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Karine Bizzi Schlemmer

SUSCETIBILIDADE *IN VITRO* A ANTIFÚNGICOS, ÓLEOS ESSENCIAIS E MODELO EXPERIMENTAL DE INFECÇÃO POR *Malassezia pachydermatis*

Santa Maria, RS
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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Farmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de Doutora em Farmacologia.

Orientador: Prof. Dr. Janio Morais Santurio

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RESUMO

SUSCETIBILIDADE *IN VITRO* A ANTIFÚNGICOS, FRAÇÕES DE ÓLEOS ESSENCIAIS E MODELO EXPERIMENTAL DE INFECÇÃO POR *Malassezia pachydermatis*

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Malassezia pachydermatis é uma levedura zoofílica encontrada principalmente no conduto auditivo de várias espécies de animais, podendo, também, ser isolada da pele de seres humanos. Antifúngicos azólicos e poliênicos são o tratamento de escolha para infecções de pele, relacionadas à *Malassezia.* No entanto, estudos têm demonstrado à ocorrência de resistência ou menor suscetibilidade desta levedura à algumas drogas. Neste contexto, este estudo teve como objetivos: avaliar a combinação *in vitro* de antifúngicos entre si e antifúngicos e frações de óleos essenciais (OEs), com base no protocolo M27-A3, com modificações, pelo método de "*checkerboard*". Em adição buscou-se avaliar os efeitos citotóxicos do carvacrol (CRV), cinamaldeído (CIN) e timol (THY) em fibroblastos de camundongos (linhagem celular 3T3) e desenvolver um modelo de infecção experimental em camundongos para *M. pachydermatis*. As combinações entre antifúngicos apresentaram um predomínio de resultados indiferentes, com as maiores taxas de sinergismo para as combinações de itraconazol+caspofungina e clotrimazol+caspofungina (55,17%). As combinações de antifúngicos e frações de OEs apresentaram 80% de interações sinérgicas para as combinações de CRV + nistatina, THY + nistatina e CRV + miconazol. Nos testes de citotoxicidade utilizando o MTT ((3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina) CRV e THY não mostraram-se citotóxicos na maioria das concentrações testadas (1-50 µg / mL), mas o CIN reduziu a viabilidade celular em todas as concentrações. Houve uma diminuição nas concentrações de espécies reativas de oxigênio na presença de CRV, CIN e THY em 24 e 72 h, mas observou-se um aumento na concentração de 50 µg / mL de CIN em 24 h de incubação. Foi observado um aumento nos níveis de fragmentação do DNA nas concentrações de 50 e 100 µg / mL de CRV, 25-100 µg / mL de CIN e na maioria das concentrações de THY. Observou-se um aumento (até 5%) na apoptose após exposição a CRV e THY (25-50 µg / mL) em 24-72 h e um aumento no nível de apoptose tardia (até 90%) com CIN (25 µg / mL) em 24-72 h. Foi desenvolvido um modelo de infecção para *M. pachydermatis* em camundongos Swiss imunocomprometidos com ciclofosfamida (CYP) e acetato de hidrocortisona (HCA). Esse protocolo de imunossupressão já é usado em diversos estudos para facilitar infecções fúngicas experimentais. A imunossupressão prévia dos camundongos permitiu a infecção da derme e da orelha de todos os animais. Além disso, os achados histopatológicos mostraram leveduras, inflamação e hiperqueratose, confirmando otite e dermatite por *M. pachydermatis*. Os resultados do presente estudo mostraram algumas combinações com altos percentuais de sinergismo, as quais, permitem a elaboração de novas hipóteses de tratamento e apontam algumas combinações candidatas a serem avaliadas em modelos experimentais *in vivo*.

Palavras-chave: *Malassezia pachydermatis*; Suscetibilidade; Antifúngicos; Óleos essenciais; Modelo de infecção.

ABSTRACT

IN VITRO SUSCETIBILITY TO ANTIFUNGALS, FRACTIONS OF ESSENTIAL OILS AND EXPERIMENTAL MODEL OF INFECTION BY *Malassezia pachydermatis*

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ADVISOR: Janio Morais Santurio

Malassezia pachydermatis is a zoophilic yeast mainly found in the auditory canal of various animal species, and may also be isolated from the skin of humans. Azole and polyene antifungals are the treatment of choice for skin infections, related to *Malassezia*. However, studies have demonstrated the occurrence of resistance or lower susceptibility of this yeast to some drugs. In this context, the objective of this study was to evaluate the in vitro combination of antifungal agents and antifungals and essential oils fractions (EOs), based on the M27-A3 protocol, with modifications by the checkerboard method. In addition, we sought to evaluate the cytotoxic effects of carvacrol (CRV), cinnamaldehyde (CIN) and thymol (THY) in mouse fibroblasts (3T3 cell line) and to develop an experimental infection model in mice for *M. pachydermatis*. Combinations of antifungals showed a predominance of indifferent results, with the highest synergism rates for combinations of itraconazole+ caspofungin and clotrimazole+caspofungin (55.17%). Combinations of antifungal and OE fractions showed 80% synergistic interactions for combinations of CRV+nystatin, THY+ nystatin and CRV+miconazole. In cytotoxicity tests using MTT (β- (4,5-dimethylthiazol-2yl) -2,5-diphenyl tetrazoline bromide) CRV and THY were not cytotoxic at most of the concentrations tested (1-50μg/mL), but CIN reduced cell viability at all concentrations There was a decrease in the concentrations of reactive oxygen species in the presence of CRV, CIN and THY at 24 and 72h, but an increase of 50μg/ml of CIN in 24h of incubation. An increase in DNA fragmentation levels at concentrations of 50 and 100μg/mL of CRV, 25-100μg/mL of CIN and at most THY concentrations was observed. an increase (up to 5%) in apoptosis after exposure to CRV and THY (25-50μg/mL) in 24-72h and an increase in the level of late apoptosis (up to 90%) with CIN (25μg/mL) in 24-72h An infection pattern for M. pachydermatis in Swiss mice immunocompromised with cyclophosphamide (CYP) and acetate hidrocortisone (HCA). This immunosuppressive protocol is already used in several studies to facilitate experimental fungal infections. Previous immunosuppression of the mice allowed infection of the dermis and ear of all animals. In addition, histopathological findings showed yeast, inflammation and hyperkeratosis, confirming otitis and dermatitis by *M. pachydermatis*. The results of the present study showed some combinations with high percentages of synergism, which allow the elaboration of new treatment hypotheses and point out some candidate combinations to be evaluated in experimental models *in vivo*.

Keywords: *Malassezia pachydermatis*. Susceptibility. Antifungals. Essencial oils. Model of infection.

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ARTIGO CIENTÍFICO

MANUSCRITO 1

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LISTA DE ABREVIATURAS E SIGLAS

SNC	sistema nervoso central
H^+	íons de hidrogênio
K^+	íons de potássio
Ca^+	íons de cálcio
CLSI	*Clinical and Laboratory Standards Institute*
EUCAST	*European Committee on Antimicrobial Susceptibility Testing*
H^+- ATPase	bomba de prótons
FLC	fluconazol
ITZ	itraconazol
KTZ	cetoconazol
CLZ	clotrimazol
MCZ	miconazol
TRB	terbinafina
NYS	nistatina
CSP	caspofungina
SDB	sabouraud dextrose
CUB	caldo ureia de Christensen
DXB	caldo dixon
CRV	carvacrol
CIN	cinamaldeído
THY	timol
DCFH - DA	dicloro-dihydro-fluoresceína diacetato
ROS	espécie reativa de oxigênio
DNA	ácido desoxirribonucleico
COX	cicloxigenase
ATP	adenosina trifosfato
HCA	acetato de hidrocortisona
CYP	ciclofosfamida

SUMÁRIO

APRESENTAÇÃO

A seção **INTRODUÇÃO** inclui uma apresentação sobre o assunto investigado e sua relevância, bem como, uma revisão bibliográfica sobre os temas discutidos nesta Tese. Os resultados encontram-se nos tópicos **ARTIGO CIENTÍFICO** e **MANUSCRITO**, os quais englobam as seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências, representando a íntegra deste estudo. Ambos os trabalhos estão formatados de acordo com o periódico aos quais foram publicados e/ou submetidos.

Os tópicos **DISCUSSÃO** e **CONCLUSÕES** apresentam interpretações e comentários gerais acerca do conteúdo abordado nesta tese, assim como sugestões de abordagens futuras. As **REFERÊNCIAS** remetem somente às citações que aparecem nos tópicos **INTRODUÇÃO** e **DISCUSSÃO**. Na seção **ANEXOS** encontram-se a carta de permissão do artigo 1, os comprovantes de submissão dos manuscritos e a carta de aprovação do Comitê de Ética e Pesquisa da Universidade Federal de Santa Maria (UFSM).

1 INTRODUÇÃO

Devido à capacidade de *Malassezia* spp. em produzir infecções superficiais e sistêmicas tanto em indivíduos imunocomprometidos como imunocompetentes, estas leveduras são consideradas importantes patógenos emergentes (ANGIOLELLA et al., 2017). *Malassezia pachydermatis*, por sua vez, é comumente associada à etiologia de otite externa e dermatite seborreica, mais raramente, a infecções sistêmicas principalmente em cães e gatos, dentre outros animais domésticos e selvagens (VELEGRAKI et al., 2015).

Apesar de *M. pachydermatis* se tratar de um micro-organismo zoofílico e geralmente considerado um agente não transmissível, tem ocorrido relatos de micoses sistêmicas em indivíduos imunocomprometidos com transmissão a partir de animais. Além disso, também há a ocorrência de formação de biofilmes por esta levedura em materiais hospitalares, principalmente em UTIs neonatais (BIRCHARD & SHERDING, 2008; CHANG et al., 1998; MORRIS et al., 2005; FIGUEIREDO et al., 2012). *M. pachydermatis* tem sido associada principalmente a infecções em neonatos, cujos principais fatores de risco incluem o uso de nutrição parenteral total, uso prolongado de cateter ou casos de pacientes imunocomprometidos (ROMAN et al., 2016).

O gênero *Malassezia* caracteriza-se por leveduras lipofílicas que são encontradas na superfície cutânea e nas mucosas de mamíferos e aves. São conhecidas atualmente 17 espécies, sendo elas: *Malassezia furfur*, *Malassezia sympodialis*, *Malassezia pachydermatis*, *Malassezia globosa*, *Malassezia obtusa*, *Malassezia restricta*, *Malassezia slooffiae*, *Malassezia caprae*, *Malassezia equina*, *Malassezia dermatis*, *Malassezia japonica*, *Malassezia yamatoensis*, *Malassezia nana*, *Malassezia cuniculi*, *Malassezia brasiliensis*, *Malassezia psittaci* e *Malassezia arunalokei* (CABANES et al., 2011; CABAÑES et., 2016; HONNAVAR et al., 2016; LOPES, 2008). *M. pachydermatis* é a única espécie não lipodependente, sendo frequentemente isolada da microbiota da pele e conduto auditivo de cães e gatos, apresentando caráter oportunista (GIRÃO et al., 2004; GUILLOT & GHÉHO, 1995). Essas leveduras se apresentam morfologicamente como esféricas, elipsoidais ou alongadas, que se reproduzem por brotamento unipolar (GUILLOT et al., 1998).

As leveduras do gênero *Malassezia* utilizam lipídios como fonte de carbono e necessitam da suplementação com ácidos graxos de cadeia longa para o crescimento *in vitro*, sendo assim denominadas lipodependentes. A exceção é *M. pachydermatis*, cuja suplementação é desnecessária (GUÉHO et al., 1996).

Os antifúngicos azólicos são os principais fármacos utilizados no tratamento da malasseziose (FARIA, 2010), entretanto, tem sido descrito, que o uso indiscriminado dos azólicos têm gerado o desenvolvimento de cepas resistentes ou menos suscetíveis a alguns fármacos desta classe (BRITO et al., 2009). Além disso, tem se observado o surgimento de resistência cruzada entre azólicos de estrutura similar, a exemplo, de itraconazol e posaconazol (FERREIRA et al., 2005; GOODMAN & GILMAN, 1996; QIAO et al., 2008; WILLIAMS et al., 2002).

Neste contexto, a busca de novos agentes antifúngicos continua sendo um assunto relevante e digno de investigação, uma vez que são poucas as classes de agentes antifúngicos disponíveis e o desenvolvimento de resistência antifúngica tem aumentado ao longo dos anos nesta espécie. Diante disso, a combinação *in vitro* entre antifúngicos e o estudo de fitoquímicos isolados de óleos essenciais com ação antifúngica são importantes. Além disso, até o momento não existe um modelo experimental para malasseziose. O uso de modelos experimentais são importantes para avaliar esses tratamentos *in vivo*.

2 REVISÃO BIBLIOGRÁFICA

2.1. O GÊNERO *Malassezia*

O gênero *Malassezia* pertence ao Filo *Basidiomycota*, Classe *Malasseziomycetes*, Ordem *Malasseziales* e Família *Malasseziaceae* (WANG et al., 2014). De forma geral, as espécies desse gênero são associadas a uma variedade de doenças da pele, incluindo a pitiríase versicolor, dermatite seborreica, foliculite e alguns subconjuntos de psoríase, dermatite atópica e otite externa (GAITANIS et al., 2012). O gênero *Malassezia* caracteriza-se por apresentar células esféricas ou elipsoides (formato de garrafa), com brotamento único em base larga. As características morfológicas do gênero incluem parede celular espessa, com diversas camadas, apresentando protuberâncias na parte interna da parede, correspondendo à invaginação da membrana plasmática. A reprodução é assexuada com produção de blastoconídeos por um processo monopolar repetitivo ou por brotamento, formando uma célula globosa, oval ou cilíndrica, adquirindo formato alongado quando se desliga da célula-mãe (COUTINHO, 2003).

Em 1846, Eichstedt reconheceu a etiologia fúngica da pitiríase versicolor, sendo considerada uma micose superficial, benigna e crônica. No entanto, o agente etiológico da pitiríase versicolor não recebeu nenhuma designação até 1853, quando Robin denominou o agente causador da pitiríase versicolor em humanos de *Microsporum furfur*, por associá-lo ao *Microsporum audoumii* e causar lesões com características furfuráceas relacionando-o com dermatófitos (GUILLOT et al., 1995; SLOOF, 1971). Em 1847, Sluyer descreveu detalhadamente essas estruturas fúngicas que receberam a denominação descrita por Robin (GUILLOT & GUÉHO, 1995).

Em 1925, Weidman isolou a levedura de um rinoceronte indiano (*Rhinoceras unicornis)* com lesões de pele, sendo primeiramente denominada *Pityrosporum pachydermatis* devido à semelhança com *Pityrosporum* sp. humano e com a característica de não apresentar lipodependência (GUILLOT & BOND, 1999). Em 1934, Lodder estudou essa característica e concluiu que a levedura isolada por Weidman crescia razoavelmente bem em meios de cultura sem suplementação com lipídios, diferindo das espécies *Pityrosporum ovale* e *Pityrosporum orbiculare* (GUILLOT & BOND, 1999).

Em 1955, Gustafson substituiu a nomenclatura de *P. pachydermatis* por *P. canis* e foi estabelecido, em 1974, que todas as leveduras do gênero que crescessem sem suplementação

de lipídios seriam agrupadas em um único táxon, *Pityrosporum canis,* que mais tarde foi substituído por *Malassezia pachydermatis* (GUILLOT & BOND, 1999).

As primeiras espécies reconhecidas do gênero *Malassezi*a foram *Malassezia furfur*, lipodependente e *Malassezia pachydermatis*, não lipodependente (SCHIOTTFELDT et al., 2002). Em 1990, Simmons e Guéo identificaram a terceira espécie do gênero: *Malassezia sympodialis*, reconhecida através de técnicas moleculares.

