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**Wylly Araújo de Oliveira**

# Atividade do óleo essencial de *Cymbopogon winterianus* Jowitt ex Bor contra *Candida albicans*, *Aspergillus flavus* e *Aspergillus fumigatus*

**João Pessoa-PB**

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# Atividade do óleo essencial de *Cymbopogon winterianus* Jowitt ex Bor contra *Candida albicans*, *Aspergillus flavus* e *Aspergillus fumigatus*

Tese de doutorado apresentada ao Programa de Pós-Graduação em Produtos Naturais e Sintéticos Bioativos, Centro de Ciências da Saúde, Universidade Federal da Paraíba, em cumprimento aos requisitos necessários para a obtenção do título de Doutor em Produtos Naturais e Sintéticos Bioativos, área de concentração: farmacologia

**Orientadora: Prof.ª Dr.ª Edeltrudes de Oliveira Lima**

**João Pessoa-PB**

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Tese de Doutorado aprovada em 22/06/2011

**Banca examinadora**

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## RESUMO

Oliveira, W. O. Atividade do óleo essencial de *Cymbopogon winterianus* Jowitt ex Bor contra *Candida albicans*, *Aspergillus flavus* e *Aspergillus fumigatus.* 2011. Tese (Doutorado em Produtos Naturais e Sintéticos Bioativos, área de concentração: Farmacologia) - UFPB/CCS, João Pessoa.

Devido ao aumento na incidência de infecções fúngicas por *Candida albicans* e *Aspergillus* spp. em pacientes imunocomprometidos, e ao surgimento de cepas resistentes aos antifúngicos convencionais, é crescente a necessidade de novos antifúngicos. Óleos essenciais têm sido testados contra fungos, e possuem potencial como agentes antifúngicos. Este trabalho investigou a atividade do óleo essencial de *Cymbopogon winterianus* contra *Candida albicans*, *Aspergillus flavus* e *A. fumigatus*, através da Concentração Inibitória Mímima (CIM), da Concentração Fungicida Mínima (CFM), do tempo de morte e de mudanças morfológicas em *C. albicans*; da inibição do crescimento radial fúngico e da análise da germinação dos conídios de *Aspergillus flavus* e *A. fumigatus* quando expostos a várias concentrações do óleo e através da lise celular e da ligação do óleo essencial com o ergosterol. Os resultados mostraram que o óleo essencial tem atividade antifúngica dependente da concentração, altera a morfologia de *C. albicans,* inibe a germinação de conídios e o crescimento micelial radial de *Aspergillus flavus* e *Aspergillus fumigatus*, causa lise celular de todas as cepas dos fungos testados e se liga ao ergosterol. Estes resultados fazem do óleo essencial de *Cymbopogon winterianus* um potencial agente no controle do crescimento de fungos que podem causar infecções, como aquelas que acontecem no ambiente hospitalar.



Palavras-chave: *Cymbopogon winterianus*, *Candida albicans*, *Aspergillus flavus*, *Aspergillus fumigatus*, antifúngico.

## ABSTRACT

Oliveira, W. A. Activity of essential oil from *Cymbopogon winterianus* Jowitt ex Bor against *Candida albicans*, *Aspergillus flavus* and *Aspergillus fumigatus.* 2011. Thesis (PhD in Bioactive Synthetic and Natural Products, concentration area: Pharmacology) - UFPB/ CCS, João Pessoa.

Due increase of incidence of fungal infections for *Candida albicans* and *Aspergillus* spp. in immunocompromised patients, and emerging resistant strains to conventional antifungals, it is accentuated the need for new antifungal. Essential oils have been tested for antimycotic activity and possess potential as antifungal agents. This work investigated the activity of essential oil of *Cymbopogon winterianus* against *Candida albicans*, *Aspergillus flavus* and *A. fumigatus*, by MIC and MFC; time-kill methods and morphological changes in the *C. albicans,* inhibition of the fungal mycelial growth and analysis of conidia germination of *Aspergillus flavus* and *A. fumigatus* when exposed to several concentrations of oil and by cellular leakage and assessed the bind of essential oil with ergosterol. The results shown that essential oil had concentration-dependent antifungal activity, alters the morphology in *C. albicans*, inhibits conidia germination and the radial mycelia growth of *Aspergillus flavus* and *Aspergillus fumigatus*, causes cellular leakage of all strains of fungal species tested and binds to ergosterol. These results make essential oil of *Cymbopogon winterianus* a potential agent in controlling fungi growth that cause infections, like hospital-acquired infections.



Key words: *Cymbopogon* winterianus, *Candida albicans*, *Aspergillus flavus*, *Aspergillus fumigatus*, antifungal.

## LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

ASD- Ágar Sabouraud Dextrose

ATCC- American Type Culture Collection

CIM- Concentração Inibitória Mínima

CFM- Concentração Fungicida Mínima

MIC- Minimum Inhibitory Concentration

MFC- Minimum Fungicidal Concentration

UFC- Unidade Formadora de Colônia

CTT- Cloreto de Trifenil Tetrazólio

EO- Essential Oil

$HClO_4$- Ácido Perclórico

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**Antifungal activity of *Cymbopogon winterianus* Jowitt ex Bor against *Candida albicans***



**Investigation of mechanism of action from *C. winterianus* against *Candida albicans***

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# 1- Introdução

## 1.1- Fungos como patógenos primários e oportunistas

Os fungos de interesse médico são de dois tipos morfológicos: leveduras, que são unicelulares e fungos filamentosos que são multicelulares. As leveduras, de maneira geral, são unicelulares, esferoidais ou ovais e podem se reproduzir assexuada ou sexuadamente. Os fungos filamentosos são multicelulares, com células tubulares denominadas hifas, cujo conjunto é denominado micélio (ZAITZ et al., 2010).

Os fungos que são patógenos podem ser divididos em duas classes, patógenos primários e patógenos oportunistas. Os fungos formam uma classe de microrganismos que possuem reservatório ambiental e infecta indivíduos que são expostos a grandes quantidades do microrganismo ou que estão imunologicamente deficientes. Patógenos primários são aqueles fungos que causam doenças em indivíduos imunocompetentes. Patógenos oportunistas causam infecções em pacientes que possuem o sistema imunológico comprometido, local ou sistemicamente. A patogênese das infecções oportunistas envolve a produção de fatores de virulência que permite ao organismo do indivíduo ser comensal durante o período em que o sistema imunológico está normal. Quando o sistema imune torna-se comprometido, o microrganismo multiplica-se mais que o normal. Este crescimento desregulado pode levar a infecções invasivas. Alguns fungos são classicamente oportunistas, como *Cryptococcus neoformans*, porém às vezes podem causar doenças em indivíduos saudáveis; e por vezes fungos que são classicamente patógenos primários, como *Coccidioides immitis*, são muito mais virulentos em

pacientes imunocomprometidos. Exemplos de patógenos primários incluem *Coccidioides immitis* e *Histoplasma capsulatum*, e entre os patógenos oportunistas estão várias espécies de *Candida*, *C. neoformans* e espécies de *Aspergillus*. Os patógenos oportunistas humanos têm ganhado importância nos últimos 20 anos, paradoxalmente por causa do sucesso da prática médica moderna, que aumenta o tempo de vida de pacientes debilitados e imunocomprometidos (BURIK; MAGEE, 2001).

As infecções fúngicas invasivas causam aumento da mortalidade, principalmente em pacientes imunosuprimidos. Os principais fatores de risco incluem neutropenia prolongada induzida por quimioterápicos citotóxicos, terapia com altas doses de costicosteróides, prematuridade extrema, cateteres, terapia prolongada com antibióticos, transplante de órgãos sólidos ou de células tronco hematopoiéticas, queimaduras, disfunção dos macrófagos e imunodeficiências congênitas ou adquiridas (HA et al., 2011; BARRET, 2002; RODRIGUEZ; PATRICK, 2001).

Pacientes imunocomprometidos são altamente susceptíveis à infecções hospitalares causados por microrganismos, como os fungos. Infecções fúngicas nestes pacientes são frequentemente severas, de progressão rápida, e ainda podem possuir o diagnóstico e o tratamento difíceis (FRIDKIN; JARVIS, 1996). A incidência de infecções hospitalares têm aumentado ao longo das décadas, com espécies fúngicas representando mais de 25% das infecções sanguíneas adquiridas em hospitais (RICHARDSON, 2005). Dentre as infecções fúngicas hospitalares, *C. albicans* e *Aspergillus* spp. possuem maior incidência entre as leveduras e os fungos filamentosos, respectivamente (FRIDKIN; JARVIS, 1996). O gênero *Candida* é responsável por cerca de 80%

das infecções fúngicas (COLOMBO; GUIMARÃES, 2003). A taxa de mortalidade por infecção oportunista por espécies de *Candida* é de aproximadamente 34% enquanto a de *Aspergillus* é de 23% (PFALLER; DIEKEMA, 2007).

## 1.2-*Candida albicans*

Entre as características micromorfológicas de *C. albicans*, estão a presença de clamidoconídios globosos, terminais e de parede celular espessa, blastoconídios ovóides ou globosos, hifas e pseudo-hifas. Em presença de soro humano ou de clara de ovo, *Candida albicans* produz tubo germinativo (ZAITZ et al., 2010, MINAMI, 2003, LACAZ et al., 2002).

Dentre as centenas de espécies descritas, leveduras do gênero *Candida* são os maiores agentes de infecção hospitalar e representam um desafio para a sobrevida de pacientes com doenças graves e aqueles em período pós-operatório. Hospitais norte-americanos com sistema de vigilância operante, notificaram *Candida* como 6° patógeno nosocomial e a 4° causa mais comum de infecções de corrente sanguínea, adquiridas em hospitais (ANVISA, 2004). Em hospitais de São Paulo e do Rio de Janeiro, *Candida albicans* (37%), *C. parapsilosis* (25%) e *C. tropicalis* (24%) foram as espécies mais frequentemente isoladas (COLOMBO; GUIMARÃES, 2003)

Dentre os principais fatores de virulência de *Candida albicans* estão as adesinas, produção de tubos germinativos, já que hifas têm maior capacidade de aderir e penetrar nas células epiteliais humanas que os blastoconídios e a produção de exoenzimas, como proteinases e fosfolipases (ÁLVARES et al., 2007).

Algumas das principais manifestações clínicas da candidíase são a candidíase cutâneo-mucosa (que acomete a pele, unhas e mucosas), a candidíase sistêmica ou visceral (que se dissemina a vários órgãos por via hematogênica) e a candidíase alérgica (lesões cutâneas estéreis, principalmente nos espaços interdigitais das mãos e em outras partes do corpo) (SIDRIM; ROCHA, 2004).

### 1.3- *Aspergillus* spp

Espécies do gênero *Aspergillus* 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 (ou esterigmas) com aspecto de garrafas e disposição radiada no seu conjunto. Na extremidade de cada uma forma-se uma cadeia de esporos basipeta (conídio ou fialósporo), globosos, secos e de cor variável (MINAMI, 2003).

Os principais quadros clínicos observados devido a 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; SIDRIM; ROCHA, 2004).

A incidênicia 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 (TEKAIA; LATGÉ, 2005).

Os principais fatores de virulência do *Aspergillus* são adesinas, micotoxinas, enzimas (como por exemplo, proteases) e a versatilidade morfológica (BURIK; MAGEE, 2001; LATGÉ, 1999). As principais micotoxinas sintetizadas por *Aspergillus* são aflatoxinas (B1, B2, G1, G2) que podem levar a câncer hepático, cirrose hepática e a hemorragias do trato gastrintestinal (LACAZ et al., 2002).

**1.4- Antifúngicos**

Vários antifúngicos com mecanismos de ação diferentes são utilizados no tratamento das micoses.

A griseofulvina penetra na célula, e no núcleo interage 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).

Os derivados azólicos (miconazol, cetoconazol, fluconazol, itraconazol) inibem a síntese do ergosterol, interferindo na enzima 14-α-demetilase, o que causa dano a função da membrana celular. Alguns triazólicos mais novos como voriconazol e ravuconazol também possuem esta mesma atividade. Outras classes de drogas também atuam inibindo a síntese do ergosterol, são elas as

alilaminas (naftifina e terbinafina) que atuam inibindo a esqualeno epoxidase e as morfolinas (amorolfina), que inibem 2 etapas da via bioquímica da síntese do ergosterol bloqueando a $\Delta^{14}$-redutase e a $\Delta^{8}$-$\Delta^{7}$ isomerase (ODDS et al., 2003).

As equinocandinas inibem a síntese de 1,3-β-glicano, um polímero de glicose que é necessário para manter a estrutura das paredes celulares fúngicas. Na ausência deste polímero, as células fúngicas perdem a integridade e a lise rapidamente se segue. A caspofungina é um membro deste grupo, sendo efetiva no tratamento da candidíase e de formas de aspergilose invasiva que são refratárias à anfotericina B (RANG et al., 2007).

Além do aumento da freqüência, infecções fúngicas invasivas estão ainda associadas com altas taxas de mortalidade, que é acima de 40% para infecções na corrente sanguínea causadas por *C. albicans* e mais de 50% em aspergilose invasiva. Dados epidemiológicos têm confirmado o aumento na importância de infecções causadas por espécies de fungos resistentes, particularmente espécies de *Candida* resistentes ao fluconazol (CANUTO; RODERO, 2002). Devido ao aumento da resistência aos antifúngicos, é urgente a necessidade de novos antifúngicos com faixa de ação fungicida ampla e poucos efeitos colaterais que limitem a dose (BARRET, 2002).

Os principais mecanismos de resistência aos derivados azólicos são a diminuição da permeabilidade da membrana aos antifúngicos, mutações ou o aumento da expressão da lanosterol 14α-demetilase e a diminuição do acúmulo intracelular das drogas devido a sistemas de efluxo. Modificações

qualitativas e quantitativas no ergosterol da membrana é a principal causa de resistência aos poliênicos (CANUTO; RODERO, 2002).

### 1.5- Plantas como fontes de antimicrobianos

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 colaterais e terapia ineficaz) ou por uma grande parte da população não ter acesso ao tratamento farmacológico convencional. Contudo, o uso potencial de plantas como fonte de drogas é ainda pouco explorado. Apenas uma pequena proporção das espécies de plantas estimadas tem sido investigadas fitoquimicamente, e uma fração menor ainda têm suas propriedades farmacológicas estudadas. No Brasil as plantas medicinais são amplamente usadas nas zonas rurais e urbanas. A maioria é usada de acordo com a tradição popular desenvolvidas pelos nativos ou trazida para o país pelos europeus, africanos e asiáticos (RATES, 2001). Dentre os compostos com atividade antimicrobiana extraídos de plantas estão os compostos fenólicos (como flavonóides e taninos), os alcalóides, os terpenos e os óleos essenciais (COWAN, 1999).

### 1.6- Óleos essenciais

Óleos essenciais são produtos obtidos de partes de plantas através de destilação a vapor. De forma geral são misturas complexas de substâncias voláteis, lipofílicas, geralmente odoríferas e líquidas. Outra característica importante é o aroma agradável e intenso da maioria dos óleos essenciais, sendo, por isso, também chamados de essências. Seus constituintes variam

desde hidrocarbonetos terpênicos, álcoois simples e terpênicos, aldeídos, cetonas, fenóis, ésteres, éteres óxidos, peróxidos, furanos, ácidos orgânicos, lactonas, cumarinas, até compostos com enxofre. Quimicamente, a grande maioria dos óleos é formada de derivados fenilpropanóides ou de terpenóides, sendo que estes últimos preponderam (SIMÕES; SPITZER, 2004).

Os óleos essenciais são populares como ingredientes de perfumes, cosméticos, produtos de limpeza doméstica assim como aromatizantes de comidas e bebidas. Eles também são úteis no tratamento de várias doenças e sua aplicação medicinal tem se tornado popular, sendo que isto é verdadeiro para muitos de seus constituintes. De maneira geral, os óleos são eficazes contra cânceres, contra a dor, possuem atividade antiinflamatória, são repelentes de insetos, têm atividade antiviral e antioxidante (ADORJAN; BUCHBAUER, 2010). Óleos essenciais podem diminuir a oxidação de LDL, o que inibe um dos fatores de risco para a aterosclerose; eles inibem ainda a agregação plaquetária, o que previne a trombose. Outras propriedades que os óleos essenciais possuem são a antifúngica (BAKKALI et al., 2008) e a antibacteriana (BURT, 2004).

A composição dos óleos essenciais de uma planta é determinada geneticamente, sendo geralmente específica para um determinado órgão e característica para o seu estágio de desenvolvimento, mas as condições ambientais são capazes de causar variações significativas. Alguns aspectos que determinam a variabilidade são o ciclo vegetativo, pois os constituintes podem variar durante o desenvolvimento do vegetal; fatores extrínsecos como a temperatura, a umidade relativa, a duração total de exposição ao sol, o regime de ventos, a disponibilidade hídrica e os nutrientes; e o processo de

obtenção, pois este pode provocar hidrólise de ésteres, isomerizações, racemizações e oxidações (GOBBO-NETO; LOPES, 2007, SIMÕES; SPITZER, 2004).

Algumas formas de extração dos óleos essenciais são por efloração, onde pétalas são depositadas sobre uma camada gordurosa, em seguida a gordura é tratada com álcool, depois este é destilado em baixas temperaturas; por arraste de vapor de água, onde os óleos que possuem tensão de vapor mais elevada que a da água são arrastados pelo vapor e em seguida separados da água; outros métodos são por extração com solventes orgânicos e por prensagem (BIZZO, 2009; SIMÕES; SPITZER, 2004).

### 1.7- *Cymbopogon winterianus*

A família Poaceae possui mais de 600 gêneros e 9.000 espécies, onde fazem parte plantas com raízes fibrosas e pouca ocorrência de arbustos ou árvores (EVANS, 1996).

O gênero *Cymbopogon* (Poaceae) é composto por mais de 100 espécies, encontradas em países tropicais. Cerca de 56 espécies são aromáticas e algumas delas têm importância medicinal, farmacológica e industrial. *Cymbopogon winterianus*, popularmente conhecida como citronela, é uma importante fonte de óleos essenciais cultivada na Índia e no Brasil. Os óleos voláteis extraídos das folhas são usados na perfumaria, cosméticos, nas indústrias farmacêutica e de aromas (QUITANS-JÚNIOR et al., 2008). O óleo essencial de *C. winterianus* é rico em citronelal, citronelol e geraniol (PEREIRA, 2009; BLANK et al., 2007).

Figura 1. *C. winterianus* no horto do Centro de Formação de Tecnólogos, Campus III da UFPB (Foto: Pereira, F. O., 2010).

