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POTENCIAL ANTIMICROBIANO E ANTIBIOFILME DO ÓLEO ESSENCIAL DE FOLHAS DE *Croton blanchetianus* Baill. SOBRE MICRORGANISMOS DE INTERESSE CLÍNICO

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RESUMO

O uso inadequado de antibióticos favorece que os microrganismos adquiram resistência,sendo considerado um grave problema recorrente de saúde pública, podendo causar morbidade e mortalidade entre os pacientes. A formação de biofilmes é um dos fatores de resistência dos microrganismos, podendo estar associados em fatores bióticos e abióticos. Diante disso, é importante a busca por novas moléculas bioativas que minimizem tais problemas. Produtos naturais, como os óleos essenciais (OEs), têm ganhado destaque como fontes de novos agentes antimicrobianos. Esse estudo teve como objetivo avaliar a composição química, toxicidade, e o potencial antimicrobiano e antibiofilme do OE extraído de folhas de *Croton blanchetianus* Baill., bem como seus mecanismos de ação sobre microrganismos de interesse clínico. A análise por CG/MS revelou a presença de compostos majoritários como *espatulenol* (20,03%); *biciclogermacreno* (5,92%); *óxido de cariofileno* (5,81); *eucaliptol* (5,62%). O óleo na concentração de 50 μg mL^{-1} foi capaz de inibir o crescimento planctônico de *Candida albicans* e *C. parapsilosis* em 78,55 e 75,94%, respectivamente. Também houve inibição na formação de biofilme de *C. albicans* e *C. parapsilosis* sob ação do óleo com 44,12 e 74,13%, respectivamente. Não houve inibição satisfatória sob ação do óleo contra bactérias, *Cryptococcus neoformans*, *Candida krusei* e *Candida tropicalis*. Análises de microscopia de fluorescência detectaram a superprodução de espécies reativas de oxigênio (ROS), a formação de poros na membrana celular e morte celular programada nas leveduras. Análise de microscopia eletrônica de varredura mostrou que o tratamento com o OE causou dano na morfologia em *C. albicans* e *C. parapsilosis*. Além disso, foi avaliado que o óleo não é tóxico à eritrócitos do tipo A, B e O+. Assim, o óleo de *C. blanchetianus* apresenta potencial antifúngico e antibiofilme contra infecções causadas por *C. albicans* e *C. parapsilosis*.

Palavras-chave: biofilmes; infecções; mecanismo de ação; produtos naturais.

ABSTRACT

The inappropriate use of antibiotics favors the acquisition of resistance by microorganisms, and is considered a serious recurring public health problem, which can cause morbidity and mortality among patients. The formation of biofilms is one of the factors of microorganism resistance and may be associated with biotic and abioticfactors. Therefore, the search for new bioactive molecules that minimize such problems is important. Natural products, such as essential oils (EOs), have gained prominence as sources of new antimicrobial agents. This study aimed to evaluate the chemical composition, toxicity, and the antimicrobial and antibiofilm potential of EOs extracted from *Croton blanchetianus* Baill. leaves, as well as their mechanisms of action on microorganisms of clinical interest. GC/MS analysis revealed the presence of major compounds such as *spatulenol* (20.03%); *bicyclogermacrene* (5.92%); *caryophyllene oxide* (5.81); *eucalyptol* (5.62%). The oil at the concentration of 50 µg mL^{-1} was able to inhibit the planktonic growth of *Candida albicans* and *C. parapsilosis* by 78.55 and 75.94%, respectively. There was also inhibition in biofilm formation of C. albicans and *C. parapsilosis* under oil action with 44.12 and 74.13%, respectively. There was no satisfactory inhibition under oil action against bacteria, *Cryptococcus neoformans*, *Candida krusei* and *Candida tropicalis*. Fluorescence microscopy analyses detected the overproduction of reactive oxygen species (ROS), the formation of pores in the cell membrane and programmed cell death in the yeasts. Scanning electron microscopy analysis showed that treatment with the EO caused damage to morphology in *C. albicans* and *C. parapsilosis*. Furthermore, it was evaluated that the oil is not toxic to type A, B and O+ erythrocytes. Thus, *C. blanchetianus* oil has antifungal and antibiofilm potential against infections caused by *C. albicans* and *C. parapsilosis*.

Keywords: biofilms; infections; mechanism of action; natural products.

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LISTA DE ABREVIATURAS E SIGLAS

EROS Espécies reativas de oxigênio

FAO Organização das Nações Unidas para a Alimentação e a Agricultura

IRAS Infecções Relacionadas à Assistência de Saúde

NIH Instituto Nacional de

OE Óleo essencial

OMS Organização Mundial da Saúde

QS *Quorum sensing*

RAM Resistência antimicrobiana

SPE Substâncias poliméricas extracelulares

SUMÁRIO

1 INTRODUÇÃO

O uso indevido ou excessivo de antibióticos vem sendo bastante prejudicial à população, uma vez que os microrganismos possuem mecanismos que favorecem o aumento da resistência aos fármacos existentes (THORNBER *et al.*, 2020), o que acarreta doenças infecciosas capazes de intensificar a morbidade e mortalidade depacientes. Ademais, o custo torna-se mais oneroso quando é realizado um tratamento antimicrobiano a fim de erradicar os microrganismos resistentes (FISHER *et al.*, 2022). O século XXI está sendo marcado pelo aumento significativo de resistência antimicrobiana (RAM), visto que os tratamentos estão sendo ineficazes contra bactérias e fungos (MURRAY *et al*., 2022). Com isso, a Organização Mundial de Saúde (OMS) declarou que a RAM está sendo considerada uma das 10 ameaças globais a humanidade (WHO, 2022).

A produção de biofilmes é um dos fatores de resistência aos microrganismos, onde estão embebidos em uma superfície que pode ser biótica ou abiótica (FALANGA *et al.*, 2022), aumentando a RAM. Os biofilmes podem se estabelecer em próteses, válvulas cardíacas, dispositivos intrauterinos e cateteres (OLIVARES *et al.*, 2020). De acordo com o Instituto Nacional de Saúde (NIH) dos EUA, os biofilmes podem ser responsáveis por 80% das complicações causadas por infecções humanas (ZARNOWSKI *et al.*, 2014; KUMAR *et al.*, 2017).

Uma das estratégias para minimizar esses problemas é a busca de novos compostos antimicrobianos, com ênfase em produtos naturais. Os óleos essenciais (OEs) são produtos naturais definidos como misturas de compostos voláteis e hidrofóbicos (GUERRA *et al.*, 2019), que podem apresentar propriedades antimicrobianas e efeitos promissores contra biofilmes (POLI *et al*., 2018). Isso pode ser atribuído pelas propriedades de terpenos/terpenóides, fenólicos que os OEs possuem, pois estes compostos são capazes de romper a membrana celular e induzir a morte celular nos patógenos, devido ao seu caráter lipofílico e baixo peso molecular (NAZZARO *et al.*, 2017; GUIMARÃES *et al.*, 2021).

Uma espécie vegetal produtora de OE é a *Croton blanchetianus*, conhecida como “marmeleiro preto”, que pertence à família Euphorbiaceae (FIRMINO *et al*.,

2019). Essa planta possui propriedades antibacteriana, antibiofilme, antifúngica, antioxidante, entre outras (AQUINO et al., 2017; NUNES *et al.*, 2022).

Pelo exposto, esse estudo visou avaliar a composição química, toxicidade, e o potencial antimicrobiano e antibiofilme do OE extraído de folhas de *Croton blanchetianus* Baill., bem como seus mecanismos de ação sobre microrganismos de interesse clínico.

2 CAPÍTULO I – REVISÃO DE LITERATURA

2.1 Resistência antimicrobiana

Em 1928, o cientista Alexander Fleming descobriu a penicilina. Contudo, somente em 1942 houve sua aplicação nas terapias de doenças microbianas, na qual houve um avanço significativo na saúde pública (STEKEL, 2018). Desde então, muitos antibióticos foram introduzidos no mercado farmacêutico para tratar doenças em humanos. As décadas entre 1950 e 1970 ficaram conhecidas como “Era de Ouro” do desenvolvimento de antibióticos (LUEPKE *et al.*, 2017), surgindo fármacos compotencial terapêutico muito importante para a saúde, como foi o caso das cefalosporinas.

Dentre os anos de descobertas de novos antibióticos, Selman Abraham Waksman, em 1947, definiu a expressão “antibiótico” como "Um material químico, que é produzido por microrganismos, é um antibiótico que retém bactérias e outros microrganismos, e até os mata" (WAKSMAN, 1947). Desde então, os sistemas de vigilância nacionais e regionais de vários países apontam um crescimento de aproximadamente 30% do uso de antibióticos em todo o mundo (GELBAND *et al.*, 2015). No entanto, o uso indevido ou excessivo de antibióticos tem levado ao surgimento de resistência, considerados pela OMS como uma ameaça a saúde humana esegurança alimentar das pessoas (DADGOSTAR, 2019). A previsão é que ocorra 10 milhões de mortes por ano até 2050 devido a RAM (O’NEILL, 2014; TAGLIABUE; RAPPUOLI, 2018).

Na medida em que ocorre um aumento na RAM, as Infecções Relacionadas à Assistência de Saúde (IRAS) (LIU; DICKTER, 2020) também possui um aumento significativo. E isso é bastante prejudicial aos pacientes com comorbidades, sejam eles

com doenças autoimunes, problemas respiratórios, idade avançada, feridas crônicas,diabetes mellitus (FERREIRA *et al*., 2017; DESPOTOVIC *et al*., 2020).

As bactérias patogênicas são responsáveis por mais de 70% de infecções hospitalares e são as mais resistentes aos antibióticos (RAHMAN *et al.*, 2022), assim como os fungos patogênicos que são responsáveis por causar doenças em bilhões de pessoas e matar cerca de 1,5 milhão anualmente (FISHER *et al.*, 2018; LEE *et al.*, 2020).

2.2 Bactérias de interesse clínico

As bactérias são organismos unicelulares e procariontes que podem ser classificadas em Gram-positivas e Gram-negativas (VARGHESE; BALACHANDRAN, 2021). Zhang e colaboradores (2023) conceituam que as bactérias Gram-positivas possuem uma parede celular rígida e espessa composta de peptidoglicano, além disso, possuem ácido teicóico e ácido lipoteicóico ligadas ao peptidoglicano; enquanto asbactérias Gram-negativas, a camada de peptidoglicano é fina e coberta por uma membrana externa com lipopolissacarídeos aderidas.

De acordo com a OMS, a maioria das infecções clínicas são causadas por bactérias Gram-negativas, estas por serem mais resistentes a penetração de fármacos antimicrobianos por conta da sua membrana externa rica em lipopolissacarídeos (THEURETZBACHER *et al.*, 2020; ZHU *et al.*, 2021). Bactérias Gram-negativas como *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella enterica* e *Enterobacter aerogenes* são mais tolerantes aos medicamentos existentes, com isso, inspira maiores cuidados do sistema público de saúde.

