UNIVERSIDADE FEDERAL DE UBERLÂNDIA

Instituto de Ciências Biomédicas

Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas

Artabotrys brachypetalus Benth.: potencial antifúngico, antibiofilme e antivirulência frente a patógenos de interesse em saúde pública e avaliação da toxicidade em *Caenorhabditis elegans*

Camila de Paula Siqueira

Uberlândia – MG

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Dissertação apresentada ao Colegiado do Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas como requisito parcial para obtenção do título de Mestre.

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Orientador: Prof. Dr. Carlos Henrique Gomes Martins

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Reuniu-se no dia 31 de agosto, às 9 horas, por vídeo conferência, a Banca Examinadora designada pelo Colegiado do Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas, assim composta pelos titulares: Reginaldo dos Santos Pedroso - ESTES/UFU; Regina Helena Pires - UNIFRAN; Carlos Henrique Gomes Martins - PPGIPA/ICBIM/UFU (Presidente) e orientador do(a) candidato(a).

Iniciando os trabalhos o presidente da mesa, Prof. Carlos Henrique Gomes Martins, apresentou a Comissão Examinadora e o(a) candidato(a). Agradeceu a presença do público e concedeu ao(à) discente a palavra para exposição do seu trabalho. A duração da apresentação do(a) discente e o tempo de arguição e resposta foram conforme as normas do Programa.

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‘‘A verdadeira colheita de meu dia a dia é algo de tão intangível e indescritível quanto os matizes da aurora e do crepúsculo. O que tenho na mão é um pouco de poeira das estrelas e um fragmento do arco-íris.” -Na natureza selvagem”

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Lista de Figuras

Lista de Tabelas

Sumário

Apresentação

Segundo dados da Organização Mundial da Saúde (OMS), 85% da população faz uso de plantas como alternativa de tratamento de patologias, o Brasil possui uma extensa diversidade de plantas com baixo custo, o que torna uma forma terapêutica acessível a toda população. Com o crescente uso de plantas medicinais, no Brasil existem políticas que incentivam a implementação dessa terapia, principalmente a implementação no Sistema Único de Saúde (SUS).

O uso de produtos naturais nos proporciona uma eficácia no tratamento de infecções causadas por microrganismo, tanto a biodiversidade da savana, brasileira quanto africana, nos proporciona plantas com extratos potentes que podem ser utilizadas contra patógenos. Bioativos de Annonacea são específicos e responsáveis por benefícios para a saúde incluindo diferentes classes de acetogeninas.

A presente dissertação de Mestrado, foi realizada no Laboratório de Ensaios Antimicrobianos (LEA) do Instituto de Ciências Biomédicas da Universidade Federal de Uberlândia (UFU). Embora a maioria das atividades realizadas nesse estudo tenham sido executadas nesse laboratório, a equipe contou com a colaboração fundamental de outros laboratórios, como do Insituto Federal do Triângulo Mineiro (IFTM), do Instituto de Química da UFU e ainda do Departamento de Ciências Exatas e Tecnológicas da Universidade Pùngué (Moçambique).

O objetivo deste trabalho foi identificar e purificar extrato bruto de folhas, raiz e caule de *A. brachypetalus* e determinar a Concentração Inibitória Mínima (CIM) e Concentração Bactericida/Fungicida Mínima (CBM/CFM), avaliar a inibição e erradicação do biofilme formado, avaliar a atividade antivirulência de leveduras do tipo *Candida* e a toxicidade em modelo *in vivo*.

Esta dissertação foi dividida em dois capítulos, conforme apresentado a seguir: **Capítulo I** – **Revisão Bibliográfica** - Apresenta uma revisão de literatura atualizada sobre doenças bucais e atividade antimicrobianas de produtos naturais.

Capítulo II *Artabotrys brachypetalus* Benth.: potencial antifúngico, antibiofilme e antivirulência frente a patógenos de interesse em saúde pública e avaliação da toxicidade em *Caenorhabditi elegans*. Artigo para ser submetido para publicação no periódico Microbial Pathogenesis (Fator de Impacto 3.738, Qualis CAPES B2- Ciências Biológicas III), em 2022.

Resumo

A mucosa bucal é composta por diversos microrganismos como bactérias e leveduras, ao entrar em desequilíbrio neste microambiente podem causar infeções. Microrganismos patogênicos possuem a capacidade formar biofilme em superfície rígida como os dentes, que podem disseminar para outros órgãos através da corrente sanguínea, essas infecções são agravadas em imunodeprimidos e em pacientes hospitalizados. Ainda assim, alguns isolados fúngicos possuem a capacidade de produzirem fatores de virulência importantes que favorecem o processo infeccioso, como expressão de enzimas. Além disso, a utilização de antifúngicos por longos períodos podem induzir a resistência às drogas usuais e gerar falha terapêutica, além de alta toxicidade para o hospedeiro, que dificulta o tratamento. Nesse contexto, *Artabotrys brachypetalus* Benth. tem sido utilizada na medicina popular para tratamento de infecções bacterianas, malária, inchaço ganglionar, dentre outras, através do uso de infusões com cascas, raiz e folhas dessa planta. O uso de produtos naturais nos proporciona uma eficácia no tratamento de infecções com efeitos colaterais minimizados. O objetivo desse estudo foi avaliar o perfil químico e a atividade antimicrobiana, antibiofilme e antivirulência do extrato bruto e frações de *A. brachypetalus* frente a bactérias causadores de cárie, doença periodontal e leveduras do *Candida* spp., bem como sua toxicidade. Foram avaliadas a concentração inibitória mínima (CIM) e a concentração bactericida mínima (CBM), e concentração fungicida mínima de *Candida* spp. (CFM), a capacidade de inibir ($CIMB_{50}$) e/ou erradicar ($CEMB_{50}$) o biofilme pré-formados, a capacidade de reduzir e/ou inibir a expressão exoenzimática por *Candida* sp. Por fim, tratou-se da toxicidade das amostras em modelo *in vivo* de *C. elgans*. A análise de LC-MS das frações mais ativas revelou a presença de metabólitos como alcalóides, flavonóides, terpenos e ácidos graxos. O extrato etanólico da raiz apresentou os melhores resultados, sendo fungicida para *C. glabrata* com CIM e CFM de 375 µg/mL. Para inibição da formação de biofilme, $CIMB_{50}$ e IC_{50} de 1,46 e 1,59 µg/mL foram obtidos para *C. glabrata*, enquanto a erradicação do biofilme apresentou resultado de IC_{50} de 0,00028 µg/mL frente a *C. tropicalis*. Ao promover a expressão de exoenzimas redutoras (½ MIC) das amostras, foi verificado, uma atividade hemolítica significativa de 42,7% ($p \leq 0,03$) frente a *C. albicans.* Para fosfolipase uma redução de 15,03% ($p \leq 0,03$) na fração diclorometano para *C. albicans*. Para proteinase, redução de 35,8% ($p \leq 0,03$) frente a *C. tropicalis*. O extrato etanólico da raiz demonstrou sobrevivência acima de 50% em concentrações acima de 1000µg/mL para *C. elegans*.

Podemos concluir com os extratos e frações de *A. brachypetalus* apresenta baixa toxicidade em ensaios realizados com *C. elegans* e alto potencial antifúngico, antibiofilme e antivirulência contra *Candida* sp.

Palavras-chave: Cárie, Periodontite, *Candida* spp., Biofilme, Atividade antimicrobiana; *A. brachypetalus*, Antivirulência.

Abstract

The oral mucosa is composed of several microorganisms such as bacteria and yeasts, when they get out of balance, these can cause infections. Pathogenic microorganisms have the ability to form biofilms on a hard surface like as teeth, causing inflammatory processes that can spread to other organs through the bloodstream, these infections are aggravated in immunocompromised and hospitalized patients. Still some isolates have the ability to produce important virulence factors that favor the infectious process and make treatment difficult, such as enzyme expression. This difficulty implies the use of drugs for long periods, inducing resistance to the usual drugs, in addition to high toxicity to the host, which makes treatment difficult. In this context, *Artabotrys brachypetalus* Benth. has been used in folk medicine for the treatment of some diseases such as bacterial infections, malaria, larvicide, lymph node swelling, among others through infusions with bark, root and leaves of the plant. The use of natural products provides us with an effectiveness in the treatment of infections with minimized. The objective of this study was to analyze the effectiveness of the root extract and fractions of *A. brachypetalus* against bacteria to causing caries and periodontal disease and *Candida* sp. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) and minimum fungicide concentrations (MFC) and the ability to inhibit and/or eradicate preformed biofilm in extracts with MIC < 100µg/mL and >1000µg/mL were evaluated. The ability to reduce and/or inhibit exoenzymatic expression by *Candida* sp. Finally, the toxicity of the samples was analyzed using the methodology of *C. elgans*. The L-CMS analysis of the most active fractions revealed the presence of metabolites such as alkaloids, flavonoids, terpenes and fatty acids. The ethanolic extract of the root showed the best results, being CFM fungicidal for *C. glabrata* with MIC and CFM of 375 µg/ml. For inhibition of biofilm formation, $CIMB_{50}$ and IC_{50} of 1.46 and 1.59 µg/ml were obtained against *C. glabrata*, while biofilm eradication resulted in IC_{50} of 0.00028 µg/ml against *C. tropicalis*. By promoting the expression of reducing exoenzymes (½MIC) from the samples, or root extract, a significant hemolytic activity of 42.7% ($p \leq 0.03$) against *C. albicans*. For phospholipase a 15.03% ($p \leq 0.03$) reduction of phospholipase to dichloromethane against *C. albicans*. For proteinase, reduction of 35.8% ($p \leq 0.03$) against *C. tropicalis*. The ethanolic extract of the root showed survival above 50% at concentrations above 1000µg/mL. We can conclude that the extracts and fractions of *A.*

brachypetalus show low toxicity in assays performed with *C. elegans* and high antifungal, antibiofilm and antivirulence potential against *Candida* sp.

Keywords: Oral diseases, *Candida* sp., Biofilm, Antimicrobial activity, *A. brachypetalus*, Antivirulence.

Capítulo I

Fundamentação Teórica

1. **Revisão da literatura**

1.1 Doença periodontal

A microbiota bucal é representada por uma variedade de microrganismos com mais de 700 espécies e tem como função a proteção contra a colonização de bactérias extrínsecas, que podem afetar a saúde sistêmica. Por outro lado, as doenças bucais mais comuns cárie, gengivite e periodontite são causadas pelos próprios microrganismos da microbiota, quando ocorre a disbiose (ARWEILER ANDA NETUSCHIL, 2016). As infecções bucais estão ligadas a diversas condições sistêmicas, e o comprometimento da integridade natural da microbiota oral que pode levar a desencadear doenças em outros órgãos do hospedeiro (HENZ et al., 2019).

Existe uma grande quantidade de doenças bucais consideradas polimicrobianas, ou seja, causadas pelo conjunto de microrganismos, como a cárie que é proveniente da fermentação de carboidratos por microrganismos e bastante associada ao *Streptococcus* spp., sendo *S. mutans* o principal microrganismo desenvolvedor desta patologia (WOELBER; AL-AHMAD AND ALT, 2022). Já a periodontite é uma doença infecciosa gengival, na qual ocorre danos nos tecidos moles e nos ossos que sustentam os dentes, normalmente causada pela falta ou precária higiene bucal (JAMAL et al., 2018). O processo inflamatório é desencadeado e perpetuado por bactérias Gram-negativas como *Porphyromonas gingivalis*, *Tannerella forsythensis*, *Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Fusobacterium nucleatum, Campylobacter rectus* e *Eikenella corrodens* e Gram-positivas como *Peptostreptococcus micros* e *Streptococcus intermedius* [5].

A cavidade bucal ainda abriga outros microrganismos como as leveduras do gênero *Candida*, que também afetam a saúde bucal especialmente de indivíduos imunocomprometidos. *C. albicans* são encontradas em biofilmes polimicrobianos associados com a cárie dental, doenças periodontais e outras doenças bucais. Assim a importância em desvendar as interações entre a formação de biofilmes bucais precisam de estratégias terapêuticas atualizadas com a capacidade de controlar interações entre espécies distintas diminuindo a virulência causadas por esses microrganismos (NEGRINI et al., 2019).

1.2 *Candida* spp.