Os praticantes da medicina popular no nordeste brasileiro utilizam a infusão das folhas frescas para o tratamento de epilepsia e ansiedade. Estudos mostraram que esta planta possui atividade depressora no sistema nervoso central (QUITANS-JÚNIOR et al., 2008). O óleo essencial da planta é ainda utilizado como repelente e possui atividade antifúngica (PEREIRA et al., 2011, DUARTE et al., 2005) e antibacteriana (DUARTE et al., 2007).

Foi demonstrado que esta planta possui menor rendimento de óleos voláteis durante o inverno e que a colheita pela manhã fornece maior rendimento. A composição química do óleo é afetada pela estação do ano (BLANK et al., 2007; CASSEL; VARGAS, 2006,). O óleo essencial da citronela está entre os 18 óleos essenciais mais importantes do mundo sob o ponto de vista comercial (BIZZO, 2009).

## 2- Objetivos

### 2.1- Objetivo geral

- Investigar o efeito do óleo essencial de *Cymbopogon winterianus* como agente antifúngico.

### 2.2- Objetivo específico

- Avaliar a atividade antifúngica e estudar como o óleo essencial de *C. winterianus* atua contra as cepas de *Candida albicans*, *Aspergillus flavus* e *A. fumigatus*.

## 3- Materiais e métodos

### 3.1- Local do trabalho

Este trabalho foi realizado no Laboratório de Micologia do Departamento de Ciências Farmacêuticas, do Centro de Ciências da Saúde da Universidade Federal da Paraíba e no Laboratório de Tecnologia Farmacêutica da Universidade Federal da Paraíba.

### 3.2- Material botânico

A planta foi identificada pela Dra. Rita Baltazar de Lima, no Laboratório de Botânica, do Departamento de Sistemática e Ecologia do Centro de Ciências Exatas e da Natureza, da Universidade Federal da Paraíba. A exsicata foi depositada no Herbário Prof. Lauro Pires Xavier, do Departamento de Sistemática e Ecologia, na Universidade Federal da Paraíba com o código JPB 41387.

*Cymbopogon winterianus* Jowitt ex Bor foi cultivada, coletada (em fevereiro de 2007) e teve o seu óleo essencial extraído por destilação a vapor, utilizando aparelho tipo Clevenger, no Centro de Tecnologia da Universidade Federal da Paraíba, na cidade de Bananeiras (Paraíba, Brasil) pelo Dr. Paulo Alves Wanderley.

### 3.3- Óleo essencial

O óleo essencial foi analisado no Laboratório de Química Fundamental da Universidade Federal de Pernambuco (UFPE), por cromatografia gasosa acoplada a espectrometria de massa (CG-EM), utilizando o instrumento QP-5050A equipado com GC-17A (Shimadzu, Japão). A diluição da amostra foi

1:1000 em hexano (v/v), o volume de injeção da amostra foi 1µL, o gás de arraste foi o Hélio com vazão de 0,9mL/min, a pressão na cabeça da coluna foi de 48,9psi e a temperatura do detector foi de 280°C. A coluna utilizada era apolar de sílica fundida de 30m de comprimento, 0,25mm de diâmetro e 0,25µm de diâmetro do filme da fase estacionária líquida. A temperatura inicial foi de 60°C, com aumento gradual até 240°C a uma taxa de 3°C por minuto. A temperatura permaneceu em 240°C por 10 minutos. Com relação às condições de uso do espectrômetro para a detecção e identificação, a temperatura das linhas de transferência era de 170°C, a voltagem de ionização 70eV, a faixa de scanning (scan time) 0,5s e a demora no início de atuação do espectrômetro (delay) foi 1,5 min. Os componentes do óleo essencial foram identificados por comparação de seus padrões de fragmentação registrados nos espectros de massa com aqueles presentes na biblioteca de espectrômetros de massas NIST 98 (National Institute of Standards and Technology, EUA) que está instalada no computador, e ainda com relatos na literatura. A quantificação dos componentes foi realizada com base na percentagem da área do pico de cada componente em relação a área total de todos os picos do cromatograma.

Na preparação da emulsão do óleo essencial, foi adicionado a um tubo de ensaio estéril a quantidade de óleo essencial necessária para obter a concentração desejada, 20µL de Tween 80 e o volume foi completado para 3mL com água destilada estéril. A mistura foi agitada por 5 minutos (ALLEGRINI et al., 1973).

Os antifúngicos anfotericina B e cetoconazol foram utilizados como controles nas concentrações indicadas no trabalho.

### 3.4- Cepas fúngicas

As cepas testadas pertencem à coleção do Laboratório de Micologia da Universidade Federal da Paraíba e incluem: *Candida albicans* (ATCC 76485, ATCC 76615, ATCC 13803, LMV 42, ICB 12, M101, LM 968, LM 68, LM 16, LM 018, LM 023, LM 601, LM 290, LM 052, LM 087), *Aspergillus flavus* (ATCC 16013,LM 247, LM 02, LM 704, LM 257, LM 907 e LM 13) e *Aspergillus fumigatus* (ATCC 46913, ATCC 40640, IPP 21).

### 3.5- Determinação da concentração inibitória mínina (CIM) e da concentração fungicida mínima (CFM) do óleo essencial contra *C. albicans*

A CIM foi determinada através do método da microdiluição (CLEELAND; SQUIRES, 1991; ELOFF, 1998). Culturas de *Candida albicans* foram semeadas em placas com ágar Sabouraud Dextrose (ASD) e incubadas durante 24-72 horas, a temperatura de 37ºC. Colônias desta cultura foram suspensas em de NaCl 0,85% estéril e o inóculo foi padronizado de acordo com a escala 0,5 de McFarland (1-5 x $10^6$ UFC/mL). Em uma placa com 96 cavidades, foi adicionado caldo Sabouraud e óleo essencial de *C. winterianus* nas concentrações de 10.000 a 39µg/mL, em diluições 1:2. A determinação da CIM foi conduzida com aproximadamente 1-5 x$10^5$ UFC/mL do microrganismo em cada cavidade. As placas foram incubadas a 37°C por 24-48 horas. Em 24-48 horas houve a observação do crescimento fúngico. Foi acrescentado 20 µL de CTT (cloreto de trifenil tetrazólio) a 0,5% em cada uma das 96 cavidades para determinação da CIM, sendo a placa incubada por mais 24 horas (COSTA et al., 2008). A CIM foi considerada como a menor concentração do óleo que

inibiu o crescimento do microrganismo, como também indicado pelo CTT através da leitura das placas de 96 cavidades. Foram realizados três experimentos independentes em ocasiões diferentes com resultados consistentes. Foram utilizados anfotericina B e cetoconazol como controles.

O cloreto de trifenil tetrazólio é incolor na forma oxidada e vermelho quando reduzido. Microrganismos vivos reduzem o CTT por ação enzimática, originando trifenil formazan, o qual é mantido dentro de grânulos nas células, as quais se tornam vermelhas (RAMOS et al., 2006).

Para determinar a CFM, 10µL de cada uma das cavidades onde não houve crescimento fúngico foi semeado em uma placa contendo ASD, estas placas foram incubadas à 37°C por 24-48 horas. A CFM foi considerada a menor concentração semeada em placa com ASD em que houve crescimento menor que 3 UFCs (Unidades Formadoras de Colônia)(ZAROR; ESPINEL-INGROFF, 1989; KLEPSER et al., 1998; ERNST et al., 1999). Óleos essenciais com CIM entre 50 e 500µg/mL são considerados com forte atividade antimicrobiana, com CIM entre 600 e 1500µg/mL possuem atividade moderada e CIM acima de 1500µg/mL é considerado com atividade fraca (SARTORATTO et al., 2004).

### 3.6- Determinação da concentração inibitória minima (CIM) e concentração fungicida mínima (CFM) do óleo essencial contra *Aspergillus flavus e A. fumigatus*

Para preparar o inóculo usado no experimento, os fungos filamentosos foram cultivados em ASD inclinado por 7 dias a 25-28°C. Depois do período de incubação, os conídios foram suspensos pela adição de NaCl 0,85% estéril ao

meio com *Aspergillus* spp, em seguida o meio de cultura com o microrganismo mais a solução salina foram suavemente agitados por 30 segundos. Os conídios foram contados utilizando-se hemocitômetro. A suspensão de conídios foi ajustada utilizando salina estéril para conter 5 x $10^6$ conídios/mL.

A CIM foi determinada pelo método da microdiluição utilizando microplacas com 96 cavidades (CLEELAND; SQUIRES, 1991; ELOFF, 1998). Em uma placa de 96 cavidades foi adicionado caldo Sabouraud e o óleo essencial de *C. winterianus* entre 10.000 e 39µg/mL, em diluições 1:2. A CIM foi realizada com um inoculo de aproximadamente 2,5 x$10^5$ conídios /mL do microrganismo em cada cavidade. As placas foram incubadas a 25 - 28℃ por 72 horas. Após 72h (e confirmada em 7 dias) houve observação visual do crescimento fúngico. A CIM foi considerada como a menor concentração do óleo essencial que inibiu o crescimento do microrganismo. Para determinar a CFM, uma alíquota de 10µL de cada cavidade onde não houve crescimento fúngico foi subcultivada em uma placa com ASD, que foi incubada a 25 - 28 ℃ por 72 horas. A CFM foi considerada como a menor concentração semeada em ASD em que houve crescimento menor que 3 UFC. Foram realizados três experimentos independentes em diferentes ocasiões com resultados consistentes (PEREIRA et al., 2011, ESPINEL-INGROFF et al., 2002). Foram utilizados anfotericina B e cetoconazol como controles. Os seguintes critérios foram utilizados, CIM entre 50 e 500µg/mL representa forte atividade antimicrobiana, CIM entre 600 e 1500µg/mL atividade moderada e CIM acima de 1500µg/mL é considerada com atividade fraca (SARTORATTO et al., 2004).

**3.7- Tempo de morte**

O tempo de morte de *C. albicans* frente ao óleo essencial foi realizado de acordo com trabalho de Klepser (1997) e colaboradores, com algumas modificações. Antes do teste, o microrgamismo foi cultivado em meio ASD. Colônias oriundas da cultura foram suspensas em NaCl 0,85% e a turvação foi ajustada de acordo com a escala 0,5 de McFarland (1-5 x $10^6$ UFC/mL). Um mililitro da suspensão fúngica foi adicionada a 9 mL de caldo Sabouraud com ou sem o óleo essencial em várias concentrações apropriadas. O inóculo inicial foi de aproximadamente 1-5 x $10^5$ UFC/mL. As concentrações do óleo essencial de *C. winterianus* testadas foram 1/2, 1, 2, 4 e 8 vezes a CIM. Estas culturas foram incubadas a 37°C e em vários períodos de tempo (0, 1, 2, 4, 6, 8, 12 e 24 horas) e uma alíquota de 100µL foi removida de cada solução e diluída dez vezes. Uma alíquota de 10µL de cada diluição foi removida e semeada em placas com ASD. As placas foram incubadas a 37°C por 24-48 horas e o número de unidades formadoras de colônias (UFC) foi contado. Quando era esperado menos de 1000 UFC/mL, a amostra de 10 µL foi semeada diretamente em ASD, sem diluição. Foram realizados dois experimentos independentes em diferentes ocasiões e os resultados representam a média dos dois experimentos. O limite mínimo de detecção deste método é 100 UFC/mL (KLEPSER et al., 1998).

O $\log_{10}$ UFC/mL foi plotado em um gráfico em função do tempo e usado para comparar a taxa e a extensão de atividade antifúngica em várias concentrações do óleo essencial (KLEPSER et al., 1997). Foi considerada atividade fungicida quando houve uma diminuição maior ou igual a 3 $\log_{10}$UFC/mL do inóculo inicial, o que resulta em uma redução de 99,9% ou

mais de UFC/mL em 24 horas, comparado com o inóculo inicial. Atividade com redução menor do que $3\log_{10}$UFC/mL comparado com o inóculo inicial foi considerada fungistática (ERNST et al., 1996; LEWIS et al., 2000, ROLING et al., 2002).

### 3.8- Crescimento micelial radial

A inibição do crescimento micelial fúngico (de *A. flavus* e *A. fumigatus*) foi determinada através de medida diária do crescimento radial do micélio em ASD adicionado com o óleo essencial de *C. winterianus* nas concentrações CIM/2, CIM e CIMx2. Para isso, um micélio de 2 mm de diâmetro foi tomado de uma cultura em meio ASD inclinado incubado a 25-28°C por 10 dias, e colocado no centro de uma placa de Petri com meio ASD contendo o óleo essencial. Em diferentes intervalos (0, 2, 4, 6, 8, 10 e 12 dias) após incubação a 25-28°C, o crescimento micelial radial foi medido em milímetros. Os controles incluídos neste experimento foram as medidas do crescimento radial em ASD sem a adição do óleo essencial, ou adicionado com anfotericina B (CIM) ou cetoconazol (CIM). Foram realizados três experimentos independentes em diferentes ocasiões e os resultados representam a média ± desvio padrão dos três experimentos (DAFERERA et al., 2003; ADAM et al., 1998).

### 3.9- Efeito do óleo essencial sobre a micromorfologia de *C. albicans*

Para observação das mudanças morfológicas em *C. albicans* ICB 12, foi usada a técnica do microcultivo para leveduras, utilizando agar-arroz em câmara úmida (SIDRIM; ROCHA, 2004; MINAMI, 2003; KURTZMAN; FEEL, 1998; GUNGI et al., 1983).

O óleo essencial foi adicionado ao meio de cultura agar-arroz, para se obter várias concentrações do óleo (CIM, CIMx2 e CIMx4). Então, em uma lâmina foi depositada 1mL de agar-arroz associado com o óleo essencial. Depois da solidificação do meio, a levedura foi semeada em duas estrias paralelas. Para evitar o ressecamento do meio a lâmina foi colocada em uma câmara úmida. Após 24, 48 e 72 horas a preparação foi examinada em um microscópio óptico. Em cada lâmina foi observada a presença de estruturas características como pseudohifas, blastoconídios e clamidoconídios.

**3.10- Germinação de conídios**

Diferentes concentrações do óleo essencial foram usadas para testar a germinação de conídios de *A. flavus* e *A. fumigatus*. Alíquotas de 0,5mL de emulsão do óleo em diferentes concentrações foram misturadas com 0,5mL de suspensão de conídios (aproximadamente 5 x $10^6$ conidios/ mL), e em seguida foram misturadas por 30 segundos. 0,1mL da mistura foi colocada em uma lâmina que em seguida foi incubada em uma câmara úmida a 25-28℃ por 24 horas. No final do período de incubação, cada lâmina foi corada com azul de lactofenol e observada no microscópio óptico. Assim, 200 conídios foram contados e a porcentagem de conídios germinados foi calculada. Cada análise foi realizada três vezes e os resultados foram expressos como a média das três repetições (RANA et al., 1997).

**3.11- Lise celular**

Suspensões de células de *C. albicans,* de conídios de *A. flavus* ou conídios de *A. fumigatus* foram lavados com solução NaCl 0,85% estéril e resuspendidas em salina estéril para preparação do inoculo. Tubos (volume final

de 12mL) contendo o inóculo de *C. albicans* (5 x $10^6$ UFC/ mL), de *A. flavus* ou *A. fumigatus* (5 x $10^6$ conídios/ mL) mais o óleo essencial em diferentes concentrações (CIM/2, CIM, CIMx2 e CIMx4) foram incubados. Em cada período de tempo (2, 4 e 24 horas), uma alíquota de 3 mL foi removida de cada um dos tubos e centrifugada a 3000rpm por 10 minutos. Os sobrenadantes foram coletados para análise de absorbância em 260nm, considerando como 100% de lise a absorbância produzida por células tratadas com 1.2 N de $HClO_4$ a 100°C por 30 min. Os resultados foram expressos como média ± desvio padrão da porcentagem de lise comparado com o controle de $HClO_4$ (100% de lise) de três experimentos independentes (ESCALANTE et al., 2008; SVETAZ et al., 2007).

**3.12- Ligação ao ergosterol**

A CIM do óleo essencial foi determinada por microdiluição utilizando microplacas com 96 cavidades como descrito anteriormente, na ausência e na presença de diferentes concentrações de ergosterol incorporados ao meio (150, 250 and 400 µg/ mL). Anfotericina B foi utilizada como controle. A CIM foi determinada após 48 horas de incubação para *C. albicans* e após 72 horas de incubação (confirmada em 7 dias) para *A. flavus* e *A. fumigatus*. Foram realizados dois experimentos independentes em diferentes ocasiões (SILVA JÚNIOR et al., 2010; ESCALANTE et al., 2008).

# 4- Resultados e discussão

## 4.1-Antifungal activity of *Cymbopogon winterianus* Jowitt ex Bor against *Candida albicans*

# Antifungal activity of *Cymbopogon winterianus* Jowitt ex Bor against *Candida albicans*

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## Abstract

*Candida albicans* is an opportunistic yeast and a member of the normal human flora that commonly causes infections in patients with any type of deficiency of the immune system. The essential oils have been tested for antimycotic activity and pose much potential as antifungal agents. This work investigated the activity of the essential oil of *Cymbopogon winterianus* against *C. albicans* by

MIC, MFC and time-kill methods. The essential oil (EO) was obtained by hydrodistillation using a Clevenger-type apparatus. It was tested fifteen strains of *C. albicans*. The MIC was determined by the microdilution method and the MFC was determined when an aliquot of the broth microdilution was cultivated in SDA medium. The phytochemical analysis of EO showed presence of citronellal (23,59%), geraniol (18,81%) and citronellol (11,74%). The EO showed moderate antifungal activity, and the concentrations 625 µg/mL and 1250 µg/mL inhibited the growth of all strains tested and it was fungicidal, respectively. The antimicrobial activity of various concentrations of EO was analyzed over time, it was found concentration-dependent antifungal activity, whose behavior was similar to amphotericin B and nystatin.

Keywords: *Candida albicans*, antifungal, *Cymbopogon winterianus.*

**Introduction**

*Candida albicans* is an opportunistic yeast and a member of the normal human flora. The individual is colonized at birth or near birth and transmission is primarily through physical contact. This yeast normally colonizes the skin and mucosal epithelium in healthy individuals (25). In patients hospitalized with cancer, immunocompromised or who use immunosuppressive drugs colonization may progress to invasion, resulting in severe systemic disease (38,26).

Systemic infection by *C. albicans* is often associated with catheters, surgery, parenteral nutrition and damage the skin, mucous membranes and the digestive tract (39). Invasive fungal infections are associated with high mortality rates and over 40% in bloodstream infections caused by *C. albicans* (8). The

common sites for superficial candidiasis include under the breasts, in the groin, in skin folds of obese persons and the perineal area (18). The major antifungal agents used in the therapy of these infections are azoles such as fluconazole and itraconazole, and the polyene compounds such as amphotericin B and nystatin (6, 30).

