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AVALIAÇÃO DA ATIVIDADE ANTIFÚNGICA DOS COMPOSTOS CUMARÍNICOS FRENTE ÀS CEPAS DO GÊNERO *Aspergillus*.

FELIPE QUEIROGA SARMENTO GUERRA

JOÃO PESSOA
2016

Felipe Queiroga Sarmento Guerra

AVALIAÇÃO DA ATIVIDADE ANTIFÚNGICA DOS COMPOSTOS CUMARÍNICOS FRENTE ÀS CEPAS DO GÊNERO *Aspergillus*.

Tese de Doutorado apreseentada ao Programa de Pós-Graduação em Produtos Naturais e Sintéticos Bioativos do Centro de Ciências da Saúde da Universidade Federal da Paraíba, em cumprimento aos requisitos necessários para obtenção do título de doutor em Produtos Naturais e Sintéticos Bioativos, área de concentração: Farmacologia.

Orientadora: Profa. Dra. Edeltrudes de Oliveira Lima

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Tese de doutorado defendida em 18 de Fevereiro de 2016**.**

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“Para se ter algo que você nunca teve, é preciso fazer algo que você nunca fez”

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GUERRA, F. Q. S. Avaliação da atividade antifúngica dos compostos cumarínicos frente às cepas do gênero *Aspergillus*. Tese (Doutorado em Produtos Naturais e Sintéticos Biotaivos, área de concentração: farmacologia), Centro de Ciências da Saúde, Universidade Federal da Paraíba, João Pessoa.

RESUMO

As infecções fúngicas, nas últimas décadas, têm se destacado devido ao seu crescente aumento e suas elevadas taxas de morbidade e mortalidade. Pacientes imunocomprometidos são os que possuem maior susceptibilidade a estes micro-organismos. Dentre estas infecções fúngicas, as espécies pertencentes ao gênero *Aspergillus* spp. possuem alta incidência entre os fungos filamentosos. A terapêutica antifúngica tem sido alvo de numerosos estudos e nestes tem sido destacado o crescente aumento de espécies fúngicas resistentes. Dessa forma, destaca-se a importância da busca de novas fontes terapêuticas que se apresentem mais eficazes e com menos tóxicas ao hospedeiro. Assim o objetivo deste estudo foi avaliar a atividade antifúngica *in vitro* e relação estrutura atividade (SAR) dos compostos cumarínicos frente às espécies do gênero *Aspergillus.* Para tal foi determinada a CIM de 24 compostos cumarínicos e posteriormente realizado estudos SAR com softwares computacionais. Dos 24 compostos ensaiados, 12 demonstraram possuir atividade antifúngica e 5 obtiveram forte atividade antifúngica. Destes derivados cumarínicos com forte atividade antifúngica, dois merecem destaque, 7-hidroxi-6-nitrocumarina (Cou-UNO_2) e 4-acetóxicumarina (Cou-UMB16), com melhores valores de CIM, foram avaliados via testes de inibição do crescimento micelial, germinação dos conídios e testes de determinação do mecanismo de ação. Por fim os produtos foram avaliados em combinação com antifúngicos padrões. Os resultados demonstraram que 12 derivados cumarínicos dos 24 ensaiados possuem atividade antifúngica, com valores de CIMs variando entre 1024-16µg/mL. A SAR demonstra que a adição de grupos eletronegativos, como substituintes do anel benzopirona, favorece a atividade antifúngica destes compostos. Os derivados cumarínicos com melhores CIM (16µg/mL), 7-hidroxi-6-nitrocumarina (Cou-UNO_2) e 4-acetóxicumarina (Cou-UMB16) foram capazes de inibir o crescimento micelial e a germinação dos conídios de *Aspergillus* spp. E estes possuem ação sobre a estrutura da parede celular fúngica. Em uma concentração subinibitória, Cou-UNO_2 potencializou a ação *in vitro* dos derivados azólicos e em combinação com os derivados azólicos (voriconazol e itraconazol) observou-se um efeito aditivo. Já Cou-UMB16 em combinação com os derivados azólicos (voriconazol e itraconazol) obteve um efeito sinérgico a aditivo, dependendo da linhagem utilizada. Logo este estudo conclui que os derivados cumarínicos possuem atividade antifúngica

contra as espécies de *A. flavus e A. fumigatus*. As cumarinas Cou-UNO$_2$ e Cou-UMB16 foram capazes de inibir o crescimento micelial e a germinação dos conídios e que esta atividade seja devido a sua ação sobre a parede celular fúngica. E que esta atividade é favorecida pela presença de moléculas eletronegativas no anel básico da cumarina (1,2-benzopirona).

Palavras chave: *Aspergillus flavus*, *Aspergillus fumigatus*, atividade antifúngica, cumarinas, relação estrutura-atividade.

GUERRA, F. Q. S. Avaliação da atividade antifúngica dos compostos cumarínicos frente às cepas do gênero *Aspergillus*. Tese (Doutorado em Produtos Naturais e Sintéticos Biotaivos, área de concentração: farmacologia), Centro de Ciências da Saúde, Universidade Federal da Paraíba, João Pessoa.

ABSTRACT

In recent decades fungal infections have increased, and their high rates of morbidity and mortality have brought them much attention. Immunocompromised patients are more susceptible to microorganism infections; in particular, fungal species of the genus *Aspergillus* spp. Among the current infectious filamentous fungi they have a rather high incidence. Antifungal treatments have been subjected to numerous studies, and the increasing number of resistant fungal species has been highlighted. Thus, we see the importance of seeking new, more effective, and less toxic therapeutic sources. The aim of this study was to evaluate the in vitro antifungal activity and structure activity relationships of coumarin compounds against *Aspergillus* species. For this the MIC of 24 coumarin compounds was determined and subsequent SAR studies were performed with computer software. 12 of the 24 tested coumarin derivatives have antifungal activity, with 5 has shown excelent activity. Thus two derivatives, 7-hydroxy-6-nitrocoumarin (Cou-UNO_2), and 4-acetoxycoumarin (Cou-UMB16), with better MIC values were evaluated via inhibition of mycelial growth and conidial germination, and by mechanism of action testing. The products were evaluated in combination with antifungals standards. The results showed that 12 of the 24 tested coumarin derivatives have antifungal activity, with MIC values ranging from 1024-16μg/ml. SAR studies have showed that the presence of a short aliphatic chain, and/or electronegative groups like ring substituents are favorable for antifungal activity. The coumarin derivatives with better MIC values (16μg/ml); Cou-UNO_2 and Cou-UMB16 were capable of inhibiting both the mycelial growth and conidial germination of *Aspergillus* spp. Their activity is on the structure of the fungal cell wall. Cou-NO_2, in a sub-inhibitory concentration, enhanced the *in vitro* effects of azoles, and in combination with azoles (voriconazole and itraconazole) there was an additive effect. Cou-UMB16 in combination with azoles (voriconazole and itraconazole) had synergistic or additive effects depending on the strain used. Thus this study concludes that coumarin derivatives have antifungal activity against the species *A. flavus and A. fumigatus*. The coumarin Cou-UMB16 and Cou-UNO_2 have able to inhibit the mycelial growth and conidio germination and that this activity is due to its action on the fungal cell wall and the presence of electronegative groups as substituents of the benzopyrone ring favors this activity.

Keywords: *Aspergillus flavus*, *Aspergillus fumigatus*, antifungal activity, coumarin, structure-activity relationship.

LISTA DE ABREVIATURAS E SIGLAS

AB: Agar Batata

ASD: Agar Sabouraud dextrose

ATCC: *American Type Culture Collection*

CFM: Concentração Fungicida Mínima

CIF: Concentração Inibitória Fracionada

CIM: Concentração Inibitória Mínima

DMSO: Dimetilsulfóxido

OMS: Organização Mundial de Saúde

RPMI: *Roswell Park Memorial Institute*

SAR: *Structure Activity relationship*

sp: espécie

spp: espécies

UFC: Unidade Formadora de Colônia

UTIs: Unidades de Terapia Intensiva

µg/mL: microgramas por mililitro

µg: micrograma

mL:mililitro

OBS.: Os termos não citados nesta relação se encontram descritos no texto.

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INTRODUÇÃO

1 INTRODUÇÃO

As infecções fúngicas, nas últimas décadas, têm se destacado devido ao seu crescente aumento e suas elevadas taxas de morbidade e mortalidade. A incidência de infecções hospitalares por espécies fúngicas representam mais de 25% das hemoculturas hospitalares. Pacientes imunocomprometidos são os que possuem maior susceptibilidade a estes micro-organismos, causando uma grande preocupação entre os profissionais da saúde. Sendo as infecções invasivas com maior índice de mortalidade, principalmente nestes indivíduos, por serem frequentemente severas, de progressão rápida, e ainda possuírem um diagnóstico e tratamento complicados (CRUZ et al., 2007; CARVALHO, 2013).

Dentre os agentes etiológicos das infecções fúngicas hospitalares destaca-se as espécies pertencentes ao gênero *Aspergillus* spp. possuindo maior incidência entre os fungos filamentosos (RICHARDSON, 2005). A incidência de aspergilose invasiva pode chegar a 15% em pacientes transplantados, sendo que a taxa de mortalidade varia entre 60-90%, dependendo do tipo de paciente infectado, tornando-se uma grande preocupação entre os profissionais de saúde (TEKAIA; LATGÉ, 2005).

1.1 *Aspergillus* spp.

O termo *Aspergillus*, derivado do latim (asperge), provém da semelhança entre as cabeças aspergilares (conidióforo do fungo) e o instrumento usado para aspergir água benta em cerimônias religiosas. O gênero foi descrito em 1729 pelo padre Micheli, caracterizado por fungos ubiquitários do solo, água, e organismos em decomposição (BENNETT, 2010)

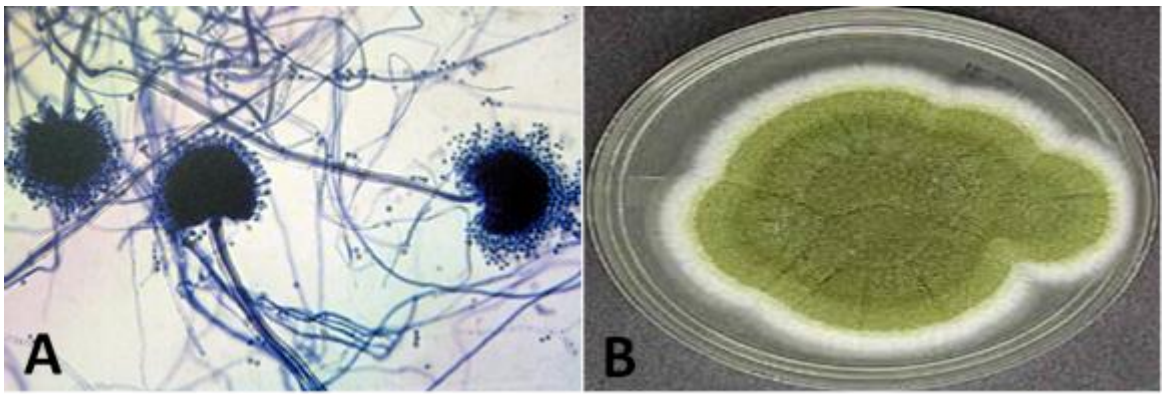
Os fungos filamentosos do gênero *Aspergillus* pertencem à família dos *Aspergillaceae* e à classe dos *Ascomicetos*, suas espécies possuem hifas ramificadas, septadas, incolores e refringentes, conidióforo com haste simples, saindo de uma célula base, com extremidade globosa ou clavada (vesícula). Da superfície da vesícula saem fiálides com aspecto de garrafas e disposição radiada no seu conjunto (figura 1). Na extremidade de cada uma, forma-se uma cadeia de conídeos basipetos, globosos, secos e de cor variável (MURRAY; ROSSENTHAL; PFALLER, 2006).

Com relação ao aspecto macroscópico, as colônias apresentam uma superfície de cor branca, na fase inicial de maturação com textura algodonosa. Posteriormente, dependendo das

espécies, sua cor pode evoluir para verde, amarelo, castanho ou preto e com textura pulverulenta com a produção dos conídeos (figura 1) (LARONE, 2002)

Apesar de grande variedade de espécies de *Aspergillus*, somente cerca de 20 foram isoladas e implicadas como patógenos em humanos e em animais domésticos. Apesar de cosmopolitas, o clima é um fator importante na predominância de algumas espécies em relação a outra, por exemplo, *Aspergillus fumigatus* é a espécie mais isolada em países de clima temperado, já as *A. flavus* e *A. niger* tem maior incidência em países tropicais (SIDRIM, 2004).

Figura 1: Aspecto microscópico (A): hifas hialinas septadas e ramificadas com cabeças aspergilares: Conidióforos, vesículas, fiálides e fialoconídeos. Aspecto macroscópico (B): colônias de *Aspergillus* sp. em meio Agar Saubouraud dextrose, incubadas por 5 dias a 25^0C.



Fonte: Disponível em http://www.moldremoval.com/*Aspergillus*.html, acessado em 15/07/2015.

Aspergillus fumigatus é o agente etiológico responsável por aproximadamente 90% das aspergiloses invasivas diagnosticadas. Esta espécie foi descrita pela primeira vez pelo médico Georg W. Fresenius, em 1863. O nome *Fumigatus* é derivado do latim " *fumigave* ", que significa fumaça referindo-se ao micélio azul-cinza esfumaçado (ARAÚJO; PINA-VAZ; RODRIGUEZ, 2005).

Apresentando mecanismos únicos de resistência ao stress, *A. fumigatus* possui a capacidade de ser termotolerante em temperaturas que podem variar entre 15ºC a 53ºC. Assim, esta característica sugere uma grande adaptação do fungo ao crescimento no hospedeiro (MARTINS et al., 2005; BHABHRA; ASKEW, 2005; CARVALHO, 2013).

Aspergillus fumigatus é caracterizado micromorfologicamente por possuir conidióforos hialinos, de parede lisa, com vesícula em forma de balão, apresentando uma fila

única de fiálides. Já a macromorfologia das colônias, em meio agar Sabouraud dextrose pós 5 dias de incubação, geralmente de aspecto aveludado e de coloração cinza-esverdeado ou cinza-azulada (figura 2). Pode ocorrer produção de pigmento em algumas amostras, figura 2 (SIDRIM, 2004; MARTINS et al., 2005).

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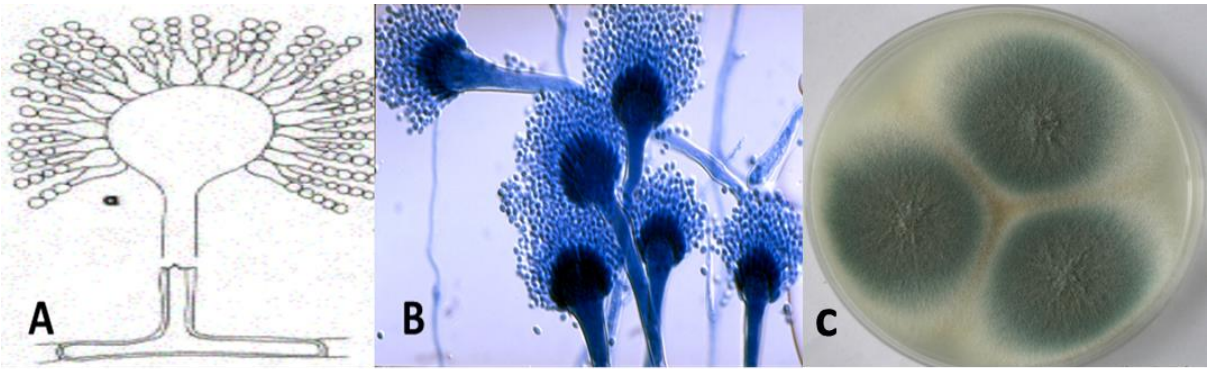


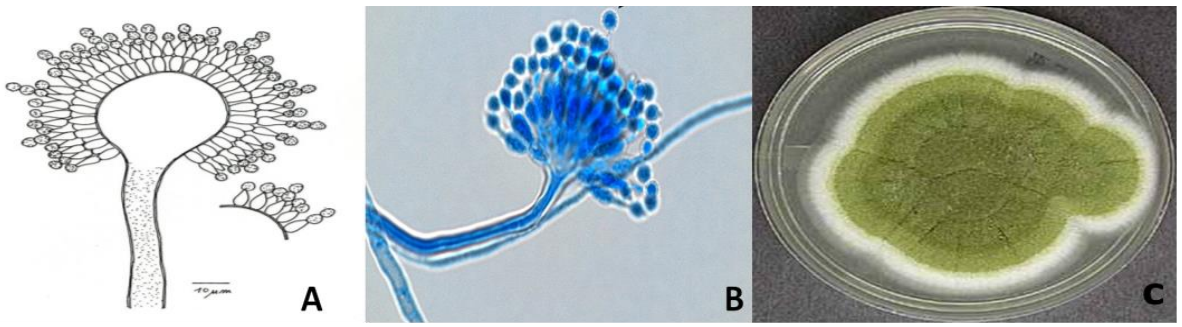
Fonte: disponível em http://www.mold.ph/*Aspergillus*.html., acessado em 20/07/15

Aspergillus flavus foi descrito pela primeira vez por Link em 1809 e caracteriza-se por ter o conidióforo com haste longa, grossa, vesícula globosa coberta com uma camada de fiálides seguida de conídios em cadeia que formam cabeças conidiais radiadas, com 300 μm a 400 μm de diâmetro e as fiálides são unisseriadas ou bisseriadas (figura 3). Os conídios são globosos e equinulados (MARTINS et al., 2005; CARVALHO, 2013)

As colónias de *A. flavus*, em agar sabouraud dextrose (ASD) por 5 dias à temperatura ambiente, apresenta uma textura cotonosa, plana com sulcos radicais e coloração amarelo-esverdeada (figura 3) (MARTINS et al., 2005; CARVALHO, 2013).

Espécies de *Aspergillus* produzem uma grande variedade de condições crônicas e alérgicas. O processo de infecção do fungo no hospedeiro ocorre principalmente através da via respiratória, devido à grande veiculação no ar atmosférico de conídios produzidos por *Aspergillus* spp. No entanto, há a necessidade de uma predisposição do hospedeiro, em relação a um défice imunológico, para que ocorra o desenvolvimento patológico. Outra porta de entrada relatada é a pele (SIDRIM, 2004; WALSH et al., 2008).

Figura 3: Representação gráfica microscópica das cabeças aspergilares de *A. flavus* (A). Aspecto micromorfológico de *A. flavus*, em aumento de 400x (B). Macroscopia da colônia de *A. flavus* em meio Agar Sabouraud dextrose pós 5 dias de incubação (C).



Fonte: disponível em http://www.mold.ph/*Aspergillus*.htm., acessado em 20/07/15

Os principais quadros clínicos observados devido à infecção por *Aspergillus* spp. são aspergilose cutânea (infecção na pele), otomicose aspergilar (infecção do conduto auditivo externo), onicomicose aspergilar (infecções nas unhas), aspergiloma ou bola fúngica (infecção nos pulmões), sinusite aspergilar (infecção dos seios paranasais), acometimento neurológico, e micotoxicoses, que representam intoxicações crônicas por produtos do metabolismo do fungo, as aflatoxinas (AMORIM et al., 2004).

A aspergilose pulmonar invasiva acomete principalmente imunossuprimidos em fase de neutropenia prolongada. As manifestações clínicas nem sempre são específicas, lembrando outros quadros infecciosos, como pneumopatias bacterianas ou virais. Tendo os sintomas bases: febre, dor torácica, hemoptise, tosse e dispneia. Doença que possui um prognóstico complicado devido à dificuldade no diagnóstico e pela própria condição do paciente (CARVALHO, 2013; MONTEIRO et al., 2012).

Em alguns indivíduos pode ocorrer o desenvolvimento abundante do fungo na cavidade pulmonar, gerando uma massa micelial compacta, conhecida como aspergiloma ou bola fúngica. Tendo como principal manifestação clínica a hemoptise recidivante, no entanto pode ser observado outros sintomas como tosse, anorexia e febre (SIDRIM, 2004; WALSH et al., 2008).

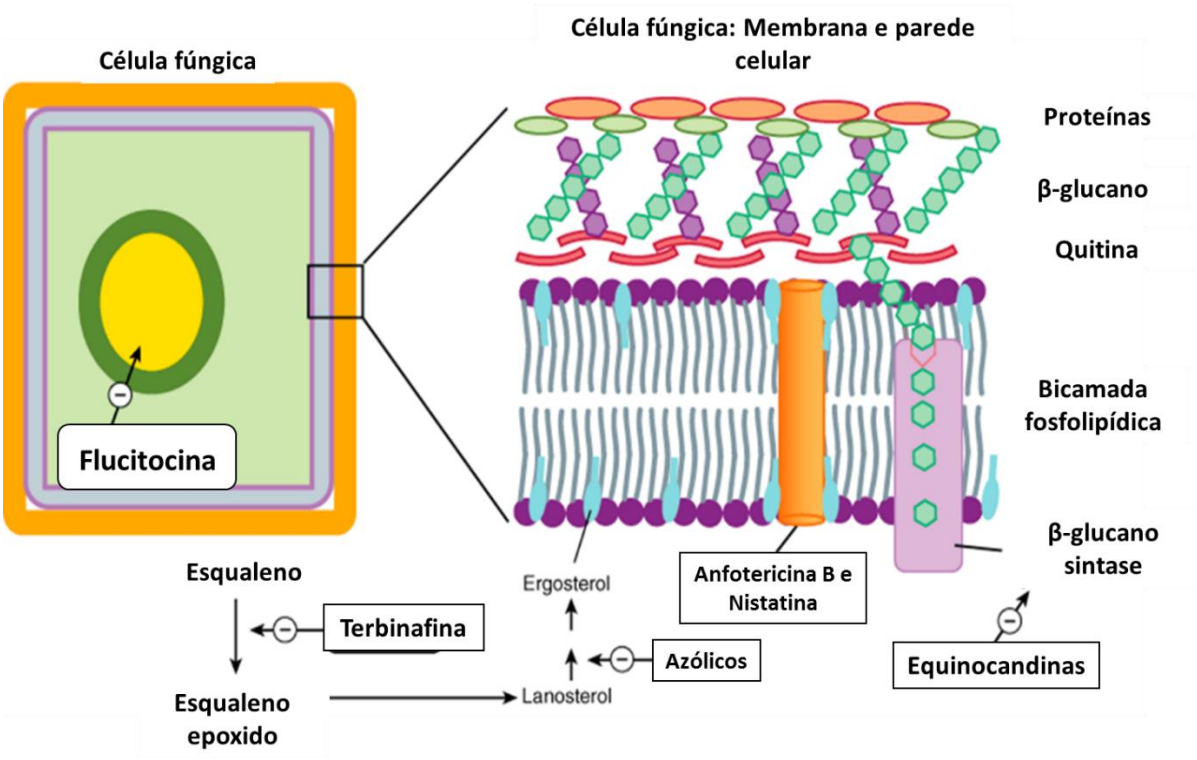
A aspergilose cutânea pode ser primária, resultante de um traumatismo, como em pacientes que sofreram queimaduras, ou via disseminação hematogênica a partir de um foco primário, normalmente pulmonar. Outras infecções estão relacionadas a disseminação

secundária do fungo para o conduto auditivo, otomicose aspergilar, ou nos seios paranasais, desenvolvendo a sinusite aspergilar (SIDRIM, 2004; OZER, 2009).

1.2 TERAPÊUTICA MEDICAMENTOSA DAS ASPERGILOSES

Vários antifúngicos com mecanismos de ação diferentes são utilizados no tratamento das micoses. A griseofulvina penetra na célula, e no núcleo interagindo com os microtúbulos desfazendo o fuso mitótico, o que inibe a multiplicação do fungo. Os derivados poliênicos (nistatina e anfotericina B) alteram a permeabilidade da membrana celular pela ligação ao ergosterol presente na membrana da célula. A flucitosina gera na célula a 5-fluorouracil, que inibe a enzima timidilato-sintetase e consequentemente a síntese de DNA. Geralmente esta droga é empregada em associação com a anfotericina B (ODDS et al., 2003).

Figura 4: Mecanismo de ação das principais classes antifúngicas.



Fonte: Modificado de KATSUNG et al., 2012.

Cada caso clínico de aspergilose deve ter enfoque particularizado na terapêutica. Os antifúngicos mais comuns usados contra este micro-organismo são os derivados azólicos (intraconazol, voriconazol e posaconazol), anfotericina b em várias formulações e a

caspofungina, como alternativa as cepas resistentes (BADDLEY et al., 2013; JACOBS et al., 2011).

Os antifúngicos azóis compreendem duas grandes classes, os imidazóis e os triazóis que compartilham o mesmo espectro antifúngico e o mesmo mecanismo de ação. Os primeiros possuem dois átomos de nitrogênio no anel azólico, enquanto os triazólicos possuem três (quadro 1). Os triazóis são metabolizados lentamente e exercem menos efeitos sobre a síntese de esteróis humanos, logo são os mais utilizados atualmente (JACOBS et al., 2011; PEREIRA, 2009).

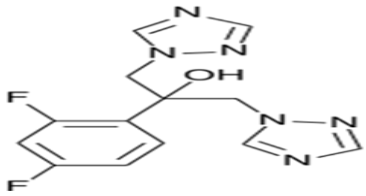
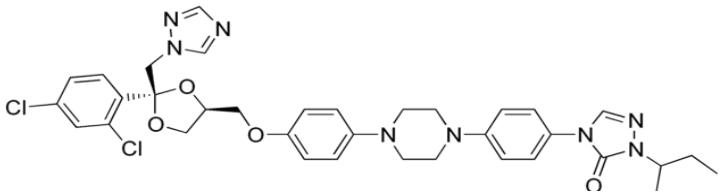
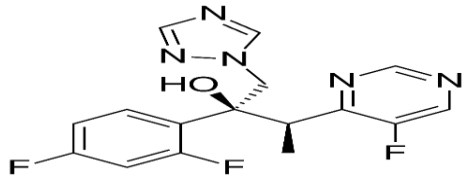
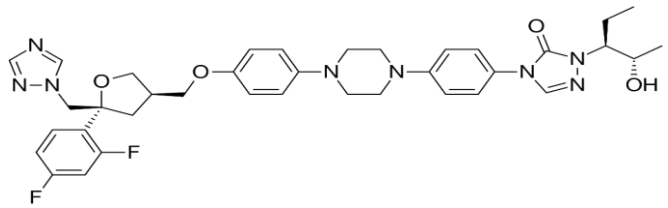
O seu principal mecanismo é a inibição da enzima 14-α-desmetilase do lanosterol, uma enzima ligada ao citocromo p450. Esta enzima está envolvida na conversão do lanosterol em ergosterol. Desta forma, estes fármacos inibem a síntese de ergosterol da membrana celular fúngica e levam à acumulação de 14-α-metilesteróis, esteróis tóxicos à estrutura fúngica (quadro 1) (BRUNTON; CHABNER; KNOLLMANN, 2012; WALSH et al., 2008)

A inibição da síntese de ergosterol resulta na inibição do crescimento da célula fúngica (fungistático) ou na morte celular (fungicida). Este mecanismo é específico porque estes antifúngicos têm maior afinidade para as enzimas da célula fúngica do que para as enzimas do citocromo p450 humanas quando comparado a outros azóis (CHANDRASEKAR, 2010; MURRAY et al., 2006).