O gênero *Candida* também compõe a microbiota da cavidade oralbucal de aproximadamente 50% da população adulta, sendo *C. albicans, C. prapsilosis, C. tropicalis* as mais comuns, ainda podem ser encontradas espécies *C. glabrata* e *C. krusei* (ATAÍDES et al., 2010). No entanto, em indivíduos com algum comprometimento imunológico essas espécies podem sair do estado comensal e tornar-se patogênicas, desencadeando infecções mais simples como a candidíase oral até infecções graves como infecção de corrente sanguínea, impactando de forma significativa nas taxas de morbimortalidade (DOI et al., 2016).

Por muito tempo *C. albicans* foi a principal espécie isolada de candidíase invasiva. No entanto, nos últimos anos o número de infecções por *Candida* não *albicans* aumentou significativamente. Atrelado a isso, temos o surgimento de isolados resistentes, tornando a situação ainda mais preocupante devido ao impacto na morbimortalidade (DOI, et al., 2016). Sendo *Candida* um dos patógenos de maior importância, o uso de medicamentos antifúngicos se torna imprescindível, que vêm sendo utilizados para antissepsia, à base de tintura de iodo, iodoquinol, violeta de genciana, ácido salicílico e benzoico, derivados sulfamídicos, corantes, quinonas e antifúngicos poliênicos (nistatina, anfotericina B). Além desses também temos os antifúngicos azóis que são: agentes do imidazol (cetoconazol, clotrimazol) e os agentes triazóis (fluconazol e Itraconazol). O uso excessivo e indiscriminado destes fármacos favorece o surgimento de microrganismos resistentes, principalmente em pacientes imunossuprimidos, susceptíveis a infecções recorrentes (ABEBE et al., 2021).

Para Cushnie et al., 2014 algumas estratégias são necessárias para ação antivirulência dos microrganismos, a eficácia de vacinas para hospedeiros imunocomprometidos, drogas com moléculas pequenas para tratamento e prevenção dos patógenos ou ainda uma droga com a capacidade de inativar a virulência direta do patógeno. Assim o estudo de plantas com atividade antimicrobiana tem-se aprimorado para extração de compostos refinados com a capacidade de ser um fitoterápico eficiente e de baixa toxicidade para o hospedeiro, sendo estes muitas vezes tóxicos para o próprio hospedeiro.

1.2.3 Exoenzimas

Candida pode se tornar patogênica em condições de imunodepressão do hospedeiro, como portadores de HIV, tratamento de câncer, hospitalizados por longos períodos, dentre outros, assim são consideradas como oportunistas. Para causar infecções a levedura necessita de ultrapassar os tecidos, e provocar alterações bioquímicas e físico-químicas. Os principais fatores de virulência causados por *Candida* incluem a expressão de exoenzimas hidrolíticas (DA ROCHA et al., 2021).

A atividade hemolítica corresponde a um fator de virulência de alguns microrganismos que torna possível o crescimento dos mesmos no hospedeiro pela utilização de proteínas ligadas ao ferro, leveduras do gênero *Candida* tem a capacidade de secretar um fator hemolítico conhecido como hemolisina, o qual desregula os eritrócitos e permite a assimilação de ferro do grupo hemoglobina, e promove a sobrevivência através da capacidade de sequestrar ferro. A atividade hemolítica desempenha papel importante na infecção de corrente sanguínea por *Candida*, no qual eritrócitos são expostos as células das leveduras, desencadeando candidíase sistêmica (FURNETO et al., 2015).

As fosfolipases estão relacionadas com o processo de ruptura da membrana das células do hospedeiro no momento da invasão, com capacidade para hidrolisar ésteres ligados a glicerofosfolipídeos, que promovem a clivagem dos fosfolipídeos, ocorre a instabilidade da membrana, com subsequente lise celular, esse mecanismo torna o biofilme por *Candida* mais virulento, pois se aderem melhor a superfície tecidual do hospedeiro tornando o biofilme mais rígido (SILVA et al., 2012).

As proteinases clivam proteínas relacionadas com o mecanismo de defesa do hospedeiro e promovem a evasão das defesas por degradação de imunoglobulinas (DA ROCHA et al., 2021). Essa virulência causada pela expressão de exoenzimas juntamente com a adesão e produção de ácidos que são os principais componentes da matriz extracelular agravam a patogenicidade de infecções bucais (ARDIZZONI et al., 2022).

O tratamento farmacológico de pacientes com candidíase envolve o uso de agentes antifúngicos imidazóis e triazóis, incluindo fluconazol, cetoconazol, miconazol, itraconazol e clotrimazol, além do agente poliêniconistatina. Esses medicamentos são eficazes no controle da candidíase, no entanto, o aumento do uso de antifúngicos e o tratamento prolongado são fatores de risco para o surgimento de espécies de *Candida*

resistentes a essas drogas, e ainda a toxicidade ao hospedeiro, visto isso, a busca por fármacos menos tóxicos é uma realidade (DE OLIVEIRA AND SCHMIDT, 2020).

1.3 Biofilme

Biofilmes são grupos de microrganismos agregados a uma superfície, formando uma barreira rígida, que fornece proteção ideal a esta comunidade, formado por uma massa sólida, rica em polissacarídeos (BOARI et al.; 2009). No que diz respeito ao biofilme dental, este apresenta-se como agente determinante na expressão de cárie e periodontite, que se forma constantemente nas superfícies dentárias e mucosa bucal, sendo formados a partir da adesão de células bacterianas e fúngicas, classificados como problema de âmbito odontológico e saúde pública (CHEN, et al., 2020). Existem evidências de que espécies diferentes possam se agregar nos biofilmes, aumentando a resistência a antimicrobianos usuais, sendo capaz de degradar ou inativar o antimicrobiano (KOKARE et al., 2009).

Outro microrganismo capaz de formar biofilme é a *Candida*, mais comumente encontrada em dispositivos médicos, possui a capacidade de resistência aos fármacos usuais devido a diversos fatores, e secreção de enzimas hidrolíticas (proteases, fosfolipases e hemolisinas) (TAFF, et al., 2013).

A capacidade da microbiota bucal em tornar-se patogênica envolve diversos processos, sendo a formação e manutenção do biofilme um processo infeccioso como protagonista desta doença. Alguns ácidos são produzidos pelas bactérias do biofilme juntamente com baixo pH, o que intensificam a virulência causadas por esses patógenos (ABEBE, 2021). Outras infecções superficiais causadas por leveduras do tipo *Candida* são associadas a dispositivos implantados, como a estomatite protética, que é uma infecção por *Candida* da mucosa, o biofilme é formado na superfície da prótese acrílica e contém grande número de bactérias além de leveduras. Outros biofilmes polimicrobianos contaminados por *Candida* spp. se instalam em próteses vocais que são instaladas em pacientes laringectomizados (DOUGLAS, 2003).

Portanto, os biofilmes são apresentados com características de desenvolvimento e traços fenotípicos únicos, o que os tornam mais resistentes a agentes antimicrobianos e fatores imunes do hospedeiro (ABEBE et al., 2021). São um grande problema em diversos cenários, pois possuem mecanismos que inativam ou impossibilitam a entrada de

antimicrobianos, aumentando a patogenicidade dessa comunidade, sejam eles na saúde ou até mesmo em meio ambiente (DAMACENO AND FARIAS, 2016). Hoje é estimado que 80% das infecções são causadas por biofilmes devido a resistência aos fármacos usuais, ao considerarmos que compostos naturais exercem funções antimicrobianas, tornando as plantas alvo de estudos para utilização alternativa de fármacos usuais (NOUBAMKHSH et al., 2022).

1.4 Produtos naturais

Desde os tempos remotos, os seres humanos buscam nos recursos ambientais uma solução para o tratamento de doenças que acometem a saúde, sendo a utilização de plantas uma das principais opções para este fim (FERREIRA et al., 2017). Compostos naturais são utilizados na medicina popular com ação terapêutica e possuem uma grande importância na manutenção nas condições de saúde das populações, sendo utilizadas de acordo com o conhecimento tradicional e possibilitando assim a cura de enfermidades. Populações que residem em localidades que sejam consideradas de difícil acesso as unidades públicas de saúde básica, como populações indígenas, quilombolas, ribeirinhos, extrativistas e produtores rurais utilizam de produtos naturais como tratamento de doenças (MORESKI et al., 2018).

É sabido que 80% da população utiliza-se de práticas tradicionais nos seus cuidados básicos de saúde tendo 85% destes a utilização de plantas ou preparações destas como alternativa no tratamento de saúde (MINISTÉRIO DA SAÚDE, 2016). Ao final da década de 1970, foi criado pela Organização Mundial da Saúde um programa para incentivar o uso de plantas medicinais em tratamentos tradicionais, o Programa de Medicina Tradicional, que recomenda aos estados-membros, que desenvolva políticas públicas com intuito de facilitar a integração entre a medicina tradicional e a medicina complementar. Com essa alternativa nos sistemas nacionais de atenção à saúde, possibilitaria promover o uso racional dessa integração (MINISTÉRIO DA SAÚDE, 2016).

Produtos naturais são utilizados na medicina como antibióticos, antifúngicos, ainda como agente anti-cancerígenos (GARG et al., 2017). Diversos compostos são descritos na literatura com ação benéfica a saúde, como flavonoides, terpenos, alcalóides, dentre outros. A procura por produtos naturais com atividade antimicrobiana combate a

doenças bucais, antifúngicas e antiparasitárias, tem merecido destaque, devido ao baixo custo, e sua eficácia frente à diversas doenças, assim a busca por novas substâncias de interesse farmacológico presentes em plantas envolve uma série de fatores, dentre eles o uso popular e a presença de metabólitos secundários com atividade biológica eficaz [5].

Nessa via de entendimento, muitas plantas foram e são utilizadas de forma popular, despertando o interesse na descoberta de compostos capazes de curar doenças. No Brasil o estudo por plantas como utilização de medicamentos compõe uma grande variedade, devido aos seus diversos Biomas, o que ocorre em outros continentes como a África (FERREIRA, et al., 2019).

1.5 *Artabotrys Brachypetalus* Benth.

A família Annonaceae consiste em 130 gêneros e aproximadamente 2500 espécies (Pumiputavon et al., 2019) é um grupo de árvores aromáticas, arbustos e cipós, que crescem principalmente nas regiões tropicais. Algumas espécies desta família possuem componentes com diversas substâncias farmacológicas, com atividades antibióticas e antiparasitárias. Espécies de Annonaceae possuem em seu metabolismo, alcaloides, terpenoides, flavonoides, acetogeninas e hidrocarbonetos monoterpênicos (PUMIPUTAVON et al., 2019).

As espécies *Artabotrys* são encontradas principalmente na Asia e África (RAVI AND SUNDARAM, 2020). Alguns estudos relatam que espécies de *Artabotrys* possuem derivados químicos naturais, que foram tradicionalmente usados na medicina chinesa para o tratamento de malária e inchaço glandular. Na Tanzânia, uma mistura da casca de *A. brachypetalus* Benth. é utilizada no tratamento de gonorreia. Além disso, a espécie *A. modestus* Diels. é utilizada através de infusões de suas folhas como medicamento para tratar náuseas e vômitos, enquanto suas raízes são utilizadas no tratamento de dor estomacal e diarreia. Já o extrato de casca das raízes de *A. brachypetalus* Oliv., possui ação analgésica e anti-inflamatória, e ainda agente antimalárico (NYANDORO et al., 2013).

Um dos compostos químicos presente em *A. brachypetalus* Benth. são os alcalóides, que podem abrigar importantes propriedades de cicatrização de feridas (CHINGWARU et al., 2019). Alcalóides são compostos de baixa massa molecular contendo nitrogênio, geralmente são alcalinos e apresentam baixa toxicidade (SAINI et

al., 2022). Esses compostos são os possíveis responsáveis pela atividade antimicrobiana da planta, sendo relatada na literatura como fitoterápicos naturais utilizados no mundo oriental, para tratamento de diversas doenças e com ação farmacológica como, analgésicos, antissépticos, sedativos dentre outros (BRIBI, 2018).

Existem poucos estudos para ensaios antimicrobianos de espécies *Artabotrys*, Nyandoro et al., (2013) relata que compostos alcalóides possuem propriedade antimicrobiana utilizada na medicina Chinesa, sendo utilizados popularmente como infusões no tratamento de gonorréia, náuseas, outras espécies são listadas com ação antinflamatória e antimalárica. Em seu trabalho os ensaios antimicrobianos revelaram-se promissores para *Staphylococcus aures* (ATCC25923) com atividade bactericida em até 40µg/mL, porém não revelou atividade bactericida para a espécie *Escherichia coli* (DSM1103). Já ao testarem para fungos *C. albicans (*DSM1665) e *Cryptococcus neoformans* (ATCC90112) não foram encontrados resultados promissores. Já a toxicidade de espécies *Artabotrys* foi analisada por Sichaemem (2011) através células HeLa e e KB apresentando IC_{50} de 17,2 e 20,8 µg/mL, tais achados demonstram que espécies de *Artabotrys* possuem potencial fitoterápico de baixa toxicidade.