The increasing resistance of fungi to azole derivatives as a result of long-term therapies may limit the use of these drugs in the future (10, 17, 30). Due to limitations of current antifungal therapy and the emergence of resistant strains, it is necessary the search for new antifungal drugs through the testing of substances from plants (3, 34). Among the candidates, the essential oils are known to possess several biological activities, such as antibacterial and antifungal action (2, 7).

*Cymbopogon winterianus* is a plant belonging to the family Poaceae, popularly known as citronella, which is cultivated in India and Brazil. This plant demonstrated depressant effect on the central nervous system, anticonvulsant effect (33), larvicidal effect against *Aedes aegypti* (27), antibacterial (14,31) and antifungal activity (18), including anti-*Candida* action (13).

This study aimed to evaluate the antimicrobial activity of the essential oil of *Cymbopogon winterianus* against *Candida albicans* and to characterize the relationship between the concentration this essential oil with the rate and extent of its antifungal activity.

## Material and Methods

### Essential oil

*Cymbopogon winterianus* Jowitt ex Bor was grown, collected and had the essential oil extracted by hydrodistillation using a Clevenger-type apparatus at the Center for Technology Training at the Federal University of Paraíba, Bananeiras city (Paraiba, Brazil) by Prof. Dr. Paulo Alves Wanderley.

The plant was identified by Prof. Dr. Rita Baltazar de Lima in Laboratory of Botany, Department of Systematics and Ecology of the Center of Exact and Nature Sciences of the Federal University of Paraíba. The voucher specimen was deposited in the Herbarium Prof. Lauro Pires Xavier of the Department of Systematics and Ecology, Federal University of Paraíba under the code JPB 41387.

### Essential oil analysis

The essential oil was analyzed using a gas chromatograph (GC) fitted to a mass spectrometer (MS) (GC-MS-Schimadzu QP-5050A) instrument equipped with a GC Schimadzu 17A. Fused silica capillary column was 30 m x 0.25 mm i.d., with film thickness 0.25 µm. Helium was used as the carrier gas at 0,9 mL/min, with inlet pressure 48,9 psi. Injector and MS transfer line temperatures were at 280 and 170℃, respectively. The initial column temperature was 60℃, and then gradually increased to 240℃ at the rate of 3℃/min. It was kept at 240℃ for 10 minutes. For GC-MS detection an electron ionization system was used with ionization energy of 70eV. Samples were diluted 1/1000 (v/v) in hexane and 1.0 µL were injected in the splitless mode (1). The compounds were identified by comparing their fragmentation patterns reported in the mass

spectra with those present in the library of mass spectrometers NIST 98 (National Institute of Standards and Technology, USA) and with reports from the literature. The components quantification was based on the area percentage of the peak of each component in relation to the total area of all standardized peaks in the chromatogram.

**Fungal samples**

The strains of *C. albicans* tested belong to the collection of the Mycology Laboratory, Federal University of Paraíba and include ATCC 76485, ATCC 76615, ATCC 13803, LMV 42, ICB 12, M101, LM 968, LM 68, LM 16, LM 018, LM 023, LM 601, LM 290, LM 052, LM 087.

**Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)**

The MIC was determined by the microdilution method. Cultures of *Candida albicans* were placed on Sabouraud Dextrose Agar (SDA) and incubated for 24-72 hours at temperature 37ºC. Colonies of this culture were suspended in sterile 0.85% NaCl and the inoculum was standardized according to the scale of 0.5 McFarland (1-5 x $10^6$ CFU/mL). In a 96-well plate was added Sabouraud broth and essential oil of *C. winterianus* concentrations of 10.0000 to 39µg/mL. The MIC determination was conducted with approximately 1-5 x$10^5$ CFU/mL of the microorganism in each well. The plates were incubated at 37°C for 24-48 hours. In 24-48 hours there was a visual observation of fungal growth. To determine the MFC, 10µL of each of the wells without fungal growth was seeded on a plate containing SDA, the SDA plating were incubated at 37°C for 24-48 hours. The MFC was considered as the lowest concentration cultivated in

plate with SDA in which growth was less than 3 CFU. Afterwards, 20 µL of 0.5% triphenyl tetrazolium chloride (TTC) was added to each of 96 wells for MIC determination, and the plate incubated for 24 hours. The MIC was determined as the lowest oil concentration that inhibited visible growth of the microorganism, as also indicated by the TTC by reading the plates of 96 wells. There were three independent experiments on different occasions (11, 15, 21, 40).

**Time-kill**

The time-kill of *C. albicans* in presence of the essential oil was performed according to Klepser *et al*. (22), with some modifications. Before testing, the microrgamism was cultivated in SDA. Colonies derived from culture were suspended in 0.85% NaCl and turbidity adjusted to the range of 0.5 McFarland (1-5 x $10^6$ CFU/mL). One milliliter of fungal suspension was added to 9 mL of broth Sabouraud with or without the essential oil in various appropriate concentrations. The initial inoculum contains 1-5 x $10^5$ CFU/mL. Concentrations of essential oil of *C. winterianus* tested were 0.5, 1, 2, 4 and 8 times the MIC. These cultures were incubated at 37°C and at various time periods (0, 1, 2, 4, 6, 8, 12 and 24 hours), an aliquot of 100 µL was removed from each solution and diluted 1:10. An aliquot of 10µL of each dilution was removed and plated on SDA. The plates were incubated at 37°C for 24-48 hours and the number of colony forming units (CFU) was counted. When less than 1000 CFU/mL was expected, 10 µL sample was plated directly into SDA without dilution. The experiment was performed in duplicate. The minimum detection limit of this method is 100 CFU/mL (23).

The $\log_{10}$ CFU/mL was plotted on a graph as function of time and used to compare the rate and extent of antifungal activity in various concentrations of essential oil. It was considered fungicidal activity when there was a decrease greater than or equal to 3 $\log_{10}$ CFU/mL of the initial inoculum, resulting in reduction of 99.9% or more CFU/mL in 24 hours compared with the initial inoculum. Activity lower than that described was considered fungistatic (16, 24, 35).

## Results

### Chemical characterization of oil constituents

Essential oil of *C. winterianus* was subjected to GC and GC-MS analysis. The phytochemicals and their respective percentage in the essential oil composition and retention times are shown in Table 1. The majority constituents were citronellal (23,59%), geraniol (18,81%), citronellol (11,74%) and eugenol (10,34%).

### Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

By the broth microdilution technique was determined the MIC and the MFC, which are shown in Table 2.

As seen in Table 2, the MIC of the essential oil tested ranged between 78 and 625 µg/mL. The concentration of 625 µg/mL inhibited the growth of all strains, while 312 µg/mL was able to inhibit 60% of the strains tested. MFC of microorganisms ranged between 312 and 1250 µg/mL, being the latter fungicidal for all strains tested.

**Time-kill**

The microorganism growth was analyzed over time when it was subjected to various concentrations of essential oil of *C. winterianus*. Two strains of *C. albicans* (ICB 12 and ATCC 13803) were submitted to the experiment of microbial time-kill (Figures 1 and 2).

The graphs show the $\log_{10}$ of CFU/mL versus time for several multiples of the MIC (625 µg/mL) and the control without essential oil. The analysis of the graph to point out that at concentrations less than or equal to 1xMIC, the essential oil has fungistatic activity (reduction of less than 99.9% or $3\log_{10}$ the number of CFU/ml of initial inoculum), and at concentrations greater than or equal to 2xMIC it has fungicidal activity (reduction greater than or equal to 99.9% or $3\log_{10}$ the number of CFU/mL of initial inoculum), therefore, the essential oil of *C. winterianus* has concentration-dependent fungicidal activity.

Although the rate and extent of antifungal activity has varied slightly between the two strains tested, their behavior in front of the essential oil was similar. The antifungal activity improved with increasing concentration of essential oil, like this, when the higher concentration of essential oil, the lower time is required for fungicidal activity.

**Discussion**

Substances from plants are used in the treatment of various diseases, but the potential of plants as a source of new drugs is still poorly explored. The estimated number of plant species, only a small percentage had their pharmacological properties studied (34), like this, there is need to search for new drugs from plants that have therapeutic potential. Therefore, the essential

oils are important because their several pharmacological activities including anti-fungal, antibacterial, antiparasitic (2,4,37).

The GC-MS analysis resulted in the identification of 18 components. Among the phytochemicals, citronellal, geraniol and citronellol are the majority constituents. Other studies recorded that citronellal, citronellol and geraniol are the main constituents of essential oil of *C. winterianus* (5, 9, 13, 33).

Among the identified compounds, some were previously reported to have antibacterial activity, including geraniol against *E. coli* (14), *Listeria monocytogenes, Salmonella enterica* and *Salmonella typhimurium* (36). Mesa-Arango *et al*. (28) showed that citronellal and geraniol were active against *Candida parapsilosis, C. krusei, Aspergillus flavus and A. fumigatus.*

The MIC and the MFC in all strains were 625 µg/mL and 1250 µg/mL, respectively. It was reported that the essential oil of this plant had antibacterial activity against *Escherichia coli* O157: H7 (MIC > 0,8% v/v), *Salmonella typhimurium* (MIC 0,4% v/v), *Staphylococcus aureus* (MIC 0,05% v/v), *Listeria monocytogenes* (MIC 0,4% v/v) (31); against enterotoxigenic, enteroinvasive and enteropathogenic serotypes of *E. coli*, with MICs ranging between 200 and 800 µg/mL (14) and antifungal activity against *Trichophyton* species (18) and against *Candida albicans* (MIC 600µg/mL) (13). MIC found in this study is according to values obtained in the other studies.

Due to its pronounced anti-*C. albicans* activity, the essential oil of *C. winterianus* had its action studied in more detail through the time-kill method. When analyzing the graphic $\log_{10}$ CFU/mL versus time, it can identify the transition between the levels of fungistatic and fungicidal activity of a substance,

the essential oil of *C. winterianus* showed concentration-dependent fungicidal activity. Another type of antifungal activity occurs for example with fluconazole, whose activity is independent of concentration, because there is no significant increase in activity with increasing concentration (6). Among the drugs commonly used to treat fungal infections, amphotericin B and nystatin are fungicidal concentration-dependent (22, 29).

It was reported that the essential oil of *Ocimum gratissimum* L. had antifungal activity against *C. albicans*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* with MICs ranging between 750 and 1500 µg/mL and in the test of time-kill this oil showed concentration-dependent antifungal activity (29).

The MFC found when an aliquot of the broth microdilution was cultivated in SDA medium was 1250µg/mL (2xMIC). This value is related to the concentration able to inhibit the growth of 99.9% or more CFU/mL in 24 hours compared with the initial inoculum, information that can be observed analyzing the time-kill graphs.

Clinically, differences in fungal dynamics can influence the selection of optimal regimes of doses of an antifungal. Agents in which the rate and extent of antifungal activity improve with increasing concentration (e.g. amphotericin B) can be optimized by the administration of large doses. In contrast, the activity of antifungal agents like fluconazole does not improve with increase in the concentration higher than the MIC. Therefore, the administration of large doses of fluconazole cannot change the rate and extent of fungal eradication (16).

Pauli and Schilcher (32) suggest that oral administration of essential oil compounds is not suitable to cure severe infections in children. Topical

application or inhalation of selected compounds for the treatment or additional treatment of mild infections is reasonable. Dorman and Deans (12) reported that administered orally, volatile oils may be able to control a wide range of microbes, but there is also the possibility that they may cause an imbalance in the gut microflora, allowing opportunistic pathogens coliforms to become established in the gastrointestinal tract with resultant deleterious effects. Other possible side effects of essential oils are irritation, allergic contact dermatitis and spasmolytic or spasmogenic properties (7). The essential can to be used for treatment some diseases like dermatomycoses and superficial candidiasis mainly in topical applications (18). The essential oils can be used alone or in combination with other drugs which can exhibit synergism (19,20).

The increased resistance of *C. albicans* and the nature of the fungistatic azoles have valued the search for new antifungal agents, once the activity against this type of pathogen is essential (16). The antifungal activity demonstrated by the essential oil of *C. winterianus* makes it a potential candidate as a major agent in controlling fungi growth that cause infections or are present in the environment.

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Table 1. Chemical composition of essential oil from *C. winterianus.*

| Peak No. | Compounds | Composition (%) | Retention time (min) |
|---|---|---|---|
| 1 | 2-methyl-2-hepten-6-one | 0.13 | 6,99 |
| 2 | β-myrcene | 0.07 | 7.18 |
| 3 | Limonene | 3.39 | 8.58 |
| 4 | Linalool | 1.34 | 11.13 |
| 5 | Citronellal | 23.59 | 13.81 |
| 6 | Citronellol | 11.74 | 17.00 |
| 7 | Geraniol | 18.81 | 18.65 |
| 8 | Citronellyl acetate | 5.29 | 22.30 |
| 9 | β-elemene | 6.40 | 22.50 |
| 10 | Eugenol | 10.34 | 23.96 |
| 11 | Germacrene | 2.63 | 27.69 |
| 12 | Δ-cadinene | 2.27 | 29.44 |
| 13 | Elemol | 6.73 | 30.67 |
| 14 | Endo-1-bourbonanol | 1.01 | 31.52 |
| 15 | Farnesol | 0.60 | 33.01 |
| 16 | γ-eudesmol | 1.00 | 33.66 |
| 17 | Torreyol | 1.65 | 34.08 |
| 18 | Trans-farnesol | 3.01 | 34.69 |

Table 2. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of essential oil of *C. winterianus* against strains of *C. albicans.*

| *C. albicans* samples | MIC (µg/mL) | MFC (µg/mL) |
|---|---|---|
| 1- ATCC 76485 | 78 | 312 |
| 2- ATCC 76615 | 78 | 312 |
| 3- ATCC 13803 | 625 | 1250 |
| 4- LMV 42 | 312 | 625 |
| 5- ICB 12 | 625 | 1250 |
| 6- M101 | 312 | 625 |
| 7- LM 968 | 156 | 312 |
| 8- LM 68 | 625 | 1250 |
| 9- LM 16 | 625 | 1250 |
| 10- LM 018 | 312 | 625 |
| 11- LM 023 | 312 | 625 |
| 12- LM 601 | 312 | 625 |
| 13- LM 290 | 625 | 1250 |
| 14- LM 052 | 312 | 625 |
| 15- LM 087 | 625 | 1250 |

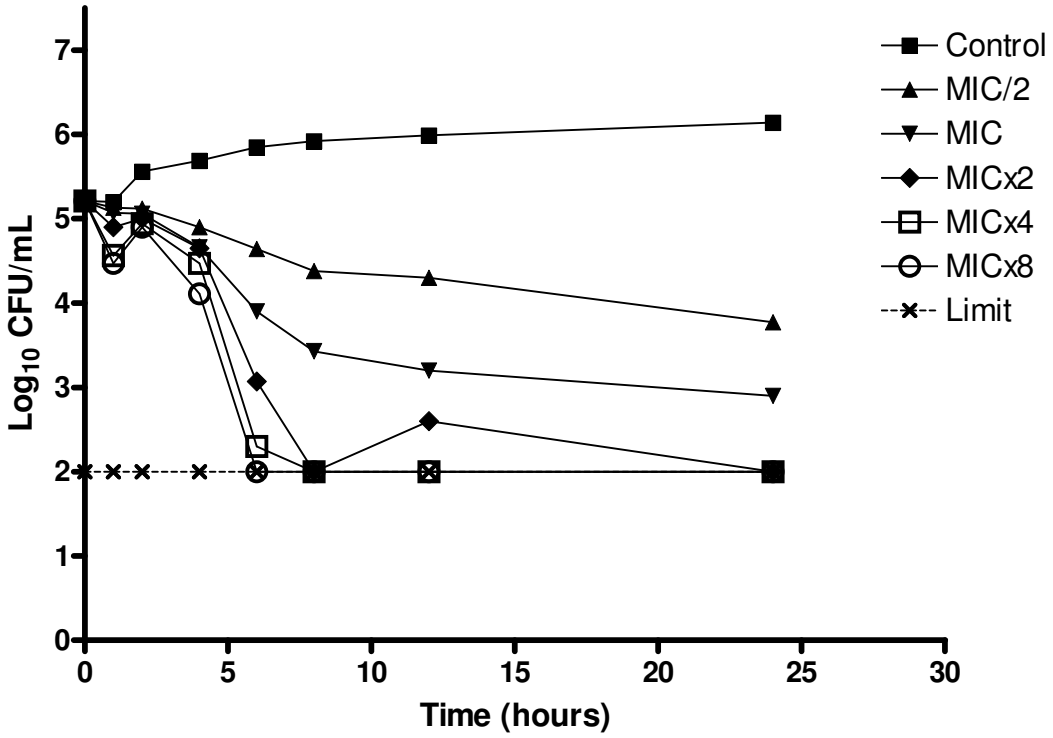


Figure 1: Time-kill of *C. albicans* ATCC 13803 when exposed to various concentrations of essential oil of *C. winterianus*.

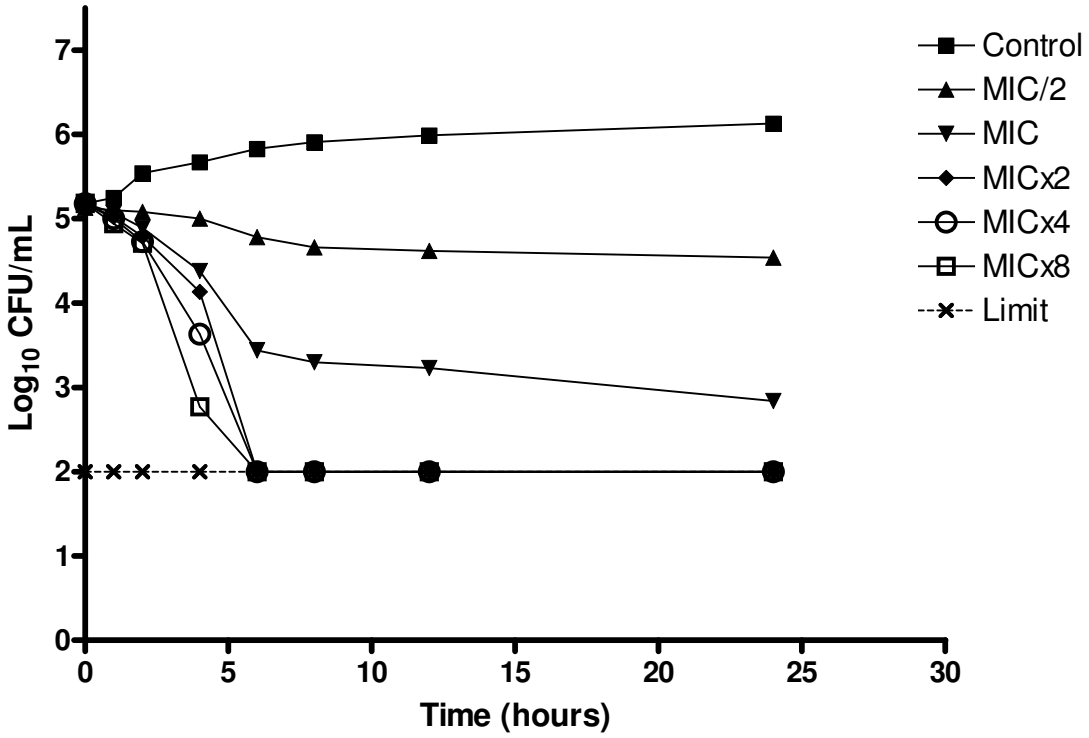


Figure 2: Time-kill of *C. albicans* ICB 12 when exposed to various concentrations of essential oil of *C. winterianus*.