A bactéria *E. coli* possui resistência contra vários tipos de antibióticos (LOOFT *et al.*, 2012). *P. aeruginosa* é uma bactéria que possui uma alta tolerância as mudanças do ambiente, podendo ser encontrada em solo e reservatórios de água poluída (DIGGLE; WHITELEY, 2020) e sendo capaz de causar IRAS como fibrose cística (OLIVARES *et al*., 2020). *K. pneumoniae* pode se associar ao trato gastrointestinal ou nasofaringe e causar infecção (WANG *et al.*, 2020). É responsável por cerca de 83% daspneumonias hospitalares (ALI *et al.*, 2022). *E. aerogenes* pode causar infecções neonatais (NAHER; AL-SA'ADY, 2020) e infecções graves a pacientes imunocomprometidos devido à sua resistência a antibióticos(SHANTIAE; TAJBAKHSH; MOMTAZ, 2022). A *S. enterica* pode colonizar os humanos, animais e pode ser encontrada no ambiente (KNODLER; ELFENBEIN, 2019). Ao ingerir algum alimento contendo *S. enterica*, esta invade o intestino no íleo e cólon, podendo causar gastroenterite neutrófila que pode disseminar para sítios sistêmicos e causar septicemia (KNODLER; ELFENBEIN, 2019).

2.3 Fungos relacionados à saúde humana

Os fungos podem causar diversas doenças em humanos, como síndromes alérgicas, infecções mucocutâneas e sistêmicas (BRANCO; MIRANDA; RODRIGUES, 2023). As infecções fúngicas são problemas recorrentes à população. A falta de orientação ou acesso aos tratamentos iniciais à infecção aumenta a taxa de mortalidade quando estas são tratadas tardiamente (REDDY, PADMAVATHI, NANCHARAIAH, 2022).

De acordo com Rokas (2022), as infecções superficiais como a pele, cabelo, unhas e olhos pode atingir 1 milhão de pessoas, já as infecções das mucosas como oral e vaginal podem afetar cerca de 135 milhões, e as infecções alérgicas, crônicas graves e agudas invasivas afetam aproximadamente 23,3 milhões de indivíduos. São números impressionantes e que causa certas preocupações quando os fungos patogênicos se tornam resistentes aos fármacos comprometendo a profilaxia e o tratamento de infecções.

Existem quatro classes de antifúngicos utilizados continuamente há muito tempo, dentre eles: os polienos, os azóis, as equinocandinas e o análogo de pirimidina 5- flucitosina (FISHER *et al.*, 2022). Mas com o passar dos tempos, a falha destes para tratamento pode ser comum por conta da AMR. A resistência se dá por diversos fatores, seja por alterações genéticas no sítio de ligação ao alvo, por via de superexpressão da quantidade de alvo disponível e/ou alteração da concentração efetiva do fármaco ou inibição da ativação do pró-fármaco para a flucitosina (EDLIND; KATIYAR, 2010; ROBBINS; CAPLAN; COWEN, 2017).

Diante disso, os fungos do gênero *Candida*, *Cryptococcus* e *Aspergillus* apresentam grande relevância clínica pois estes podem causar infecções sistêmicas graves e presentes entre os três patógenos humanos mais letais (BASTOS *et al.*, 2021). *C. neoformans* era conhecido como agente causador de uma doença infecciosa,

silenciosa e inicialmente com um diagnóstico difícil (SALAZAR *et al.*, 2020). Geralmente, é encontrado no ambiente associado a excrementos de pombos. Pode causar meningite criptocócica, que é uma das principais causas de morbidade e mortalidade em indivíduos imunocomprometidos (BERMAS; GEDDES-MCALISTER, 2020).

As leveduras podem estar presentes em diversas partes do corpo humano, sejam elas internas ou externas. O gênero *Candida* pertence ao filo Ascomycota, e podem estar presentes em meios aquáticos, solos e alguns colonizam os seres humanos e animais. As infecções causadas por esses agentes podem ser: candidíase oral, vaginal, orofaríngea, onicomicose e sistêmica (KHODADADI *et al.*, 2021). Algumas espécies de *Candida* como *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis* e *C. krusei* são responsáveis por cerca de 90% de casos de candidíase na população global (SINGH; TÓTH; GÁCSER, 2020) e isso intensifica quando ocorre o uso inadequado de antibióticos em pessoas com sistema imunológico comprometido. Uma das doenças quemais se agrava dentre as diversas estirpes de *Candida* é a candidemia, doença nosocomial sistêmica que pode estar atribuída a 30-40% de mortalidade (CUERVO *et al.*, 2019; KOHELER *et al.*, 2019). Dentre as mais variadas espécies de *Candida, C. albicans, C. tropicalis C. parapsilosis* e *C. krusei* são responsáveis por cerca de 10 a 47% do aumento mundial de mortalidade de crianças hospitalizadas (MOTTA *et al.*, 2017).

C. albicans é um organismo comensal que coloniza o trato gastrointestinal, trato reprodutivo feminino e cavidade oral (LIMON; SKALSKI; UNDERHILL, 2017), mas em um sistema imune baixo, causa infecções sistêmicas ou localizadas (BOJANG*et al.*, 2021). Essa espécie é capaz de mudar morfologicamente de levedura para forma de hifas e pseudohifas, num processo denominado morfogênese, e, por isso, podeinvadir as membranas mucosas até a corrente sanguínea (JOSHI *et al.*, 2022), o que a torna um patógeno com uma importância clínica a ser trabalhada.

C. parapsilosis é um patógeno da pele humana que pode estar associado em superfícies mucosas, unhas e trato gastrointestinal (PAMMI *et al.*, 2013), causando doenças no trato urinário, endocardite, sistema nervoso central (CHEN *et al.*, 2006; BHALLA *et al.*, 2018). A principal via de infecção por essa espécie é através de implantes cirúrgicos ou cateteres (COBO *et al.*, 2017).

C. tropicalis pode ser encontrado comumente na natureza, sendo um colonizador comum da pele humana, cavidade oral e trato digestivo (GÓMEZ- GAVIRIA; RAMÍREZ-SOTELO; MORA-MONTES, 2023). Entretanto, também é um patógeno oportunista que pode causar infecções nosocomiais, e é considerada uma segunda espécie mais recorrente isolada depois de *C. albicans* (ZUZA-ALVES; SILVA- ROCHA; CHAVES, 2017; WANG *et al.*, 2021). Possui resistência aos medicamentos o que contribui para a candidíase invasiva (FAN *et al.*, 2017; WANG *et al.*, 2021).

Doenças causadas por *C. krusei* podem atingir uma taxa de mortalidade entre 20 e 67%, o que pode ser atribuída a sua baixa resposta as terapias antifúngicas (NAVARRO-ARIAS *et al.*, 2019), além disso, pode causar espondilite (OVERGAAUW *et al.*, 2020).

2.4 Biofilmes microbianos

Um dos fatores que podem influenciar na AMR por bactérias ou fungos patogênicos é a formação de biofilmes. Os biofilmes são definidos como comunidades microbianas inseridas em uma matriz polimérica extracelular (CIOFU *et al.*, 2022), tornando-os capazes de suportar diversos fatores, tal como, fagocitose, exposição a UV, entre outros (GUPTA *et al.*, 2016), além disso, os biofilmes podem ser prejudiciais à saúde humana, já que conseguem se aderir em meios bióticos e abióticos (CIOFU *et al.*, 2022). Os biofilmes são mais estáveis devido as substâncias poliméricas extracelulares (EPS), que produz polímeros tridimensionais (3D) que se ligam às células do biofilme e os mobilizam (MISHRA *et al.*, 2023). Outros fatores que estabilizam os biofilmes sãoas interações intensas de comunicação entre célula-célula, criação de microconsórcios sinérgicos, exopolissacarídeos que contribuem para a interação de coesão e adesão de proteínas e DNA (MISHRA *et al.*, 2023).

Bactérias patogênicas também podem formar biofilmes. Elas são capazes de apresentarem resistência de aproximadamente 1000 vezes aos fármacos quando comparados a sua forma planctônica (LÓPEZ; SOTO, 2020). A formação de *quorum sensing* (QS) ocorre quando existem uma comunicação das células bacterianas entre si,e isso pode potencializar a formação de biofilmes. Existem moléculas químicas denominadas “autoindutores” que induzem a comunicação entre as células, colaborando

para uma maior interação entre elas (NOURBAKHSH *et al.*, 2022). Os acúmulos dessas moléculas químicas induzem alterações em genes de expressão para a formação de biofilme, além de induzir ou reduzir fatores de virulência (NOURBAKHSH *et al.*, 2022).

Os fungos ao formarem biofilmes acaba dificultando as terapias, tornando- as mais longas e intensas. Rajendran *et al.* (2016) mostraram que de 134 pacientes com candidemia, 41% vieram a óbito num intervalo de 30 dias, e isso foi associado a habilidade das candidas em formar biofilmes.

De acordo com Harding e colaboradores (2009), espécies do gênero *Candida* são capazes de formar biofilmes: no primeiro estágio ocorre a adsorção de células em uma superfície, ocorrendo interações físico-químicas; no segundo estágio ocorre uma adesão entre as células através da matriz extracelular polimérica; no terceiroestágio ocorre a formação da camada basal de microcolônias, além do desenvolvimento de hifas e um aumento mais organizado da matriz extracelular; já no quarto estágio ocorre a maturação do biofilme; e no quinto e último estágio ocorre a dispersão das células, podendo haver uma nova colonização.

Alguns mecanismos adquiridos de resistência também são fundamentais para a tolerância dos biofilmes às drogas antifúngicas, dentre eles: mutações nos genes que codificam enzimas, mutações na perda de função em genes na via da biossíntese de ergosterol e superexpressão da bomba de efluxo (KAUR; NOBILE, 2023).

Com o intuito de abranger mais opções no mercado e ter um menor custo, é imprescindível a busca de novos compostos capazes de atenuar esse problema. Compostos oriundos de plantas medicinais pode ser uma alternativa viável para aindústria farmacêutica.

2.5 Produtos Naturais

Desde as antigas civilizações que as plantas medicinais são utilizadas como uma alternativa para o tratamento de doenças (DUTRA *et al.*, 2016). O semiárido brasileiro, caracterizado por altas temperaturas, baixa umidade relativa do ar durante grande parte do ano e baixos índices pluviométricos (REIS *et al.*, 2015), detém uma ampla variedade de espécies vegetais com potencial terapêutico. Esse bioma tem uma

exploração científica reduzida e possui uma biodiversidade elevada, podendo vir a ofertar novas descobertas de espécies com potencial medicinal (OLIVEIRA, 2020).