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Capítulo II

Artabotrys brachypetalus Benth.: antifungal, antibiofilm and antivirulence potential against pathogens of public health interest and their toxicity in *Caenorhabditis elegans*

Artabotrys brachypetalus Benth.: antifungal, antibiofilm and antivirulence potential against pathogens of public health interest and their toxicity in *Caenorhabditis elegans*

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Abreviation

½ MIC Sub-inhibitory Concentrations

ºC Celsius

ASD Agar Dextrose Sabouraud

CBM Minimum Bactericidal Concentration

CFM Minimum Fungicidal Concentration

CFU Colony Forming Units

CLSI Clinical and Laboratory Standards Institute

DMSO Dimethyl Sulfoxide

EE Extract Ethanolic

HPLC-MS-ESI High Performance Liquid Chromatography Coupled to Mass Spectrometry

IC_{50} Minimum Biofilm Eradication Concentration able to reduce by 50% or more the cell viability of the preformed biofilm.

LCMS Liquid Chromatography to Mass

$MBIC_{50}$ Inhibition of Biofilm Formation able to reduce by 50% or more to biofilm

MIC Minimum Inhibitory Concentration

MTT 2,3-bis (2-methoxy-4- nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

NGM Nematode Growth Medium

OD values of the biomass in relation

PBS Phosphate-buffered Saline

Abstract

Oral disease can happen as a result of microorganism's interaction, low immune system and/or poor hygiene. Resistance to the treatment may occur, since the standard procedure consists of use of highly toxic drugs. The use of natural products provides us with an effective treatment of infections with minimized side effects. The antimicrobial activity of *Artabotrys brachypetalus* Benth is described in the literature, and therefore, this study aims to evaluate the toxicity and antimicrobial activity from root extract and fractions. The tests were carried out against microorganisms that cause caries, periodontal disease and *Candida* sp. There were evaluated the minimum inhibitory concentration (MIC) and the minimum bactericidal/fungicidal concentration (MBC/MFC), as well as the ability to inhibit and/or eradicate the preformed biofilm and the ability to reduce and/or inhibit exoenzymatic expression by *Candida* sp. After that, the samples toxicity by the methodology of *C. elgans* was also evaluated. LC-MS analysis of the most active fractions revealed the presence of metabolites such as alkaloids, flavonoids, terpenes and fatty acids. The ethanolic extract from the root showed the best results, with CFM being fungicidal for *C. glabrata* with MIC and CFM of 375 µg/ml. Regarding inhibition of biofilm formation, $MBIC_{50}$ and IC_{50} of 1.46 and 1.59 µg/ml were obtained against *C. glabrata*, whereas biofilm eradication presented an IC_{50} result of 0.00028 µg/ml against *C. tropicalis*. Concerning the expression of reducing exoenzymes (½MIC), root extract revealed a significant hemolytic activity of 42,7% ($p \leq 0.03$) against *C. albicans*. The following results were found: for phospholipase a reduction of 15,03% ($p \leq 0.03$) phospholipase to dichloromethane against *C. albicans*; for proteinase, a reduction of 35,8% ($p \leq 0.03$) against *C. tropicalis*. The ethanolic root extract revealed a survival rate above 50% for concentrations above 1000µg/mL. We can conclude, from the extracts and fractions of *A. brachypetalus* Benth, that it has a low toxicity in assays carried out with *C. elegans* and a high antifungal, antibiofilm and antivirulence potential against *Candida* sp.

Keywords: Oral diseases, Antimicrobial activity; Biofilm, *A. brachypetalus*, Antivirulence

1. **Introduction**

Oral biofilm is defined by microbial communities adhered to a surface, surrounded by a polymeric substance called extracellular matrix, forming a rigid barrier that provides ideal protection to this community. The fermentation of carbohydrates by microorganisms is closely associated with *Streptococcus* spp., with *S. mutans* being the main initiating microorganism of caries, affecting 90% of individuals, whereas *S. sobrinus* is presented with lower prevalence in the population, with caries reaching about 20% [1,2]. Other microorganisms are also associated with caries formation, such as *Actinomyces* sp, *Lactobacillus* sp, *Bifidobacterium* sp and still species of *Scardovia* sp [3].

Other infectious periodontal diseases also damage the soft tissues and bones that support the teeth [4].This pathology is triggered and perpetuated by Gram-negative bacteria such as *Porphyromonas gingivalis*, *Tannerella forsythensis*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Campylobacter* rectus and *Eikenella corrodens*, Gram-positive bacteria such as *Peptostreptococcus micros*, *Streptococcus intermedius* and spiral bacteria such as *Treponema denticola* [5]. Caries and periodontitis occur from the adhesion of bacterial cells to the tooth and are classified as the main dental and public health oral diseases [6].

The genus *Candida* spp. is present in the oral microbiota, which can be found in the microbiota of up to 35% of the adult population, *C. albicans*, *C. tropicalis* and *C. parapsilosis* being the most common [7]. In immunocompromised individuals, *Candida* spp. can go from from a commensal state to a pathogenic one, spreading to the individual's internal organs [8]. Although commensal, *Candida* spp. shows several virulence factors important in its pathogenicity, such as: production of hemolysins, proteinases, phospholipases (considered essential for the invasion process into the host cells) and also the ability to form biofilms (which are capable of adhering and invading epithelial cells and endothelial) [9,10].

In light of this, the search for natural products with antimicrobial and antifungal activities has been highlighted. The plant species Annonaceae consists of 112 genera and approximately 2500 species, from which we have *Artabotrys*, belonging to this family and having natural chemical derivatives, like protoberberine and alkaloids, forming compounds with medicinal actives [11,12]. According to Lee [11], bark and roots of *A.*

brachypetalus were traditionally used in Chinese medicine. Antimicrobial activities of *Artabotrys* species are described with antifungal action by *A. modestus* and *A. brachypetalus* for *C. albicans* and *Aspergillus* spp., respectively, and antibacterial for *S. aureus* due to alkaloid compounds, showing antimicrobial action [13,12].

Given the few studies found in the literature and the search for new substances with antimicrobial and antivirulence action, our study seeks to determine the antimicrobial activity of the crude extract and fractions of *Artabotrys brachypetalus* against oral bacteria and *Candida* yeasts. Besides that, it seeks to evaluate the ability to inhibit biofilm formation and eradication of pre-formed biofilm, as well as the production of exoenzymes produced by *Candida* species.

2. Material and Methods

2.1. Plant material

The twigs, root and leaves of *A. brachypetalus* were collected in Maputo Province, Marracuene District, Mozambique. The species was identified by a specialist and a voucher specimen was deposited at the Herbarium of the Institute of Traditional and Alternative Medicine in Misau, Maputo-Mozambique under identification number 24. Certificate on Nº S4/PIF/2018. The samples were dried at room temperature for ten days and taken to Brazil to the Federal University of Uberlândia.

2.2. Extracts and fractions preparation

Samples of roots, twigs and leaves of *A. brachypetalus* were dried in an oven with air circulation at 35°C to moisture 8.3%. The dried and ground plant material from the root (173.46g), twigs (85.32g) and leaves (66.13g) was extracted with ethanol (98% v/v) by maceration at room temperature for 48h. The ethanolic extract (EE) was filtered and concentrated on a rotary evaporator (IKA, RV 10). This extraction procedure was repeated four times. Then, the ethanolic extracts were frozen and lyophilized to remove water yielding 18.9g, 1.98g and 13.67g of extract for the root, twigs and leaves, respectively.

Due to the fact that the ethanolic extract of the root showed better results in biological assays, it was used for liquid-liquid extraction. The ethanolic extract of the root

(15g) was solubilized with 300 mL of a methanol solution: water (9:1 v/v), then it was partitioned with solvents of increasing polarity: hexane (5 x 200mL), dichloromethane (5 x 200mL), ethyl acetate (5 x 200mL) and butanol (5 x 200mL). The fractions were concentrated by rotary evaporator under reduced pressure at 40 °C yielding 1.48 g, 2.56g, 3.05g, 2.73g and 4.98 g for the hexane, dichloromethane, ethyl acetate, n-butanol and hydromethanolic remainder fractions, respectively. Then, they were stored in a freezer (-18 °C) for further analysis.

Samples of crude ethanolic extract from the root, stem and leaves, as well as fractions in hexane, dichloromethane, ethyl acetate, *n*-butanol and the remaining hydromethanolic were used in the tests.

2.3. High performance liquid chromatography coupled to mass spectrometry (HPLC-MS-ESI)

HPLC-MS analysis of the bioactive fractions of *A. brachypetalus* were carried out on a liquid chromatograph (Agilent, model Infinity 1260), coupled to a high-resolution mass spectrometer QTOF (Quadrupole Time of Flight – Agilent, model 6520 B), with electrospray ionization source (ESI) according to João et al [14]. A volume of 1.0 µL of the sample was injected into the chromatograph using an Agilent Zorbax C18 column (2.1mm x 50 mm, 1.8 µm). The chromatographic conditions consisted of ultrapure water with formic acid (0.1% v/v) (mobile phase A) and methanol (mobile phase B). The gradient system was: 10% B (0 min), 98% B (0–10 min), remaining with 98% B (10 – 17 min) with a 0.6 mL/min flow. The ionization parameters were: 58 psi nebulizer pressure, drying gas at 8L/min, 220 ºC temperature and an energy of 4.5KV was applied to the capillary. The analysis was performed in the negative mode $[M-H]^-$ in high resolution (MS). The molecular formula was proposed for each compound according to a list suggested by the MassHunter® Software following the lowest difference between the experimental mass and the exact mass, error in ppm, unsaturation equivalence and nitrogen rule. The sequential mass spectrometry (MS2) of the molecular ions was performed at different collision energies. The chemical composition of the extract was proposed comparing the mass spectra of fragments and the high-resolution mass obtained from other studies in the literature and from Metlin library.

2.4 Antimicrobial activities

2.4.1. Microorganisms

Standard strains from the American Type Culture Collection (ATCC) were used for the antimicrobial assays, listed below: *Candida albicans* (ATCC 90028), *C. glabrata* (ATCC 2001); *C. krusei* (ATCC 6258); *C. parapsilosis* (ATCC 22019) and *C. tropicalis* (ATCC 13803); *S. mitis* (ATCC 49456); *S. mutans* (ATCC 25175); *S. sanguinis* (ATCC 10556); *Aggregatibacter actinomycetemcomitans* (ATCC 43717); *Actinomyces naeslundii* (ATCC 19039), *Fusobacterium nucleatum* (ATCC 2586); *Porphyromonas gingivalis* (ATCC 33277). All isolates belong to the culture collection of the Antimicrobial Testing Laboratory of the Federal University of Uberlândia (LEA-UFU) and are kept under cryopreservation at -20ºC.

2.4.2. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal/Bactericidal Concentration (MFC/CBM)

• MIC/MFC for *Candida* spp.

In a 96-well microplate (Kasvi, São José dos Pinhais, Paraná, Brazil) containing RPMI 1640 broth (Gibco, Paisleu, Scotland, UK), buffered with 3-[N-Morpholino-Propanel]-Sulfonic – MOPS (Sigma-Aldrich, St. Louis, Missouri, USA) and the samples dissolved in methanol (Anidrol, Diadema, São Paulo, Brazil), according to Clinical and Laboratory Standards Institute (CLSI) document M27-A3[15] the suspension of the fungal isolate was added, whose turbidity was equivalent to the 0.5 tube on the McFarland scale 1-5x10^6, and the final concentration of the inoculum in the well was adjusted to a final concentration of 0.5 x 10^3 – 2.5 x 10^3 CFU/mL. Final sample concentrations from 0.98 to 3000 μg/mL were obtained. Then, the plates were incubated at 37°C for 24 hours. The antifungal Amphotericin B (Sigma-Aldrich) was used as a positive control. After incubation, the interpret the results, 30 μL of a 0.02% aqueous solution of resazurin (Sigma-Aldrich) was used. The microplate was re-incubated for 30 min and analyzed according to the color variations between blue (without bacterial growth) and pink (with bacterial growth). The lowest concentration of the test compound for which of

the wells remained blue was considered the MIC. The tests were evaluated in independent triplicates[16].