## 4.2- Investigation of mechanism of action from *Cymbopogon winterianus* against *Candida albicans*

# Investigation of mechanism of action from *Cymbopogon winterianus* against *Candida albicans*

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## Abstract

Fungal diseases have become increasingly important in the past few years and *Candida* species are commensal and opportunistic pathogens present on virtually all humans. *Candida albicans* is pathogenic for man and turned resistant to antifungal agents, so it is important the search of new antifungals. The study aimed to investigate the mechanism of action of essential oil of *C. winterianus* against *C. albicans*. For this, it was observed morphological changes in the *C. albicans* when exposed to several concentrations of oil by the technique of microculture for yeasts, using agar-rice in moist chamber. Cellular leakage was analyzed after cell lysis by absorbing of compounds at 260nm; also it was assessed the bind of essential oil with ergosterol. The essential oil of *C. winterianus* inhibited the formation of pseudohyphae and chlamydoconidia in

all concentrations tested (MIC, 2xMIC and 4xMIC), it caused cellular lysis at concentrations MIC, 2xMIC and 4xMIC; and its MIC in presence of ergosterol increase until 8 times, which indicate binding to ergosterol.

Key words: *Candida albicans*, antifungal, *Cymbopogon winterianus*

**Introduction**

Fungal diseases have become increasingly important in the past few years. Because few fungi are professional pathogens, fungal pathogenic mechanisms tend to be highly complex. Fungal pathogens can be divided into two general classes, primary pathogens and opportunistic pathogens. Model examples of such primary pathogens include *Coccidioides immitis* and *Histoplasma capsulatum*, whose characteristic diseases have been known for many years. Opportunistic human fungal pathogens have become increasingly important over past 20 years, paradoxically because the success of modern medical practice has led to the survival of debilitated and immunosuppressed patients. Such patients are highly susceptible to infections by opportunistic pathogens such as *Candida* species, *Cryptococcus neoformans*, *Aspergillus fumigatus* and other *Aspergillus* species (BURIK; MAGEE, 2001).

*Candida* species are commensal and opportunistic pathogens present on virtually all humans, these species frequently isolated from mucosal sites where they are generally considered to be colonizing organisms. However, bloodstream infection due to *Candida* spp. with or without disseminated candidiasis are now major causes of morbidity and mortality in hospitalized patients, including severe burns patients (HA et al., 2011). *Candida albicans* is a fungus that could be located on the skin and in mucous membranes such as

the vagina, mouth, or rectum. This fungus can also travel through the blood stream and affect the throat, intestines, and heart valves. Among the various human fungal pathogens which attack immunocompromised patients, *C. albicans* accounts for the majority of systemic infections with mortality rates ranging from 50% to 100% (TAYEL et al., 2010).

The limited efficacy of the available antifungal drugs against the fungi which cause serious mycoses, mainly in AIDS hosts, added to the rapid emergence of resistant organisms which further diminishes therapeutic capabilities, have highlighted the need of new antifungal compounds which could constitute alternatives to the existing drugs (SVETAZ et al., 2007, PATTERSON, 2005).

Over the next decade, antifungal resistance may become a crucial determinant of the outcome of antifungal treatment. While the incidence of systemic fungal infections has been increasing, the choice of suitable antifungal agents remains relatively limited, although the advent of the new echinocandin class is a welcome development as is the expansion of members of the triazole antifungals. While historically the principal pathogenic species in hospitalized patients have been *Candida albicans* and *Aspergillus fumigatus*, clinicians dealing with high-risk patient populations now also need to consider a number of emerging fungal species (ROGERS, 2006).

*Candida albicans* and related species pathogenic for man become resistant to antifungal agents, in particular triazole compounds, by expression of efflux pumps that reduce drug accumulation, alteration of structure or concentration of antifungal target proteins, and alteration of membrane sterol

composition. The clinical consequences of antifungal resistance can be seen in treatments failures in patients and in changes in the prevalence of *Candida* species causing disease (SANGLARD; ODDS, 2002). For that reason is important the search of new antifungals.

The medicinal plants have been an important source to the development of drugs with biological activity. Many plant extracts and essential oils isolated from plants have been shown to exert biological activity in vitro and in vivo, which justified research on traditional medicine focused on the characterization of antimicrobial activity of these plants. Since plants produce a variety of compounds with antimicrobial properties, may yield candidate compounds for developing new antimicrobial drugs. It is expected that plant compounds show target sites other than those currently used by antibiotics, which will be active against drug-resistant microbial pathogens (DUARTE et al., 2005). Interest in plants with antifungal properties has increased as a consequence of current problems associated with the fungal therapy. Since antiquity, medicinal plants have been used to treat common infectious diseases and the essential oils of these plants have been widely used in treatment of infectious pathologies in many body parts (PEREIRA et al., 2011). In addition, the essential oils are natural products that exhibit a variety of biological properties, such as analgesic, anticonvulsant, antibacterial and antifungal (QUITANS-JÚNIOR et al., 2008; BAKKALI et al., 2008).

*Cymbopogon winterianus* Jowitt ex Bor (Poaceae) is popularly known as "citronella" or "java citronella", is a perennial herb which cultivated in India and Brazil. This medicinal plant is used by population as repellent, antimycotic and acaricide activities (PEREIRA et al., 2011; BLANK et al., 2007). This study

aimed to evaluate the activity of essential oil of *C. winterianus* against *C. albicans,* analysing the alterations in morphological forms, measurement of cellular leakage, and determine if oil binds to the ergosterol.

## Material and methods

### Essential oil

*Cymbopogon winterianus* Jowitt ex Bor was grown, collected and had the essential oil extracted by hydrodistillation using a Clevenger- type apparatus at the Center for Technology Training at the Federal University of Paraíba, Bananeiras city (Paraiba, Brazil) by Dr. Paulo Alves Wanderley.

The plant was identified by Dra. Rita Baltazar de Lima in Laboratory of Botany, Department of Systematics and Ecology of the Center of Exact and Nature Sciences of the Federal University of Paraíba. The voucher specimen was deposited in the Herbarium Prof. Lauro Pires Xavier of the Department of Systematics and Ecology, Federal University of Paraíba under the code JPB 41387.

### Fungal samples

The strains of *C. albicans* tested were ICB-12 and ATCC 13803, they belong to the collection of the Mycology Laboratory, Federal University of Paraíba.

**Effect of essential of *C. winterianus* on the micromorphology of *C. albicans***

For observation of morphological changes in the *C. albicans* strain ICB 12, it was used the technique of microculture for yeasts, using agar-rice in moist chamber (SIDRIM; ROCHA, 2004; MINAMI, 2003; KURTZMAN; FEEL, 1998; GUNGI et al., 1983).

Concentrations of essential oil of *C. winterianus* were added to the culture medium agar-rice, in order to obtain various concentrations of the products (MIC, MICx2, MICx4). Then, on a slide glass were deposited 1mL of agar-rice associated with the essential oil. After culture medium solidification, the yeast was seeded in 2 parallel striations. The striations were covered with sterile coverslip. To avoid desiccation of the medium, it was made a moist chamber. After 24h, 48h and 72h the preparation was examined under optical microscope. In each slide was observed presence of characteristic structures as pseudohyphae, blastoconidia and chlamydoconidia.

**Leakage Effect**

Cultures of *Candida albicans* were placed on Sabouraud Dextrose Agar (SDA) and incubated for 24-72 hours at temperature 37ºC. Colonies of this culture were suspended in sterile 0.85% NaCl and the inoculum was standardized according to the scale of 0.5 McFarland (1-5 x $10^6$ CFU/mL).

Cell suspension of *C. albicans* was washed with sterile NaCl 0,85%, resuspended in sterile NaCl 0,85% and used to prepare the inoculum. Tubes (final volume 12mL) containing inoculum of 5 x $10^6$ UFC/ mL and essential oil of *C. winterianus* at MIC/2, 1xMIC, 2x MIC or 4x MIC were left from 2 to 24 h.

Amphotericin B (MIC) and ketoconazole (MIC) were used as control drugs, MICs this drugs were determinated according to OLIVEIRA et al., 2011. At each period of time (2, 4 and 24h), 3mL aliquots were removed from each test solution and centrifuged at 3000 rpm for 10 min. The supernatants were collected for absorbance analysis at 260nm, considering as 100% release the absorbance produced by cells treated with 1.2 N $HClO_4$ at 100°C, 30 min. Results were expressed as means ± standard deviation of the percentage of leakage compared with the control with $HClO_4$ (100%), values were obtained from three independent assays (ESCALANTE et al., 2008; SVETAZ et al., 2007).

**Ergosterol Effect**

The MIC of essential oil of *C. winterianus* against *C. albicans* ICB 12 was determined by the microdilution method using microplates of 96 wells as explained in OLIVEIRA et al., 2011, in the absence and in the presence of different concentrations (150, 250 and 400 µg/ mL) of ergosterol added to the medium, in different lines of the same microplate. Amphotericin B was used as a control drug. The MIC was determined after 48h of incubation. This assay was carried out in two independent assays (SILVA JUNIOR et al., 2010; ESCALANTE et al., 2008).

**Results and Discussion**

Despite the introduction of improved antifungal drugs for treatment and prophylaxis, invasive fungal infections remain a significant clinical problem. Infections caused by eukaryotic organisms like yeasts generally present more difficult therapeutic problems than do bacterial infections. There are relatively few antifungals agents that can identify unique targets not shared with human

hosts. Current drug treatments are effective, but resistant strains and intrinsically resistant species are emerging fast. Moreover, the treatment cost, associated host cytoxicity and the fact which most available antifungal drugs have only fungistatic activity point to search for new strategies. Natural products provide an unparalleled source of chemical scaffolds with diverse biological activities, and have a profound impact on antimicrobial drug discovery (KHAN et al., 2010).

In study of our team, we demonstrated that the essential oil of *C. winterianus* was active against the yeast *Candida albicans,* minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were 625µg/mL and 1250µg/mL, respectively. When antimicrobial activity of various concentrations was analysed over time, it was found concentration-dependent fungicidal activity in time-kill assay. In the phytochemical analysis, citronellal (23,59%), geraniol (18,81%), citronellol (11,74%) and eugenol (10,34%) were the majority constituents (OLIVEIRA et al., 2011).

Some antifungal agents are generally fungistatic rather than fungicidal, which poses several problems. Because fungistatic agents merely arrest fungus growth, a quick relapse or incomplete eradication of the infecting organism can occur. Achieving a cure is particularly difficult in patients with an impaired immune system, because stopping fungus growth may not suffice to prevent dissemination. Fungicidal agents should demonstrate increase clinical efficacy, a short duration of therapy, and a reduced relapse rate compared with fungistatic agents such as griseofulvin and ketoconazole, which typically results in prolonged treatment with frequent relapses (ELEWSKI, 1993). As essential

oil of *C. winterianus* had concentration-dependent fungicidal activity, it can be a candidate to antifungal.

Essential oils have strong activity with the MIC values between 50-500µg/mL, moderate activity with MIC values between 600-1500µg/mL and weak activity above 1500µg/mL (SARTORATTO et al., 2004). MIC of essential oil of *C. winterianus* against *C. albicans* was 625µg/mL, this essential oil shown moderate activity (OLIVEIRA et al., 2011).

In this work, we studied the activity of essential oil of *C. winterianus* against *C. albicans*, we analysed the alterations in morphological forms, measurement of cellular leakage and determine if oil binds to the ergosterol.

The formation of hyphae and pseudohyphae is related to virulence factor expressed by *C. albicans*. As these structures represent a barrier to phagocytosis and allow the settlement of yeasts on epithelial tissue. Morphological change is associated with microrganism's pathogenicity, and it is believed that environmental factors influence the physiological state of the commensal yeast (ROMANI et al., 2003).

The results (table 1) show that essential oil of *C. winterianus* at concentrations MIC, MICx2 and MICx4 inhibited the formation of pseudohyphae and chlamydoconidia *of C. albicans*.

It was demonstrated that essential oils of *Origanum vulgare* L. and *Cinnamomum zeylanicumI,* and also thymol inhibited the pseudohyphae and chlamydoconidia formation of *C. albicans* (CASTRO, 2010; LUNA, 2007)

For *Candida*, it is the ability of hyphae to grow through host cell that is proposed to account for the importance of polymorphism in virulence. In *Candida albicans*, both the yeast and hyphal forms are found at the site of infection. Recent virulence studies of filamentation regulatory mutants argue that both yeast and filamentous forms have roles in infection. Several antifungal agents have morphological effects on *C. albicans*. Amphotericin B prevents filamentous growth. In the presence of the azole antifungals, cells appear as aggregates or chains of swollen structures, many of them with a bud-like formation. Pneumocandins can cause profound changes in hyphal growth, light micrographs show abnormally swollen germ tubes, highly branched hyphal tips, and many cells with distended ballon shapes (BURIK; MAGEE, 2001). Therefore, it is important that an antifungal inhibits the formation of hyphae, pseudohyphae and/or chlamydoconidia, because contribute for reduce a virulence factor which contribute to pathogenesis. Thus, as essential oil of *C. winterianus* acted on polymorphism of *C. albicans*, it can be a promiser antufungal agent.

Considering the lipophilic character of essential oils and its possibility of interacting with fungal membranes, we studied the effect of several concentrations of essential oil (MIC/2, 1x, 2x and 4x MIC) on the irreversible damage to the fungal membrane by measuring the leakage of 260nm-absorbing intracellular materials released to the medium at different time intervals (2, 4 and 24 hours). The figures 1 and 2 shows leakage from cells over 2, 4 and 24 hours period of interaction with essential oils at MIC/2, MIC, MICx2 and MICx4 concentrations. The results showed that essential oil at 2xMIC and 4xMIC produced damage to the fungal membrane, evidenced by increase of

percentage of cellular leakage. *C. albicans* ICB 12 exposed at MICx2 and MICx4 in 24 hours had 71% and 82% of cellular leakage, respectively. The oil caused less leakage at MIC (about 40%) than at 2x MIC and 4x MIC; and produced much less damage at MIC/2, approximately 16%. Perchloric acid was considered to produce 100% cellular leakage.

The amphotericin B (MIC 2µg/mL) and cetoconazol (MIC 16µg/mL) also caused cellular leakage (Figures 1 and 2). Probably, it is due amhotericin B binds ergosterol of the membrane and increase the permeability and ketoconazol inhibits lanosterol 14α-demetilase which inhibit the synthesis of ergosterol, this changes the normal permeability and fluidity of the fungal membrane is altered (ODDS et al., 2003).

The damage to the cells could be observed by measuring the release of intracellular components to the medium from treated cells. Cellular components which absorb at 260nm represent one class of leakage components, primarily nucleotides, of which uracil-containing compounds exhibit the strongest absorbance (LUNDE; KUBO, 2000; ESCALANTE et al 2008, SVETAZ et al 2007).

When a drug disrupt membranes, as amphotericin B, it acts at concentrations such as 1x and 4xMFC (SVETAZ et al., 2007; LUNDE; KUBO, 2000). In our study, essential oil of *C. winterianus* caused cellular leakage at 2xMIC (1xMFC) and at 4xMIC (2xMFC), which suggests the activity of the essential oil on damage of the membranes. Essential oil of *C. winterianus* was demonstrated to cause cellular leakage against *Trichophyton rubrum*, suggesting its actuation could probably involve the fungal plasma membrane

(PEREIRA, 2009). It was reported that other essential oils compromise the structural and functional integrity of cytoplasmic membranes, which suggests that the antimicrobial mechanism is due the membrane damage. Essential oil of *Syzygium aromaticum* shown fungicidal effect that resulted from a extensive lesion of the cell membrane on *Candida*, *Aspergillus* and dermatophyte species, as *T. rubrum* and *T. mentagrophytes* (PINTO et al., 2009). Essential oil of *Ocimum sanctum* has antifungal activity, and the mechanism of fungicidal activity is resulted from extensive lesions of the plasma membrane, it target the structural and functional integrity of the cytoplasmic membrane an *Candida*, leading to cell death (KHAN et al., 2010). The essential oil of *Melaleuca alternifolia* exhibited antimicrobial activity against *Staphylococcus aureus*, and its components compromise the cytoplasmic membrane (CARSON et al., 2002). Geraniol was shown to enhance the rate of potassium leakage out of whole cells and also was shown increase bilayer permeability (BARD et al., 1988).

The cytotoxic capacity of the essential oils can make them excellent antiseptic and antimicrobial agents. Cytotoxicity appears to include such membrane damage. In bacteria, the permeabilization of the membranes is associated with loss of ions and reduction of membrane potencial, collapse of the proton pump and depletion of the ATP pool. Essential oils can coagulate the cytoplasm and damage lipids and proteins. Damage to the cell wall and membrane can lead to the leakage of macromolecules and to lysis (BAKKALI et al., 2008, HELANDER et al., 1998, SIKKEMA et al., 1994).

The accumulation of lipophilic compounds in the cytoplasmic membrane of microorganisms has considerable effects on the structural and functional properties of these membranes. The numerous observations of toxic effects of

terpenes, aromatics, cycloalkanes, alkanes, alcohols and phenols can be explained largely by the interactions of these compounds with the membrane constituents. As a result of accumulated lipophilic molecules, the membrane loses its integrity, and an increase in permeability to protons and ions can be observed (SIKKEMA et al., 1995). Scanning and transmission electron microscopy observations reveal cell ultrastructural alterations in several compartments such as plasma membrane, cytoplasm (swelling, shriveling, vacuolations, leakage) and nucleus by essential oils (BAKKALI et al., 2008).

To determine if essential oil of *C. winterianus* binds to the fungal membrane sterol, the MIC of this compound for *Candida albicans* was determined with and without the addition of ergosterol. If the activity of compound was caused by binding to ergosterol, the exogenous ergosterol would prevent the binding to the fungal membrane's ergosterol. As a consequence, MIC enhancement of essential oil in the presence of exogenous ergosterol with respect to the control assay should have been observed (ESCALANTE et al., 2008).