Da classe triazol, o voriconazol mostrou maior eficácia contra *Aspergillus* spp. e possui menos efeitos tóxicos quando comparado a anfotericina B, logo este é o antifúngico de primeira escolha no tratamento da aspergilose invasiva. Este possui uma estrutura química (quadro 1) semelhante à do fluconazol (bistriazol fluorado), porém com atividade aumentada *in vitro*, espectro ampliado e hidrossolubilidade baixa. (BRUNTON; CHABNER; KNOLLMANN, 2012)

Baddley et al., 2013, relatam que algumas drogas utilizadas para o tratamento de tuberculose ou epilepsia reduzem os níveis sanguíneos do voriconazol. E este pode necessitar de um ajuste da dose para maximizar o sucesso principalmente em crianças, idosos e doentes com problemas de fígado ou cirrose.

Quadro 1. Estrutura química dos principais derivados azólicos.

Estrutura química	Nomeclatura
	Fluconazol
	Itraconazol
	Voriconazol
	Posoconazol

Fonte: Disponível em http://www.google.com/patents/EP2343053A1?cl=en, acessado em 20/07/15.

O intraconazol é utilizado como alternativa terapêutica secundária para o tratamento dos casos de aspergilose invasiva. Este é um triazol lipofílico (quadro 1) de alto peso molecular que pode ser usado por via oral ou intravenosa. A absorção do itraconazol pela via de oral é irregular e necessita de um pH gástrico ácido, logo este pode sofrer interferência do uso concomitante com antiácidos (WALSH et al., 2008).

O posoconazol (quadro 1) possui estrutura química semelhante ao intraconazol mas tem sido estudado no tratamento da aspergilose invasiva somente em formulação oral. Atualmente é utilizado de forma profilática contra *Aspergillus* para pacientes neutropênicos com leucemias e mielodisplasia (BADDLEY et al., 2013; BRUNTON; CHABNER; KNOLLMANN, 2012)

Pacientes na qual a aspergilose é resistente ao voriconazol, a opção terapêutica incluem o uso de formulações da anfotericina B ou uma equinocandina, tal como a caspofugina (WALSH et al., 2008).

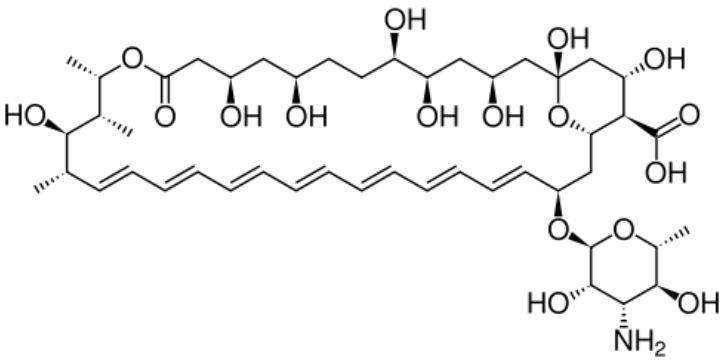
Anfotericina B foi descoberta, em 1955, por Gold e colaboradores, quando investigavam uma estirpe de *Streptomyces nodosus*, esta pertence à família dos macrolídeos poliênicos com atividade antifúngica. Esta contém sete ligações duplas conjugadas na posição *trans* e uma 3-amino-3, 6-didesoximanose ligada ao anel principal, como descrito na figura 5, conferindo certa hidrossolubilidade em valores extremos de pH. (FILIPPIN; SOUZA, 2006).

Seu mecanismo de ação antifúngico está relacionado a sua ligação ao ergosterol, o principal esterol da membrana celular fúngica. Quando a anfotericina B se liga ao ergosterol, formam-se canais iónicos na membrana celular que destroem a integridade osmótica da membrana celular fúngica e levam à perda dos constituintes intracelulares e à morte celular (figura 4) (CHANDRASEKAR, 2010).

A terapêutica com anfotericina B é condicionada pela sua toxicidade, particularmente a nível renal. Esta limitação levou ao desenvolvimento de formulações lipídicas do fármaco, com o objetivo de reduzir a toxicidade. Existem atualmente quatro formulações de anfotericna B comercialmente disponíveis: anfotericina B convencional (C-AMB), anfotericina B lipossomal (L-AMB), complexo lipídico da anfotericina B (ABLC) e dispersão coloidal de anfotericina B (ABCD) (BRUNTON; CHABNER; KNOLLMANN, 2012).

Aspergillus terreus é mais resistente à anfotericina B do que outras espécies de *Aspergillus*, no entanto o mecanismo dessa resistência ainda não é bem esclarecido (BLUM et al., 2013; LASS-FLORL, 2012).

Figura 5: Estrutura química da Anfotericina B



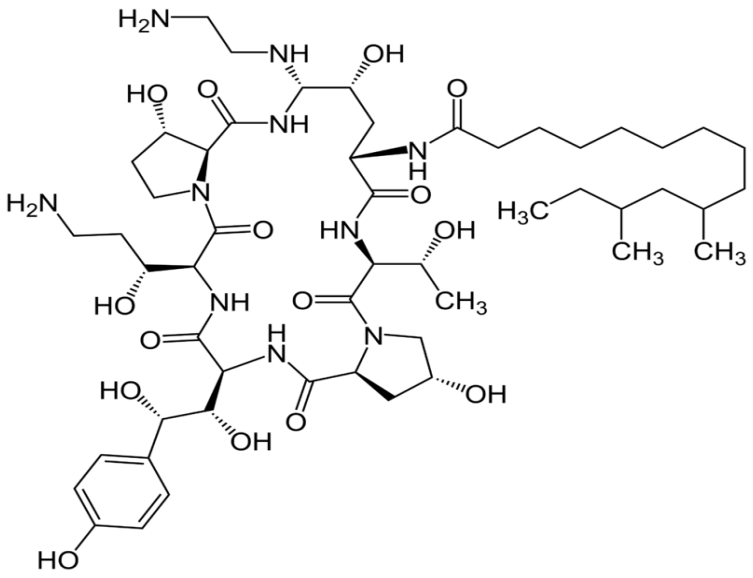
Fonte: Disponível em http://www.iqb.es/cbasicas/farma/farma04/a042.htm, acessado em 15/07/2015.

A procura de produtos naturais de fermentação fúngica na década de 1970 levou à descoberta das equinocandinas. Três equinocandinas estão aprovadas para uso clínico: a caspofungina, a anidulafungina e a micafungina. Todas são lipopeptídeos cíclicos com um núcleo hexapeptídico (figura 6). As equinocandinas inibem a síntese de 1,3-glicano, um polímero de glicose que é necessário para manter a estrutura das paredes celulares fúngicas (figura 4). Na ausência deste polímero, as células fúngicas perdem a integridade e a lise rapidamente se segue (BRUNTON; CHABNER; KNOLLMANN, 2012).

A caspofungina como membro deste grupo, é efetiva no tratamento das formas de aspergilose invasiva que são refratárias à anfotericina B. O acetato de caspofungina é um lipopeptídeo semissintético hidrossolúvel, sintetizado a partir do produto da fermentação de *Glarea lozoyensis* (WALSH et al., 2008; BRUNTON; CHABNER; KNOLLMANN, 2012)

O aumento de cepas resistentes às drogas azólicas e os vários efeitos adversos associados ao uso da anfotericina B, incluindo nefrotoxicidade e neurotoxicidade, faz com que o tratamento das enfermidades fúngicas seja muitas vezes ineficaz, sendo assim motivo de grande preocupação entre profissionais da saúde (MICELI; LEE, 2011).

Figura 6: Estrutura química da Caspofungina



Fonte: http://www.iqb.es/diccio/c/ca5.htm, acessado em 15/07/2015.

Diversos mecanismos bioquímicos contribuem para o fenótipo de resistência a drogas nos fungos. Os principais mecanismos de resistência aos derivados azólicos são a diminuição da permeabilidade da membrana aos antifúngicos, mutações ou aumento da expressão da lanosterol 14-demetilase e a diminuição do acúmulo intracelular das drogas devido a sistemas de efluxo. Verificando na prática clínica cada vez mais um aumento na incidência de cepas resistentes aos antifúngicos azólicos (BOWYER et al., 2011). Modificações qualitativas e quantitativas no ergosterol da membrana é a principal causa de resistência aos poliênicos (FUOLI et al., 2008).

1.3 PRODUTOS NATURAIS E SEUS DERIVADOS: Cumarinas

A terapêutica antifúngica tem sido alvo de numerosos estudos e nestes tem sido destacado o crescente aumento de espécies fúngicas resistentes aos antimicrobianos convencionais e a incidência de infecções fúngicas por *Aspergillus* spp. em pacientes imunocomprometidos. Dessa forma, destaca-se a importância da busca de novas fontes terapêuticas para o tratamento das infecções fúngicas, que se apresentem mais eficazes e com menos efeitos adversos para o hospedeiro.

O uso terapêutico das plantas medicinais é um dos pontos mais característicos da espécie humana, tão antigo quanto à própria humanidade e encontrado praticamente em todas as civilizações e grupos culturais conhecidos. No tocante às aplicações das plantas medicinais

com propósito antimicrobiano, a ideia de que certas plantas tinham potencial dito cicatrizante ou curativo era bem aceita muito antes da descoberta dos próprios ‘micróbios’ pela humanidade. Atualmente, sabe-se que essas plantas continham o que é caracterizado como propriedades antimicrobianas (PEREIRA, 2009).

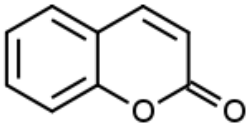
As atividades biológicas de substâncias antimicrobianas existentes em vegetais encontram-se associadas à presença de compostos químicos oriundos do metabolismo secundário das espécies vegetais como alcalóides, flavonóides, taninos, cumarinas, glicosídios e os óleos essenciais. É nesse sentido que o estudo apropriado da química e farmacologia de plantas medicinais ou de seus derivados se apresentam como um instrumento relevante de investigação de suas propriedades (PEREIRA, 2009).

Além de suas propriedades antimicrobianas intrínsecas, substâncias derivadas de produtos naturais podem alterar o efeito de antifúngico, seja aumentando a atividade antimicrobiana ou revertendo à resistência aos antifúngicos convencionais. A utilização destas substâncias pode representar um avanço contra os mecanismos de resistência que inativam antifúngicos padrões (CASTRO, 2010).

Assim, o estudo e a descoberta de produtos naturais e com princípios ativos que apresentem atividade antimicrobiana intrínseca ou combinada com antifúngicos de uso comum podem representar uma nova ferramenta de fazer frente aos micro-organismos multidroga resistentes, além de impedir o contato destes com os produtos sintéticos, diminuindo o risco de se selecionar novos ou melhores mecanismos de resistência. Dessa forma, pode-se direcionar a indústria farmacêutica de forma a favorecer a produção e o uso de fitoterápicos como adjuvantes para o combate aos agentes infecciosos (COUTINHO et al., 2008).

Dentre os metabólitos derivados de plantas medicinais, destacam-se as cumarinas, grupo de compostos, derivados do metabolismo da fenilalanina, que consiste no anel benzeno unidos ao anel pirano, sendo o representante mais simples e o mais encontrado, a cumarina (1,2-benzopirona), figura 9. O primeiro representante foi isolado por Vogel em 1820 na espécie *Coumarona adorata*. As moléculas cumarínicas podem estar presentes em várias partes das plantas e também são isoladas de fungos e bactérias (RIBEIRO, 2002; CELEGHINI et al., 2001).

Figura 7: Estrutura química básica da cumarina (1,2-benzopirona)



Fonte: Disponível em http://www.cosmeticaemfoco.com.br/2008/02/alerta-sobre-as-cumarinas.html, acessado em 25/07/15

Estes metabólitos são largamente encontrados em todo o mundo, tem-se isolado cumarinas em várias partes da planta tanto nas folhas como nas raízes e frutos, de várias famílias diferentes, como por exemplo *Fabaceae, Asteraceae, Rutaceae, Saxifragaceae* e *Thymelaceae* (RAZAVI, 2011).

Pode ser encontrada na natureza uma grande variedade de substituintes do anel básico, 1,2 – benzopirona, das cumarinas, o que pode explicar a extensa variedade de efeitos farmacológicos destes compostos, incluindo: anticoagulantes, estrogênios, fotossensibilizante, anti-helmíntico, sedativo, vasodilatador, analgésico, hipotérmicos e antimicrobianos (ONUMA et al., 2011; HAMDI et al., 2012; STEIN et al., 2006; DURAIPANDIYAN; IGNACIMUTHU, 2009; GARCIA et al., 2011).

Diversas cumarinas e os produtos derivados delas já tiveram comprovados quanto a sua atividade antifúngica. E acredita-se que estas propriedades são normalmente um reflexo do seu próprio papel nos vegetais, como fitoalexinas (WIDODO et al., 2012; ZEID, 2002; RAZAVI, 2011).

A atividade biológica interessante das cumarinas tem feito estes compostos alvos atraentes para síntese orgânica. Várias estratégias de síntese foram já desenvolvidas. As cumarinas podem ser sintetizado pela reação de Perkin, reação de Pechmann ou por condensação de Knoevenagel saliciladeídos com ácido malônico (ROSITCA et al., 2002; AL-AMIERY et al., 2012; AGRODY et al., 2001)

4.3 ESTUDOS DE RELAÇÃO ESTRUTURA ATIVIDADE (SAR)

As cumarinas são extremamente variáveis em sua estrutura química, a sua atividade biológica é influenciada devido aos vários tipos de substituições no seu núcleo básico. Logo um estudo cuidadoso da relação entre sua estrutura e a atividade antifúngica observada, pode

aumentar a comprensão dos mecanismos pelos quais estas cumarinas agem (SOLTANI et al., 2009; WIDODO et al., 2012; RAZAVI, 2011).

A pesquisa e desenvolvimento de novos agentes antimicrobianos e/ou a modificação molecular de compostos químicos já existentes para descoberta de novos compostos-protótipo com atividade biológica potencial são importantes ferramentas para se garantir o combate ao crescente número de fungos patogênicos resistentes e levar ao uso clínico de compostos cada vez mais eficientes e menos tóxicos (ALMEIDA et al., 2010).

Os estudos de relações estrutura-atividade (SAR: *Structure-Activity Relationships*) são utilizados para definir os parâmetros importantes para a ação de uma classe de compostos análogos contra certa patologia, pois parte da premissa que estruturas semelhantes tendem então a possuir a mesma atividade farmacológica e interagir com o mesmo sítio (NETO et al., 2006).

Estes estudos procuram estabelecer uma relação matemática, sob a forma de uma equação, entre a atividade biológica e os parâmetros (ou propriedades) físico-químicos mensuráveis que possuam influência sobre a atividade terapêutica de uma classe de compostos. Estes estudos são realizados utilizando programas de computador que calculam as propriedades físico-químicas mensuráveis de substâncias químicas (quando os cálculos são realizados com as estruturas tridimensionais dos compostos que estão sendo estudados, tem-se o processo denominado modelagem molecular) e que avaliam a relação destas com a atividade biológica, gerando equações de regressão ou modelos de previsão (planejamento de fármacos auxiliado por computador, CADD: *Computer Aided Drug-Design*) (ALMEIDA et al., 2010).

Esses modelos devem ser capazes de explicar o fenômeno observado bem como de proporcionar previsões dentro e, se possível, fora dos limites observados, sendo utilizadas técnicas estatísticas multivariadas que podem gerar modelos lineares ou não. Para os modelos lineares, a técnicas de Mínimos Quadrados Parciais (MQP ou PLS - *Partial Least Squares*) é a função matemática mais utilizada (ALMEIDA et al., 2010).

Os compostos cumarínicos são objetos de vários estudos SAR observados na literatura científica. Destacando aqueles no qual se observa a busca de melhores substituintes no anel benzopirano para aumentar sua atividade antimicrobiana. Mladenović et al., 2010, demostrou que a adição de substituintes hidroxila e acetil nas posições 4 e 3, respctivamente tinham relação com a tividade antimicrobiana. Jha et al., 2009, destaca que a adição de ligações rotativas ao anel central pode ser favorável para o efeito antifúngico.

Um número crescente de infecções fúngicas disseminadas causadas por espécies do gênero *Aspergillus* tem alarmado a comunidade científica por dois motivos: primeiro, sua fácil disseminação em ambientes hospitalares e segundo, a grande dificuldade no tratamento das enfermidades causadas por este micro-organismo relacionado ao tempo prolongado do uso do antifúngico para um tratamento efetivo e seus efeitos adversos, além da baixa sucetibilidade de algumas cepas aos antifúngicos utilizados na prática clínica, sendo observada uma alta taxa de mortalidade em pacientes imunocomprometidos.

Atualmente, há aumento do interesse por terapias alternativas e o uso terapêutico de produtos naturais, especialmente por derivados de plantas. Este interesse por drogas oriundas de plantas se deve a várias razões, como pela ineficiência da medicina convencional (efeitos adversos e terapia ineficaz) ou por uma grande parte da população não ter acesso ao tratamento farmacológico convencional. Sendo o Brasil o detentor da maior diversidade genética do mundo, com cerca de 55 mil espécies catalogadas. Apesar da riqueza da flora brasileira, nos últimos 20 anos, o número de informações sobre plantas medicinais tem crescido apenas 8% anualmente (PEREIRA, 2009; CARNEIRO et al., 2015).

A pesquisa de um novo protótipo antifúngico sobre fungos potencialmente patogênicos ou oportunistas se faz necessário, fator de significativo estímulo para a realização desta proposta de trabalho. Há a possibilidade de contribuir para a pesquisa científica quanto à investigação farmacológica e a relação estrutura atividade de um novo antifúngico, com a perspectiva de amplo espectro de ação, pouco tempo de uso e mínimos efeitos adversos sobre o hospedeiro.

OBJETIVOS

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a atividade antifúngica *in vitro* e relação estrutura atividade dos compostos cumarínicos frente às espécies do gênero *Aspergillus.*

2.2 OBJETIVOS ESPECÍFICOS

- Determinar a Concentração Inibitória Mínima (CIM) dos compostos cumarínicos;
- Avaliar o efeito das cumarinas, com melhores resultados de CIM, sobre a cinética de crescimento micelial e na germinação dos conídeos;
- Analisar a ação das respectivas cumarinas sobre a parede celular fúngica e sobre a membrana celular fúngica.
- Avaliar a interferência das respectivas cumarinas sobre o efeito dos antifúngicos padrões.
- Realizar estudos de CADD (Computer-Aided Drug Design) com a série de cumarinas avaliada, através de ferramentas de modelagem molecular, SAR e quimiometria.

MATERIAL E MÉTODOS

3 MATERIAL E MÉTODOS

3.1. LOCAL DA PESQUISA

O trabalho foi realizado nos Laboratórios de Micologia do Departamento de Ciências Farmacêuticas, do Centro de Ciências da Saúde (CCS), da Universidade Federal da Paraíba (UFPB). E parcerias, para o apoio no desenvolvimento do trabalho foram realizadas no Laboratório de Ecologia Química – UFPB - Campus IV, no Laboratório De Síntese Orgânica do Departamento de Ciências Biológicas da Universidade Estadual da Paraíba. Período: Agosto de 2012 a Dezembro de 2014.

3.2 ANTIFÚNGICOS PADRÕES

Os produtos utilizados na execução das metodologias foram adquiridos da Sigma-Aldrich®. Os fármacos de escolha foram anfotericina B, fluconazol, intraconazol e o voriconazol, comumente utilizados como produtos de escolha para o tratamento de infecções por *Aspergillus* spp. As soluções também foram preparadas no momento de execução dos testes.

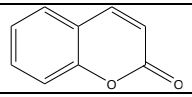
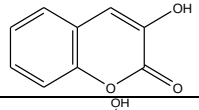
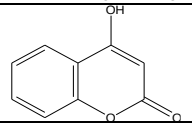
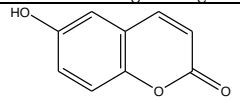
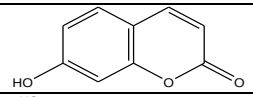
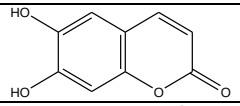
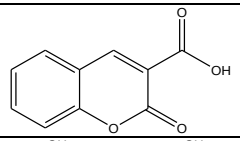
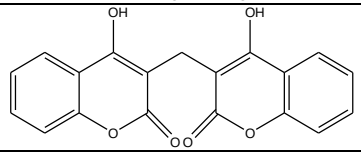
3.3 MICRO-ORGANISMOS

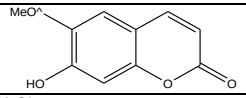
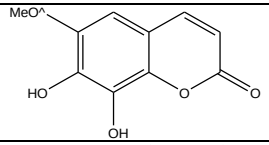
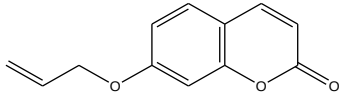
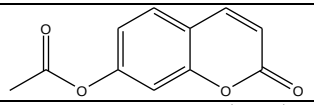
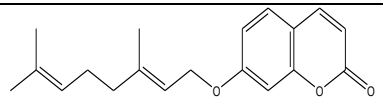
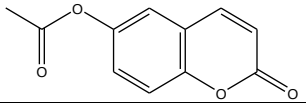
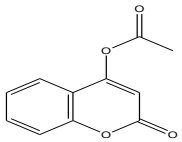
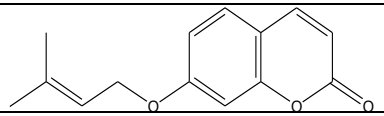
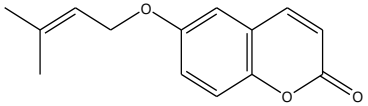
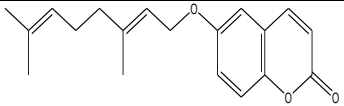
Para os ensaios de atividade antifúngica foram selecionadas um total de 14 cepas de fungos do gênero *Asperlillus*, especificamente 7 cepas de *A. fumigatus* (ATCC 16913, ATCC 40640, ATCC 46913, IPP 210, LM 121, LM 743 e LM 135) e sete cepas de *A. flavus* (ATCC 16013, LM-247, LM-210, LM-26, LM 35, LM 36 e LM 23) obtidas da coleção do Laboratório de Micologia. As cepas estoques dos fungos utilizados nos ensaios foram mantidas em tubos de ensaio contendo agar batata inclinado, sob refrigeração (4°C) e a temperatura ambiente (28-30°C).

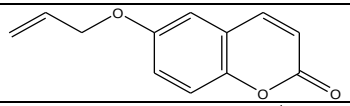
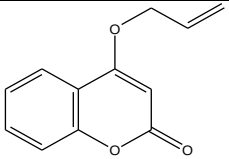
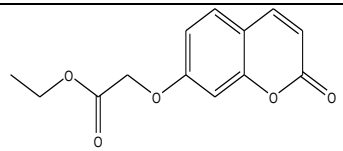
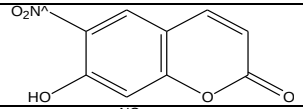
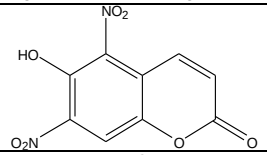
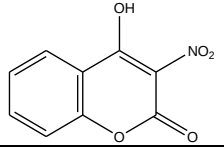
3.4 COMPOSTOS CUMARÍNICOS

Foram utilizadas para avaliação da atividade antifúngica, uma série de cumarinas obtidas de fontes naturais e sintéticas, uma parte comprada via Sigma-Aldrich® e outra parte sintetizada pelo grupo do laboratório de fitoquímica sob responsabilidade dos professores Dr. José Maria Barbosa Filho e Dr. Francisco J. B. Mendonça Junior, fazendo parte da dissertação do aluno Rodrigo S. A. de Araújo, processo de síntese está descrito no artigo de Araújo et al., 2013. Estes estão dispostos no quadro abaixo:

Quadro 2: Estrutura química, nomenclatura e origem das 24 cumarinas analisadas.

N^0	**Estrutura química**	**Nomenclatura**	**Origem de aquisição**
1		1,2-benzopirona ou Cumarina	Sigma-Aldrich®
2		3-hidroxicumarina	Sigma-Aldrich®
3		4-hidroxicumarina	Sigma-Aldrich®
4		6-hidroxicumarina	Sigma-Aldrich®
5		7-hidroxicumarina	Sigma-Aldrich®
6		6,7-diidroxicumarina	Sigma-Aldrich®
7		Cumarina-3-ácido carboxílico	Sigma-Aldrich®
8		3,3′-metileno-bis (4-hidroxicumarina)	Sigma-Aldrich®

9		6-metóxi-7-hidroxicumarina	Sigma-Aldrich®
10		7,8-diidroxi-6-metóxicumarina	Sigma-Aldrich®
11		7alilóxicumarina	Sigma-Aldrich®
12		7-acetóxicumarina	Sigma-Aldrich®
13		7-geranilóxicumarina	Sigma-Aldrich®
14		6-acetóxicumarina	Sintética*
15		4-acetóxicumarina	Sintética*
16		7-prenilóxicumarina	Sintética*
17		6-prenilóxicumarina	Sintética*
18		6-geranilóxicumarina	Sintética*

19		6-alilóxicumarina	Sintética*
20		4-alilóxicumarina	Sintética*
21		7-O-(etóxi de etila)-cumarina	Sintética*
22		7-hidróxi-6-nitro-2H-1-benzopiran-2-ona ou 6-nitro-7-hidroxicumarina	Sintética*
23		5,7-binitro-6-hidroxicumarina	Sintética*
24		2-nitro-3-hidroxicoumarina	Sintética*

* Derivados sintetizados no laboratório de fitoquímica sob responsabilidade dos professores Dr. José Maria Barbosa Filho e Dr. Francisco J. B. Mendonça Junior.