For MFC determination, a 10 µL aliquot of the inoculum was removed from each well, before the addition of resauzurin (Sigma-Aldrich) and seeded on a plate with Sabouraud Dextrose Agar (ASD). The presence of colonies on the aliquoted inserts indicates microbial growth in the aliquoted well. The experiments were performed in triplicate. The PBE (percentual bacteriostatic efficiency, %) was obtained as fungicidal/fungistatic activity (MFC/MIC). The ratio MFC/MIC was calculated in order to determine if the compound had a fungistatic (MFC/MIC≥4) or fungicidal (MFC/MIC≤4) activity[17].

• MIC/CBM for Facultative Anaerobic bacteria

Determination of the samples MIC was performed by dilution in a 96-well plate (Kasvi), according to the methodology recommended by the CLSI in document M07-A9[18], with adaptations. The culture medium used was Brain Heart Infusion – BHI broth (Kasvi). The samples were solubilized in methanol (Anidrol) and diluted in BHI broth. Concentrations ranged from 0.0115 to 400 µg/mL and chlorhexidine (0.0115 – 59.0 µg/mL) (Sigma-Aldrich) was used as a positive control. The final methanol content was up to 5% (v/v). The inoculum was adjusted to a concentration of 5×10^6 colony forming units (CFU) mL, whose turbidity was equivalent to the 0.5 tube on the McFarland scale. The strains were incubated in a microaerophilic jar system at 37ºC for 24 hours. The reading of results was performed as mentioned above, but inside an anaerobic chamber.

After incubation, 30 µL of aqueous resazurin (Sigma-Aldrich) solution to 0.02% was added to the wells to observe bacterial growth [16]. To determine the MBC, an aliquot (10 µL) of the inoculum was removed from each well before the addition of resazurin and placed on a plate with 5% horse blood agar, incubated in a microaerophilic jar system at 35°C for 24 hours. The presence of colonies on the aliquoted inserts indicated microbial growth in the aliquoted well. The experiments were performed in triplicate.

• CIM/CBM for Anaerobic bacteria

Determination of the samples MIC was performed by dilution in a 96-well microplate (Kasvi). The broth used in the tests was Schadler broth (Merck, Darnstadt, Hesse, DE) supplemented with hemin (5.0 mg/mL, Sigma-Aldrich) and vitamin K1 (1.0 mg/mL, Sigma), as recommended by CLSI in document M11-A7[19]. The samples were solubilized in methanol and diluted in Schadler broth, obtaining a final concentration between 0.0115 and 400 μg/mL. The antimicrobial Metronidazole (0.031 – 16 μg/mL) (Sigma-Aldrich) was used as a positive control. The final inoculum concentration was adjusted to 5×10^6 CFU/mL and incubated in an anaerobic chamber (miniMACS Anaerobic WORKSTATION) under an atmosphere containing 5 – 10% H_2, 10% CO_2 and 80 – 85% N_2 for 72 hours.

Analysis of results and MBC determination were done in the same way as for facultative anaerobic bacteria, considering the culture medium and incubation conditions for anaerobic bacteria. The experiments were performed in triplicate.

According to Holetz et al.,[20] natural products extracts with antibacterial activity are considered good when showing MIC below 100 μg/ml, moderate from 100 to 500 μg/ml, and weak from 500 to 1000 μg/ml and above 1000 μg/ml, respectively.

2.5. Antibiofilm assay

The ability to inhibit formation and eradication of preformed biofilm by *Candida* spp. was checked through the evaluation of cell viability and biomass, with samples of crude root extract, fractions of acetate ethyl, dichloromethane, hydromethanolic and *n*-butanol.

The assay performed followed the proposed MIC methodology by the M27-A3 CLSI [15] in 96-well plates. The fungal inoculum suspension was then added, with a final concentration adjusted to $1x10^6$ cel/mL [21]. Subsequently, RPMI 1640 (Gibco) broth was added with MOPS and the samples were diluted in 96-well plates, obtaining final concentrations in each well ranging from 0.98 to 3000 μg/mL. Then, the plates were incubated at 37°C for 24 hours for biofilm formation.

For biomass assessment, the contents of the wells were removed and washed three times with phosphate-buffered saline (PBS) to remove non-adhered cells. In sequence,

aliquots of 100 µL of methanol were added for 15 minutes to fix the formed biofilm. After this time, methanol was removed and crystal violet was added (Sigma-Aldrich) for 20 minutes. Finally, the plates were gently washed with distilled water, 33% acetic acid (Merck) was added for 30 minutes to solubilize the crystal violet (Sigma-Aldrich) and the absorbance of each well was determined through a spectrophotometer (Glomax Discover Microplate Reader (PROMEGA)) with a wavelength of 595 nm. The minimum biofilm inhibitory concentration was determined as the lowest concentration capable of causing 50% or more inhibition of biofilm formation ($MBIC_{50}$) [21].

To assess the samples ability to inhibit biofilm metabolic activity, the assay followed the Pierce et al.[21] methodology, in which the contents of the wells, after incubation, were washed with PBS three times to remove non-adhered cells, and following that it was added 50 µL of menadione and 2,3-bis (2-methoxy-4- nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (MTT) (Sigma-Aldrich) according with Menezes et al., 2022[22] with final concentration of 0.5mg/mL and incubated for 6 hours at 37ºC. The plates were packed with aluminum foil to prevent MTT (Sigma-Aldrich) degradation. Then, the formazan product was solubilized with the addition of 100 µL of DMSO (Sigma-Aldrich) for 10 minutes and 80 µL from each well were transferred to another plate for a spectrophotometer reading (Mindray MR-96AN) with a 490 nm wavelength.

To assess the samples ability to eradicate the preformed biofilm, 100µL of the isolate suspension with a concentration of $1x10^6$CFU/mL were added to the wells of the 96-well plates so that the microorganism could adhere to the plate and form a biofilm. The plates were incubated at 37°C for 24 hours. After biofilm formation, non-adherent cells were removed by washing the wells with PBS three times, adherent biofilms were exposed to aliquots of *A. brachypetalus* samples diluted in RPMI 1640 (Gibco) for 24 hours and incubated at 37ºC. Then, the wells were washed three times with PBS to remove non-adherent cells and 50µL of MTT and menadione solution, with a final concentration of 0.5mg/mL, were added and incubated for 6 hours at 37ºC. Then, the formazan product was solubilized by adding 100 µL of DMSO (Sigma-Aldrich) for 10 minutes, and 80µL from each well were transferred to another plate for a spectrophotometer reading (Mindray MR-96A) with a 490 nm wavelength. The Biofilm Eradication Minimum Concentration ($BEMC_{50}$) was determined as the lowest concentration capable of eliminating 50% or more of the biofilm formed. The classification followed Menezes et

al.,[22] in which low, moderate and high refer to the inhibition of biofilm formation in terms of biomass, being: low inhibition – OD < 0.44, moderate inhibition – 0, 44 < OD, 1.17 and high inhibition – OD > 1.17.

The tests were performed in triplicate, in independent experiments. Thus, it was possible to calculate the concentration capable of inhibiting 50% of the cell viability of the biofilm and eradicating 50% or more of the viable cells from the preformed biofilm.

2.6. Inhibition of enzyme activity

The samples ability to inhibit and/or reduce the hemolytic, phospholipase and proteinase activity of *Candida* spp. was evaluated at sub-inhibitory concentrations (½ MIC), from crude root extract samples, fractions of acetate ethyl, dichloromethane, hydromethanolic and *n*-butanol according to El.Houssaini et al.,[23]. Briefly, a suspension was carried out in tubes containing PBS; pH=7.4, with turbidity equivalent to McFarland 0.5 scale. Then, 500µL aliquots of this suspension were added to tubes containing 500 µL of RPMI 1640 (Gibco) plus the tested samples, so that the final concentrations of the compounds in the tube were equivalent to the values of ½ MIC and the final amount of fungal cells was $1x10^6$ – $1x10^7$ cells/mL in each tube. The material was incubated at 37ºC for 24 hours.

After this exposure, the tubes were centrifuged at 3000 rpm for 10 minutes, the supernatant discarded and the pellet washed with PBS and centrifuged again under the same conditions, the procedure being repeated twice more and the pellet resuspended in PBS was 1 x 10^6 cells/mL.

Subsequently, 5 µL of the fungal inoculum suspension were placed at equidistant points of 90x15 mm petri dishes containing ASD agar plus 7% defibrinated horse blood and incubated at 37ºC for 48 hours. For phospholipase determination, 5 µL of the fungal suspension were placed in 90x15 mm petri dishes containing egg yolk agar [24] incubated at 37 °C for 96 hours. Also, for proteinase determination, fungal inoculum was added to bovine serum albumin agar (GibcoBRL, Grand Island, New York, USA), incubated at 37 °C for 7 days. The control was performed with isolates and RPMI 1640 (Gibco) as a positive control and Amphotericin B used as a negative control.

The plates were checked for precipitation zone appearance around the colonies, being expressed by Hi (hemolytic zone), Pz (phospholipase zone) and Prz (proteinase zone). The activity was observed by halo presence around the colonies that were measured to calculate the inhibition index (Hc), determined by the ratio between the total diameter (halo + colony) and colony diameter [24]. The results were classified as negative (Hi, Pz, Prz = 1), moderate (0.63 < Hi, Pz, Prz < 1) and severe (Hi, Pz, Prz ≤ 0.63), according to the methodology proposed by Price et al.,[24]. The suspension containing only the isolate and RPMI1640 (Gibco) broth was used as a positive control and Amphotericin B was used as a control for the assays. The tests were performed in triplicate, in two independent experiments. The significance of enzyme inhibition was determined by analyzing the ANOVA One Way test followed by the Kruskal Wallis test with the aid of the GraphPad Prism software, version 8.2. P, in which values <0.05 were considered significant.

2.7. Toxicity assessment in *Caenorhabditis elegans*

The toxicity evaluation was performed for the root ethanolic crude extract of *A. brachypetalus* Benth and the most promising fractions (ethyl acetate, dichloromethane, hydromethanolic and *n*-butanol) in the MIC, using the in vivo model of *C. elegans* proposed according to Andrade et al.,[25] and Singulani et al.,[26]. The mutant strain *C. elegans* AU37 was grown on plates containing Nematode Growth Medium (NGM) agar seeded with *Escherichia coli* OP_{50} and incubated at 16 ºC for 72 hours. After incubation, the NGM plates containing larvae and eggs were washed with M9 buffer and the supernatant was placed in 15 mL Falcon tubes. Bleaching solution (hypochlorite + NaOH) (Sigma-Aldrich) was added to kill the adult larvae. The eggs were placed on plates containing NGM agar and incubated at 15 °C for 24 h. Subsequently, the NGM (plates containing the hatched larvae stages L1/L2 were washed with M9 buffer, and the supernatant was transferred to new plates containing NGM seeded with *E. coli* OP_{50} and incubated at 16ºC for 24 hours. Subsequently, the larvae were in the L4 stage, which 20 µL of washed content (10 – 20 larvae) were added to each well in 96-well plates and 100 µL of each sample was added, along with 80 µL of Brain Heart Infusion broth (BHI) (Kasvi). After that, there were added antibiotics (streptomycin, ampicillin and kanamycin) (Sigma-Aldrich) and incubated at 16 ºC for 72 hours. Extracts and fractions were solubilized in methanol.

Larvae were counted every day for 3 consecutive days, in which they were touched and those who moved were accounted for as live larvae. As a result, a lower concentration capable of killing 50% or more of the larvae was determined, called Lethal Concentration (LC_{50}). The experiments were performed in triplicate.

3. Results

3.1. Identification of the compounds by HPLC-MS-ESI

The crude extract, dichloromethane, *n*-butanol, hydromethanolic and ethyl acetate fractions showed considerable results in the biological assays so they were analyzed by HPLC-MS-ESI in positive and negative modes. The chromatogram proposals for identifying compounds are represented in Table 1. The crude extract and fractions showed mainly the presence of alkaloids, in addition to that, terpenes and fatty acids were also found. Regarding the ethyl acetate fraction, organic acids, alkaloids, flavonoids and fatty acids were the main classes of metabolites identified.

3.2. Determination of Minimum Inhibitory Concentration (MIC), Minimum Fungicidal/Bactericidal (MFC/CBM)

The crude ethanolic root extract and its fractions of *A. brachypetalus* Benth. showed action against *Candida* spp., especially *Candida glabrata* ATCC2001 which showed a fungicidal MFC for the ethanolic root extract, with MIC and MFC values of 375 µg/mL (Table 2). However, crude extracts and fractions did not show antibacterial action against the isolates tested for concentrations – MIC >400 µg/mL (Table 2).