Em 1806, Friedrich Serturner isolou a morfina um alcalóide da papoula, e isso estimulou novos estudos sobre medicamentos derivados de plantas (DUTRA *et al.*, 2016). De acordo com a Organização das Nações Unidas para a Alimentação e a Agricultura (FAO), estima-se que em 2050, cerca de 80% da população utilizará as plantas medicinais para tratamento de saúde (RAMAKRISHNAN *et al.*, 2017).

Alguns fatores abióticos podem estimular as plantas da Caatinga a produzir metabólitos secundários com atividades antioxidantes (MORAIS *et al.*, 2006) e com potencial terapêutico (OLIVEIRA-JUNIOR *et al.*, 2019) e essas plantas, também caracterizadas como medicinais, são importantes para investigações farmacológicasfuturas. Elas possuem diversos tipos de princípios ativos que curam enfermidades e previnem as infecções (JESUS *et al.*, 2019). Dentre os metabólitos secundários destes vegetais, os produtos naturais podem possuir princípios ativos comprovados. Estes foram amplamente visados pela academia e empresas farmacêuticas com o uso de novas tecnologias (SHERIDAN, 2012; HARVEY; EDRADA-EBEL; QUINN, 2015). Um dos produtos naturais capazes de minimizar os problemas recorrentes de bactérias e fungos resistentes a antibióticos são óleos essenciais (OEs).

2.5.1 Óleos Essenciais

Os OEs são considerados odoríferos, voláteis (LIN *et al.*, 2022). Podem ser sintetizados em diferentes partes das plantas, como as folhas, o caule, as sementes, os frutos e raízes (BHAVANIRAMYA *et al.*, 2019). Estas substâncias formam-se no citoplasma e podem ser encontradas como gotículas pequenas nas glândulas da planta (ANGANE *et al.*, 2022). Sua composição pode ser influenciada pela sazonalidade, temperatura, umidade, luz, entre outros (NASCIMENTO JÚNIOR *et al.*, 2020; BORGES *et al.*, 2019).

Os OEs podem ser extraídos por diferentes técnicas de extração, como hidrodestilação, destilação por vapor, maceração e extração por solvente (HOU *et al.*, 2022). Sua composição química pode ser analisada por cromatografia gasosa e espectrometria de massa (CG-MS) (PARRISH *et al.*, 2020), para o reconhecimento de

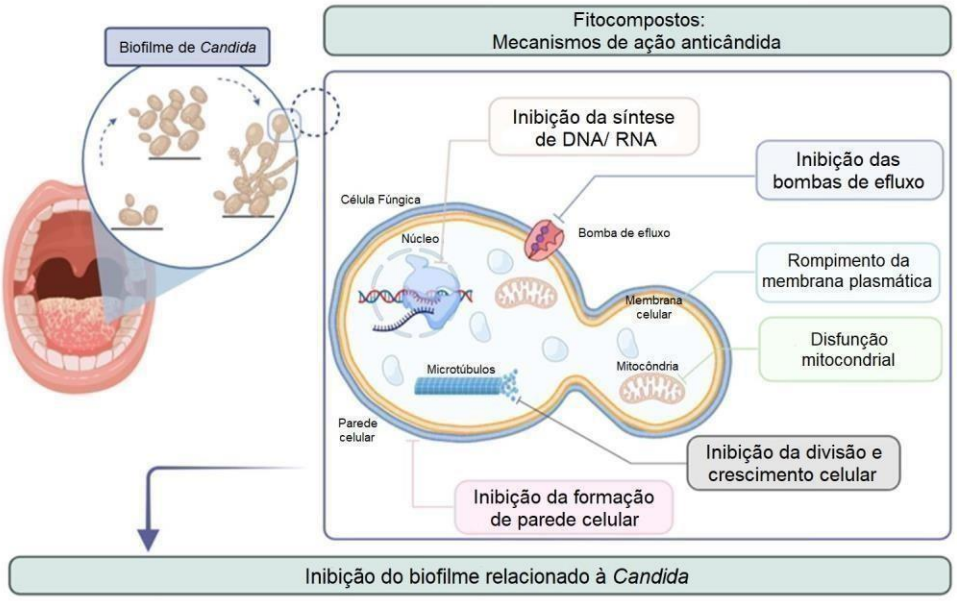
diferentes compostos com distintas atividades biológicas. No entanto, vale ressaltar que, as propriedades de cada óleo dependem de grande parte de sua composição química (ABD RASHED *et al.,* 2021).

Podem ser utilizados como cosméticos, perfumes, na horticultura ou na indústria farmacêutica como um produto vegetal (SIMÕES *et al.*, 2016; SHARMEEN *et al.*, 2021). Interessantemente, são degradáveis ao contato de luz e calor, ou seja, podem oxidar facilmente (CAMPOLO *et al.*, 2020), por isso que a extração deve ser realizada em temperaturas moderadas para evitar sua rápida oxidação (FORNARI *et al.*, 2012).

O modo de ação dos OEs em células bacterianas e fúngicas em sua forma planctônica ou biofilme pode estar relacionado aos diferentes mecanismos que cada composto presente no óleo pode atuar (BURT, 2004). A parte hidrofílica dos grupos funcionais e a parte lipofílica da estrutura dos hidrocarbonetos nos OEs são os que desempenham os principais papéis nos efeitos antimicrobianos (BURT, 2004; KHORSHIDIAN *et al.,* 2018).

Em relação ao modo de ação contra bactérias, supõem-se que as Gram-negativas sejam menos sucetíveis aos Oes com relação as Gram-positivas, pela presença da membrana celular externa que contém cadeias de polissacarídeos hidrofílicos, os quais atuam como barreira aos compostos hidrofóbicos presentes nos Oes (AL-MAQTARI *et al*., 2020). Os OEs podem interagir com a membrana celular e passar para o citoplasma, com isso, ocorre a alteração da permeabilidade da membrana e perturbação de enzimas que são essenciais no metabolismo energético (BASIM; YEGEN; ZELLER, 200; KHORSHIDIAN *et al*., 2018). Compostos presentes nos óleos como terpenóides e fenólicos possuem atividade antimicrobiana, podendo inibir a síntese de DNA/RNA, inibir a ação de bombas de efluxo, divisão e crescimento celular, como também provocam danos à parede celular e disfunção mitocondrial (Figura 1) (GUIMARÃES *et al*., 2021). Os terpenos são os principais componentes dos OEs e apresentam diversas propriedades, como atividade antimicrobiana, antifúngica e antiviral (PICHERSKY;GERSHENZON, 2002). Os compostos fenólicos são encontrados em todas as partes das plantas e possuem propiedades antioxidante, anticancerígena e anti-inflamatória (RUBIÓ; MOTILVA; ROMERO, 2013; ALUDATT *et al.*, 2018). Os alcalóides possuem propriedades antimicrobianas e antimaláricas (OTHMAN; SLEIMAN; ABDEL-MASSIH, 2019).

Figura 1. Mecanismos de ação de fitocompostos contra *Candida spp.*



Fonte: Adaptado de Guimarães *et al.*, (2021).

Para fungos, os OEs podem inibir tanto o crescimento do micélio como a produção de toxinas. Segundo Nogueira *et al.*, (2010), os OEs podem atuar sobre a membrana interna e afetar as mitocôndrias, causando morte celular. Os terpenóides podem diminuir o número de mitocôndrias e afetar as espécies reativas de oxigênio (ROS) e a síntese de ATP (HAQUE *et al.*, 2016). Outros estudos mostraram que os Oes poderiam alterar as proteínas de membrana, destruir a integridade das membranas celulares, além disso, podem aumentar a fluidez e/ou permeabilidade, como a saída de substâncias intracelulares (JIN *et al.*, 2019).

A investigação de como os OEs podem agir nas estruturas de bactérias e fungos podem servir como um bom potencial biotecnológico e com novas perspectivas na indústria farmacêutica afim de substituir os agentes antimicrobianos sintéticos.

2.6 Gênero Croton

No Brasil, a família Euphorbiaceae possui aproximadamente 63 gêneros e

945 espécies de plantas (CORDEIRO *et al.,* 2015). As espécies desta família são usadas como fontes de moléculas fungicidas, antibacterianas e no tratamento de doenças gastrointestinais (AZUAJE, 2017). Um gênero muito conhecido desta família é o *Croton*, que foi identificado por Linnaeus em 1753 (SILVA; SALES; CARNEIRO- TORRES, 2009). No Brasil, o gênero apresenta aproximadamente 68 espécies endêmicas no bioma Caatinga (CORDEIRO *et al.*, 2015).

Espécies do gênero *Croton* podem ser encontradas em diversos locais, podendo apresentar flores e frutos na maior parte do ano, além disso, podem servir para restaurar áreas degradadas (LIMA; PIRANI, 2008). Possui uma variedade defitoquímicos, como: alcalóides, terpenóides (diterpenos, triterpenóides e esteróides) e flavanóides (GARCÍA-DÍAZ *et al.*, 2019).

Souza e colaboradores (2017) ressaltaram em seus estudos, que os horários e períodos do ano podem causar diferenças nas composições químicas, bem como os rendimentos do óleo de espécies de *Croton argyrophylloides, C. jacobinensis* e *C. sincorensis*. Os autores evidenciaram também que, espécies do gênero *Croton* produzem constituintes com propriedades biológicas.

2.6.1 Croton blanchetianus Baill.

A espécie *C. blanchetianus* é um arbusto que pode ser encontrado no Nordeste brasileiro, conhecido popularmente como “marmeleiro preto” (OLIVEIRA *et al.,* 2022). A presença de tricomas glandulares é uma característica atribuída à espécie (BARROS; SOARES, 2013). Essa espécie pode ocupar diversas áreas desmatadas, tendo o seu crescimento e sua propagação de maneira silvestre, já que sobrevivem em períodos secos (LORENZI; MATOS, 2002), além disso, pode apresentar mecanismo de brotação após o corte (COSTA, 2014). Durante o período seco, perde todas as folhas, enquanto no período chuvoso, brota-se logo, possuindo um aroma específico, no qual, caracteriza-o (SILVEIRA; PESSOA, 2005). Apresenta uma altura variável, podendo chegar a aproximadamente 6 m, possui folhas simples e estipulas grandes presentes nos ramos jovens (LORENZI; MATOS, 2002).

C. blanchetianus possui ação anti-inflamatória, antibacteriana, gastroprotetora, anticonceptiva e antiparasitária (AQUINO *et al.*, 2017; CAVALCANTI;

SILVEIRA; SILVA, 2020; FREITAS *et al.,* 2020). Alguns extratos das folhas desta espécie apresentam em sua composição, alcalóides, flavonóides, saponinas, taninos, terpenos e esteróides (FREITAS *et al.*, 2020).