Em 1993, a taxonomia do gênero *Malassezia* foi reconhecida através do sequenciamento do rRNA (GUILLOT & GUÉHO, 1993). Em 1996, Guého et al. descreveram e nomearam sete espécies de *Malassezia*: *Malassezia furfur*, *Malassezia sympodialis*, *Malassezia obtusa*, *Malassezia globosa*, *Malassezia restricta*, *Malassezia slooffiae* e *Malassezia pachydermatis*, a única lipídeo não dependente. Mais tarde, outras espécies de *Malassezia* foram descritas, incluindo *Malassezia dermatis* (SUGITA et al., 2002), *Malassezia japonica* (SUGITA et al., 2003), *Malassezia nana* (HIRAI et al., 2004), *Malassezia yamotoensis* (SUGITA et al., 2004*), Malassezia eqüina*, *Malassezia caprae* (CAFARCHIA et al., 2008) e *Malassezia cuniculi* (CABAÑES et al., 2011).

Recentemente, foram isoladas e identificadas três novas espécies. *Malassezia brasiliensis* e *Malassezia psittaci* foram isoladas de papagaios (CABAÑES et al., 2016) enquanto que a *Malassezia arunalokei* foi isolada de pacientes imunocompetentes com dermatite seborreica (HONNAVAR et al., 2016). Atualmente, o gênero *Malassezia* inclui 17 espécies, a maioria delas são dependentes de lipídios, enquanto que, a espécie *M. pachydermatis*, é considerada lipofílica não dependente (CABAÑES, 2014).

2.2 *Malassezia pachydermatis*

M. pachydermatis é uma levedura zoofílica encontrada principalmente no conduto auditivo de várias espécies de animais, podendo, entretanto, ser isolada da pele de seres humanos. É um fungo lipofílico, porém não-lipodependente, sendo, assim, capaz de crescer em ágar Sabouraud sem a necessidade da adição de fonte de ácidos graxos de cadeia longa, o que o diferencia das outras espécies. (CABAÑES et al., 2007; SCHLOTTFELDT et al., 2002; VARGAS et al., 2004).

M. pachydermatis é considerada um habitante normal e patógeno oportunista do meato acústico externo de cães e gatos. Pela alta frequência de isolamento no conduto auditivo de cães com otite externa e na pele de animais com dermatite pruriginosa, torna-se um importante invasor patogênico secundário em várias espécies animais (BOND et al., 1995).

Assim como os ácaros do gênero *Demodex* spp. e as bactérias do gênero *Staphylococcus* spp., a levedura *M. pachydermatis* é constituinte da microbiota sapróbia cutânea de cães e gatos, embora seja um agente oportunista (NAHAS, 1997).

Os sinais clínicos são caracterizados por prurido moderado a intenso, alopecia local ou generalizada, escoriações, eritema e seborréia, geralmente apresentando odor corporal desagradável, rançoso e seborreico, pele espessada e áspera (WILKINSON & HARVEY, 1996). Nos casos crônicos podem ser observados hiperpigmentação, liquenificação e hiperceratose. As lesões podem se desenvolver nos espaços interdigitais, parte ventral do pescoço, axilas, região perineal e dobras cutâneas (MEDLEAU & HNILICA, 2003).

M. pachydermatis também tem sido associada a infecções sistêmicas no homem, particularmente em pacientes imunocomprometidos e em neonatos (ROMAN et al., 2016). As leveduras do gênero *Malassezia* em humanos estão associadas a quadros patológicos como pitiríase versicolor, dermatite seborreica e dermatite atópica, que anteriormente eram apenas associadas à espécie *M. furfur* (SCHIOTTFELDT et al., 2002).

Morfologicamente, *M. pachydermatis* apresenta células ovais pequenas (2µm-2,5µm x 4µm-5µm). Os brotos, que são os maiores entre todas as espécies, surgem na base larga, onde pode ser observado um colarete ou cicatriz devido a sucessivos brotamentos (SCHLOTTFELDT et al., 2002; VARGAS et al., 2004).

As colônias são opacas de coloração amarelo creme ou marrom alaranjado e a textura é seca e granulosa, algumas vezes gordurosa (GUILLOT et al., 1996). *M. pachydermatis* é particularmente sensível ao frio e a maioria das cepas tornam-se inviáveis após três meses em temperatura à 4ºC (GUILLOT & BOND, 1999). Após sete dias de incubação à 37ºC as leveduras são mantidas vivas em temperatura ambiente.

O isolamento de *M. pachydermatis* é realizado em meio de cultivo ágar Saboraud dextrose, acrescido de cloranfenicol e ciclohexamida, incubado sob temperaturas entre 27°C e 37°C. Este meio permite o isolamento da maioria das espécies de fungos responsáveis por dermatopatias em carnívoros, tais como os dermatófitos e as leveduras. Na rotina laboratorial, meios de cultivo suplementados com uma fonte de ácidos graxos têm sido utilizados para cultivo de *Malassezia* spp., tais como o ágar Dixon modificado (GUILLOT et al., 1998).

Apesar dessas características, pode-se também identificar a espécie *M. pachydermatis* através de técnicas moleculares (AIZAWA et al., 1999, 2001; CARFACHIA et al., 2007; GUILLOT et al., 1997).

2.3 MODELOS EXPERIMENTAIS

O modelo murino é um dos modelos experimentais mais utilizados para o estudo das infecções fúngicas, principalmente devido à similaridade dos sistemas imunológicos e fisiológicos com o dos humanos. No entanto, até o momento não existe um modelo experimental estabelecido para malasseziose. Estudos *in vivo* sobre patogênese ou tratamento da doença foram realizados em cães infectados experimentalmente.

A suscetibilidade de cães como modelo experimental foi demonstrada através da inoculação de *Malassezia paqhydermatis* na pele normal de dez cães da raça Beagle. Quatro dos seis cães desafiados sem oclusão desenvolveram lesões transitórias geralmente caracterizadas clinicamente por eritema e pápulas, e nos achados histológicos por hiperplasia epidérmica e dermatite. A oclusão induziu lesões mais persistentes, que desapareceram em 24 dias. Em quatro cães desafiados com oclusão, ocorreram lesões na pele. Eritema e pápulas foram mais graves em três cães. Este estudo sugere que a resistência da pele canina saudável à infecção por *M. pachydermatis* é mediada por respostas locais de hipersensibilidade tardia e / ou mecanismos imunes epidérmicos inatos (BOND et al., 2004).

Outro estudo realizado por ROSENBERG et al. (1980) utilizaram coelhos como modelo de infecção para indução de *Malassezia ovale.* Os coelhos desenvolveram lesões semelhantes à psoríase humana.

Van Cutsem et al. (1990) induziram infecção de *Pytirosporum ovale* em cobaias. Após sete dias consecutivos de infecção, observaram-se lesões com características de caspa, dermatite seborreica, inflamação, eritema, crostas, descamação, hiperceratose e paraqueratose.

Outro estudo foi realizado utilizando cobaias e camundongos infectados com *Pityrosporum ovale* e *Pityrosporum orbiculare.* Aspectos clínicos e histológicos mostraram o desenvolvimento de leveduras no estrato córneo, com hiperqueratose no óstio folicular e no bulbo piloso, características semelhantes à dermatite seborreica humana (DROUHET et al., 1980).

2.4 AGENTES ANTIFÚNGICOS

O número de fármacos antifúngicos disponíveis para o tratamento das infecções fúngicas ainda é reduzido, comparado com a grande variedade de fármacos e associações antibacterianas. Devido às semelhanças filogenéticas, fungos e mamíferos possuem algumas vias metabólicas homólogas, isso faz com que exista maior dificuldade no desenvolvimento

de agentes antifúngicos seletivos, havendo a necessidade de descobrir novos alvos fúngicos exclusivos que poderão ser explorados (PAPPAS et al, 2009).

Diferentes classes de agentes antifúngicos estão disponíveis no mercado, como as alilaminas (terbinafina e naftifina), derivados poliênicos (anfotericina B e nistatina), antimetabólitos (5-flucitosina), azólicos (cetoconazol, clotrimazol, miconazol, itraconazol, fluconazol, voriconazol, ravuconazol e posaconazol) e inibidores da síntese de glucana (caspofungina, anidulafungina e micafungina) (DERESINSKI & STEVENS, 2003; JOHNSON et al, 2004). Os fármacos com ação sobre os esteróides da membrana plasmática dos fungos (ergosterol) incluem os derivados poliênicos, azólicos e alilaminas.

Azólicos e poliênicos são freqüentemente empregados no tratamento das infecções de pele causadas por *Malassezia* em humanos e animais (TEELEN et al., 2018). Em infecções sistêmicas, a terapia oral com fluconazol ou itraconazol pode ser utilizada (GUPTA & FOLEY, 2015; HALD et al., 2015). Além disso, o tratamento intravenoso com anfotericina B tem sido utilizado para tratar infecções sistêmicas em bebês prematuros (TEELEN et al., 2018).

2.4.1 Poliênicos

Os antifúngicos poliênicos ligam-se diretamente ao ergosterol, formando canais (poros), que aumentam a permeabilidade da membrana, com perda do material citoplasmático, o que pode resultar em morte celular (DREW, 2010).

Os poliênicos são fungicidas de amplo espectro, produzidos por bactérias do gênero *Streptomyces*. Fazem parte desta classe de antifúngicos, a nistatina e a anfotericina B. A nistatina foi descoberta em 1950 por Hazen e Brown, pesquisadoras dos Laboratórios de Pesquisas do Departamento de Saúde do Estado de Nova Iorque, EUA (GROESCHKE et al., 2006; HAC-WYDRO & DYNAROWICZ-LATKA, 2006a; HAC-WYDRO & DYNAROWICZ-LATKA, 2006b; TAVARES, 2001). Devido a sua toxicidade, a nistatina é usada somente para o tratamento tópico (BEM-AMI et al., 2008). A anfotericina B foi desenvolvida em 1956 e utilizada como principal agente antifúngico até o início da década de 1990 para tratamento sistêmico (MAERTENS, 2004).

2.4.2 Azólicos

Os derivados azólicos interferem na síntese de ergosterol através da inibição da C-14-α-desmetilase, bloqueando uma etapa precursora na síntese de ergosterol (DREW, 2010). Os azólicos são antifúngicos caracterizados por apresentarem um anel pentagonal na sua estrutura molecular, unido por uma ligação carbono-nitrogênio com outros anéis aromáticos. Estes agentes antifúngicos são classificados em imidazólicos quando o anel pentagonal possui três átomos de carbono e dois de nitrogênio (miconazol e cetoconazol) e triazólicos quando o anel possui dois átomos de carbono e três de nitrogênio (fluconazol, itraconazol, voriconazol, posaconazol e ravuconazol) (CATALÁN & MOONTEJO, 2006). Os triazólicos representam um novo grupo de azólicos com grande eficiência e baixa toxicidade. Possuem alta afinidade pelo citocromo P450 fúngico e baixa afinidade pelo citocromo P450 dos mamíferos (SPINOSA, 2002).

O fluconazol é um fármaco fungistático, com ampla distribuição em todo o organismo. Ao contrário dos imidazóis e do itraconazol, o fluconazol atinge concentrações elevadas no líquido cefalorraquidiano, sendo, portanto, o medicamento de escolha para o tratamento da maioria das meningites fúngicas (SLAGLE, 2005). Pode ser administrado tanto por via oral, quanto intravenosa. Possui uma excelente biodisponibilidade, apresenta boa penetração cérebro-espinhal e alcança níveis de quase 80% no sangue (COLOMBO et al., 2007; HAJJEH et al., 2004). A concentração plasmática máxima é de 4 a 8 μg/mL após doses repetidas de 100 mg. A excreção renal representa mais de 90% da eliminação, e a meia-vida de eliminação é de 25 a 30 horas (BENNETT, 2006).

O itraconazol apresenta atividade antifúngica contra espécies de *Candida* spp., *Malassezia* spp., *Cryptococcus neoformans*, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides immitis* e dermatófitos (BOSSCHE et al., 2003). Possui consideráveis vantagens sobre o fluconazol no tratamento de aspergilose e esporotricose, entretanto, o fluconazol demonstra um perfil farmacológico e toxicológico mais favorável (TERRELL, 1999). Além disso, é usado no tratamento de doenças causadas por fungos em humanos e também no tratamento da dermatofitose em gatos (RIGOPOULOS et al., 2004). Apresenta eficácia após administração de 5 mg / kg por via oral a cada dois dias, durante três semanas (terapia em pulso) no tratamento da dermatite causada por *Malassezia* spp. A terapia em pulso tem a vantagem de reduzir os custos e os efeitos colaterais, melhorando a adesão ao tratamento. Entretanto, as infecções graves podem exigir um tratamento mais prolongado ou doses mais elevadas (DRENO et al., 2003). O itraconazol é administrado por via oral e, após

absorção, sofre extenso metabolismo hepático. Estudos de farmacocinética mostraram que níveis terapêuticos ativos de itraconazol em humanos são mantidos por muito mais tempo em alguns tecidos infectados do que no plasma (BOSSCHE et al., 2003). É altamente lipossolúvel, com meia vida de 36 horas, sendo excretado na urina (BENNETT, 2003).

O cetoconazol mostra-se eficiente contra micoses superficiais e profundas (LACAZ et al., 2002). Entretanto, é comum a ocorrência de recidiva após tratamento aparentemente bem sucedido (SILVA, 2006). Abrange desde *Malassezia* spp. e outros dermatófitos, resistentes ou não à griseofulvina (LACAZ et al., 2002). Foi o primeiro azólico de uso oral no tratamento de micoses sistêmicas e até os dias de hoje, é utilizado em micoses dermatológicas e não dermatológicas na medicina veterinária de pequenos animais (FARIAS & GIUFRIDA, 2002; JAHAM et al., 2000). Distingue-se dos triazóis pela sua maior capacidade em inibir as enzimas do citocromo P450 dos mamíferos, ou seja, é menos seletivo para o citocromo P450 fúngico do que os mais novos derivados azólicos. Distribui-se amplamente por todos os tecidos e líquidos teciduais, porém só atinge concentrações terapêuticas no SNC quando administrado em doses altas (SILVA, 2006). O principal efeito adverso é a hepatotoxicidade, que é rara, mas que pode se tornar fatal. Outros efeitos colaterais que podem ocorrer consistem em distúrbios gastrintestinais e prurido (BENNETT, 2006). Em cães, tem-se relatado inapetência, prurido e alopecia como efeitos indesejáveis produzidos pelo cetoconazol. Observa-se ainda a elevação de enzimas hepáticas, aconselhando-se, portanto, monitorar os efeitos hepatotóxicos do cetoconazol através de dosagem sérica de transaminases hepáticas (APPELT & CAVALCANTE, 2008).

O clotrimazol é utilizado topicamente no tratamento de dermatofitose, candidose e malasseziose (SAWYER et al., 1975). Além disso, o clotrimazol a 1% é indicado no tratamento de otites externas (LOBELL et al., 1995).

O miconazol é bastante utilizado como antifúngico tópico ou por via oral para o tratamento das infecções fúngicas do trato gastrintestinal (BENNETT, 2006). Entretanto, já foi muito utilizado por via parenteral para o tratamento de micoses sistêmicas (MCDOUGALL et al., 1982; NEGRONI et al., 1977; ROLAN et al., 1983; SUNG et al., 1977). O miconazol é um imidazol de amplo espectro de atividade antifúngica e antibacteriana, particularmente em cocos Gram-positivos (*Staphylococcus* spp. e *Streptococcus* spp.). Este antifúngico é comumente utilizado por via tópica e, raramente, por via intravenosa, sendo a administração por esta última via restrita ao tratamento de infecções sistêmicas graves, pois desencadeia muitas reações adversas. Não é administrado por via oral, pois a absorção é muito pequena (COSTA & GÓRNIAK, 2006).

2.4.3 Alilaminas

Os antifúngicos da classe das alilaminas (terbinafina e naftifina) também atuam na biossíntese do ergosterol, porém inibem a enzima esqualeno-epoxidase (CARRILLO-MUÑOZ et al., 2006).

A terbinafina é um antifúngico oral ou tópico usado para tratar dermatófitos e onicomicose, e tem sido avaliado também em combinações com outros agentes (VAZQUEZ, 2003). Terbinafina é um composto fungicida ceratinofílico, altamente lipofílico, pertencente ao grupo das alilaminas (COSTA & GÓRNIAK, 2002). É altamente efetiva contra dermatófitos *in vitro* e *in vivo* (DAVIS & BALFOUR, 1995; GHANNOUM & RICE, 1999), bem como contra fungos filamentosos, dimórficos e dematiáceos, e algumas espécies de leveduras (BALFOUR & FAULDS, 1992).