3.5 MEIOS DE CULTURA

Os meios de cultura utilizados nos ensaios para avaliação da atividade antifúngica foram o meio sólido Ágar Sabouraud Dextrose (ASD) e o meio líquido RPMI (*Roswell Park Memorial Institute*) 1640 adquiridos da Difco® e Sigma Aldrich®, respectivamente, preparados de acordo com as instruções do fabricante. Para conservação das cepas e preparação do inóculo foi utilizado o ágar batata (AB), também adquirido da Difco®. Os meios foram solubilizados com água destilada e esterilizados em autoclave, a 121°C por 15 minutos.

3.6 INÓCULO:

Na preparação do inóculo dos fungos, primeiramente os isolados foram cultivados em meio AB a 28°C por 8 dias, para atingirem um bom crescimento. As recentes colônias fúngicas foram devidamente cobertas com 5 mL de solução salina estéril (NaCl 0,85 % p/v). A mistura resultante de conídios e fragmentos de hifas foi retirada e transferida para tubos de ensaio esterilizados. Em seguida, essas suspensões foram agitadas por 2 minutos com auxílio do aparelho Vortex. A padronização do inóculo foi realizada através da contagem das unidades formadoras de colônias (UFC) em câmera de Neubauer, no qual se obteve um inóculo de aproximadamente 10^6 UFC/mL (CLEELAND; SQUIRES, 1991; HADACEK; GREGER, 2000; SAHIN et al., 2004).

3.7 AVALIAÇÃO DAS ATIVIDADES ANTIFÚNGICAS *in vitro*

3.7.1 Determinação da Concentração Inibitória Mínima (CIM)

A determinação da CIM dos produtos foi realizada pela técnica de microdiluição, utilizando placas contendo 96 cavidades com fundo em forma de “U” e em duplicata (CLEELAND; SQUIRES, 1991; HADACEK; GREGER, 2000; SAHIN et al., 2004; Moreira et al., 2010). Em cada orifício da placa, foi adicionado 100 µL do meio líquido RPMI 1640 duplamente concentrado. Posteriormente, 100 µL da solução dos produtos, também duplamente concentrado, foram dispensados nas cavidades da primeira linha da placa. E por meio de uma diluição seriada a uma razão de dois, foram obtidas concentrações de 1024 µg/mL até 4 µg/mL,

de modo que na primeira linha da placa se encontrará a maior concentração e na última, a menor concentração. Por fim, foi adicionado 10 µL do inóculo das espécies nas cavidades, onde cada coluna da placa refere-se a uma cepa fúngica, especificamente.

Um controle de micro-organismo foi realizado colocando-se nas poços 100 µL do mesmo RPMI 1640 duplamente concentrado, 100 µL de água destilada estéril e 10 µL do inóculo de cada espécie. Para verificar a ausência de interferência nos resultados pelo solvente utilizado na preparação das soluções, no caso o DMSO, foi feito um controle no qual foi colocado nas cavidades 100 µL do caldo duplamente concentrado, 100 µL do DMSO (10% em água destilada estéril) e 10 µL da suspensão. Um controle de esterilidade também foi realizado, onde foi colocado 200 µL do RPMI 1640 em um orifício sem a suspensão dos fungos. As placas foram seladas e incubadas a 25-28°C por até 72 horas para ser realizada a leitura. Paralelamente, foi realizado o mesmo experimento com o antifúngico Anfotericina B, o qual foi testado nas mesmas concentrações dos produtos.

Define-se a CIM para os produtos testados como a menor concentração capaz de inibir visualmente o crescimento fúngico verificado nos orifícios, quando comparado com o crescimento controle. Os resultados foram expressos em CIM_{90} para aquelas concentrações que cada produto conseguiu inibir 90% das cepas ensaiadas, respectivamente. Logo, os testes posteriores foram utilizadas as duas cumarinas com melhores CIM_{90}.

A atividade antimicrobiana dos produtos foi interpretada e considerada ativa ou não, conforme os seguintes critérios CIM até 500µg/mL são considerados com forte atividade antimicrobiana, com CIM entre 600 e 1600µg/mL possuem atividade moderada e CIM acima de 1600µg/mL é considerado com atividade fraca (SARTORATTO et al., 2004).

3.7.2 Efeitos sobre o crescimento micelial

A análise da interferência do produto natural sobre o crescimento micelial foi realizada pela determinação da massa micelial seca dos fungos em estudo, utilizando-se a técnica de diluição em caldo (RASOOLI et al., 2006; SHARMA; TRIPATHI, 2008). Em um tubo de ensaio esterilizado foram adicionados 10 mL do RPMI 1640, previamente acrescido da solução dos produtos cumarínicos, anfotericina B com a concentração de CIM_{90} em seguida foi

adicionado um fragmento de aproximadamente 1 cm^2 das colônias fúngicas crescidas em ASD a 28°C por 8 dias. No tubo controle correspondente, o fragmento foi inoculado em 10 mL de RPMI 1640. Todo o sistema foi incubado a 28°C por um tempo total de 10 dias.

Para isto, as culturas foram autoclavadas a 121°C por 30 segundos com a finalidade de causar inativação das estruturas fúngicas, seguida pela filtração da cultura através de papel de filtro estéril e lavagem com água destilada estéril. O micélio retido no papel de filtro foi submetido à secagem em estufa a 60°C por 4 horas e a 40°C "overnight". Ao término, o papel de filtro contendo o micélio seco foi pesado e o percentual de inibição do crescimento micelial foi calculado, comparando os resultados obtidos no experimento teste com os resultados do experimento controle. Os ensaios foram realizados em duplicata e os resultados expresso pela média aritmética obtidas nos dois ensaios.

3.7.3 Efeitos sobre a germinação dos conídeos fúngicos

Aspergillus spp. produzem numerosos conídeos assexuados os quais são veiculados no ambiente e, portanto, são considerados um fator importante no desencadeamento das infecções no hospedeiro. Pensando nisto, julga-se importante avaliar quantitativamente o poder dos produtos em interferir na germinação dos conídeos fúngicos das cepas de *Aspergillus* spp.

Assim, os produtos foram testados em suas respectivas CIM_{90} e um controle apenas com água destilada foram utilizados. A partir de repiques recentes dos fungos em AB, foi preparado o inóculo dos mesmos com solução salina estéril, contendo uma mistura resultante de conídios e fragmentos de hifas. Em seguida, essas suspensões foram agitadas por 2 minutos com auxílio do aparelho Vortex, deixando em repouso por 20 minutos, tempo para que os fragmentos de hifas se sedimentem e o sobrenadante, contendo os conídios ser então recolhido. O número de conídios foi determinado em uma câmara de Neubauer e ajustado a 10^6 conídeos/mL. Em tubos de ensaio estéreis, 500 µL do RPMI 1640 duplamente concentrado acrescido do composto cumarínico nas concentrações finais desejadas, foram homogeneamente misturadas com 500 µL da suspensão dos conídios fúngicos e imediatamente incubado a temperatura de 28°C. Amostras dessa mistura foram tomadas no tempo de 24 h para análise. Todo o experimento foi feito em duplicata, onde o número de conídios foi determinado em uma câmara de Neubauer e o percentual de inibição das alterações dos conídeos foi calculado,

comparando os resultados obtidos no experimento teste com os resultados do experimento controle, nos diversos intervalos de tempo. A análise foi realizada em microscópio óptico comum (Zeiss® model Primo Star) (SURENDER et al., 1987; RANA et al., 1997; SHARMA; TRIPARTHI, 2008).

3.8 INVESTIGAÇÃO DO MECANISMO DE AÇÃO ANTIFÚNGICO

3.8.1 Ação do produto na parede celular fúngica (ensaio com sorbitol)

Este método se baseia na medida dos danos que os compostos cumarínicos com atividade antifúngica produzem aos componentes da parede celular fúngica. Caso o produto atue de alguma forma sob a parede celular do fungo, ele provocará lise de suas células quando na ausência de um estabilizador osmótico, mas permitirá seu crescimento na presença desse suporte osmótico. Dessa maneira, este ensaio compara as CIM's dos produtos antifúngicos na ausência e presença de sorbitol a 0,8 M, um protetor osmótico usado para estabilizar os protoplastos de fungos.

A determinação da CIM dos produtos, na presença do sorbitol, foi realizada pelo microdiluição, utilizando placas de microtitulação contendo 96 cavidades, com fundo em forma de "U" e em duplicata, semelhante ao item 3.7.1. Em cada orifício da placa, foram adicionados 100 µL do meio líquido RPMI 1640 previamente adicionado de sorbitol (PM = 182,17) (VETEC Química Fina Ltda – Rio de Janeiro/RJ), ambos duplamente concentrados. Posteriormente, 100 µL da solução dos produtos, também duplamente concentrados, foram dispensados nas cavidades da primeira linha da placa. E por meio de uma diluição seriada a uma razão de dois, foram obtidas concentrações de 1024 µg/mL até 4 µg/mL dos produtos e, no caso do sorbitol, uma concentração final de 0,8 M em cada cavidade. Por fim, foram adicionados 10 µL do inóculo das espécies nas cavidades, onde cada coluna da placa refere-se a uma cepa fúngica, especificamente.

Um controle de micro-organismo foi realizado colocando-se nas cavidades 100 µL do mesmo RPMI 1640 e sorbitol (0,8 M), 100 µL de água destilada estéril e 10 µL do inóculo de cada espécie. Um controle de esterilidade também foi realizado, onde foi colocado 200 µL do RPMI 1640 em um orifício sem a suspensão dos fungos. Por último, foi realizado o mesmo

procedimento com o antifúngico padrão (Anfotericina B). As placas foram seladas e incubadas a 28°C por até 8 dias para ser realizada a leitura (FROST et al., 1995; ZACCHINO, 2001).

3.8.2 Ação dos produtos naturais na membrana celular (Interação com ergosterol)

Muitos fármacos disponíveis para o uso clínico interagem diretamente com o ergosterol, ocasionando danos à membrana celular fúngica (VALGUS, 2003). Caso os efeitos dos compostos cumarínicos sobre a célula fúngica sejam devido à ligação ao ergosterol presente na membrana, pode-se verificar se esses produtos interagem diretamente com o esterol. Pois, na presença de ergosterol exógeno no meio de cultura ocorrerá prevenção na ligação dos produtos ao ergosterol da membrana. Dessa maneira, a CIM dos produtos tendem a aumentar na presença do ergosterol exógeno, porque precisará de uma concentração muito maior deles para que possam interagir com ergosterol da membrana fúngica.

A determinação da CIM dos produtos contra cepas de *Aspergillus* spp**.** foi realizada por microdiluição, utilizando placas de microtitulação contendo 96 cavidades, com fundo em forma de "U" (ALAMAR®) e em duplicata semelhante ao protocolo exposto na determinação da CIM, item 3.7.1. O meio de cultura (RPMI 1640) foi utilizado na ausência e na presença de 400 µg/mL de ergosterol (Sigma-Aldrich®). Um controle de micro-organismo foi realizado colocando-se nas cavidades 100 µL do mesmo RPMI 1640 e ergosterol nas mesmas concentrações e 10 µL do inóculo de cada espécie. Um controle de esterilidade também foi realizado, onde foi colocado 200 µL do RPMI 1640 em um orifício sem a suspensão dos fungos. Por último, foi realizado o mesmo procedimento com a Anfotericina B visto que possui mecanismo de ação conhecido, no qual ocorre interação com ergosterol da membrana e outro (fluconazol) que atua por mecanismo de ação diferente, para servir de controles positivos dos resultados. As placas foram seladas e incubadas a 28°C por até 8 dias para ser realizada a leitura (ESCALANTE et al., 2008).

3.9 ANÁLISES DA ASSOCIAÇÃO DOS COMPOSTOS CUMARÍNICOS SOBRE O EFEITO DOS ANTIFÚNGICOS PADRÕES

3.9.1 Ensaio de modulação dos compostos sobre os antifúngicos padrões.

O protocolo a ser utilizado foi adaptado de Coutinho et al., 2008. Para a avaliação da modulação do composto cumarínico sobre os antifúngicos padrões foram utilizados os antifúngicos Anfotericina B e os derivados azólicos (Voriconazol, Intraconazol e fluconazol), a CIM foi avaliada na presença e na ausência do composto cumarínico contra cepas de *Aspergillus* spp**.**, utilizando placas de microdiluição contendo 96 cavidades, com fundo em forma de "U" (ALAMAR®) e em duplicata semelhante ao protocolo exposto na determinação da CIM, item 3.7.1.

Os compostos cumarínicos foram misturados em RPMI 1640 em concentrações subinibitórias (aproximadamente CIM/8). As soluções dos antifúngicos foram solubilizadas e preparadas diluições na concentração de 1024 µg/mL e volumes de 100 µL foram diluídos seriadamente 1:2 em RPMI 1640. Em cada cavidade com 100 µL do meio de cultura terá 10 % do inóculo fúngico (aproximadamente, 5 x 10^6 conídeos/mL) previamente preparado como demonstrado no item 3.6. Foram utilizados os mesmos controles que foram usados para avaliação da CIM. As placas foram seladas e incubadas a 25-28°C por 72 horas para ser realizada a leitura. (COUTINHO et al., 2008).

3.9.2 Ensaio de associação dos compostos com antifúngicos padrões frente cepas de *Aspergillus* spp.

As soluções do composto cumarínico e as soluções dos antifúngicos foram solubilizadas e preparadas diluições em concentrações dobradas (2048 e 1024 µg/mL respectivamente) em relação à concentração inicial definida e volumes de 100 µL foram diluídos seriadamente 1:2 em RPMI 1640 em cada cavidade com 100 µL do meio de cultura terá 10 % da suspensão fúngica que foi previamente preparada como descrito no item 3.6. Cada microplaca estéril foi preenchida no sentido vertical e horizontal com o composto cumarínico e um antifúngico por vez para a obtenção do índice de Concentração Inibitória Fracionada (índice CIF). As placas preenchidas foram incubadas a 28 ^{0}C por 72 horas.

O índice CIF foi calculado através da soma do CIF^A + CIF^B, onde A representa o antifúngico e B o composto cumarínico em teste. O CIF^A, por sua vez, foi calculado pela relação CIM^A combinado/ CIM^A sozinho, enquanto que o CIF^B = CIM^B combinado/ CIM^B sozinho.

Este índice foi interpretado da seguinte maneira: sinergismo (< 0.5), aditividade (0,5 – 1,0), indiferença (>1,0) ou antagonismo (>4,0) (ELIOPOULOS; MOELLERING, 1991).

3.10 ESTUDOS DE CADD E MODELAGEM MOLECULAR

Os estudos foram realizados em colaboração dos professores doutores Luciana Scotti, Marcus T. Scotti. A partir dos valores encontrados das concentrações inibitórias mínimas (item 3.7.1) foram realizadas diversas metodologias do planejamento racional de fármacos resultando na proposição do provável grupo farmacofórico responsável pela atividade antifúngica exibida pelos derivados cumarínicos.

3.10.1 Otimização das geometrias moleculares

Inicialmente, foi utilizado o programa HyperChem 8.0, as estruturas químicas dos compostos em estudo foram desenhadas e as geometrias otimizadas utilizando-se campo de força de mecânica molecular MM+ (Allinger, 1977). Em seguida, uma nova otimização da geometria foi feita, adotando-se o método semi-empírico AM1 (Austin Model 1) (DEWAR et al., 1985).

As estruturas otimizadas das espécies foram submetidas à análise conformacional, usando-se o método randômico (COHEN, 1996; LEACH, 2001). O confôrmero de menor energia no estado gasoso foi então selecionado e utilizado em diversas ferramentas do estudo computacional.

3.10.2 Análise das relações quantitativas estrutura-atividade biológica (QSAR) e estrutura-propriedade (QSPR)

Os arquivos das moléculas otimizadas e com cargas calculadas foram, posteriormente, importados para serem utilizados no programa Pentacle 1.5 (Pentacle, 2012), que calculará os descritores moleculares e construirá modelos matemáticos a fim de avaliar as relações quantitativas estrutura-atividade biológicos (QSAR) e estrutura-propriedade (QSPR) do conjunto em estudo.

Os modelos obtidos pelo Pentacle 1.5 foram analisados estatisticamente pelo programa Pentacle GRIND (MOBYDIGS DESCRIPTION, 2010).

3.10.3 Análise PCA, PLS e CPCA.

As metodologias de análise explanatória de dados que foram aplicadas neste estudo foram PCA (Análise de Componentes Principais) e PLS (regressão por mínimos quadrados parciais) presentes no programa Pentacle GRIND (Pentacle, 2012).

O método PCA está baseado na correlação entre variáveis e na realidade agrupa aquelas que estão altamente correlacionadas. Uma das vantagens destas transformações é que ruídos experimentais podem ser eliminados, pois estes não estão correlacionados com as informações contidas na matriz de dados original. Outra vantagem é que podem ser escolhidas as variáveis originais mais importantes do ponto de vista estatístico.

O método de calibração ou de regressão empregado foi o PLS (*Partial least squares)*, regressão por mínimos quadrados parciais, que tende ao melhor ajuste entre as variáveis dependentes e a independente. Nestas análises, pretende-se investigar os ligantes e espera-se com a interpretação dos descritores holísticos calculados o esclarecimento mais detalhado do farmacóforo dos investigados.

Abordagens qualitativas de visualização gráfica, obtidas com o campo de força GRIND incluso no VolSurf+, foram também aplicadas para obtenção de informações adicionais na interpretação das interações das moléculas em estudo com *probes* DRY, H_20, O e N.

Efetuou-se a construção de mapas de superfícies de HOMO, LUMO; densidades eletrônicas totais em relação à HOMO e LUMO e mapas de potencial eletrostático e de lipofilicidade, utilizando o programa de modelagem molecular Spartan for Windows v. 6.0. (SPARTAN MODEL HOMEPAGE FOR WINDOWS, 2010).

3.11 ANÁLISES ESTATÍSTICAS

Para a análise estatística foram utilizados testes matemáticos definidos de acordo com a característica do experimento. Os resultados foram considerados significativos quando apresentarem valores de $p < 0,05$.

RESULTADOS E DISCUSSÕES (ARTIGOS)

4 RESULTADOS E DISCUSSÕES

4.1 Synthesis, Structure-Activity Relationships (SAR) and *In Silico* studies of coumarin derivatives with antifungal activity*

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Synthesis, Structure-Activity Relationships (SAR) and *In Silico* Studies of Coumarin Derivatives with Antifungal Activity

Abstract: The increased incidence of opportunistic fungal infections, associated with greater resistance to the antifungal drugs currently in use has highlighted the need for new solutions. In this study twenty four coumarin derivatives were screened *in vitro* for antifungal activity against strains of *Aspergillus*. Some of the compounds exhibited significant antifungal activity with MICs values ranging between 16–32 µg/mL. The structure-activity relationships (SAR) study demonstrated that *O*-substitutions are essential for antifungal activity. It also showed that the presence of a short aliphatic chain and/or electron withdrawing groups (NO_2 and/or acetate) favor activity. These findings were confirmed using density functional theory (DFT), when calculating the LUMO density. In Principal Component Analysis (PCA), two significant principal components (PCs) explained more than 60% of the total variance. The best Partial Least Squares Regression (PLS) model showed an r^2 of 0.86 and q^2_{cv} of 0.64 corroborating the SAR observations as well as demonstrating a greater probe *N1* interaction for active compounds. Descriptors generated by TIP correlogram demonstrated the importance of the molecular shape for antifungal activity.

Keywords: coumarin derivatives; antifungal activity; *Aspergillus* sp.; structure-activity relationships (SAR); molecular modeling; principal component analysis (PCA); partial least squares regression (PLS); density functional theory (DFT)

1. Introduction

The increased incidence of fungal infections, especially dangerous hospital-acquired infections and infections in immunocompromised patients has highlighted the need for new antifungal treatments. Drug-resistant fungal isolates have been reported for all known classes of antifungal drugs. As a result, mortality, morbidity, and the associated cost of medical care for fungal infections are all steadily rising. In addition, because many of the currently available drugs are toxic and have other drawbacks involving spectrum, tissue distribution, central nervous system penetration, and high cost, the number of efficacious anti-mycotic drugs is limited [1]. Many of these drugs actually produce infection recurrence, for being fungistatic and not fungicidal.

In particular, *Aspergillus* infections have been increasing, and while the most frequent pathogen to cause aspergillosis is *Aspergillus fumigatus*, *A. terreus*, *A. flavus* and *A. niger* are becoming increasingly common [2]. *A. fumigatus* is an opportunistic pathogen which incites

disease in hosts whose local or systemic immune response has been impaired, damaged, or is innately dysfunctional. After *Aspergillus fumigatus*, *A. flavus* is the second leading cause of invasive aspergillosis, and it is the most common cause of superficial infections. Common clinical syndromes associated with *A. flavus* infections include chronic granulomatous sinusitis, keratitis, cutaneous aspergillosis, wound infections, and osteomyelitis following trauma and inoculation. In addition, *A. flavus* produces aflatoxins, the most toxic natural hepatic-carcinogens ever characterized [3].

There is an urgent need for discovery and testing of new compounds with antifungal properties. Natural products are inexhaustible as a source of chemical structures and for more than a century have been used as scaffolds for the synthesis of new drugs. The coumarins, phenolic compounds which possess a benzopyranone nucleus [4] are one of the major classes of secondary metabolites, which has been highlighted in biological studies as being (anti-HIV [5], hepato-protective [6], anti-inflammatory [7], antimicrobial [8], antimitotic [9] and antitumor [10]), and in part, due to their antifungal properties [11–13] they are believed to exert a role in plant protection against herbivorous, fungal, and bacterial infections [14].

It was recently reported that coumarin derivatives may be used effectively as antifungal agents. Johann *et al.*. [11] demonstrated that coumarins isolated from plant extracts exhibit activity against certain strains of *Sporothrix schenckii* (MICs of 125–250 µg/mL), and *Cryptococcus gattii* (MIC of 250 µg/mL). Daoubi *et al.*. [13] showed their antifungal activity against *Botrytis cinerea* in concentrations ranging from 25 to 200 mg/L with IC_{50} values of 33.3–157.5 mg/L. Creaven *et al.*. [15] demonstrated that coumarin derivatives are active against *Candida albicans* (MIC_{80} of 4.5–246.6 µM). Jurd *et al.*. [16,17] studied several coumarin derivatives showing inhibitory activity against varied strains including Candida (*Candida albicans, C. tropicalis, C. chalmersi), Saccharomyces cerevisiae, Aspergillus flavus, A. niger, A. oryzae, A. glaucus,* and *Penicillium chrysogenum*.

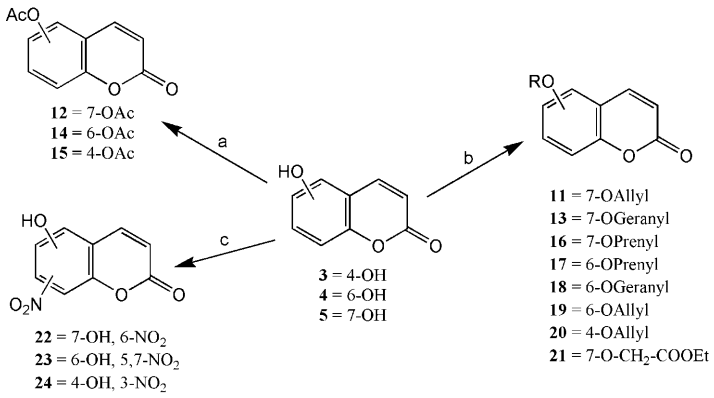
Pursuing our research in the field [18–20], we herewith describe the synthesis and *in vitro* antifungal evaluation of coumarin derivatives against *Aspergillus* species. After antifungal evaluation, the derivatives were subjected to structure-activity relationship (SAR) analysis, electronic surface analyses, molecular modelling, and chemometrics (Principal Component Analysis (PCA), and Partial Least Square Regression (PLS)), all to extract information on structure and its relation to antifungal properties [21,22].

2. Results and discussion

2.1 Synthesis

As shown in Scheme 1, the coumarin derivatives (**11–24**) were synthesized by alkylation, acetylation, and nitration of commercial coumarins: 4-hydroxy- (**3**), 6-hydroxy- (**4**) and 7-hydroxy-coumarin (**5**). Alkylation reactions were done according to procedures previously described with small modifications [23,24], using different alkyl halides; (allyl bromide, geranyl bromide, prenyl bromide and ethyl chloro-acetate) which gave coumarin derivatives: **11** [25], **13** [26], **16** [26], **17** [27], **18** [28], **19** [29], **20** [30] and **21** [31] in satisfactory yields (45.5%–98%, except **18**). Acetylation was carried out under ultrasonic irradiation (which increases the rate, speed and yield of chemical reactions, by liquids emulsification [32]), using an acetic anhydride and pyridine mixture afforded derivatives **15** [33], **14** [17], and **12** [34], in good yields (77%–90%). Nitro-coumarins **22** [35], **23**, and **24** [36] were synthesized by standard nitration procedures, using a mixture of nitric and acetic acids in an ice bath [37].

Scheme 1. Synthesis of alkyl-, acetyl- and nitro-coumarin derivatives.



Reagents and Conditions: (a) Acetic Anhydride, Pyridine, rt., ultrasound irradiation; (b) Allyl Bromide, Geranyl Bromide, Prenyl Bromide, or Ethyl Chloroacetate, K_2CO_3, Acetonitrile, reflux; (c) HNO_3/AcOH, 0–5 °C for 30 min, then 90 min at rt.

The chemical structures of **11**–**22** and **24** were confirmed by comparing their physical and spectral data with the respective literatures. The purity of all synthesized compounds were >98% determined by HPLC.

Structural confirmation of **23** was based on its NMR, mass spectra and elemental analysis. In the ^{1}H NMR we observed only three signals related to three hydrogens which indicated a probable bi-nitration. There were two doublets coupled together (J = 10.0 Hz) at

6.75 and 7.77 ppm, which were attributed to H-3 and H-4, and one singlet at 8.23 ppm (related to H-8). These signals provide good evidence that a bi-nitration occurred on the aromatic ring at the C-5 and C-7 positions, both were ortho-positioned with respect to the 6-hydroxyl group, as also observed in previous works [38]. The unequivocal determination of the carbons was done by interpretation of their ^{13}C NMR, ^{13}C–^{1}H HMQC and ^{13}C–^{1}H HMBC spectra.