Trabalhos que mostram atividade antimicrobiana e seus mecanismos de ação ainda são escassos na literatura, portanto, torna-se indispensável um estudo mais aprofundado sobre o OE de *C. blanchetianus* e sua atividade antimicrobiana contra bactérias e fungos.

3 HIPÓTESE

Óleo essencial extraído de folhas de *C. blanchetianus* Baill. possui em sua composição química substâncias com propriedades antibacteriana, antifúngica e antibiofilme e com diferentes mecanismos de ação, além disso, possui uma baixa toxicidade a eritrócitos humanos.

4 OBJETIVOS

4.1 Objetivo Geral

- Caracterizar e avaliar o efeito antibacteriano, antifúngico e antibiofilme do óleo essencial extraído de folhas de *C. blanchetianus*, bem como identificar possíveis mecanismos de ação.

4.2 Objetivos Específicos

- Identificar os componentes químicos presentes no óleo através de CG/MS;
- Avaliar a concentração inibitória mínima e a inibição da formação de biofilmesdo óleo de *C. blanchetianus* sobre bactérias e fungos de interesse clínico;
- Determinar os mecanismos de ação, através dos estudos de danos na membrana celular, indução da superprodução de espécies reativas de oxigênio e indução de apoptose;
- Identificar prováveis modificações morfológicas e estruturais dos microrganismos na presença do óleo através da microscopia eletrônica de varredura;
- Averiguar se o óleo essencial é tóxico para eritrócitos humanos.

5 CAPÍTULO II

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Essential Oil from *Croton blanchetianus* Leaves: Anticandidal Potential and Mechanisms of Action

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ABSTRACT

Antimicrobial drugs are becoming ineffective given the resistance acquired by microorganisms. As such, it is imperative to seek new antimicrobial molecules that could provide a basis for the development of new drugs. Therefore, this work aimed to evaluate the antimicrobial potential and the mechanisms of action of the essential oil extracted from leaves of *Croton blanchetianus* (named *Cb*EO) on different fungi and bacteria of clinical importance in both planktonic and biofilm lifestyles. GC-MS/MS analysis revealed the presence of twenty-three different compounds in the *Cb*EO, which were identified using the Kovats retention index. Among these, the most abundant were sphatulenol (20.03%), bicyclogermacrene (5.92%), caryophyllene oxide (5.81%), and eucalyptol (5.62%). *Cb*OE (50 µg mL^{-1}) barely inhibited the growth of *Bacillus subtilis* (23%), *Pseudomonas aeruginosa* (27%), and *Salmonella enterica* (28%), and no inhibition was obtained against *Enterobacter aerogenes* and *Klebsiella pneumoniae*. Additionally, no activity against bacterial biofilm was detected. In contrast, *Cb*EO was active against Candida species. *C. albicans* and *C. parapsilosis* were inhibited by 78and 75%, respectively. The antibiofilm potential also was favorable against *C. albicans* and *C. parapsilosis*, inhibiting 44 and 74% of biofilm formation and reducing around 41 and 27% of the preformed biofilm, respectively. *Cb*OE caused membrane damage and pore formation, overproduction of ROS, and apoptosis on *C. albicans* and *C. parapsilosis* cells, as well as not inducing hemolysis in human red cells. The results obtained in this work raise the possibility of using the essential oil of *C. blanchetianus* leaves as an alternative to fight infections caused by *C. albicans* and *C. parapsilosis*.

Keywords: essential oil; GC-MS/MS; biotechnological potential; *Candida* genus; antibiofilm activit

1. Introduction

The misuse of antimicrobial drugs has dramatically intensified, generating a huge concern in health systems because of the emergence of multidrug-resistant pathogens, affecting around 5 million people worldwide [1]. Such conditions have driven humanity to an era referred to as post-antibiotic, making infections stronger and antibiotics useless [2]. For instance, infections caused by drug-resistant human pathogenic yeasts from the *Candida* genus, which affects healthy and immunocompromised people, are hard to treat. These infections range from superficial candidiasis localized on the skin to systemic and invasive bloodstream infections [3,4].

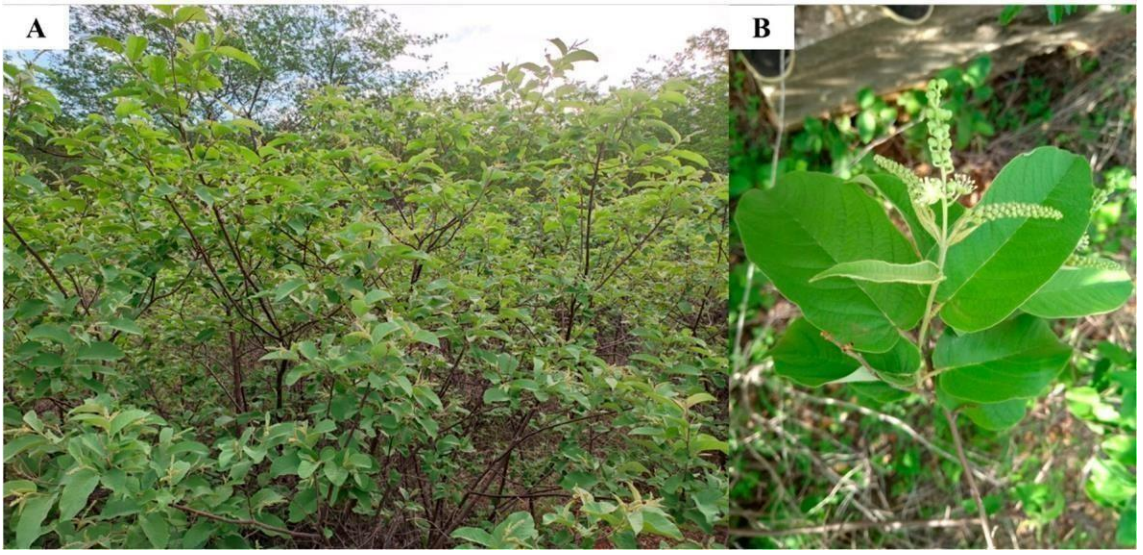
In addition, the formation of biofilms is another factor that enhance resistance of microorganisms to drugs [5]. Biofilm cells show differences in morphology, physiology, and gene expression compared to the planktonic form [6]. Moreover, they can be resistant to UV exposure, phagocytosis, and dehydration [7]. Thereby, drug- resistant microorganisms and their biofilms are problems that requires urgent attention and efforts to find new molecules to fight them back.

One of the alternatives for minimizing these problems is the search for new natural molecules, such as secondary metabolites produced by plants, which can inhibit the growth of resistant pathogens [8]. The Brazilian biomes are rich in many medicinal plants acting as biological sources for new molecules with therapeutic potential. Aromatic plants produce essential oils (EOs), defined as volatile and with strong odor characteristic, can be extracted from the leaves, barks, seeds, and fruits. In addition, some plant essential oils have been used to overcome microbial resistance [9,10].

C. blanchetianus (Figure 1) Baill is an oil-producing plant belonging to the Euphorbiaceae family that have plants with several medicinal properties [11]. It has antibacterial, anti-inflammatory, and gastroprotective actions [9,10]. For instance, Melo et al. [9] reported that the essential oil from *C. blanchetianus* has a potent antibacterial activity against *Aeromonas hydrophila*, *Listeria monocytogenes*, and *Salmonella enteritidis* [9]. Additionally, oil of *C. blanchetianus* applied on meat was shown to prevent the growth of the foodborne pathogen *S. enteritidis*, thus showing the potential application of the oil in industry. Works with the essential oil from *C. blanchetianus* on planktonic and biofilm cells of bacteria and fungi of clinical importance and its respective mechanisms of action are still scarce. Based on the application of essential oil

from *C. blanchetianus*, we hypothesized that the oil could have application against human pathogens. Therefore, this work aims to characterize and evaluate the antimicrobial and antibiofilm potential of the essential oil from *C. blanchetianus* leaves (*Cb*EO), as well as its mechanisms of action.

Figure 1. *C. blanchetianus* in the field. (A) Many *C. blanchetianus* trees and (B) *C. blanchetianus* leaves used in the oil extraction.



2. Resultds and Discussion

2.1 GC-MS/MS Analysis

Gas chromatography coupled with mass spectrometer (GC-MS/MS) analysis revealed the presence of twenty-three different compounds in the C*b*EO, which were identified using the Kovats retention index. Among these, the most abundant were sphatulenol (20.03%), bicyclogermacrene (5.92%), caryophyllene oxide (5.81%), and eucalyptol (5.62%). Almost all the compounds found in C*b*EO have been reportedto present some biological activity. Although sphatulenol is the most abundantcompound in the C*b*EO (**Table 1**), to date, it has not been related to antimicrobial or antifungal activity. Usually, the biological activities attributed to sphatulenol are

antioxidant and anti-leishmanial activity. Usually, the biological activities attributed to amorphene are antioxidant and anti-leishmanial activity [12–14]. However, other abundant compounds have been associated with antimicrobial activity. For instance, spathulenol displays antifungal and antibacterial activities [12], bicyclogermacrene possesses antifungal and antioxidant activities [13], caryophyllene oxide has been reported to possess antioxidant, anticancer, and antimicrobial properties [14], and eucalyptol presents anticandidal activity [15].

Table 1. Compounds identified in essential oil from *C. blanchetianus* leaves (*Cb*EO).

Compound	Retention Time	Area (%)
limonene	6.02	0.61
eucalyptol	6.14	5.62
borneol	9.84	0.64
terpinen-4-ol	10.13	1.32
α-terpineol	10.52	1.16
myrtenol	10.70	0.70
δ-elemene	14.61	0.46
α-ylangene	15.67	0.51
β-bourbonene	15.92	0.95
sativene	16.15	1.99
E-caryophyllene	16.86	1.95
aromadendrene	17.37	0.56
6,9-guaiadiene	17.47	1.31
α-humulene	17.75	0.73
alloaromadendrene	17.95	1.10
germacrene D	18.47	0.70
γ-himachalene	18.61	0.57
bicyclogermacrene	18.90	5.92
δ-amorphene	19.54	1.49
spathulenol	21.08	20.03
caryophyllene oxide	21.16	5.81
shyobunone	21,65	4.55
epi- α-muurolol	22.79	2.98

In addition to these most abundant compounds, other components (Table 1) detected in *Cb*EO have also been reported with respect to their antimicrobial activity, such as limonene, borneol, α-terpineol, and sativene, presenting, respectively, anticandidal, antibacterial, and antifungal activities [15–20]. Furthermore, proteomic analysis of *C. albicans* cells treated with limonene revealed an up-accumulation of proteins involved with oxidative stress, DNA damage, nucleolar stress, and apoptosis [16].