A terbinafina não interfere no metabolismo de hormônios ou outros mecanismos, liga-se fortemente às proteínas plasmáticas, difundindo-se rapidamente através da derme e concentrando-se no estrato córneo lipofílico. Em humanos, menos de 5% da dose são absorvidos após aplicação tópica e a biotransformação resulta em metabólitos sem atividade fúngica excretados pela urina, com meia-vida de eliminação de 17 horas. Os efeitos colaterais são leves ou moderados e temporários e os sintomas mais frequentes são gastrointestinais ou reações cutâneas sem gravidade (RICHARDSON & WARNOCK, 1993).

2.4.4 Equinocandinas

As equinocandinas são a mais nova classe de antifúngicos para uso clínico, representada pela anidulafungina, caspofungina e micafungina. São lipopeptídeos semissintéticos, com estrutura química de hexapeptídeos cíclicos ligados a uma cadeia lateral de ácido graxo. Seu mecanismo de ação é através da inibição da enzima β-1,3-glucano sintase, localizada na parede celular do fungo e responsável pela síntese de β-1,3-glucano, resultando em um desequilíbrio osmótico e prejudicando a viabilidade do micro-organismo (BOWMAN et al., 2002; DERESINSKI et al., 2003; KARTSONIS et al., 2003). A toxicidade deste grupo de antifúngicos é limitada. Possuem um amplo espectro de atividade fungistática contra espécies de *Candida* azóis-resistentes tornando-os a terapia de escolha para muitas formas de candidíase invasiva, principalmente em pacientes oncológicos (CORNELY et al., 2012; KULLBERG et al., 2011; PAPPAS et al., 2009).

A caspofungina emergiu como a primeira equinocandina a ser comercializada no Brasil no ano de 2000. Possui ação fungistática e demonstrou ser tão eficaz quanto, e menos tóxica do que, anfotericina B no tratamento de doença invasiva causada por *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata*, *Candida krusei*, *Candida guillermondii*, *Candida lipolytica* e *Candida rugosa* (MORA-DUARTE et al., 2002; SPELLBERG et al., 2006), incluindo amostras resistentes ao fluconazol e à anfotericina B. Metabolizada por hidrólise hepática e N-acetilação, seus metabólitos inativos são eliminados pela urina. Assim, a disfunção hepática severa, deve ser considerada para diminuição da dose de caspofungina durante o tratamento. A caspofungina possui interação com agentes que são metabolizados pelo sistema citocromo P450 e, seu nível sérico é reduzido na presença de rifampicina (ASHLEY et al., 2006).

2.5 ÓLEOS ESSENCIAIS

Diversos óleos voláteis são conhecidos por possuírem propriedades antifúngicas e, desta forma, são potencialmente aplicáveis como agentes antimicóticos. Rao et al. (2010) ao estudarem o mecanismo de ação e atividade antifúngica de terpenóides fenólicos concluíram que sua atividade antimicrobiana estão relacionadas a modificações na estrutura da parede celular do micro-organismo. Mais especificamente, alteram a permeabilidade da membrana citoplasmática pela modificação no gradiente de íons de hidrogênio (H^+), potássio (K^+) e cálcio (Ca^{++}). Esta alteração conduz na deterioração de processos essenciais para a sobrevivência da célula como o transporte de elétrons, de proteínas, passos da fosforilação e outras reações dependentes de enzimas (AHMAD et al., 2011; AHMAD et al., 2013; CAMELE et al., 2012; HOMEYER et al., 2015; RAO et al., 2010). Desta forma ocorre perda do controle quimiosmótico e morte do organismo. Além disso, o rompimento da parede celular deve-se ao caráter lipofílico dos óleos essenciais que se acumulam nas membranas (RAO et al., 2010).

Salgueiro et al. (2003) estudaram a composição e atividade antifúngica dos óleos essenciais de *Origanum virens* frente a espécies de *Candida*. O óleo caracterizado pelo alto conteúdo de carvacrol (68,1%) mostrou ter efeito fungicida através de extensa lesão na membrana celular. Da mesma forma Pina Vaz et al. (2004) ao analisarem a atividade antifúngica do óleo de tomilho (*Thymus vulgaris*), suas frações majoritárias (carvacrol e timol), anfotericina B e fluconazol concluíram que e o principal mecanismo de ação observado foi na membrana celular. Corrobora com os dados obtidos por Pinto et al. (2009)

que avaliaram a composição, a atividade antifúngica e o mecanismo de ação do óleo essencial de *Thymus pulegioides* sobre *Candida* spp., *Aspergillus* spp. e dermatófitos demontrando CIMs mais baixas que o fluconazol e a anfotericina B. As análises químicas do óleo demonstraram alto conteúdo de carvacrol e timol. Neste mesmo estudo, a análise de citometria de fluxo e quantificação do ergosterol da membrana fúngica, demonstraram que os principais danos ocorreram na membrana celular.

Um estudo realizado por Vinciguerra et al. (2018) avaliou a atividade antifúngica dos óleos essenciais de *Origanum vulgare* e *Thymus vulgaris* e seu componente principal, carvacrol, contra 27 isolados clínicos de *Malassezia furfur.* Os óleos essenciais e o carvacrol foram mais ativos contra isolados de *M. furfur* resistentes ou dose-dependentes ao fluconazol.

Nardoni et al. (2014) avaliaram a eficácia de uma mistura de *Citrus aurantium*, *Lavandula officinalis*, *Origanum vulgare*, *Origanum majorana*, *Mentha piperita* e *Helichrysum italicum*, em 20 cães com *M. pachydermatis*. O tratamento alcançou um bom resultado clínico e não houve recidiva da doença. A eficácia de toda mistura e de cada componente dos óleos essenciais foram avaliados também através do teste de microdiluição. As menores CIMs foram observadas para *O. vulgare* seguida de *M. piperita*, *O. majorana*, *C. aurantium* e *L. officinalis*, enquanto *H. italicum* não produziu efeito antimicótico. Os principais compostos ativos foram timol, carvacrol, p-cimeno, 1,8-cineol, limoneno e mentol.

Barac et al. (2017) avaliaram a atividade antifúngica do óleo essencial de *Myrtus communis* contra *Malassezia* sp., isolado da pele de pacientes com pitiríase versicolor. A maior atividade inibitória foi demonstrada no crescimento de *Malassezia furfur* e *Malassezia sympodialis*.

Outro estudo avaliou a atividade antifúngica dos óleos essenciais de *Zataria multiflora*, *Thymus kotschyanus*, *Mentha spicata*, *Artemisia sieberi*, *Rosmarinus officinalis* e *Heracleum persicum* contra isolados patogênicos de *Malassezia* isolados da pele e mucosas de cães com dermatite atópica. M. pachydermatis foi a espécie mais isolada e os óleos essenciais de *Z. multiflora* e *T. kotschyanus* exibiram os maiores efeitos inibitórios (KHOSRAVI et al., 2016).

A atividade antifúngica do óleo essencial de *Thapsia villosa* e seus principais componentes, limoneno (57,5%) e metileugenol (35,9%), foram avaliados contra *Candida* spp., *Cryptococcus neoformans*, *Malassezia furfur*, *Aspergillus* spp. e *dermatófitos.* Também foi avaliada a combinação de *T. villosa*, limoneno e metileugenol com fluconazol. A combinação de limoneno e fluconazol apresentou sinergismo. Enquanto que as combinações de *T. villosa* e fluconazol e metileugenol e fluconazol demonstraram indiferença (PINTO et al., 2017).

Um estudo realizado por Sadhasivam et al. (2016) mostrou que o óleo de *Boswellia serrata* possui potente atividade antimicrobiana contra *Propionibacterium acnes*, *Candida albicans*, *Malassezia* spp. e especialmente *Trichophyton* spp. Além disso, o óleo de Boswellia serrata demonstrou atividade sinérgica contra cepas de *C. albicans* resistentes a azólicos.

Um estudo avaliou a atividade antifúngica do óleo de *Cinnamomum cassia* (alto teor de cinamaldeído, 92,2%) e também sua atividade combinada à anfotericina B. O óleo essencial exibiu potente atividade frente a *C. albicans* e potencializou o efeito da anfotericina B (GIORDANI et al., 2006). Sinergismo *in vitro* também foi observado na associação de cinamaldeído e fluconazol frente à *Aspergillus fumigatus* e *Trichophyton rubrum* (KHAN & AHMAD, 2011).

Pozzatti et al. (2008), relataram que isolados de *C. albicans* resistentes ao fluconazol demonstraram sensibilidade ao óleo de canela. Um achado interessante nesse estudo foi o fato de que as CIMs dos isolados resistentes foram menores que para os isolados sensíveis ao fluconazol.

Gucwa et al. (2018) relataram alta atividade do óleo de canela contra isolados clínicos de *Candida albicans* e *Candida glabrata*. Outro estudo avaliou a atividade do óleo da casca da canela, exibindo atividade inibitória potente (CIM 62,5 μg/mL), contra *Candida albicans*. A microscopia de força atômica revelou danos à parede celular e defeitos no fuso mitótico, comprometendo a membrana celular e permitindo o vazamento de componentes celulares. Os múltiplos alvos do óleo da casca da canela podem ser atribuídos aos seus componentes, incluindo o cinamaldeído (74%) e componentes menores (<6%), como linalol (3,9%), acetato de cinamila (3,8%), α-cariofileno (5,3%) e limoneno (2%). A inibição completa do conjunto do fuso mitótico foi observada em *C. albicans* tratados com cinamaldeído na CIM (112 μg/mL) (SHAHINA et al., 2018).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar a suscetibilidade *in vitro* de agentes antifúngicos associados e com frações de óleos essenciais frente a *Malassezia pachydermatis* e desenvolver um modelo experimental de infecção de malasseziose.

3.2 OBJETIVOS ESPECÍFICOS

3.2.1 Avaliar as associações dos antifúngicos azólicos fluconazol, itraconazol, cetoconazol, clotrimazol e miconazol com os antifúngicos terbinafina, nistatina e caspofungina frente a isolados de *M. pachydermatis*.

3.2.2 Avaliar a atividade antifúngica *in vitro* das associações de carvacrol, cinamaldeído e timol com os agentes antifúngicos fluconazol, itraconazol, cetoconazol, clotrimazol, miconazol, terbinafina e nistatina, frente a isolados de *M. pachydermatis.*

3.2.3 Investigar os efeitos citotóxicos do carvacrol, cinamaldeído e timol em células de fibroblastos embrionários de ratos (linhagem celular 3T3).

3.2.4 Desenvolver um modelo experimental de infecção de *M. pachydermatis* utilizando camundongos Swiss imunocomprometidos.

4 ARTIGO 1

In vitro combination of antifungal agents against *Malassezia pachydermatis*

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In vitro combination of antifungal agents against *Malassezia pachydermatis*

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Abstract

The yeast *Malassezia pachydermatis* is a common commensal and occasional opportunistic pathogen of theskin microbiota of animals and humans. In this study, the susceptibility of *M. pachydermatis* isolates to fluconazole (FLC), itraconazole (ITZ), ketoconazole (KTZ), clotrimazole (CLZ), and miconazole (MCZ) alone and in combination with terbinafine (TRB), nystatin (NYS), and caspofungin (CSP) was evaluated *in vitro* based on the M27-A3 technique and the checkerboard microdilution method using Sabouraud dextrose broth with 1% tween 80 (SDB). Based on the mean FICI values, the main synergies observed were combinations of ITZ+CSP and CLZ+CSP (55.17%). The most significant combinations deserve *in vivo* evaluations because might provide effective alternative treatments against *M. pachydermatis* due to their synergistic interactions.

Key words: *Malassezia pachydermatis*, susceptibility test, antifungal drugs, synergism, combination of drugs.

Introduction

Malassezia pachydermatis is a lipophilic, and nonmycelial yeast that is part of the normal skin microbiota of animals and humans. Although regarded as a commensal microorganism, this species can become an opportunistic pathogenand cause several forms of dermatitis in both animals and humans, otitis in pets, and systemic infections in humans, particularly in neonates and immunocompromised patients.[1–4]

Amphotericin B and liposomal amphotericin B are indicated in systemic cases of *Malassezia* infection, while azole and echinocandinsantifungals and flucytosine are suggested for any other population/manifestation of the disease.[3,5,6] Topical and systemic azole antifungal drugs are frequently used in the localized skin disease caused by *Malassezia* species. However, considering the increasing number of *M. pachydermatis* infections[1,2,7,8] and the emergence of its azole-resistant,[1,9–13] there is a growing interest in both *in vitro* antifungal susceptibility testing and in combination therapy options against *M. pachydermatis* infections. However, studies devoted to the evaluation of the combination of antifungals agents against *M. pachydermatis* are still limited.[14] In this context, this study aims to evaluate the *in vitro* combination of azole antifungal drugs with terbinafine, nystatin, and caspofungin against *M. pachydermatis*.

Methods

Microorganisms

M. pachydermatis strain ATCC 14522 and 29 *M. pachydermatis* strain from our collection (Mycological Research Laboratory – LAPEMI, Federal University of Santa Maria – UFSM, Santa Maria, Brazil), isolated from cases of canine otitis, were used.

Table 1. *In vitro* combinations of antifungal drugs against 30 *Malassezia pachydermatis* isolates.

MIC range (GM)		Drug combination				
		Drugs	FICI mean range (GM)	Interpretation (%)		
				Syn	Ind	Ant
Fluconazole	2–64 (9.23)	FLZ+TRB	0.09–4.125 (0.94)	27.58	58.62	13.79
Itraconazole	0.125–4 (1.53)	FLZ+NYS	0.14–4.25 (1.22)	24.14	55.17	20.69
Ketoconazole	0.03–4 (0.22)	FLZ+CSP	0.12–4.25 (0.98)	24.14	68.96	6.89
Clotrimazole	2–16 (6.60)	ITZ+TRB	0.13–4.00 (0.75)	34.48	55.17	10.34
Miconazole	0.5–16 (2.25)	ITZ+NYS	0.13–2.25 (0.51)	48.28	51.72	0.00
Terbinafine	0.06–8 (0.43)	ITZ+CSP	0.12–2.06 (0.54)	55.17	44.83	0.00
Nystatin	1–16 (6.15)	KTZ+TRB	0.15–4.5 (1.07)	27.58	62.07	10.34
Caspofungin	32–128 (67.13)	KTZ+NYS	0.04–2.33 (0.60)	44.83	55.17	0.00
		KTZ+CSP	0.03–4.00 (0.62)	48.28	34.48	17.24
		CLZ+TRB	0.07–4.00 (0.65)	41.37	51.72	6.89
		CLZ+NYS	0.25–4.00 (0.88)	31.03	58.62	10.34
		CLZ+CSP	0.12–2.00 (0.52)	55.17	44.83	0.00
		MCZ+TRB	0.09–4.5 (0.98)	20.69	62.07	17.24
		MCZ+NYS	0.18–4.25 (0.88)	20.69	68.96	10.34
		MCZ+CSP	0.12–8 (0.75)	44.83	34.48	20.69

Ant, antagonism; CLZ, clotrimazole; CSP, caspofungin; FICI, fractional inhibitory concentration index; FLZ, fluconazole; GM; mean geometric; Ind, indifference; ITZ, itraconazole; KTZ, ketoconazole; MCZ, miconazole; MIC, minimum concentration inhibitory; NST, nystatin; Syn, synergic; TRB, terbinafine.

The isolates were identified phenotypically based on its macroscopic and microscopic morphology, and by polymerase chain reaction (PCR)-based assays.[15,16] The isolates were stored at −80°C until the time of use. Before performance of the tests, the isolates were subcultured on Dixon agar supplemented with tween 80.

Antifungal drugs

The antifungal drugs fluconazole (FLC; Pfizer, Gladstone, NJ, USA), itraconazole (ITZ) and ketoconazole (KTZ) (Janssen Pharmaceutica, Brazil), clotrimazole (CLZ; Bayer Schering Pharma, Berlin, Germany), miconazole (MCZ; Labware, Brazil), terbinafine (TRB; Bristol-Myers Squibb Pharmaceuticals Research Institute, Princeton, NJ, USA), nystatin (NYS), and caspofungin (CSP) (Bristol-Myers Squibb Pharmaceuticals Research Institute, USA) were obtained commercially and were diluted in dimethyl sulfoxide or distilled water to generate stock solutions (1:100 work solutions). The tested concentration (μg/ml) ranged from 0.125 to 32 for FLC; 0.01 to 2 for ITZ and KTZ; 0.06 to 8 for CLZ, MCZ, and NYS; 0.03 to 4 for TRB and 0.06 to 16 for CSP.