3.3. Biofilm inhibition

The evaluated samples had a high effectiveness in the inhibition of biofilm formation by *C. albicans* (ATCC 90028), with emphasis on the ethanolic root extract and the hydromethanolic fraction, whose $MBIC_{50}/IC_{50}$ values were 23.47 and 1.46µg/mL, respectively (Figures 1A, 1E). Still on the biomass, a high inhibition of formation by *C. albicans* (ATCC 90028) was observed from a 93.75 µg/mL concentration for the

hydromethanolic fraction and 187.5µg/mL for the *n*-butanol fraction (Figures 1D, 1E). In addition, the concentrations that reduced by at least 50% (IC_{50}) the cell viability of the biofilm of *C. albicans* (ATCC 90028) ranged between 45.45 and 111.8µg/mL, with dichloromethane fraction being the sample with the lowest value (Figure 1C).

As for *C. glabrata* (ATCC 2001), the crude ethanolic root extract samples, for the ethyl acetate and n-butanol fraction, were able to inhibit the formation of 50% or more of the biofilm from $MBIC_{50}$ (1.46 µg/mL) (Figure 2A, 2B, 2D). Cell viability of *C. glabrata* (ATCC 2001) biofilm was reduced by at least 50% in 1.598µg/mL (crude ethanolic root extract) (Figure 2A).

Regarding *C. tropicalis* (ATCC 13805), the best values obtained for $MBIC_{50}$ were 2.92µg/mL for the ethyl acetate and dichloromethane fractions (Figure 2B, 2C). The cell viability of the *C. tropicalis* (ATCC 13805) biofilm was reduced by at least 50% in 0.056µg/mL of the ethyl acetate fraction (Figure 2B).

3.4. Eradication of the formed biofilm

The ethanolic crude extract of the root shows a variation in the ability to eradicate biofilm preformed by *C. albicans* (ATCC 90028), *C. glabrata* (ATCC 2001) and *C. tropicalis* (ATCC 13805) – Figure 3A. It is observed that the most effective samples in reducing the cell viability of the biofilm preformed by *C. albicans* (ATCC 90028) were the fractions of hydromethanolic and dichloromethane, which presented IC_{50} of 16.77 and 27.96µg/mL, respectively.

In relation to *C. glabrata* (ATCC 2001), the ability to reduce the cell viability of the pre-formed biofilm was observed in all samples, with emphasis on the crude ethanolic root extract, which presented an IC_{50} of 4.46 µg/mL (Figure 3B).

Finally, regarding the biofilm preformed by *C. tropicalis* (ATCC 13805), all analyzed samples showed a IC_{50} with high inhibition capacity, especially the ethanolic crude extract of the root, which presented an IC_{50} of 0.00028 µg/mL, thus noting its high efficacy in the eradication of preformed biofilm for *C. tropicalis* (ATCC 13805) – Figure 3A. The ethanolic crude extract of the root demonstrates that at an IC_{50} of 0.00028µg/mL for C tropicalis, it eradicates 50% or more of the pre-formed biofilm (Figure 3A).

3.5. Exoenzyme inhibition

3.5.1. Hemolysin inhibition

The exposure of *C. albicans* (atcc 90028), *C. glabrata* (atcc2001) and *C. tropicalis* (ATCC 13805) to ½ MIC of the samples promoted a significant reduction of hemolytic activity in relation to the control, reaching an inhibition of 42,7% ($p \leq 0.03$) of the activity of *C. albicans* after exposure to the root extract and 45,9% ($p \leq 0.04$) after exposure to the hydromethanolic fraction (Table 3).

3.5.2. Phospholipase inhibition

The phospholipase inhibition capacity of the samples was evaluated against *C. albicans* (ATCC 90028) as it is the only isolate producing this enzyme. It was observed that only the dichloromethane fraction promoted a significant reduction of the phospholipase activity ($p \leq 0.03$) in relation to the control, reaching 15.4% activity inhibition after treatment (Table 4).

3.5.3. Proteinase inhibition

The exposure of *C. albicans* (ATCC 90028) and *C. tropicalis* (ATCC 13805) to ½ MIC concentrations reduced proteinase activity, with emphasis on the dichloromethane fraction that caused activity inhibition of 35.8% of the ($p \leq 0.03$) (Table 5).

3.6. Toxicity assessment in *Caenorhabditis elegans*

The present study demonstrated that the ethanolic crude extract of the root and the ethyl acetate, dichloromethane, hydromethanolic and n-butanol fractions have low toxicity when exposed to C. elegans nematodes, especially the hydromethanolic fraction which has a survival rate of 80 % at a concentration of 1000µg/mL (Figure 5).

4. Discussion

In this study, samples of *A. brachypetalus* Benth. were analyzed against *Candida* species and other bacteria that cause oral diseases. These problems are aggravated by the microorganisms' resistance to usual drugs, and also by the high toxicity caused to the host by prolonged drugs use. For this reason, we focused on natural alternatives due to their effectiveness in the treatment also linked to lower toxicity [27].

The antifungal efficacy of *Annonaceae* species is described in the literature, due to alkaloid metabolites, which are naturally bioactive, demonstrating cytotoxic, antitumor, insecticidal, vermicide, antimicrobial, immunosuppressive, antiemetic and antimalarial activities [28,12]. Assays performed by Nyandoro [12] reveal that *Artabotrys* obtained MIC results of 2.5 – 20µg/mL against *S. aureus* (ATCC 25923), with efficacy comparable to the antimicrobial Ampicillin (MIC = 2.5µg/mL).

The analysis by HPLC-MS-ESI allowed us to see the chemical profile of *A. brachypetalus* bioactive fractions. Alkaloids were found in the ethanol extract and in all fractions . However, the dichloromethane fraction showed to have a more evident content of terpenes and fatty acids, while in the ethyl acetate, *n*-butanol and hydromethanolic fraction it was possible to identify some organic acids and flavonoids.

Regarding the chemical composition of *A. brachypetalus*, some alkaloids identified in crude extract and fractions have already been isolated from the stem bark of the species. Among them are norjuziphine, N-methylcoclaurine, armepavine, anonain, 10-demethyldiscretine, and discretine [29].

Some alkaloids have also been reported in other species of the *Annonaceae* family, other classes of compounds were identified in the species, such as amides derived from phenolic acids, which are reported in Annonaceae. From the species *A. hexapetalus*, flavonoids and phenolic acids, long-chain fatty acids [11] and sesquiterpenes were isolated. Flavonoids have also been isolated from *A. uncinatus* [30]. Lignanamides (grossamide) and sesquiterpernos were isolated from *Mitrephora thorelii* (Annonacea) [31]. Triterpenes have been well described in *A. odoratissimus* [32]. These studies show that the proposed identification performed by HPLC-MS of *A. brachypetalus* roots corroborates the chemical profile of other *Artabotrys* species as well as the Annonaceae family.

This study obtained effective results, showing fungicidal action against *C. glabrata* with MIC/MFC of 375µg/mL by the crude ethanolic extract of the root of *A. brachypetalus*, the same extract was fungistatic for the other species studies. The different results from drugs antifungal action used for different *Candida* species can be attributed to their inherent morphological and pathological characteristics. Species differ in terms of ploidy, morphology, phylogeny, mitochondrial function, aspartyl protease secretion and biofilm architecture [33]. The MIC results for bacteria were higher than 400µg/mL, being considered unpromising.

C. albicans has the ability to adhere in environments with higher concentration of lipids and proteins, being able to form more complex biofilms with pseudohyphae, this adhesion allows for greater virulence of the yeast [33]. The antibiofilm effects of natural products are based on aspects such as inhibition of polymer matrix formation, suppression of cell adhesion and attachment, decreased production of signaling molecules and biofilm development [32]. When performing biofilm inhibition tests, we obtained results capable of inhibiting 50% or more of the biofilm formation of *C. albicans*, promising for the hydromethanolic fraction, which presented a $MBIC_{50}$ result of 1.46µg/mL, indicating a possibility of alternative and complementary use of these plant extracts in microbial control.

To eradicate the pre-formed biofilm, the compound needs to disrupt the cell matrix and cause the enzyme to lyse[10]. When observing the reduction of the cellular viability of the biofilm preformed by *C. albicans* in the samples, the fractions of Hydromethanolic and Dichloromethane showed better results, since the eradication exceeds 70% in higher concentrations, especially *C. tropicalis*, with lower results to MIC, being 0.00028µg/mL, concentration found by the ethanolic extract of the plant root, such results can be characterized by the presence of alkaloids, as reported by Nyandoro et al.,[12].

Drugs used in fungal infections treatment can become toxic when administered long term, thus, the use of extracts and fractions of *A. brachypetalus* can be used because they present reduced toxicity, due to the results found with low concentrations compared to Amphotericin B, traditionally used. In this study, the effectiveness of the antifungal action by crude extract of the root and fractions of *A. brachypetalus* was demonstrated. The nematode *C. elegans* had already being used in the evaluation of compounds against *Candida* yeasts [26], by testing other species. Corroborating with this study, it was revealed that the compounds evaluated have a low toxicity even at concentrations higher than those evaluated at the MIC found with fungicidal activity. Such findings demonstrate the high efficacy of *A. brachypetalus* compounds against *Candida* species, still with low toxicity.

Alkaloids have gained prominence in natural products studies, showing themselves to be possible alternatives in biofilm eradication, due to their chemical structure with nitrogen presence [34,35]. Therefore, the possible responsible for inhibiting and eradicating *Candida* spp. can be justified by the presence of alkaloid compounds in the plant, as described by Nyandoro et al.,[12]. Another aggravating factor in the difficulty

in treating *Candida* species is the ability to produce enzymes that help in their pathogenicity, especially hemolysins, proteinases and phospholipases [36,37].

Another aggravating factor in the difficulty in treating *Candida* species is the ability to produce enzymes that help in their pathogenicity, especially hemolysins, proteinases and phospholipases [36,37]. The extracts evaluated significantly inhibited the production of hemolysin, phospholipase and proteinase by *Candida* spp. at sub-inhibitory concentrations, compared to Amphotericin B, revealing the extracts antivirulence potential, with a greater reduction than that caused by exposure to Amphotericin B. The study presented by Cesar et al.[38], reveals findings on *A. muricata*, its extracts have bioactives similar to *A. brachypetalus*, belonging to the same family. In their study, significant inhibition values were found against *Candida* species. Our study reveals alternatives in the use of low-cost plants, in terms of production, for public health, since the crude root extract and its fractions have a high potential for antifungal activity against the biofilms formed by *Candida* spp.

The relevant results found from the extract and fractions may be related to their chemical compositions. Alkaloids, extracts containing flavonoids and terpenes as well as these isolated compounds have shown potential effects against a broad spectrum of microorganisms, and therefore can be considered potential antimicrobial agents [39]. Fatty acids were also found in the dichloromethane and ethyl acetate fractions, they have well-known antimicrobial properties and therefore may play a role in the observed pathologies [40].

The root ethanolic extract and fractions of *A. brachypetalus* root contain compounds from different classes of metabolites with recognized antifungal potential. According to Cushnie et al.[41], among them, the alkaloids stand out because, in addition to their great biological potential, they have been models for the development of various antibiotics.

5. Conclusion

Root extract and fractions of *A. brachypetalus* revealed promising antifungal activity *in vitro*. The evaluation of inhibition of biofilm formation showed significant results against *Candida* sp., as well as the eradication of biofilm formation. The reduction of homolytic, phospholipase and proteinase was found, demonstrating the possibility of

decreasing virulence caused by *Candida* sp. *C elegans* test *in vivo* revealed low toxicity in the extract and its fractions, and even at higher concentrations *A. brachypetalus* did not demonstrate antibacterial activity.