2.2 Antimicrobial Activity

*Cb*OE was tested against several human-pathogenic bacteria and yeasts (Table 2). The antibacterial activity of *Cb*OE barely inhibited the growth of *Bacillus subtilis* (23%), *Pseudomonas aeruginosa* (27%), and *Salmonella enterica* (28%), and no inhibition was observed against *Enterobacter aerogenes* and *Klebsiella pneumoniae*, even using the highest concentration tested (50 μg mL^{-1}). Likewise, no activity against the bacterial biofilms was detected at 50 μg mL^{-1} (Table 2). In a previous study [21], it was shown that the aromadendrene present in the EO of *Eucalyptus globulus* has remarkable activity on strains of methicillin-resistant *S. aureus* (MRSA), vancomycin- resistant *Enterococcus faecalis* (VRE), and *Acinetobacter baumanii*. However, the inefficiency of *Cb*EO against bacteria can be explained by the low concentration of potent antibacterial molecules, such as borneol, α-terpineol, and sativene found in other essential oils [15–20].

Table 2. Evaluation of *Cb*EO on planktonic cells and biofilm formation of some microorganisms.

	***Cb*EO (50 μg mL^{-1})**			
	Antifungal activity			
	Inhibition of planktonic cells growth (%)	Inhibition of biofilm formation (%)	Biomass reduction of preformed biofilm (%)	Nystatin
icans ATCC10231	78.55 ± 0.3	44.12 ± 0.2	41.12 ± 0.4	85.75 ± 0.1
C. krusei ATCC 6258	9.58 ± 0.5	00.00	00.00	75.00 ± 0.6
psilosis ATCC22019	75.94 ± 0.1	74.13 ± 0.7	27.75 ± 0.8	86.66 ± 0.3
icalis clinicalisolate	00.00	00.00	00.00	93.85 ± 0.9
rmans ATCC32045	00.00	00.00	00.00	75.02 ± 0.5
Antibacterial activity				**Ciprofloxacin**
B, subtilis ATCC 6633	23.00 ± 0.3	00.00	00.00	85.42 ± 0.4
E. aerogenes ATCC 13048	00.00	00.00	00.00	84.23 ± 0.4
moniae ATCC10031	00.00	00.00	00.00	81.68 ± 0.7
ginosa ATCC25619	27.01 ± 0.7	00.00	00.00	87.39 ± 0.8
erica ATCC14028	28.12 ± 0.5	00.00	00.00	87.74 ± 0.2

Regarding anticandidal activity, *Cb*EO presented better results against *C. albicans* and *C. parapsilosis,* with inhibition of 78 and 75%, respectively, at 50 μgmL^{-1} (Table 2). Otherwise, *C. krusei* was barely susceptible (9.5% of inhibition) and *C.*

tropicalis was not affected by treatment with *Cb*EO even at the highest concentration tested (50 μg mL^{-1}). The antibiofilm potential of *Cb*OE (50 μg mL^{-1}) was also favorable against *C. albicans* and *C. parapsilosis*, inhibiting 44 and 74% of biofilm formation and reducing preformed biofilm by 41 and 27%, respectively (Table 2). Theseresults are in agreement with higher concentrations of spathulenol, bicyclogermacrene, caryophyllene oxide, and eucalyptol, which have been described as potent antifungal and anticandidal compounds [12–15]. Keymaram et al. [22] showed in their study that eucalyptol possesses antibiofilm activity against *C. albicans* at concentrations ranging from 125 to 8000 μg mL^{-1}. Although detected at a low concentration in *Cb*EO, limonene could also contribute to anticandidal activity, specifically against *C. albicans*, since this compound has already shown anticandidal activity [16]. Al-Ghanayem [23] showed that lemongrass leaf oil (*Cymbopogon flexuosus*) was active against *C. albicans* planktonic cells, and complete biofilm reduction could be observed at 0.5 μL mL^{-1} and 30% at a concentration of 0.03 μL mL^{-1}.

In another study, the Pistachio hull essential oil completely inhibited the growth of *C. parapsilosis* at a concentration of 2.50 mg mL^{-1} and slightly inhibited *C. glabrata*and *C. albicans* at the same concentration [24]. It seems that the results presented by *Cb*EO are exciting when compared to those above. Compared to D'Arrigo et al. [24], EO was effective against *C. parapsilosis* at a concentration 50 times lower than that required for essential oil from Pistachio hull. In addition, *Cb*EO was also effective against *C. parapsilosis* biofilm (Table 2). Natural products are considered strong inhibitors when they have an MIC_{50} of up to 0.5 mg mL^{-1}, moderate inhibitors between 0.6 and 1.5, and weak inhibitors when this value is higher than 1.6 mg mL^{-1} (Madeira et al., 2016). Corroborating this work, *Cb*EO is considered a strong inhibitor of *C. albicans* and *C. parapsilosis*, since it shows activity at 0.05 mg mL^{-1} (Table 2).

C. citrate oil with silicone rubber coating inhibited *C. tropicalis* biofilm formation by between 45 and 76% [25]. Here, our results revealed that *Cb*EO was not effective against *C. tropicalis*. This, indeed, is not a negative result. However, it is an interesting result because shows selectivity. The literature has already discussed that different essential oils may present distinct biological activities based on their composition [23]. The activity of *Cb*EO against *C. albicans* and *C. parapsilosis* biofilms has a great potential to drive further studies to develop new drugs against

biofilms. As already mentioned, biofilm formation is one of the mechanisms of fungal resistance occasioned by *Candida* species that may be associated with medical devices such as cardiovascular, venous, and urinary catheters [26].

2.3 Mechanisms of Action

2.3.1 Membrane Damage and Pore Formation

There are other studies evaluating the anticandidal activity of essential oils from many plants. However, few have shown the mechanism of action. Thakre et al. [16] revealed by proteomic analysis the up accumulation of proteins related to damage to DNA and nucleolar region, suggesting induction of apoptosis in *C. albicans* cells treatedwith limonene. Recently, Yu et al. [19] reported that limonene induced mitochondrial membrane depolarization and cell membrane pore formation.

Here, we provide evidence about how the *Cb*EO displays anticandidal activity. The propidium iodide (PI) uptake assay was employed to evaluate the membrane damage in *C. albicans* and *C. parapsilosis* planktonic and biofilm cells. PI interacts with DNA, releasing red fluorescence. However, it can only move through damaged membrane, thus indicating pore formation. Therefore, healthy membranes do not allow the passage of PI through them. The data obtained regarding the planktonic cells showed no fluorescence of PI on control (5% DMSO) cells (Figure 2). In contrast, *C. albicans* and *C. parapsilosis* cells treated with *Cb*EO showed red fluorescence, indicating membrane damage (Figure 2). The fluorescence was quantified and showed that *C. parapsilosis* cells were more susceptible to membrane damage caused by *Cb*EO than *C. albicans* (Figure 2). Similarly, *Cb*EO was also able to induce membrane permeabilization in both *C. albicans* and *C. parapsilosis* biofilms (Figure 3). Indeed, *Cb*EO was more efficient against *C. albicans* biofilms than in planktonic cells (Figure 3). The membrane damage induced by essential oils from plants has already been discussed [27]. However, there are no studies indicating the size of the pore formed.

Although PI indicates damage to the cell membrane, it does not give any information about pore size. Thus, new assays were performed using a fluorophore with a size of 6 kDa (Dextran-FITC). Only *C. parapsilosis* planktonic cells showed green fluorescence, indicating that *Cb*EO allowed the movement of 6 kDa FITC-dextran(Figure 3). In contrast, green fluorescence was observed in biofilm cells of both *C.*

albicans and *C. parapsilosis* (Figure 2). Etxaniz et al. [28] revealed that in some cases, the cell membrane can recover from the formation of a pore. For example, the pores revealed by PI have a size of around 0.1 nm, making it possible for the cell to recover. However, pores revealed by FITC-Dextran have a size of 1.0 nm, and are thus classifiedas large pores, making it quite difficult for the cells to recover [28], since there may be extravasation of various cellular molecules, membrane depolarization, and induction of apoptosis, as reported by Thakre et al. [16].

The *Zanthoxylum schinifolium* essential oil had activity on membrane permeabilization against the fungus *Malassezia restricta* [29]. Terpenoids have already been reported to alter membrane fluidity and modulation of proteins linked to signaling and transport [30]. In a previous study [31], it was shown that terpinen-4-ol can impair the integrity and physiology of fungal cells by inducing membrane loss.

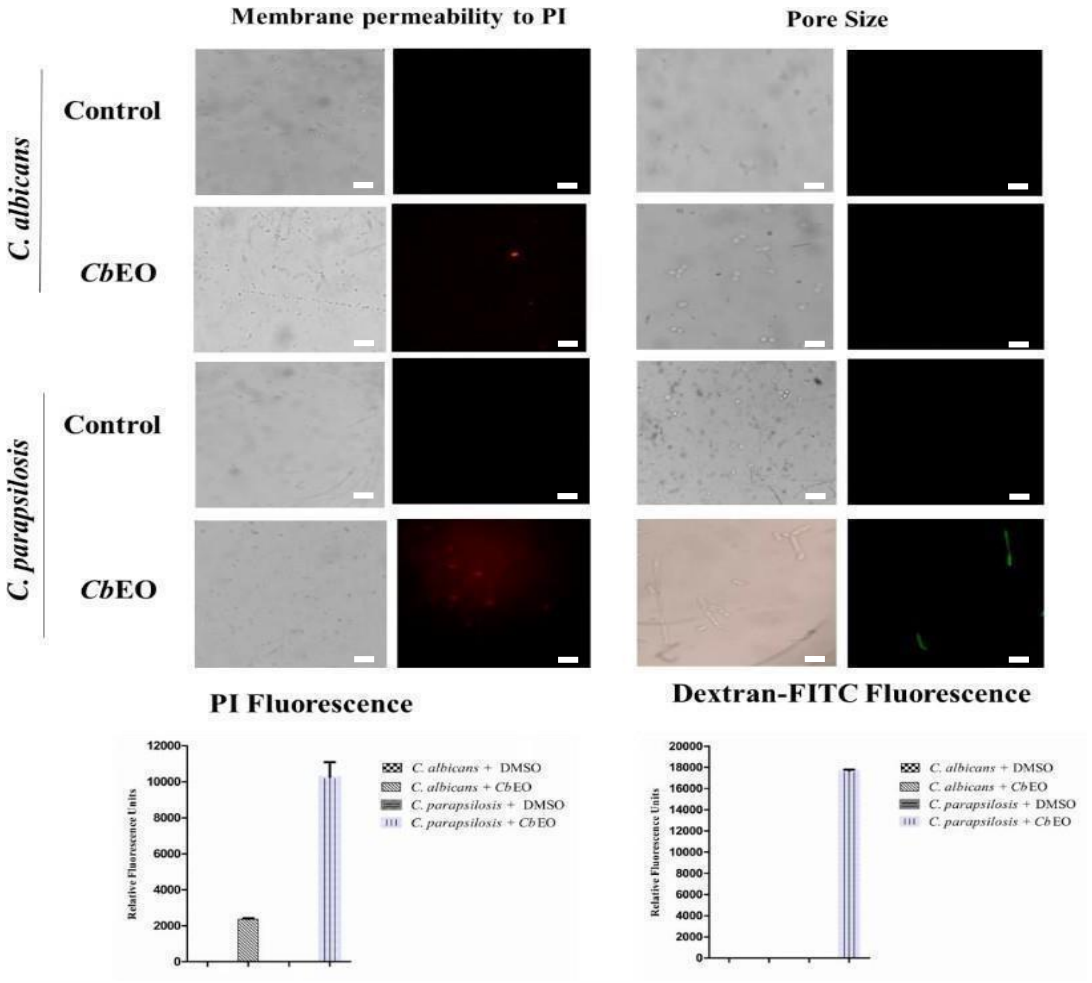


Figure 2. Fluorescence images showing membrane damage and pore size on planktonic cells of *C. albicans* and *C. parapsilosis*. The membrane damage was assayed by propidium iodide (PI) uptake and pore size by using a 6 kDa dextran-FITC. The control was 5% DMSO. Bars indicates 100 µm.