In the ^{13}C–^{1}H HMQC was possible to determine unambiguously the three primary carbons at 116.3 (C-8), 124.0 (C-3) and 136.7 ppm (C-4). Signals from quaternary carbons were determined by analysis of the correlations observed in the ^{13}C–^{1}H HMBC, which are described in Table 1.

The mass spectrum also confirmed the proposed structure, where it is observed as the base peak, the fragment $[M-1]^{+\bullet}$ at 250.9, characteristic for phenolic compounds.

Table 1. ^{13}C–^{1}H HMBC correlations for **23**.

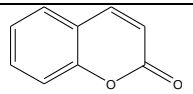
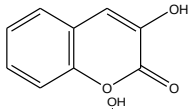
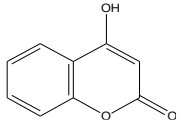
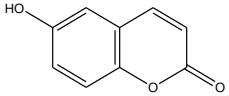
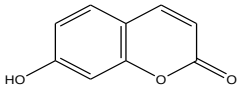
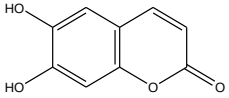
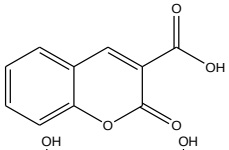
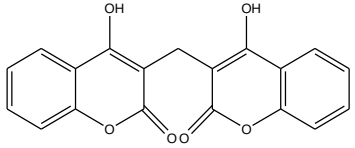
Hydrogen	Carbon	C-2	C-3	C-4	C-4'	C-5	C-6	C-7	C-8	C-8'
	(ppm)	159.5	124.0	136.7	118.8	139.6	138.9	143.1	116.3	146.3
H-3	6.75	α	linked	α	β	-	-	-	-	-
H-4	7.77	β	α	linked	α	β	-	-	-	β
H-8	8.23	-	-	-	β	-	β	α	linked	α

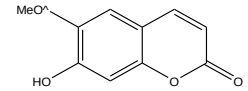
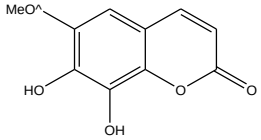
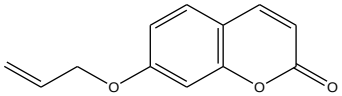
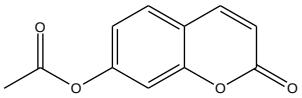
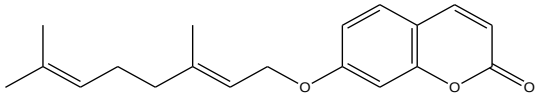
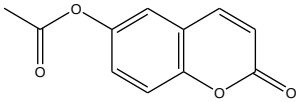
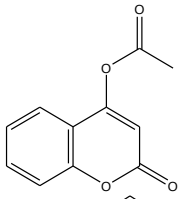
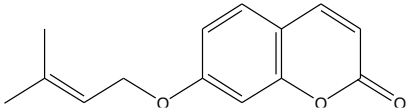
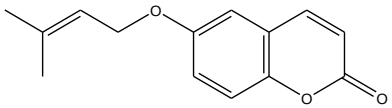
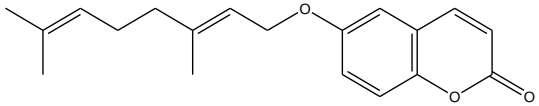
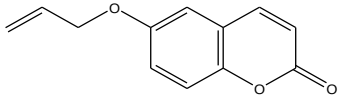
2.2. In vitro *Antifungal Evaluation and SAR Study*

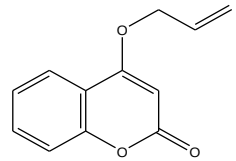
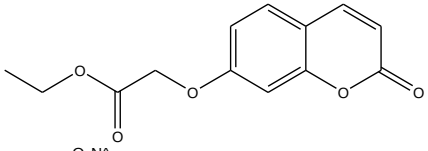
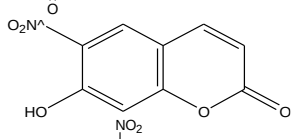
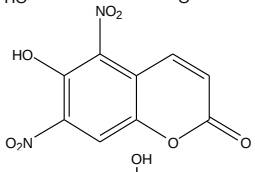
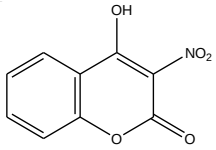
From Table 2, the *in vitro* antifungal activity of the commercial (**1**–**10**) and synthesized coumarins (**11**–**24**) was investigated using two *Aspergillus* species, including four *Aspergillus fumigatus* strains (ATCC 16913, ATCC 40640, ATCC 46913, and IPP 210) and four *Aspergillus flavus* strains (ATCC 16013, LM-247, LM-210, and LM-26). The chemical structure, and Minimum Inhibitory Concentration (MIC) values, expressed in micrograms per milliliter compared with amphotericin B (AmpB) are presented.

The data presented in Table 2 show that commercial coumarins (**1**–**10**) are generally ineffective against the tested strains (MIC 1024 μg/mL), with the exception of coumarin **2** which presented moderately strong activity (MIC = 64 μg/mL for *A. fumigatus*, and 128 μg/mL for *A. flavus*). This difference in sensitivity between the two species was also observed for derivative **16** (MIC = 128 μg/mL for *A. fumigatus*, and 1024 μg/mL for *A. flavus*), and for the reference drug Amphotericin B (AmpB) with an MIC of 2 μg/mL for *A. fumigatus*, and between 2 and 512 μg/mL for *A. flavus*).

Table 2. Chemical structures and *in vitro* antifungal activity of compounds **1**–**24** against *A. fumigatus* and *A. flavus*.

Compound	Chemical Structures	*A. fumigatus*				*A. flavus*			
		ATCC 16913 ($\log 1/c_{MIC}$)	IPP 210	ATCC 46913	ATCC 40640	LM 247	ATCC 16013	LM 210	LM 26
1		1024 (2.15)	1024	1024	1024	1024	1024	1024	1024
2		64 (3.40)	64	64	64	128	128	128	128
3		≥2048 (1.89)	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048
4		≥2048 (1.89)	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048
5		≥2048 (1.89)	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048
6		≥2048 (1.93)	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048
7		1024 (2.26)	1024	1024	1024	≥2048	1024	1024	1024
8		≥2048 (2.21)	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048

9		≥2048 (1.97)	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048
10		≥2048 (2.00)	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048
11		64 (3.49)	64	64	64	256	64	64	64
12		64 (3.50)	64	64	64	128	64	64	64
13		≥2048 (2.16)	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048
14		256 (2.89)	256	256	256	256	256	256	256
15		16 (4.10)	16	16	16	16	16	16	16
16		128 (3.25)	128	128	128	1024	1024	1024	1024
17		≥2048 (2.05)	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048
18		≥2048 (2.16)	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048
19		≥2048 (1.99)	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048	≥2048

20		32 (3.80)	64	32	32	1024	32	32	64
21		512 (2.68)	512	512	512	512	512	512	512
22		16 (4.11)	16	16	16	16	16	16	16
23		512 (2.69)	512	512	512	512	512	512	512
24		1024 (2.30)	1024	1024	1024	1024	1024	1024	1024
AmpB *		2	2	2	2	8	2	2	512

***** AmpB, Amphotericin B.

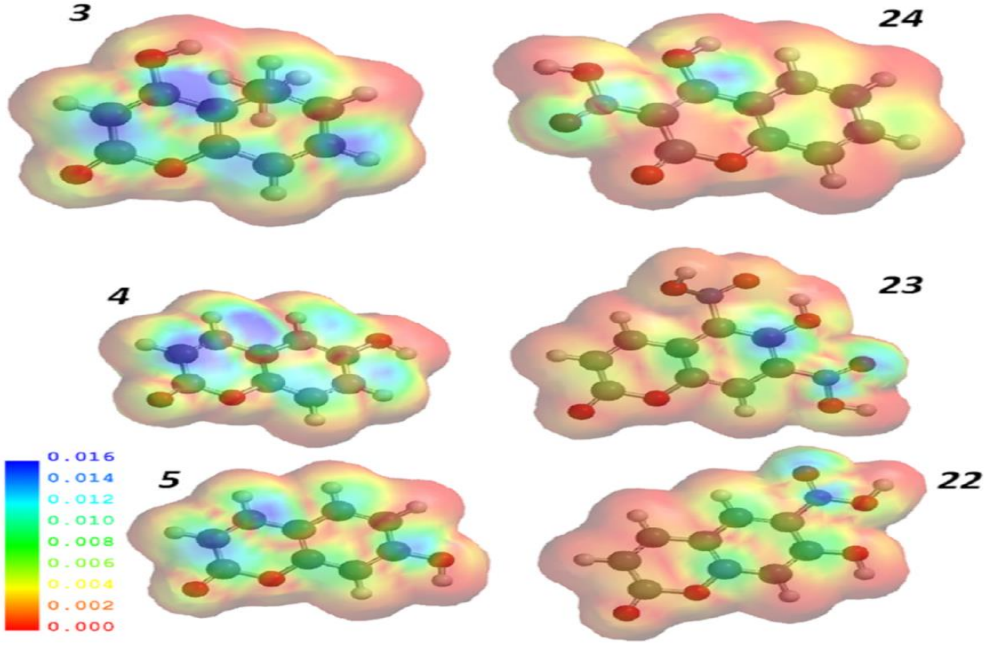
\

Despite the apparent similar sensitivity profile, it is observed that for one sixth of compounds (**7**, **11**, **12** and **20**) the *Aspergillus flavus* strain LM-247 proved to be more resistant than the other *A. flavus* strains, with MIC values of 2 to 32 times higher.

Most derivatives did not compare well to the antifungal activity of AmpB, however nine compounds showed MIC values that were either lower (**2**, **11**, **12**, **14**, **15**, **20**, **22**), or equal (**21**, **23**) to the reference drug (AmpB) for the strain LM-26 which is AmpB-resistant).

The introduction of electron withdrawing groups to the coumarin skeleton appears to contribute positively to the fungicidal activity, as seen with all the nitro-derivatives evaluated. Nitration of the inactive compounds (**3**, **4** and **5**) resulted respectively in derivatives: **24** (MIC = 1024 µg/mL), **23** (MIC = 512 µg/mL), and **22** the most active compound (MIC = 16 µg/mL) each derivative being (respectively) 2, 4, and 128 times more active than its precursor. DFT confirmed these findings. In the LUMO density surfaces, we highlight the differences between active (nitro-coumarins) and inactive compounds. The active compounds have stronger red regions (lower electronic concentration). Although the electrostatic potential map also represents the electronic concentration, LUMO density better demonstrates the ability of one molecule to receive electrons (Figure 1).

Figure 1. Representation of LUMO density surfaces for active (**22**–**24**) and inactive (**3**–**5**) compounds. Red regions represent lower electronic concentrations.



The positive influence of electron withdrawing groups can also be observed for the three series (4-, 6- and 7-hydroxycoumarins) of synthesized compounds. In every case it was

observed that the compounds resulting from acetylation (respectively **15**, **14**, and **12**), are the most active from each series. Compound **15** was as active as compound **22** (the most active compound). The hypothesis that electron withdrawing groups favor antifungal activity corroborates the observations found with PLS analysis (Section 2.3.), where it was found that the interaction of compounds with the probe *N1*, increases with antifungal activity.

Analyzing the other replacements performed (introduction of geranyl, prenyl and allyl groups), we observed differing patterns of activity for the 6-hydroxy- and 7-hydroxy-coumarin derivatives. With the 6-hydroxy-coumarin derivatives, substitutions did not result in increased fungicidal activity, all compounds showed to be inactive (**17**, **18** and **19**). This was also observed in previous studies [17]. However, for the 7-hydroxy-coumarin series, there is a clear relationship between the size of the introduced group and the fungicidal activity. Reduction of the alkenyl side-chain length increases the activity proportionately, so that the compound having the largest radical (geranyl) is inactive (**13** MIC ≥ 2048 µg/mL), the compound with an intermediate side-chain (prenyl) showed strong to moderate activity (**16** MIC = 1024 µg/mL for *A. fumigatus* and 128 µg/mL for *A. flavus*),and the compound with the smallest aliphatic chain displayed the best activity profile (**11** MIC = 64 µg/mL).These results are in agreement with studies observed by Jurd *et al.*. [16] who observed that the antifungal activity of umbelliferone (7-hydroxy-coumarin) may be increased by *O*-alkylation, and *O*-acylation with shorter alkyl and acyl groups.

2.3. Computational Studies

For performing chemometric analysis (Principal Component Analysis (PCA)), and Partial Least Square Regression (PLS) the MIC values for *Aspergillus fumigatus* ATCC-16913 were used [21,39,40]. Inhibitory activity data of the investigated compounds determined as micrograms per milliliter were converted to the negative logarithms of molar MICs (log1/*c*MIC) (Table 2) which was used as a dependent variables set in this study. Principal component analysis (PCA) and partial least squares (PLS) are chemometric tools for extracting and rationalizing the information from any ultivariate description of a biological system. Complexity reduction and data simplification are two of the most important features of such tools. These chemometric tools were developed in the Pentacle software [41]. The Pentacle software is a computational tool for computing alignment-free molecular descriptors, also called GRid-INdependent descriptors or GRIND. The software is based on Molecular Interaction Fields, describe the ability of the molecules to interact with other molecules and do

not require to superimpose the compounds. GRIND descriptors are highly relevant for describing biological properties of compounds [42] (see item 3.4. Molecular modeling and electronic surfaces).2.3.1. Principal Component Analysis (PCA) and Partial Lest Squares Regression (PLS)

The PCA is a technique used to reduce the principal matrix information, splitting into two smaller matrices called loading and score: the matrix loading (P) contains information about the variables and is composed of vectors (principal components, PCs) which are obtained from the original variables; the matrix score (T) contains information about the objects. Each object is described in terms of the projections from the PC instead of the original variables.

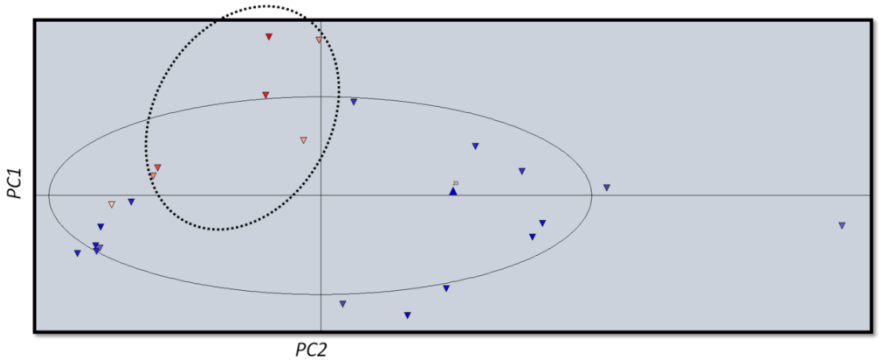
Preliminary exploratory analysis was developed using PCA and considering 405 independent variables or descriptors. Two significant principal components (PCs) explain more than 60% of the total variance (Table 3).

Table 3. Explained variance using PCA.

PC	% explained variance from original data
1	50.04
2	10.62
3	7.96
4	5.80
5	3.54

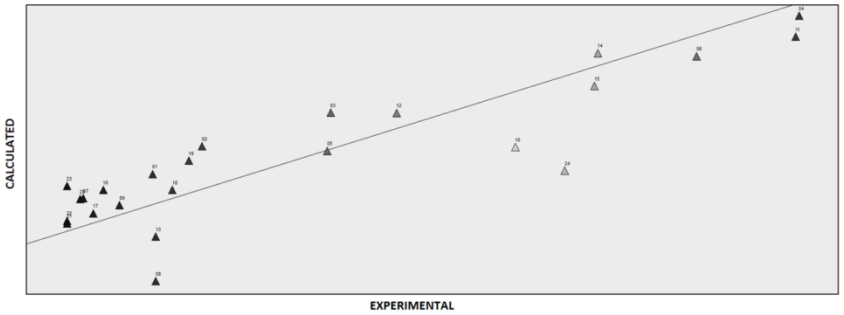
Score and loading plots are interconnected until any descriptor change in the loading plot is reflected by changes in the position of compounds in the score plot [43]. The score plot exhibits satisfactory discrimination between more potent (blue) and less potent (red) compounds. The less active compounds are concentrated in the upper left quadrand (Figure 2).

Figure 2. Scores plot from PCA. Red triangle represents less active and blue triangle represents more active compounds.



The best model from PLS was obtained with three LVs and 72 descriptors selected from 405. The variable selection via fractional factorial design (FFD), which evaluates the effect on the model standard derivation of error of prediction (SDEP) of every single variable and variable combination. The GRIND descriptors condensed represent a small number of principal properties (GRIND-PP), requiring a biologically relevant description of the molecular similarity. Two significant latent variables emerged from PLS model and *leave-one-out* [44]. The best model showed an r^2 of 0.86, and a q^2_{cv} of 0.64. Figure 3 represent the score plot obtained with PLS.

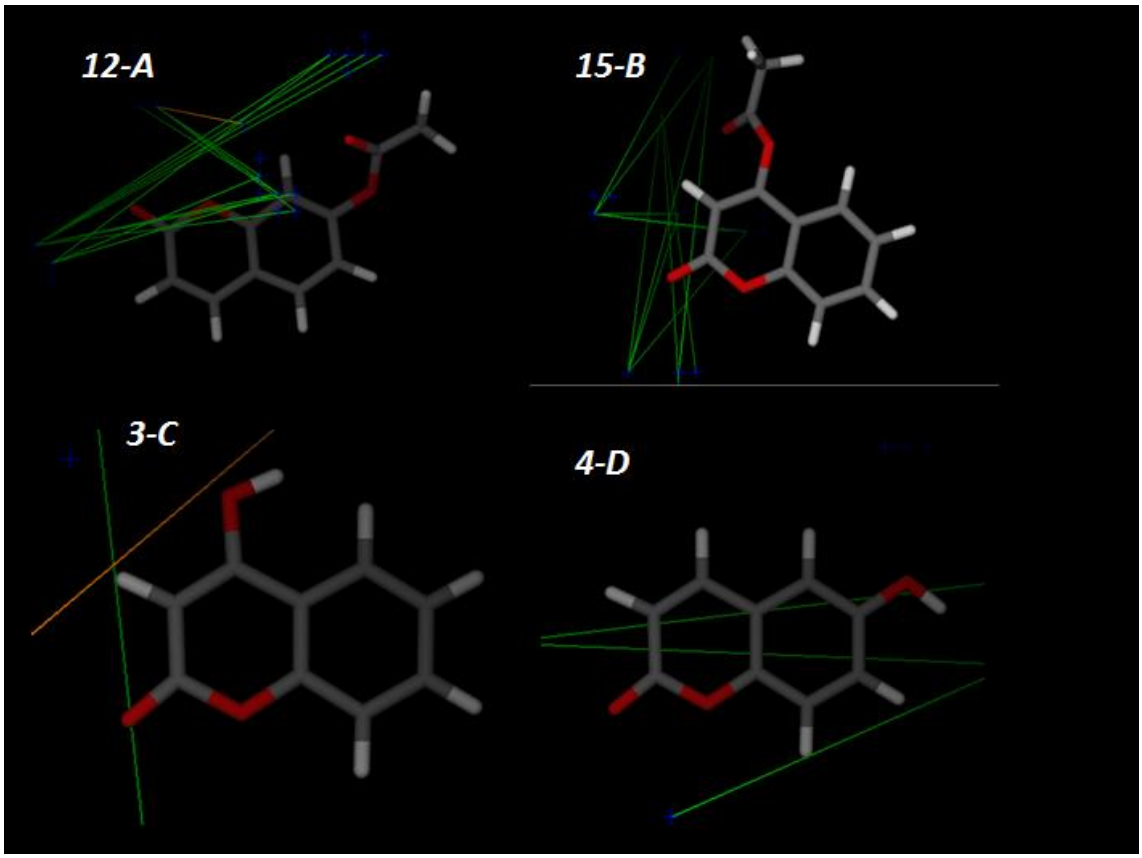
Figure 3. Best fit model obtained in PLS.



The principal descriptors highlighted in the PLS model were: *350* (5.6–6 A), *531* (10.8–11.2 A), *527* (7.2–7.6 A), and *201* (13.2–13.6 A) were generated through interactions with the probe *TIP* (shape), the molecular contour also appears to be important. The molecular descriptors obtained can be observed in graphical diagrams called "correlograms". We observed the largest number of interactions of active compounds with the probe *N1*. This may indicate that coumarins devoid of electrons have greater attraction with electronegative atom of this probe. Otherwise occurs in inactive compounds. The Figure 4 shows the active compounds **12** and **15** and their interactions with *N1*. Comparatively, we can see the inactive compounds

(**3** and **4**). Inactive interactions are smaller than active ones, corroborating the fact that electron withdrawing groups increases the antifungal activity.

Figure 4. Examples of important structural features for antifungal activity, highlighted by the PLS analysis: (**A**) and (**B**) **12** and **15,** respectively—active compounds; (**C**) and (**D**) **3** and **4**—inactive compounds, respectively. *N1–TIP* interactions (orange), *N1–N1* (green) interactions.



3. Material and Methods

3.1. General Methods

All synthetic coumarins (1,2-Benzopyrone **1**; 3-Hydroxycoumarin **2**; 4-Hydroxycoumarin **3**; 6-Hydroxycoumarin **4**; 7-Hydroxycoumarin **5**; 6,7-Dihydroxycoumarin **6**; Coumarin-3-carboxylic acid **7**; 3,3'-Methylene-bis-(4-hydroxycoumarin) **8**; 6-Methoxy-7-hydroxycoumarin **9** and 7,8-Dihydroxy-6-methoxycoumarin **10**), reagents and solvents were purchase from Sigma-Aldrich (Seelze, Germany), and used without further purification. All reactions were monitored by thin-layer chromatography (TLC) on pre-coated silica gel GF254 plates (Fluka, St. Gallen, Switzerland). Melting points were determined on a Fisaton 430 apparatus (Fisaton, São Paulo, Brazil) using open capillaries, and the reports values are uncorrected. Acetylation reactions were performed with an ultrasoundUSC-1400A (40 kHz,

Unique, Indaiatuba, Brazil). Infrared (IR) spectra were recorded using potassium bromide pellets on a Bruker IFS-66 IR spectrometer (Bruker, San Francisco, CA, USA), with the frequencies expressed in cm−1. NMR were recorded on a Varian Unity Plus 300, 400 and 500 MHz spectrometer (Varian, Palo Alto, CA, USA), using TMS as an internal standard. Peak assignment in ^{13}C spectra are based on 2D HSQC and HMBC spectra. Chemical shifts were reported in ppm (δ), and coupling constants (*J*) were reported in Hertz. Signals were designated as follows: s, singlet; d, doublet and bs, broad. HRMS was recorded on a MicroTOF mass spectrometer (ESI) (Bruker). Low-resolution ESI mass spectra was recorded on a Amazon X (Bruker). Elemental analysis was performed using an EA 1110 CHNS-O elemental analyzer (CE instruments, Wigan, UK).3.2. Synthesis

3.2.1. General Alkylation Procedure

A mixture of commercial coumarins (**3**, **4** or **5**) (3 mmol), K_2CO_3 (3.9 mmol), and an alkyl halides (3.9 mmol) in anhydrous acetonitrile (20 mL) was stirred in reflux for 22–46 h, and monitored by TLC. The reaction was filtered and washed two times with ethyl acetate (10 mL). The organic phase was washed with water (50 mL), dried over Na_2SO_4, and then evaporated in vacuum. The residue obtained was recrystallized using Methanol/THF mixtures to give the compounds (by % yield/time reaction): **11** [25] (56.7/37 h); **13** [26] (45.5/27 h); **16** [26] (90/22 h); **17** [27] (86/22 h); **18** [28] (13.5/46 h); **19** [29] (66.7/35 h); **20** [30] (78/44 h), and **21** [31] (98/38 h) as white solids.

3.2.2. General Acetylation Procedure

Coumarins (**3**, **4** and **5**) (5 mmol), Ac_2O (50 mmol), and pyridine (5.5 mmol) were mixed in a Schlenk in an ultrasound bath, at room temperature for the appropriate time (monitored by TLC). After completion, the reaction was quenched with cold water (20 mL). The mixtures were filtered, washed three times with water (20 mL), and dried in a desiccator giving pure solid products (% yield/time reaction):**12** [34] (90/15 min);**14** [17] (76.7/15 min) and **15** [33](86/30 min).

3.2.3. General Nitration Procedure

Nitro-coumarins were synthesized by standard nitration procedures [37], using a mixture of nitric/acetic acids in an ice bath for 30 min., and then at room temperature for an additional 90 min. affording: **22** [35] (84%), **23** (70%) and **24** [36] (53.3%).

5,7-Dinitro-6-hydroxycoumarin (**23**): Yield 70%; m.p.: 155–157 °C; ^{1}H NMR [500 MHz, δ (ppm), cd3od]: 6.75 (d, *J* = 10.0 Hz, 1H, H-3), 7.77 (d, *J* = 10 Hz, 1H, H-4), 8.23 (s, 1H, H-8), 10.65 (bs, OH); ^{13}C NMR [100MHz, δ (ppm), cd3od]: 116.3 (C-8), 118.8 (C-4´), 124.0 (C-3), 136.7 (C-4), 138.9 (C-6), 139.6 (C-5), 143.1 (C-7), 146.3 (C-8'), 159.5 (C-2); MS (EI) *m/z* (relative intensity) 250.9 ($[M-1]^{+\bullet}$, 100), 220.9 (40), 190.9 (10). HRMS [ESI (*m*/*z*)] calcd for $C_9H_4N_2O_7$ = 252.0019, found for $[M-1]^{+\bullet}$ = 250.9517; Anal. Calcd for $C_9H_4N_2O_7$: C, 42.87; H, 1.60; N, 11.11; O, 44.42; Found: C, 42.87; H, 1.61; N, 11.14; O, 44.38.

3.3. Antifungal Activity

The *in vitro* antifungal activity of the studied compounds was investigated using eight *Aspergillus* strains, including four strains of *Aspergillus fumigatus* (ATCC 16913, ATCC 40640, ATCC 46913 and IPP 210), and four strains of *Aspergillus flavus* (ATCC 16013, LM-247, LM-210 and LM-26). These strains were supplied by the URM Culture Collection of the Department of Mycology, Department of Pharmaceutical Sciences of the Federal University of Paraiba, Brazil. The fungi were maintained on potato dextrose agar (PDA)—Difco®—at 28 °C and 4 °C until testing procedures.