The results of figure 3 had shown that MIC of amphotericin B against *C. albicans* ICB 12 increased to 4xMIC, 8xMIC and 16xMIC with addition of 150, 250 and 400µg/mL of ergosterol, respectively (MIC found to the amphotericin B was 2µg/mL). The MIC of the *C. winterianus* raised in the presence of ergosterol to 2xMIC, 4xMIC and 8xMIC with addition of 150, 250 and 400µg/mL of ergosterol respectively.

A 4-fold increase of MIC was observed for the positive control drug amphotericin B, whose interaction with ergosterol has been demonstrated (ESCALANTE et al., 2008). In addition, MIC of amphotericin B against

*Saccharomyces cerevisiae* increased with the change to addition of ergosterol (SILVA JUNIOR et al., 2010). Amphotericin B is a well-known membrane-disruptive agent which has a direct membrane effect and is lethal to yeast. It forms stable pores in the membrane by complexing with ergosterol, which results in permeability to ions such as potassium and protons (LUNDE;KUBO, 2000).

Most therapies, designed to treat fungal infections, target the ergosterol biosynthesis pathway or its end product, ergosterol, a membrane sterol that is unique to fungi. Ergosterol is the main sterol of yeasts and other fungi, and it is necessary for growth and normal functions of cells. Besides serving as a bioregulator of membrane fluidity, asymmetry and membrane integrity, ergosterol contributes to the proper function of membrane-bound enzymes (KHAN et al., 2010, LUPPETI et al., 2002). When antifungal agents bind to the membrane ergosterol, leads to fungal membrane disruption and loss of the intracellular content (ESCALANTE et al., 2008). Regarding their biological properties, essential oils are complex mixtures of numerous molecules; their biological effects are the result of a synergism of all molecules or reflect only those of the main molecules present at the highest levels according to gas chromatographical analysis. It is possible that the activity of the main components is modulated by other minor molecules (BAKKALI et al., 2008).

Considering the large number of different groups of chemical compounds present in essential oils, it is most likely that their activity is not attributable to one specific mechanism but that there are several targets in the cell. The locations or mechanisms in the cell thought to be sites of action for essential oil components are damage to cytoplasmic membrane, damage to membrane

proteins, leakage of cell contents, degradation of the cell wall, coagulation of cytoplasm and depletion of the proton motive force. Not all of these mechanisms are separate targets, some are affected as a consequence of another mechanism being targeted (BURT, 2004, HELANDER et al., 1998).

The results in this work suggest that essential oil of *C. winterianus* interact with ergosterol of membrane; in addition, there is possibility of interactions between several compounds of essential oil with other targets in the fungal. Considering that the incidence of both community-acquired and nosocomial fungal infections has significantly increased over the past few decades, accompanying the growing number of high risk patients, particularly those with impaired immunity (PINTO et al., 2009), the search of new alternatives for disease antifungal treatment is important, and natural products, especially the plants, can be the source for new drugs.

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Table1: Micromorphological changes promoted by essential oil of *C. winterianus* against *C. albicans* strain ICB 12. Presence (+) and absence (-) of the structure.

| | Pseudohyphae | Blastoconidia | Chlamydoconidia |
|---|---|---|---|
| Control (distilled water) | + | + | + |
| MIC | - | + | - |
| MICx2 | - | + | - |
| MICx4 | - | + | - |

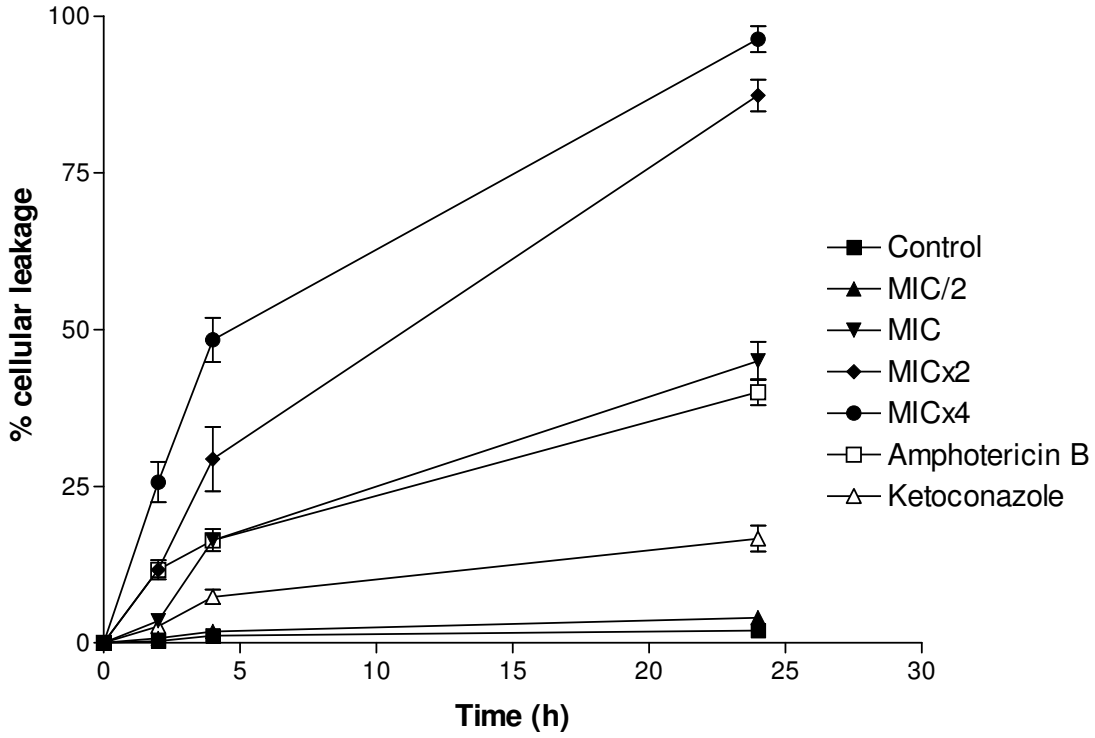


Figure 1- Percent of leakage of *C. albicans* ATCC 13803 cells untreated (control) or treated with essential oil of *C. winterianus* at MIC/2, MIC, MICx2 or MICx4; amphotericin B (MIC) or ketoconazole (MIC). Cells treated with perchloric acid was considered to produce 100% cellular leakage.

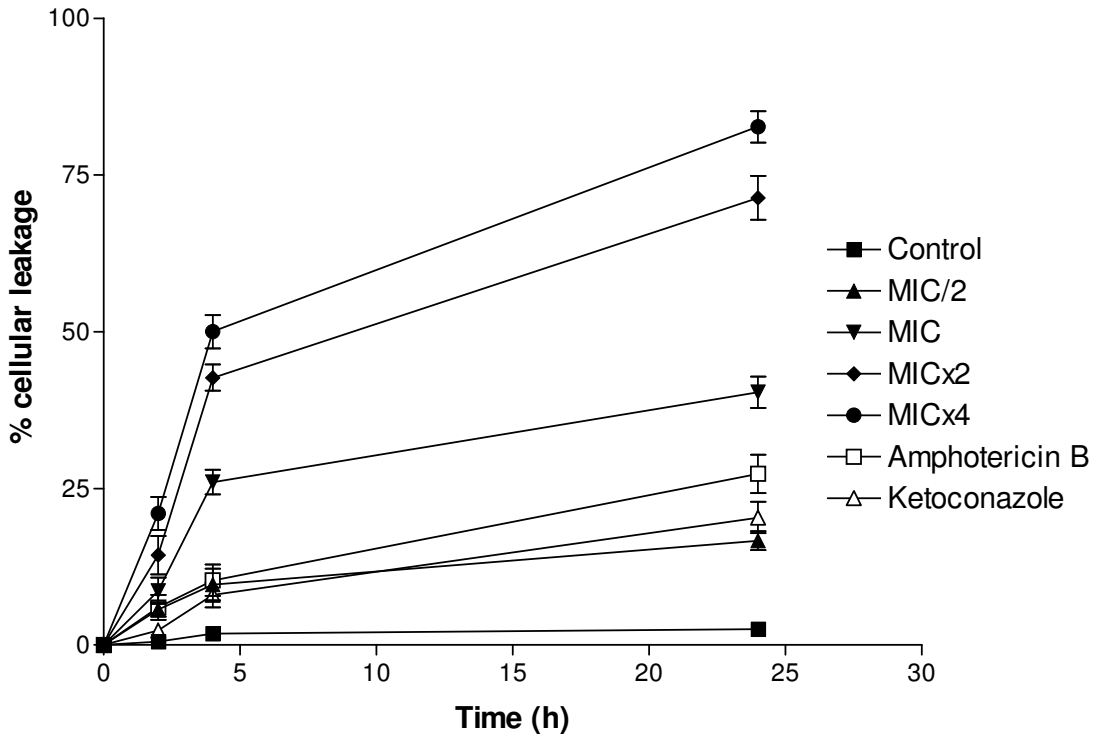


Figure 2- Percent of leakage of *C. albicans* ICB 12 cells untreated (control) or treated with essential oil of *C. winterianus* at MIC/2, MIC, MICx2 or MICx4; amphotericin B (MIC) or ketoconazole (MIC). Cells treated with perchloric acid was considered to produce 100% cellular leakage.

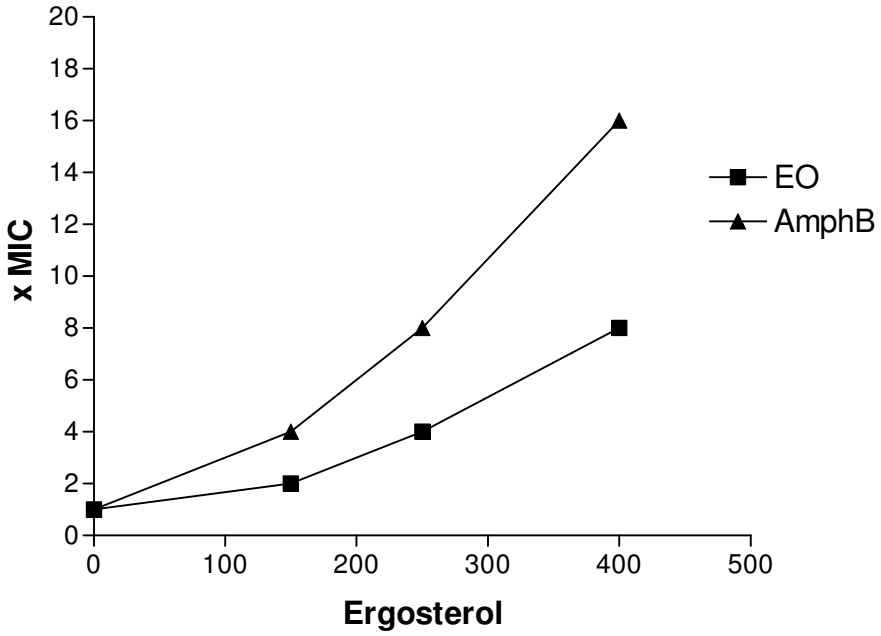


Figure 3- Effect of different concentrations of exogenous ergosterol (150-400µg/mL) on the MIC of both essential oil of *C. winterianus* and amphotericin B for *Candida albicans* ICB 12. EO= essential oil of *C. winterianus*, AmphB= amphotericin B.

## 4.3- Activity of essential oil from *Cymbopogon winterianus* Jowitt ex Bor against *Aspergillus flavus*

# Activity of essential oil from *Cymbopogon winterianus* Jowitt ex Bor against *Aspergillus flavus*

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## Abstract

Commom clinical syndromes associated with *Aspergillus flavus* include chronic granulomatous sinusitis, keratitis, cutaneous aspergillosis, wound infections and osteomyelitis following trauma and inoculation. The increased incidence of fungal infections, especially dangerous hospital-acquired infections and infections in immunocompromised patients, has accentuated the need for new antifungal treatments. There has been growing interest in alternative therapies as the therapeutic use of natural products, especially those derived from plants. This study aimed to evaluate the antifungal activity of essential oil of *Cymbopogon winterianus* against *Aspergillus flavus* strains. The MIC was determined by microdilution assay and the MFC was determined when an aliquot of the broth microdilution was cultivated in SDA medium. Inhibition of the

fungal mycelial growth was determined by the daily measure of the radial mycelial growth on SDA with different concentrations of the oil. Several concentrations of the essential oil were tested on conidia germination. Compounds which absorb at 260nm avaluated cellular leakage, and more, it was analyzed the bind of essential oil to ergosterol. The MIC ranged between 156,25µg/mL and 312,5µg/mL and the MFC ranged between 312,5µg/mL and 625µg/mL. The oil provided reduction in the mycelia growth at MIC and MICx2 and exhibited a strong inhibition of conidia germination at concentrations MIC, MICx2 and MICx4. The oil at MIC, 2xMIC and 4xMIC produced damage to the fungal membrane. The oil interacts with ergosterol, because increasing of the MIC of essential oil in the presence of ergosterol.

Key words: *Cymbopogon winterianus, Aspergillus flavus*, antifungal

**Introduction**

There is a crisis in health care for effectively treating and preventing infections caused by fungi. The increased incidence of fungal infections, especially dangerous hospital-acquired infections and infections in immunocompromised patients, has accentuated 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 cost of medical care associated with fungal infections are steadily rising. The vast majority of this infections are due to species of *Candida, Aspergillus, Cryptococcus* and *Coccidioides*. The incidence and severity of fungal infections are increasing at an alarming rate; for example, in some patient populations the mortality rate is in excess of 80% (GEORGE; SELITRENNIKOFF, 2006).

Among about 150 fungal species that are able to cause disease in a mammalian host, *Aspergillus* species are exceptional because they can cause allergic responses or harm immunocompromised individuals, in most severe cases with a fatal outcome (KRAPPMANN, 2006). *Aspergillus* infections have grown in importance in the last years. After *Aspergillus fumigatus*, *A. flavus* is the second leading cause of invasive aspergillosis and it is the most common cause of superficial infection. Particularly commom 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 hepatocarcinogenic natural compounds ever characterized (HEDAYATI et al., 2007).

Until recent years, the only drugs available to treat aspergillosis were amphotericin B and itraconazole, the latter in oral and intravenous formulations. Recently voricanazole, posaconazole and caspofungin have also been approved for the treatment of aspergillosis. Although resistance to antifungal drugs is not as great as resistance to antibacterial agents, there has been an increase in the number of reported cases of fungal resistance. Some investigators have reported isolates of *A. flavus* resistant to amphotericin B and itraconazole mycoses (HEDAYATI et al., 2007; DENNING et al., 1997). For these reasons is important the search for new antifungals.

In recent years, there has been growing interest in alternative therapies as the therapeutic use of natural products, especially those derived from plants (RATES, 2001). Essential oils are aromatic oily liquids obtained from plant material (flowers, seeds, leaves, fruits and roots). They are secondary

metabolites that are highly enriched in compounds based on an isoprene structure. They are called terpenes and they occur as diterpenes, triterpenes and tetraterpenes, as well as hemiterpenes and sesquiterpenes. When the compounds contain additional elements, usually oxygen, they are termed terpenoids (BURT, 2004; COWAN, 1999). Essential oils are known for their antiseptic, i.e. bactericidal, virucidal and fungicidal, and medicinal properties and their fragrance, they are used in preservation of foods and as antimicrobial, analgesic, sedative, anti-inflamatory, spasmolytic and locally anesthesic remedies (BAKKALI et al., 2008). Therefore, the essential oils are important because their several pharmacological activities. *Cymbopogon winterianus* (Poaceae), popularly known as "citronella" and "java grass", is an important essential oil yielding aromatic grass cultivated in India and Brazil (QUITANS-JÚNIOR et al., 2008). It was demonstrated that essential oil of this plant has antifungal activity against *Candida albicans* (OLIVEIRA et al., 2011) and *Trichophyton rubrum* (PEREIRA et al., 2011).

Due the antifungal properties of this essential oil, the aim of this study was to evaluate the antifungal activity of essential oil of *Cymbopogon winterianus* against *Aspergillus flavus* strains.

## Material and Methods

### Essential Oil

*Cymbopogon winterianus* Jowitt ex Bor was grown, collected and had the essential oil extracted by hydrodistillation using a Clevenger-type apparatus at the Center for Technology Training at the Federal University of Paraíba, Bananeiras city (Paraiba, Brazil) by Dr. Paulo Alves Wanderley.

The plant was identified by Dra. Rita Baltazar de Lima in Laboratory of Botany, Department of Systematics and Ecology of the Center of Exact and Nature Sciences of the Federal University of Paraíba. The voucher specimen was deposited in the Herbarium Prof. Lauro Pires Xavier of the Department of Systematics and Ecology, Federal University of Paraíba under the code JPB 41387.

**Fungal samples**

The strains of *A. flavus* tested belong to the collection of the Mycology Laboratory, Federal University of Paraíba and include ATCC 16013,LM 247, LM 02, LM 704, LM 257, LM 907 and LM 13.

**Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)**

Stock cultures were kept on sterile Sabouraud Dextrose Agar (SDA) slants under 7℃ (± 1℃). For preparing the inoculum used in antimould assays were used 7 days-old culture grown on sterile SDA at 25-28℃. After the incubation period, the mould conidia were taken by adding sterile NaCl 0,85% on the growth media followed for gentle shaking for 30 seconds. Mould conidia was counted using hemocytometer. The inoculum of conidial suspension was adjusted using sterile NaCl 0,85% to contain approximately 5 x $10^6$ conidia/mL.

The MIC was determined by the microdilution method using microplates of 96 wells (CLEELAND; SQUIRES, 1991; ELOFF, 1998). Conidial suspension from 7-day-old of *A. flavus* culture was prepared and standardized by hemocytometer in sterile 0,85% NaCl for susceptibility testing as described previously. In a 96-well plate was added Sabouraud broth and essential oil of *C.*

*winterianus* at concentrations of 10.000 to 39µg/mL. The MIC determination was conducted with an inoculum of approximately 2,5 x$10^5$ conidia/mL of the microorganism in each well. The plates were incubated at 25 - 28℃ for 72 hours. In 72 hours (and confirmed at 7 days) there was a visual observation of fungal growth. The MIC was considered as the lowest oil concentration that inhibited growth of the microorganism. To determine the MFC, 10µL sample from each well where no fungal growth was subcultured on a plate containing SDA, the SDA plating were incubated at 25 - 28 ℃ for 72 hours. The MFC was defined as the lowest concentration that showed either no growth or fewer than three colonies on ASD. There were three independent experiments on different occasions with consistent results (PEREIRA et al., 2011, ESPINEL-INGROFF et al., 2002). Amphotericin B and ketoconazole were used as control.