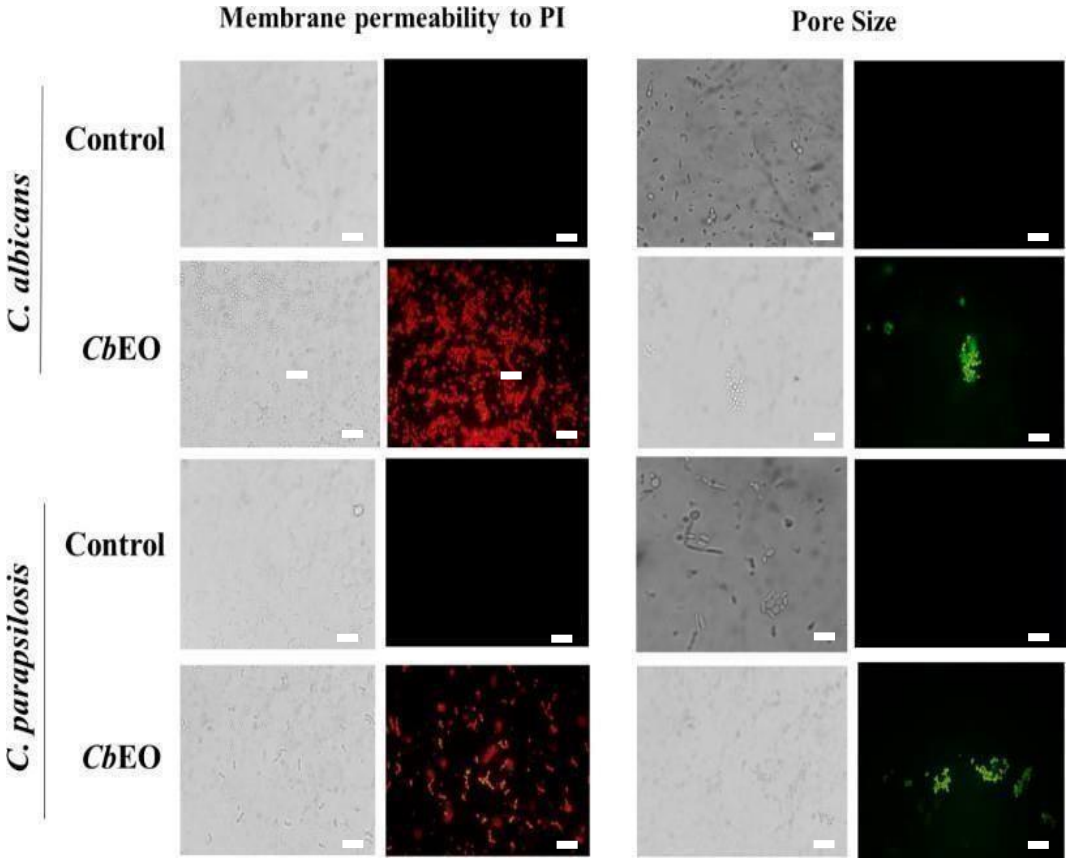


Figure 3. Fluorescence images showing membrane damage and pore size on biofilm cells of *C. albicans* and *C. parapsilosis*. The membrane damage was assayed by propidium iodide (PI) uptake and pore size by using a 6 kDa FITC-Dextran. The controlwas 5% DMSO. Bars indicates 100 µm.

2.3.2 Overproduction of Reactive Oxygen Species (ROS)

To further explore the mechanism of action, the ROS overproduction induced by *Cb*EO in both *C. albicans* and *C. parapsilosis* cells was evaluated. The *Cb*EO (50 µg mL^{-1}) induced ROS overproduction (Figure 4) in planktonic cells of both yeasts (green

fluorescence). However, there was a difference in the intensity of ROS produced byboth species. The quantification of fluorescence revealed a higher amount of ROS produced by *C. parapsilosis* (Figure 4). As expected, the controls did not have an overproduction of ROS. *Cb*EO also induced ROS overproduction in biofilms of both *C. parapsilosis* and *C. albicans* (Figure 5— white arrows), albeit to a lesser extent.

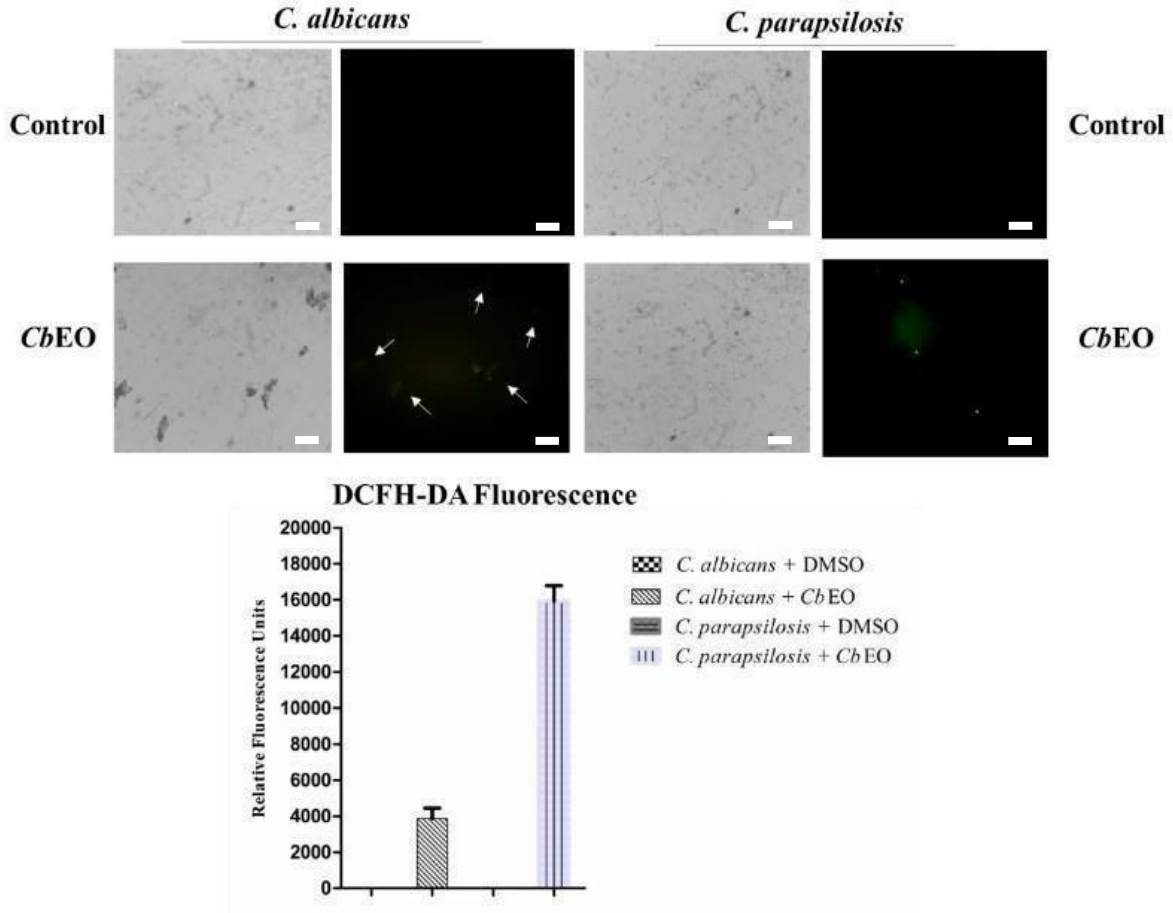


Figure 4. Fluorescence images showing ROS overproduction (Green Fluorescence) by planktonic cells of *C. albicans* and *C. parapsilosis*. The control was 5% DMSO. White arrows indicate cell with green fluorescence. Bars indicates 100 µm.

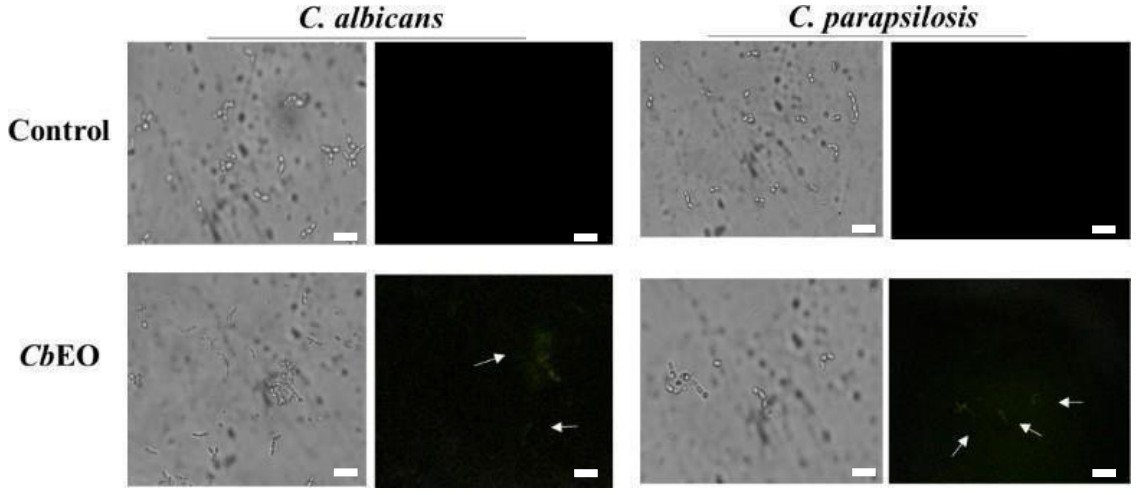


Figure 5. Fluorescence images showing ROS overproduction (Green Fluorescence) by biofilm cells of *C. albicans* and *C. parapsilosis*. The control was 5% DMSO. White arrows indicate cells with green fluorescence. Bars indicates 100 µm.