Stock cultures were kept on sterile Sabouraud Dextrose Agar (SDA) slants under 7 °C (±1 °C). For preparing the inoculum of the anti-mold assays, we used 7 day-old cultures grown on sterile SDA at 25–28 °C. After the incubation period, the mold conidia were removed by adding sterile NaCl 0.85% to the growth media followed by gentle shaking for 30 s. Mold conidia were counted using a hemocytometer. The conidial suspension inoculum was adjusted using sterile NaCl 0.85%, for approximately 5×10^6 conidia/mL.

MIC values were determined by the microdilution broth method using 96-wells microplates [45,46]. Conidial suspension from 7-day-old *A. flavus* culture was prepared and standardized by hemocytometer in sterile NaCl 0.85% for susceptibility testing as described previously. To a 96-well plate Sabouraud broth and coumarins were added at concentrations of 2048 to 8 µg/mL. The MIC determination was conducted with an inoculum of approximately 2.5×10^5 conidia/mL microorganism in each well. The plates were incubated at 25–28 °C for 72 h. Within 72 h (and confirmed at 7 days) there was visible fungal growth. The MIC was defined as the lowest concentration of antifungal agent that completely inhibited the growth of the fungi, as detected by the unaided eye [47].Amphotericin B was used as the reference fungicide.

3.4. Molecular Modeling and Electronic Surfaces

Using the program Hyperchem version 8.0 [48], the chemical structures of the compounds of interest were drawn and their geometry was optimized using MM+ force field [49]. Afterwards, we performed a new geometry optimization using the semi-empiric method RM1 (Recife Model 1) [50]. The RHF (*Restricted Hartree-Fock*) level was used. The optimised structures were subjected to conformational analyses using the random search method [22,51] with 1000 interactions, 100 cycles of optimisation, and 10 lowest minimum energy conformers. The selected dihedrals were evaluated by rotation in accordance with the standard (default) conditions of the program, in which the number of simultaneous variations was 1 to 8, and acyclic chains were submitted to rotations from 60 to 180º, while torsion rings were in the range of 30° to 120°.

The lowest energy conformers selected were saved in .sdf format with the Spartan for Windows 8.0 program [52], and then exportedto the Pentacle 1.5 program [41], PCA and PLS methodologies were carried out. The Pentacle software is a computational tool for computing alignment-free molecular descriptors, also called GRid-Independent descriptors or GRIND. The software is based on Molecular Interaction Fields and describes the ability of the molecules to interact with other molecules without requiring compound superimposition. Aims to find the underlying relationship between the structure of a molecule and its binding affinity (or other biological properties) using information extracted from molecular descriptors. The calculation of descriptors includes the hydrophobic probe (*DRY*), the hydrogen bond acceptor probe (*O*), the shape probe (*TIP*), and the hydrogen bond donor probe (*N1*) [42].

The electronic surfaces were calculated in Spartan for Windows 8.0. The compared gradient was 1.02893 ×10−7 (red) 0.0161204 ×10−7 (blue).

4. Conclusions

Synthesis and antifungal evaluation of twenty-four coumarin derivatives against *Aspergillus fumigatus* and *A. flavus* are described. Some derivatives showed significant antifungal activities with MICs values ranging between 16–32 µg/mL, including seven compounds (**2**, **11**, **12**, **14**, **15**, **20** and **22**) which were more active than the reference drug Amphotericin B for the LM-26 strain (*A. flavus*).

SAR study permitted two conclusions: *O*-substitution is essential for antifungal activity and the presence of a short aliphatic chain (geranyl<prenyl<allyl), and/or electron withdrawing groups (NO_2 and/or acetate) favors activity.

In parallel, the compounds were submitted to the chemometric tools: PCA and PLS, using the program Pentacle. The results were satisfactory and corroborated the SAR analysis. The most active compounds showed greater interaction with the probe *N1*, which shows greater electronic concentration. These findings were also confirmed by density functional theory (DFT), calculating the LUMO density. Descriptors generated by the probe *TIP* were also highlighted, indicating that the molecular contour is indeed important for the antifungal activity, as observed in the SAR analysis.

These results suggest derivatives of coumarin as promising compounds for the development of new anti-*Aspergillus* agents.

Acknowledgments

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Sample Availability

Samples of the compounds 11–24 are available from the authors.

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4.2 Evaluation of Antifungal activity and mode of action of new coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one, against *Aspergillus* spp.

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Abstract

Aspergillus spp. produces a wide variety of diseases. For the treatment of such infections, the azoles and Amphotericin B are used in various formulations. The treatment of fungal diseases is often ineffective, because of increases in azole resistance and their several associated adverse effects. To overcome these problems, natural products and their derivatives are interesting alternatives. The aim of this study was to examine the effects of coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one (Cou-NO_2), both alone, and with antifungal drugs. Its mode of action against *Aspergillus* spp. Cou-NO_2 was tested to evaluate its effects on mycelia growth, and germination of fungal conidia of *Aspergillus* spp. We also investigated possible Cou-NO_2 action on cell walls (0.8 M sorbitol), and on Cou-NO_2 to ergosterol binding in the cell membrane. The study shows that Cou-NO_2 is capable of inhibiting both the mycelia growth and germination of conidia for the species tested, and that its action affects the structure of the fungal cell wall. At sub-inhibitory concentration, Cou-NO_2 enhanced the *in vitro* effects of azoles. Moreover, in combination with azoles (voriconazole and itraconazole) Cou-NO_2 displays an additive effect. Thus, our study supports the use of coumarin

derivative 7-hydroxy-6-nitro-2H-1-benzopyran-2-one as an antifungal agent against *Aspergillus* species.

Keywords: Coumarin, antifungal, ergosterol, *Aspergillus* spp, aspergillosis.

1. Introduction

The incidence and prevalence of invasive fungal infections have increased in recent years, especially in the currently large population of immunocompromised patients, and those hospitalized with serious underlying diseases. Fungal species represent 25% of the microorganisms isolated in blood cultures of hospitalized patients. Of these, species of the genus *Aspergillus spp*. have the highest incidence among the filamentous fungi [1, 2].

Aspergillus spp. produces a wide variety of diseases. The main route of infection is penetration by air. In cases of invasive aspergillosis *Aspergillus fumigatus* is the most common species isolated in the world. In Brazil, the species *A. flavus* is the most common [3]. The main clinical manifestations observed due to *Aspergillus spp* infections are cutaneous aspergillosis, otomycosis, aspergilloma, and sinusitis [4].

For the treatment of such infections, the azoles (Fluconazole, Itraconazole, and Voriconazole), and Amphotericin B are used in various formulations. However, with the increase of azole resistance, and the several adverse effects associated with the use of Amphotericin B, (which include nephrotoxicity and neurotoxicity [5]), the treatment of fungal diseases is often ineffective, which has caused alarm among health professionals.

To overcome these problems, natural products and their derivatives are interesting alternatives. The coumarins (phenolic compounds which possess a benzopyranone nucleus and are one of the major classes of secondary metabolites), have been highlighted in antimicrobial activity studies [6-8].

Recently reported by our group, the antifungal activity against *Aspergillus fumigatus*, and *A. flavus* of twenty-four coumarin derivatives was described. Some of these derivatives showed significant antifungal activity with Minimum Inhibition Concentration (MIC) values ranging from 16 to 32 μg/mL. 7-hydroxy-6-nitro-2H-1-benzopyran-2-one (figure 1) revealed an MIC value of 16 μg/mL [9]. Despite the promising results of the study, a need remains for better assessment of the effects of coumarin derivatives on the fungus structure, and possible mechanisms action.

Increases in the availability of antifungal compounds have induced searches for better therapeutic strategies, such as the use of two or more antifungal drugs in combination [10]. Combination therapy of antifungal drugs with natural products and their derivatives has been little explored; this is especially true for the coumarin derivatives, which promise an alternative against strains de *Aspergillus* spp.

The aim of this study was to examine the effects and mode of action of coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one, both alone, and together with antifungal drugs, against *Aspergillus* spp.

2. Material and Methods

2.1. Microorganisms:

Aspergillus spp. used in the antifungal assay was obtained from the archival collection of the Federal University of Paraíba Laboratory of Mycology (LM). They included *A. fumigatus* (ATCC 46913, LM 121, LM 743, and LM 135), and *A. flavus* (ATCC 16013, LM 35, LM 36, and LM 23). Stock inoculates (suspensions) of *Aspergillus* spp. were prepared from 8 day old potato dextrose agar (Difco Lab., USA), the cultures grown at room temperature. Fungal colonies were covered with 5 mL of sterile saline solution (0,9%), the surface was gently agitated with vortexes, fungal elements with saline solution were transferred to sterile tubes. Inoculate was standardized at 0.5 tube of McFarland scale (10^6 CFU/mL). The final concentration confirmation was done by counting the microorganisms in a Neubauer chamber [11-13].

2.2. Chemicals

The product tested was the coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one (Cou-NO2), obtained by biosynthesis [9]. Amphotericin B, Flucanozole, Itraconazole and Voriconazole were obtained from Sigma Aldrich®, Brazil. The drugs were dissolved in DMSO (dimethylsulfoxide), and sterile distilled water was used to obtain solutions of 1024 µg/mL for each antifungals. The concentration of DMSO did not exceed 0.5% in the assays.

2.3. Culture media

To test the biological activity of the products, Potato Agar (AP), and Sabouraud dextrose agar (SDA) were purchased from Difco Laboratories (Detroit, MI, USA), and RPMI-1640-L-glutamine (without sodium bicarbonate) (Sigma-Aldrich, São Paulo, SP, Brazil) culture media were used. They were prepared and used according to the manufacturers' instructions.

2.4 Minimum Inhibitory Concentration (MIC)

The determination of minimum inhibitory concentration against ATCC strains was demonstrated in an article previously published by our group [9]. We also carried out determinations of the coumarin derivative CIM in clinical strains of *A. flavus* and *A. fumigatus*. Broth microdilution assays were used to determine the MICs of coumarin derivative 7-hydroxy-6-nitro-2H-1-benzopyran-2-one (Cou-NO2), and amphotericin B. RPMI-1640 was added to all the wells of 96-well plates. Two-fold serial dilutions of the three agents were prepared to obtain concentrations varying between 4 μg/ml and 1024 μg/ml. Finally, 10 μl aliquots of the inoculate suspension were added to the wells, and the plates were incubated at 28ºC for 3 days. Negative controls (without drugs) were used to confirm conidia viability, and sensitivity controls (for DMSO) were also included in the studies. At 72 h there were visual observations for fungal growth. The MIC was defined as the lowest concentration capable of visually inhibiting fungal growth by 100 %. The results were expressed as the arithmetic mean of three experiments [14, 15].

2.4. Effects on mycelia growth

Analysis of the interference of coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one, on mycelia growth was performed by determining the dry mycelia weight of *A. fumigatus* (ATCC 46913) and *A. flavus* (ATCC 16013) [15, 16]. Flasks containing MIC (16 μg/mL) and MIC x 2 (32 μg/mL) of coumarin derivative in RPMI-1640 medium, were inoculated with suspension of the test *A. fumigatus* (ATCC 46913) and *A. flavus* (ATCC 16013) strains. In the corresponding control, the same amount of coumarin derivative was replaced by distilled water. The system was incubated at 28 °C for 8 days. Flasks containing mycelia were filtered through Whatman® Grade 1 Qualitative Filtration Paper (particle retention: 11 μm), and then washed with distilled water. The mycelia were dried at 60 °C for 6 h, and kept at 40 °C overnight. The filter paper containing dry mycelia from two independent assays were weighed, and the mean values obtained. Percentage growth inhibition based on the dry weight of each at time of analysis, was calculated according to Sharma and Tripathi [15].

2.5. Conidial germination assay

The coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one (Cou-NO_2), and Amphotericin B were tested to evaluate effects on the germination of fungal conidia of *A. fumigatus* (ATCC 46913), and *A. flavus* (ATCC 16013). Flasks containing MIC (16 μg/mL)

and MIC x 2 (32 μg/mL) of coumarin derivative, and a control with distilled water, were used. In sterile test tubes, 500 μL of RPMI-1640 plus the Cou-NO_2 were evenly mixed with 500 μL of fungal conidia suspension and immediately incubated at 28 °C. Samples of this mixture were taken after 24 h of incubation for analysis. The whole experiment was performed in duplicate, where the number of conidia was determined in a Neubauer chamber, and the inhibition percentage of spore germination at each time point was calculated by comparing the results obtained in the test experiments with the results of the control experiment. The analysis was conducted under an optical microscope (Zeiss® Primo Star) [17, 18].

2.6. Sorbitol assay effects.

The assay was performed using medium with and without sorbitol (control), to evaluate possible mechanisms involved in the antifungal activity of the test product on the *Aspergillus* spp. cell wall. The sorbitol was added to the culture medium in a final concentration of 0.8M. The assay was performed by microdilution method in 96-well plates in a "U" (Alamar, Diadema, SP, Brazil) [11, 12]. The plates were sealed aseptically, incubated at 28°C, and readings were taken at 3 days. Based on the ability of sorbitol to act as a fungal cell wall osmotic protective agent, the higher MIC values observed in the medium with added sorbitol compared to the standard medium suggest the cell wall as one of the possible cell targets for the product tested [19, 20]. Amphotericin B was used as the control drug. The assay was performed in duplicate and expressed as the geometric mean of the results.

2.7. Ergosterol Binding Assay - MIC Value Determination in Presence of Ergosterol.

To assess if the product binds to the fungal membrane sterols, an experiment was performed according to the method described by Escalante et al.. [21], with some modifications. The ergosterol was prepared as was described by Leite et al.. [20]. The MIC of coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one (Cou-NO_2) against *Aspergillus* spp. was determined by using broth microdilution techniques [11,12], in the presence and absence of exogenous ergosterol (Sigma-Aldrich, São Paulo, SP, Brazil) added to the assay medium, in different lines of the same micro-plate. Briefly, a solution of Cou-NO_2 was doubly diluted serially with RPMI-1640 (volume = 100 μL) containing plus ergosterol at a concentration of 400μg/mL. A volume of 10 μL yeast suspension (0,5 McFarland) was added to each well. The same procedure was realized for amphotericin B, whose interaction with membrane ergosterol is already known, serving as a control drug. The plates were sealed and incubated at 28∘C.The plates were read after 3 days of incubation, and the MIC was determined as the lowest concentration of test agent inhibiting the visible growth. The assay was carried out in duplicate

and the geometric mean values were calculated. Thus, this binding assay reflected the ability of the compound to bind with ergosterol.

2.8. Checkerboard assay

A checkerboard micro-titer test was performed to evaluate the interaction of coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one with the antifungal drugs (azoles and Amphotericin B) against *A. fumigatus,* ATCC 46913, and *A. flavus,* ATCC 16013. A series of 2-fold dilutions, in eight for each coumarin derivative and antifungal drug were made in RPMI-1640 to obtain four times the final concentration being achieved in the micro-titer well. Furthermore, 50 µl of each dilution of coumarin derivative was added to the 96-well micro-titer plates in the vertical direction, while 50 µl of each dilution of antifungal drugs was added in the horizontal direction, so that various combinations of coumarin derivative and antifungal drugs could be achieved. In addition, 10 µl of inoculum from the spore suspension (1.5×10^5 CFU mL−1) was added to each well, and the plates were incubated at 30 °C for 3 days.

In order to evaluate the activity of the combinations of drugs, fractional inhibitory concentration (FIC) indices were calculated as $FIC^A + FIC^B$, where FIC^A and FIC^B represent the Minimum Concentrations inhibiting the fungal growth for drugs A and B, respectively: $FIC^A = MIC^A$ combination/ MIC^A alone and $FIC^B = MIC^B$ combination/MIC^B alone. A mean FIC index was calculated based on the following equation: FIC index = $FIC^A + FIC^B$

In addition, the interpretation was made as follows: synergistic (< 0.5), additivity (0.5-1.), indifferent (> 1), or antagonistic (> 4) [22, 23].

2.9. Drug susceptibility test

The minimum inhibitory concentrations (MIC) of coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one (Cou-NO2), and antifungal drug were determined in RPMI-1640 by microdilution assay using spore suspension (1.5×10^5 CFU mL−1), and a drug concentration range of 1024 to 2,5 µg/mL (two-fold serial dilutions) [23]. MIC was defined as the lowest concentration at which no growth was observed. For the evaluation of the coumarin derivative as a modulator of antifungal properties, MICs of the antifungal drugs were determined in the presence of Cou-NO2 (2 µg/mL), and at sub-inhibitory concentrations (MIC/8); the plates were incubated for 3 days at 30°C [25].

2.10. Data analysis

The results were expressed in mean ± SE. Statistical analyses were performed with *t*-test. P < 0, 05 was considered significant.

Results and Discussion

The difficulty in treating infections caused by *Aspergillus* spp. is in part related to the rather small arsenal of antifungal agents currently in use. The secondary metabolites derived from plants or biosynthesis serve as important fields of research for new antifungal agents. The coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one (Cou-NO_2) a biosynthetic compound has been reported for its antifungal activity [9].

The Minimum inhibitory concentration of the coumarin derivative (Cou-NO2) on the clinical strains was similar to that observed previously by our [9] group, however now achieving an MIC of 32 mg / mL against the clinical strains *A. fumigatus* LM 743 and LM 135 (Table 1).

This study also verified Cou-NO2 action against *Aspergillus* mycelial growth and spore germination (*A. fumigatus* ATCC 46913 and *A. flavus* ATCC 16013).

The effect of differing concentrations of the test drug (MIC and MIC x 2) on mycelia growth was determined by measurement of mycelium dry weights, and the results are shown in Figure 2. With respect to effects on *A. fumigatus* ATCC 46913, and *A. flavus* ATCC 16013, it can be seen that Cou-NO_2 in MIC concentrations of (16 µg / mL), and MIC x 2 (32 µg / mL) inhibited normal mycelia growth ($p < 0.05$) when compared to the control. The results show that Cou-NO_2 at its MIC concentration was more potent than amphotericin B at its respective MIC concentration ($P < 0.05$).

The production of hyphae, and consequent mycelium formation are important virulence factors for *Aspergillus* spp., Hyphae are more difficult to phagocytize, and can induce apoptosis in macrophages, since they often form inside these cells after phagocytosis [26]. Thus, reductions in mycelia growth as an effect of the Cou-NO_2 coumarin derivative interfering with the fungal virulence of *Aspergillus* spp., proved superior to amphotericin B in its respective MIC concentrations.

Aspergillus spp. produces numerous asexual conidia which are spread throughout the environment, and are also considered an important factor in triggering infections in the host. Thus, it is deemed important to quantitatively evaluate the power of a product to interfere with fungal spore germination [26].

The conidial percentage of *A. fumigatus* ATCC 46913, and *A. flavus* ATCC 16013, germinated in the presence and absence (control) of the test-drugs is shown in figure 3. In the two test concentrations (MIC and MIC x 2), Cou-NO_2 displayed significant inhibitory action for *Aspergillus* spp. ($P < 0.05$), as compared to the control. However, this action was shown to be less potent ($P < 0.05$) when compared with respective concentrations of Amphotericin B.

The great challenge when developing new antifungal drugs is in the similarity between fungal micro-organisms cells and human cells. Thus, the desired targets for a new antifungal's action must be unique, or at least sufficiently different from the host [27, 28]. Based on this, two important fungal structures become targets for detecting antifungal drugs: the fungal cell wall, and ergosterol present in the plasma membrane.

Many drugs available for clinical use interact directly with ergosterol, causing damage to the fungal cell membrane [29]. If the effects of coumarin compounds on the fungal cell are due to ergosterol binding in the membrane, one can verify if they interact directly. In the presence of exogenous ergosterol in the culture medium, decreased binding of the product to the ergosterol of the membrane occurs. Thus, the product's MIC tends to increase in the presence of exogenous ergosterol, needing a much higher concentration to interact with the fungal membrane ergosterol [16, 20].

In this study, the MIC values for both Cou-NO_2 experiments, with or without exogenous ergosterol in the culture media, were identical; suggesting that the coumarin derivative tested does not act via binding to ergosterol in the plasma membrane.

To investigate the action of the coumarin derivative (Cou-NO_2) on the cell wall we carried osmotic shield testing with sorbitol, the test results are shown in Table 2. The MIC values of the Cou-NO_2 increased 4-fold in the presence of sorbitol in the culture medium when compared to medium without sorbitol. This suggests that Cou-NO_2 acts on the fungal cell wall structure. This is the first report of such activity on filamentous fungi.

According to Widodo et al.. [30] coumarin acts by forming pores in the cell wall, with consequent release of cytoplasmic contents and cell death, confirming the results found in this study. However, other studies show that coumarins alter the morphology of the fungal mitochondrial cell, and induce apoptosis [31, 32].

In addition to their inherent antimicrobial properties, natural products and their derivatives may alter the effects of standard antifungal agents (those used in clinical practice). The use of two or more antifungal combinations can lead to a reduction in the required drug dosages and decrease the normally produced adverse event profile [33, 34].

The addition of coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one, (Cou-NO_2) to the growth medium at a sub-inhibitory concentration of 2 ug / ml (MIC / 8), resulted in a decreased MIC for fluconazole 256 mg / mL to 128 mg / mL in both *A. flavus* as for of *A. fumigatus*. The MIC of voriconazole decreased for *A. fumigatus* 4 ug / ml to 0.5 ug / ml, and for *A. flavus* 2 ug / ml to 1 ug / ml for the respective species. Also, the MIC of itraconazole decreased from 128 ug / ml to 32 ug / ml, yet only for *A. fumigatus*, as

demonstrated in Table 4. There was however, no significant modulatory effect on the MIC of amphotericin B (table 3).

In tables 4 and 5, the results are observed for combinations of the coumarin derivative 7-hydroxy-6-nitro-2H-1-benzopyran-2-one (Cou-NO_2) with antifungal agents (amphotericin B, and azole derivatives) against *A. fumigatus* ATCC 46913, and *A. flavus* ATCC 16013. Additive effects were observed for the combinations of Cou-NO_2 with itraconazole and voriconazole, resulting in a Fractional Inhibitory Concentration (FIC) index of equal to 0.75 against both respective species tested. However, the combination of the Cou-NO_2 and Amphotericin B and fluconazole showed CIF index of 2 and 2 respectively for each species tested.

According to these results, coumarin derivatives positively modulated the *in vitro* action of the azole derivatives, and the combinations with voriconazole and itraconazole obtained additive effects, suggesting future pharmacological use as an adjuvant for these drugs.

Several reports have been made concerning different antifungal combinations assayed *in vitro* and applied in the clinic [33, 35-37], and with other plant derivatives [10], but combinations of a coumarin derivative with synthetic drugs against *Aspergillus* spp., are reported here for the first time.

Conclusion

Based on these results, the present study demonstrates that 7-hydroxy-6-nitro-2 H-1-benzopyran-2-one (Cou-NO2) is capable of inhibiting both the mycelial growth, and germination of conidia for the *Aspergillus* species tested, thus interfering in its virulence. The results also suggest that the action of coumarin derivatives affects the structure of the fungal cell wall. At sub-inhibitory concentration, Cou-NO2 enhanced the in vitro effects of azoles. In addition, in combination with the azoles (voriconazole and itraconazole) Cou-NO2 displays an additive effect. Thus, our studies support the potential use of 7-hydroxy-6-nitro-2H-1-benzopyran-2-one as an antifungal agent against *Aspergillus* species.

Conflict of Interests

The author(s) declare(s) that there is no conflict of interests regarding the publication of this paper.

Author's Contributions

The authors Felipe Queiroga Sarmento Guerra, Rodrigo S. A. de Araújo, Janiere Pereira de Sousa, Fillipe de Oliveira Lima, Francisco J. B. Mendonça-Junior, José M. Barbosa-Filho and Edeltrudes de Oliveira Lima are responsible for drafting the paper and

listed below are the individual contributions of each author to the paper. Felipe Queiroga Sarmento Guerra participated in the project design, collection and analysis of data, drafting the paper, and critical revision of the intellectual content. Janiere Pereira de Sousa participated in data collection, data analysis, and revision of the paper. Fillipe de Oliveira Lima participated in data collection, data analysis, and revision of the paper. Rodrigo S. A. de Araújo participated in coumarin synthesis, data analysis, and revision of the paper. Francisco J. B. Mendonça-Junior participated in coumarin synthesis, data analysis, and revision of the paper. José M. Barbosa-Filho participated in coumarin synthesis, data analysis, and revision of the paper. Edeltrudes de Oliveira Lima guided all stages of the work and participated in both review and drafting of the project and the paper, including final approval of the version to be published.

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Figure 1. Chemical structure for 7-hydroxy-6-nitro-2H-1-benzopyran-2-one (Cou-NO_2).