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Table 1: Proposal for identification of the compounds in the ethanolic extract and fractions of *A. brachypetalus* root.

t_R (min)	$[M - H]^-$	$[M+H]^+$	Exact Mass	Error (ppm)	Fragment ions (*m/z*) MS/MS	Molecular formula	Tentative identify	Sample	Reference
0.72	179.0557	--	179.0561	– 2.23	5 eV: 161, 143, 119, 113, 101	$C_6H_{12}O_6$	Fructose or glucose	BF	[402,43]
0.78	191.0564	--	191.0561	1.57	20 eV: 191, 173, 127, 109	$C_7H_{12}O_6$	Quinic acid	AEF, BF, WF	[44]
0.86	133.0141	--	133.0142	– 0.75	20 eV: 115	$C_4H_6O_5$	Malic acid	AEF, BF, EE, WF	[45]
0.87	--	180.1020	180.1019	0.56	20 eV: 163, 145, 117, 115	$C_{10}H_{13}NO_2$	Salsolinol	AEF, BF, EE, WF	[45]
1.41	--	194.1178	194.1176	1.03	20 eV: 177, 151, 145, 117, 115	$C_{11}H_{15}NO_2$	Heliamine	BF, WF	[45]
2.42	153.0189	--	153.0193	– 2.61	15 eV: 109	$C_7H_6O_4$	Protocatechuic acid	BF	46,47]
2.68	357.1193	--	357.1191	0.56	20 eV: 195, 171, 165	$C_{16}H_{22}O_9$	3-(2-Hydroxy-4-methoxyphenyl)propanoic acid hexose	AEF, BF, WF	[46]
2.75	--	314.1750	314.1751	– 0.32	20 eV: 269, 237, 209, 175, 107	$C_{19}H_{24}NO_3$	Lotusine	BF, WF	[48]
2.90	270.1136	272.1282	270.1136 / 272.1281	0 / 0.37	20 eV: 255, 237, 209, 161, 143, 123, 107	$C_{16}H_{17}NO_3$	Norcoclaurine	AEF, BF, WF	[48]
3.15	312.1243	314.1386	312.1241 / 314.1387	0.64 / – 0.32	20 eV: 299, 269, 209, 191, 178, 163, 151, 119	$C_{18}H_{19}NO_4$	Pessoine	AEF, BF, DF, EE, WF	[49]
3.53	289.0717	291.0870	289.0718 / 291.0874	– 0.34 / – 1.37	15 eV: 289, 245, 205, 203, 179, 151, 125, 109	$C_{15}H_{14}O_6$	(Epi)catechin	AEF, BF	[50,51]
3.66	--	300.1590	300.1594	– 1.33	25 eV: 269, 237, 209, 175, 143, 121, 115, 107	$C_{18}H_{21}NO_3$	N-methyl-coclaurine*	AEF, BF, DF, EE, WF	[2]
3.73	--	286.1442	286.1438	1.40	20 eV: 269, 237, 209, 175, 143, 107	$C_{17}H_{19}NO_3$	Norjuziphine*	AEF, BF, WF	[52]
3.78	326.1395	328.1541	326.1398/ 328.1543	– 0.92/ – 0.61	(-)25 eV: 311, 296, 268 / (+)25 eV: 313, 297, 265, 237, 191, 178, 163, 151, 137, 119	$C_{19}H_{21}NO_4$	10-demethyldiscretine*	AEF, ,BF, DF, EE, WF	[9]

t_R (min)	$[M-H]^-$	$[M+H]^+$	Exact Mass	Error (ppm)	Fragment ions (*m/z*) MS/MS	Molecular formula	Tentative identify	Sample	Reference
3.96	--	344.1492	344.1492	0.0	20 eV: 326, 299, 284, 267, 194, 176, 151, 101	$C_{19}H_{21}NO_5$	N-formylreticuline	AEF, BF, DF, WF	MoNA[c]
4.08	--	330.1700	330.1700	0.0	25 eV: 299, 267, 235, 207, 192, 175, 151, 143, 137	$C_{19}H_{23}NO_4$	Reticuline	AEF,BF, DF, EE, WF	[2]
	312.1605	314.1753	314.1751	0.64	15 eV: 297, 280, 265 / 25 eV: 298, 283, 269, 252, 237, 209,151, 121, 115, 137, 107	$C_{19}H_{23}NO_3$	Armepavine*	AEF,, BF, DF, EE, WF	[29,48]
4.21	--	342.1701	342.1700	0.29	25 eV: 311, 192, 178, 163, 151, 119	$C_{20}H_{23}NO_4$	Discretine*	AEF, BF, DF, EE, WF	[29]
	--	328.1541	328.1543	– 0.61	20 eV: 311, 297, 265, 279, 237, 233, 178, 151	$C_{19}H_{21}NO_4$	3'-demethyl-romneine	AEF, BF, DF, EE, WF	[52]
4.59	--	342.1701	342.1700	0.29	20 eV: 192, 178, 165, 163, 151	$C_{20}H_{23}NO_4$	Discretine (isomer)	AEF, DF	[29,49]
4.64	--	356.1855	356.1856	– 0.28	30 eV; 192, 177, 149, 148	$C_{21}H_{25}NO_4$	Tetrahydropalmatine or N-methyltetrahydro-Columbamine	AEF,BF, DF, EE, WF	[52]
4.70	--	268.1330	268.1332		20 eV: 251, 236, 219, **191**, 107	$C_{17}H_{17}NO_2$	Asimilobine	BF, EE, WF	[50]
4.74	--	286.1437	286.1438	– 0.35	25 eV: 269, 237, 209, 175, 178, 143, 121, 107	$C_{17}H_{19}NO_3$	Norjuziphine (isomer)	AEF, BF, DF, EE, WF	[29,49]
4.94	--	312.1594	312.1594	0.0	20 eV: 281, 266, 249, 234, 206	$C_{19}H_{21}NO_3$	Thebaine	AEF, DF	[45,53, Pubchemd]
5.04	--	282.1126	282.1125	0.35	25 eV: 264, 234, 206, 179	$C_{17}H_{15}NO_3$	Norushinsunine	AEF, BF, DF, WF	[49]
	--	264.1019	264.1019	0.0	20 eV: 264, 234, 206, 179	$C_{17}H_{13}NO_2$	Dehydroanonaine	AEF, BF, DF, WF	Tentative
5.14	--	298.1440	298.1438	0.67	15 eV: 281, 266, 249, 234, 221, 206	$C_{18}H_{19}NO_3$	Stepharine or isopiline	AEF, BF, DF, WF	[56,49]
5.24	303.0512	--	303.0510	0.65	10 eV: 285, 275, 241, 217, 177, 153, 125	$C_{15}H_{12}O_7$	Taxifolin	AEF	[57,58]
5.32	298.1088	--	298.1085	1.0	25 eV: 178, 161, 135	$C_{17}H_{16}NO_4$	N-Caffeoyltyramine I	AEF	[59]

t_R (min)	$[M-H]^-$	$[M+H]^+$	Exact Mass	Error (ppm)	Fragment ions (*m/z*) MS/MS	Molecular formula	Tentative identify	Sample	Reference
5.44	--	328.1539	328.1543	– 1.21	20 eV: 311, 296, 279, 251, 178	$C_{19}H_{21}NO_4$	Laurotetanine	AEF, DF	MoNA[e]
5.64	--	324.1229	324.1230	– 0.31	30 eV: 309, 294, 281, 266, 237	$C_{19}H_{17}NO_4$	Dehydrodiscretamine	AEF, DF	[55,52]
	--	300.1592	300.1594	– 0.67	20 eV: 283, 251, 223, 175, 159, 143, 121	$C_{18}H_{21}NO_3$	O-methylcoclaurine ou magnococline	AEF, DF	[49]
	--	338.1386	338.1387	– 0.30	30 eV: 323, 308, 294, 279	$C_{20}H_{19}NO_4$	Columbamine or Jatrorrhizine	AEF, BF, DF	[55,52,60]
5.76	--	282.1490	282.1489	0.35	15 eV: 265, 250, 234	$C_{18}H_{19}NO_2$	Nornuciferina	AEF, DF	[49,48]
5.95	--	266.1176	266.1176	0.0	15 eV: 249, 219, 191	$C_{17}H_{15}NO_2$	Anonaine	AEF, BF, DF	[49,48]
6.00	298.1086	--	298.1085	0.34	25 eV: 178, 161, 148, 135	$C_{17}H_{17}NO_4$	N-trans-Caffeoyltryramine	AEF,BF, DF	[61]
6.06	312.1252	314.1387	312.1258 / 314.1387	1.92 / 0.0	(-)20 eV: 297, 178, 148 / (+)20 eV: 177, 145, 121	$C_{18}H_{19}NO_4$	N-trans-Feruloyltyramine I	AEF, BF, DF	[61]
	--	338.1387	338.1387	0.0	30 eV: 323, 308, 294, 279	$C_{20}H_{19}NO_4$	Columbamine or Jatrorrhizine	AEF, BF, DF	[55,52,60]
6.16	328.1191	330.1336	328.1190 / 330.1136	0.30 / 0.0	30 eV: 328, 313, 178, 148, 135	$C_{18}H_{19}NO_5$	Feruloyloctopamine	AEF, BF	[59]
6.26	--	322.1072	322.1074	– 0.62	30 eV: 307, 306, 292, 261	$C_{19}H_{15}NO_4$	Thalifendine ou Berberrubine	DF	[55,52]
6.50	--	296.1282	296.1281	0.34	20 eV: 279, 264, 249, 234, 221	$C_{18}H_{17}NO_3$	Xylopine	AEF, BF, DF, WF	[54,63]
6.60	255.1027	--	255.1027	0.0	10 eV: 237, 183, 149, 134	$C_{16}H_{16}O_3$	Pterostilbene	AEF	[64]
6.72	312.1243	314.1387	312.1241 / 314.1387	0.64 / 0.0	(-)20 eV: 297, 190, 178, 148, 135 / (+)20 eV: 177, 145, 121	$C_{18}H_{19}NO_4$	N-trans-Feruloyltyramine II	AEF, BF, DF, EE	[61]
	342.1347	344.1492	342.1347 / 344.1492	0.0 / 0.0	(-)20 eV: 327, 312, 208, 178, 163 / (+)25 eV: 207, 177, 145, 121	$C_{18}H_{21}NO_5$	N-Feruloyltyramine derivate	AEF, , BF, DF	[65,64]
7.04	623.2400	625.2545	623.2399/625.2544	0.16	30 eV: 566, 460 / 20 eV: 488, 432, 460, 326, 299, 201, 121	$C_{36}H_{37}N_2O_8$	*N-cis*-grossamide	AEF, BF, DF	[66]

t_R (min)	$[M-H]^-$	$[M+H]^+$	Exact Mass	Error (ppm)	Fragment ions (*m/z*) MS/MS	Molecular formula	Tentative identify	Sample	Reference
8.06	306.0772	308.0917	306.0772/ 308.0917	0.0 / 0.0	(-)25 ev: 290, 276, 262, 248/ (+)30 eV: 293, 275, 264, 247	$C_{18}H_{13}NO_4$	Isomoschatoline	AEF, BF, DF	[67]
	623.2398	625.2551	623.2399/ 625.2544	0.16 / 1.12	(-)25 eV: 460, 297 / (+)25 eV: 488, 462, 351, 325, 307	$C_{36}H_{37}N_2O_8$	*N-trans-*grossamide	AEF, BF, DF	[64,66]
8.67	--	237.1853	237.1849	1.67	10 eV: 237, 219, 201, 191, 161, 145, 121, 109	$C_{15}H_{24}O_2$	Sesquiterpene	DF	Tentative
9.06	--	306.0761	306.0761	0.0	30 eV: 291, 263, 235, 207	$C_{18}H_{11}NO_4$	Oxoxylopine ou oxoputerine	AEF, BF, DF	[67]
9.17	329.2334	--	329.2333	0.30	20 eV: 311, 293, 229, 171, 157, 139, 127	$C_{18}H_{34}O_5$	Trihydroxyoctadecedienoic acid I	AEF	[59]
9.27	329.2334	--	329.2333	0.30	--	$C_{18}H_{34}O_5$	Trihydroxyoctadecedienoic acid II	AEF	[59]
9.54	--	235.1694	235.1693	0.43	25eV: 202,175, 147, 133, 119	$C_{15}H_{22}O_2$	Sesquiterpene Derivated	DF	Tentative
9.75	--	237.1848	237.1849	– 0.42	25 eV: 179, 161, 151, 139, 121, 107	$C_{15}H_{24}O_2$	Sesquiterpene Derivated	DF	Tentative
9.89	--	219.1745	219.1743	0.91	20 eV: 201, 159, 133, 119, 105	$C_{15}H_{22}O$	Sesquiterpene Derivated	DF	Tentative
10.19	--	219.1745	219.1743	0.91	20 eV: 219, 201, 176, 163, 159, 145, 137	$C_{15}H_{22}O$	Sesquiterpene Derivated	DF	Tentative
10.21	309.2072	--	309.2071	0.32	20 eV: 291, 225, 197, 169	$C_{18}H_{30}O_4$	Linolenic acid 13-hydroperoxide	DF	[44,47]
10.86	--	293.2122	293.2122	0.00	20 eV: 275, 235, 171, 121	$C_{18}H_{30}O_3$	Hydroxy-octadecatrienoic acid	DF, EE	[46,68,47]
	--	277.2162	277.2162	0.0	20 eV: 149, 135, 121, 107	$C_{18}H_{28}O_2$	Stearidonic acid	DF	[47,69]
11.05	--	457.3678	457.3676	0.44	10 eV: 457, 439, 421, 325, 223, 159, 109	$C_{30}H_{50}O_3$	Triterpene derivated	DF	[70,71]
11.19	295.2278	--	295.2279	– 0,34	20 eV: 277, 195, 171	$C_{18}H_{32}O_3$	Hydroxy-octadecadienoic Acid	DF, EE	[44,47]
11.45	--	205.1951	205.1951	0.0	20 eV: 149, 135, 121, 109, 107, 105	$C_{15}H_{24}$	Prespatane	EE, DF	MONA[f]

t_R (min)	$[M-H]^-$	$[M+H]^+$	Exact Mass	Error (ppm)	Fragment ions (*m/z*) MS/MS	Molecular formula	Tentative identify	Sample	Reference
12.09	277.2172	--	277.2173	– 0.36	25 eV: 271, 244, 208, 158, 130, 121	$C_{18}H_{30}O_2$	Octadecatrienoic acid	DF, EE	[46,64]