Ding et al. [32] reported that ROS levels increased in *C. albicans* cells after incubation with a quinoline compound. Yang et al. [33] showed that lavender essential oil induced oxidative stress in *K. pneumonia*, leading to membrane permeabilization and cell death. Indeed, the high level of ROS could be lethal to cells because it can lead to damage to critical molecules such as DNA, protein, and lipids [34]. Looking the composition of lavender essential [33], the presence of limonene, borneol, and caryophyllene was also present in *Cb*EO. These compounds, present in both oils, could be involved in ROS overproduction in microbial cells. ROS is essential to biofilm biogenesis, development, and formation, as well as the genetic variability of cells [35]. However, the line between benefits and lethal effects is thin and easy to cross. A slight imbalance of ROS levels can lead to its accumulation, which is lethal, because itinactivates vital molecules such as carbohydrates, nucleic acids, proteins, and lipids, triggering programmed cell death [36].

2.3.3 Caspase 3/7-Mediated Apoptosis

*Cb*EO induced caspase 3/7-mediated apoptosis in planktonic cells of *C. albicans* and *C. parapsilosis* and weakly in biofilms of *C. albicans* only (Figures 6 and 7). The exact mechanism of how *Cb*EO induced apoptosis in the *C. parapsilosis* cells has notyet been elucidated. Caspase-3/7 starts apoptotic DNA fragmentation by activating of a protein called DNA fragmentation factor-45 (DFF45) and an inhibitor of caspase- activated DNase (ICAD). Thakre et al. [16] revealed by proteomic analysis that the treatment with limonene increase the accumulation of proteins involved with DNA damage and apoptosis in *C. albicans* cells. However, as happens here, the authors didnot understand the mechanism involved.

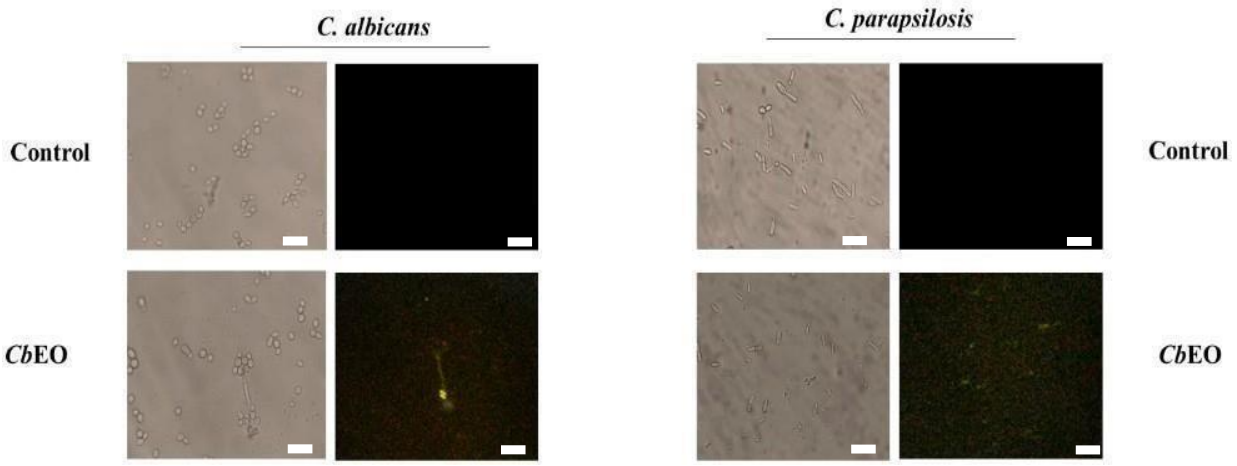


Figure 6. Evaluation of apoptosis in *C. albicans* and *C. parapsilosis* strains under the action of *Cb*EO at the concentration of 50 µg mL^{-1}. Bars indicates 100 µm.

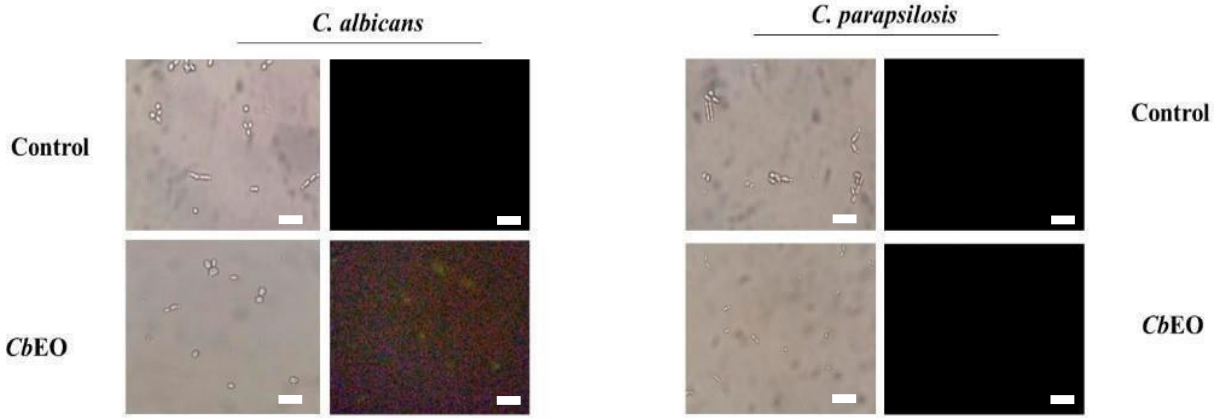


Figure 7. Evaluation of apoptosis in *C. albicans* and *C. parapsilosis* biofilms under the action of *Cb*EO at the concentration of 50 µg mL^{-1}. Bars indicates 100 µm.

2.3.4 Scanning Electron Microscopy (SEM)

SEM was also used to evaluate the damage on *C. albicans* and *C. parapsilosis* cells caused (Figure 8) after treatment with *Cb*EO (50 µg mL^{-1}). *Cb*EO caused changes in the morphology of cells, scars, roughness, and depletions leading to loss of internal content corroborating with the data from fluorescent microscopy. In contrast, control cells did not present any damage (Figure 8). The SEM analysis provided important

results, because there were not many works evaluating the cellular damage caused byEOs in yeast cells.

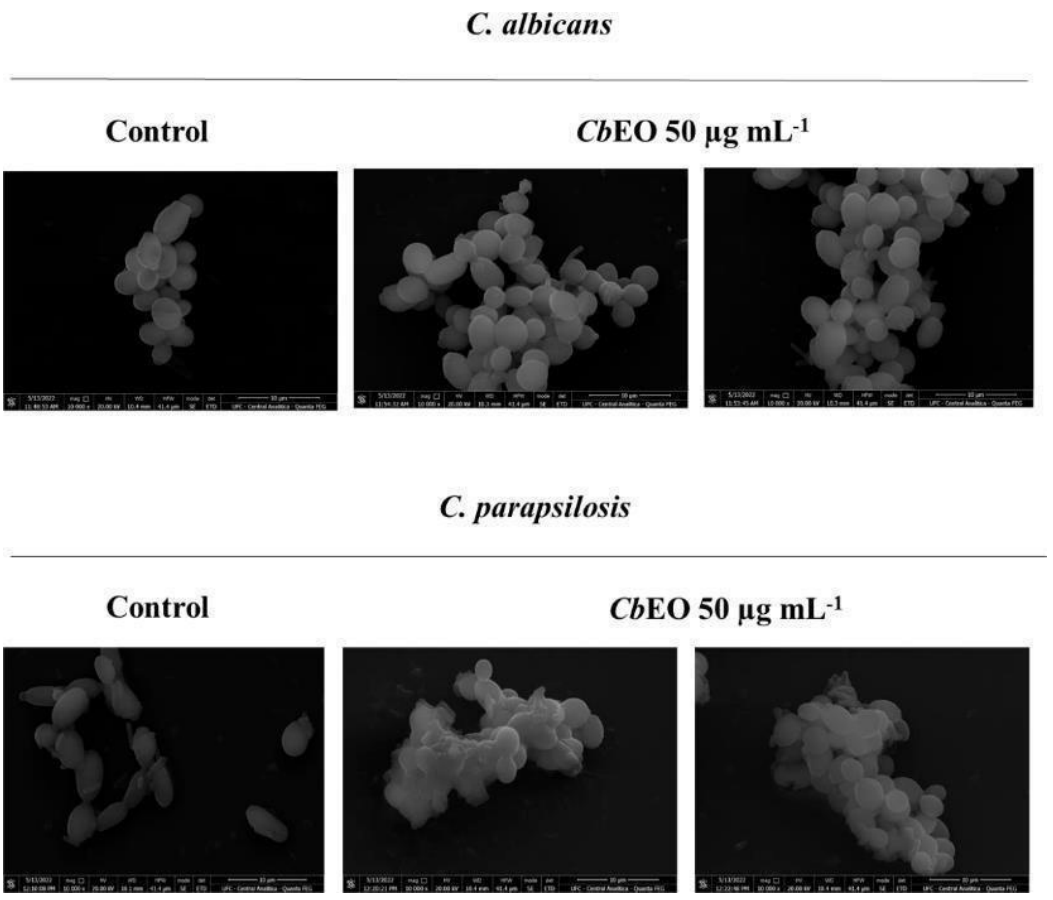


Figure 8. Scanning electron microscopy of *C. albicans* and *C. parapsilosis* cells afterthe action of *Cb*EO at the concentration of 50 µg mL^{-1}. Magnification 10,000×.

2.3.5 Hemolytic Activity

To be considered as potential molecules for the development of new drugs, the candidate should not present any or very low toxicity to hosts [37]. Based on the data obtained, *Cb*EO does not show toxicity to blood types A, B and O, even at the highest concentration tested (250 µg mL^{-1}), when compared to the control (DMSO 5%) (Table 3).

Table 3. Hemolytic activity of *Cb*EO against different human red blood cells

Blood Type	Hemolysis (%)						
	0.1% Triton	5% DMSO	*Cb*EO 250 $\mu g\ mL^{-1}$	*Cb*EO 150 $\mu g\ mL^{-1}$	*Cb*EO 100 $\mu g\ mL^{-1}$	*Cb*EO 50 $\mu g\ mL^{-1}$	*Cb*EO 25 $\mu g\ mL^{-1}$
Type A	100	0	0	0	v	0	0
Type B	100	0	0	0	0	0	0
Type O	100	0	23	0	0	0	0

3. Conclusions

This study highlights relevant results about the potential of *Cb*EO as a source of anticandida molecules. These data are essential for understanding the possible application of *Cb*EO in treating infections caused by *C. albicans* and *C. parapsilosis*. A future perspective is essential to notice that *Cb*EO is not toxic to human red blood cells, which, together with anticandidal activity, opens up great prospects for its application in the future. The main conclusion is that *Cb*EO possesses potent anticandida activity in both planktonic and biofilm lifestyles and presents no danger to human red blood cells.

