

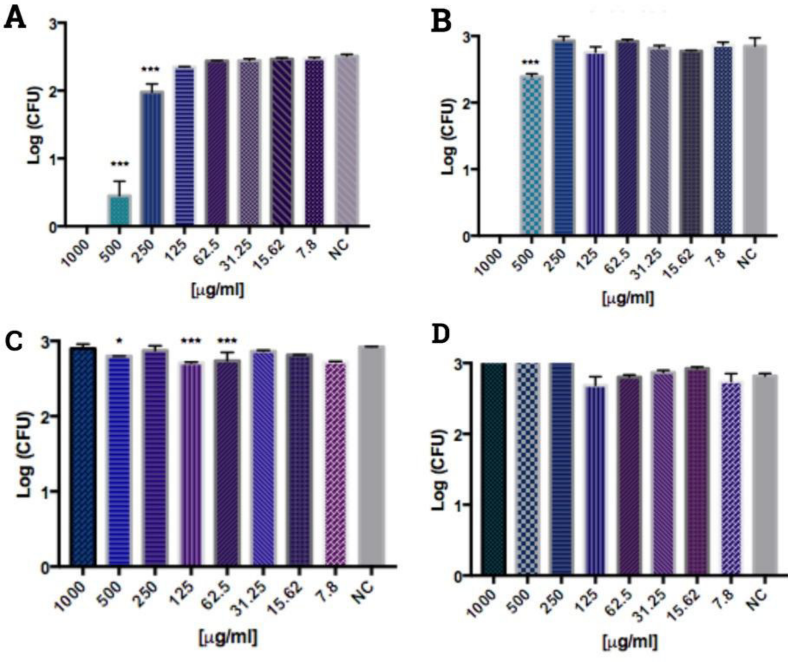


Figure 3. Logarithm of number of Colony Forming Units (CFUs) of the tested microorganisms in the presence of decreasing concentrations of IRPE from *T. marneffei*.(A) *E. coli*ATCC 25922. (B) *S. aureus* ATCC 25923. (C) *C. tropicalis* ATCC 1369. (D) *C. albicans* ATCC 10231. Error bars represent standard deviation. Statistical p value (represented by * or ***) indicate concentrations that are significantly different from control. *$p < 0.05$; ***$p < 0.001$.

2.5 Cytotoxic activity

2.5.1. Effect of the ethyl acetate intracellular red pigment extract on cell viability of MCF-7

The intracellular red pigment extract (IRPE) from *T. marneffei* promoted cell viability reduction after 72 hours of treatment in the lowest concentration tested: 0.25 µg/mL, compared to the other treatments, with a mean cellular viability of 63.13%, although it showed no statistical difference compared to the negative control (p=0.0713) (Figure 4). The IC50 value after 72 hours of treatment was 45.43 ± 1.657 µg/mL.

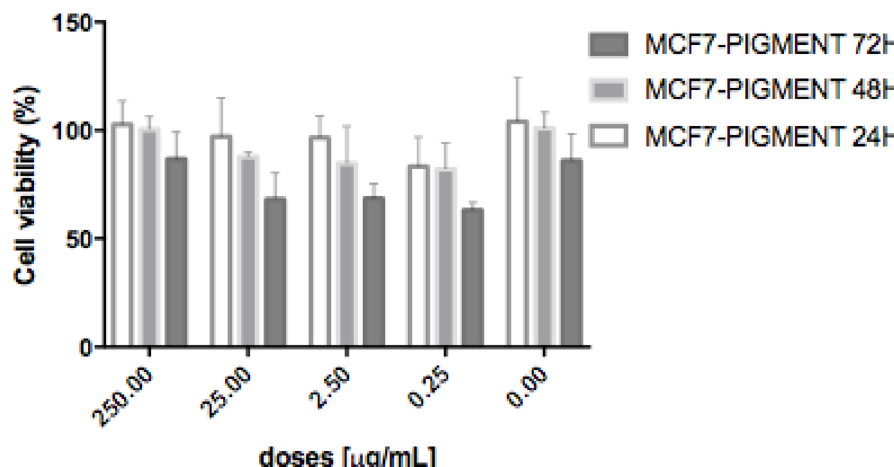


Figure 4. Reduction of cell viability of MCF-7 throughout all the treatments, with a greater cell viability reduction after 72 hours of treatment. 0.00: negative control.

2.5.2 Effect of the ethyl acetate intracellular red pigment extract on cell viability of GM07492

The intracellular red pigment extract (IRPE) from *Talaromyces marneffei* did not exert any cytotoxicity on human lung fibroblasts cell line (GM07492) compared to the negative control. Instead, it increased the cell viability on all treatments (Figure 5).