MIC values between 50-500µg/mL indicates strong activity, moderate activity is MIC values between 600-1500µg/mL and weak activity is MIC above 1500µg/mL (SARTORATTO et al., 2004).

**Mycelial growth inhibition**

Inhibition of the fungal mycelial growth of *A. flavus* was determined using the poisoned substrate technique (diluition in solid medium) by the daily measure of the radial mycelial growth on SDA added essential oil at MIC/2, MIC and MICx2. For this, a 2 mm plug taken from a 10 days-old mould culture grown on SDA slants at 25 - 28 ℃ were placed on the center of sterile SDA Petri dishes containing the essential oil. At different intervals (0, 2, 4, 6, 8, 10, and 12 days) after incubation at 25 - 28 ℃, the radial mycelial growth was measured (mm). Control included in this assay was the observation of the mould radial

growth on SDA without adding essential oil, or added of amphotericin B (at MIC) or ketoconazole (at MIC). Results were expressed as means ± standard deviation of three independent experiments realizated on different occasions (DAFERERA et al., 2003; ADAM et al., 1998).

**Germination analysis**

Different concentrations of the essential oil were tested for conidia germination. Aliquots of 0.5 mL of the essential oil emulsions at different concentrations were mixed with 0.5 mL of the mould conidia suspension (approximatelly 5 x $10^6$ conidia/ mL) followed by shaking using Vortex for 30 seconds. 0.1mL of the mixture was placed on separated glass slides which were incubated in a moist chamber at 25-28℃ for 24 hours. At the end of the incubation period, each slide was fixed with lactophenol-cotton blue stain and observed under the microscope for conidia germination. About 200 conidia were counted and the percentage of conidia germination was calculated. Each assay was performed three times and the results were expressed as the average of the three repetitions (RANA et al., 1997).

**Leakage Effect**

The inoculum of conidial suspension was prepared as described previously in sterile NaCl 0,85% to contain approximately 5 x $10^6$ conidia/mL.

Conidia suspension of *A. flavus* was washed with sterile NaCl 0,85% and resuspend in sterile NaCl 0,85% to prepare the inoculum. Tubes (final volume 12mL) containing inoculum of 5 x $10^6$ conidia/ mL and essential oil of *C. winterianus* at MIC/2, MIC, MICx2 and MICx4 were left from 2 to 24 h. Amphotericin B (MIC) and ketoconazole (MIC) were used as control drugs. At

each period of time (2, 4 and 24 hour), 3mL aliquots were removed from each test solution and centrifuged at 3000 rpm for 10 min. The supernatants were collected for absorbance analysis at 260nm, considering as 100% release the absorbance produced by conidia treated with 1.2 N $HClO_4$ at 100°C, 30 min. Results were means ± standard deviation of the percentage of leakage compared with the control with $HClO_4$ (100%), values were obtained from three independent assays (ESCALANTE et al., 2008; SVETAZ et al., 2007; LUNDE; KUBO, 2000).

**Ergosterol Effect**

The MIC of essential oil of *C. winterianus* against *A. flavus* was determined by the microdilution method using microplates of 96 wells as explained above, in the absence and in the presence of different concentrations (150, 250 and 400 µg/ mL) of ergosterol added to the medium, in different lines of the same microplate. Amphotericin B was used as a control drug. MICs were read at 3 and 7 days of incubation. This assay was carried out in two independent assays (SILVA JUNIOR et al., 2010; ESCALANTE et al., 2008).

**Results and discussion**

The antifungal drugs are limited and suffer from a number of limitations that can render their use difficult; for example, dose-limiting nephrotoxicity associated with amphotericin B, rapid development of resistence with flucytosine and fungistatic mode of action and resistence development with the azoles. There is thus an urgent need for new antifungals with a broad, fungicidal spectrum of action, and with fewer dose-limiting side effects (BARRET, 2002). The essential oils are known by their antibacterial and

antifungal properties (BAKKALI et al., 2008), and it was demonstrated that essential oil of *C. winterianus* has fungicidal activity against *Trichophyton rubrum* (PEREIRA et al., 2011), which do it a potencial candidate as an agent in controlling fungi growth.

The essential oil of *C. winterianus* showed antifungal activity against *A. flavus*, the MIC ranged between 156,25µg/mL and 312,5µg/mL. The MFC ranged between 312,5µg/mL and 625µg/mL, which results represent 2xMIC (table 1). Essential oils have strong activity with the MIC values between 50-500µg/mL, moderate activity with MIC values between 600-1500µg/mL and weak activity above 1500µg/mL (SARTORATTO et al., 2004), so essential oil of *C. winterianus* against *A. flavus* had strong activity.

To be fungicidal rather than fungistatic is an important finding since antifungal agents that kill fungi (cidal) have demonstrated to be, in most cases, clinically more useful than those that merely inhibit (static) fungal growth (ESCALANTE et al., 2008; LEWIS; GRAYBIL, 2008). It was found concentration-dependent fungicidal activity in time-kill assay of essential oil of *C. winterianus* against *Candida albicans* (OLIVEIRA et al., 2011).

It was reported that essential oil of *C. winterianus* exhibited activity against *Trichophyton rubrum*, MIC and MFC were 312µg/mL and 2500µg/mL respectively (PEREIRA et al., 2011), and against other *Trichophyton* species (HARRIS, 2002). Essential oil of this plant displayed antifungal activity against *Candida albicans* (DUARTE et al., 2005) and antibacterial activity against *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes* (OUSSALAH et al., 2007, DUARTE et al., 2007).

Other essential oils, such as *Origanum vulgare* (CARMO et al., 2008b), *Cinnamomum zeylanicum* (CARMO et al., 2008a), *Hyptis suaveolens* (MOREIRA et al., 2010), *Satureja hortensis* (DIKBAS et al 2008) exhibited antifungal activity against *Aspergillus* species including *Aspergillus flavus, A. fumigatus and A. niger*. Tatsadjieu et al., 2009 demonstrated that MIC of essential oil of *Lippia rugosa* against *A. flavus* was 1000 mg/L. Geraniol (51,5%), nerol (18,6%) and geranial (10,4%) were the main components of *Lippia rugosa* oil and its radial growth was significantly influenced by incubation time and essential oil concentration.

The analysis of chemical composition of essential oil used in this work demonstrated that citronellal (23,59%), geraniol (18,81%), citronellol (11,74%) and eugenol (10,34%) are the majority constituents (OLIVEIRA et al., 2011). The antimicrobial effect of some essential oil components of *C. winterianus* has been investigated. It was found that geraniol was effective in suppressing *A. flavus* growth at 500mg/L (MAHMOUD, 1999) and had antimicrobial activity against *Escherichia coli* (DUARTE et al., 2007). Geraniol and citronellol appear to be active against *Cryptococcus neoformans* (VIOLLON; CHAUMONT, 1994).

Effect of the essential oil on the radial mycelial growth in solid medium is showed in figure 1 and 2. To both strains, essential oil provided reduction in the growth at MIC and MICx2 concentrations when compared with the control assay. The MIC/2 concentration allowed the mycelial mould growth. The MIC found to amphotericin B and ketoconazole against *Aspergillus flavus* (to both strains ATCC 16013 and LM 907) was 4µg/mL and 16µg/mL, respectively. Amphotericin B and ketoconazole at MIC also inhibited the mycelial growth.

Mycelia growth was considerably reduced with increasing concentration of essential oil, while in general their growth increased with incubation time. 91,17% and 97,6% inhibition of mycelia growth were respectively observed at MIC and 2xMIC after 12 days of incubation when compared with the control to *A. flavus* strain ATCC 16013.

Several essential oils demonstrated activity against mycelia growth of fungi. Essential oil of *Cinnamomum zeylanicum* inhibited mycelial growth of *Cladosporium cladosporioides* and *Fonsecaea compacta* (MOREIRA et al., 2007) and the while essential oil of *C. winterianus* inhibited the mycelium development of *Trichophyton rubrum* (PEREIRA et al., 2011). Essential oils of *Citrus lemon*, *Citrus sinensis*, *Citrus reticulata* and *Citrus paradis* all showed capacity to reduce or inhibit the growth of the moulds *Aspergillus flavus*, *A. niger*, *Penicillium chrysogenum* and *P. verrucosum* (VIUDA-MARTOS et al., 2008). Oil of *Lippia rugosa* inhibited colony growth of *A. flavus* (TATSADJIEU et al 2009).Venkateswarlu and Kelly, 1996 shown that ketoconazole inhibited concentration-dependent the mycelial growth of *Aspergillus fumigatus*.

The activity to different concentrations of essential oil against *A. fumigatus* conidia germination is presented in the table 2 and 3. As can be noted, the essential oil exhibited a strong inhibition of conidia germination at concentrations MIC, MICx2 and MICx4 to both strains. The oil showed very weak inhibition at concentration MIC/2, and absence of inhibition at MIC/4. Pereira, 2009 reported that essential oil of *C. winterianus* showed a strong inhibitory power on the conidial germination of *T. rubrum* and *T. mentagrophytes*. Other essential oils, as essential oil of *Aegle mermelos* caused total inhibition of conidial germination of *Alternaria alternate*, *A.*

*brassicae*, *A. carthami*, *Curvularia lunata*, *Fusarium ciceri* and *F. udum* (RANA et al., 1997). It was demonstrated also, that essential oil of *Origanum vulgare* inhibited spore germination of *A. flavus*, *A. fumigatus* and *A. niger* (CARMO et al., 2008b).

*Aspergillus* species, which are classical filamentous molds, have morphological versatility and form conidia that are the infectious agent. Many molds form conidia, or vegetative spores, acattered by wind or water, these small, resistant cells serve as a mode of dissemination. This morphological versatility is an important virulence factor (BURIK; MAGEE, 2001). So, it is important antifungals inhibit the morphological change.

Considering the lipophilic character of essential oil of *C. winterianus* and its possibility of interacting with fungal membranes, we studied the effect of several concentrations of essential oil (MIC/2, MIC, 2x and 4x MIC) on the irreversible damage to the fungal membrane by measuring the leakage of 260nm-absorbing intracellular materials released to the medium at different time intervals (2, 4 and 24 hours). The figure 3 shows leakage from conidia cells over 2, 4 and 24 hours period of interaction with essential oil. The result showed that essential oil at MIC (39,66% of cellular leakage), 2xMIC (59,3% of cellular leakage) and 4xMIC (88% of cellular leakage) produced damage to the fungal membrane at 24 hours, as evidenced by increasing of absorbance at 260nm. It produced very few damage at MIC/2 (7,66% of cellular leakage). The antifungals amphotericin B (MIC 4µg/mL) and ketoconazole (MIC 16µg/mL) also caused cellular leakage, the amphotericin B (27,33%) more than ketoconazole (17,33%). Perchloric acid was considered to produce 100% cellular leakage.

This results suggest that essential oil of *C. winterianus* caused conidia leakage of *A. flavus*.

The amphotericin B and cetoconazol also altered the permeability of the membrane, which can have caused cellular lysis (Figure 3). Amhotericin B is known for binds ergosterol and increase the permeability of the membrane, and ketoconazol inhibits lanosterol 14α-demetilase which changes the normal permeability and fluidity of the fungal membrane due decrease of production of ergosterol (ODDS et al., 2003).

The damage to the conidia could be observed by measuring the release of intracellular components to the medium from treated conidia. Conidia components that absorb at 260nm represent one class of leakage components, primarily nucleotides, of which uracil-containing compounds exhibit the strongest absorbance (LUNDE; KUBO, 2000; ESCALANTE et al 2008, SVETAZ et al 2007).

It was demonstrated that *C. winteruanus* caused cellular leakage of *T. rubrum* and *T. mentagrophytes* (PEREIRA, 2009). Geraniol was shown to enhance the rate of potassium leakage out of whole cells and also was shown increase bilayer permeability (BARD et al., 1988). The substances 2',4'-dihydroxy-3'-methoxychalcone and 2',4'-dihydroxychalcone isolated from *Zuccagnia punctata* produced a time-dependent damage to the fungal membrane of *S. cerevisiae* only at 10xMIC, it did not produce any damage either at 1x or at 4x MFC, this result suggested that substances did not produce any damage in the membrane (SVETAZ et al., 2007). In this study, we demonstrated that essential oil of *C. winterianus* caused cellular leakage at 2x

and 4xMIC, therefore it suggests that this oil damage the membranes of conidia. In study of polygodial, a sesquiterpene dialdehyde isolated from *Polyganum hydropiper*, against *S. cerevisiae*, shows that the MIC and MFC of polygodial were no more than twofold different, demonstrating its fungicidal activity presenting cellular leakage effects; and yet, amphotericin B and miconazole also was able to cause cellular leakage effects, in addition, amphotericin B caused more cellular leakage than miconazole (LUNDE; KUBO, 2000).

An important characteristic of essential oil and their components is their hydrophobicity, which enables them to partition in the lipids of the cell membrane and mitochondria, disturbing the structures and rendering them more permeable. Leakage of ions and other cell contents can then occur. Although a certain amount of leakage from bacterial cells may be tolerated without loss of viability, extensive loss cell contents or the exit of critical molecules and ions will lead to death (BURT, 2004; SIKKEMA et al., 1995; SIKKEMA et al., 1994).

The changes in the integrity of the membrane, as a result of the interactions of lipophilic solutes with different components of the membrane, also affect the functioning of the membrane. The passive proton (ion) flux can increase as a result of alterations in membrane structure caused by an increase in temperature, mechanical stress, interaction with lipophilic molecules, and other factors (SIKKEMA et al., 1995).

To investigate whether the essential oil interact with ergosterol, it was analyzed the values of the MIC in the presence of increasing concentrations of

ergosterol. The MIC of the essential oil in the presence of the ergosterol increased from 1xMIC in 150µg/mL of ergosterol to 2x and 4xMIC, in 250 µg/mL and 400µg/mL of ergosterol, respectively (Figure 4). In addition, the MIC of the amphotericin B increased from 2xMIC at 150µg/mL, to 4x and 8x MIC at 250 and 400µg/mL of exogenous ergosterol, respectively. This suggests an interaction between essential oil and the ergosterol.

Sterols are essential structural and regulatory components of eukaryotic cell membranes. Ergosterol is the end product of the sterol biosynthetic pathway and is the major sterol in fungus. Like cholesterol in mammalian cells, it is responsible for membrane fluidity, integrity of the membranes and permeability (IWAKI et al., 2008). When antifungal agents bind to the membrane ergosterol, leads to fungal membrane disruption and loss of the intracellular content (ESCALANTE et al., 2008).

The mechanism of action of amphotericin B, which is shared in common with other polyenes, is based on the binding of the hydrophobic moiety of this molecule to the fungal cell membrane ergosterol moiety, producing an aggregate that forms transmembrane channels. This defect causes depolarization of the membrane and an increase in membrane permeability to protons and monovalent cations (LENIADO-LABORÍN; CABRALES-VARGAS, 2009). It was shown that amphotericin B in the presence of exogenic ergosterol shown MIC enhancement against *T. mentagrophytes*, confirming the mode of action of amphotericin B, by binding to membrane ergosterol (YAMAGUCHI et al., 2009). It was demonstrated that compounds with antifungal activity by ergosterol binding, form large pores in cell membranes causing lysis, as

cecropin A against *Aspergillus flavus, A. fumugatus, A. niger*, *Fusarium moniliforme* and *F. oxysporum* (DeLUCCA et al., 1997).

Considering the large number of different groups of chemical compounds present in essential oils, it is most likely that their activity is not attributable to one specific mechanism but that there are several targets in the cell. The locations or mechanisms in the cell to action for essential oil components are damage to cytoplasmic membrane, damage to membrane proteins, leakage of cell contents, degradation of the cell wall, coagulation of cytoplasm and depletion of the proton motive force. Not all of these mechanisms are separate targets, some are affected as a consequence of another mechanism being targeted (BURT, 2004; HELANDER et al., 1998), and is possible that their biological effects are the result of a synergism of several molecules, and the activity of the main components is modulated by minor molecules (BAKKALI et al., 2008).

With the emergence of the pathogens resistant to antifungals, make necessary the search for new drugs, and as plants have an almost limitless ability to synthesize substances (COWAN, 1999), the natural products can be a source of new drugs. The essential oil of *C. winterianus* can be a promising alternative to antimicrobial compound, supporting its possible use as a therapeutic alternative for the treatment of infectious caused by *Aspergillus flavus*.

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Table 1. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of essential oil *C. winterianus* against strains of *A. flavus*.

| *A. flavus* samples | MIC (µg/mL) | MFC (µg/mL) |
|---|---|---|
| 1- ATCC 16013 | 312,5 | 625 |
| 2- LM 247 | 312,5 | 625 |
| 3- LM 02 | 312,5 | 625 |
| 4-LM 704 | 156,25 | 312,5 |
| 5-LM 257 | 156,25 | 312,5 |
| 6-LM 907 | 312,5 | 625 |
| 7-LM 13 | 156,25 | 312,5 |

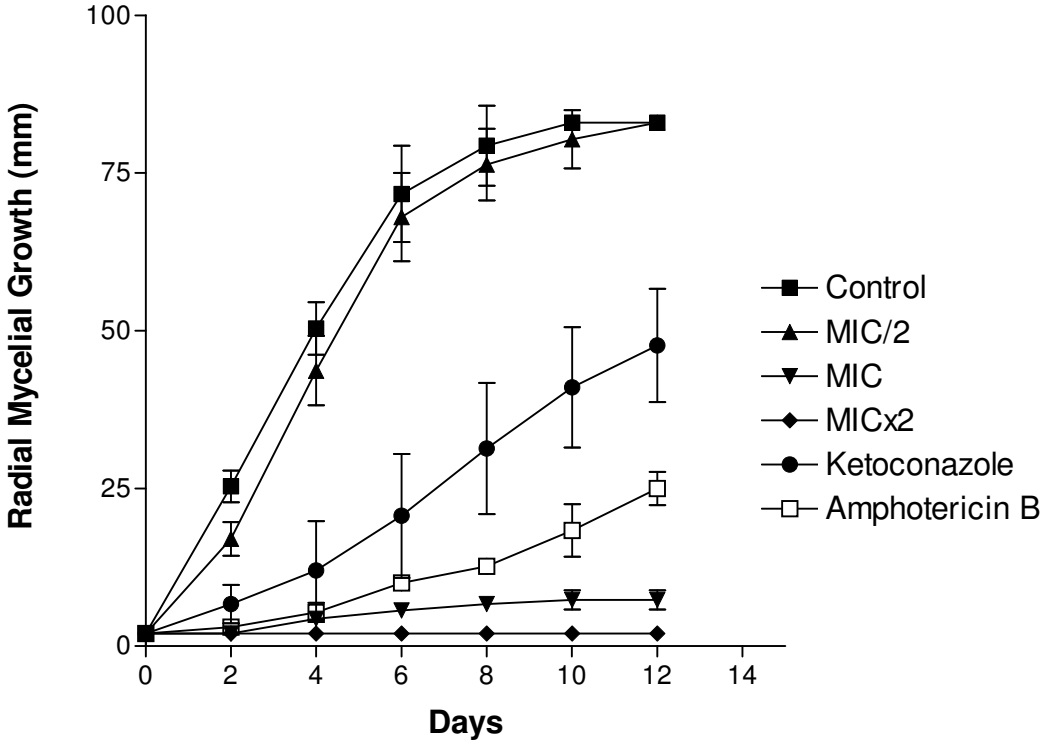


Figure 1. Effect of essential oil of *C. winterianus* on radial mycelial growth of *A. flavus* ATCC 16013.