4. Experimental Section

4.1 Biological Material

The leaves of *Croton blanchethianus* Baill. were collected in the city of Mossoró, Rio Grande do Norte, Brazil (latitude: −5.201324, longitude: −37.320572). Regarding microorganisms, the yeasts *C. albicans* (ATCC 10231), *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019), *Cryptococcus neoformans* (ATCC 32045) and *C. tropicalis* (Clinical isolate) and the bacteria *B. subtilis* (ATCC 6633), *E. aerogenes* (ATCC 13048), *K. pneumoniae* (ATCC 10031), *P. aeruginosa* (ATCC 25619), and *S. enterica* (ATCC 14028) were obtained of the Laboratory of Plant Toxins of the Federal University of Ceará, Brazil.

4.2 Oil Extraction

The original equipment was provided by the Laboratory of Plant Physiology and Biochemistry (LFBP). For the extraction process, the methodology proposed by Oliveira et al. was followed [38]. We decided to use the leaves because they have the

largest amount of glandular trichomes, for the storage and synthesis of bioactive metabolites such as the terpenoids and flavonoids that are present. The essential oil fromthe leaves of *C. blanchethianus* (*Cb*EO) was extracted by the hydrodistillation method using a Clevenger apparatus. In the Clevenger apparatus, samples are diluted in water, which is boiled to evaporate volatile components in the steam. In this way, two layers (aqueous and oil-rich) are obtained, and oil is separated using separating funnels. For extraction, the leaves were weighed (586 g) on a precision balance and then placed in a volumetric flask containing 2 L of distilled water. The flask was attached and heated on a heating mantle. After boiling, the steam generated was condensed, and oil and water were collected in a Dean–Stark apparatus. As they are immiscible, two phases were formed, and it was possible to separate oil and water. The oil was extracted for a period of 2 h, controlling the temperature to approximately 100 °C. The essential oil was dehydrated 6 g of anhydrous sodium sulfate (PM:142.04) and 10 mL of ethyl ether, resulting 8 mL of oil, which was stored under refrigeration (4 °C).

4.3 Characterization of CbEO by GC-MS/MS Analysis

The chemical composition of *Cb*EO was examined by gas chromatography coupled with mass spectrometry (GC-MS/MS) (Shimadzu GCMS-QP2010 SE, Kyoto, Japan), which was equipped with an Rtx®-5MS capillary column (30 m × 0.25 mm × 0.25 µm). The operating conditions of the GC-MS/MS were optimized as follows: 70 eV, carrier gas (He), flow rate of 1.7 mL.min^{-1}, and pressure 53.5 KPa. The temperatures of the injector and the interface of the detector were 25 °C and 230 °C, respectively. The oven temperature program was 100 ◦C for 3 min, and then 310 °C at a heating rate of 3.5 °C/min and maintained at 310 °C for 5 min. The identification of the constituents of the essential oils was investigated by comparing the mass spectra and Kovats index values (IK) with those of the library search references.

4.4 Antimicrobial and Antibiofilm Activities

The anticandidal activity was evaluated using the microdilution method described by the Clinical and Laboratory Standards Institute [39] with some

modifications. To evaluate cell growth inhibition, an aliquot (50 µL) of yeast cell suspensions (0.5–2.5 × 10^6 CFU mL^{-1}) in Saboraud liquid medium was mixed in 96- well plates with 50 µL of *Cb*EO (ranging from 50 to 0.008 µg mL^{-1}, diluted in 5% DMSO). The antibacterial activity was determined according to the method described by Oliveira et al. [40] with modifications. The experimental assay was similar to the previous one, with the bacterial cells being cultivated in Mueller–Hinton Broth medium. The positive controls were nystatin and ciprofloxacin and the negative control was 5% DMSO. After 24 h at 37 °C, the cell growth was measured using a microplate reader at 600 nm (Epoch, BioTek Instruments Inc., Winooski, VT, USA). Each experiment was performed three times, with three replicates per treatment.

The antibiofilm assays were performed according to the method described by Dias et al. [41]. The same controls and concentrations of *Cb*EO used in planktonic cells were applied in the biofilm assays. After 24 h of incubation, the supernatant wasremoved from the wells, followed by three washes with 0.15 M NaCl, and then 200 µL of methyl alcohol was added to each well for 15 min to allow fixation of the adhered cells to occur. Then, 200 µL of 0.1% crystal violet was added for another 15 min. To dissolve the dye attached to the biofilm, 200 µL of 33% acetic acid was added and left on the plate for reading the absorbance at 590 nm using a plate reader.

4.5 Mechanisms of Actions

4.5.1 Membrane Damage

For cell membrane integrity assay, 50 µL of C. *albicans* or *C. parapsilosis* cell suspension (2.5 × 10^3 CFUmL^{-1}, in Saboraud liquid medium) was mixed with 50 µL of *Cb*EO (50 µg mL^{-1}) and incubated for 24 h at 37 °C. Then, the samples were centrifuged at 5000× g for 5 min at 4 °C, and the cells were washed three times with100 µL of 0.15 M NaCl. Subsequently, 50 µL of NaCl and 3 µL of 1 mM propidium iodide (PI) were added and the mixture was incubated for 30 min in the dark at 37 °C. Afterward, the samples were washed twice with 0.15 M NaCl to remove excess PI and the cells were resuspended in 50 µL of 0.15 M NaCl to be analyzed on a fluorescence microscope (Olympus System BX 41, Tokyo, Japan) with an excitation wavelength of 535 nm and emission wavelength of 617 nm.

Additionally, the samples were treated similarly to the previous analysis and incubated with 3 µL of 1 mM 6 kDa FITC-Dextran (Sigma Aldrich, Sao Paulo, Brazil) in the dark for 30 min, according to Oliveira et al. [40]. The result was observed under a fluorescence microscope (Olympus System BX60) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

4.5.2 Induction of Reactive Oxygen Species (ROS)

To perform ROS overproduction, the methodology described by Dikalov and Harrison [42] was followed. The experimental design was similar to the previous assays of antimicrobial activity. First 50 µL of *C. albicans* or *C. parapsilosis* cell suspension (2.5×10^6 CFU mL^{-1}, in Saboraud liquid medium) was mixed with 50 µL of *Cb*EO (50 µg mL^{-1}) and incubated for 24 h at 37 °C. The samples were washed three times with 0.15 M NaCl solution and incubated with 50 µL of 0.2 M DCFH-DA for 20 min in the dark. A fluorescence microscope (Olympus System BX60) was used with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

4.5.3 Induction of Apoptosis

The caspase activity was measured after cell incubation for 24 h, in the presence and absence of essential oil, according to the manufacturer's instructions. The cells were treated as above and then incubated using 3 µL of 2 mM CellEvent® reagent (ThermoFisher, São Paulo, SP, Brazil) for 30 min in the dark. Then, cells were washed and centrifuged as described above. Finally, the cells were observed under a fluorescence microscope (Olympus System BX60) at an excitation wavelength of 342 nm and an emission wavelength of 441 nm.

4.6 Scanning Electron Microscopy (SEM)

For SEM analysis, the samples were mixed under the same conditions described previously, and then glutaraldehyde in 0.15 M sodium phosphate buffer pH 7.2 was added for 16 h at 25 °C for fixation. After that, each sample was washed three times with 0.15 M sodium phosphate buffer pH 7.2. For dehydration, it was washed with

ethanol at different concentrations (30, 50, 70, and 100%) leaving it for 10 min, except for the last concentration, which was dehydrated twice for 10 min. Subsequently, 50% hexamethyldisilane (HMDS, Sigma, St. Louis, MI, USA) was diluted in ethanol for 10 min and then 100% HMDS was added. A 15 µL aliquot was added to coverslips and made it possible to dry at room temperature. For observation of the cells, they were coated with gold on aluminum surfaces and observed under a scanning electron microscope (Everhart–Thornley) [43].

4.7 Hemolytic Activity

The hemolytic activity of *Cb*EO was tested against red blood cells (A, B and O+) according to Oliveira et al. [40]. Blood types were provided by the Center for Hematology and Hemotherapy of Ceará (Brazil). Cells were centrifuged at 5000× g for 5 min at 4 °C, and dissolved in 0.15 M NaCl. Six washes were performed, and then the bloods were diluted to 2.5% in 0.15 M NaCl. Subsequently, an aliquot of 300 µL of each blood type was incubated with 300 µL of *Cb*OE at concentrations ranging from 50 to 12.5 µg mL^{-1}, while the negative control contained 5% DMSO and the positive control 0.1% (v/v) Triton X-100. Then, the samples were incubated for 30 min at 37 °C, followed by centrifugation (5000× g for 5 min at 4 °C). After that, the supernatants were collected and transferred to 96-well plates. Hemolysis (%) was calculated by measuring the absorbance of the supernatant at 414 nm using a microplate reader.

4.8 Statistical Analysis

All tests were performed in three biologically independent experiments. The difference between the means of the triplicates was verified by applying the ANOVA test followed by the Tukey method using the GraphPad Prism program version 5.01 (GraphPad Software company, Santa Clara, CA, USA). Values of $p < 0.05$ were considered statistically significant.

Conflicts of Interest: The authors report no conflict of interest. The authors alone are responsible for the content and the writing of the paper.

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5 CONCLUSÃO

O óleo essencial extraído de folhas de *C. blanchetianus* Baill. mostrou em sua composição química vinte e três compostos, sendo os mais abundantes: *espatulenol* (20,03%); *biciclogermacreno* (5,92%); *óxido de cariofileno* (5,81); *eucaliptol* (5,62%). Não foi possível obter valores de inibição significativos sobre as bactérias Gram-negativas, *C. neoformans, C. tropicalis* e *C. krusei* testados nesse trabalho. No entanto, os resultados foram satisfatórios tanto em sua inibição de células planctônicas como em biofilmes para *C. albicans* e *C. parapsilosis*.

Dentre os mecanismos de ação investigados, podemos concluir que houve danos à membrana e espécies reativas de oxigênio. Também foi avaliado que o óleo não possui toxicidade sobre eritrócitos humanos.

Assim, pode-se concluir que o óleo essencial de *C. blanchetianus* é uma fonte natural promissora para a busca de novos compostos ao combate de infecções causadas por *C. albicans* e *C. parapsilosis*.

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