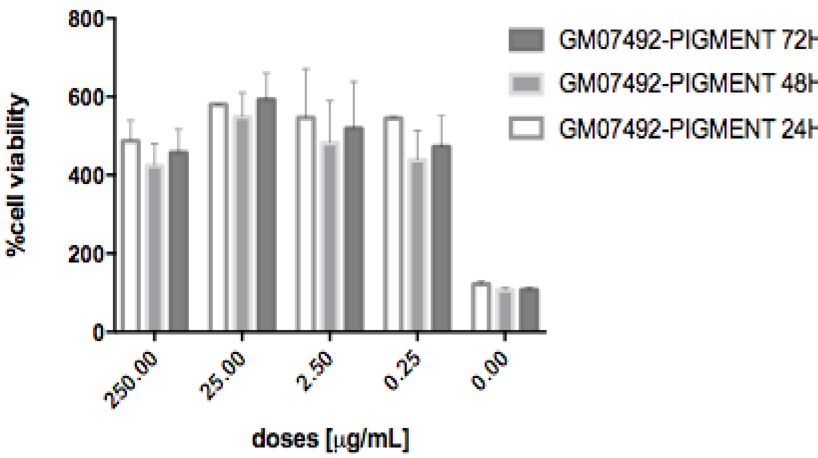


Figure 5. Cell viability of GM07492 on all tested concentrations throughout all the treatments. 0.00: negative control.

3 Discussion

A study performed by Rostami[25] reported that the MBC (Minimum Bactericidal Concentration) of the pigments obtained from *Micrococcus roseus* (PEM) and *Rhodotorula*

glutinis (PER) for *S. aureus* was 16 mg/mL, while the MBCs of these substances differed from each other for *E. coli*: 64 and 32 mg/mL, respectively. The extract tested here showed a more prominent bactericidal activity against these bacteria, since the MBC was much lower: 1 mg/mL. Literature reports that some triterpenes compounds possesses good antimicrobial activity[26,27], which can explain the satisfactory results found.

Another research analyzed the antibacterial and antifungal activities of rubropunctatin, monascorubrin, monascin and ankaflavin isolated from *Monascus purpureus*. It was found that these compounds caused a strong inhibition of *Bacillus subtilis* at 1mg/ml[28], similar to the result found in the present study, in which IRPE from *T. marneffei* exhibited strong inhibition in the growth of *S. aureus*, also a gram-positive bacterium, at the same concentration. Rubropunctatin and monascorubrin were also active against *Candida pseudotropicalis*, but monascin and ankaflavin were not. This suggests that the former substances may be also present in *T. marneffei*'s red pigment extract, since it was active against *C. tropicalis*, that belongs to the same complex as *C. pseudotropicalis*. Literature reports that the latter substances (monascin and ankaflavin) are also present in *T. marneffei*'s pigment[29,30], therefore, it can also explain the weak anti-*Candida* activity found in the present study.

Kim et al.[31] analyzed the antimicrobial activity of amino-acids derivatives of *Monascus* pigments. Its antibacterial activity against *E. coli* was due to the decrease of the oxygen uptake rate of *E. coli* in culture. Since *T. marneffei*'s pigment is a *Monascus*-like pigment, this may explain its bactericidal property in the highest concentration tested.

The data presented here also indicate the cytostatic property, with dose/time-dependent effect of IRPE from *T. marneffei* in hormone receptor-positive breast cancer cells (MCF-7). It was able to reduce cell viability after 72 hours of treatment, when compared to the other treatments (24 and 48 hours), at the lower concentration tested.

Soumya[32] found an IC50 value of a solid red pigment of oily nature produced by *Fusarium chlamydosporum*, also a filamentous fungus, of 62 µg/mL MCF-7. Our red pigment extract had a lower IC50 value (45.43 µg/mL) against the same tumor cell line, which shows its cytostatic potential and points to the possibility of the isolation and purification of its bioactive compounds.

The secondary metabolites purpurogemutanin and purpureuututantidine from *Penicillium purpurogenum* G59, a fungus also derived from a marine environment, significantly inhibited proliferation of K562 (tumor cells from chronic human leukemia), HeLa (cervical cancer), BGC-823 (gastric adenocarcinoma) and MCF-7 (breast cancer), with

inhibition rates ranging from 62.8% to 88% at 100 µg/mL by the MTT test. These promissory results were obtained after a mutation induced in the fungus by diethyl sulfate (DES)[33]. It is worthy to highlight that, despite the fact that we did not obtain such high inhibition rates in the proliferation of MCF-7, we did not induce any mutation in *T. marneffei*, and still we obtained a inhibition rate of 28.71% in the lowest concentration tested from a crude red pigment extract.