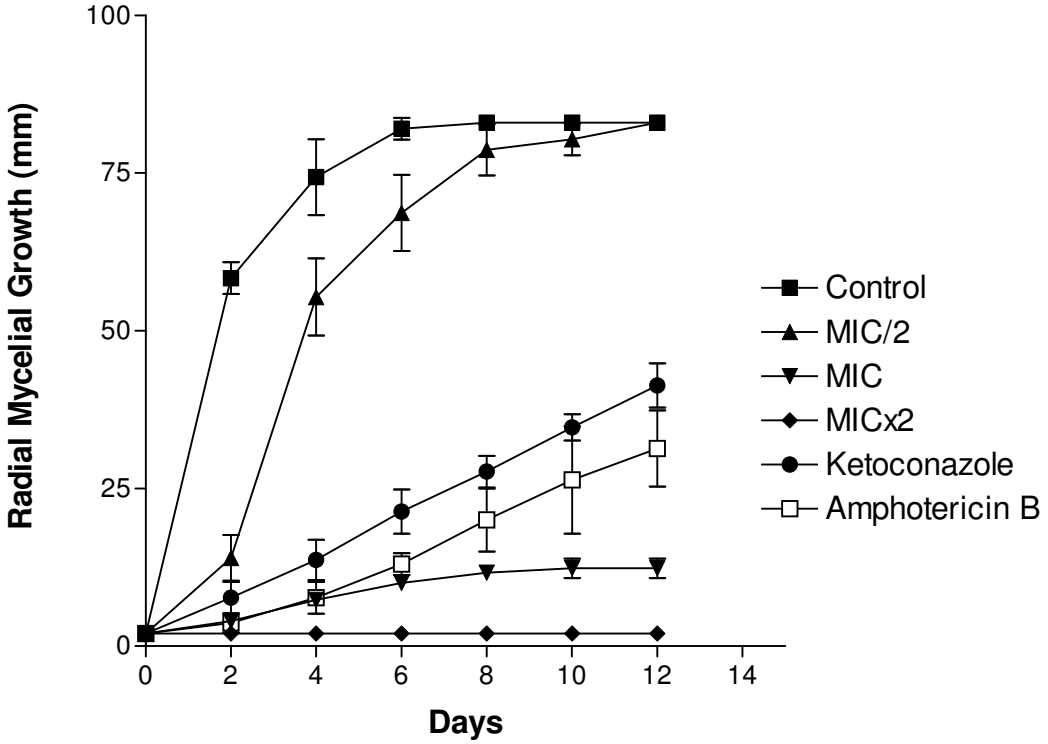


Figure 2. Effect of essential oil of *C. winterianus* on radial mycelial growth of *A. flavus* LM 907.

Table 2. Effect of essential oil of *C. winterianus* on conidia germination of *A. flavus* ATCC 16013 (results expressed in percentage of conidia germination).

| Concentrations of essential oil | Conidial germination |
|---|---|
| Control | 100% |
| MIC/4 | 100% |
| MIC/2 | 93% |
| MIC | 3% |
| MIC x 2 | 0% |
| MIC x 4 | 0% |

Table 3. Effect of essential oil of *C. winterianus* on conidia germination of *A. flavus* LM 907 (results expressed in percentage of conidia germination).

| Concentrations of essential oil | Conidial germination |
|---|---|
| Control | 100% |
| MIC/4 | 100% |
| MIC/2 | 94% |
| MIC | 5% |
| MIC x 2 | 0% |
| MIC x 4 | 0% |

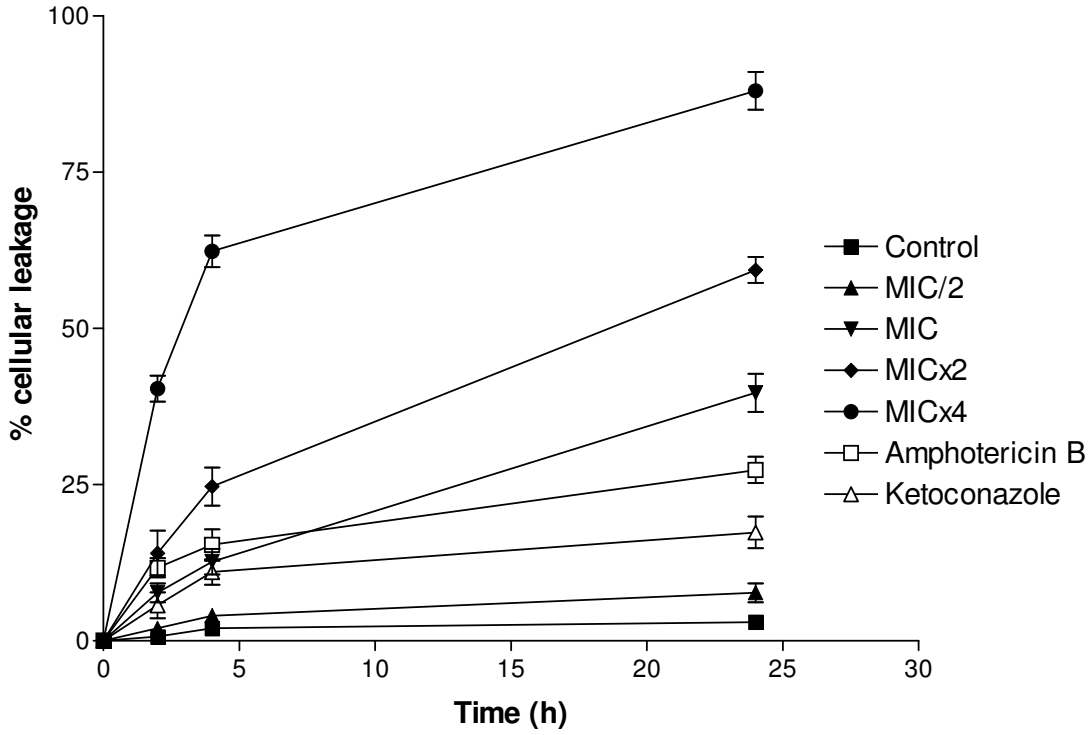


Figure 3- Percentage of leakage of conidia of *A. flavus* ATCC 16013 untreated (control) or treated with essential oil of *C. winterianus* at MIC/2, MIC, MICx2 and MICx4 and with amphotericin B (MIC) and ketoconazole (MIC). Cells treated with perchloric acid was considered to produce 100% cellular leakage.

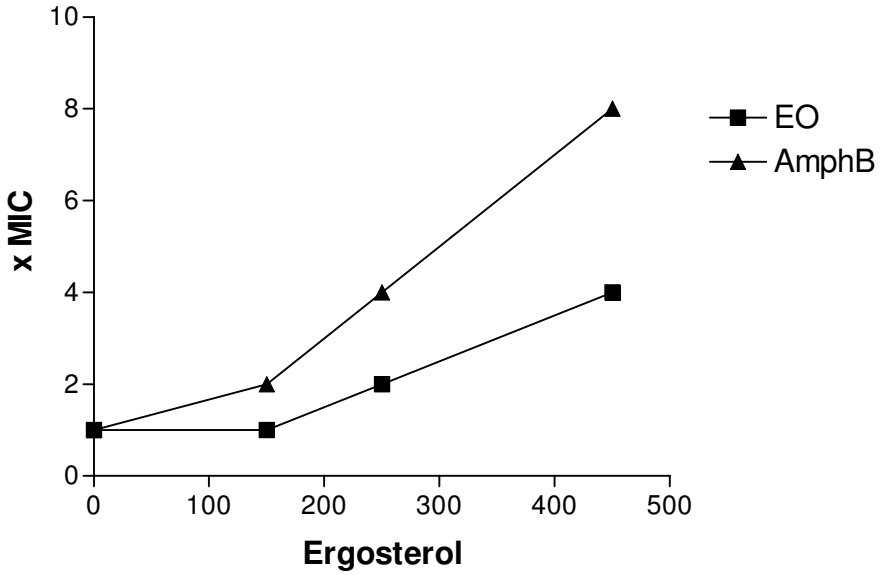


Figure 4- Effect of different concentrations of exogenous ergosterol (150-400µg/mL) on the MIC of both essential oil of *C. winterianus* and amphotericin B for *A. flavus* ATCC 16013. EO= essential oil of *C. winterianus*, AmphB= amphotericin B.

## 4.4- Activity of essential oil from *Cymbopogon winterianus* Jowitt ex Bor against *Aspergillus fumigatus*

# Activity of essential oil from *Cymbopogon winterianus* Jowitt ex Bor against *Aspergillus fumigatus*

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## Abstract

The most frequent pathogen to cause aspergillosis is *Aspergillus fumigatus* but species such as *A. terreus, A. flavus* or *A. niger* have also become increasingly evident in infections. Plants with medicinal properties have been focus of scientific studies all over the world in order to discovery new and effective antimicrobial compounds. The aim of this work was to investigate the antifungal activity of essential oil of *C. winterianus* against *A. fumigatus*. The MIC was determined by microdilution assay and the MFC was determined when an aliquot of the broth microdilution was cultivated in SDA medium. Inhibition of the fungal mycelial growth was determined by the daily measure of the radial mycelial growth on SDA with different concentrations of the oil. Several

concentrations of the essential oil were tested on conidia germination. Compounds which absorb at 260nm were detected in cellular leakage, and more, it was analyzed the bind of essential oil to ergosterol. The MIC and the MFC were 312,5µg/mL and 625µg/mL, respectively for all strains tested. The oil reduced in the mycelia growth at MIC and MICx2. The oil inhibited of conidia germination at concentrations MIC, MICx2 and MICx4, there was weak inhibition at concentration MIC/2. Essential oil at MIC, 2xMIC and 4xMIC produced damage to the fungal membrane, it is confirmed for increase of absorbance. The MIC of the oil increased in the presence of ergosterol, which suggests binding to ergosterol.

**Introduction**

The fungal kingdom comprises about 1,5 million species, but only a few of them have evolved to be pathogenic in mammals. Rapid progress in medicine accompanied by an increase in numbers of immunosuppressed patients has put fungal pathogens in the focus of interest for clinicians and medical microbiologists. The most frequent pathogen to cause aspergillosis is *Aspergillus fumigatus* but species such as *A. terreus, A. flavus* or *A. niger* have also become increasingly evident in infections (KRAPPMANN, 2006). *Aspegillus fumigatus* is an opportunistic pathogen, which incite disease in hosts whose local or systemic immune attributes have been impaired, damaged, or are innately dysfunctional. On the contrary, primary pathogens are those fungi that cause disease in noncompromised patients (BURIK; MAGEE, 2001).

*Aspergillus fumigatus* is a worldwide saprotrophic species that plays an essential role in recycling carbon and nitrogen. This fungus has a high

sporulating capacity which results in the ubiquitous presence of high concentrations of conidia (1-100 conidia/$m^3$) in the atmosphere indoors and outdoors. Conidia of *A. fumigatus* are continuously inhaled by humans and are eliminated efficiently by innate immune mechanisms. *A. fumigatus* is a prevalent airborne fungal pathogen, and causes severe and usually fatal invasive infections in immuno-compromised hosts (LATGÉ, 2003). Diseases caused by *Aspergillus fumigatus* can be divided into three categories: allergic reactions, colonization with restricted invasiveness are observed in immunocompetent individuals and systemic infections with high mortality rates occur in immunocompromised patients (BRAKHAGE; LANGFELDER, 2002).

Because of the dramatic rise in fungal infections and the current trend toward increasing awareness in traditional medicine, plant-derived antifungal compounds are attracting much interest as natural alternatives owing to their versatile applications (KHAN et al., 2010). Plants with medicinal properties have been focus of scientific studies all over the world in order to discovery new and effective antimicrobial compounds (OLIVEIRA et al., 2007).Essential oils possess a broad spectrum of antimicrobial activities attributed to the high content of phenolic derivatives, but a few have been reported to have significant antifungal activity (KHAN et al., 2010, BAKKALI et al., 2008).

*Cymbopogon winterianus* is a plant popularly known as citronella, which is cultivated in India and Brazil. Its essential oil shown many biological activities, like anticonvulsivant properties (QUITANS-JÚNIOR et al., 2008), antibacterial (DUARTE et al., 2007; OUSSALAH et al., 2007) and antifungal (PEREIRA et al., 2011, DUARTE et al., 2005) properties.

The aim of this work was, therefore, to investigate the antifungal activity of essential oil of *C. winterianus* against *A. fumigatus* strains.

## Material and Methods

### Essential Oil

*Cymbopogon winterianus* Jowitt ex Bor was grown, collected and had the essential oil extracted by hydrodistillation using a Clevenger-type apparatus at the Center for Technology Training at the Federal University of Paraíba, Bananeiras city (Paraiba, Brazil) by Dr. Paulo Alves Wanderley.

The plant was identified by Dra. Rita Baltazar de Lima in Laboratory of Botany, Department of Systematics and Ecology of the Center of Exact and Nature Sciences of the Federal University of Paraíba. The voucher specimen was deposited in the Herbarium Prof. Lauro Pires Xavier of the Department of Systematics and Ecology, Federal University of Paraíba under the code JPB 41387.

### Fungal samples

The strains of *A. fumigatus* tested belong to the collection of the Mycology Laboratory, Federal University of Paraíba and include ATCC 46913, ATCC 40640, IPP 21.

### Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

Stock cultures were kept on sterile Sabouraud Dextrose Agar (SDA) slants under 7℃ (± 1℃). For preparing the inoculum used in antimould assays

were used 7 days-old culture grown on sterile SA at 25-28℃. After the incubation period, the mould conidia were taken by adding sterile NaCl 0,85% on the growth media followed for gentle shaking for 30 seconds. Mould conidia was counted using hemocytometer. The conidial suspension was adjusted using sterile NaCl 0,85% to contain approximately 5 x $10^6$ conidia/mL.

The MIC was determined by the microdilution method using microplates of 96 wells (CLEELAND; SQUIRES, 1991; ELOFF, 1998). Conidial suspension from 7-day-old of *A. fumigatus* culture was prepared and standardized by hemocytometer in sterile 0,85% NaCl for susceptibility testing as described previously. In a 96-well plate was added Sabouraud broth and essential oil of *C. winterianus* at concentrations of 10.0000 to 39µg/mL. The MIC determination was conducted with an inoculum of approximately 2,5 x$10^5$ conidia /mL of the microorganism in each well. The plates were incubated at 25 - 28℃ for 72 hours. In 72 hours (and confirmed at 7 days) there was a visual observation of fungal growth. The MIC was considered as the lowest oil concentration that inhibited growth of the microorganism. To determine the MFC, 10µL sample from each well where no fungal growth was subcultured on a plate containing SDA, the SDA plating were incubated at 25 - 28 ℃ for 72 hours. The MFC was defined as the lowest concentration that showed either no growth or fewer than three colonies on ASD. There were three independent experiments on different occasions with consistent results (PEREIRA et al., 2011, ESPINEL-INGROFF et al., 2002). Amphotericin B and ketoconazole were used as control.

Values of MIC between 50-500µg/mL indicates strong activity, moderate activity is MIC values between 600-1500µg/mL and weak activity is MIC above 1500µg/mL (SARTORATTO et al., 2004).

**Mycelial growth inhibition**

Inhibition of the fungal mycelial growth of *A. fumigatus* was determined using the poisoned substrate technique (diluition in solid medium) by the daily measure of the radial mycelial growth on SDA added essential oil at MIC/2, MIC and MICx2. For this, a 2 mm plug taken from a 10 days-old mould culture grown on SDA slants at 25 - 28 °C were placed on the center of sterile SDA Petri dishes containing the essential oil. At different intervals (0, 2, 4, 6, 8, 10, and 12 days) after incubation at 25 - 28 °C, the radial mycelial growth was measured (mm). Control included in this assay was the observation of the mould radial growth on SDA without adding essential oil, or added of amphotericin B (at MIC) or ketoconazole (at MIC). Results were expressed as means ± standard deviation of three independent experiments realizated on different occasions (DAFERERA et al., 2003; ADAM et al., 1998).

**Germination analysis**

Different concentrations of the essential oil were tested for conidia germination. Aliquots of 0.5 mL of the essential oil solutions at different concentrations were mixed with 0.5 mL of the mould conidia suspension (approximatelly 5 x $10^6$ conidia/ mL) followed by shaking using Vortex for 30 seconds. 0.1mL of the mixture was placed on separated glass slides which were incubated in a moist chamber at 25-28°C for 24 hours. At the end of the incubation period, each slide was fixed with lactophenol-cotton blue stain and observed under the microscope for conidia germination. About 200 conidia were counted and the percentage of conidia germination was calculated. Each assay

was performed three times and the results were expressed as the average of the three repetitions (RANA et al., 1997).

**Leakage Effect**

The inoculum of conidia suspension was prepared as described previously in sterile NaCl 0,85% to contain approximately 5 x $10^6$ conidia/mL.

Conidia suspension of *A. fumigatus* was washed with sterile NaCl 0,85% and resuspend in sterile NaCl 0,85%, was used to prepare the inoculum. Tubes (final volume 12mL) containing inoculum of 5 x $10^6$ conidia/ mL and essential oil of *C. winterianus* at MIC/2, MIC, MICx2 and MICx4 were left from 2 to 24 h. Amphotericin B (at MIC) and ketoconazole (at MIC) were used as control drugs. At each period of time (2, 4 and 24 hour), 3mL aliquots were removed from each test solution and centrifuged at 3000 rpm for 10 min. The supernatants were collected for absorbance analysis at 260nm, considering as 100% release the absorbance produced by conidia treated with 1.2 N $HClO_4$ at 100°C, 30 min. Results were means ± standard deviation of the percentage of leakage compared with the control with $HClO_4$ (100%), values were obtained from three independent assays (ESCALANTE et al., 2008; SVETAZ et al., 2007; LUNDE; KUBO, 2000).