Rt: Retention time (minutes); NI: not identified; --: not obtained; *: isolated in the species; Metlin - Online library available at: https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage; [a]https://pubchem.ncbi.nlm.nih.gov/compound/Salsolinol#section=MS-MS [b]https://mona.fiehnlab.ucdavis.edu/spectra/display/CCMSLIB00005436068 [c]https://mona.fiehnlab.ucdavis.edu/spectra/display/CCMSLIB00005436050; [d]https://pubchem.ncbi.nlm.nih.gov/compound/Thebaine#section=Other-MS [e]https://mona.fiehnlab.ucdavis.edu/spectra/display/CCMSLIB00005436056. [f]https://mona.fiehnlab.ucdavis.edu/spectra/display/CCMSLIB00001055095.

Table 2: Minimum Inhibitory Concentration and Minimum Fungicide Concentration of ethanol and root, twigs and twig extracts, and fractions against *Candida* spp.

	Minimum Inhibitory Concentration - MIC/ Minimum Fungicidal Concentration - MFC (µg/mL)							
Isolados	*Candida albicans* ATCC 90028		*Candida glabrata* ATCC 2001		*Candida tropicalis* ATCC 13805		*Candida parapsilosis**	*Candida krusei**
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MIC**
Root extract	23.43	750	375	375	375	750	-	-
Leaves extract	-	-	-	-	-	-	-	-
Twigs exctrat	-	-	-	-	-	-	-	-
Acetate ethyl	23.43	-	23.43	-	23.43	-	-	-
Dichloromethane	46.87	187.5	23.43	750	93.75	750	-	-
Hexane	-	-	-	-	-	-	-	-
Hydromethanolic	93.75	750	93.75	-	93.75	-	-	-
n-butanol	11.72	1500	11.72	-	23.43	-	-	-
Anfotericin B	-	-	-	-	-	-	0.5	1.0

*: Technique control strains: *Candida Krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 – Anfotericin B 0.5 and 1.0 µg/mL, respectively. - : MIC/CFM≥3000 µg/mL.

Table 3: Hemolytic index and percentage of inhibition of hemolytic activity of *C. albicans* (ATCC 90028), *C. glabrata* (ATCC 2001) and *C. tropicalis* (ATCC 13805) after exposure to inhibitory concentrations and ½MICs of the root extract and fractions.

			***C. albicans* ATCC 90028**				
	Controle	Root extract	Acetate ethyl	Diclorometh ane	Hydromethanolic	*n*-butanol	Anfotericina B
Média Hi	0.36	0.51	0.41	0.37	0.41	0.41	0.43
% inibição	-	-42.7	-14	-2.8	-13.3	-13.3	-20.2
p valor		**0.03**	0.99	0.99	0.99	0.99	0.39
			***C. glabrata* ATCC 2001**				
	Controle	Root extract	Acetate ethyl	Diclorometh ane	Hydromethanolic	*n*-butanol	Anfotericina B
Média Hi	0.37	0.51	0.52	0.43	0.54	0.46	0.55
% inibição	-	-37.8	-40.5	-16.2	-45.9	-21.6	-48.6
p valor		0.15	0.12	0.99	**0.04**	0.99	**0.03**
			***C. tropicalis* ATCC 13805**				
	Controle	Root extract	Acetate ethyl	Diclorometh ane	Hydromethanolic	*n*-butanol	Anfotericina B

Média Hi	0.37	0.54	0.55	0.48	0.47	0.47	0.64
% inibição	-	-35	-37.5	-20	-17.5	-17.5	-60
p valor		0.09	0.05	0.86	0.76	0.9	**0.01**

p<(0.05) statistically significant.

Table 4: Index and percentage of inhibition of phospholipase activity of *C. albicans* (ATCC 90028) after exposure to ½MICs concentrations of root extract and fractions.

	***C. albicans* ATCC 90028**						
	Controle	Root extract	Acetate ethyl	Dicloromethane	Hydromethanolic	*n*-butanol	Anfotericina B
Média Hi	0.39	0.42	0.42	0.45	0.43	0.41	0.41
% inibição	-	-7.7	-7.7	-15.4	-10.3	-5.2	-7.7
p valor		0.99	0.99	**0.03**	0.24	0.99	0.99

p<(0.05) statistically significant.

Table 5: Index and percentage of inhibition of proteinase activity of *C. albicans* (ATCC 90028) and *C. tropicalis* ATCC 13805 after exposure to ½MICs concentrations of root extract and fractions.

***C. albicans* ATCC 90028**							
	Controle	Root extract	Acetate ethyl	Diclorometh ane	Hydromethanolic	*n*-butanol	Anfotericina B
Média Hi	0,59	0.59	0.72	0.73	0.61	0.78	0.92
% inibição	-	0	-25.4	-23.7	-3.4	-25.4	-55.9
p valor		0.99	0.38	0.41	0.99	0.28	**0.01**
***C. tropicalis* ATCC 13805**							
	Controle	Root extract	Acetate ethyl	Diclorometh ane	Hydromethanolic	*n*-butanol	Anfotericina B
Média Hi	0.57	0.63	0.67	0.77	0.66	0.70	0.78
% inibição	-	-10.5	-17.5	-35.8	-15.8	-22.8	-36.8
p valor		0.99	0.48	**0.03**	0.66	0.12	**0.03**

p<(0.05) statistically significant.

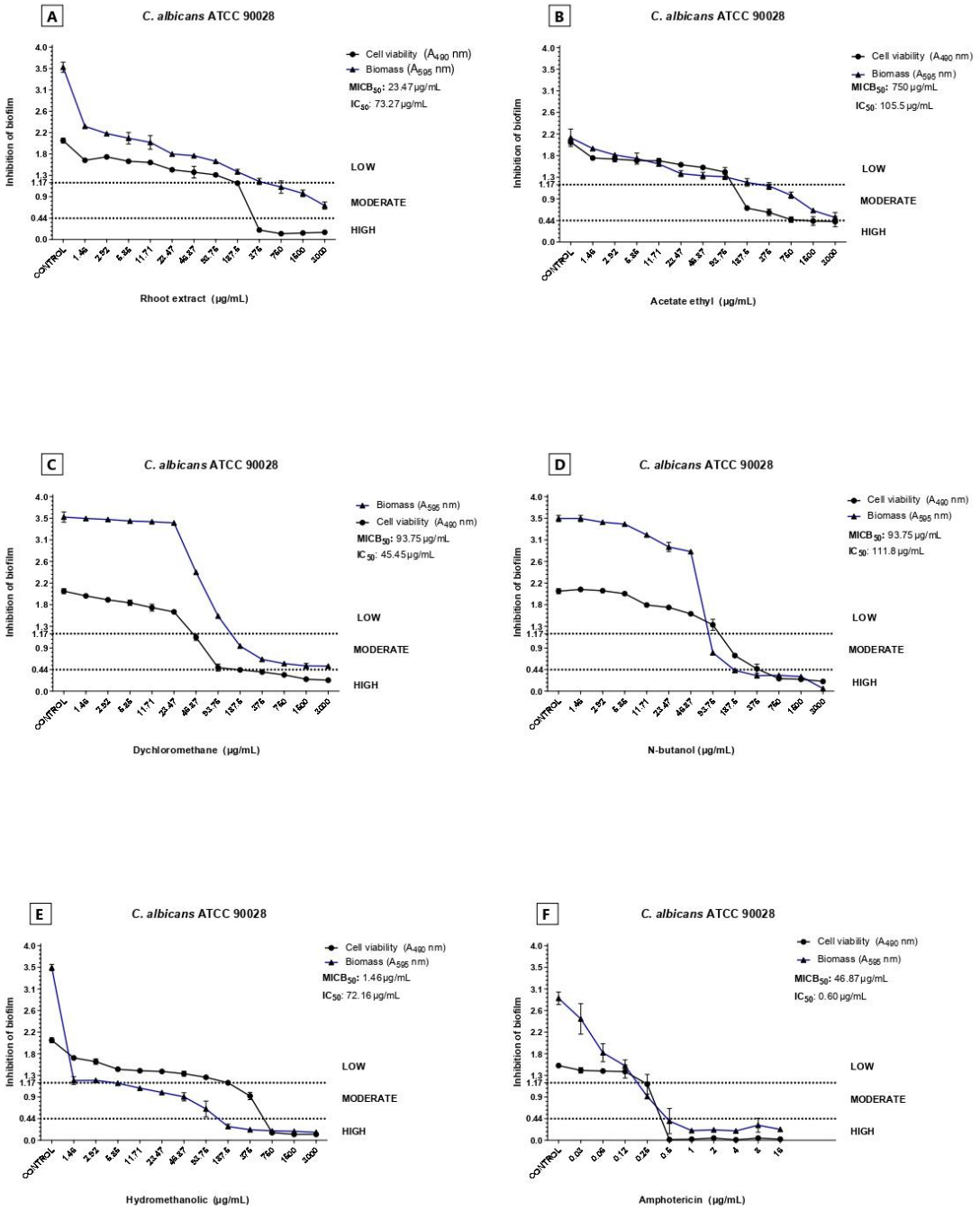

Figure 1: Inhibition of *C. albicans* (ATCC90028) biofilm by extracts and fractions of *A. brachypetalus* Benth. (ABCDE) and Amphotericin B (F).

Note: The blue line refers to the curve of OD values of the biomass in relation to the sample tested. The black line refers to the OD values of the metabolic activity of the biofilm in relation to the sample tested. The $CIMB_{50}$ values refer to the concentration of the sample that was able to inhibit 50% or more of biofilm formation in relation to the biomass (WEI et al., 2006). The IC_{50} values indicate the concentration of the sample that was able to inhibit by half the metabolic activity of the biofilm, compared to the control group (SEBAUGH, 2011). The low, moderate and high classification refers to the inhibition of biofilm formation in terms of biomass was made of according to the classification of *Candida* spp. regarding the biofilm formation proposed by Menezes et al. (2022).