In view of the results found, it suggested the usage of IRPE as a food coloring and conservative, since *S. aureus* and *E. coli* are also food contaminants, causing intestinal infections in humans[34]. Besides, IRPE exerted no cytotoxicity in the normal cell line tested, therefore, it may be utilized by food, pharmaceutical, cosmetic and textile industries, but further *in vivo* studies need to be done to corroborate these results.

Also, more studies are needed to elucidate the cytostatic and antibacterial activities of the probable bioactive compounds further isolated from the extract, in order to identify the molecular targets of the mechanisms of bacterial and cancer cells' death and inhibition of proliferation, respectively, to aid in the acquisition of new therapeutic drugs or preventive combinations in the treatment of breast cancer and/or bacterial infections.

It is suggested that triterpenes may be responsible for both cytotostatic and antibacterial activities displayed and may be useful for synthesis of chemopreventive drugs and/or antibiotics.

4 Materials and methods

4.1 Collection of the fungus

T. marneffei was isolated from the soil of a marine environment in São Luís, Maranhão, Brazil, named Jansen Lagoon State Park. This ecosystem has been suffering for decades from anthropogenic pollution (dumping of domestic sewage into its water body). Its geographic coordinates are: 2º 30' 13" S 44º 17' 53" W. The fungus is deposited in the Collection of Fungi of Federal University of Maranhão.

4.2 Molecular identification

The filamentous fungus was initially grown in potato-dextrose broth for about 4 days. After this incubation period, the fungal biomass was divided into two samples: the

first consisted of 425 mg of fungal biomass that was macerated with sterile beach sand and then the instructions of the DNEasy Plant Mini Kit from Qiagen were followed, and the other consisted of the same quantitiy of fungal biomass that was then macerated with liquid nitrogen and then the instructions of the Genomic DNA Extraction Biopur Kit were followed.

The regions of the transcribed internal spacer (ITS1 and ITS2) of the ribosomal DNA and the 5.8S rDNA gene specific for *T. marneffei* were amplified by polymerase chain reaction. The pair of primers TM1 utilized was: 5' CGT AAC AAG GTT TCC GTA GGT 3' (forward) and 5'GTG CTT GAG GGC AGA AAT GA 3'. PCR reactions were done in 25 µl. The DNA was amplified by PCR using an automated thermocycler, following the following parameters: initial denaturation of 5 minutes at 95 °C, 30 seconds denaturation at 95°C, followed by 35 cycles of 50 seconds of annealing of the primers at 55°C, 1 minute of extension at 72°C and a final extension period of 10 minutes at 72°C. Five microliters of each PCR reaction were subjected to electrophoresis in 2% agarose gel at 120 V for 45 minutes in 1X TBE. PCR products were visualized in UV after staining with SYBR Safe (0.5 g/ml)[35].

4.3 Production and extraction of intracellular pigment

To obtain the intracellular extract of the *T. marneffei*'s pigment, the fungus was grown in Potato-Dextrose Agar (PDA) for seven days at 25ºC. Five blocks of approximately 6 mm containing the mycelium were then transferred to 1,000-mL Erlenmyers containing 500 mL of Potato-Dextrose Broth (PDB). The flasks were incubated without shaking for 21 days at 25°C. Thereafter, 300 ml of Ethyl Acetate were added to 500 ml of the fermented broth and left overnight for decantation. The mycelium was then separated from the liquid mediumby filtration on Whatman paper no. 4. Then, 300 ml of Ethyl Acetate was added to the fungal biomass to provide the intracelular pigment. This solution was placed in a separation funnel to obtain a purer solution. It was then concentrated in a rotavaporator and stored at 4°C for further analysis of antibacterial and antifungal activities *in vitro*[36].

4.4 Chemical composition of the fungal pigment extract

The intracellular extract (PMI) was analyzed on a liquid chromatogram with a diode array detector (LC-20AD, SPD-M20A). For the separation of the compounds a column C18-C Shin-Pack VP-ODS (250 mm X 4.6 ID mm, diameter of particular 5μm) was utilized. The flux of the mobile phase was 1 mL/min and the temperature of the oven was 40°C. The mobile phase was composed of acetonitrile: 0.1% solution of formic acid and the gradient mode is described in the table below. It was injected 20 μL of the extract.

4.5 Biological activities

4.5.1 Antibacterial and antifungal activities of fungal pigment

4.5.1.1 Broth microdilution assay

The Minimal Inhibitory Concentration (MIC) of the ethyl acetate extract of the intracellular red pigment from *T. marneffei* for the tested microrganisms was determined based on the microdilution method in a 96-well microplate.