In vitro susceptibility and drug interaction tests

The minimal inhibitory concentrations (MICs) were performed according to the CLSI M27-A3 guidelines for antifungal susceptibility testing of yeasts[17] using Sabouraud dextrose broth (SDB) with 1% tween 80.[18] The interactions between azole antifungal drugs and TRB, NYS, and CSP were assessed by the checkerboard microdilution method.[19,20] The fractional inhibitory concentration (FIC) was calculated for each agent by dividing the MIC of each drug combination by the MIC of the drug alone. The FIC values were then totaled to determine the fractional inhibitory concentration index (FICI) that resulted from the drug combinations. Synergism was defined as an FICI $\leq$ 0.5, indifference was defined as 1.0 < FICI $\leq$ 4, and antagonism was defined as FICI > 4.[20]

Results

The *in vitro* susceptibilities of the 30 *M. pachydermatis* isolates are listed in Table 1. The MIC (geometric mean MIC, in μg/ml) values for the antifungal drugs ranged from 2 to 64 μg/ml (9.23) for FLZ, 0.125 to 4 μg/ml (1.53) for ITZ, 0.03 to 4 μg/ml (0.22) for KTZ, 2 to 16 μg/ml (6.60) for CLZ, 0.5 to 16 μg/ml (2.25) for MCZ, 0.06 to 8 μg/ml (0.43) for TRB, 1 to 16 μg/ml (6.15) for NYS and 32 to 128 μg/ml (67.13) for CSP. Most interactions between the azole antifungal drugs with other antifungals drugs were indifferent (Table 1). The highest synergistic interactions, ranging from 41.37% to 55.17%, were observed for the combinations ITZ+NYS, ITZ+CSP, KTZ+NYS, KTZ+CSP, CLZ+TRB, CLZ+CSP, and MCZ+CSP. However, excepting ITZ+NYS, ITZ+CSP, KTZ+NYS, and CLZ+CSP, all other combinations resulted in antagonistic interactions ranging from 6.89% to 20.69%.

Discussion

This study adds data about *in vitro* antifungal activities and synergisms of combinations of different antifungal drugs against *M. pachydermatis*. Individually, the lowest MICs against *M. pachydermatis* were observed with ITZ, KTZ, MCZ, and TRB. Regarding the synergistic interactions of the azole antifungal drugs with the other antifungal drugs, the main synergies observed were combinations of ITZ+CSP and CLZ+CSP (55.17%). However, a predominance of indifferent interactions was observed.

The susceptibility tests were performed according to the CLSI M27-A3, but the SDB with 1% tween 80 was used instead of Roswell Park Memorial Institute (RPMI) 1640 broth because of the description that SDB is most suitable for the evaluation of the *in vitro* susceptibility of *M. pachydermatis*.[18,21] Previously studies using SDB to evaluate the susceptibility of *M. pachydermatis*[11,18, 22–24] showed similar results when compared with the results of this study.

Several studies also described the reduced susceptibility of *M. pachydermatis* to antifungal azole drugs.[1,2,7,8] Moreover, this species is also able to produce biofilm,[25–27] which directly impact on the persistence of the disease as consequence of the resistance to antifungal treatment. The *in vitro* susceptibility testing of *M. pachydermatis* is an important tool to detect the decreased susceptibility of isolates, treatment suggestion and establishment of breakpoints.[22]

In the context, combination therapy is an important alternative when monotherapy is not effective because may reduce treatment toxicity through the administration of lower doses and may potentiate the antifungal activity of conventional agents. In addition, can decrease the potential of microorganisms acquiring resistance to certain antimicrobials.[28]

In conclusion, our *in vitro* results are a preview of the effects of antifungal drugs used in combination. Although *in vitro* studies have limitations, they are necessary for the development of valid hypotheses regarding new treatments against *M. pachydermatis*. The antifungals that we tested in this study are frequently usedin the treatment of fungal infections. Therefore, they are promising candidates for new studies on combination treatments of malasseziosis.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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5 MANUSCRITO 1

Antifungal activities of carvacrol, cinnamaldehyde and thymol against *Malassezia pachydermatis* and cytotoxicity in embryonic fibroblast 3T3 cell line

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Title: Antifungal activities of carvacrol, cinnamaldehyde and thymol against *Malassezia pachydermatis* and cytotoxicity in embryonic fibroblast 3T3 cell line

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Abstract

We investigated the *in vitro* activities of carvacrol (CRV), cinnamaldehyde (CIN) and thymol (THY) alone and in combination with antifungal agents against *Malassezia pachydermatis*. The mean fractional inhibitory concentration index (FICI) mean showed primary synergies for the combinations CRV + nystatin, THY + nystatin, and CRV + miconazole (80%). The cytotoxic effects of CRV, CIN and THY on the fibroblast 3T3 cell line were examined. At 24-72h, the MTT assay results showed no cytotoxicity for CRV or THY (1-50µg/mL), but CIN displayed a large decrease in cell viability at all concentrations tested. Reactive oxygen species concentrations decreased at 24-72h of incubation in the presence of CRV, CIN and THY however increased with 50µg/mL CIN at 24h. DNA fragmentation levels increased at 24-72h with CRV (50-100µg/mL), CIN (25-10µg/mL) and THY (almost all concentrations). Annexin V/propidium iodide staining indicated a slight increase (up to 5%) in apoptosis following exposure to CRV and THY (25-50µg/mL) for 24-72h and a large increase in the level of late apoptosis (up to 90%) with CIN (25µg/mL, 24-72h). We observed with the inhibitory concentrations tested low toxicity in citotoxicity assays.

Keywords: susceptibility testing; malasseziosis; essential oil; phytochemicals

Introduction

The *Malassezia* genus includes 17 species (1), three were recently proposed (2, 3). *Malassezia pachydermatis* is a commensal and occasional opportunistic yeast that is isolated from the skin of wild and domestic carnivores, that may become pathogenic under the influence of predisposing factors, and lead to the development of dermatitis and otitis. These diseases are common in dogs and less frequently in other animals (4).

The dermatitis and otitis caused by *Malassezia* spp. usually require treatment with high doses of azole drugs for prolonged periods. These drugs are mainly combined with antibiotics and glucocorticoids to control concurrent bacterial infection and inflammation. Clinical date suggest thatazole failure treatments are growing as well as the resistance is a well documented (5, 6). These information justify support the development of new therapies for use in clinical practice.

Phytochemicals isolated from essential oils are effective alternatives to inhibit microbial pathogens (7). The interest in isolated monoterpenes has grown over the past several years, particularly for carvacrol and thymol, which show significant biological activities, including antibacterial, antifungal, antiviral, antioxidant, and anti-inflammatory activities (7, 8, 9). Cinnamaldehyde is also an effective inhibitor of the growth of bacteria, yeast and filamentous fungi (10). The association between essential oil compounds and antifungal drugs demonstrated a synergistic effect on growth inhibition (11, 12).

Therefore, investigations of the actions of carvacrol, cinnamaldehyde and thymol alone or in combination with antifungal agents against *M. pachydermatis* are warranted. We performed parallel investigations to evaluate whether these compounds exhibit cytotoxic effects using the mouse embryonic fibroblast 3T3 cell line as an *in vitro* experimental model.

Materials and methods

Isolates

A total of 30 *M. pachydermatis* isolates were tested. Twenty-nine *M. pachydermatis* strains were isolated from dogs with otitis, and CBS 6542 was included as a reference strain. *M. pachydermatis* isolates were primarily recovered in Sabouraud dextrose agar containing chloramphenicol. Before testing, each isolate was sub-cultured onto modified Dixon agar to ensure its purity and viability. The identities of the isolates were confirmed using PCR (13, 14).

Susceptibility testing

Carvacrol (CRV, Sigma Aldrich, USA), cinnamaldehyde (CIN, Sigma Aldrich, USA) and thymol (THY, Sigma Aldrich, USA) were solubilized in ethanol (3.2×10^4 µg/mL stock solution) and diluted in Sabouraud dextrose (working solution). Terbinafine (TRB, Bristol-Myers Squibb Pharmaceuticals Research Institute, USA), nystatin (NYS, Bristol-Myers Squibb Pharmaceuticals Research Institute, USA) ketoconazole (KTZ, Janssen Pharmaceutica, Brazil), itraconazole (ITZ, Janssen Pharmaceutica, Brazil), clotrimazole (CTZ, Bayer Schering Pharma, USA), miconazole (MCZ, Labware, Brazil) and fluconazole (FLZ, Pfizer, USA) were solubilized in dimethyl sulfoxide or distilled water to generate stock solutions. The minimal inhibitory concentrations (MICs) were performed according to the CLSI M27-A3 guidelines for antifungal susceptibility testing of yeasts (15) using Sabouraud dextrose broth (SDB) with 1% tween 80 as previously reported (16). Interactions between the antifungal drugs and CRV, CIN and THY were evaluated using the broth microdilution checkerboard method, and the lowest fractional inhibitory concentration (FIC) was calculated for each agent by division of the MIC of each drug used in combination and the MIC of the drug alone. FIC values were then summed up to determine the fractional inhibitory concentration index (FICI) resulting from the combination. The results after 72h of incubation at 32°C were interpreted according to the lowest FICI. Interactions were determined as follows: FICI ≤ 0.5, synergism; FICI > 0.5 to < 4, indifference and FICI ≥ 4, antagonism (17).

Evaluation of phytochemical compound toxicity

Cell culture

This study used the Swiss albino mouse embryonic fibroblasts 3T3 cell line (ATCC® 3T3). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) containing 10% fetal bovine serum and penicillin and streptomycin. The cells were maintained in an incubator at 37°C, 5% CO_2 and subcultured until a monolayer was achieved (approximately 85% confluence) to perform the experiments.

Phytochemical compounds

CRV, CIN, and THY were first dissolved in 99% ethanol solution. Further dilutions were made with DMEM containing 10% fetal bovine serum and penicillin and streptomycin. Working solutions were prepared to obtain final concentrations of 0, 1, 5, 10, 25, 50 and 100µg/mL for cell culture treatments. A vehicle control was prepared in the same manner as component-treated samples, including the addition of the vehicle (0.5% ethanol) instead of components.

Cellular proliferation evaluation by MTT assay

Cellular proliferation was evaluated using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (18). The MTT assay measures the level of NAD(P)-H-dependent cellular oxidoreductase enzymes, which reflects the number of viable cells. To perform the MTT assay, cells were plated in a 96-well microplate at a cell density of 1×10^5 cells/mL and treated with different concentrations of CRV, CIN and THY for 24h and 72h. Cells were stained for 2h at 37°C with 20 µL/well MTT reagent (5 mg/mL diluted in PBS). After incubation, the plates were centrifuged at 400 × g for 5 min, and the medium was carefully removed. Then 100 µl of DMSO was added to each well to solubilize the purple formazan crystals that were produced. The absorbance of each well was measured at 590 nm in a plate reader (Bio-Rad Laboratories, USA), and the results are expressed as the average percentage for each treatment concentration compared to the control.

Intracellular reactive oxygen species (ROS)

Intracellular ROS concentrations were determined using the fluorescence probe dichlorofluorescein diacetate (DCFH-DA), which is a well-established compound for the detection and quantification of free radicals, particularly intracellular hydrogen peroxide (H_2O_2). DCFH-DA is transported across the cell membrane, and it is deacetylated by cytosolic esterases to form non-fluorescent DCFH, that is trapped within the cells. DCFH is converted to fluorescent DCF by the action of peroxidase (19, 20).

After each time point of exposure (24 and 72h) the cells were treated with DCFH-DA (10 µM) for 60 min at 37°C. Fluorescence was measured at 488 nm excitation and 525 nm emission (SpectraMax M2/M2e Multi-mode Plate Reader, Molecular Devices Corporation, USA). All tests were performed in 96-well microplates in sextuplicate for each of the tested samples, and the results are expressed as fluorescence intensity (nm).

Evaluation of DNA damage

To evaluate the potential cytotoxic or cytoprotective effects of CRV, CIN, and THY, the concentration of free double-stranded DNA (dsDNA) in cell culture was measured using the specific Quant-IT™ PicoGreen® dsDNA kit (Invitrogen – Life Technologies). This fluorochrome dye creates a highly stable complex with dsDNA under alkaline conditions but does not form a complex with single-stranded DNA (dsDNA), proteins, SDS or urea. This particular characteristic is used to identify free dsDNA in culture medium, which indicates cell death. The test was performed as previously described by Parra et al. (21). Briefly, the cells were treated as described above and incubated for 24h and 72h. After incubation, the dsDNA was measured using 50µl of the cell supernatant and 50µl of DNA PicoGreen dissolved in TE buffer (1:1; v/v), followed by an incubation for 5 min in the dark. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm at room temperature (SpectraMax M2/M2e Multi-mode Plate Reader, Molecular Devices Corporation, USA). The results are expressed as a percentage of dsDNA calculated for each treatment in relation to the untreated control samples. Values below 100% indicated a decrease, and values above 100% indicated an increase in cell mortality compared to the control group.

Detection of apoptosis

Apoptosis was detected by flow cytometry as described by Cárdeno et al. (22) with modifications. Briefly, 3T3 cells (5×10^5 cells/mL) were seeded in 24-well plates. After 24h of incubation, the cells were treated with different concentrations of CRV (25 and 50µg/mL), CIN (10 and 25µg/mL), and THY (25 and 50µg/mL). A negative control without any compound was prepared. The cells were exposed for 24 and 72h and media were collected. The cells were detached by trypsinization (0.05% Trypsin–EDTA; Sigma–Aldrich, USA) and collected in 0.5 mL of DMEM with 10% fetal bovine serum. After, the cells were centrifuged at 1500 rpm for 5 min at 4°C, resuspended and washed with ice-cold PBS. Cells were then centrifuged again and resuspended in ice-cold 1× binding buffer (BB) at a final concentration of 5×10^5 cells/mL. The cells were incubated with 20 µL/mL annexin V-FITC and 50µg/mL of a propidium iodide (PI) solution (Annexin V-FITC Apoptosis Detection Kit, BD Biosciences, USA). Four different groups of cells were obtained based on their stainability: cells not stained with annexin V or PI [annexin(-)/PI(-)]: viable cells (quadrant E3); cells

stained with annexin V but not stained with PI [annexin(+)/PI(-)]: early apoptotic cells (quadrant E4); cells stained with both annexin V and PI [annexin(+)/PI(+)]: late apoptotic cells (quadrant E2); and cells not stained with annexin V but stained with PI [annexin(-)/ PI(+)]: primary necrotic cells (quadrant E1). The untreated population was used to define the basal level of apoptotic and dead cells. Following the acquisition of sample data (channel FL2-A) on a BD Accuri^{TM} C6 flow cytometer (BD Biosciences' USA), the sample results were generated in a graphic and tabular format using FCAP array v3.0.1 software.

Statistical analysis

The data for the experiments are presented as the arithmetic mean percentage ± standard error of the mean (SEM) in relation to the control. Statistical analyses were performed using SigmaPlot software version 12.5, and one-way ANOVA was performed followed by Dunnett's post hoc test. Differences were considered significant at $p < 0.05$ (*) and $p < 0.001$ (**) compared to the control group.

Results

Susceptibility testing

The *in vitro* susceptibilities of the 30 *M. pachydermatis* isolates are listed in Table 1. The geometric means (GM) of the MIC values of CRV, CIN and THY ranged from 10 to 320 µg/mL (GM 64.98), 2.5 to 640µg/mL (GM 12.89) and 10 to 640µg/mL (GM 66.50), respectively. The MIC values for the antifungal drugs ranged from 1 to 64µg/mL (9.40) for FLZ, 0.015 to 4µg/mL (0.08) for ITZ, 0.0039 to 1µg/mL (0.02) for KTZ, 0.03 to 64µg/mL (4.50) for CTZ, 0.03 to 64µg/mL (8.96) for MCZ, 0.03 to 64µg/mL (2.57) for TRB, and 4 to 64 µg/mL (41.26) for NYS.