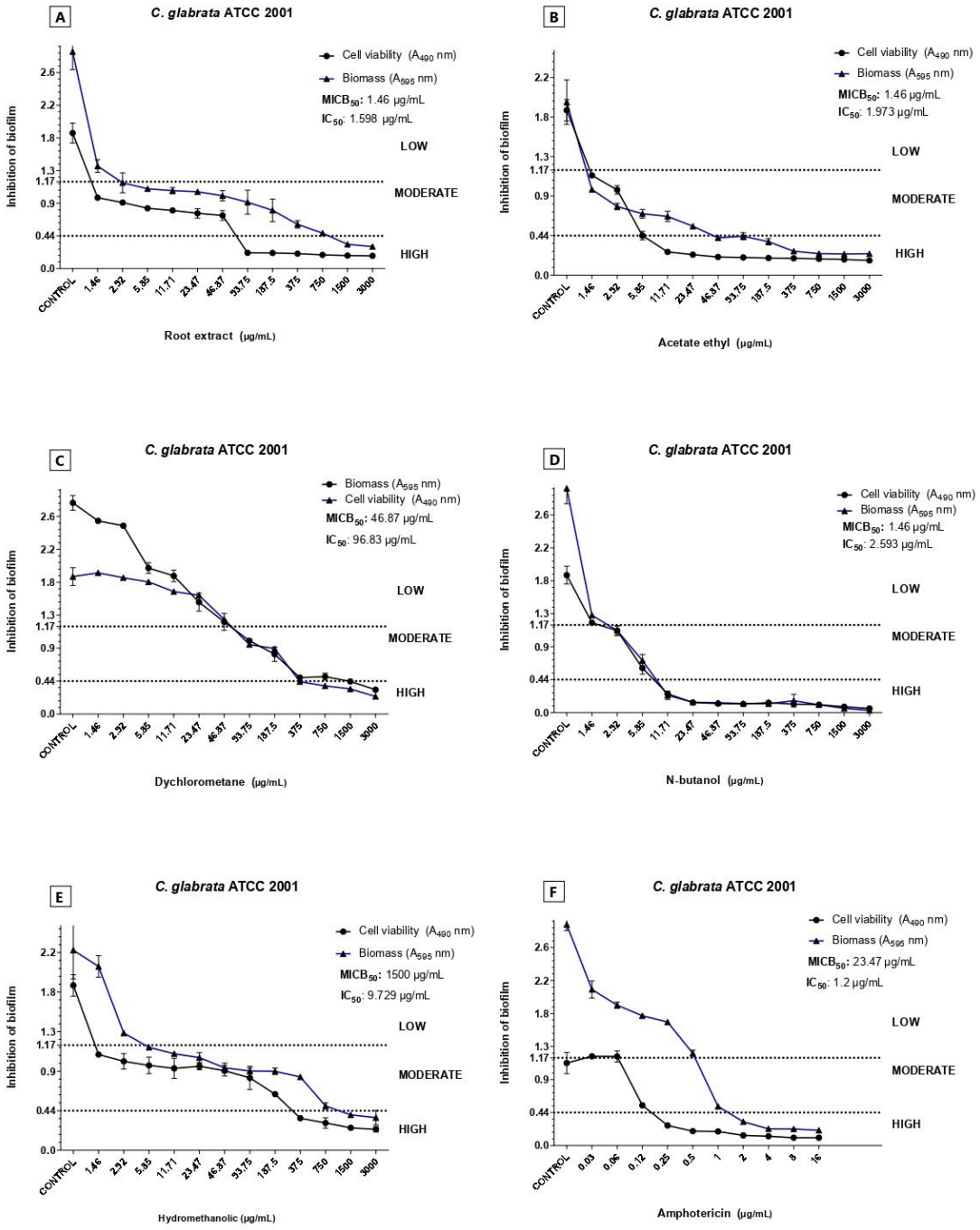

Figure 2: Inhibition of *C. glabrata* (ATCC2001) biofilm by extracts and fractions of *A. brachypetalus* Benth. (ABCDE) and Amphotericin B (F).

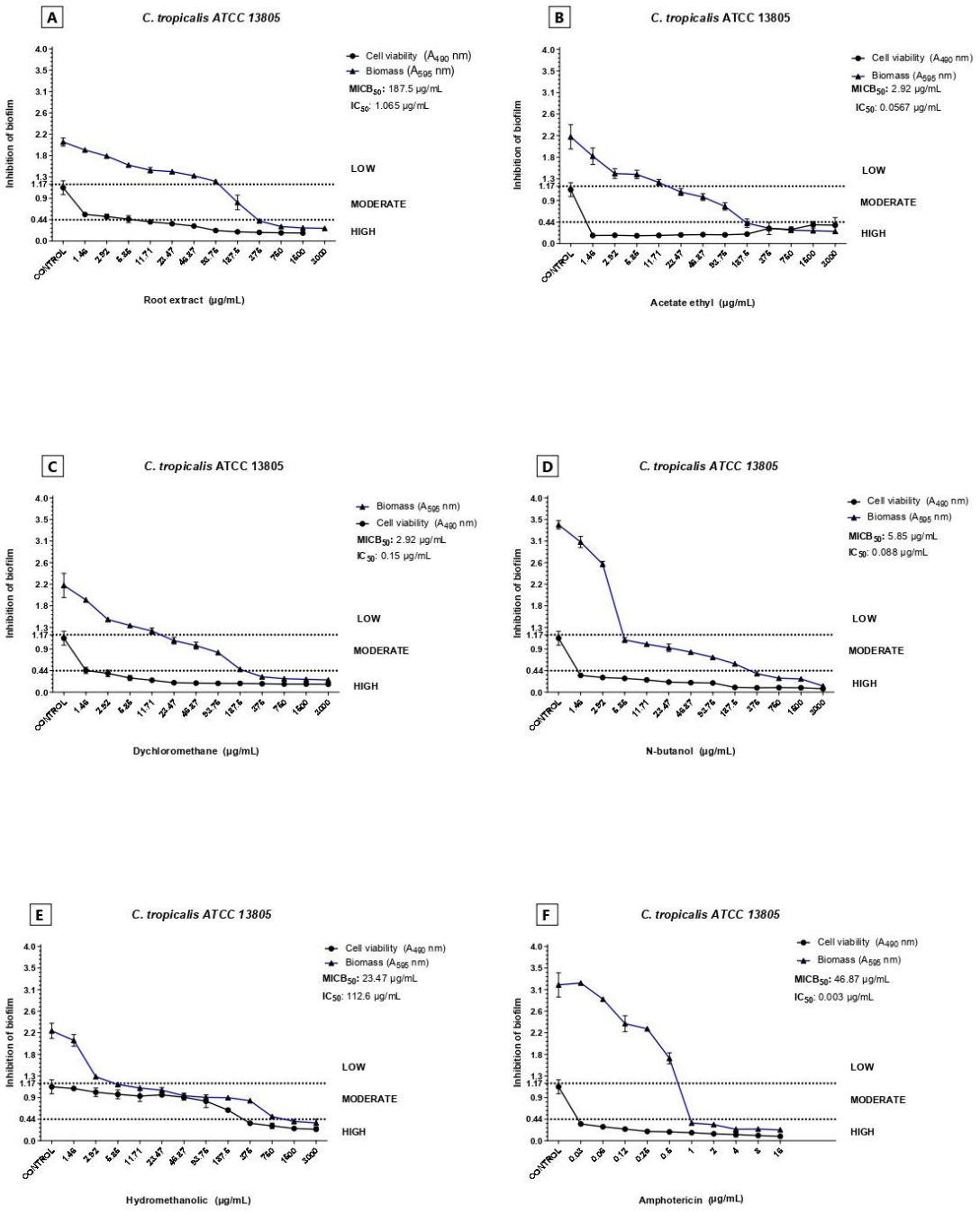

Figure 3: Inhibition of *C. tropicalis* (ATCC13805) biofilm by extracts and fractions of *A. brachypetalus* Benth. (ABCDE) and Amphotericin B (F).

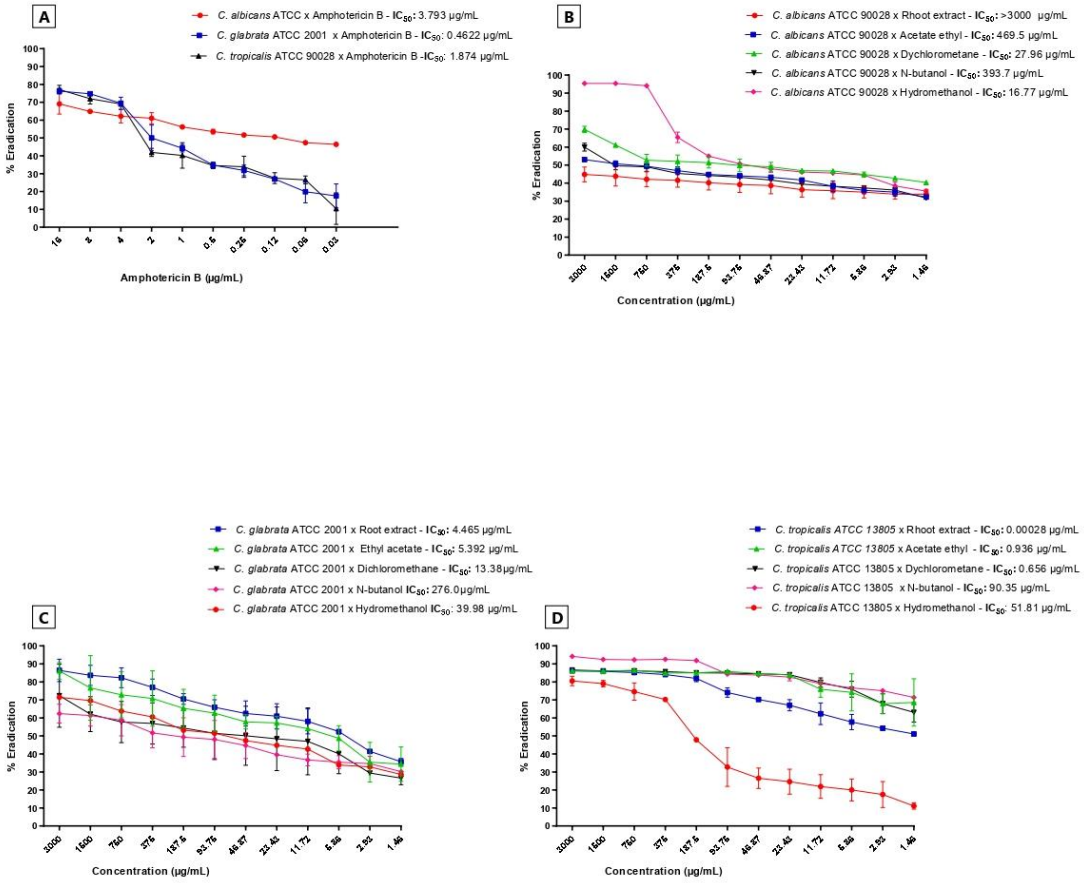

Figure 4: Percentage of eradication of viable cells from preformed biofilm of *C. albicans* (ATCC 90028) (B) *C. glabrata* (ATCC 2001) (C), *C. tropicalis* (ATCC 13805) (D) at different concentrations of extract and fractions of *Artabotrys brachypetalus* Benth. and Amphotericin B (A).

Note: The IC_{50} values (Minimum Biofilm Eradication Concentration) refer to the concentration of the sample that was able to reduce by 50% or more the cell viability of the preformed biofilm.

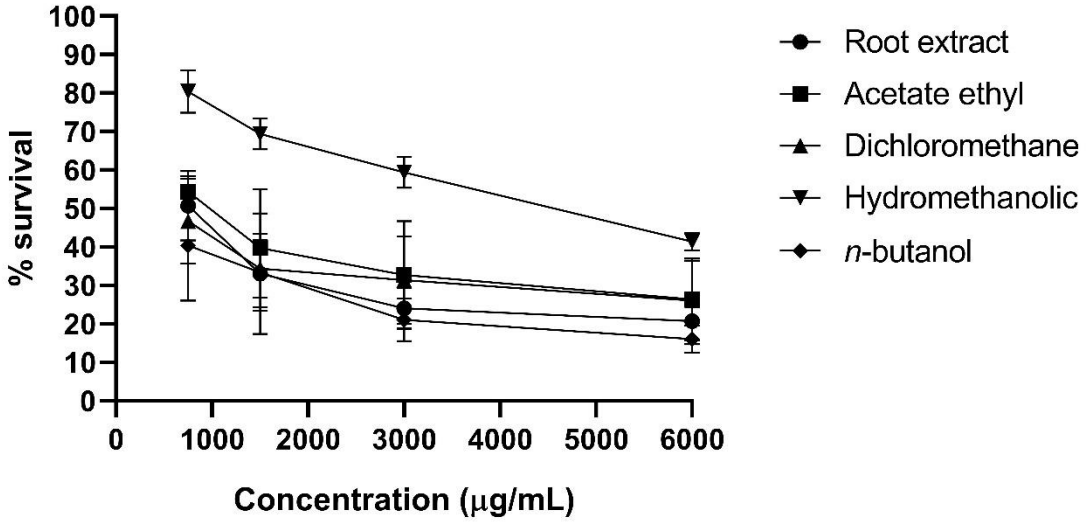

Figure 5: Survival curve in response to ethanolic crude root extract and its ethyl acetate, dichloromethane, hydromethanolic and *n*-butanol fractions for the *C. elegans* model.