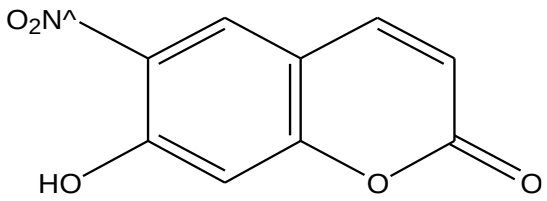
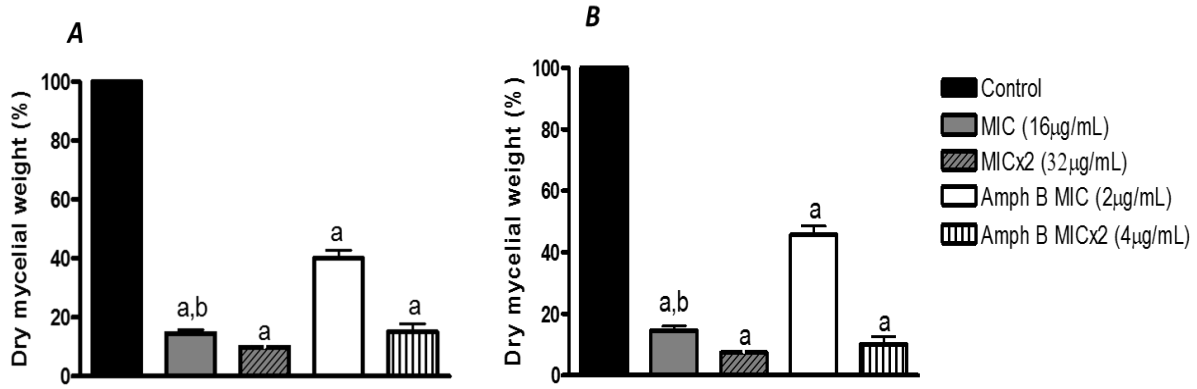
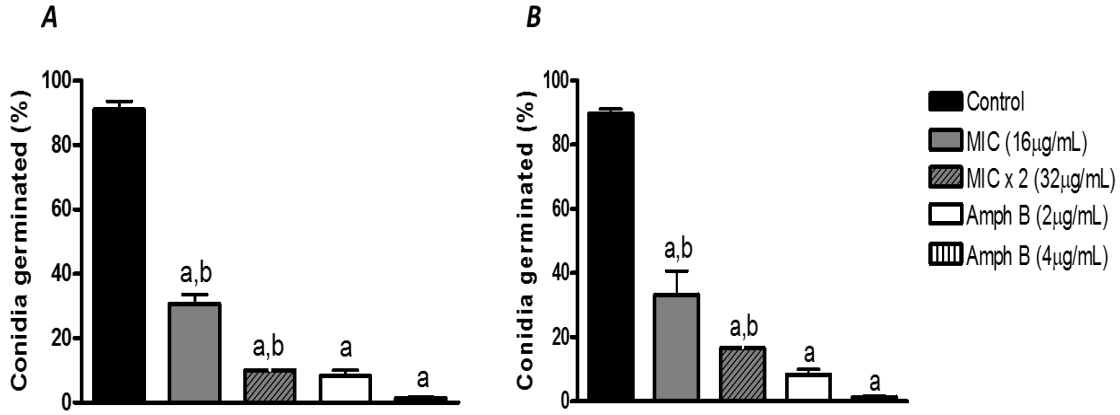


Figure 2: Percentage of dry mycelia weight produced by *A. fumigatus* (ATCC 46913), **2A**, and *A. flavus* (ATCC 16013), **2B**, in the absence (control) and presence of 7-hydroxy-6-nitro-2H-1-benzopyran-2-one (MIC: 16 μg/ml; 2 x MIC: 32 μg/ml) and Amphotericin B (MIC: 2 μg/ml; 2 x MIC: 4 μg/ml). Control produced 100% of dry mycelia weight.



a: $p < 0.05$ compared to control. b: $p < 0.05$ compared to Amphotericin B with respective concentration.

Figure 3: Percentage of conidial germination of *A. fumigatus* (ATCC 46913), **3A**, and *A. flavus* (ATCC 16013), **3B**, in the absence (control) and presence of 7-hydroxy-6-nitro-2H-1-benzopyran-2-one (MIC: 16 μg/ml; 2 x MIC: 32 μg/ml) and Amphotericin B (MIC: 2 μg/ml; 2 x MIC: 4 μg/ml).



a: $p < 0.05$ compared to control. b: $p < 0.05$ compared to Amphotericin B with respective concentration

Table 1: MIC values (µg/mL) of 7-hydroxy-6-nitro-2H-1-benzopyran-2-one against clinical strains of *Aspergillus* spp.

	MIC values (µg/mL)					
Microorganisms	*A. fumigatus* (LM 121)	*A. fumigatus* (LM 135)	*A. fumigatus* (LM 743)	*A. flavus* (LM 35)	*A. flavus* (LM 36)	*A. flavus* (LM 23)
Cou-UNO_2	16	32	32	16	16	16
Amphotericin B	2	2	2	2	2	2
Viability control	+	+	+	+	+	+
Negative control	-	-	-	-	-	-
Sensitivy control	+	+	+	+	+	+

Note: Cou-UNO_2: 7-hydroxy-6-nitro-2H-1-benzopyran-2-one.

Table 2: MIC values (µg/mL) of drugs in the absence and presence of sorbitol (0.8 M) and ergosterol (400 µg/mL) against *Aspergillus* spp.

	MIC values (µg/mL)					
Microorganisms	*A. fumigatus* ATCC 46913	*A. flavus* ATCC 16013	*A. fumigatus* ATCC 46913	*A. flavus* ATCC 16013	*A. fumigatus* ATCC 46913	*A. flavus* ATCC 16013
Drugs	**- Sterols**		**+ sorbitol**		**+ ergosterol**	
Cou-UNO_2	16	16	256	256	16	16
Amphotericin B	2	2	-	-	16	16
Flucanozole	256	512	512	512	256	512

Note: Cou-UNO_2: 7-hydroxy-6-nitro-2H-1-benzopyran-2-one.

Table 3. MIC values of antifungals in the absence and presence of coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one, MIC/8, against *Aspergillus* spp.

	MIC alone		**Combined MIC (Cou-NO_2)**	
Drugs	*A. fumigatus* ATCC 46913	*A. flavus* ATCC 16013	*A. fumigatus* ATCC 46913	*A. flavus* ATCC 16013
Amphotericin B	2	2	2	2
Flucanozole	256	256	128	128
Itraconazole	128	128	32	128
Voriconazole	4	2	0,5	1

Table 4: MIC of Antifungal drugs and effect of combination with coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one (Cou-UNO_2), against *A. flavus*, ATCC 16013.

Antifungal + Cou-UNO_2	MIC (µg/mL)	FIC INDEX (Type of interaction)
Cou-UNO_2	16	
Amphotericin B	2	
Flucanozole	256	
Itraconazole	128	
Voriconazole	4	
Cou-UNO_2/ Amphotericin B	16 / 2	2 (Indifferent)
Cou-UNO_2/ Flucanozole	16 / 256	2 (Indifferent)
Cou-UNO_2/ Itraconazole	8 / 32	0,75 (Additivity)
Cou-UNO_2/ Voriconazole	8 / 1	0,75 (Additivity)

Note: Cou-UNO_2: 7-hydroxy-6-nitro-2H-1-benzopyran-2-one. FIC*: F*ractional Inhibitory Concentration. MIC: Minimal Concentration Inhibitory.

Table 5: MIC of Antifungal drugs and effect of combination with coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one (Cou-1), against *A. fumigatus*, ATCC 46913.

Antifungal + Cou-UNO_2	MIC (µg/mL)	FIC INDEX (Type of interaction)
Cou-UNO2	16	
Amphotericin B	2	
Flucanozole	256	
Itraconazole	128	
Voriconazole	2	
Cou-UNO2/ Amphotericin B	16 / 2	2 (Indifferent)
Cou-UNO2/ Flucanozole	16 / 256	2 (Indifferent)
Cou-UNO2/ Itraconazole	4 / 64	0,75 (Additivity)
Cou-UNO2/ Voriconazole	8 / 0,5	0,5 (Additivity)

Note: Cou-UNO_2: 7-hydroxy-6-nitro-2H-1-benzopyran-2-one. FIC*: F*ractional Inhibitory Concentration. MIC: Minimal Concentration Inhibitory

New coumarin derivative, 4-acetatecoumarin, with antifungal activity against *Aspergillus* spp.

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New coumarin derivative, 4-acetatecoumarin, with antifungal activity against *Aspergillus* spp.

Abstract

In recent years fungal infections have become a concern for health professionals, the emergence of resistant strains has been reported for all known classes of antifungal drugs. Among the fungi causing disease, we highlight those that belong to the genus *Aspergillus.* Although resistance to antifungal drugs is not as common as resistance to antibacterial agents, there has been a significant increase in the number of reported cases of fungal resistance. For these reasons, the search for new antifungals is important. Thus, the aim of this study was to examine the effects of coumarin derivative, 4 acetatecoumarin (Cou UMB16), both alone and together with antifungal drugs and its mode of action against *Aspergillus spp*. Its mode of action against *Aspergillus* spp. Cou UMB16 was tested to evaluate its effects on mycelia growth, and germination of fungal conidia of *Aspergillus* spp. We also investigated possible Cou UMB16 action on cell walls (0.8 M sorbitol), and on Cou UMB16 to ergosterol binding in the cell membrane. Our results suggest that the coumarin derivative 4-acetatecoumarin (Cou-UMB16) has antimicrobial activity, which inhibits virulence factors in the *Aspergillus* species and is on the structure of the fungal cell wall. When applying Cou-UMB16 in combination with azoles has both synergistic and additive effects were observed

Keywords: Coumarin, antifungal, sorbitol, *Aspergillus* spp., aspergillosis.

1. Introduction

In recent years fungal infections have become a concern for health professionals, the emergence of resistant strains has been reported for all known classes of antifungal drµgs. As a result, especially in immunocompromised patients, mortality, morbidity and the cost of treatment against fungal pathogens have all increased. Among the fungi causing disease, we highlight those that belong to the genus *Aspergillus* [1].

Aspergillus fumigatus is the most common species isolated in cases of invasive aspergillosis, the species *Aspergillus flavus, Aspergillus niger* and *Aspergillus terreus* soon follow. In certain locations, the species *A. flavus* and *A. terreus* are frequently isolated [2].

Aspergillus fumigatus, being a saprotrophic species, plays an essential role in recycling carbon and nitrogen worldwide. This fungus has a high sporulation capacity which results in an ubiquitous presence of high atmospheric conidia concentrations (1-100 conidia/m3) both indoors and outdoors. *A. fumigatus* is a prevalent airborne fungal pathogen, and causes severe and usually fatal invasive infections in immunocompromised hosts [3, 4].

A. flavus is the second leading cause of invasive aspergillosis and is the most common cause of superficial fungal infections. Particularly common clinical syndromes associated with *A. flavus* include chronic granulomatous sinusitis, keratitis, cutaneous aspergillosis, wound infections, and osteomyelitis (following trauma and inoculation). In addition, *A. flavus* produces aflatoxins, the most toxic and potent natural hepatocarcinogenic compounds ever characterized [5].

Until recently, the only drµgs available to treat aspergillosis were amphotericin B and itraconazole, the latter in oral and intravenous formulations. Recently voriconazole, posaconazole, and caspofungin have also been approved for treatment. Althoµgh resistance to antifungal drµgs is not as common as resistance to antibacterial agents, there has been a significant increase in the number of reported cases of fungal resistance. Some investigators have reported mycoses isolates of *A. flavus* resistant to amphotericin B and itraconazole [1, 5]. For these reasons, the search for new antifungals is important.

In work recently reported by our group, the antifungal activity of twenty-four coumarin derivatives against *Aspergillus fumigatus* and *A. flavus* were described. Certain derivatives showed significant antifungal activity with Minimum Inhibition Concentration (MIC) values ranging from 16 to 32 µg/mL, including 4-acetetatecoumarin (figure 1) with an MIC value of 16 µg/mL [6]. These results motivated us to more thoroµghly assess the activity of this coumarin derivative on the fungal cell.

Another promising alternative is the possibility of combining coumarin derivatives with antifungals against strains of *Aspergillus* spp. Combination therapy of the available antifungal drµgs with natural products and their derivatives is even less explored, especially in the case of coumarin derivatives [7].

Thus, the aim of this study was to examine the effects of coumarin derivative, 4-acetetatecoumarin, both alone and together with antifungal drµgs and its mode of action against *Aspergillus* spp.

2. Material and Methods

2.1 Microorganisms:

Aspergillus spp. used in the antifungal assay was obtained from the archival collection of the Federal University of Paraíba Laboratory of Mycology (LM). They included *A. fumigatus* (ATCC 46913, LM 121, LM 743, and LM 135), and *A. flavus* (ATCC 16013, LM 35, LM 36, and LM 23). Stock inoculates (suspensions) of *Aspergillus* spp. were prepared from 8 day old potato dextrose agar (Difco Lab., USA), the cultures grown at room temperature. Fungal colonies were covered with 5 mL of sterile saline solution (0,9%), the surface was gently agitated with vortexes, fungal elements with saline solution were transferred to sterile tubes. Inoculate was standardized at 0.5 tube of McFarland scale (10^6 CFU/mL). The final concentration confirmation was done by counting the microorganisms in a Neubauer chamber [8-10].

2.2 Chemicals

The product tested was the coumarin derivative, 4-acetatecoumarine (Cou-UMB16), obtained by biosynthesis [6]. Amphotericin B, Flucanozole, Itraconazole and Voriconazole were obtained from Sigma Aldrich®, Brazil. The drµgs were dissolved in DMSO (dimethylsulfoxide), and sterile distilled water was used to obtain solutions of 1024 µg/mL for each antifungals. The concentration of DMSO did not exceed 0.5% in the assays.

2.3 Culture media

To test the biological activity of the products, Potato Agar (AP), and Sabouraud dextrose agar (SDA) were purchased from Difco Laboratories (Detroit, MI, USA), and RPMI-1640-L-glutamine (without sodium bicarbonate) (Sigma-Aldrich, São Paulo, SP, Brazil) culture media were used. They were prepared and used according to the manufacturers' instructions.

2.4 Minimum Inhibitory Concentration (MIC)

The determination of minimum inhibitory concentration against ATCC strains was demonstrated in an article previously published by our group [6]. We also carried out determinations of the coumarin derivative MIC in clinical strains of *A. flavus* and *A. fumigatus*. Broth microdilution assays were used to determine the MICs of coumarin derivative 4-acetatecoumarine (Cou-UMB16), and amphotericin B. RPMI-1640 was added to all the wells of 96-well plates. Two-fold serial dilutions of the three agents were prepared to obtain concentrations varying between 4 μg/ml and 1024 μg/ml. Finally, 10 μl aliquots of the inoculate suspension were added to the wells, and the plates were incubated at 28ºC for 3 days. Negative controls (without drμgs) were used to confirm conidia viability, and sensitivity controls (for DMSO) were also included in the studies. At 72 h there were visual observations for fungal growth. The MIC was defined as the lowest concentration capable of visually inhibiting fungal growth by 100 %. The results were expressed as the arithmetic mean of three experiments [11, 12].

2.5 Effects on mycelia growth

Analysis of the interference of coumarin derivative, 4-acetatecoumarine, on mycelia growth was performed by determining the dry mycelia weight of *A. fumigatus* (ATCC 46913) and *A. flavus* (ATCC 16013) [12, 13]. Flasks containing MIC (16 μg/mL) and MIC x 2 (32 μg/mL) of coumarin derivative in RPMI-1640 medium, were inoculated with suspension of the test *A. fumigatus* (ATCC 46913) and *A. flavus* (ATCC 16013) strains. In the corresponding control, the same amount of coumarin derivative was replaced by distilled water. The system was incubated at 28 °C for 8 days. Flasks containing mycelia were filtered throμgh Whatman® Grade 1 Qualitative Filtration Paper (particle retention: 11 μm), and then washed with distilled water. The mycelia were dried at 60 °C for 6 h, and kept at 40 °C overnight. The filter paper containing dry mycelia from two independent assays were weighed, and the mean values obtained. Percentage growth inhibition based on the dry weight of each at time of analysis, was calculated according to Sharma and Tripathi [12].

2.6 Conidial germination assay

The coumarin derivative, 4-acetatecoumarine (Cou-UMB16), and Amphotericin B were tested to evaluate effects on the germination of fungal conidia of *A. fumigatus* (ATCC 46913), and *A. flavus* (ATCC 16013). Flasks containing MIC (16 μg/mL) and MIC x 2 (32

µg/mL) of coumarin derivative, and a control with distilled water, were used. In sterile test tubes, 500 µL of RPMI-1640 plus the Cou-UMB16were evenly mixed with 500 µL of fungal conidia suspension and immediately incubated at 28 °C. Samples of this mixture were taken after 24 h of incubation for analysis. The whole experiment was performed in duplicate, where the number of conidia was determined in a Neubauer chamber, and the inhibition percentage of spore germination at each time point was calculated by comparing the results obtained in the test experiments with the results of the control experiment. The analysis was conducted under an optical microscope (Zeiss® Primo Star) [14, 15].

2.7 Sorbitol assay effects.

The assay was performed using medium with and without sorbitol (control), to evaluate possible mechanisms involved in the antifungal activity of the test product on the *Aspergillus* spp. cell wall. The sorbitol was added to the culture medium in a final concentration of 0.8M. The assay was performed by microdilution method in 96-well plates in a "U" (Alamar, Diadema, SP, Brazil) [8, 9]. The plates were sealed aseptically, incubated at 28◦C, and readings were taken at 3 days. Based on the ability of sorbitol to act as a fungal cell wall osmotic protective agent, the higher MIC values observed in the medium with added sorbitol compared to the standard medium sµggest the cell wall as one of the possible cell targets for the product tested [16, 17]. Amphotericin B was used as the control drµg. The assay was performed in duplicate and expressed as the geometric mean of the results.

2.8 Ergosterol Binding Assay - MIC Value Determination in Presence of Ergosterol.

To assess if the product binds to the fungal membrane sterols, an experiment was performed according to the method described by Escalante *et al.* [17], with some modifications. The ergosterol was prepared as was described by Leite *et al.* [18]. The MIC of coumarin derivative, 4-acetatecoumarine(Cou-UMB16) against *Aspergillus* spp. was determined by using broth microdilution techniques [8, 9], in the presence and absence of exogenous ergosterol (Sigma-Aldrich, São Paulo, SP, Brazil) added to the assay medium, in different lines of the same micro-plate. Briefly, a solution of Cou-UMB16was doubly diluted serially with RPMI-1640 (volume = 100 μL) containing plus ergosterol at a concentration of 400μg/mL. A volume of 10 μL yeast suspension (0,5 McFarland) was added to each well. The same procedure was realized for amphotericin B, whose interaction with membrane ergosterol is already known, serving as a control drµg. The plates were sealed and incubated at 28◦C.The plates were read after 3 days of incubation, and the MIC was determined as the lowest concentration of test agent

inhibiting the visible growth. The assay was carried out in duplicate and the geometric mean values were calculated. Thus, this binding assay reflected the ability of the compound to bind with ergosterol.

2.9 Checkerboard assay

A checkerboard micro-titer test was performed to evaluate the interaction of coumarin derivative, 4-acetatecoumarine with the antifungal drμgs (azoles and Amphotericin B) against *A. fumigatus*, ATCC 46913, and *A. flavus*, ATCC 16013. A series of 2-fold dilutions, in eight for each coumarin derivative and antifungal drμg were made in RPMI-1640 to obtain four times the final concentration being achieved in the micro-titer well. Furthermore, 50 μl of each dilution of coumarin derivative was added to the 96-well micro-titer plates in the vertical direction, while 50 μl of each dilution of antifungal drμgs was added in the horizontal direction, so that various combinations of coumarin derivative and antifungal drμgs could be achieved. In addition, 10 μl of inoculum from the spore suspension (1.5×10^5 CFU mL−1) was added to each well, and the plates were incubated at 30 °C for 3 days.

In order to evaluate the activity of the combinations of drμgs, fractional inhibitory concentration (FIC) indices were calculated as $FIC^A + FIC^B$, where FIC^A and FIC^B represent the Minimum Concentrations inhibiting the fungal growth for drμgs A and B, respectively: $FIC^A = MIC^A$ combination/ MIC^A alone and $FIC^B = MIC^B$ combination/MIC^B alone. A mean FIC index was calculated based on the following equation: FIC index = $FIC^A + FIC^B$

In addition, the interpretation was made as follows: synergistic (< 0.5), additivity (0.5-1.), indifferent (> 1), or antagonistic (> 4) [19, 20].

*2.10 Dr*μg *susceptibility test*

The minimum inhibitory concentrations (MIC) of coumarin derivative, 4-acetatecoumarine (Cou-UMB16), and antifungal drμg were determined in RPMI-1640 by microdilution assay using spore suspension (1.5×10^5 CFU mL−1), and a drμg concentration range of 1024 to 2,5 μg/mL (two-fold serial dilutions) [20]. MIC was defined as the lowest concentration at which no growth was observed. For the evaluation of the coumarin derivative as a modulator of antifungal properties, MICs of the antifungal drμgs were determined in the presence of Cou-UMB16 (2 μg/mL), and at sub-inhibitory concentrations (MIC/8); the plates were incubated for 3 days at 30°C [21].

Data analysis

The results were expressed in mean ± SE. Statistical analyses were performed with *t*-test. P < 0, 05 was considered significant.

3. Results

The minimum inhibitory concentration of coumarinic derivative, 4-acetoxycoumarin (Figure 1) was determined on the tested strains, and the clinical outcomes were found to be similar to those observed by our group in a previous study [6]. However, the MIC for strains of *Aspergillus fumigatus* LM 121, and LM 135 were respectively 32 and 128 mg/mL. The MIC of *Aspergillus flavus* (strain LM 36) was also 32 mg/ml (Table 1).

It was also observed that the coumarinic derivative Cou-UMB16 has inhibitory activity against spore germination and mycelial growth of Aspergillus strains (*A. fumigatus* ATCC 46913 and *A. flavus* ATCC 16013).

The percentage of conidia germinated by *A. fumigatus* ATCC 46913 and *A. flavus* ATCC 16013 in the presence and absence (control) of test products is described in Figure 2. In the test with two concentrations (MIC and MIC x 2), Cou-UMB16 showed significant inhibitory action against *Aspergillus* spp. spore germination (P <0.05) when compared to the control. However, the activity was less potent (P <0.05) compared with respective concentrations of Amphotericin B.

The effect of different concentrations of test drµgs (at MIC and MIC x 2) on mycelial growth was determined by dry mycelial mass quantification; the results are shown in Figure 3. With respect to effects on *A. fumigatus* ATCC 46913 and *A. flavus* ATCC 16013, concentrations of Cou-UMB16 at 16 µg/mL (MIC), and 32/mL (MIC x 2) inhibited mycelial growth of normal strains (p <0.05) when compared to the control. Further analysis demonstrated that Cou-UMB16 at its MIC x 2 was more potent than amphotericin B at its respective MIC x 2 (P <0.05).

In this study, the MIC values of Cou-UMB16 in culture media with or without exogenous ergosterol were identical in both experiments, sµggesting that the coumarin derivative tested does not act via binding to plasma membrane ergosterol; results shown in Table 2.

To investigate the action on the cell wall of the coumarin derivative Cou-UMB16 we carried out a test with sorbitol (as an osmotic shield); the test results are shown in Table 2. The Cou-UMB16 MIC values increased 8 times in the presence of culture medium with sorbitol compared to culture medium without sorbitol, sµggesting that Cou-UMB16 indeed

acts on the structure of the fungal cell wall, results similar to other coumarins analyzed by our group [22].

No modulatory effect on the standard antifungal agents' (fluconazole, voriconazole, itraconazole and Amphotericin B) MICs was observed when coumarin derivative, 4-acetoxycoumarin was added to growth medium (Table 3) at a sub-inhibitory concentration of 2 µg/ml (MIC/8).

Tables 4 and 5 show the results of coumarin derivative 4-acetoxycoumarin (Cou-UMB16) in combination with the antifungal drµgs (Amphotericin B, and azole derivatives), against *A. flavus* ATCC 16013 and *A. fumigatus* ATCC 46913.

The combination of Cou-UMB16 with voriconazole against *A. flavus* ATCC 16013 revealed synergistic effects. Against *A. flavus* ATCC 16013 additive effects were observed for the combination of Cou-UMB16 with Itraconazole; the Fractional Inhibitory Concentration index (FIC) being equal to 0.63. With voriconazole against *Aspergillus fumigatus* ATCC 46913, the FIC index was 1.0. However, the combination of Cou-UMB16 and Amphotericin B and fluconazole revealed an FIC index greater than 1.0 for all strains tested.

4. Discussion

Current antifungal treatments against *Aspergillus* spp. are still effective, but resistant strains and intrinsically resistant species are emerging fast. Current antifungal drµgs suffer from a number of limitations that can render their use difficult; e.g. amphotericin B´s dose-limiting nephrotoxicity; and azoles' simple fungistatic mode of action (with the development of fungal resistance), this reveals the urgent need for new antifungals with broad fungicidal spectrums of action, and with fewer dose-limiting side effects [23, 24].

An important field of study for new antifungal drµgs is plant biosynthesis of secondary metabolites (and their derivatives). The coumarin derivative, 4-acetatecoumarin (Cou-UMB16); a biosynthetic compound, has antifungal activity already reported [6].

Coumarins, as a group of compounds are derived from phenylalanine metabolism. They reveal a benzene ring attached to a pyran ring, the simplest and the most common representative found being coumarin (1,2-benzopyrone). These metabolites are found throughout the world. Coumarins have been isolated in various plant parts; the leaves, roots, and fruits of several different families such as; *Fabaceae*, *Asteraceae*, *Rutaceae*, *Saxifragaceae* and *Thymelaceae* [24].

Various coumarins and their derivatives have already proven their antifungal activity. It is believed that these properties are usually a reflection of their initial roles in vegetables; such as the phytoalexins [24-25]. The interesting biological activities of the coumarins have made them attractive targets for organic synthesis [6, 26-27].

This study confirmed the MIC_{90} of 4-acetatecoumarin (Cou-UMB16) at 16 µg/mL, and its activity against mycelial growth and spore germination for *A. fumigatus* ATCC 46913 and *A. flavus* ATCC 16013.

Research indicate a compound with MIC ranging from 50 to 500 µg/mL as having optimal antimicrobial activity, and attribute moderate activity to compounds which have a variation of 500 to 1500 µg/mL in MIC. Thus, according to these parameters, it can be stated that 4-acetatecoumarin showed an optimal antimicrobial activity [27, 28].

As reported in the introduction, *Aspergillus* spp. are ubiquitous to the soil and produce large numbers of asexual conidia (conveyed by the environment), which then become important factors for the spread of the fungus and consequent host infections [29].

The coumarin derivative, Cou-UMB16 displayed inhibitory action against *Aspergillus* spp. spore germination, thus interfering in this important virulence factor. Hyphae are difficult to phagocytize structures, which can induce apoptosis in macrophages (after phagocytosis) if forming inside. The resulting mycelium formation is an important virulence factor for *Aspergillus* spp. [29].

Reductions in mycelial growth produced by Cou-UMB16 interfere with *Aspergillus spp*. virulence. This result was similar to that found in our previous study group [22], with other coumarin derivatives; e.g. 7-hydroxy-6-nitro-2H-1-benzopyran-2-one.

The MIC values for Cou-UMB16 were multiplied 4 times in the presence of sorbitol dosed culture medium when compared to normal culture medium, sµggesting that Cou-UMB16 acts on the structure of the fungal cell wall. The same results were reported in a study with secondary coumarin 7-hydroxy-6-nitro-2H-1-benzopyran-2-one [22].

Studies show that coumarin acts via formation of pores in the cell wall, a consequent release of cytoplasmic contents and cell death, this corroborates the results found in our study [25]. However, other studies show that coumarins alter the mitochondrial morphology of the fungal cell, thus inducing apoptosis [24, 30].