Table 1 shows the percentages of synergism, indifference, and antagonism that resulted from the combinations of CRV, CIN or THY with antifungal drugs against *M. pachydermatis* strains. The highest synergistic interaction (80.0%) based on the MIC values was observed for the following combinations: THY + NYS, CRV + NYS and CRV + MCZ. The other combinations produced synergistic interactions that ranged from 16.6% to 70.0%. The highest percentage of indifference (70%) was observed for CIN + FLZ, THY+ TRB, and

CIN + TRB. The highest antagonistic effects were detected from the combinations CRV + KTZ, THY + KTZ (40.0%) and CIN + KTZ (46.6%).

Cellular proliferation evaluation by MTT assay

The cellular proliferation analysis data are shown in Figure 1. The cells treated with CRV demonstrated a significant decrease in viability at only 100µg/mL at both exposure times (Fig. 1A). There was a significant decrease in cell viability at all concentrations of CIN at both incubation times, with the exception of 1µg/mL CIN at 72h (Fig. 1B). Almost all tested concentrations of THY presented increased cell viability at 24 and 72h of incubation. A significant decrease was observed at only 100µg/mL of THY at 24h (Fig. 1C).

ROS measurement

The ROS levels generated in response to CRV, CIN and THY are shown in Table 2. At 24h of incubation, CRV and THY significantly decreased ROS levels at almost all concentrations, and CIN significantly increased ROS at only 50µg/mL. At 72h of incubation, the ROS levels significantly decreased for the three treatments at almost all concentrations tested.

Evaluation of DNA damage

The fibroblast DNA damage caused by *in vitro* exposure to increasing concentrations of CRV, CIN and THY at 24h and 72h of incubation is shown in Fig. 2. CRV (50 and 100 µg/mL) at 24h and 100µg/mL CRV at 72h significantly increased dsDNA levels compared with the control (Fig. 2A). Similarly, the cells exposed to 25 to 100µg/mL CIN for 24h and 50 – 100µg/mL CIN for 72h exhibited increased dsDNA levels compared with the control (Fig. 2B). Fibroblasts exposed to THY presented a significant increase at almost all concentrations tested at 24h (5 to 100µg/mL) and a significant increase from 25 to 100µg/mL after 72h of incubation (Fig. 2C).

Detection of apoptosis

We quantified the extent of apoptosis in cells labelled with Annexin-V/PI stain using flow cytometry. Representative dot plots revealed that only 2-4% of control cells were dead or undergoing apoptosis, which is a normal event for cells growing in culture. An increase in cell death stages was observed after exposure to different concentrations of CRV, CIN, and THY for 24h and 72h (Fig. 3). A significant increase in early and late apoptosis appeared at only 50 µg/mL CRV at 24h compared to the control group. At 72h, significant changes in late apoptosis appeared at the two CRV concentrations assayed (Fig. 3A). CIN produced a significant increase in early and late apoptosis stages at 25µg/mL at 24h. The necrosis stage exhibited an increase at 10µg/mL CIN at 24h. A significant increase in the necrosis stage was observed at 10µg/mL CIN at 72h, and increased late apoptosis occurred with 25µg/mL CIN at 72h (Fig. 3B). A significant increase in early apoptosis was observed in cells treated with 25 and 50µg/mL THY for 24h. Only the late apoptosis stage was significantly higher than the control group with 50µg/mL THY at 72h (Fig. 3C).

Discussion

Similar to previous findings, the susceptibility results found here (Table 1) showed high *in vitro* antifungal activity for the tested drugs (23-25). The individual MICs obtained for CRV, CIN and THY ranged from 2.5 to 640µg/mL and are in accordance with the MICs described in studies that used essential oils (EOs) with a high percentage of these compounds against *M. pachydermatis* (26, 27). Many studies have demonstrated that EOs and their isolated compounds achieved their effects by inhibition of membrane ergosterol and signalling pathways involved in yeast to hyphae morphogenesis (7, 11, 28). A previous study demonstrated that CRV and THY inhibited ergosterol biosynthesis and consequently affected cell membrane integrity (28).

The results of the present study provide the first demonstration of synergism between CRV, CIN and THY in combination with antifungal drugs against *M. pachydermatis*. The highest synergistic interactions (80%) were observed for CRV+NYS, CRV+MCZ, and THY+NYS. Synergistic results from the combination of THY+NYS had already showed by Castro et al. (11) against *Candida* strains. The authors attributed these outcomes to the inhibition of ergosterol formation and/or an increase in cell permeability, which allowed the passage of one or both agents. The same mechanism was also hypothesized for *in vitro* synergism observed for CIN+FLZ against *Aspergillus fumigatus* and *Trichophyton rubrum* (29).

EOs or their constituents can exert cytotoxic effects on eukaryotic cells. Cytotoxicity is an important property for the chemotherapeutic applications of EOs against microorganisms, but it is also responsible for the undesirable side effects towards hosts (7). We investigated the cytotoxic effects of CRV, CIN and THY *in vitro* using mouse fibroblasts. The MTT cell viability assay demonstrated cytotoxic effects at only the highest CRV and THY concentration (100µg/mL) after 24h and 72h of incubation, but these agents induced no cytotoxicity at lower concentrations. CIN exhibited the highest cytotoxic effect with a significant decrease in cell viability at all concentrations in both incubation times. Our findings are in accordance with a recent study that concluded that CRV exposure (at concentrations above 100µg/mL) increased the cell death rates of human lymphocytes (30). Another study showed moderate cytotoxicity for CRV and THY in cultured murine B16-F10 melanoma cells (31). These discrepancies may be partially explained due to the different metabolic abilities of each cell line (32).

Our ROS production results showed that CRV, CIN and THY effectively prevented oxidative stress in 3T3 mouse fibroblasts at the exposure times used by a decrease in DCFH-DA oxidation levels after exposure. These results are corroborated by other studies that have shown that CRV exhibits an inhibitory effect on COX (33) in addition to its antioxidant and free radical scavenger activities (34), which could reduce oxidative stress and inflammation. Ündeğer et al. (8) observed a slight decrease in ROS generation in the V79 cell line in the presence of THY (1–100µM) and CRV (5µM).

In addition to the antioxidant effects exhibited by EOs and their constituents, some of them (e.g., phenolic compounds) also exhibit pro-oxidant effects at high concentrations, which can damage DNA, lipids and other biological molecules (7). Our results suggest that CRV prevented DNA damage at low concentrations (at 1, 5, 10 and 25µg/mL) and exerted a cytoprotective effect by decreasing dsDNA levels. However, THY induced DNA damage at almost all tested concentrations (5, 10, 50 and 100µg/mL) at 24h, but CIN induced DNA damage at only 50 and 100µg/mL. THY and CIN induced DNA damage in concentrations above 25µg/mL after 72h of incubation. In support of our findings, Ündeğer et al. (8) reported that CRV concentrations up to 25µM and THY concentrations up to 5µM did not induce significant DNA strand breakage in V79 cells but that 25µM THY increased DNA damage. Llana-Ruiz-Cabello et al. (32) studied for the first time the *in vivo* genotoxic effects produced in rats orally exposed to CRV, and they reported that CRV did not induce *in vivo* genotoxicity or oxidative DNA damage in the investigated tissues. Using COMET assay, Slamenová et al. (35) demonstrated that CRV and THY did not have any genotoxic effect on the human cell

lines HepG2 and Caco-2.

Cell death induced by EOs and their components was investigated extensively in several cultured cells. In our study, we observed a slight increase (up to 5%) in early and late apoptosis after CRV and THY exposure at concentrations of 25µg/mL and 50µ/mL after 24h and 72h of exposure. However, the late apoptosis level increased tremendously (up to 90%) in the presence of 25µg/mL CIN after 24h and 72h of exposure, and a smaller increase in necrosis (up to 5%) was observed at 10µg/mL. Previous studies demonstrated that CRV and THY slightly increased the incidence of apoptotic cell death at concentrations used for antimicrobial purposes in Caco-2 cells (36). Another study also using Caco-2 cells demonstrated that CRV increased late apoptosis and necrosis at concentrations greater than 230µM but that THY did not produce any apoptotic or necrotic cells at any of the concentrations assayed (32). Cancer research has demonstrated that CIN induces apoptosis in a variety of tumour cell lines (37).

In conclusion, we demonstrated synergistic interactions for combinations of CRV, CIN or THY and antifungal agents, which suggests that these combinations provide an effective alternative treatment for malasseziosis. Our results for CRV and THY revealed cytotoxic effects only at the highest concentrations. These findings support promising investment in *in vitro* and *in vivo* research to further assess the potential use of these agents alone or in combination with antifungal drugs against *M. pachydermatis*.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The transparency document associated with this article may be found in the online version.

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Table 1- *In vitro* activity and combinations of carvacrol, thymol, cinnamaldehyde and antifungals against 30 *Malassezia pachydermatis* isolates.

Drugs alone				Drug Combinations					
	MIC range (GM)	MIC_{50}	MIC_{90}	Drugs	MIC associated range (GM)	FICI mean range (GM)	Interpretation (%)		
Phytochemicals							Syn	Ind	Ant
carvacrol (CRV)	10-320 (64.98)	80	160	CRV+FLZ	1.25-160 (9.33) + 0.25-32 (1.66)	0.125-4.062 (0.46)	53.3	43.3	3.3
thymol (THY)	10-640 (66.50)	80	160	THY+FLZ	1.25-160 (9.62) + 0.25-32 (1.44)	0.031-1.125 (0.42)	46.6	53.3	0.0
cinnamaldehyde (CIN)	2.5-640 (12.89)	10	40	CIN+FLZ	1.25-40 (6.64) + 0.25-16 (0.79)	0.066-12 (0.73)	26.6	70.0	3.3
Antifungals				CRV+ITZ	1.25-160 (9.18) + 0.016-1 (0.02)	0.07-3.083(0.53)	33.3	66.6	0.0
fluconazole (FLZ)	1-64 (9.40)	4	64	THY+ITZ	1.25-160 (7.57) + 0.016-0.5 (0.02)	0.093-4.52(0.54)	36.6	60.0	3.3
itraconazole (ITZ)	0.015-4 (0.08)	0.06	0.5	CIN+ITZ	1.25-160 (4.66) + 0.016-0.125 (0.02)	0.007-16.52 (0.85)	30.0	56.6	13.3
ketoconazole (KTZ)	0.0039-1 (0.02)	0.15	0.25	CRV+KTZ	1.25-160 (10) + 0.016-1 (0.02)	0.25-6.006 (1.29)	23.3	36.6	40.0
clotrimazole (CTZ)	0.03-64 (4.50)	4	32	THY+KTZ	1.25-160 (8.50) + 0.016-0.25 (0.02)	0.078-8.006 (1.27)	20.0	40.0	40.0
miconazole (MCZ)	0.03-64 (8.96)	8	32	CIN+KTZ	1.25-160 (5.48) + 0.016-0.062 (0.02)	0.093-6.006(1.55)	30.0	30.0	46.6
terbinafine (TRB)	0.03-64 (2.57)	2	32	CRV+CTZ	1.25-160 (4.35) + 0.063-8 (0.57)	0.039-2.062 (0.27)	70.0	30.0	0.0
nystatin (NYS)	4-64(41.26)	64	64	THY+CTZ	1.25-160 (6.01) + 0.063-4 (0.46)	0.039-3 (0.29)	70.0	30.0	0.0
				CIN+CTZ	1.25-40 (3.15) + 0.063-8 (0.52)	0.064-2.125(0.52)	40.0	60.0	0.0
				CRV+MCZ	1.25-160 (3.96) + 0.063-8 (0.89)	0.023-3 (0.21)	80.0	16.6	3.3
				THY+MCZ	1.25-160 (4.25) + 0.063-8 (0.87)	0.031-2.031(0.25)	70.0	30.0	0.0
				CIN+MCZ	1.25-40 (2.17) + 0.016-8 (0.72)	0.039-2.003 (0.31)	66.6	33.3	0.0
				CRV+TRB	1.25-160 (22.44) + 0.125-16 (0.29)	0.019-5 (0.68)	30.0	66.6	3.3
				THY+TRB	1.25-160 (21.43) + 0.125-16 (0.67)	0.062-2.5 (0.89)	23.3	70.0	6.6
				CIN+TRB	1.25-40 (8.31) + 0.125-8 (0.29)	0.046-4.5 (0.97)	16.6	70.0	13.3
				CRV+NYS	1.25-160 (4.06) + 0.125-16 (1.86)	0.023-2.015 (0.18)	80.0	20.0	0.0
				THY+NYS	1.25-160 (3.15) + 0.25-32 (3.10)	0.035-4.003 (0.17)	80.0	16.6	3.3
				CIN+NYS	1.25-20 (2.22) + 0.25-64 (29.17)	0.062-1.25 (0.31)	70.0	30.0	0.0

MIC, Minimum inhibitory concentration; GM, geometric mean; MIC_{50} and MIC_{90}, Concentrations required to inhibit the growth of 50 and 90%, respectively, of the microorganisms used; FICI, mean fractional inhibitory concentration index; Syn, synergic; Ind, indifference; Ant, antagonism.

Table 2. Effect of different concentrations of carvacrol, cinnamaldehyde and thymol on reactive oxygen species (ROS) levels in the mouse fibroblast 3T3 cell line.

Treatments	Concentration µg/mL	ROS levels (% of control)	
		24 h	72 h
Control		100.00 ± 1.34	100.00 ±0.78
Carvacrol	1	93.88 ± 0.78 **	100.98 ±0.73
	5	91.75 ± 0.32 **	99.38 ± 0.78
	10	96.91 ± 0.49 *	97.43 ± 0.61 *
	25	91.15 ± 0.29 **	98.34 ±0.49
	50	96.61 ± 0.44 *	95.81 ± 0.66 **
	100	92.16 ± 1.04 **	95.21 ± 0.42 **
Control		100.00 ±0.39	100.00 ±0.52
Cinnamaldehyde	1	101.50 ± 0.87	97.40 ± 0.29 **
	5	100.24 ± 0.49	97.55 ± 0.41 **
	10	100.29 ± 0.58	96.24 ± 0.52 **
	25	100.92 ± 0.41	98.31 ± 0.27 *
	50	103.35 ± 0.20 **	96.59 ± 0.29 **
	100	101.68 ± 0.34	94.99 ± 0.28 **
Control		100.00 ±0.57	100.00 ±0.50
Thymol	1	93.14 ± 0.40 **	98.09 ± 0.69
	5	95.41 ± 0.59 **	96.52 ± 0.87 *
	10	95.34 ± 0.32 **	96.10 ± 0.82 *
	25	94.56 ± 0.62 **	90.14 ± 0.73 **
	50	90.29 ± 0.33 **	93.03 ± 1.15 **
	100	89.00 ± 0.70 **	87.37 ± 0.31 **