**Ergosterol Effect**

The MIC of essential oil of *C. winterianus* against *A. fumigatus* was determined by the microdilution method using microplates of 96 wells as explained above, in the absence and in the presence of different concentrations (150, 250 and 400 µg/ mL) of ergosterol added to the assay medium, in different lines of the same microplate. Amphotericin B was used as a control drug. MICs

were read at 3 and 7 days of incubation. This assay was carried out in two independent assays (SILVA JUNIOR et al., 2010; ESCALANTE et al., 2008).

**Results and discussion**

Part of the difficulty in treating fungal infections is the lack of a number of effective antifungal drugs. Current treatments include a variety of azoles that inhibit the synthesis of ergosterol, polyenes, that bind ergosterol resulting in a fungal membrane distortion, 5-flucytosine that inhibits nucleic acid biosynthesis and, most recently, the echinocandin lipopeptides that inhibit (1,3)β-glucan synthase activity, resulting in fungal cell death (GEORGE; SELITRENNIKOFF, 2006). Sikkema et al., 1995 attributed the lipophilic character of some substances (including essential oils) and it ability to interaction with hydrophobic parts of the cell, as an important role in the mechanism of the toxic action.

In this work, we studied the activity of essential oil of *C. winterianus* against *Aspergillus fumigatus*. Here we determined MIC and MFC of the essential oil, analysed the radial mycelial growth, the germination of conidia, the alterations in morphological forms of microorganism, measurement of cellular leakage,and determine if oil binds to the fungal membrane sterol.

The essential oil of *C. winterianus* was active against the filamentous fungi *Aspergillus fumigatus* with minimum inhibitory concentrations (MICs) equals 312,5µg/mL. The MFC found was 625µg/mL for all strains tested (table 1). This essential oil had strong activity because the MIC was between 50-500µg/mL (SARTORATTO et al., 2004).

Effect of the essential oil on the radial mycelial growth in solid medium is showed in figure 1**.** The essential oil provided reduction in the mycelial growth at MIC (reduction 82% of growth) and MICx2 (reduction 97,6% of growth) concentrations when compared with the control assay at 12 days of incubation. The MIC/2 concentration allowed the mycelial mould growth. The MIC found to amphotericin B and ketoconazole against *Aspergillus fumigatus* (strain ATCC 46913) was 2µg/mL and 8µg/mL, respectively. It can be observed that both antifungals inhibited the radial mycelia growth.

It was reported that other essential oils were active against moulds. Essential oils of *Hyptis suaveolens* and *Cinnamomum zeylanicum* strongly inhibited the mycelial growth of *A. fumigatus* and *A. parasiticus* (MOREIRA et al., 2010); and *A. fumigatus*, *A. flavus* and *A. niger* (CARMO et al., 2008a), respectively. The oil of *Origanum vulgare* acted on mycelia growth of *A. fumigatus, A. flavus*, and *A. niger* (CARMO et al., 2008b).

The activity to different concentrations of essential oil against *A. fumigatus* conidia germination is presented in the table 2. As can be noted, the essential oil exhibited a strong inhibition of conidia germination at concentrations MIC, MICx2 and MICx4 and weak inhibition at concentration MIC/2. It was reported that essential oil of *C. winterianus* showed a strong inhibitory power on the conidial germination of *T. rubrum* and *T. mentagrophytes* (PEREIRA, 2009). Essential oil of *Cinnamomum zeylanicum* inhibited spore germination of *A. fumigatus, A. flavus* and *A. niger* (CARMO et al 2008a).

About virulence factors (penetration and dissemination factors) of *A. fumigatus* and other true molds, they are able to penetrate blood vessels and grow along the vessel lumen as they invade tissue. Almost all pathogenic fungi can grow in more than one form. *Aspergillus* species, which are classical filamentous molds, have morphological versatility and form conidia that are the infectious agent. Many molds form conidia, or vegetative spores, acattered by wind or water, these small, resistant cells serve as a mode of dissemination. In the case of aspergillosis, conidia serve as the propagule that infects debilitated patients. Conidia bind to fibrinogen and laminin, but the binding properties are lost as the conidia germinate and produce hyphae. Hence, this cell form may play a role more complicated than that of a simple vector in establishing the infection (BURIK; MAGEE, 2001). The virulence of *A. fumigatus* can then be caused either by the production of fungal proteins that promote mycelia growth into the lung parenchyma or by structural features of the conidia that confer resistance to the host's antifungal mechanisms (LATGÉ, 2001).

Considering the lipophilic character of essential oil of *C. winterianus* and its possibility of interacting with fungal membranes, we studied the effect of several concentrations of essential oil (MIC/2, 1x, 2x and 4x MIC) on the irreversible damage to the fungal membrane by measuring the leakage of 260nm-absorbing intracellular materials released to the medium at different time intervals (2, 4 and 24 hours). The figure 2 shows leakage from mould cells over 2, 4 and 24 hours period of interaction with essential oils at MIC/2, MIC, MICx2 and MICx4 concentrations. The result showed that essential oil at MIC, 2xMIC and 4xMIC at 24 hours produced damage to the fungal membrane, respectively 40,66%, 67,33% and 85,33% of cellular leakage. It did not produce any damage

at MIC/2 (6,66% of cellular leakage). Perchloric acid was considered to produce 100% cellular leakage. The amphotericin B and the ketoconazol also caused cellular lysis, probably due their interference with the membrane, because one binds and the other inhibits the synthesis of ergosterol. They can alter the permeability and fluidity of the membrane (ODDS et al., 2003).

*A. fumigatus* conidia (spores) are gray-green in color and only 2.5-3.0 µm in diameter, allowing them to reach the lung alveoli. Conidia contain a single haploid nucleus. The conidiophores extends from a foot cell at right angles to the mycelium and culminates in a broadly clavate vesicle (20-30µm in diameter). A single layer of cells (phialides) is attached to the apical side of the vesicle. The conidia result from a constriction of an elongated portion of the phialide (BRAKHAGE; LANGFELDER, 2002).The damage to the conidia could be observed by measuring the release of intracellular components to the medium from treated conidia. Conidia components that absorb at 260nm represent one class of leakage components, primarily nucleotides, of which uracil-containing compounds exhibit the strongest absorbance (LUNDE; KUBO, 2000; ESCALANTE et al., 2008, SVETAZ et al., 2007).

The inherent activity of an oil can be expected to relate to the chemical configuration of the components, the proportions in which they are present and to interactions between them. An additive effect is observed when the combined effect is equal to the sum of the individual effects. Antagonism is observed when the effect of one or both compounds is less when they are applied together than when individually applied. Synergism is observed when the effect of the combined substances is greater than the sum of the individual effects. Some studies have concluded that whole essential oils have a greater

antibacterial activity than the major components mixed, which suggests that the minor components are critical to be activity and may have a synergistic effect or potentiating influence (BURT, 2004).

The most important characteristic that these compounds have in common is that they are more or less hydrophobic. Lipophilicity is a physicochemical feature of a compound. Cell lysis can be observed on the interactions between lipophilic compounds and membrane, it changes lipid-protein and lipid-lipid interactions (SIKKEMA et al., 1995).

The interactions of lipophilic compounds with the phospholipid bilayer causes dramatic changes in the structure of the membrane. Accumulation of such compounds in the hydrophobic part of the membrane will disturb the interactions between the acyl chains of the phospholipids, leading to modification of membrane fluidity, and eventually may result in swelling of the bilayer (SIKKEMA et al., 1995).

To verify whether essential oil of *C. winterianus* acts as an antifungal agent by acting on ergosterol, it was observed the values of the MIC in the presence of increasing concentrations of ergosterol. If this mechanism operates, the MIC of the test compounds will be much higher in the presence of ergosterol than that in its absence (SILVA JUNIOR et al., 2010). The results had shown that MIC of amphotericin B against *A. fumigatus* increased with the increase of ergosterol to 4x, 8x and 8x MIC, when the concentration of exogenous ergosterol were 150, 250 and 400µg/mL, respectively. The MIC of the *C. winterianus* was raised to 2x, 4x and 4x MIC in the presence of ergosterol (150, 250 and 400µg/mL). This suggests an interaction between

essential oil and the ergosterol, since it is known that *Aspergillus fumigatus* has ergosterol, this oil can be target for study to new antifungal (ALCAZAR-FUOLI et al., 2008).

The target of polyene antifungal agents is the fungal membrane, on which they act directly. Amphotericin B binds to ergosterol, and this interaction leads to the formation of aqueous pores consisting of an annulus of eight amphotericin B molecules linked hydrophobically to the membrane ergosterol, which is thereby displaced from its normal phospholipid interactions (CANUTO; RODERO, 2002). Other polyene antifungals which bind to ergosterol are natamycin (WELSCHER et al., 2008) and nystatin (ODDS et al., 2003). Amphotericin B in the presence of exogenic ergosterol shown MIC enhancement against *T. mentagrophytes*, confirming the mode of action of amphotericin B, by binding to membrane ergosterol (YAMAGUCHI et al., 2009). It was reported that cecropin A, a member of a family of lytic peptides, has antifungal activity by ergosterol binding and forming large pores in cell membranes causing lysis in *Aspergillus flavus, A. fumugatus, A. niger*, *Fusarium moniliforme* and *F. oxysporum* (DeLUCCA et al., 1997).

Unfortunately, resistance to older antifungal drugs has gained more and more importance in the past years. Therefore, new antifungals agents are still needed, in particular active compounds which might not cause development of resistance very rapidly. However, the development of resistance is more probable, if an antifungal inhibits only one essential function of the pathogen, e.g. fungal reproduction or membrane synthesis. In particular, new antifungals targeting several functions of the fungus simultaneously are urgent needed (BORELLI et al., 2008). Essential oils comprise a large number of components

and it is likely that their mode of action involves several targets in the cell (BURT, 2004). Despite in this study shows the interaction of the essential oil of *C. winterianus* with ergosterol, other interactions with *A. fumigatus* are possible. In conclusion, a broad inhibition of fungal growth in addition to its availability as natural volatile product, justifies its possible rational use of the essential oil of *C. winterianus* as an alternative antifungal.

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Table 1. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of essential oil *C. winterianus* against strains of *A. fumigatus*.

| *A. fumigatus* samples | MIC (µg/mL) | MFC (µg/mL) |
|---|---|---|
| 1- ATCC 46913 | 312,5 | 625 |
| 2- ATCC 40640 | 312,5 | 625 |
| 3- IPP21 | 312,5 | 625 |

Table 2. Effect of essential oil of *C. winterianus* on conidia germination of *A. fumigatus* ATCC 46913 (results expressed in percentage of conidia germination).

| Concentrations of essential oil | Conidia germination |
|---|---|
| Control | 100% |
| MIC/4 | 100% |
| MIC/2 | 90% |
| MIC | 3% |
| MIC x 2 | 0% |
| MIC x 4 | 0% |

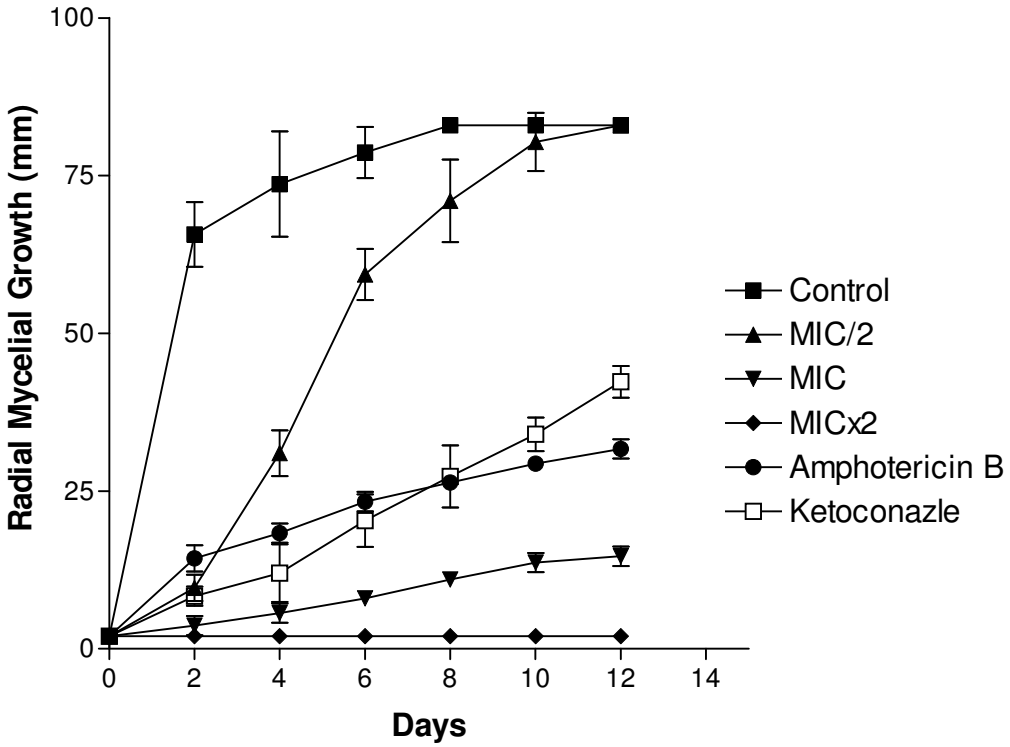


Figure 1. Effect of essential oil of *C. winterianus* on radial mycelial growth of *A. fumigatus* ATCC 46913.

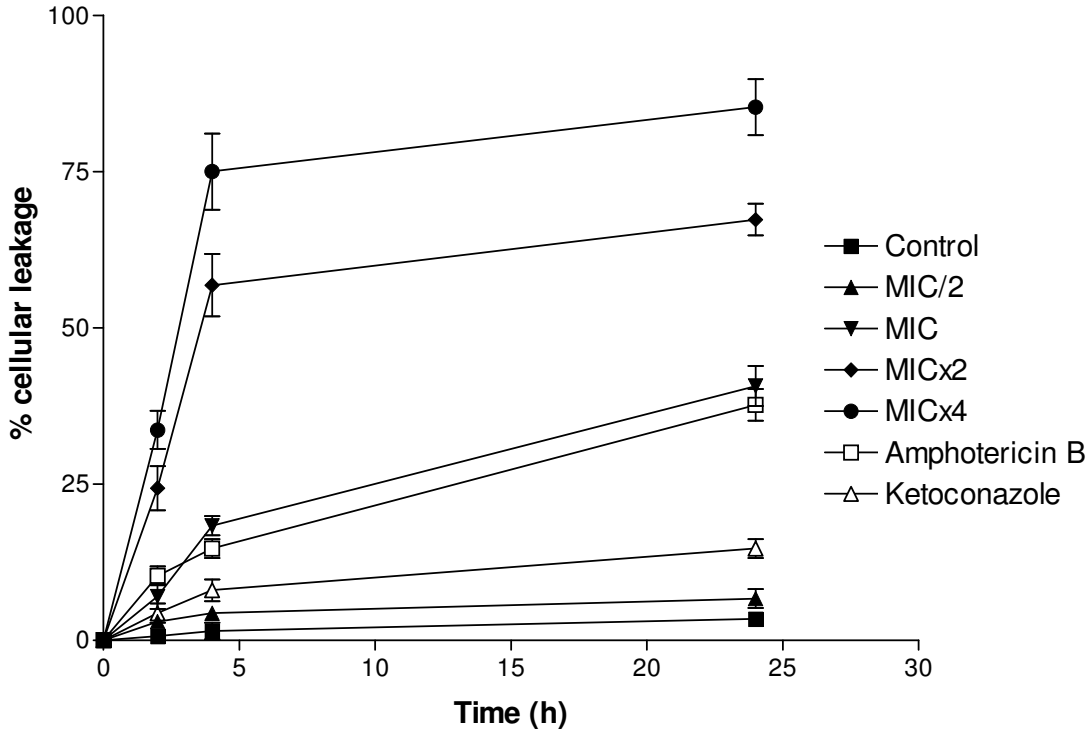


Figure 2- Percent of leakage of conidia of *A. fumigatus* ATCC 46913 untreated (control) or treated with essential oil of *C. winterianus* at MIC/2, MIC, MICx2 and MICx4 and with amphotericin B (MIC) or ketoconazole (MIC). Cells treated with perchloric acid was considered to produce 100% cellular leakage.

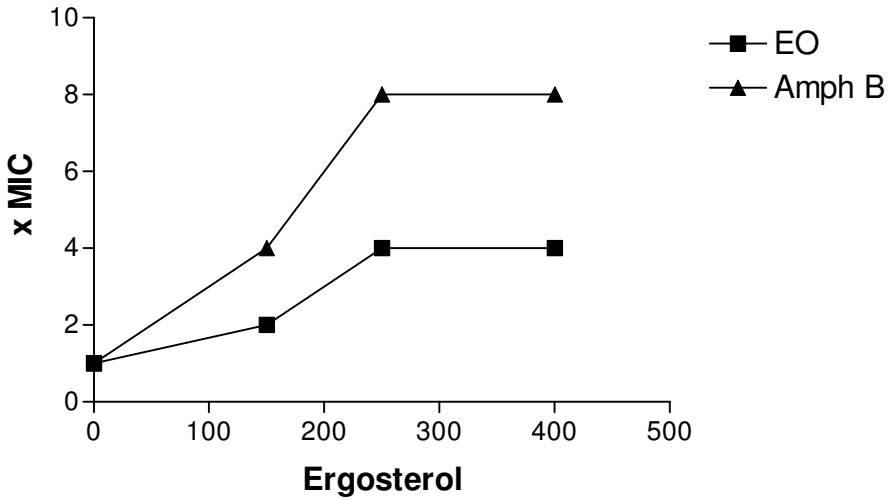


Figure 3- Effect of different concentrations of exogenous ergosterol (150-400µg/mL) on the MIC of both essential oil of *C. winterianus* and amphotericin B for *A. fumigatus* ATCC 46913. EO= essential oil of C. winterianus, AmphB= amphotericin B.

## 5- Conclusões

- O óleo essencial (OE) de *C. winterianus* possui atividade contra *Candida albicans, Aspergillus flavus e A. fumigatus*;
- O óleo possui atividade fungicida dependente da concentração contra *C. albicans* induzindo alterações na micromorfologia, com a inibição da formação de pseudo-hifa e de clamidoconídio;
- O OE inibe o crescimento micelial radial, como também a germinação de conídios de *A. flavus e A. fumigatus;*
- O óleo (CIM, CIMx2 e CIMx4) causa lise de células de *C. albicans* e de conídios de *A. flavus e A. fumigatus ;*
- O OE liga-se ao ergosterol, o que pode aumentar a permeabilidade da membrana e levar à lise das células de *C. albicans* e de *A. flavus e A. fumigatus*.

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