The synergistic effect observed against *A. flavus* ATCC 16013 when combining Cou-UMB16 and voriconazole; and the additive effects against *A. flavus* ATCC 16013 when combined with itraconazole; and against *A. fumigatus* ATCC 46913 when combined with

voriconazole, sµggest that Cou-UMB16 may be used in future combinations with these drµgs.

Several reports have indicated yet other antifungal combinations, being assayed *in vitro* and then applied in clinics [31 -34], and with differing plant derivatives [7], but against *Aspergillus* spp., combinations of synthetic drµgs with coumarin derivatives are little explored.

Conclusion

Our results sµggest that the coumarin derivative coumarin 4-acetate (Cou-UMB16) has antimicrobial activity which inhibits virulence factors in the *Aspergillus* species tested. The results also demonstrate that the action of coumarin derivatives is on the structure of the fungal cell wall. When applying Cou-UMB16 in combination with azoles (voriconazole and itraconazole), both synergistic and additive effects were observed, depending on the bacterial strain used.

Conflict of Interests

The author(s) declare(s) that there is no conflict of interests regarding the publication of this paper.

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Figure 1. Chemical structure for 4-acetatecoumarine (Cou-UMB16).

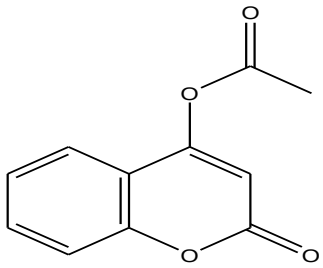
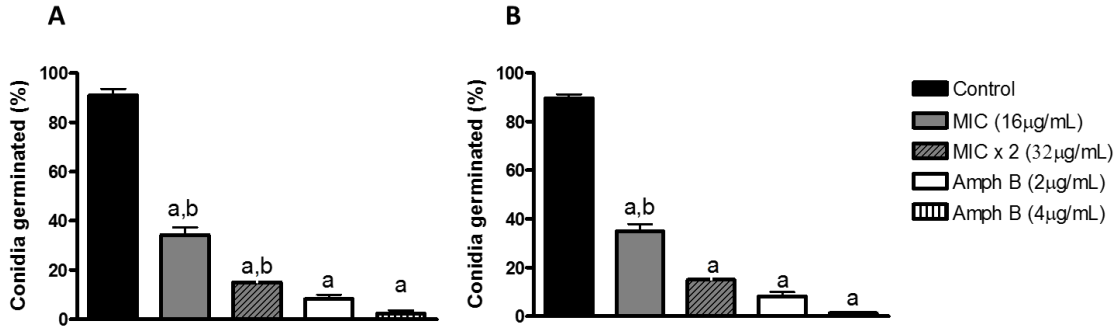
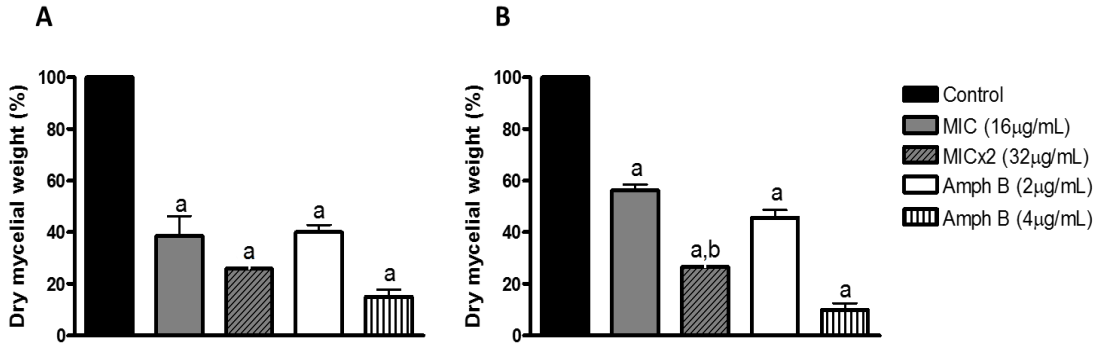


Figure 2: Percentage of conidial germination of *A. fumigatus* (ATCC 46913), **3A**, and *A. flavus* (ATCC 16013), **3B**, in the absence (control) and presence of 4-acetatecoumarine (MIC: 16 μg/ml; 2 x MIC: 32 μg/ml) and Amphotericin B (MIC: 2 μg/ml; 2 x MIC: 4 μg/ml).



a: $p < 0.05$ compared to control. b: $p < 0.05$ compared to Amphotericin B with respective concentration

Figure 3: Percentage of dry mycelia weight produced by *A. fumigatus* (ATCC 46913), **2A**, and *A. flavus* (ATCC 16013), **2B**, in the absence (control) and presence of 4-acetatecoumarine (MIC: 16 μg/ml; 2 x MIC: 32 μg/ml) and Amphotericin B (MIC: 2 μg/ml; 2 x MIC: 4 μg/ml). Control produced 100% of dry mycelia weight.



a: $p < 0.05$ compared to control. b: $p < 0.05$ compared to Amphotericin B with respective concentration.

Table 1: MIC values (µg/mL) of 4-acetatecoumarine (Cou-UMB16) against clinical strains of *Aspergillus* spp.

	MIC values (µg/mL)					
Microorganisms	*A. fumigatus* (LM 121)	*A. fumigatus* (LM 135)	*A. fumigatus* (LM 743)	*A. flavus* (LM 35)	*A. flavus* (LM 36)	*A. flavus* (LM 23)
Cou-UMB16	32	128	16	32	16	16
Amphotericin B	2	2	2	2	2	2
Viability control	+	+	+	+	+	+
Negative control	-	-	-	-	-	-
Sensitivy control	+	+	+	+	+	+

Note: Cou-UMB16: 4-acetatecoumarin

Table 2: MIC values (µg/mL) of coumarin derivative, 4-acetatecoumarin (Cou-UMB16), in the absence and presence of sorbitol (0.8 M) and ergosterol (400 µg/mL) against *Aspergillus* spp.

	MIC values (µg/mL)					
Microorganisms	*A.fumigatus* ATCC(46913)	*A.flavus* ATCC(16013)	*A.fumigatus* ATCC(46913)	*A.flavus* ATCC(16013)	*A.fumigatus* ATCC(46913)	*A.flavus* ATCC(16013)
Drugs	**- Sterols**		**+ sorbitol**		**+ ergosterol**	
Cou-UMB16	16	16	128	128	16	32
Amphotericin B	2	2	-	-	16	16
Flucanozole	256	512	512	512	256	512

Table 3. MIC values of antifungals in the absence and presence of 4-acetatecoumarin (Cou-UMB16) (MIC/8) against *Aspergillus* spp.

	MIC alone		**Combined MIC**	
Drugs	*A.fumigatus* ATCC(46913)	*A.flavus* ATCC(16013)	*A.fumigatus* ATCC(46913)	*A.flavus* ATCC(16013)
Amphotericin B	2	2	2	2
Flucanozole	256	256	256	256
Itraconazole	128	128	128	128
Voriconazole	4	2	4	2

Table 4: MIC of Antifungal drugs and effect of combination with coumarin derivative, 4-acetatecoumarine (Cou-UMB16), against *A. flavus,* ATCC 16013.

Antifungal + Cou-UMB16	MIC (µg/mL)	FIC INDEX (Type of interaction)
Cou-UMB16	16	
Amphotericin B	2	
Flucanozole	256	
Itraconazole	128	
Voriconazole	2	
Cou-UMB16/ Amphotericin B	16 / 2	2 (Indifferent)
Cou-UMB16/ Flucanozole	16 / 256	2 (Indifferent)
Cou-UMB16/ Itraconazole	2 / 64	0,63 (Additivity)
Cou-UMB16/ Voriconazole	2 / 0,5	0,38 (Synergism)

Note: Cou-UMB16: 4-acetatecoumarin. FIC*:* Fractional Inhibitory Concentration. MIC: Minimal Concentration Inhibitory

Table 5: MIC of Antifungal drugs and effect of combination with coumarin derivative, 4-acetatecoumarine (Cou-UMB16), against *A. fumigatus*, ATCC 46913.

Cou-UMB16	MIC (µg/mL)	FIC INDEX (Type of interaction)
Cou-UMB16	16	
Amphotericin B	2	
Flucanozole	256	
Itraconazole	128	
Voriconazole	2	
Cou-UMB16/ Amphotericin B	16 / 4	1,5 (Indifferent)
Cou-UMB16/ Flucanozole	16 / 2	2 (Indifferent)
Cou-UMB16/ Itraconazole	4 / 256	1,25 (Indifferent)
Cou-UMB16/ Voriconazole	8 /128	1,0 (Additivity)

Note: Cou-UMB16: 4-acetatecoumarin. FIC*:* Fractional Inhibitory Concentration. MIC: Minimal Concentration Inhibitory

CONCLUSÃO

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Os resultados demonstraram que:

- 12 derivados cumarínicos ensaiados, total de 24 compostos, contra as cepas de *Aspergillus* spp., demostraram atividade antifúngica, com valores de CIM_{90} variando entre 1024-16µg/mL.

- Dois derivados cumarínicos obtiveram melhores CIM_{90} (16µg/mL), 7-hidroxi-6-nitrocumarina (Cou-UNO_2) e 4-acetóxicumarina (Cou-UMB16).

- Os derivados cumarínicos com melhores CIM, Cou-UNO_2 e Cou-UMB16, foram capazes de inibir o crescimento micelial e a germinação dos conídios das espécies de *Aspergillus* ensaiadas, interferindo, assim, na sua virulência.

- Os resultados também sugerem que a ação dos derivados cumarínico (Cou- UNO_2 e Cou-UMB16) é sobre a estrutura da parede celular fúngica.

- Em uma concentração subinibitória, Cou-UNO_2 potencializou a ação *in vitro* dos derivados azólicos.

- O derivado cumarínico Cou-UNO_2 em combinação com os derivados azólicos (voriconazol e itraconazol) observou-se um efeito aditivo.

- O derivado cumarínico Cou-UMB16 em combinação com os derivados azólicos (voriconazol e itraconazol) obteve um efeito sinérgico a aditivo, dependendo da linhagem utilizada.

- Estudo SAR e química computacional permitiu observar que a cumarina (1,2-benzopirona) não apresentou antividade antifúngica e hidroxilações no anel básico nas posições 4, 6 e 7 não alterou esta atividade. A adição de grupos prenilados nessas posições alterou a atividade

- A O-acetilação nas posições 4, 6 e 7 favorecem a atividade antifúngica dos compostos cumarínicos. A adição de radicais NO_2 nas posições orto-hidroxi melhora a atividade

antifúngica. Estes dados sugerem que grupos eletronegativos adicionados ao anel básico das cumarinas favorecem positivamente a atividade antifúngica.

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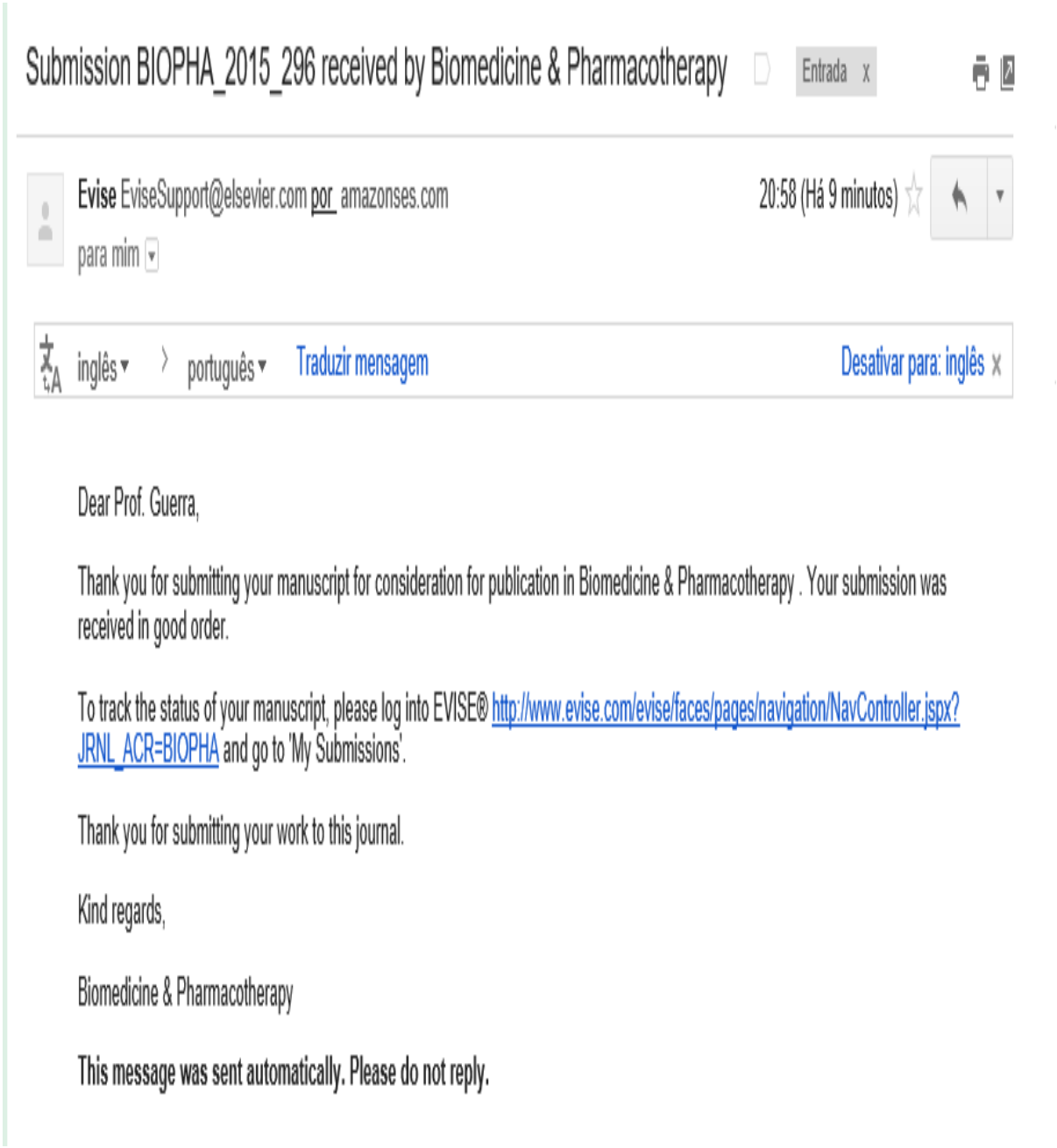
ANEXOS

Anexo1: Artigo submetido:



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Anexo 2: Artigos publicados relacionados à tese

Int. J. Mol. Sci. **2013**, *14*, 1293-1309; doi:10.3390/ijms14011293

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Article

Synthesis, Structure-Activity Relationships (SAR) and *in Silico* Studies of Coumarin Derivatives with Antifungal Activity

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Abstract: The increased incidence of opportunistic fungal infections, associated with greater resistance to the antifungal drugs currently in use has highlighted the need for new solutions. In this study twenty four coumarin derivatives were screened *in vitro* for antifungal activity against strains of *Aspergillus*. Some of the compounds exhibited significant antifungal activity with MICs values ranging between 16 and 32 µg/mL. The structure-activity relationships (SAR) study demonstrated that *O*-substitutions are essential for antifungal activity. It also showed that the presence of a short aliphatic chain and/or electron withdrawing groups (NO_2 and/or acetate) favor activity. These findings were

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Hindawi

Research Article

Evaluation of Antifungal Activity and Mode of Action of New Coumarin Derivative, 7-Hydroxy-6-nitro-2H-1-benzopyran-2-one, against *Aspergillus* spp.

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Aspergillus spp. produce a wide variety of diseases. For the treatment of such infections, the azoles and Amphotericin B are used in various formulations. The treatment of fungal diseases is often ineffective, because of increases in azole resistance and their several associated adverse effects. To overcome these problems, natural products and their derivatives are interesting alternatives. The aim of this study was to examine the effects of coumarin derivative, 7-hydroxy-6-nitro-2H-1-benzopyran-2-one (Cou-NO_2), both alone and with antifungal drugs. Its mode of action against *Aspergillus* spp. Cou-NO_2 was tested to evaluate its effects on mycelia growth and germination of fungal conidia of *Aspergillus* spp. We also investigated possible Cou-NO_2 action on cell walls (0.8 M sorbitol) and on Cou-NO_2 to ergosterol binding in the cell membrane. The study shows that Cou-NO_2 is capable of inhibiting both the mycelia growth and germination of conidia for the species tested, and that its action affects the structure of the fungal cell wall. At subinhibitory concentration, Cou-NO_2 enhanced the in vitro effects of azoles. Moreover, in combination with azoles (voriconazole and itraconazole) Cou-NO_2 displays an additive effect. Thus, our study supports the use of coumarin derivative 7-hydroxy-6-nitro-2H-1-benzopyran-2-one as an antifungal agent against *Aspergillus* species.

1. Introduction

The incidence and prevalence of invasive fungal infections have increased in recent years, especially in the currently large population of immunocompromised patients and those hospitalized with serious underlying diseases. Fungal species represent 25% of the microorganisms isolated in blood cultures of hospitalized patients. Of these, species of the genus *Aspergillus* spp. have the highest incidence among the filamentous fungi [1, 2].

Aspergillus spp. produce a wide variety of diseases. The main route of infection is penetration by air. In cases of invasive aspergillosis *Aspergillus fumigatus* is the most common species isolated in the world. In Brazil, the species *A. flavus* is the most common [3]. The main clinical manifestations observed due to *Aspergillus* spp. infections are cutaneous aspergillosis, otomycosis, aspergilloma, and sinusitis [4].

For the treatment of such infections, the azoles (Fluconazole, Itraconazole, and Voriconazole) and Amphotericin B are used in various formulations. However, with the

Anexo 3: Artigos publicados durante o período da tese

BioFar

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ATIVIDADE ANTIBACTERIANA DE ÓLEOS ESSENCIAIS DE ESPECIARIAS SOBRE CEPAS DE *ACINETOBACTER* SPP. MULTIDROGAS-RESISTENTES

Felipe Queiroga Sarmento Guerra[1], Juliana Moura Mendes[2], Wylly Araújo de Oliveira[3], Luis Alberto de Sousa Rodrigues[4], Bernadete Helena Cavalcante Santos[5], Edeltrudes de Oliveira Lima[6]

RESUMO - A resistência aos antimicrobianos tem se tornado um dos problemas mais alarmantes deste século, pois a mesma se modifica de forma muito dinâmica, afetando microrganismos que antes não eram considerados patógenos graves, como é o caso das bactérias do gênero *Acinetobacter* spp. Nos últimos anos tem sido isolado com frequência cepas multidrogas-resistentes de *Acinetobacter* relacionadas com surtos hospitalares. Por isso, tem aumentado o interesse por novas alternativas terapêuticas, a exemplo o uso de produtos naturais, especialmente os óleos essenciais (OEs), que estão presentes nas plantas medicinais, ervas e condimentos. Os OEs de especiarias possuem inúmeras ações biológicas descritas, das quais se destaca a atividade antimicrobiana. O objetivo desse trabalho foi investigar a atividade antibacteriana dos óleos essenciais das especiarias *Coriandrum sativum* L.(coentro), *Ocimum basilicum* L. (basilicão), *Origanum majorana* L. (manjerona) e *Rosmarinus officinalis* L. (alecrim) sobre cepas multidrogas-resistentes de *Acinetobacter* spp. Foram utilizadas 24 cepas de *Acinetobacter* spp provenientes de ambiente hospitalar de diferentes períodos, estas foram previamente isoladas, identificadas e realizado o teste de sensibilidade a antibióticos pelo método de disco difusão. Para avaliar a atividade antibacteriana determinou-se a Concentração Mínima Inibitória (CIM) dos óleos essenciais sobre as cepas citadas, utilizando o método de microdiluição. Os quatro OEs demonstraram efeito inibitório do crescimento bacteriano, sendo o coentro com a menor CIM (1,25-2,5 mg/mL). Dessa forma pode-se concluir que OEs das especiarias investigadas apresentaram efetiva atividade antibacteriana contra cepas de *Acinetobacter* spp. multidrogas-resistentes de ampla importância clínica e epidemiológica, principalmente, devido as infecções hospitalares causadas pela mesma.

Unitermos: Produtos Naturais, Concentração Inibitória, Resistência bacteriana

ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS OF SPICES ON MULTIDRUG-RESISTANT STRAINS OF *ACINETOBACTER* SPP.

ABSTRACT- Antimicrobial resistance has become one of the most alarming of this century, because it changes very dynamic, affecting microorganisms that were not considered serious pathogens, such as bacteria of the genus *Acinetobacter* spp. Recent years have often been isolated multidrug-resistant strains of *Acinetobacter* outbreaks related to hospital. Therefore, there has been increasing interest in new therapies, such as the use of natural products, especially essential oils (EOs), which are present in medicinal plants, herbs and spices. The EOs of spices have numerous biological actions described, of which stands the antimicrobial activity. The aim of this study was

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Artículo Original | Original Article

Actividad antifúngica del aceite esencial de *Eugenia caryophyllata* sobre cepas de *Candida tropicalis* de aislados clínicos

[Antifungal activity of the essential oil of Eugenia caryophyllata on Candida tropicalis strains from clinical isolates]

Juliana MOURA MENDES[1], Felipe Queiroga SARMENTO GUERRA[1], Fillipe de OLIVEIRA PEREIRA[1], Janiere PEREIRA de SOUSA[1], Vinicius NOGUEIRA TRAJANO[1] & Edeltrudes de OLIVEIRA LIMA[1]

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Abstract
Candidiasis is an opportunistic fungal infection caused by *Candida* yeasts. In Brazil, *C. tropicalis* is the second most frequently isolated microorganism after *C. albicans*. The arising of strains resistant to conventional antifungal agents has increased the search for new alternatives from natural products, especially essential oils. This research investigated essential oil activity against strains of *C. tropicalis* by disk diffusion method. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were also determinate. In the disk diffusion, the essential oils of *Cinnamomum zeylanicum*, *Eugenia caryophyllata* and *Origanum vulgare* had the highest inhibition zones values. MIC and MFC values of *E. caryophyllata* essential oil were 512 and 1024 µg/mL, respectively. MIC and MFC amphotericin B values were identical (2 µg/mL). Therefore, it was concluded that *E. caryophyllata* essential oil has strong antifungal activity and may be subject to further studies.

Keywords: Candidiasis, *Candida tropicalis*, clove, clinical isolates, strong activity, essential oil, antifungal activity

Resumen
La candidiasis es una infección fúngica oportunista causada por levaduras del género *Candida*. En Brasil, la especie *C. tropicalis* esta siendo aislada frecuentemente, es el segundo microorganismo más aislado después de *C. albicans*. La aparición de cepas resistentes a los antifúngicos convencionales ha aumentado la búsqueda de nuevas alternativas provenientes de productos naturales, especialmente los aceites esenciales. En este estudio se investigó la actividad de los aceites esenciales contra las cepas de *C. tropicalis*, utilizando el método de difusión en disco, la concentración inhibitoria mínima (CIM) y la concentración fungicida mínima (CFM). En el método de difusión en disco, con los aceites esenciales de *Cinnamomum zeylanicum*, *Eugenia caryophyllata* y *Origanum vulgare* se obtuvieron mayores valores de inhibición. La CIM y CFM del aceite esencial de *Eugenia caryophyllata* fueron 512 y 1024 µg/mL, mientras que los de la anfotericina B fueron idénticos, 2 µg/mL. Por lo tanto, se puede concluir que el aceite esencial de *E. caryophyllata* tiene potente actividad antifúngica y puede ser objeto de nuevos estudios sobre esta actividad.

Palabras Clave: Candidiasis, *Candida tropicalis*, clavo de la India, Aislado clínico, aceite esencial, actividad antifúngica

Natural Product Research: Formerly Natural Product Letters

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http://www.tandfonline.com/loi/gnpl20

Increasing antibiotic activity against a multidrug-resistant Acinetobacter spp by essential oils of Citrus limon and Cinnamomum zeylanicum

Felipe Queiroga Sarmento Guerra [a] , Juliana Moura Mendes [a] , Janiere Pereira de Sousa [a] , Maria F.B. Morais-Braga [b] , Bernadete Helena Cavalcante Santos [c] , Henrique Douglas Melo Coutinho [b] & Edeltrudes de Oliveira Lima [a]

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CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITY OF *Cinnamomum zeylanicum* Blume ESSENTIAL OIL ON MULTI-DRUG RESISTANT *Acinetobacter* spp. strains

Felipe Queiroga Sarmento Guerra[1*], *Juliana Moura Mendes*[1], *Wylly Araújo de Oliveira*[1], *José Galberto Martins da Costa*[2], *Henrique Douglas Melo Coutinho*[3], *Edeltrudes Oliveira Lima*[1].

ABSTRACT - The genus *Acinetobacter* comprises a group of Gram-negative bacteria and non-fermenters of glucose that are important nosocomial pathogens, many of which being resistant to many antimicrobial agents. Studies on the antibacterial activity of new products have been conducted, including the essential oils. The essential oil analysis resulted in the identification of five components with eugenol as the major component. The essential oil at 625 µg/ml concentration produced inhibition on the growth of 71% strains of *Acinetobacter* and the MBC was established as 1250 µg/mL. It was observed from the time kill experiment that the essential oil of *Cinnamomum zeylanicum* has concentration-dependent bactericidal activity. The results of this study have shown the antibacterial potential of the essential oil and may lead to future researches on this essential oil or your chemical constituents, aiming its possible clinical application in the treatment of infections caused by multi drug resistant *Acinetobacter* species.

COMPOSIÇÃO QUÍMICA E ATIVIDADE ANTIMICROBIANA DO ÓLEO ESSENCIAL DE *Cinnamomum zeylanicum* Blume SOBRE CEPAS MULTI-RESTISTENTES DE *Acinetobacter* spp.

RESUMO - O gênero *Acinetobacter* compreende um grupo de bactérias Gram-negativas, imóveis e não fermentadoras de glicose que estão se firmando cada vez mais como importantes patógenos hospitalares, sendo muitas delas resistentes a inúmeros agentes antimicrobianos. Estudos sobre a atividade antibacteriana de novos produtos têm sido realizados, incluindo os óleos essenciais. A análise de óleo essencial resultou na identificação de cinco componentes, tendo o eugenol como o componente majoritário. O óleo essencial, em uma concentração de 625 µg/ml, inibiu o crescimento de 71% das cepas de *Acinetobacter* spp. e sua Concentração Bactericida Mínima foi estabelecida em 1250 µg/ml. Foi observado, no experimento da cinética de morte microbiana, que o óleo essencial de *Cinnamomum zeylanicum* possui uma atividade antimicrobiana concentração dependente. Os resultados deste estudo demonstram o potencial antibacteriano do referido óleo essencial e podendo conduzir a futuras pesquisas sobre este óleo essencial ou seus constituintes químicos, visando a sua aplicação na prática clínica para o possível tratamento de infecções causadas por espécies multidrogas resistentes de *Acinetobacter*.