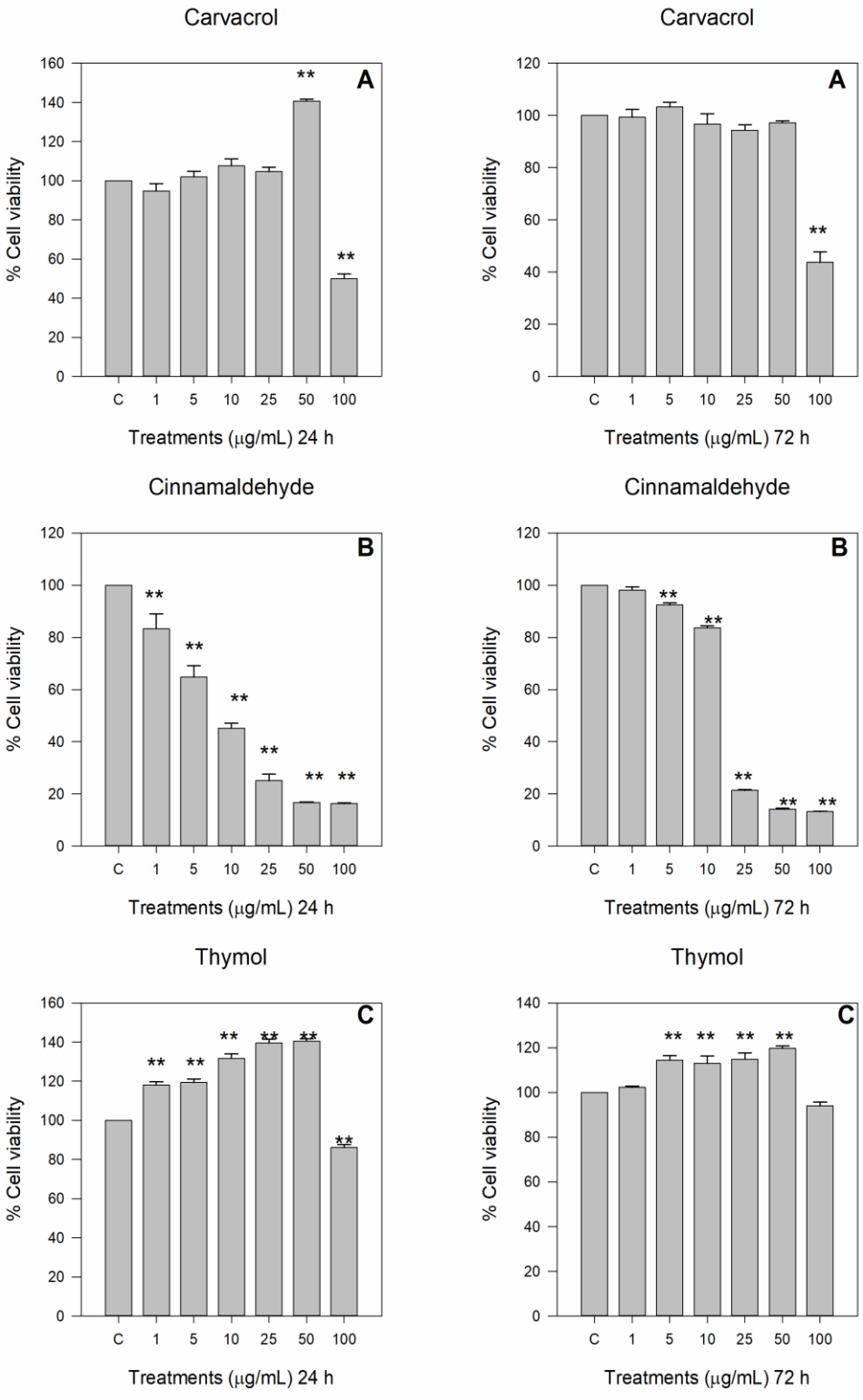
Data are reported as the means ± SEM and are representative of six replicates. * $p < 0.05$, ** $p < 0.001$ compared to the respective control group.

Figure legends

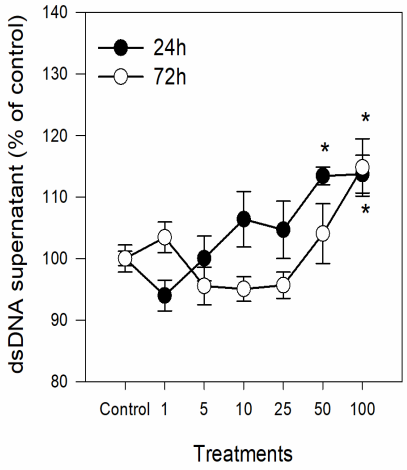
Figure 1. Cytotoxicity evaluation by MTT reduction in mouse fibroblasts (3T3 cell line) treated with different concentrations of carvacrol (A), cinnamaldehyde (B) and thymol (C) for 24h and 72h. Values are the means ± SD (n=4). ** indicates significant differences between treatments and control (C) groups at $p \leq 0.001$

Figure 2. Evaluation of DNA damage at 24h and 72h after exposing mouse fibroblasts (3T3 cell line) to increasing concentrations of carvacrol (A), cinnamaldehyde (B), and thymol (C). Values are expressed as the means ± SEM and are representative of six replicates. * $p < 0.05$, ** $p < 0.001$ compared to the respective control group

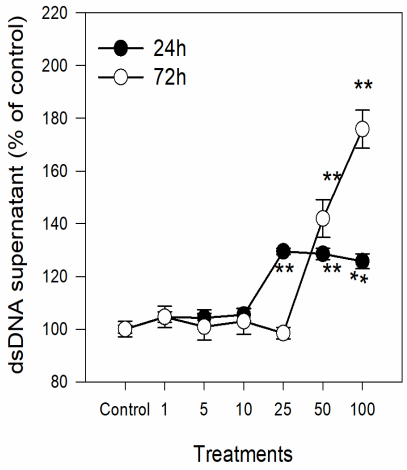
Figure 3. Percentages of apoptotic (early and late) and necrotic mouse fibroblasts (3T3 cell line) after exposure to different concentrations of carvacrol (A), cinnamaldehyde (B), and thymol (C) for 24h and 72h. Data are presented as the means ± SEM of 3 independent experiments carried out in duplicate. * $p < 0.05$, ** $p < 0.001$ compared to the respective control group


Carvacrol
A
% Cell viability
Treatments (µg/mL) 24 h
Carvacrol
A
% Cell viability
Treatments (µg/mL) 72 h
Cinnamaldehyde
B
% Cell viability
Treatments (µg/mL) 24 h
Cinnamaldehyde
B
% Cell viability
Treatments (µg/mL) 72 h
Thymol
C
% Cell viability
Treatments (µg/mL) 24 h
Thymol
C
% Cell viability
Treatments (µg/mL) 72 h
C 1 5 10 25 50 100

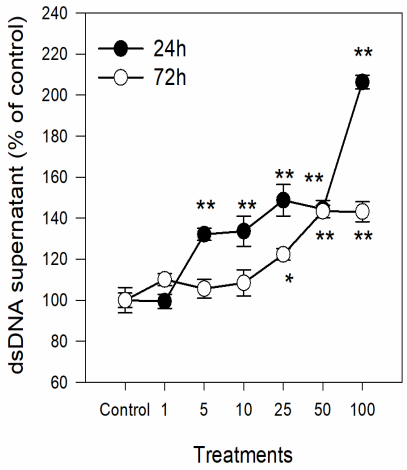
Carvacrol

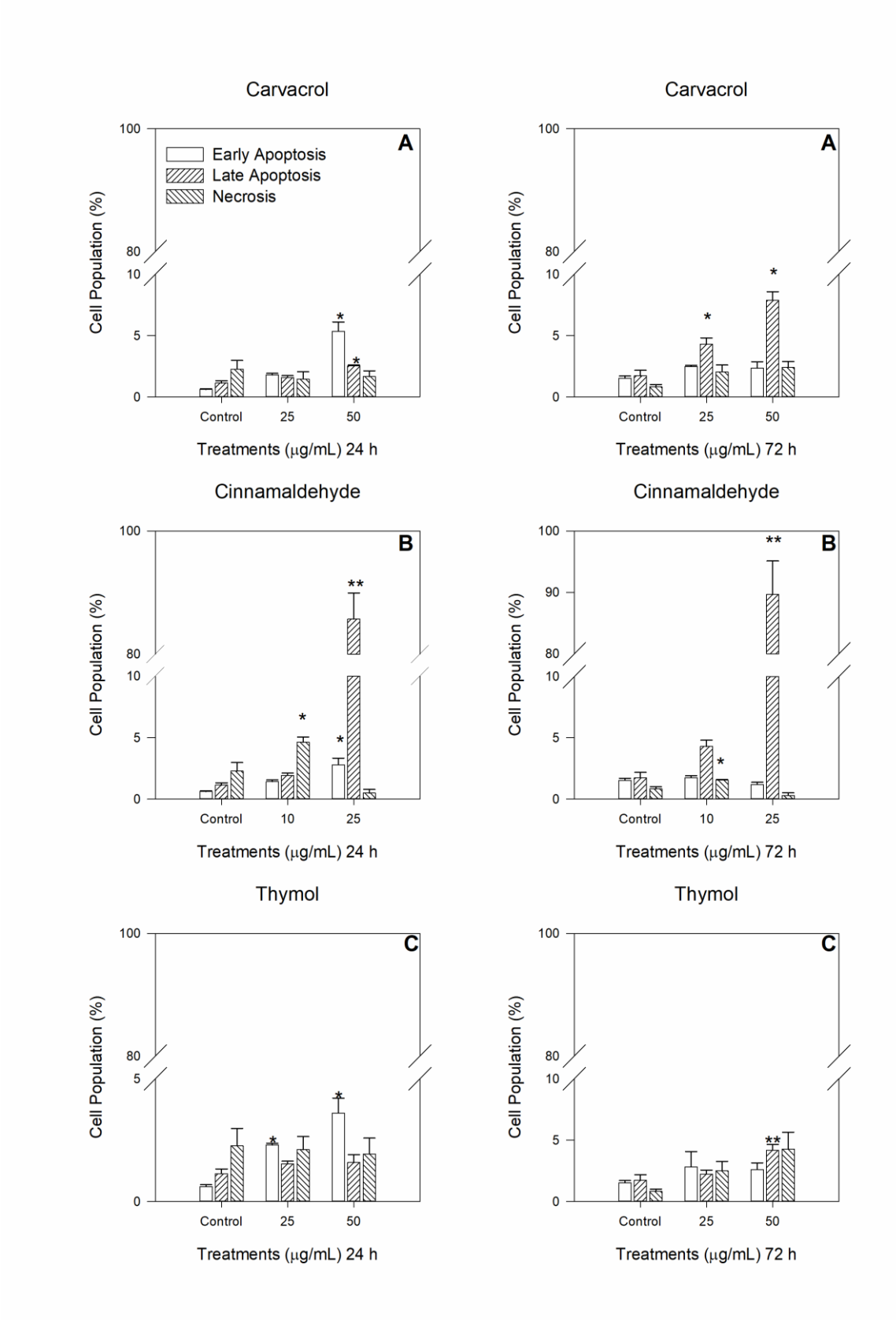


Cinnamaldehyde



Thymol




Carvacrol
A
Early Apoptosis
Late Apoptosis
Necrosis
Cell Population (%)
100
80
10
5
0
Control
25
50
Treatments (μg/mL) 24 h
Carvacrol
A
Cell Population (%)
Control
25
50
Treatments (μg/mL) 72 h
Cinnamaldehyde
B
Cell Population (%)
100
80
10
5
0
Control
10
25
Treatments (μg/mL) 24 h
Cinnamaldehyde
B
Cell Population (%)
100
90
80
10
5
0
Control
10
25
Treatments (μg/mL) 72 h
Thymol
C
Cell Population (%)
100
80
5
0
Control
25
50
Treatments (μg/mL) 24 h
Thymol
C
Cell Population (%)
100
80
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5
0
Control
25
50
Treatments (μg/mL) 72 h

6 MANUSCRITO 2

An experimental murine model of otitis and dermatitis caused by *Malassezia pachydermatis*

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Title: An experimental murine model of otitis and dermatitis caused by *Malassezia pachydermatis*

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Abstract

We report a malasseziosis model in immunocompromised Swiss mice. For this model, the mice were immunosuppressed with a combination of cyclophosphamide at 150mg/kg and hydrocortisone acetate at 250mg/kg. Two groups were formed according to the site of inoculation. Dermatitis group received an intradermal injection of 5×10^6 cell/mouse at a shaved dorsal region while the otitis group received the same inoculum in the middle ear. Five animal/group were euthanized at different times, and the skin and ear were histopathologically analyzed. During the first euthanasia, which occurred after inoculation, microscopic examination showed that all mice presented budding yeast-like in a tissue sample. The presence of yeasts decreased over time being undetected on the 17^{th} day (dermatitis group) and the 21^{st} day (otitis group) after inoculation. This is the first murine model for malasseziosis that can be useful for evaluating new treatment approaches.

Keywords: malasseziosis; new infection model; otitis; dermatitis.

INTRODUCTION

The lipophilic yeast, *Malassezia pachydermatis*, is a normal commensal of the skin of wild and domestic carnivores.[1] Despite the fact that this yeast is part of the normal microbiota of the skin and external ear of dogs and cats, under some predisposing factors it can overgrow and lead to the development of dermatitis and otitis.[1, 2] The opportunistic nature of *M. pachydermatis* can be related to alterations on the skin surface microclimate or in host response, as well as to yeast virulence factors.[3] Although *M. pachydermatis* is primarily a zoophilic species, it can be found colonizing the skin and other sites in humans and has been reported to cause systemic infections in neonates and immunocompromised patients.[1, 4, 5]

Topical and systemic therapies with different antifungal agents, mainly azole compounds, are frequently used to treat *Malassezia* infections.[2, 3] The emergence of azole-resistant *M. pachydermatis* and the increasing in infections caused by *Malassezia* in both humans and animals, emphasizes the importance of finding new treatment approaches.[4, 6] The antifungal susceptibility test for *Malassezia* spp. has not yet been standardized and several studies using different test conditions have shown contradictory results on drug susceptibility.[7-9] Since these discrepant results may lead to difficulties in clinical practice, *in vivo* studies are needed to assess the correlation between *in vitro* findings and clinical outcomes. In this context, the objective of this study was to develop a murine experimental model of *Malassezia* otitis and dermatitis.

MATERIALS AND METHODS

Microorganisms

The standard strain of *M. pachydermatis* ATCC 14522, which was obtained from a case of external otitis in a dog, was selected for this study. *M. pachydermatis* strain was subcultured for 6 or 7 days at 32°C on Dixon agar supplemented with tween 80. For the

infection model, the inoculum was prepared in saline solution with a final cell density of 5×10^6 cells/mL. According to protocol dermal/cutaneous candidiasis,[10] the inoculum was incubated with shaking at 32°C for 24h, in YPD medium with 10% fetal bovine serum (FBS). After 24 h incubation, the inoculum was centrifuged for 1 min at 800×g, room temperature. The supernatant was discarded, and cells were resuspended in sterile PBS and again centrifuged for 1 min at 800×g, room temperature. The cell suspension was quantified in a hemocytometer and adjusted to a concentration of 5×10^6 cell/mL. Viability was confirmed by counting on SDA plates.

Mice

Seventy female Swiss mice, six-week-old (Central Animal Laboratory of the Federal University of Santa Maria - UFSM, Santa Maria, Brazil) weighing 24.7 g ± 2.67 were used in this study. The mice were housed at a temperature of 22ºC with 12-h light/dark cycles and provided food and sterile water *ad libitum*. All procedures were approved by Ethics Committee on the Use of Animals at UFSM (protocol number: 5870100217).

Ethics statement

All standard animal husbandry practices were followed meticulously during the study. Appropriate steps were adopted to keep the mice free from stress or discomfort. In order to prevent animal distress, humane endpoints were established at the very beginning of the experiment. Throughout the study, the mice were examined 3±4 times daily for clinical signs, such as rapid or very slow, shallow or labored breathing, weight changes, ruffled fur, hunched posture, impaired ambulation, or lethargy/drowsiness. Other signs taken into consideration included physical and mental alertness, chronic diarrhea and bleeding. These signs were used

to decide whether to euthanize the animals during the study or wait until the endpoint. In this sense, euthanasia was performed by deepening anesthesia with isoflurane (Cristalia).

Experimental infection model

In order to establish the infection, mice were submitted to an immunosuppression protocol. All mice received one intraperitoneal (i.p.) dose of cyclophosphamide (CYP, Baxter, 150mg/kg) 4 days before infection and a new i.p. dose of CYP (150mg/kg) plus one subcutaneous (s.c.) dose of hydrocortisone acetate (HCA, Sigma-Aldrich, 250mg/kg) at one day before infection. Prior the infection, mice were anesthetized with an intramuscular injection (50µL) of ketamine (37.5mg/mL) and xylazine (5mg/mL), and a cell suspension (50 µL) was inoculated in the middle ear and intradermal into the deep dermis and superficial fat of the shaved dorsal region. On day 0, all mice in the otitis group (n = 35), were instilled into middle ear with 50µL of croton oil (5% in acetone; Sigma-Aldrich), and after with 5×10^6 cells/mouse (50µL). The animals in the dermatitis group (n = 35) were infected with an intradermal injection of 5×10^6 cells/mouse at dorsal region. To follow the course of infection, mice were euthanized with isoflurane (inhalation excess) on days 3, 7, 10, 14, 17, 21, and 30 after the infection (5 mice/group/day). During euthanasia, middle ear and skin were aseptically harvested and processed. The animals were monitored daily up to 30 days postinfection for signs of morbidity and mortality. Moreover, the middle ear of the mice were observed through an otoscope. The body weights of the animals were recorded daily.