Inocula of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were prepared by transferring isolated colonies to tubes containing 3 ml of sterile saline. Thereafter, they were standardized by comparing their absorbances to the absorbance of the 0.5 MacFarland scale ($1x10^8$ CFU / ml) in an ELISA microplate reader (Biotek), according to the guidelines of the National Comitte For Clinical Laboratory Standards M07-A10 document[37]. For the yeasts (*Candida albicans* ATCC 10231 and *Candida tropicalis* ATCC 1369), the same procedure was done, however, a 1:50 dilution with Brainand Heart Infusion Broth (BHI) was made to obtain a suspension containing approximately 5.0×10^2 at 2.5×10^3 cells per ml, according to the guidelines of the National Comitee For Clinical Laboratory Standards M27-A2 document (2002)[38], with some modifications.

In the 96-well microplates, 180 μl of fungal pigment solution in Mueller-Hinton broth (for bacteria) or BHI (for yeasts) at different concentrations (1000, 500, 250, 125, 62, 5, 31,25, 15,62 and 7,8 μg/mL) were dispensed to six wells, respectively. Then, 20 μL of the inocula of the bacteria and fungi tested were added to the wells. Positive controls were prepared by dispensing 180 μl of gentamicin at a concentration of 10 μg/ml (for gram-negative bacteria), vancomycin at 8 μg/ml (for Gram-positive bacteria) and Amphotericin B at 16 μg/ml (for the yeasts), and then it was added 20 μl of the inoculum of the tested micro-organisms to six wells, respectively. Negative controls consisted of 180 μl of Mueller-Hinton

broth, for the bacteria, and BHI, for the fungi and 20 µl of the bacterial and fungal inocula. Microplates were then were incubated at 37°C for 24 hours. After incubation, 10 µl of each well was plated in Sabouraud-Dextrose Agar (SDA), for the yeasts, and MHA (Mueller-Hinton Agar), for bacteria.

The minimum inhibitory concentrations were defined as the lowest concentration at which inhibition of growth of the microorganism was observed on the plates compared to the negative control by counting the Colony Forming Units (CFUs), and MBC was defined as the lowest concentration at which no growth was observed on the plates. The experiment was done in duplicate.

4.5.1.2 Statistical analysis

Results were compared using one-way analysis of variance (ANOVA) by applying the Tukey's multiple comparison post-test, using GraphPad Prism 6 (Graphpad Software, CA, USA).

4.6 Evaluation of cytotoxicactivity of IRPE from *T. marneffei*

The cytotoxic effects of the various concentrations of the intracellular red pigment extract (IRPE) from *T. marneffei* on MCF-7 and GM07492 cells were determined by MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] assay. MCF-7 and GM07492 cells (2.0 x 10^4 per well plus DMEM medium) were seeded in triplicate onto a sterile 96-well plate. After a 24-h incubation, the medium was replaced with 100 µL of fresh FBS-free medium which contained IRPE at concentrations of 250, 25, 2.5 and 0.25 µg/mL. A triplicate was also performed for negative control, containing only medium with 10% SFB. The plate was incubated for 24, 48 and 72 h at 37 C in 5% CO_2 and then the media were discarded. Afterwards, the cells were stained with 100 µL of MTT solution at 37 C for 4 h. Thereafter, the supernatant was aspirated, and 100 µL of ethyl alcohol was added to dissolve the formazan. The optical density at 540 nm (OD540) was determined. The experiment was done in duplicate.

The viability of the cells was quantified, in percentage, using the folowing equation:

Cell viability (%) = (ODtest/ODcontrol) x 100

where ODcontrol is the OD570 in the negative control wells (cells incubated with media only) and ODtest is the OD570 of the cells exposed to IRPE.

4.6.1 Statistical analysis

Results were compared using one-way analysis of variance (ANOVA) by applying the Dunnett's multiple comparisons post-test, using GraphPad Prism 6 (Graphpad Software, CA, USA).

Conclusions

The intracellular red pigment extract (IRPE) from *T. marneffei* showed intermediate antibacterial activity against two human pathogenic bacteria: *S. aureus* and *E. coli*. IRPE also exhibited an intermediate cytotoxic activity on MCF-7 and did not exert cytotoxicity on the normal cell line tested.

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Conflict of interest

All authors revised the manuscript and there is no conflict of interest.

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7 CONCLUSÕES

A análise química do extrato pigmentado vermelho intracelular (IRPE) de *T. marneffei* sugere presença de compostos das classes antraquinonas, triterpenos e ácidos fenólicos.

O extrato pigmentado vermelho intracelular (IRPE) de *T. marneffei* apresentou atividade antibacteriana intermediária contra duas bactérias patogênicas humanas*: S. aureus* e *E. coli*. O IRPE também exibiu uma atividade citotóxica intermediária em MCF-7 (linhagem de câncer de mama) e não exerceu citotoxicidade na linhagem celular normal testada. Tendo em vista os resultados encontrados, estudos futuros devem ser realizados para verificar outras possíveis aplicações biotecnológicas do pigmento, como nas indústrias cosmética, alimentícia e/ou têxtil.

Além disso, mais estudos são necessários para elucidar as atividades citostática e antibacteriana dos compostos presentes no extrato responsáveis pelas atividades biológicas encontradas, a fim de identificar os alvos moleculares do mecanismo da inibição e morte das células cancerosas e bacterianas, respectivamente, para auxiliar na aquisição de novos agentes terapêuticos, drogas ou combinações preventivas no tratamento de câncer de mama e/ou infecções bacterianas.

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ANEXOS

Anexo A: Normas de submissão da revista Marine Drugs.

Manuscript Submission Overview

Types of Publications

Marine Drugs has no restrictions on the length of manuscripts, provided that the text is concise and comprehensive. Full experimental details must be provided so that the results can be reproduced. *Marine Drugs* requires that authors publish all experimental controls and make full datasets available where possible (see the guidelines on Supplementary Materials and references to unpublished data).

Manuscripts submitted to *Marine Drugs* should neither been published before nor be under consideration for publication in another journal. The main article types are as follows:

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Submission Process

Manuscripts for *Marine Drugs* should be submitted online at susy.mdpi.com The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the author list (read the criteria to qualify for authorship) and that they have all read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to the submission website. Once you have registered, click here to go to the submission form for *Marine Drugs*. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

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Authors must use the Microsoft Word template or LaTeX template to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the editorial office marinedrugs@mdpi.com. Accepted file formats are:

- *Microsoft Word:* Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, the *Marine Drugs*Microsoft Word template file must be used. Please insert your graphics (schemes, figures, *etc.*) in the main text after the paragraph of its first citation.
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now also use the online application writeLaTeX to submit articles directly to *Marine Drugs*. The MDPI LaTeX template file should be selected from the writeLaTeX template gallery.
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A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work and why it fits the scope of the journal. Confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. Any prior submissions of the manuscript to MDPI journals must be acknowledged. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

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This journal automatically deposits papers to PubMed Central after publication of an issue. Authors do not need to separately submit their papers through the NIH Manuscript Submission System (NIHMS, http://nihms.nih.gov/).

Manuscript Preparation

General Considerations

- **Research manuscripts** should comprise:
  - Front matter: Title, Author list, Affiliations, Abstract, Keywords
  - Research manuscript sections: Introduction, Results, Discussion, Materials and Methods, Conclusions (optional).
  - Back matter: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, References.
- **Review manuscripts** should comprise the front matter, literature review sections and the back matter. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the PRISMA guidelines.
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- **Abbreviations** should be defined in parentheses the first time they appear in the abstract, main text, and in figure or table captions and used consistently thereafter.
- **SI Units** (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible
- **Accession numbers** of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on Deposition of Sequences and of Expression Data.
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Front Matter

These sections should appear in all manuscript types

- **Title:** The title of your manuscript should be concise, specific and relevant. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used.
- **Author List and Affiliations:** Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, and country. At least one author should be designated as corresponding author, and his or her email address and other details should be included at the end of the affiliation section. Please read the criteria to qualify for authorship.
- **Abstract:** The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used. 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the

article: it must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.

- **Keywords:** Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Research Manuscript Sections

- **Introduction:** The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance, including specific hypotheses being tested. The current state of the research field should be reviewed carefully and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the main conclusions. Keep the introduction comprehensible to scientists working outside the topic of the paper.
- **Results:** Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.
- **Discussion:** Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible and limitations of the work highlighted. Future research directions may also be mentioned. This section may be combined with Results.
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Back Matter

- **Supplementary Materials:** Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.
- **Acknowledgments:** All sources of funding of the study should be disclosed. Clearly indicate grants that you have received in support of your research work and if you received funds to cover publication costs. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly and correctly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to FundRef if the manuscript is finally published.
- **Author Contributions:** Each author is expected to have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or have drafted the work or substantively revised it; AND has approved the submitted version (and version substantially edited by journal staff that involves the author’s contribution to the study); AND agrees to be personally accountable for the author’s own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature.
  For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.; Methodology, X.X.; Software, X.X.; Validation, X.X., Y.Y. and Z.Z.; Formal

Analysis, X.X.; Investigation, X.X.; Resources, X.X.; Data Curation, X.X.; Writing – Original Draft Preparation, X.X.; Writing – Review & Editing, X.X.; Visualization, X.X.; Supervision, X.X.; Project Administration, X.X.; Funding Acquisition, Y.Y.", please turn to the CRediT taxonomy for the term explanation. For more background on CRediT, see here. "**Authorship must include and be limited to those who have contributed substantially to the work. Please read the section concerning the criteria to qualify for authorship carefully**".

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- **References:** References must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as EndNote, ReferenceManager or Zotero to avoid typing mistakes and duplicated references. We encourage citations to data, computer code and other citable research material. If available online, you may use reference style 9. below.
- Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

The reference list should include the full title, as recommended by the ACS style guide. Style files for Endnote and Zotero are available.

References should be described as follows, depending on the type of work:

- Journal Articles:

1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range. Available online: URL (accessed on Day Month Year).

- Books and Book Chapters:

2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.
3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.

- Unpublished work, submitted work, personal communication:

4. Author 1, A.B.; Author 2, C. Title of Unpublished Work. status (unpublished; manuscript in preparation).
5. Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name* stage of publication (under review; accepted; in press).
6. Author 1, A.B. (University, City, State, Country); Author 2, C. (Institute, City, State, Country). Personal communication, Year.

- Conference Proceedings:

7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), Proceedings of the Name of the Conference, Location of

Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).

- Thesis:

8. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.

- Websites:

9. Title of Site. Available online: URL (accessed on Day Month Year).

Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited website using a service such as WebCite. Archived websites should be cited using the link provided as follows:

10. Title of Site. URL (archived on Day Month Year).

See the Reference List and Citations Guide for more detailed information.

Preparing Figures, Schemes and Tables

- File for Figures and schemes must be provided during submission in a single zip archive and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.
- *Marine Drugs* can publish multimedia files in articles or as supplementary materials. Please contact the editorial office for further information.
- All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).
- All Figures, Schemes and Tables should have a short explanatory title and caption.
- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.
- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.

Supplementary Materials, Data Deposit and Software Source Code

Data Availability

In order to maintain the integrity, transparency and reproducibility of research records, authors must make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as supplementary information in this journal.

Computer Code and Software

For work where novel computer code was developed, authors should release the code either by depositing in a recognized, public repository or uploading as supplementary information to the publication. The name and version of all software used should be clearly indicated.

Supplementary Material

Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable, however we recommend that common, non-proprietary formats are used where possible.

Unpublished Data

Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

Remote Hosting and Large Data Sets

Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal Data also accepts submissions of data set papers.

Deposition of Sequences and of Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- *New nucleic acid sequences* must be deposited in one of the following databases: GenBank, EMBL, or DDBJ. Sequences should be submitted to only one database.
- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the GEO database or in the NCBI's Sequence Read Archive.
- *New microarray data* must be deposited either in the GEO or the ArrayExpress databases.The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.
- *New protein sequences* obtained by protein sequencing must be submitted to UniProt (submission tool SPIN).

All sequence names and the accession numbers provided by the databases should be provided in the Materials and Methods section of the article.

References in Supplementary Files

Citations and References in Supplementary files are permitted provided that they also appear in the reference list of the main text.

Research and Publication Ethics

Research Ethics

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/), revised in 2013. According to point 23 of this declaration, an approval from an ethics committee should have been obtained before undertaking the research. At a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board should be cited in the Methods Section of the article. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). Editors reserve the right to reject any submission that does not meet these requirements.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

A written informed consent for publication must be obtained from participating patients who can be identified (including by the patients themselves). Patients' initials or other personal identifiers must not appear in any images. For manuscripts that include any case details, personal information, and/or images of patients, authors must obtain signed informed consent from patients (or their relatives/guardians) before submitting to an MDPI journal. Patient details must be anonymized as far as possible, e.g., do not mention specific age, ethnicity, or occupation where they are not relevant to the conclusions. A template permission form is available to download. A blank version of the form used to obtain permission (without the patient names or signature) must be uploaded with your submission.

You may refer to our sample form and provide an appropriate form after consulting with your affiliated institution. Alternatively, you may provide a detailed justification of why informed consent is not necessary. For the purposes of publishing in MDPI journals, a consent, permission, or release form should include unlimited permission for publication in all formats (including print, electronic, and online), in sublicensed and reprinted versions (including translations and derived works), and in other works and products under open access license. To respect patients' and any other individual's privacy, please do not send signed forms. The journal reserves the right to ask authors to provide signed forms if necessary.

Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Any experimental work must also have been conducted in accordance with relevant national legislation on the use of animals for research. For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [1].

Manuscripts containing original descriptions of research conducted in experimental animals must contain details of approval by a properly constituted research ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods section.

Marine Drugs endorses the ARRIVE guidelines (www.nc3rs.org.uk/ARRIVE) for reporting experiments using live animals. Authors and reviewers can use the ARRIVE guidelines as a checklist, which can be found at www.nc3rs.org.uk/ARRIVEchecklist.

1. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Used in Scientific Procedures. Available online: http://www.official-documents.gov.uk/document/hc8889/hc01/0107/0107.pdf.

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The MLH1^{+} cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the Convention on Biological Diversity and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana, Nicotiana benthamiana, Oriza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX , institute, city, country).

Publication Ethics Statement

Marine Drugs is a member of the Committee on Publication Ethics (COPE). We fully adhere to its Code of Conduct and to its Best Practice Guidelines.

The editors of this journal enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *Marine Drugs* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *Marine Drugs* must abide to the following:

- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.
- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.
- Simultaneous submission of manuscripts to more than one journal is not tolerated.
- Republishing content that is not novel is not tolerated (for example, an English translation of a paper that is already published in another language will not be accepted).
- If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our policy regarding publication of publishing addenda and corrections.
- Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license. For further information, see the Rights and Permissions page.
- Plagiarism, data fabrication and image manipulation are not tolerated.
    - **Plagiarism is not acceptable** in *Marine Drugs* submissions.

      Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

Reuse of text that is copied from another source must be between quotes and the original source must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.

If plagiarism is detected during the peer review process, the manuscript will be rejected outright. If plagiarism is detected after publication, we will retract the paper.

- **Image files must not be manipulated or adjusted in any way** that could lead to misinterpretation of the information provided by the original image.

  Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

  If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

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The correct identification of the active components of extracts obtained from marine sources is a key scientific information; thus, the journal will not accept papers reporting only activity of extracts but lacking any chemical characterization of its components.

Reports on previously undescribed compounds must include, as supplementary data, 1H and either 13C or key 2D NMR spectra described in the discussion. The identification of known compounds in extracts should be safely supported with chromatographic and/or spectroscopic data (e.g. LC/GC retention times and/or UV, mass spectra), as well as comparison with data of authentic samples or previously published values. When previously reported compounds are isolated and used in biological activity assays, the 1H NMR spectrum should be given in supplementary data as a proof of purity. Authors should consider very carefully potential sources of artifacts and contaminants resulting from extraction procedures or sample handling.

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During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last five years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as

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Expanded and high quality conference papers can be considered as articles if they fulfil the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright of the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper. *Marine Drugs* does not publish pilot studies or studies with inadequate statistical power.

Qualification for Authorship

Each author is expected to have made substantial contributions to the conception or design of the work; acquisition, analysis, or interpretation of data; the creation of new software used in the work; and/or writing or substantively revising the manuscript. In addition, all authors must have approved the submitted version (and any substantially modified version that involves the author’s contribution to the study); AND agrees to be personally accountable for the author’s own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even those in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature. Note that acquisition of funding, collection of data, or general supervision of the research group do not, by themselves, justify authorship.Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgements.

More detailed guidance on authorship is given by the International Council of Medical Journal Editors (ICMJE). The journal also adheres to the standards of the Committee on Publication Ethics (COPE) that "all authors should agree to be listed and should approve the submitted and accepted versions of the publication. Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication (e.g. answering reviewers' comments)." [1]. We reserve the right to request confirmation that all authors meet the authorship conditions.

1. Wager, E.; Kleinert, S. Responsible research publication: international standards for authors. A position statement developed at the 2nd World Conference on Research Integrity, Singapore, July 22-24, 2010. In Promoting Research Integrity in a Global Environment; Mayer, T., Steneck, N., eds.; Imperial College Press / World Scientific Publishing: Singapore; Chapter 50, pp. 309-16.

Editorial Procedures and Peer-Review

Initial Checks

All submitted manuscripts received by the Editorial Office will be checked by a professional in-house *Managing Editor* to determine whether they are properly prepared and whether they follow the ethical policies of the journal, including those for human and animal experimentation. Manuscripts that do not fit the journal's ethics policy or do not meet the standards of the journal will be rejected before peer-review. Manuscripts that are not properly prepared will be returned to the authors for revision and resubmission. After these checks, the *Managing Editor* will consult the journals' *Editor-in-Chief* or *Associate Editors* to determine whether the manuscript fits the scope of the journal and whether it is scientifically sound. No judgment on the potential impact of the work will be made at this stage. Reject decisions at this stage will be verified by the *Editor-in-Chief*.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include *Editorial Board members* and Guest Editors of the journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past five years and should not currently work or collaborate with any of the institutions of the co-authors of the submitted manuscript.

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Editorial Decision and Revision

All the articles, reviews and communications published in MDPI journals go through the peer-review process and receive at least two reviews. The in-house editor will communicate the decision of the academic editor, which will be one of the following:

- *Accept after Minor Revisions*:
  The paper is in principle accepted after revision based on the reviewer's comments. Authors are given five days for minor revisions.
- *Reconsider after Major Revisions*:
  The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer's comments cannot be revised. Usually, only one round of major revisions is allowed. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be returned to the reviewer for further comments.
- *Reject and Encourage Resubmission*:
  If additional experiments are needed to support the conclusions, the manuscript will be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.
- *Reject*:
  The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments. The *Managing Editor* of the journal will forward the manuscript and related information (including the identities of the referees) to the Editor-in-Chief, Associate Editor, or Editorial Board member. The academic Editor being consulted will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage is final and cannot be reversed.

In the case of a special issue, the *Managing Editor* of the journal will forward the manuscript and related information (including the identities of the referees) to the *Editor-in-Chief* who will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage will be final and cannot be reversed.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the www.mdpi.comwebsite.

Clinical Trials Registration

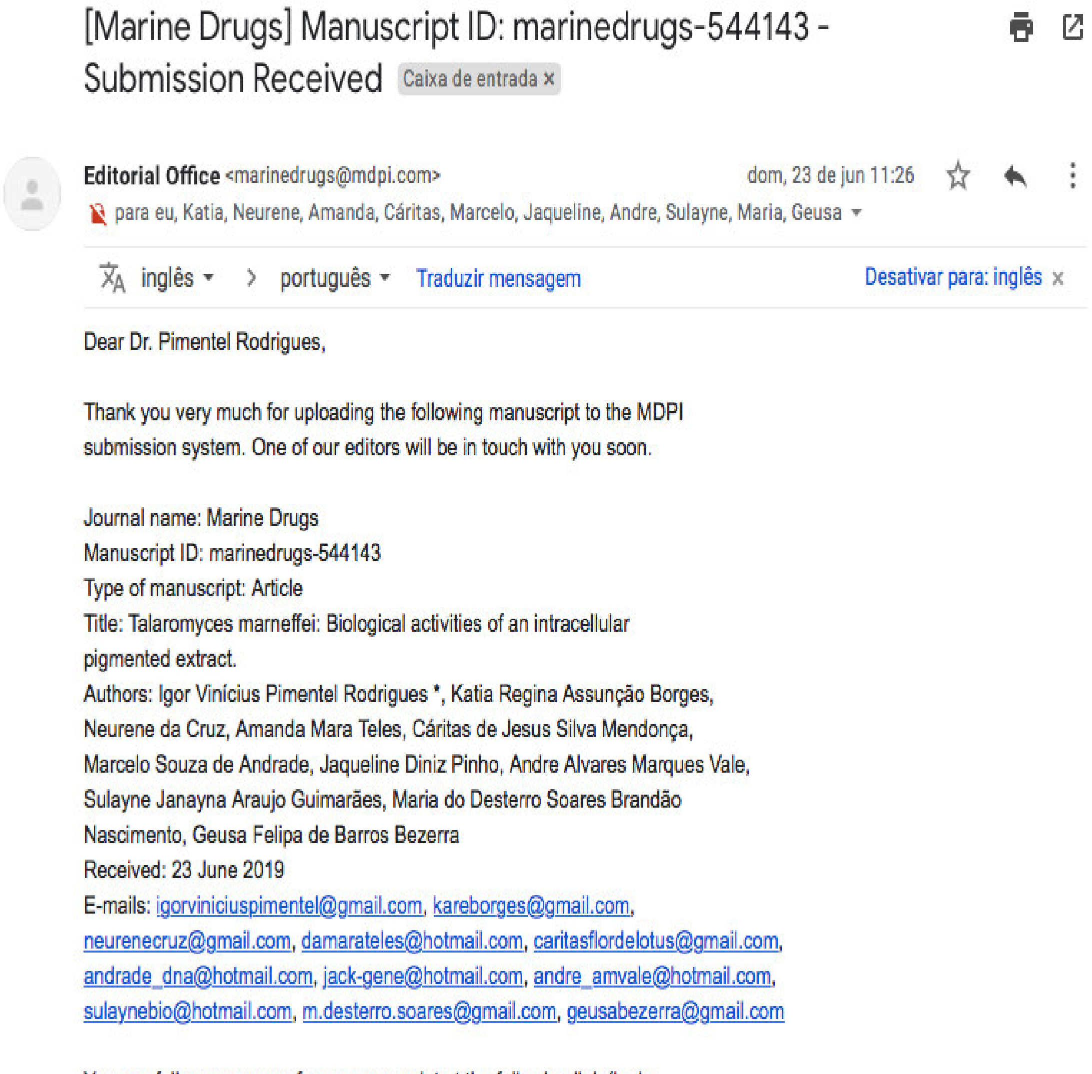
Registration

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register or and to cite a reference to the registration in the Methods section. Suitable databases include clinicaltrials.gov, the EU Clinical Trials Register and those listed by the World Health Organisation International Clinical Trials Registry Platform.

CONSORT Statement

Marine Drugs requires a completed CONSORT 2010 checklist and flow diagram as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (http://www.consort-statement.org) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist. Meeting these basic reporting requirements will greatly improve the value of your trial report and may enhance its chances for eventual publication.

Anexo B: Comprovante de submissão do manuscrito:



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