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ARTIGO ORIGINAL / RESEARCH

Atividade antifúngica de óleos essenciais sobre leveduras *Candida* não *albicans*

Antifungal activity of essential oils on non *Candida albicans* yeasts

Maiza Rocha de Abrantes[1*], Edeltrudes de Oliveira Lima[2], Mariana Araújo Paulo de Medeiros[1], Camilla Pinheiro de Menezes[2], Felipe Queiroga Sarmento Guerra[2] & Eveline Pipolo Milan[3].

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RESUMO: Os produtos oriundos de plantas medicinais com atividade antimicrobiana vêm ganhando grande perspectiva e tem como meta a obtenção de princípios ativos para uma possível aplicação prática no tratamento das infecções. Neste trabalho foi realizada uma avaliação da atividade antifúngica desses óleos essências (OE) obtidos de plantas medicinais sobre *Candida* não *albicans*, isoladas das mucosas vaginal e anal. A metodologia empregada foi à técnica de difusão com disco, em ágar Sabouraud dextrose. Os ensaios foram realizados em duplicata e os resultados considerados positivos quando as médias aritméticas dos halos de inibição apresentaram valores iguais ou superiores a 15 mm de diâmetro, em pelo menos, 50% do total das cepas testadas. Dentre os produtos testados, as leveduras foram resistentes ao OE de *Coriandrum sativum*. No entanto apresentaram boa sensibilidade aos OEs de *Citrus limonum*, *Cinnamomum zeylanicum*, *Eucalyptus globulus*, *Eugenia coryophyllata*, *Mentha arvensis*, *Mentha piperita*, *Mentha spicata*, *Origanum vulgare*, *Pimpinella anisum* com média de halos de inibição entre 18 a 49 mm de diâmetro. Considerando os resultados obtidos no estudo, conclui-se que os OEs acima citados, exceto o *C. sativum*, mostraram forte atividade anti-*Candida*. Planeja-se realizar investigações mais amplas de tais produtos para a inibição do crescimento de leveduras *Candidas* não *albicans*.

PALAVRAS CHAVES: Plantas medicinais, Produtos naturais, Resistência a medicamentos.

ABSTRACT: The products from medicinal plants with antimicrobial activity have been gaining great perspective and aims to achieve active principles for a possible practical application in the treatment of infections. This work was carried out an evaluation of the antifungal activity of these essential oils (EO) obtained from medicinal plants on non-*Candida albicans* isolated from vaginal and anal mucosa. The methodology used was the disk diffusion technique in Sabouraud dextrose agar. Assays were performed in duplicate and the results considered positive when the averages of inhibition halos show values equal or superior to 15 mm in diameter, at least 50% of strains tested. Among the products tested, the yeasts were resistant OE *Coriandrum sativum*. However showed good sensitivity to the EOs of *Citrus Limonum*, *Cinnamomum zeylanicum*, *Eucalyptus globulus*, *Eugenia coryophyllata*, *Mentha arvensis*, *Mentha piperita*, *Mentha spicata*, *Origanum vulgare*, *Pimpinella anisum* mean inhibition zones between 18 and 49 mm in diameter. Considering the results obtained in the study, it is concluded that the EOs listed above, except C. *sativum*, showed strong anti-*Candida* activity. It is planned to carry out more extensive investigation of such a product for inhibiting yeast growth *Candidas* not *albicans*.

KEYWORDS: Medicinal plants, Natural products, Drug resistance.

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DISTRIBUTION OF DERMATOPHYTES FROM SOILS OF URBAN AND RURAL AREAS OF CITIES OF PARAIBA STATE, BRAZIL

Zélia Inez Vieira da Silva PONTES[1], Aarytenn Carlos de OLIVEIRA[1], Felipe Queiroga Sarmento GUERRA[1], Luiz Ronaldo de Araújo PONTES[2] & Jeanmar Pereira dos SANTOS[3]

SUMMARY

The dermatophytes, keratinophilic fungi, represent important microorganisms of the soil microbiota, where there are cosmopolitan species and others with restricted geographic distribution. The aim of this study was to broaden the knowledge about the presence of dermatophytes in soils of urban (empty lots, schools, slums, squares, beaches and horses) and rural areas and about the evolution of their prevalence in soils of varying pH in cities of the four mesoregions of Paraíba State, Brazil. Soil samples were collected from 31 cities of Paraíba State. Of 212 samples, 62% showed fungal growth, particularly those from the Mata Paraibana mesoregion (43.5%), which has a tropical climate, hot and humid. Soil pH varied from 4.65 to 9.06, with 71% of the growth of dermatophytes occurring at alkaline pH (7.02 - 9.06) ($p = 0.0000$). Of 131 strains isolated, 57.3% were geophilic species, particularly *Trichophyton terrestre* (31.3%) and *Microsporum gypseum* (21.4%). *M. nanum* and *T. ajelloi* were isolated for the first time in Paraíba State. The zoophilic species identified were *T. mentagrophytes* var. *mentagrophytes* (31.3 %) and *T. verrucosum* (7.6 %), and *T. tonsurans* was isolated as an anthropophilic species. The soils of urban areas including empty lots, schools, slums and squares of cities in the mesoregions of Paraíba State were found to be the most suitable reservoirs for almost all dermatophytes; their growth may have been influenced by environmental factors, soils with residues of human and/or animal keratin and alkaline pH.

KEYWORDS: Dermatophytes; Keratinophilic fungi; Soil; pH conditions; Brazil.

INTRODUCTION

The dermatophytes (*Trichophyton*, *Microsporum* and *Epidermophyton*), keratinophilic fungi, represent important microorganisms of the soil microbiota, where there are cosmopolitan species and others with restricted geographic distribution[1,10,11,17,22]. There have been reports of the isolation of *T. ajelloi*, *T. rubrum*, *T. mentagrophytes*, *T. verrucosum*, *T. terrestre*, *T. tonsurans*, *T. simii*, *T. schoenleinii*, *M. gypseum*, *M. canis*, *M. audouinii*, *M. nanum*, *M. cookei* and/or *E. floccosum*, from the soils of various Brazilian states and locals around the world[4,20,24,25,30,32,38].

The occurrence of fungi in the soil can also be influenced by non-biological factors such as soil temperature, humidity, rainfall, environmental light, climate, chemical composition, quantity of organic matter in the soil and pH. Some have a wide range of tolerance for acidic to alkaline soils[5,7,14,34]. However, studies of soil pH in relation to occurrence of dermatophytes are uncommon in Brazil.

The study of the diversity of dermatophytes in the soil is important because changes in the distribution of species of dermatophytes due to ecological factors, socio-economic, therapeutic, and migration processes of livestock populations, reflect the epidemiology of dermatophytosis, which are one of the source infections of the soil[5,9,18,31]. Thus, the aim of this study was to broaden the study into the presence of dermatophytes from soils of urban and rural areas of cities of four mesoregions of Paraíba State and the influence of pH on fungi growth.

MATERIALS AND METHODS

The state of Paraíba is situated in the eastern portion of Northeast Brazil, with coordinates between 6° and 8° S and between 34° and 38° W; therefore, it is included in the tropical zone. It comprises an area of 56,372 km² and is divided into four mesoregions (Mata Paraibana, Borborema, Agreste Paraibano and Sertão Paraibano) and into 23 geographic microregions, including a total of 223 cities. In the Mata Paraibana, the predominant climate is warm, humid tropical (As') with an average annual rainfall of 1,800 mm, temperature of 26 °C and relative humidity of 80%. The soils are sandy and muddy, which are influenced by sea water and have especially coastal vegetation of mangrove swamp, rainforest and cerrado. In Borborema, the predominant climate is semi-arid (Bsh), warm and dry with average annual rainfall of 500 mm, temperature of 26 °C and relative humidity of 75%. The soils are shallow stony soil with caatinga

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PESQUISA/RESEARCH

Antibacterial activity of the essential oil of *Citrus limon* against multidrug resistant *Acinetobacter* strains

Atividade antibacteriana do óleo essencial de Citrus limon contra cepas multidroga resistente Acinetobacter

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ABSTRACT

Actinetobacter species have gained importance in recent years due to their involvement in serious infections and antimicrobial resistance. New products with antibacterial activity have been studied including the essential oil (EO) of *Citrus limon*. The present study aimed to evaluate the effect of the essential oil of *C. limon* against multidrug resistant strains of *Acinetobacter* spp. isolated from clinical material. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined by the microplate bioassay, and a time kill study of *Acinetobacter* spp. treated with EO, was performed. The oil caused the growth inhibition in 16 (67%) of 24 strains tested, showing a MIC of 625 µg/mL and MBC of 1250 µg/mL. In a time kill study, the oil displayed a concentration-dependent antibacterial activity. These results suggest that EO of *C. limon* may suppress the growth of *Acinetobacter* species.

Keywords: Drug resistant, Bacteria, *Acinetobacter, Citrus limon*

RESUMO

Espécies do gênero *Actinetobacter* ganharam importância nos últimos anos devido ao seu envolvimento em infecções graves e sua resistência antimicrobiana. Novos produtos com atividade antibacteriana foram estudados, incluindo o óleo essencial (OE) de *Citrus limon*. O presente estudo teve como objetivo avaliar o efeito do óleo essencial de *C. limon* contra cepas multirresistentes de *Acinetobacter spp.* isoladas a partir de material clínico. A concentração inibitória mínima (CIM) e Concentração Bactericida Mínima (CBM) foram determinadas pelo bioensaio com microplacas, e um estudo de tempo de morte *Acinetobacter spp.* tratado com OE, foi realizado. O óleo causou a inibição do crescimento em 16 (67%) de 24 linhagens testadas, demonstrando uma CIM de 625 ug / mL e CBM de 1250 ug / ml. Em um estudo de tempo de morte, o óleo apresentou uma atividade antibacteriana dependente da concentração. Estes resultados sugerem que o óleo essencial de *C. limon* pode suprimir o crescimento das espécies de *Acinetobacter*.

Palavras-chave: Resistências às drogas, bactérias, *Acinetobacter e Citrus limon*

INTRODUTION

Lemon has been valued as an important part of a healthy diet. It is well established that lemon fruit and its by-products constitute an interesting source of phenolic compounds (mainly flavonoids) and other nutrients and non-nutrient compounds (vitamins, minerals, dietary fiber, essential oils, organic acids and carotenoids), which are necessary for normal growth and the correct functioning of human physiological systems. Some of these known compounds are, for example, essential oils mainly used as food flavoring, perfumes and pharmaceutical formulations due to their functional properties (Gonzales *et al*, 2010). The essential oil of *Citrus limon* (Rutaceae) is rich in biologically active compounds, which are responsible for its antibacterial, antifungal, antiparasitic and antiviral activities (Prabuseenivasan *et al.*, 2006).

The volatile constituents are a mixture of monoterpene

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Research Article

Evaluation of Antifungal Activity and Mechanism of Action of Citral against *Candida albicans*

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Candida albicans is a yeast that commensally inhabits the human body and can cause opportunistic or pathogenic infections. *Objective.* To investigate the antifungal activity of citral against *C. albicans*. *Methodology.* The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) were determined by the broth microdilution technique. We also investigated possible citral action on cell walls (0.8 M sorbitol), cell membranes (citral to ergosterol binding), the time-kill curve, and biological activity on the yeast's morphology. *Results.* The MIC and MFC of citral were, respectively, 64 μg/mL and 256 μg/mL. Involvement with the cell wall and ergosterol binding were excluded as possible mechanisms of action. In the morphological interference assay, it was observed that the product inhibited pseudohyphae and chlamydoconidia formation. The MIC and the MFC of citral required only 4 hours of exposure to effectively kill 99.9% of the inoculum. *Conclusion.* Citral showed in vitro antifungal potential against strains of *C. albicans*. Citral's mechanism of action does not involve the cell wall or ergosterol, and further study is needed to completely describe its effects before being used in the future as a component of new antifungals.

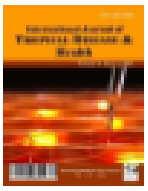
1. Introduction

The prevalence of *Candida albicans* infections is increasing at an alarming rate, and this is especially true for immunocompromised individuals, such as AIDS patients, transplant patients, and neonates [1, 2]. *C. albicans* is an opportunist pathogen that lives commensally within the human body; it is the leading cause of human fungal infections. *C. albicans* infection usually develops as a consequence of host immune response alterations [3].

The increased incidence of *Candida* infections can be attributed to a variety of factors, either exogenous or (especially) endogenous. Over 100 species of *Candida* are known; *C. albicans* is the main representative. The frequency of distribution for *Candida* spp. varies in accordance with geographical location. Among the *Candida* species, it is *C. albicans* that is involved in bloodstream infections for 44% of Latin American and 62% of European cases [4].

Conventional fungal infection treatments are unsatisfactory. Therefore, it has become essential to develop new drugs and alternative therapies (including natural products) for the treatment of *C. albicans* infections. Plants and their derivatives are known to be important in pharmacological research due to their great potential as a source for a variety of biologically active ingredients used in drug development. Amongst these products we find the terpenes, a class of natural substances of vegetable origin formed by combining five carbons called isoprene (C_5H_8). Terpenes can be classified according to their number of isoprene units: monoterpenes (C_{10}), the most representative molecules, and sesquiterpenes (C_{15}), but there are also hemiterpenes (C_5), diterpenes (C_{20}), triterpenes (C_{30}), and tetraterpenes (C_{40}) [5].

Citral (3,7-dimethyl-2-6-octadienal) is the name given to a mixture of two geometric isomers: geranial (*trans*-citral, citral A) and neral (*cis*-citral, citral B), which are acyclic α, β-unsaturated monoterpene aldehydes that occur naturally



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Investigation of *Melissa officinalis* L. Essential Oil for Antifungal Activity against *Cladosporium carrionii*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors CPM, FQSG, LSP, FOP and EOL designed in this study, performed the statistical analysis, wrote the protocol and managed the analysis of the study. Authors VNT, VGS and FSS performed the chemical analysis of the essential oil. Author CPM managed the literature searches and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Cladosporium carrionii is considered the most important pathogenic species of genus because of the numerous cases of disease which causes in the world. Due to its antifungal resistance, these fungal infections are difficult to treat. Given the broad biological activity displayed by natural products, essential oils obtained from plants are often investigated to determine their antimicrobial activity.
Aims: Therefore, we identified components of *Melissa officinalis* L. essential oil, investigating

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FIBROSE CÍSTICA: ASPECTOS GENÉTICOS, CLÍNICOS E DIAGNÓSTICOS

CYSTIC FIBROSIS: GENETICS, CLINICAL AND DIAGNOSTIC ASPECTS

INGRID LACERDA PESSOA[1*], FELIPE QUEIROGA SARMENTO GUERRA[2], CAMILLA PINHEIRO DE MENEZES[3], GREGÓRIO FERNANDES GONÇALVES[3]

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RESUMO

A fibrose cística (FC) é uma doença genética autossômica recessiva, caracterizada pela disfunção de uma proteína denominada *cystic fibrosis transmembrane conductance regulator* (CFTR). A síndrome clínica é multissistêmica e decorre do transporte deficitoso de cloreto. Em geral, apresenta-se por uma ampla gama de manifestações, dentre elas, doença pulmonar progressiva, disfunções gastrointestinais, doença hepática, infertilidade masculina, desnutrição e elevadas concentrações de eletrólitos no suor. Embora seja considerada uma doença da infância, o número de pacientes adultos com FC vem aumentando consideravelmente, graças aos avanços dos conhecimentos genéticos, das técnicas diagnósticas, acompanhamento e tratamento desta doença. Nesta revisão da literatura científica, abordam-se atualizações a respeito dos aspectos moleculares e genéticos da CFTR, bem como da fisiopatologia, clínica e diagnóstico da FC.

PALAVRAS-CHAVE: Fibrose cística, mucoviscidose, CFTR, canalopatias.

ABSTRACT

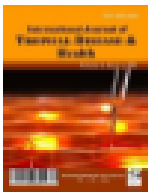
Cystic fibrosis (CF) is an autosomal recessive genetic disease characterized by dysfunction of a protein called cystic fibrosis transmembrane conductance regulator (CFTR). The clinical syndrome is multisystem and results from defective transport of chloride. In general, it features a wide range of manifestations, among them, progressive lung disease, gastrointestinal disorders, liver disease, male infertility, malnutrition, and high concentrations of electrolytes in sweat. Although it is considered a childhood disease, the number of adult patients with CF has increased considerably, due to the advances of genetic knowledge, diagnostic techniques, monitoring and treatment of this disease. In this review of the scientific literature, updates about molecular and genetic aspects of CFTR are approached, as well as the pathophysiology and diagnosis of CF.

KEYWORDS: Cystic fibrosis, CFTR, channelopathies.

1. INTRODUÇÃO

A fibrose cística (FC) ou mucoviscidose é uma doença genética cujo padrão de hereditariedade é autossômico recessivo. As manifestações clínicas resultam da disfunção de uma proteína denominada proteína reguladora da condutância transmembrana da fibrose cística ou *Cystic Fibrosis Transmembrane Conductance Regulator* (CFTR). Esta proteína atua como canal de cloreto, regulando o balanço entre íons e água através do epitélio e é encontrada na membrana apical de células do trato respiratório, de glândulas submucosas do pâncreas exócrino, do fígado, dos ductos sudoríparos, do trato reprodutivo, entre outros sítios. Assim, a expressão clínica da FC é heterogênea, caracterizada pelo transporte deficitoso de cloretos, especialmente nas células exócrinas dos pulmões, pâncreas e glândulas sudoríparas[1, 2, 3].

A incidência da fibrose cística difere de acordo com o grupo étnico, variando de um para cada 2000 ou 3500 caucasianos nascidos na Europa, nos Estados Unidos e Canadá, e com a menor incidência entre os hispânicos, afro-americanos e asiáticos, ocorrendo em 1 para 8.400, 1 para 15.300 e 1 para 32.000, respectivamente. Sendo que um indivíduo em cada 25, destas populações, é portador assintomático do gene[4].



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Antifungal Activity of Phytochemicals against Samples of *Penicillium*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors TBD and EOL did the study design, wrote the protocol and analyses of study. Authors TBD, LSP, CPM, FQSG and JPS did the processing samples while the literature searches were done by authors TBD and SBF. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: The incidence of fungal infections has increased over the last ten years and fungi of the genus *Penicillium* can be found in various substrates and affect immunocompromised people, hospitalized patients, many animals and plants, as well as compromise the quality of air indoors. The current situation of indiscriminate use of antibiotics and the consequent resistance of microorganisms to conventional antimicrobial therapy has been stimulating researchers to seek alternative sources of antimicrobial compounds, among them the medicinal plants. The tendency of getting phytochemicals from extracts, fractions, fixed or essential oils obtained from plant species is currently observed. In this context, the present study aims to evaluate the in vitro antifungal activity of seven phytochemicals (geraniol, carvacrol, thymol, linalool, p-cymene, terpinolene and citral) against twelve samples of *Penicillium*.

Place of Study: Laboratory tests were carried out at the Mycology Laboratory Department of Pharmaceutical Sciences, located in the Health Sciences Center (CCS) of the Federal

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Research Article

Antibacterial Activity of the Monoterpene Linalool: Alone and in Association with Antibiotics Against Bacteria of Clinical Importance

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ABSTRACT
Antibacterial activity studies of new molecules, either alone or in combination with existing antibiotics, are of great importance considering the resistance acquired by microorganisms in recent times. Linalool is a phyto-constituent found in the essential oils of various plant species. It is a monoterpene widely used in perfumery, cosmetics, and the food industries. Our objective was to determine the pharmacological effects produced on the bacterial strains *Staphylococcus aureus*, and *Pseudomonas aeruginosa* when combining standard antibiotics with linalool. The Minimum Inhibitory Concentration (MIC) was calculated using microdilution technique, where the linalool concentrations varied from 2 to 1024 µg /mL. Combinations with standard antibiotics were analyzed by the checkerboard method where the fractional inhibitory concentration (FIC) indices were calculated. Linalool, Imipenem, and Ciprofloxacin showed respective MIC antibacterial activities against *S. aureus* of 1024, 4, and 2 µg/mL. In *S. aureus*, the linalool with Imipenem association showed a synergistic effect (FIC = 0.0625); while with ciprofloxacin, the linalool showed additivity (FIC = 0.75). In *P. aeruginosa*, the Imipenem/linalool association was synergistic for both the ATCC and clinical strains (FIC = 0.0625). The association of *linalool* with ciprofloxacin was indifferent. We conclude that Linalool associated with existing standard antibiotics may increase antibacterial effectiveness, resulting in synergistic activity against bacterial strains of clinical importance. This makes the molecule potentially important for production of new, therapeutically effective drugs against resistant microorganisms.

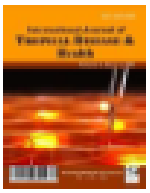
Key words: natural products, antibacterial activity, synergism, linalool.

INTRODUCTION

In the 21st century, given the growing number of multiresistant bacterial strains, and resistance exchanges between different species, bacterial resistance has become a critical challenge, (Ex.: *Neisseria gonorrhoeae, Pneumocystis pneumoniae, Pseudomonas aeruginosa and Staphylococcus aureus*)[1,2]. As a global public health problem, the theme was proposed by the World Health Organization (WHO) in 2011, and emphasized on World Health Day as a controlling target among global strategies to ensure safe healthcare. Attention was also drawn to the challenges of implementing immediate actions to control the spread of resistant microorganisms in order to minimize the progressive deterioration of therapies handling such cases.

Among the pathogens considered important in relation to bacterial resistance, one might highlight *S. aureus* and *P. aeruginosa*. Infections caused by these pathogens are a clinical challenge, due to adaptations under the selective pressures of intense antimicrobial use; *S. aureus* has achieved a great ability to develop resistance[3], and *P. aeruginosa* is already characterized by limited susceptibility to any number of antimicrobial agents[4].
Because of the great resistance that microorganisms, such as *S. aureus* and *P. aeruginosa*, have acquired to a wide range of antibiotics in recent years, the search for new compounds has been the subject of intensive research. The fight against this emerging problem of pathogenic organism resistance has in the present day employed two divergent approaches: the development of completely new antibiotics, and/or combinations of substances

*Author for Correspondence



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Antifungal Activity of Citral by Disruption of Ergosterol Biosynthesis in Fluconazole Resistant *Candida tropicalis*

Janiere Pereira de Sousa[1*], Aratã Oliveira Cortez Costa[1], Maria Clerya Alvino Leite[1], Felipe Queiroga Sarmento Guerra[1], Viviane Araújo da Silva[1], Camilla Pinheiro de Menezes[1], Fillipe Oliveira Pereira[2] and Edeltrudes Oliveira Lima[1]

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Authors' contributions

This work was carried out in collaboration between all authors. Authors JPS, AOCC, MCAL, FQSG, VAS, CPM, FOP and EOL are responsible for drafting the paper and listed below are the individual contributions of each author to the paper. Author JPS participated in the project design, collection and analysis of data, drafting the paper, and critical revision of the intellectual content. Authors AOCC, MCAL, FQSG, VAS, CPM, and FOP participated in data collection, data analysis, and revision of the paper. Author EOL guided all stages of the work and participated in both review and drafting of the project and the paper, including final approval of the version to be published. All authors read and approved the final manuscript.

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ABSTRACT

A limited number of antifungals and the emergence of resistant strains have hindered the treatment of candidiasis, making the search for new antifungals urgent. Citral is a monoterpene with known pharmacological properties, including antimicrobial action.

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Anexo 4: Premiações

9th International Congress of Pharmaceutical Sciences

CIFARP 2013

Ribeirão Preto, Brazil, November 20-23, 2013

THE BRAZILIAN YEAR OF SCIENCE INTEGRATING PHARMACEUTICAL AREAS

We certify that "RODRIGO SANTOS AQUINO DE ARAÚJO", received the CIFARP Travel Award to present the work "SYNTHESIS, SAR, DFT STUDIES AND EVALUATION OF ANTIFUNGAL ACTIVITY OF COUMARINS DERIVATIVES" at the 9th International Congress of Pharmaceutical Sciences – CIFARP, held on November 20-23, 2013, in Ribeirão Preto, SP, Brazil.

Ribeirão Preto, Brazil, November 23th, 2013

Prof. Dr. Sérgio de Albuquerque
Dean of FCFRP-USP

PROMOTION

USP

ABCF

Br. Leonardo Régis Leira Pereira
Organizing Committee

ESTADO DA PARAÍBA
CÂMARA MUNICIPAL DE CAMPINA GRANDE
(Casa de Félix Araújo)

Ofício nº 5.308 – DRE - AGS. Em 12 de dezembro de 2013.

Prezado Senhor,

Dirigimo-nos a V. Sa., a fim de comunicar-lhe que esta Câmara, atendendo ao Requerimento nº 2.826/2013, de autoria do Vereador **BRUNO CUNHA LIMA**, subscrito pelos Edis Lula Cabral e Alves Pimentel Filho, aprovado por unanimidade, fez constar na Ata de nossos trabalhos legislativos, um **VOTO DE APLAUSOS** ao Professor Rodrigo Araújo(UEPB-Campus v) pela conquista do Prêmio Internacional Travel Awad , concedido pelo 9º Congresso de Ciências Farmacêuticas neste mês de novembro.

O trabalho premiado é um estudo sobre a potencialidade de moléculas antifúngicas , voltado à analise especifica do grupo metabólico das cumarinas, que possuem propriedades farmacológicas associados com antimicrobianos, antitumorais e anticoagulantes. De acordo com Rodrigo de Araújo" o aumento da incidência de infecções fúngicas oportunistas, associadas a maior resistência às drogas antifúngicas atualmente em uso, tem destacado a necessidade de novas soluções nos estudos farmacológicos e bioquímicos. Esta pesquisa foi desenvolvida com essa finalidade.

Cordialmente,

NELSON GOMES FILHO
Presidente

ANTONIO ALVES PIMENTEL FILHO
1º Secretário

Ao Ilmo. Sr..
Professor RODRIGO DE ARAÚJO
Universidade Estadual da Paraíba - UEPB
Avenida das Baraúnas, 351 - Bodocongó.
Campus v da UEPB