Quantitative skin cultures

Fungal burdens of the skin of all mice in dermatitis group were determined by CFU counting. The tissues were homogenized with manual pressure using glass beads (25 repetitions).[11] From the homogenate, dilutions (1:10, 1:100, and 1:1000) were performed in

saline solution and placed in SDA + chloramphenicol (0.05g/L) plates. CFU were enumerated after incubation at 32ºC for 72h. The fungal burden in the tissue is given as the $\log_{10}$ CFU per gram of tissue.

Histological studies

Middle ear and skin were removed from mice that were euthanized with isoflurane (inhalation excess) from otitis and dermatitis group, respectively. The organs were fixed in 10% buffered formalin for 24h; after paraffin embedding, 3-μm sections were stained with hematoxylin and eosin (H&E). Furthermore, tissue slices were stained with methenamine silver stain and scored as 0 (if no visible) or +, ++, and +++ proportional to the increase in the fungal load of the tissue with the purpose to quantify the fungal load.

Statistical analysis

Fungal burden in skin of dermatitis group, which were converted to $\log_{10}$ per gram of skin prior to analyses, were compared using the One Way ANOVA followed by Dunnett's post-hoc. Data were analyzed using Sigma Plot software (version 12.5). Significant differences were considered at $p < 0.05$.

RESULTS

Mice in the dermatitis group showed a nodule and erythema within 24 to 72h after inoculation. Between fourth and seventh days nodule ulcerated; erythema and crusting persisted for four weeks (Figure 1A). In the otitis group, mice presented erythema within 48h; desquamation started on day 3 up to day 7; desquamation and a yellowish secretion persisted

until the 14th day after inoculation (Figure 1B). The loss of corporal weight did not exceed 20% of the initial weight during the experiment. Besides, towards the end of the study there was a gain of weight (mean ± SD = 24.7 ± 2.67 at day 0 and 28.27 ± 3.96 at day 30).

The results of CFU from dermatitis group are shown in Figure 2. *M. pachydermatis* was recovered from the skin of all infected immunosuppressed mice (n=5/day) euthanized at days 3 or 7 post infection, and at days 10 or 14 days after inoculation only three mice/day had positive isolation. Statistical analyses showed that the number of $\log_{10}$ CFU decreased significantly in mice euthanized on day 10 or 14 compared to day 3 ($p < 0.01$ for both). *M. pachydermatis* was no longer detected from day 17 after infection. Qualitative histopathological results for skin and ear demontrated that the number of infected mice and the fungal load decrease over the time in both otitis and dermatitis groups (Table 1). Figure 1C-F demonstrates the main histological findings. From the skin lesions, in the deep dermis and/or in the subcutaneous tissue, we observed focally extensive areas consisting predominantly of neutrophilic inflammatory infiltrate. A variable number of oval, budding yeast-like organisms were seen at the center of these areas and within the stratum corneum (at epidermis) (Figure 1E and F). In the stratum corneum of the ear, we can see yeast-like organisms, ranging from mild (+) to moderate (++) amount (Figure 1C and D, respectively). There were also cases of orthokeratotic and/or parakeratotic hyperkeratosis on the skin and ear tissue of almost samples.

DISCUSSION

M. pachydermatis is an opportunistic yeast that needs predisposing factors to overgrowth and cause disease, such as excessive sebum production and/or epidermal trauma, which can be associated with hypersensitivity conditions, immune imbalance, keratinization disorders, antimicrobial or corticosteroid therapy, infections and endocrine disorders.[1, 3, 12, 13]

Moreover, previous studies evaluating different fungal infections in mice had demonstrated the need of the use of chemical immunosuppression protocols to induce disease.[14]

We successfully induced experimental malasseziosis in mice immunosuppressed with a combination of cytotoxic (CYP) and corticosteroid (HCA) drugs, which is a well-documented immunosuppression protocol used to facilitate experimental fungal infections.[15-18] The previous mice immunosuppression allowed the infection of the dermis and ear of all animals with the classical signs of malasseziosis, such as crusting, scaling, and erythema which can be observed during both animal and human infections.[3] Histopathological findings showed yeast-like organisms, inflammation and hyperkeratosis, confirming otitis and dermatitis by *Malassezia*.

Since there is not a stablished animal model of malasseziosis, the few *in vivo* studies about pathogenesis or treatment of the disease were performed in both experimentally and naturally infected dogs,[19, 20] including reports of experimental *M. pachydermatis*-induced otitis externa in healthy beagle dogs.[21] Attempts of experimental infection with *Malassezia* species in others mammals include rabbits,[22] guinea pig,[23] mice and rats.[24]

Clinical data about *Malassezia* infections demonstrated that the treatment of malasseziosis usually requires prolonged periods and high dosages of antifungal azole drugs suggesting that these drugs are prophylactically ineffective.[2, 12, 13, 25, 26] Moreover, relapses in animals are frequent.[2, 12] In this context, the development of experimental models may help in the new treatments approaches of the disease and the evaluation of new anti-*Malassezia* drugs.

In conclusion, we have successfully developed an animal model to study *Malassezia* otitis and dermatitis. From this model, the correlation between *in vitro* and *in vivo* antifungal drugs activities can be assessed. Moreover, it may be useful to discover new anti-*Malassezia* drugs to help in these difficult to treat and relapsing clinical forms of malasseziosis.

Acknowledgement

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Conflict of Interest

The authors report no conflicts of interest.

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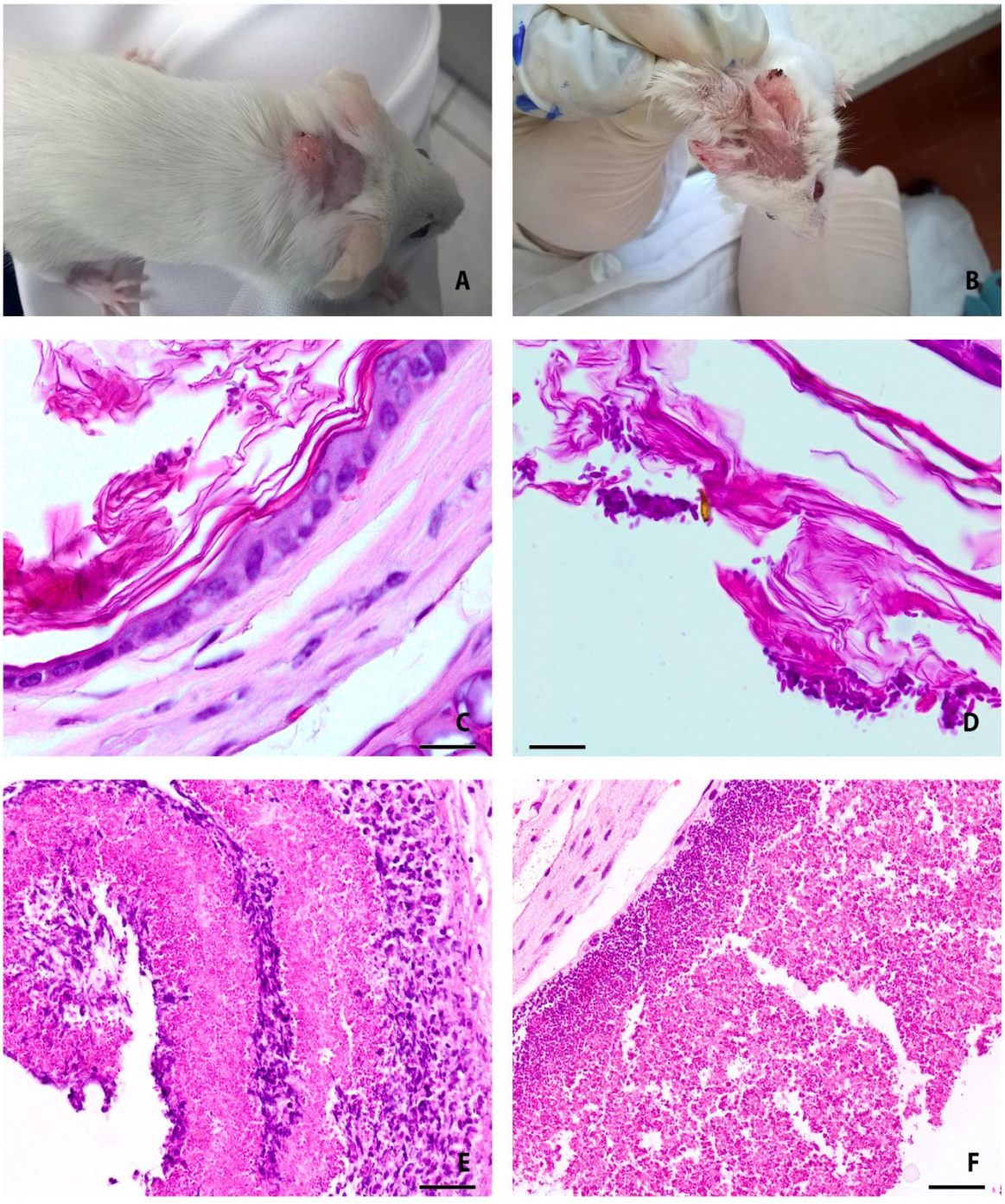
Figure Captions

Figure 1. Macroscopic and histological lesions of the skin and ear lesions of immunosuppressed mice infected with *Malassezia pachydermatis*. A) Nodular formation at day 3 after inoculation in mouse of the dermatitis group. B) Severe lesions in the ear advancing to the head in mouse of the otitis group. C) Stratum corneum of the ear canal, with mild amount of yeast (score +) (hematoxylin-eosin [HE, Bar = 10μm]). D) Stratum corneum of the ear canal, with moderate amount of yeast (score ++) (HE, Bar = 10μm). E) Skin sample: subcutaneous tissue with moderate amount of yeast (score ++) (HE, Bar = 20μm). F) Skin sample: subcutaneous tissue with moderate amount of yeast (score +++) (HE, Bar = 20μm).

Figure 2. Point and columns means plot of CFU recovered from skin of dermatitis group. Each data point corresponds to the $\log_{10}$ CFU/g of skin for an individual mouse. A point with error bars (standard deviations – SD) indicates the means for each day post infection. On days 3 and 7, n = 5; on days 10 and 14, n = 3.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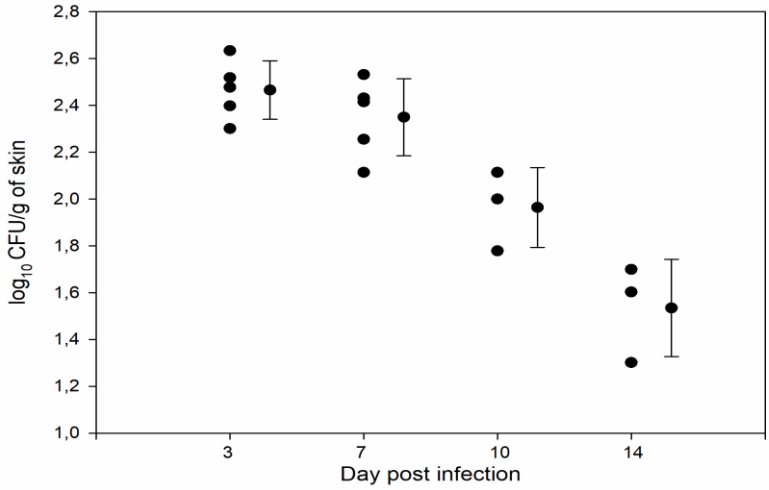
2,8
2,6
2,4
2,2
2,0
1,8
1,6
1,4
1,2
1,0
log10 CFU/g of skin
3
7
10
14
Day post infection

Table 1. Qualitative histopathological results for skin and ear from dermatitis and otitis groups.

Days post inoculation (n = 5/day)	Number of skin positive mice	Number of ear positive mice
3	5 (+++)	5 (++)
7	3 (+++)	5 (++)
10	3 (++)	3 (+)
14	0 (0)	2 (+)
17	1 (+)	1 (+)
21	0 (0)	1 (+)
30	0 (0)	0 (0)

+: used to indicate tissue fungal load; 0: without visible yeast

7 DISCUSSÃO

O gênero *Malassezia* é conhecido por incluir diferentes espécies, que estão associadas a várias doenças em humanos e animais (BOND et al., 2010; GAITANIS et al., 2012). As opções de tratamento para dermatite/otite por *Malassezia* em cães incluem a terapia sistêmica e/ou tópica com agentes antifúngicos, além de antibióticos. Derivados azólicos como itraconazol, cetoconazol, miconazol e clotrimazol são os mais utilizados, embora, a terbinafina e o tiabendazol também sejam usados (GREENE, 2006; NEGRE et al., 2009). Além disso, *M. pachydermatis* também pode causar infecções em seres humanos (AL-SWEIH et al., 2014; CHEN et al., 2017; CHRYSSANTHOU et al., 2001; GAITANIS etal., 2012). O fluconazol e a anfotericina B são geralmente utilizados nestes casos (CHRYSSANTHOU et al., 2001; GAITANIS et al., 2012).

Com base nos resultados dos testes de suscetibilidade *in vitro*, alguns estudos têm mostrado resistência à vários agentes antifúngicos (CAFARCHIA et al., 2012a; CAFARCHIA et al., 2012b; NASCENTE et al., 2003; NASCENTE et al., 2009). No entanto, esta questão permanece controversa, principalmente devido a falta de um método padrão, específico para *Malassezia* (PEANO et al., 2017). As condições empregadas nos métodos CLSI e EUCAST não são adequadas para *M. pachydermatis*, particularmente devido ao meio (caldo RPMI), que não suporta o crescimento adequado da levedura (CAFARCHIA et al., 2012c; PEANO et al., 2012). Além disso, *M. pachydermatis* apresenta um crescimento mais lento em comparação com as espécies de *Candida* (PEANO et al., 2012).

Atualmente, as técnicas padronizadas pelo o CLSI são consolidadas apenas para os gêneros *Candida* e *Cryptococcus*. Neste contexto, diversos estudos têm sido desenvolvidos, utilizando diferentes ajustes nos protocolos de acordo com as necessidades particulares do gênero (CAFARCHIA et al., 2012c; CAFARCHIA et al., 2015; GUPTA et al., 2000; VELEGRAKI et al., 2004).

Cafarchia et al. (2012c) avaliaram a suscetibilidade *in vitro* do cetoconazol, itraconazol e fluconazol, utilizando o protocolo padronizado pelo CLSI com modificações. Diferentes meio de cultivo foram testados, como caldo ureia de Christensen (CUB), RPMI 1640 contendo suplemento lipídico, Sabouraud dextrose com 1% Tween 80 (SDB) e caldo Dixon (DXB). Este estudo demonstrou um bom crescimento de *M. pachydermatis* em CUB, SDB e DXB e não em RPMI 1640.

Em nosso estudo, a suscetibilidade de *M. pachydermatis* foi avaliada *in vitro* através da combinação de agentes antifúngicos, com base na técnica M27-A3 (CLSI, 2008),

utilizando o caldo Sabouraud dextrose com 1% de tween 80. Nossos resultados demonstraram sinergismos para as combinações de ITZ + CSP e CLZ + CSP (55,17%). No entanto, houve predomínio de interações indiferentes, com percentuais acima de 60% para as combinações de KTZ+TRB, MCZ+TRB (62,07%), FLC+CSP e MCZ+NYS (68,96%).

Estudos dedicados à avaliação da combinação de agentes antifúngicos contra *M. pachydermatis* ainda são limitados. Recentemente, Álvarez-Pérez et al (2018) demonstraram o efeito da combinação de anfotericina B com azóis, em isolados de *M. pachydermatis*. Os resultados mostraram que a anfotericina B antagonizou o efeito do itraconazol.

Outro estudo avaliou o efeito combinado da polimixina B e do miconazol contra cinco cepas clínicas de *M. pachydermatis*. Este estudo demonstrou efeito sinérgico i*n vitro*, indicando o uso desta associação no tratamento da otite externa canina causada por *M. pachydermatis* (CHIAVASSA et al., 2013).

Estudos têm demonstrado, a ocorrência de cepas resistentes ou menos suscetíveis a algumas drogas. Dessa forma, o uso de fitoquímicos isolados de óleos essenciais com ação anti-*M. pachydermatis* são uma boa estratégia, conforme demonstram nossos resultados. Nesta perspectiva o segundo objetivo desse trabalho foi avaliar a suscetibilidade do carvacrol, timol e cinamaldeído associado a antifúngicos frente a isolados de *M. pachydermatis*. Além disso, avaliamos a citotoxicidade destes compostos em fibroblastos embrionários de ratos (linhagem celular 3T3).

As propriedades farmacológicas e toxicológicas destes fitoquímicos têm sido demonstrada em vários estudos (VINCIGUERRA et al., 2018; DE CASTRO et al., 2015; JESUS et al., 2015; KHAN & AHMAD, 2011; RAUT & KARUPPAYIL, 2014; LLANA-RUIZ-CABELLO et al., 2014). No entanto, nossos resultados fornecem a primeira demonstração de sinergismo entre CRV, THY e CIN com drogas antifúngicas contra *M. pachydermatis*. As maiores interações sinérgicas foram observadas para CRV + NYS, CRV + MCZ e THY + NYS (80%).

Faria et al. (2011) avaliaram a atividade da anfotericina B, fluconazol e itraconazol em combinação com 13 compostos fenólicos, frente a nove linhagens de *Candida* e uma linhagem de *Cryptococcus neoformans*. Todos os fármacos mostraram sinergismo quando foram associados ao timol frente aos isolados de *Candida albicans*. No entanto, frente aos isolados de *Cryptococcus neoformans*, apenas a combinação do timol com anfotericina B apresentou efeito sinérgico.

Guo et al. (2009) compararam a combinação do fluconazol com timol frente a 25 isolados de *Candida albicans* sensíveis e resistentes ao fluconazol. O fluconazol não

demonstrou atividade frente a *C. albicans.* Porém, quando associado ao timol, o fluconazol demostrou interações sinérgicas para 24 isolados de *C. albicans*. Taguchi et al. (2013) avaliaram o efeito isolado de cinamaldeído frente a isolados de *C. albicans*. Os resultados apresentaram atividade fungicida e fungistática contra *C. albicans*, afetando a estrutura das células.

Alguns estudos sugerem que a atividade antimicrobiana de óleos essenciais e seus componentes, pode ser atribuída à inibição de diferentes enzimas, principalmente aquelas envolvidas com a produção de energia e/ou síntese de componentes estruturais dos micro-organismos (LAMBERT et al., 2001). Outra hipótese estudada é de que estes compostos sensibilizam a bicamada fosfolipídica da membrana celular dos patógenos, causando um aumento da permeabilidade e perdas de constituintes intracelulares vitais, ou causando inibição ou danos nos sistemas enzimáticos (COX et al., 2000; SINGH et al., 2002). Alguns autores sugerem qua a alteração na atividade dos canais de cálcio é a causa do aumento da permeabilidade e liberação dos constituintes intracelulares, assim como um decréscimo no ATP intracelular nas células enquanto que, simultaneamente, há aumento no ATP extracelular, ocasionando uma ruptura na membrana celular do micro-organismo (ALIGIANNIS et al., 2001; HELANDER et al., 1998; PONCE et al., 2003; SARTORATTO et al., 2004).

Velluti et al. (2003) sugerem que a atividade antimicrobiana está ligada à estrutura de seus componentes, sendo que a presença de um núcleo aromático e um grupo OH fenólico, formariam ligações de hidrogênio com os sítios ativos de enzimas microbianas alvo. Ainda se acredita que núcleos aromáticos, contendo um grupo funcional polar, sejam os responsáveis pela atividade antimicrobiana dos óleos essenciais (LAMBERT et al., 2001; MILOS et al., 2000; PORTE & GODOY, 2001; SIKKEMA et al., 1994).

Nós investigamos os efeitos citotóxicos de CRV, CIN e THY *in vitro* usando fibroblastos de camundongos (linhagem celular 3T3). O ensaio de viabilidade celular (MTT) demonstrou efeitos citotóxicos apenas na concentração mais alta de CRV e THY (100 µg / mL). Enquanto que o CIN apresentou uma diminuição significativa na viabilidade celular em todas as concentrações, em ambos os tempos de incubação.

Nossos resultados estão de acordo com um estudo recente que concluiu que a exposição ao CRV (em concentrações acima de 100 µg / mL) aumentou as taxas de morte celular de linfócitos humanos (ARISTATILE et al., 2015).

Quando avaliamos a produção de ROS, nossos resultados mostraram que CRV, CIN e THY preveniram o estresse oxidativo através da diminuição nos níveis de oxidação de DCFH-

DA. Esses resultados são corroborados por outros estudos que mostram que o CRV exibe um efeito inibitório na COX (HOTTA et al., 2010), além de atividade antioxidante e de remoção de radicais livres (GUIMARÃES et al., 2010), o que poderia reduzir o estresse oxidativo e a inflamação. Ündeğer et al. (2009) observaram uma ligeira diminuição na geração de ROS na linhagem celular V79 na presença de THY (1-100 µM) e CRV (5 µM).

Além dos efeitos antioxidantes exibidos pelos óleos essenciais e seus constituintes, eles também podem exibir efeitos pró-oxidantes em altas concentrações, que podem danificar o DNA, os lipídios e outras moléculas biológicas (RAUT et al., 2014). Nossos resultados sugerem que o CRV preveniu danos no DNA em baixas concentrações (1, 5, 10 e 25 µg / mL). No entanto, o THY induziu danos no DNA em quase todas as concentrações testadas (5, 10, 50 e 100 µg / mL) em 24 h e o CIN induziu danos no DNA em apenas 50 e 100 µg / mL. Após 72h de incubação, o THY e o CIN induziram danos no DNA em concentrações acima de 25 µg / mL. Em apoio aos nossos achados, Ündeğer et al. (2009) relataram que as concentrações de até 25 µM de CRV e até 5 µM de THY, não induziram quebra significativa da cadeia de DNA em células V79, mas 25 µM de THY aumentaram o dano ao DNA. Llana-Ruiz-Cabello et al. (2014) estudaram pela primeira vez os efeitos genotóxicos do CRV em ratos e observaram que o CRV não induz genotoxicidade *in vivo* ou dano oxidativo ao DNA nos tecidos investigados.

Em nosso estudo também observamos um aumento (até 5%) na apoptose precoce e tardia após a exposição ao CRV e THY nas concentrações de 25 µg / mL e 50 µ / mL. No entanto, o nível de apoptose tardia aumentou (até 90%) na presença de CIN 25 µg / mL e um aumento na necrose (até 5%) foi observado em 10 µg / mL. Estudos anteriores demonstraram que o CRV e o THY aumentaram ligeiramente a incidência de morte celular por apoptose em concentrações utilizadas para fins antimicrobianos em células Caco-2 (DUSAN et al., 2006). Outro estudo utilizando células Caco-2 demonstrou que a CRV aumentou a apoptose tardia e a necrose em concentrações superiores a 230 µM, mas que THY não causou apoptose ou necrose em nenhuma das concentrações testadas (LLANA-RUIZ-CABELLO et al., 2014).

Os resultados obtidos neste estudo são encorajadores porque a faixa de concentrações testadas nos ensaios de citotoxicidade não foi prejudicial às células ou ao DNA e incluiu as CIMs obtidas para todos os isolados testados.

Estudos futuros devem ser realizados com estes compostos, pois o maior desafio a partir da descoberta da atividade antifúngica é a extrapolação para o tratamento *in vivo*, definindo a toxicidade, via de administração e posologia destes compostos. Fundamentado nesses dados, o objetivo do manuscrito 2 foi desenvolver um modelo de infecção experimental para *M.*

pachydermatis. Até o momento não existe um modelo estabelecido para *Malassezia*, dessa forma, desenvolvemos um modelo de malasseziose em camundongos Swiss imunocomprometidos com uma combinação de ciclofosfamida (CYP) e acetato de hidrocortisona (HCA). Esse protocolo de imunossupressão já é usado em diversos estudos para facilitar infecções fúngicas experimentais (DENARDI et al., 2018; GEBREMARIAM et al., 2017; LI et al., 2012; SHEPPARD et al., 2004). A imunossupressão prévia dos camundongos permitiu a infecção da derme e da orelha de todos os animais com os sinais clássicos de malasseziose (GAITANIS et al., 2012). Além disso, os achados histopatológicos mostraram leveduras, inflamação e hiperqueratose, confirmando otite e dermatite por *Malassezia pachydermatis*.

Tondolo et al. (2017) desenvolveram um novo modelo de pitiose vascular / disseminada em camundongos imunossuprimidos usando uma combinação de drogas (CYP + HCA).

Outro estudo demonstrou interação sinérgica entre posaconazole e anidulafungina em camundongos imunossuprimidos com uma combinação de CYP + HCA infectados por *Aspergillus fumigatus* (DENARDI, et al., 2018).

Neste contexto, os estudos *in vitro* são preliminares, todavia, as combinações sinérgicas merecem avaliações *in vivo*, pois podem fornecer tratamentos alternativos eficazes contra *M. pachydermatis* e consequentemente reduzir a resistência frente aos antifúngicos. Além disso, desenvolvemos com sucesso um modelo de infecção para *M. pachydermatis*. A partir deste modelo, podemos avaliar a correlação entre a atividade de drogas antifúngicas *in vitro* e *in vivo*.

8 CONCLUSÕES

Com base nos resultados deste trabalho, podemos concluir que:

- Os principais sinergismos observados foram as combinações de ITZ + CSP e CLZ + CSP (55,17%). No entanto, as combinações *in vitro* entre antifúngicos, mostraram predomínio de interações indiferentes.

- As combinações entre antifúngicos e frações de OEs apresentaram elevados percentuais de sinergismo, principalmente para CRV + NYS, THY + NYS e CRV + MCZ (80%);

- CRV e THY apresentaram toxicidade apenas nas concentrações mais altas testadas (50 e 100 µg / mL);

- CIN apresentou maior toxicidade do que o CRV e THY;

- Foi desenvolvido um modelo de infecção experimental *in vivo* de malasseziose, utilizando camundongos Swiss imunocomprometidos. A partir deste modelo, a correlação entre as atividades de drogas antifúngicas *in vitro* e *in vivo* poderá ser avaliada.

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ANEXO A – SUBMISSÃO DO MANUSCRITO 1

From: **Journal of Essential Oil Research** <onbehalfof@manuscriptcentral.com>
Date: 2018-05-28 17:05 GMT-03:00
Subject: Journal of Essential Oil Research - Manuscript ID TJEO-2018-0188 has been submitted online
To: janio.santurio@gmail.com

28-May-2018

Dear Dr Santurio:

Your manuscript entitled "Antifungal activities of carvacrol, cinnamaldehyde and thymol against Malassezia pachydermatis and cyto and genotoxicity in embryonic fibroblast 3T3 cell line" has been successfully submitted online and is presently being given full consideration for publication in Journal of Essential Oil Research.

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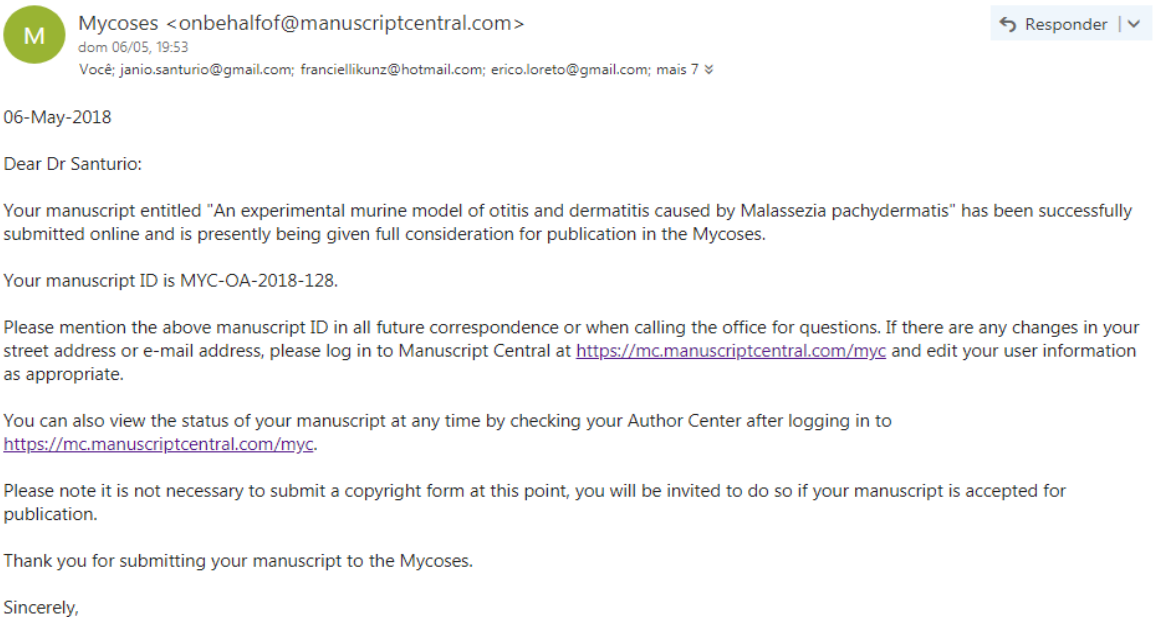
Thank you for submitting your manuscript to Journal of Essential Oil Research.

Sincerely,
Journal of Essential Oil Research Editorial Office

ANEXO B – SUBMISSÃO DO MANUSCRITO 2



Mycoses <onbehalfof@manuscriptcentral.com>
dom 06/05, 19:53
Você; janio.santurio@gmail.com; franciellikunz@hotmail.com; erico.loreto@gmail.com; mais 7

Responder

06-May-2018

Dear Dr Santurio:

Your manuscript entitled "An experimental murine model of otitis and dermatitis caused by Malassezia pachydermatis" has been successfully submitted online and is presently being given full consideration for publication in the Mycoses.

Your manuscript ID is MYC-OA-2018-128.

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Sincerely,

ANEXO C - CARTA DE APROVAÇÃO

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que a proposta intitulada "Combinação de drogas antifúngicas e não antifúngicas in vitro e in vivo para o tratamento de Malassezia pachydermatis ", protocolada sob o CEUA nº 5870100217, sob a responsabilidade de **Janio Morais Santurio** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 05/04/2017.

We certify that the proposal "Combination of antifungal and non-antifungal drugs in vitro and in vivo for the treatment of Malassezia pachydermatis", utilizing 80 Heterogenics mice (80 females), protocol number CEUA 5870100217, under the responsibility of **Janio Morais Santurio** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 04/05/2017.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 04/2017 a 05/2017 Área: Microbiologia E Parasitologia

Origem:	Biotério Central UFSM				
Espécie:	Camundongos heterogênicos	sexo: Fêmeas	Idade:	5 a 6 semanas	N: 80
Linhagem:	Swiss		Peso:	20 a 25 g	

Resumo: Malassezia pachydermatis é uma levedura pertencente à microbiota normal de animais e, usualmente, apontada como responsável por otites externas e também por diversas formas de dermatites, principalmente em cães. A instalação do quadro clínico indica uma alteração do equilíbrio existente entre o microrganismo comensal e seu hospedeiro, sendo desencadeado pela excessiva multiplicação da M. pachydermatis em função de alterações no mecanismo de defesa do hospedeiro. Em resposta às falhas da terapêutica antifúngica, vários autores têm buscado estratégias que garantam o sucesso da atividade antifúngica. Entre essas estratégias a combinação de fármacos tem merecido atenção. Neste contexto, ressalta-se a importância de analisar as interações de fármacos com atividade antifúngica, frente a fungos do gênero Malassezia, no intuito de descobrir interações sinérgicas que possam servir como possíveis alternativas mais eficazes de tratamento para esta patologia.

Local do experimento: Local onde será mantido o animal até o final do experimento: Biotério Setorial do Departamento de Farmacologia (Prédio 21).

Santa Maria, 02 de junho de 2018

Prof. Dr. Denis Broock Rosemberg
Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Saulo Tadeu Lemos Pinto Filho
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria