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ANTONIA THASSYA LUCAS DOS SANTOS

ANÁLISE QUÍMICA E EFEITO ANTIFÚNGICO DE *Spondias tuberosa* Arruda (UMBU) FRENTE A LEVEDURAS DO GÊNERO *Candida*

CRATO - CE

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Dissertação apresentada ao Programa de Pós graduação em Etnobiologia e Conservação da Natureza (UFRPE, UEPB, URCA e UFPE) como parte dos requisitos para obtenção do título de mestre.

Orientador: Prof. Dr. Henrique Douglas Melo Coutinho
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SUMÁRIO

RESUMO

SANTOS, Antonia Thassya Lucas dos. Universidade Federal Rural de Pernambuco. Fevereiro/2020. ANÁLISE QUÍMICA E EFEITO ANTIFÚNGICO DE *Spondias tuberosa* Arruda (UMBU) FRENTE A LEVEDURAS DO GÊNERO *Candida*. Henrique Douglas Melo Coutinho. Maria Flaviana Bezerra Morais Braga.

Infecções causadas por fungos tem se tornado um problema cada vez maior de saúde pública e este fato vem se agravando por conta do surgimento de novos mecanismos de resistência. Diante disso, tem se buscado nos produtos naturais, uma alternativa no combate a esses patógenos. *Spondias tuberosa* Arruda (Umbu) é uma planta do semiárido brasileiro, de importância na medicina tradicional. Baseado nesse contexto e na importância da conservação da espécie, esse trabalho objetivou analisar a composição química e avaliar de forma comparativa a ação antifúngica e o potencial inibidor de virulência dos extratos aquosos e hidroalcoólicos das folhas e raízes de *Spondias tuberosa* Arruda (umbu) frente às linhagens do gênero *Candida*. A análise dos componentes químicos das folhas e raízes foi feito por UPLC-ESI-QTOF-MS. Os ensaios antifúngicos foram realizados frente à *C. albicans* e *C. tropicalis* (tipo padrão e isolado clínico), por microdiluição, para determinação da IC_{50} e obtenção da curva de viabilidade celular sob a ação intrínseca dos extratos e do fluconazol (controle) e de forma combinada extrato/fármaco. A Concentração Fungicida Mínima (CFM) foi realizada por subcultivo. Para verificação do efeito sobre a morfologia das leveduras e inibição de sua virulência foram preparadas câmaras de microcultivo. Na identificação dos extratos constatou-se principalmente a presença de composto fenólicos e flavonoides. Os extratos apresentaram um efeito fungistático ($\geq$ 16.384 µg/mL) e, na combinação destes com o Fluconazol, em algumas concentrações, o efeito foi sinérgico. Na transição morfológica os extratos conseguiram inibir ou reduzir os filamentos das cepas testadas em concentrações progressivas. Os resultados obtidos mostraram que os extratos das folhas tem efeito semelhantes com os das raízes, e assim pode ser uma alternativa de diminuir o impacto causado pela utilização das raízes, procurando contribuir com a conservação e manejo da espécie.

Palavras-chave: *Candida albicans*, *Candida tropicalis*, Conservação, Inibidor de virulência.

ABSTRACT

SANTOS, Antonia Thassya Lucas dos. Universidade Federal Rural de Pernambuco. Fevereiro/2020. ANÁLISE QUÍMICA E ANTIFUNGAL EFFECT OF *Spondias tuberosa* Arruda (UMBU) ON GENDER YEAST *Candida*. Henrique Douglas Melo Coutinho. Maria Flaviana Bezerra Morais Braga.

Infections caused by fungi have become an increasing public health problem and this fact has been aggravated by the emergence of new resistance mechanisms. Given this, natural products have been sought as an alternative in combating these pathogens. *Spondias tuberosa* Arruda (Umbu) is a Brazilian semiarid plant of importance in traditional medicine. Based on this context and the importance of conservation of the species, this work aimed to analyze the chemical composition and comparatively evaluate the antifungal action and the potential virulence inhibitor of aqueous and hydroalcoholic extracts of leaves and roots of *Spondias tuberosa* Arruda (umbu) against strains of the genus *Candida*. The analysis of the chemical components of leaves and roots was performed by UPLC-ESI-QTOF-MS. Antifungal assays were performed against *C. albicans* and *C. tropicalis* (standard and isolated clinical type) by microdilution to determine the IC50 and to obtain the cell viability curve under the intrinsic action of extracts and fluconazole (control) and extract / drug combination form. Minimum Fungicidal Concentration (MFC) was performed by subculture. To verify the effect on yeast morphology and inhibition of yeast virulence, microculture chambers were prepared. In the identification of the extracts it was verified mainly the presence of phenolic compounds and flavonoids. The extracts had a fungistatic effect (≥ 16,384 µg / mL) and, in their combination with Fluconazole, at some concentrations, the effect was synergistic. In the morphological transition the extracts were able to inhibit or reduce the filaments of the tested strains in progressive concentrations. The obtained results showed that the leaf extracts have similar effect to the roots, and thus can be an alternative to reduce the impact caused by the use of the roots, seeking to contribute to the conservation and management of the species.

Key words: *Candida albicans*, *Candida tropicalis*, Conservation, Virulence inhibitor.

INTRODUÇÃO GERAL

Muitas das infecções fúngicas nos seres humanos podem ser causadas por fungos oportunistas, dentre os mais importantes destacam-se *Aspergillus fumigatus* e *Candida albicans*, mas outras espécies dos gêneros *Candida* e *Trichosporon,* começam a se manifestar. Infecções fúngicas sistêmicas são de difícil diagnóstico e estão associadas à elevada mortalidade e morbidade, sendo um sério problema de saúde pública (MENEZES et al., 2013).

Candidíase é uma micose causada por leveduras do gênero *Candida*, em que a lesão pode ser branda, aguda ou crônica, superficial ou profunda, e de espectro clínico variado (BARBEDO; SGARBI, 2010). A resistência fúngica representa um amplo desafio clínico no tratamento de infecções invasivas devido à escassez de antifúngicos efetivos disponíveis. Além disso, os medicamentos atuais podem ser limitados pelas interações medicamentosas e com efeitos adversos graves (WIEDERHOLD, 2017).

Neste sentido, pesquisas por novos agentes antifúngicos obtidos a partir de extratos, frações e óleos essenciais ou constituintes isolados de plantas da flora brasileira vêm sendo intensificadas, na busca de reverter a resistência de *Candida* spp. (EDDOUZI et al., 2013; CALIXTO JÚNIOR et al., 2015).

No semiárido brasileiro muitas plantas possuem potencial farmacológico, dentre estas *Spondias tuberosa* Arruda (Umbu), espécie de grande importância econômica, social e ecológica. Na medicina popular, é utilizada para diabetes, colesterol, congestão diarreia, inflamações, afta, dores de estômago e uterinas e corrimento feminino (JORGE et al., 2007; NETO; PERONI; DE ALBUQUERQUE, 2010; RIBEIRO et al., 2014; UCHÔA et al., 2015), sintomatologia que pode estar associada à infecção por *Candida* spp. denominada candidíase, micose ocasionada principalmente pela espécie *Candida albicans*.

A utilização popular da raiz tubérculo de *S. tuberosa* agrega certa ressalva, uma vez que pode futuramente causar danos aos exemplares da espécie devido à perda de propriedades pela variação genética que pode sofrer e consequente diminuição das populações nos ambientes em que habitam. A verificação da composição química da raiz, que é pouco relatada na literatura, em comparação a composição das folhas pode vir a minimizar ou mesmo cessar o uso das raízes, o que auxiliaria na conservação da espécie, haja vista que é a composição química responsável pelas atividades

farmacológicas (DE ALBUQUERQUE et al, 2011; MACÊDO; RIBEIRO; SOUZA, 2013).

Mediante o exposto, esse trabalho buscou avaliar a composição química e efeito antifúngico dos extratos aquosos (infusão) e hidroalcoólico das folhas e raízes de *S. tuberosa* Arruda (umbu) sobre cepas de *C. albicans* e *C. tropicalis* e seu potencial inibidor de virulência de levedura, trabalhando-se com a hipótese de que as folhas tenham o mesmo efeito que as raízes, o que seria interessante do ponto de vista da conservação da espécie, uma vez que a exploração do recurso folha, além de causar menos dano a planta, seria de melhor acesso e disponibilidade para as populações usuárias.

CAPÍTULO 1: FUNDAMENTAÇÃO TEÓRICA

1.1 GÊNERO *Spondias*

A família Anacardiaceae inclui várias espécies frutíferas que são exploradas no mundo, dentre elas cajarana do sertão (*Spondias* sp.), umbu (*Spondias tuberosa*) caju (*Anacardium occidentale L.*), manga (*Mangifera indica L.*) e pistache (*Pistacia vera L.*) (OLIVEIRA, 2011). Estudos químicos realizados com espécies desta família indicam que são ricas em polifenois (ASUQUO et al., 2013; SCHULZE-KAYSERS; FEUEREISEN; SCHIEBER, 2015), taninos, terpenoides (sesquiterpenos e monoterpenos), ácidos graxos, esteroides e flavonoides (BORGES et al., 2007; DANTAS et al., 2014; DE LIMA et al., 2017).

Spondias é um gênero pertencente à família Anacardiaceae. Que abrange 18 espécies distribuídas em regiões tropicais do mundo, com importância social e econômica e com determinadas espécies utilizadas na indústria agrícola (SAMEH et al., 2018; SILVA et al., 2015). E na medicina popular são empregadas para diarreia, dor de barriga, gastrite, corrimento feminino, colesterol, enxaqueca, cicatrizante, diabetes, inflamação na garganta e no fígado, dor no estômago, irritação na pele, tosse e bronquite (JÚNIOR et al., 2014; MACÊDO; RIBEIRO; SOUZA, 2013; NETO; PERONI; DE ALBUQUERQUE, 2010; RANGEL et al., 2010; RIBEIRO et al., 2014).

Diferentes espécies de *Spondias* são citadas na literatura por suas atividades biológicas como antimicrobiana, antitumoral, gastroprotetora, larvicida, antioxidante, citotóxica e trombolítica (ALBUQUERQUE et al., 2014; EZE; DANG; OKOYE, 2014; ISLAM et al., 2013; SABIU et al., 2015; SANTOS; SANTOS; MARISCO, 2017).

1.2 ESPÉCIE *Spondias tuberosa* Arruda

A espécie *S. tuberosa* (umbuzeiro) é nativa do Brasil, e pode apresentar hábito arbustivo a arbóreo, sendo distribuídas nas regiões norte, nordeste, centro-oeste e sudeste. Apresentam significativa importância ecológica e econômica, onde os frutos são utilizados na renda familiar de pequenos agricultores (CAVALCANTI et al., 2000; FLORA DO BRASIL 2020, 2018; NETO et al., 2013). Raízes e folhas podem ser usadas como alimento e a água contida nas raízes é utilizada na medicina popular (EPSTEIN, 1998). O umbuzeiro, destaca-se pela possibilidade de ser cultivado em larga escala, onde pode ser aproveitado tanto para alimentação humana quanto para a suplementação alimentar de animais, especialmente caprinos e ovinos, que constituem

os rebanhos predominantes no semiárido (ALBUQUERQUE; ANDRADE; SILVA, 2005). Também serve de mantimento para polinizadores e dispersores.

Estudo fitoquímico realizado com extrato etanólico bruto das folhas e da casca de *Spondias* sp. e *S. tuberosa* revelou a presença de esteroides, taninos, flavonoides, terpenoides, triterpenos, saponinas, cumarinas, alcaloides, monoterpenos, sesquiterpenos e diterpenos (BARBOSA et al., 2016; DE LIMA et al., 2017). No extrato hidroetanólico de folhas da planta identificou-se a presença de flavonoides, compostos fenólicos, ácido clorogênico, ácido cafeico, rutina e isoquercitrina (SIQUEIRA et al., 2016). Borges e colaboradores (2007) realizaram estudo com o óleo das sementes de umbu e constataram a presença de ácidos graxos como: ácido palmílico, ácido esteárico, ácido oleico, ácido linoleico e ácido araquídico. Os extratos metanólico e acetato de etila das folhas de *S. tuberosa* revelaram a presença de quercetina, rutina, ácido clorogênico e β-sitosterol. Extrato etanólico da casca da planta não promoveu toxicidade aguda ou citotóxica (BARBOSA et al., 2016).

Siqueira (2015) cita que *S. tuberosa* tem ação anti-inflamatória e experimentos com extrato metanólico do umbu demonstraram atividade de inibição da acetilcolinesterase e o extrato diclorometano exibiu atividade anticancerígena (ZERAIK et al., 2016), atividade antimicrobiana (DA SILVA et al., 2012; DE BRITO COSTA et al., 2013).

Spondias tuberosa é utilizada como planta medicinal por várias comunidades locais no nordeste do Brasil para tratar infecções, distúrbios digestivos e condições inflamatórias (SIQUEIRA et al., 2016). As folhas são usadas no tratamento de diabetes, inflamação, constipação, dores estomacais e uterinas (JORGE et al., 2007; UCHÔA et al., 2015). A casca do caule é utilizada para enxaqueca, baixar colesterol, cicatrizante e inflamação (SOARES; SIQUEIRA; DE ALBUQUERQUE, 2012; JÚNIOR et al., 2014). Ribeiro e colaboradores (2014) cita o uso da raiz-tubérculo (infusão) pela população para corrimento feminino. Esta planta, entretanto, enfrenta vários problemas naturais e antrópicos que podem levar a sua extinção (MERTENS et al., 2017). Apesar desses riscos, a espécie não foi avaliada quanto ao *status* de conservação, mas estudos científicos são necessários para agregar e incentivar a preservação da espécie (FLORA DO BRASIL 2020, EM CONSTRUÇÃO).

1.3 GÊNERO *Candida*

Conforme a taxonomia, as espécies do gênero *Candida*, encontram-se alocadas no reino Fungi, Divisão Ascomycota, Subdivisão Saccharomycotina, Classe Saccharomycetes, Família Saccharomycetaceae (BUTLER, 2010). O gênero supracitado compreende várias espécies que habitam uma variedade de hospedeiros, como agentes comensais e/ou patógenos (WHIBLEY; GAFFEN 2015). Além disso, são organismos eucariontes que contêm parede celular, constituída por uma camada interna de quitina e β-1-3-glucano, sobre a qual outros polissacarídeos e glicoproteínas estão aderidos (ERWIG; GOW, 2016). Apresentam morfogênese celular variada, sendo capaz de se apresentar na forma de levedura, hifa e/ou pseudohifa e podem se organizar em dois estilos de vida planctônico e em biofilme (WONG et al., 2014).

Infecções oportunistas devido a espécies de *Candida* ocorrem com frequência em ambientes de terapia intensiva (SHOKOHI et al., 2017). Pacientes imunossuprimidos com quadros clínicos de diabetes, leucemias, AIDS, e o uso exacerbado de antibióticos de amplo espectro vem contribuindo para o aumento dos casos de micoses relacionadas a este gênero (DE ALMEIDA, 2008).

A forma como um patógeno pode escapar e afetar o sistema imunológico e ocasionar uma doença está relacionado a diferença da forma ao longo da infecção, que para causar infecções invasivas alguns fungos patogênicos, transitam entre as formas de levedura e filamentos (SEMAN et al., 2018), características de algumas espécies de *Candida*.

A prevalência de infecções fúngicas por *Candida* spp., vem aumentando em seres humanos, devido a diversos fatores que podem alterar a relação hospedeiro-patógeno. Sintomas e direção da enfermidade são integrados, às condições gerais e imunológicas da pessoa contaminada, bem como as características das espécies (MODRZEWSKA; KURNATOWSKI, 2013).

Apesar do aparecimento de novos fármacos, as espécies fúngicas podem desenvolver mecanismos de resistência dificultando o tratamento de pacientes com infecções. Assim, uma otimização dos procedimentos para a detecção de resistência associada à caracterização dos seus mecanismos, permite melhorar a eficácia da terapia antifúngica (PFALLER, 2012).

Durante alguns anos, o fluconazol tem sido usado para tratar infecções causadas por *Candida*. Porém, o uso indiscriminado dessa terapia beneficiou o aparecimento de isolados resistentes. Dentre estes, mutações no gene ERG11, as quais têm sido descritas como um dos principais mecanismos de resistência em espécies de *Candida*

(CARVALHO et al., 2013). Para tanto, o processo se dar através do átomo de nitrogênio livre do anel azol que se liga um átomo de ferro dentro do grupo heme da enzima. Isso impede a ativação do oxigênio e, por sua vez, a desmetilação do lanosterol, que inibe o processo de biossíntese do ergosterol. De modo que o ergosterol é um componente essencial da membranas celulares fúngicas, essa inibição é tóxica, os esteróis metilados se concentram na membrana celular dos fungos e o crescimento celular é cessado (BERKOW; LOCKHART, 2017).

1.4 ESPÉCIE *Candida albicans*

Candida albicans é uma espécie patogênica oportunista que causa infecções superficiais e sistêmicas em indivíduos imunocomprometidos (CHANG et al., 2018). Doenças causadas por *C. albicans* varia de infecções vaginais, a infecções mais intensas em pacientes hospitalizados que podem elevar as taxas de morbidade e mortalidade (POULAIN, 2015).

A espécie apresenta fatores de virulência tais como adesividade, alterações fenotípicas e morfológicas que resultam no processo infeccioso. Outros fatores estão envolvidos na alteração da forma comensal para patogênica, como a proteção contra a lise osmótica (parede celular), liberação de proteases que danificam o epitélio do hospedeiro, formação de hifas para acrescentar a capacidade nutricional e fixação ao tecido (DE ROSSI et al., 2011). A habilidade de alternar entre formas de crescimento de levedura, pseudo-hifa e hifas (polimorfismo) é uma das características de virulência mais pesquisadas de *C. albicans* (LU et al., 2014).

A levedura está equipada com uma complexidade de características comensais e de virulência, favorecendo o fungo a colonizar dentro da microbiota na fase comensal e a invadir o tecido hospedeiro durante a infecção. As propriedades de virulência incluem a habilidade de alterar a morfologia de uma célula de levedura em desenvolvimento para forma filamentosa, a expressão de adesinas e invasinas na superfície celular, a capacidade de danificar as células hospedeiras, a produção de biofilmes, o trigmotropismo (sensor de contato), a mudança fenotípica e a secreção de enzimas hidrolíticas (MAYER et al., 2012). Alguns desses atributos, se não todos, também estão possivelmente envolvidos no crescimento comensal (HÖFS; MOGAVERO; HUBE, 2016).

Esta espécie naturalmente sensível a todas as drogas antifúngicas de uso sistêmico, e em casos de resistência a azólicos são conhecidos em pacientes expostos prolongadamente a estes medicamentos (TIRABOSCHI et al., 2007).

1.5 ESPÉCIE *Candida tropicalis*

É um agente patogênico fúngico emergente com elevadas taxas de mortalidade associadas de 40 a 70% (CHEN et al., 2014). Atualmente, a dimensão de isolados clínicos de *C. tropicalis* aumentou expressivamente. Determinadas cepas colonizam a pele ou superfícies mucosas como comensais, outras causam infecções invasivas (JIANG et al., 2016).

Candida tropicalis é uma das espécies do gênero *Candida* que está no topo da lista epidemiológica de agentes causadores de candidíase sistêmica. É considerado um importante agente causador de candidíase invasiva e coloniza 60 % a 80 % dos pacientes imunocomprometidos. Esse fungo tem sido associado a taxas de mortalidade semelhantes ou mais altas do que as relatadas para *C. albicans* (NAVARRO-ARIAS et al., 2019).

Diversos fatores de virulência podem ser responsáveis por infecções de *C. tropicalis*. A adesão a superfícies (células epiteliais e dispositivos médicos), assim como a formação de biofilme, são consideradas o primeiro passo para iniciar a infecção. Além disso, a secreção de enzimas (proteinases e fosfolipases), bem como sua atividade hemolítica, é reconhecida como importantes fatores na invasão de tecidos envolvidos (NEGRI et al., 2010).

Linhagens de *C. tropicalis* desenvolveram resistência aos azólicos devido ao aumento da expressão do gene ERG 11, associado com uma mutação missense nesse gene (MENEZES; MENDES; CUNHA, 2009).

1.6 RESISTÊNCIA E VIRULÊNCIA FÚNGICA

Um dos principais motivo de resistência em espécies de *Candida* é que apenas 4 classes de medicamentos estão disponíveis para o tratamento sistêmico das infecções por estes fungos, incluindo os azóis (fluconazol, itraconazol, isavuconazol, posaconazol e voriconazol), polienos (anfotericina B convencional e suas formulações lipídicas),

equinocandinas (anidulafungina, caspofungina, e micafungina) e, por fim, o análogo da pirimidina flucitosina. Dentre essas classes de drogas, apenas os componentes dos três primeiros são licenciados para monoterapia contra infecções por *Candida* e apenas fluconazol e equinocandinas são recomendados como agentes de primeira linha para candidíase invasiva (ARENDRUP; PATTERSON, 2017).

O desenvolvimento da resistência antifúngica é um processo complexo que envolve hospedeiros, medicamentos e fatores microbianos, que contribuem juntamente para a falha terapêutica. O estado imunológico do hospedeiro é importante, pois este sistema deve trabalhar em conjunto com os antifúngicos para controlar uma infecção. O distúrbio imunológico grave resulta em pacientes menos responsivos ao tratamento, uma vez que os encargos microbianos são maiores e o medicamento deve combater a infecção sem suporte imunológico (PERLIN; SHOR; ZHAO, 2015).

Existem poucos antifúngicos no uso clínico, e a resistência a medicamentos/toxicidade do hospedeiro comprometem a utilidade clínica. Dessa forma, a resistência às drogas antifúngicas é um fenômeno complexo que envolve múltiplos mecanismos (XIE et al., 2014). Além de que, os atuais medicamentos podem ser limitados por interações medicamentosas, efeitos adversos e toxicidades graves que impedem seu uso prolongado ou aumento da dose (WIEDERHOLD, 2017).

A medicina vem enfrentando diversos desafios emergentes na resistência antifúngica. Isso inclui taxas aumentadas de resistência ao azol e equinocandinas em vários espécies não *C. albicans* e resistência ao azol em *Aspergillus fumigatus* que podem ocorrer devido à exposição clínica ou ambiental a esses agentes (WIEDERHOLD, 2017). Dessa forma, a resistência ao azol entre as espécies de *Candida* e *Aspergillus* é um dos maiores desafios para o sucesso clínico, seguida pela resistência à equinocandina e multidrogas. A disseminação de *A. fumigatus* resistente a azóis e ameaças emergentes como *C. auris* multirresistente também são alarmantes. O mecanismos moleculares que ocasionam resistência às drogas ocorrem naturalmente em espécies menos suscetíveis e são adquiridos em linhagens de organismos suscetíveis (PERLIN; RAUTEMAA-RICHARDSON; ALASTRUEY-IZQUIERDO, 2017).

Mecanismos de resistência não são trocados entre espécies de *Candida*; assim, a resistência adquirida surge em resposta à pressão de seleção antifúngica no paciente individual ou, mais raramente, ocorre devido à transmissão horizontal de cepas resistentes entre os pacientes. Apesar de que a resistência a múltiplas drogas seja incomum, informações crescentes de resistência a múltiplas drogas aos azóis,

equinocandinas e polienos ocorreram em várias espécies de *Candida*, principalmente *C. glabrata* e, mais recentemente, *Candida auris* (ARENDRUP; PATTERSON, 2017). Alguns dos mecanismos que contribuem para as resistências aos medicamentos antifúngicos são as interações alteradas entre alvo e droga, concentrações celulares reduzidas mediadas por transportadores de efluxo de drogas e barreiras de permeabilidade associadas a biofilmes (PERLIN; RAUTEMAA-RICHARDSON; ALASTRUEY-IZQUIERDO, 2017).

A virulência de um micro-organismo é definida como sua capacidade de causar doença, que é mediada por múltiplos fatores. Ainda que certos aspectos da virulência serem determinados geneticamente, eles são expressos pelos micro-organismos apenas quando existem condições ambientais favoráveis, tais como teor nutricional, atmosfera de oxigênio e temperatura. Os principais fatores de virulência em leveduras são: aderência, hidrofobicidade de superfície celular, produção de tubos germinativos e exoenzimas, variabilidade genotípica, tigmotropismo, internalização e alteração fenotípica (ÁLVARES; SVIDZINSKI; CONSOLARO, 2007).

A capacidade de produzir enzimas hidrolíticas é considerada um importante fator de virulência. As principais enzimas desenvolvidas por leveduras do gênero *Candida* são as proteinases e as fosfolipases (RÖRIG; COLACITE; ABEGG, 2009). As proteinases produzidas por *C. albicans* são reguladas por uma família composta por 10 genes (SAP1-10), estas enzimas apresentam atividade proteolítica, degradando colágeno, queratina e peptídeos localizados na superfície de mucosas e podem, ainda, agir sobre elementos do sistema imunológico, tais como imunoglobulinas, complemento e citocinas, favorecendo a propagação das leveduras nos tecidos do hospedeiro e evasão ao sistema imune (GOULART et al., 2017)

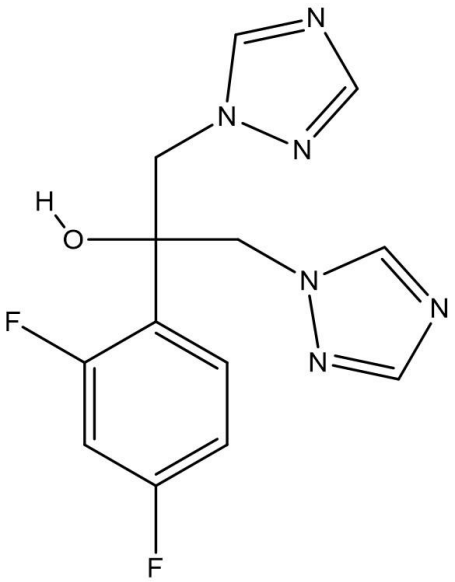
Outros fatores de virulência de *Candida* que estão associados às infecções sanguíneas incluem produção de hemolisina, produção de proteinases e formação de biofilmes. A formação de biofilmes, ainda que existente em todas as espécies, diverge significativamente entre elas, dependendo da superfície, por exemplo, e das suas características individuais (ROCHA et al., 2017).

Devido a esses fatores, são necessários estudos com produtos naturais para o desenvolvimento de medicamentos como uma nova estratégia terapêutica para atingir patógenos fúngicos e o conhecimento dos mecanismos que estes utilizam para causar as enfermidades nos seres humanos.

1.7 FLUCONAZOL

Os azóis compõem um grupo de fármacos antifúngicos viáveis que afetam o ergosterol. Atuam inibindo seletivamente a biossíntese do ergosterol e, por isso, têm atividade antifúngica de amplo espectro. A terapia com azóis resulta em membranas citoplasmáticas fúngicas anormais, que ocasionam danos e modificações críticas nas funções de transporte através da membrana (MADIGAN et al., 2016).

Dentro do grupo dos azóis se destaca o fármaco fluconazol (Figura 1) que se caracteriza por ser mais solúvel em água, sendo assim, mais fácil de usar e mais



eficiente. É o agente antifúngico utilizado como alternativa terapêutica habitual de diversos tratamentos de infecções fúngicas sistêmicas específicas (DOS SANTOS JR et al., 2005; TORTORA; CASE; FUNKE, 2016).

Figura 1 – Estrutura Química do Fluconazol

Fonte: Pubchem - adaptado por ChemBioDraw Ultra 12.0

É uma droga que tem seu espectro de ação ampliado bem como sua seletividade para o citocromo P-450 da célula fúngica, em virtude da substituição do anel imidazólico por um anel triazólico, que impede o crescimento de fungos por meio da inibição da síntese do ergosterol (esteróide), essencial à sua sobrevivência (OLIVEIRA et al., 2010).

Seu mecanismo de ação tem como base a inibição da enzima lanosterol 14-α-demetilase no complexo citocromo P-450 dos fungos, resultando na inibição da conversão de lanosterol em ergosterol. Com a depleção do ergosterol e sua substituição por esteróis incomuns, ocorre alteração da permeabilidade normal e fluidez da membrana fúngica, secundariamente há consequências para enzimas ligadas a membranas, tais como os envolvidos na síntese da parede celular (SIDRIM; ROCHA, 2010; DOS SANTOS JR et al., 2005).

1.8 IMPORTÂNCIA DE ESTUDOS FITOQUÍMICOS

Estudos das plantas vêm crescendo no Brasil e no mundo, com isso aumenta a importância e o conhecimento sobre os seus componentes químicos (DE FARIA GOES et al., 2016). Há muitos anos várias plantas apresentam uso medicinal e nos extratos destes vegetais, a atuação conjunta ou isolada de algumas substâncias é responsável pela atividade biológica, e este efeito difere de acordo com a dose (DE REZENDE et al., 2016).

Substâncias secundárias são os princípios ativos vegetais responsáveis pelo efeito medicinal de uma planta, entretanto dependendo da administração, o efeito deixa de ser terapêutico e passa a ser tóxico. O princípio ativo é uma combinação de conteúdos que proporciona a atuação farmacológica e difere de fármaco à medida que o termo designa uma substância química conhecida e de estrutura química definida (DE REZENDE et al., 2016).

Apesar de existir determinadas inovações para a descoberta de medicamentos, nenhuma pode suprir a importância dos produtos naturais na descoberta e desenvolvimento de drogas (MOGHADAMTOUSI et al., 2015). Diante disso vem sendo investigado fontes de plantas ricas em compostos bioativos para uso em diferentes aplicações da indústria alimentícia, cosmética e farmacêutica (MORAES, 2016). Diversos tipos de técnicas são usadas para detectar, quantificar e isolar compostos contidos em produtos naturais que são analisados na forma de extratos, óleos e frações polares e apolares (MATOS, 2009).

CAPÍTULO 2

ARTIGO NA NORMA DA REVISTA ANTIBIOTICS

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UPLC-MS-ESI-QTOF Analysis and Antifungal Activity of the *Spondias tuberosa* Arruda Leaf and Root Hydroalcoholic Extracts

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Abstract: The aim of this study was to identify and evaluate the chemical compositions and effects of the *S. tuberosa* leaf and root hydroalcoholic extracts (HELST and HERST) against different strains of *Candida*. Chemical analysis was performed by Ultra-Performance Liquid Chromatography Coupled to Quadrupole/Time of Flight System (UPLC-MS-ESI-QTOF). The Inhibitory Concentration of 50% of the growth (IC_{50}) as well as the intrinsic and combined action of the extracts with the antifungal fluconazole (FCZ) were determined by the microdilution method while the minimum fungicidal concentrations (MFCs) and the effect on fungal morphological transitions were analyzed by subculture and in humid chambers, respectively. From the preliminary phytochemical analysis, the phenols and flavonoids were the most abundant. The intrinsic IC_{50} values for HELST ranged from 5716.3 to 7805.8 µg/mL and from 6175.4 to 51070.9 µg/mL for the HERST, whereas the combination of the extracts with fluconazole presented IC_{50} values from 2.65 to 278.41 µg/mL. The MFC of the extracts, individually, for all the tested strains was ≥16384 µg/mL. When fluconazole was combined with each extract, the MFC against CA URM 5974 was reduced (HELST: 2048 and HERST: 4096 µg/mL). Synergism was observed against standard *C. albicans* (CA) and *C. tropicalis* (CT) strains and with the root extract against the CT isolate. The leaf extract inhibited the morphological transition of all strains while the root extract inhibited only CT strains.

Keywords: chromatography; fluconazole; *Candida* spp., morphological transition

1. Introduction

Candida species, especially *C. albicans*, are commonly found on human mucosal surfaces but are becoming an important progressive invasive pathogen due to their increased prevalence in immunocompromised patients and the increased use of antibiotics [1].

Infections caused by the *Candida* genus are associated with a high morbidity and mortality rate, where these species are responsible for superficial and systemic candidiasis, with the latter being a serious problem for health systems and patients [2]. Several *Candida* species are polymorphic and able to transition between different morphological conditions involving yeast, hyphal, and pseudohyphal forms [3].

The inappropriate use of antifungal drugs contributes to the increase in microbial resistance, thus an understanding the mechanisms of antifungal drugs, the cellular molecular mechanisms involved in the antifungal resistance process, is sought [4]. Given the few commercially available antifungals and their varied side effects, a need to produce new and more effective antifungal agents with fewer adverse effects exists [5].

Studies addressing the use of plants have been growing in Brazil as well as worldwide, increasing the importance and knowledge of their chemical components [6]. In this sense, natural products and their chemical diversity are seen as options for potentially active therapeutic sources, which can be a means of discovering new drugs [7].

Spondias tuberosa Arruda (umbu) is a native plant from Brazil, belonging to the Anacardiaceae family, with great ecological and economic importance [8], being used in popular medicine for infections, stomach disorders, and inflammatory conditions [9]. Among its biological activities, its anti-inflammatory [10], anticancer [11], antibacterial [12], antioxidant [13], and antiviral activities stand out [14].

Using ethnobiology as a guiding strategy for bioactivity research, the objective of this study was to identify the chemical composition of the *S. tuberosa* leaf and root hydroalcoholic extracts and their antifungal effect, both the intrinsic and combined effect with conventional fluconazole, as well as their action on morphological transition, a *Candida* spp. virulence factor.

2. Results

The leaf and root extract yields were, respectively, 0.74% and 0.635%. Preliminary phytochemical analysis of the extracts revealed the presence of several metabolite classes, such as: Alkaloids, steroids, phenols, flavonoids, triterpenoids, and xanthones (Table1). It is noteworthy that the presence of phenols and flavonoids prevailed in all extracts. Among the secondary metabolites investigated, coumarins, flavanonols, and tannins were not detected.

Table 1. Preliminary phytochemical analysis of extracts.

	Special Metabolite Classes (SMC)									
	SMC 1	**SMC 2**	**SMC 3**	**SMC 4**	**SCM 5**	**SMC 6**	**SMC 7**	**SMC 8**	**SMC 9**	**SMC 10**
HELST	✚	-	✚	-	-	✚	-	✚	-	✚
HERST	✚	-	-	✚	-	✚	-	-	✚	-

SMC 1: Phenols; SMC 2: Tannins; SMC 3: Flavonoids of the flavone, flavonol and xanthone type; SMC 4: Flavonoids of the anthocyanin and anthocyanidin type; SMC 5: Flavanoid of leucoanthocyanidins, catechins; SMC 6: Flavanone-type flavanoid; SMC 7: Flavanoid Flavanoid; SMC 8: Steroids; SMC 9: Triterpenoids; SMC 10: Alkaloids; (✚): positive; (-): absent.

In UPLC-MS-ESI-QTOF, identification of the compounds was based on their molecular ion mass, retention time, fragmentation pattern, and data available in the literature, as shown in Tables 2 and 3, according to the order of elution, molecular formula, error, and major fragments (MS2).

Table 2. Ultra-Performance Liquid Chromatography Coupled to Quadrupole/Time of Flight System (UPLC-MS-ESI-QTOF) identification of hydroalcoholic extract compounds of *Spondias tuberosa* leaves.

Peak no.	Rt min	[M-H]⁻ Observed	[M-H]⁻ Calculated	Product Ions (MS/MS)	Empirical Formula	Ppm (error)	Putative Name	References
1	2.82	174.9525	174.9502	175.9599	$C_6H_6O_6$		Dehydroascorbic acid	[15]
2	3.28	443.1294	443.1283	381.1805, 281.1358, 119.0361	$C_{21}H_{32}O_{10}$	2.5	Dehydrophaseic acid hexose	[16]
3	3.40	189.0021	189.0035	207,0115, 188.9965, 126.9987	$C_6H_5O_7$	−7.4	None identified	-
4	3.65	188.9986	188.9977	207.0199, 188.9996, 127.0019	$C_{13}HO_2$	4.8	None identified	-
5	4.53	191.0109	191.0133	173.0243, 127.0278, 85.0283	$C_7H_{12}O_6$	−12.6	Quinic acid (Organic acid)	[17,18]
6	4.65	343.0833	343.0818	191.0497, 169.0125, 125.0265	$C_{14}H_{16}O_{10}$	4.4	Galloyl quinic acid isomer Ia	[18]
7	4.67	343.0508	343.0513	191.0523, 169.0112, 125.0259	$C_{14}H_{16}O_{10}$	−1.5	Galloyl quinic acid isomer IIa	[18]
8	4.74	343.1045	343.1029	191.0196, 169.0011, 125.9942	$C_{14}H_{16}O_{10}$	4.9	Galloyl quinic acid isomer III	[18])
9	4.89	343.0904	343.0877	191.0540, 169.0121, 125.0247	$C_{14}H_{16}O_{10}$	7.9	Galloyl quinic acid isomer IV	[18]
10	6.28	421.2582	421.2590	331.0801, 3010357	$C_{19}H_{18}O_{11}$	−1.9	Mangiferin	[15,19]
11	6.48	939.4997	939.4989	787.4410, 277.2166, 125.0243	$C_{41}H_{32}O_{26}$	0.9	Penta-O-galloyl hexoside	[20]
12	6.56	397.1313	397.1287	502.2910, 474.2616, 277.2155	$C_{22}H_{21}O_7$	6.5	None identified	-
13	6.90	277.2142	277.2168	279.2315, 277.2151, 189.0038	$C_{18}H_{29}O_2$	−9.4	None identified	-
14	7.00	339.2007	339.2019	-	$C_{22}H_{28}O_3$	−3.5	Caffeoyl-D-glucose	[16]
15	7.50	483.2540	483.2535	271.0134, 169.0511, 125.9984	$C_{20}H_{20}O_{14}$	1.0	Digalloyl glucose (Digalloyglucose)	[16,21]

[M-H]⁻—Reference Ion on negative ion mode.

Table 4 shows the IC_{50} values of the extracts, fluconazole, and their combined action, where the concentration in which the natural product or the drug could reduce the microorganismal population by 50% can be seen. The HESTL and HESTR values were not clinically significant while fluconazole achieved a 50% reduction in the fungal population, whereas when associated with leaf extract, there was an increase in drug action. With root extract, the effect was similar to fluconazole for *C. albicans* strains, and for CT INCQS 40042, there was a decrease in the effect of the drug, while for CT URM there was an improvement in fluconazole action.

Table 3. UPLC-MS-ESI-QTOF identification of hydroalcoholic extract compounds of *Spondias tuberora* roots.

Peak no.	Rt min	$[M-H]^-$ Observed	$[M-H]^-$ Calculated	Product Ions (MS/MS)	Empirical Formula	Ppm (error)	Putative Name	References
1	2.83	272.9554	272.9578	274.9536, 273.9563, 158.9753	$C_{15}H_{12}O_5$	−8.8	(±)-Naringenin	[15]
2	3.11	341.2104	341.2117	297.2246, 295.2048, 119.0465	$C_{22}H_{30}O_3$	2.1	Anacardic acid 1	[16]
3	3.14	343.1186	343.1182	299.2344	$C_{22}H_{21}O_3$	1.2	Anacardic acid 2	[16]
4	3.16	377.0822	377.0814	379.0782, 377.0770, 341.1009	$C_{25}H_{13}O_4$	2.1	No identified	-
5	3.22	345.0010	345.0035	301.2471	$C_{22}H_{34}O_3$	−7.2	Anacardic acid 3	[16]
6	3.52	355.0232	355.0243	355.0258, 163.0371	$C_{16}H_{18}O_9$	−3.1	Chlorogenic acid	[9]
7	4.39	411.0173	411.0213	411.0232, 240.9987, 169.0123	$C_{17}H_7N_4O_9$	−9.7	None identified	-
8	4.50	461.1219	461.1236	257.0848, 229.8624, 151.0033	$C_{22}H_{21}O_{11}$	−3.7	Kaempferol-7-Oglucuronide	[16]
9	4.62	197.0424	197.0450	199.06, 198.05, 182.0187	$C_9H_{10}O_5$	−13.2	2-Hydroxy-3,4-dimethoxybenzoic acid	[15]
10	4.90	433.0583	433.0560	300.9944, 271.0716	$C_{20}H_{18}O_{11}$	5.3	Quercetin O-pentoside	[20]
11	4.99	315.0096	315.0082	394.9633, 315.0095, 299.9882	$C_{16}H_{12}O_7$	4.4	Rhamnetin	[22]
12	5.05	463.1048	463.1029	316.0255, 271.0625	$C_{21}H_{20}O_{12}$	4.1	Myricetin O-deoxyhexoside	[20]
13	5.08	449.1247	449.1236	316.0205	$C_{20}H_{18}O_{12}$	2.4	Myricetin O-pentoside	[20]
14	5.35	331.2458	331.2484	271.0428, 241.0313, 125.0252	$C_9H_{16}O_{13}$	−7.8	Monogalloyl-glucose	[16]
15	5.52	461.2616	461.2598	315.0131	$C_{21}H_{17}O_{12}$	3.9	Isorhamnetin Orhamnoside	[23]
16	6.15	833.5256	833.5262	833.5175, 507.2970, 175.0390	$C_{43}H_{77}O_{15}$	0.7	None identified	-

$[M-H]^-$—Reference Ion on negative ion mode.

Table 4. 50% inhibitory concentration (IC_{50}) of microorganisms (µg/mL) by extracts, fluconazole, and their combination.

	CA INCQS 40006	CA URM 5974	CT INCQS 40042	CT URM 4262
HELST	6211.1 *	5716.3 *	7166.8 *	7805.8 *
HERST	6264.8 *	8919.9 *	51070.9 *	6175.4 *
FLUCONAZOLE	22.79	3.97	88.08	47.30
HELST + FCZ	13.60	2.65 *	44.86 *	43.75
HERST + FCZ	25.81	4.38	278.41 *	26.15 *

CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: National Institute of Quality Control in Health; URM: University Recife Mycology; HELST: Hydroalcoolic Extract of Leaves *Spondias tuberosa*; HERST: Hydroalcoholic Extract of the Roots of *Spondias tuberosa*; FCZ: Fluconazole. * Corresponding to the ANOVA with $p < 0.0001$ for all data.

The action of the extracts and fluconazole on the cell viability curve can be seen in Figure 1. The extracts presented no relevant action against the tested strains while fluconazole presented a more effective action against CA URM 5974 (32 µg/mL), and against other strains when its concentration was increased.

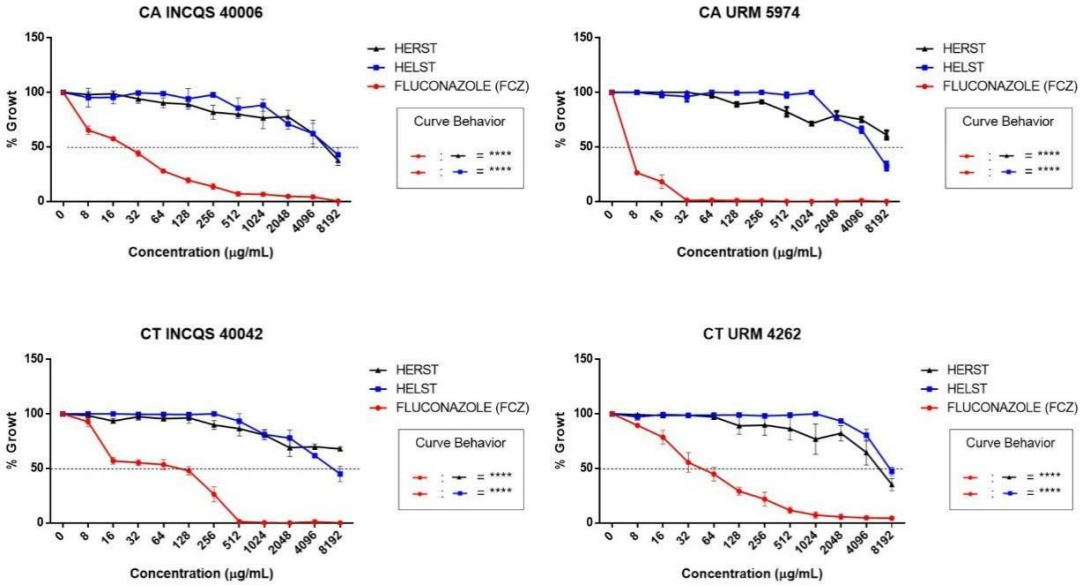

Figure 1. Cell viability curve demonstrating the inhibitory effect of fluconazole and hydroalcoholic extracts of *Spondias tuberosa* against *Candida* strains. CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: National Institute of Quality Control in Health; URM: University Recife Mycology; HELST: Hydroalcoholic Extract of Leaves of *Spondias tuberosa*; HERST: Hydroalcoholic Extract of the Roots of *Spondias tuberosa*; FCZ: Fluconazole. ****—Statistical significance with $p < 0.0001$.

The MFC of the extracts against all tested strains was ≥16,384 µg/mL, as well as the fluconazole MFC against *C. tropicalis*, thus observing a fungistatic effect. The FCZ effect was termed fungicidal at a concentration of 8192 µg/mL (Table 5) against standard and isolated *C. albicans* strains. An MFC of 2048 and 4096 µg/mL, respectively, was observed against the CA isolate in the fluconazole combination with the HELST and HERST extracts while the MFC ranged from 8192 to ≥16,384 µg/mL against the other tested strains.

Table 5. Minimal fungicidal concentration (MFC) of microorganisms (µg/mL) by extracts, fluconazole, and their combination.

	CA INCQS 40006	CA URM 5974	CT INCQS 40042	CT URM 4262
HELST	≥16,384	≥16,384	≥16,384	≥16,384
HERST	≥16,384	≥16,384	≥16,384	≥16,384
FLUCONAZOLE	8192	8192	≥16,384	≥16,384
HELST + FCZ	8192	2048	≥16,384	8192
HERST + FCZ	8192	4096	≥16,384	≥16,384

CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: National Institute of Quality Control in Health; URM: University Recife Mycology; HELST: Hydroalcoolic Extract of Leaves *Spondias tuberosa*; HERST: Hydroalcoholic Extract of the Roots of *Spondias tuberosa*; FCZ: Fluconazole.

When combined with the antifungal agent fluconazole, the HELST potentiated the effect of the drug, where a synergistic effect against CA INCQS 40006 (8 to 256 µg/mL) and CT INCQS 40042 (8 to 512 µg/mL) was observed. On the other hand, the cell viability curve for CA URM 5974 and CT URM 4262 isolates was similar to that of fluconazole (Figure 2).

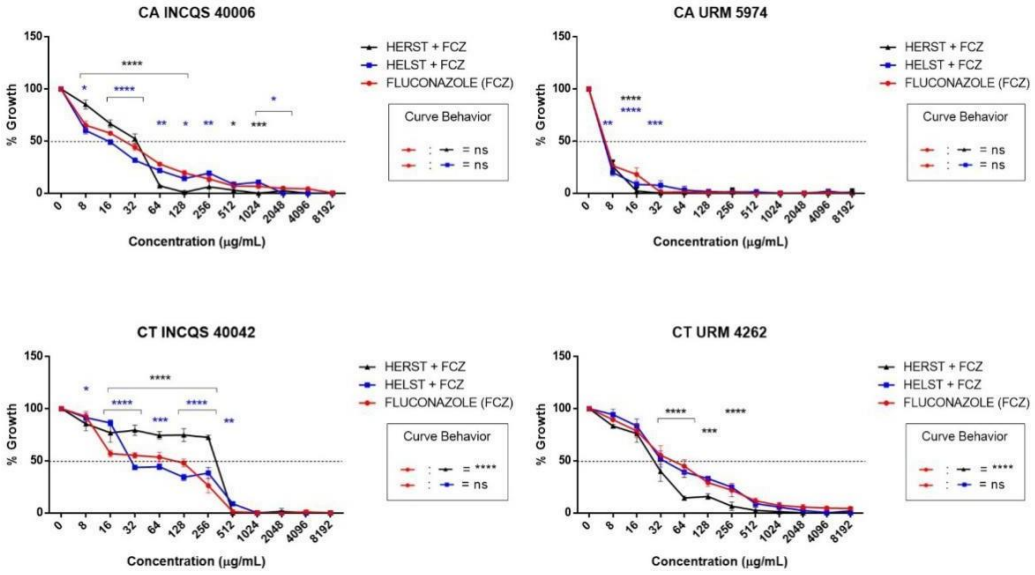

Figure 2. Evaluation of the modifying effect of fluconazole action by extracts. CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: National Institute of Quality Control in Health; URM: University Recife Mycology; HELST: Hydroalcoholic Extract of Leaves of *Spondias tuberosa*; HERST: Hydroalcoholic Extract of the Roots of *Spondias tuberosa*; FCZ: Fluconazole; NS: not significant; *—Statistical significance with $p < 0.05$; **—Statistical significance with $p < 0.01$; ***—Statistical significance with $p < 0.001$; ****—Statistical significance with $p < 0.0001$.

A synergism against CA INCQS 40006 (64 to 1024 µg/mL), CA URM 5974 (16 µg/mL), and CT URM 4262 (8 to 1024 µg/mL) can be observed when the HERST was combined with fluconazole. However, an antagonism was observed against the standard CT INCQS 40042 strain (16 to 256 µg/mL), where the combination with the extract diminished the effect of the drug.

In the antivirulence assay, the HELST was able to inhibit hyphal emission at the highest concentration (HCE) in all tested strains. At the HCE/4 concentration, the extract reduced morphological transition by 44.09% and 45.45%, respectively, against CA INCQS 40006 and CT INCQS 40042 when compared to the growth control. At lower concentrations, the HELST presented insignificant or similar results to the growth control. Fluconazole inhibited the tested strains at all concentrations (Figure 3).

The HERST caused filament inhibition only at the highest concentration for CT INCQS 40042 and CT URM 4262 while it reduced hyphal growth by 54.30% and 62.09% respectively, for CA INCQS 40006 and CA URM 5974, all relative to the growth control. At the lowest concentrations, the HERST did not obtain considerable results.

Figure 4 shows the filamentous structures in the growth control and the progressive effect of fluconazole and HELST on *C. tropicalis* yeast morphology (CT INCQS 40042). The micromorphology recordings for fluconazole presented inhibition at all tested concentrations while the HELST caused inhibition at the HCE concentration (8192 µg/mL) and a decrease in hyphae and pseudohyphae growth at the HCE/4 concentration (2048 µg/mL). The HCE/16 concentration (512 µg/mL) presented similar results to the growth control.

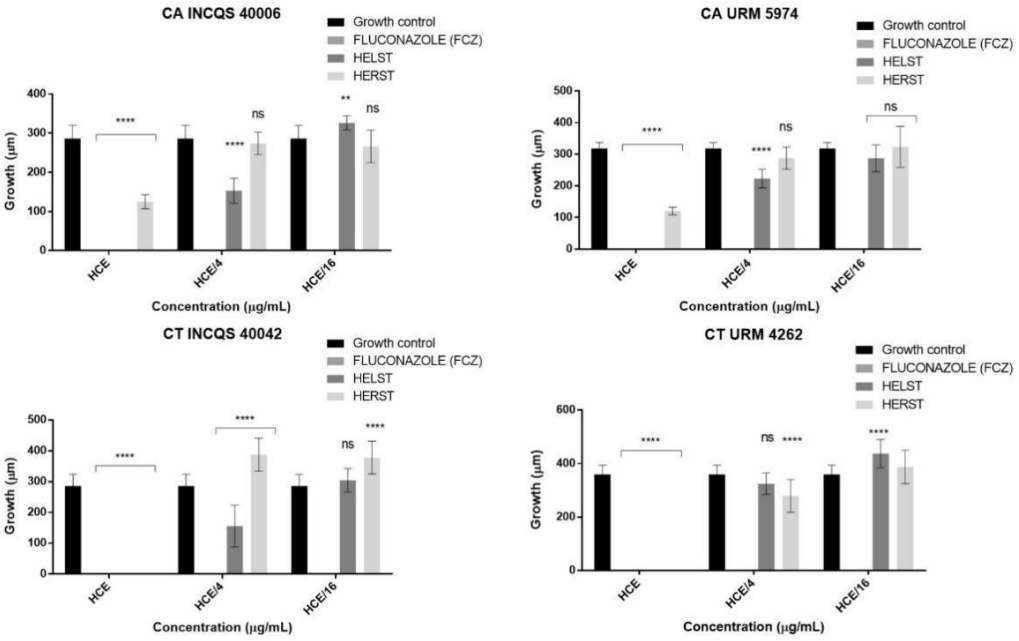

Figure 3. Effect of extracts and fluconazole on the morphological transition of *Candida* spp. CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: National Institute of Quality Control in Health; URM: University Recife Mycology; HELST: Hydroalcoholic Extract of Leaves of *Spondias tuberosa*; HERST: Hydroalcoholic Extract of the Roots of *Spondias tuberosa* FCZ: Fluconazole; HCE: Higher Concentration Evaluated; ns: not significant. NS: not significant; **—Statistical significance with $p < 0.01$; ****—Statistical significance with $p < 0.0001$.

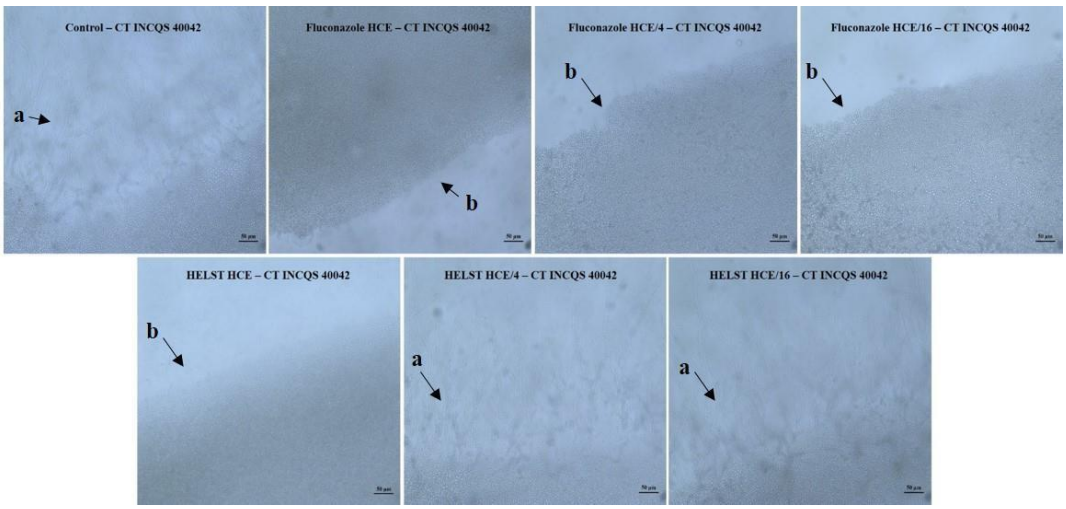

Figure 4. Demonstrative effect of HELST and fluconazole on the morphological transition of *Candida tropicalis*. CT: *Candida tropicalis*; INCQS: National Institute of Quality Control in Health; URM: University Recife Mycology; HELST: Hydroalcoholic Extract of Leaves of *Spondias tuberosa*; FCZ: Fluconazole; HCE: Higher Concentration Evaluate; a: presence of filamentous structures; b: absence of filamentous structures. A—hyphae development; B—hyphae inhibition.

Generally, the HELST obtained better results in all the performed tests, which is interesting from the point of view of the conservation of the species, as since the leaves are more accessible, the collection is practical and less harmful to the plant.

3. Discussion

Phytochemical studies using the *S. tuberosa* leaf methanolic and ethyl acetate extracts also revealed the presence of phenols, flavonoids, flavones, triterpenes, and steroid compounds, similar to those found in the present study analysis; however, these also found cinnamic derivatives, saponins, and leucoanthocyanidins, which differ from the present results [14,24]. The *S. monbin* ethanolic extract revealed the presence of polyphenols and flavonoids [25]. The authors of [9] found chlorogenic acid in *S. tuberosa* leaf hydroethanolic extract by HPLC analysis, with the same compound being identified in the root hydroalcoholic extract of the same species.

The authors of [26] inhibited *C. albicans* growth with *S. tuberosa* leaf hexanic extract using a different methodology, obtaining an IC_{50} of 2.0 mg/mL, a result that is different to those of the present study; however, the authors did not detect a fungicidal effect, a result similar to that of this study. *S. tuberosa* bark ethanolic extract did not inhibit growth of the *C. albicans*, *C. glabrata*, *C. krusei*, or *C. tropicalis* strains with the disc diffusion technique [27], whose effect may be explained by the fact that the hexane extract have a lower polarity and the extracted constituents are different from those obtained with the extracts in this study [28].

Some of the compounds identified in the *S. tuberosa* leaf and root extracts are reported in the literature for their antifungal activity. The author of [29] reports the antifungal activity of mangiferin. An inhibition in filament growth was observed with the *C. albicans* (ATCC 90028 and MTCC 186) strains and clinical isolates (CA1, CA2, CA3, and CA4) in a study addressing the synergistic association of quinic acid with undecanoic acid on *Candida* spp. morphological transition. However, only a moderate inhibition was observed for *C. tropicalis* (MTCC 184) strains and isolates (CT1, CT2, and CT3) [30]. The authors of [31] tested several compounds using the disc diffusion method where one of them was narigenin, a compound present in HERST, which demonstrated activity against *C. albicans*.

The authors of [30] showed that quinic acid, which was identified in the HELST, did not inhibit the growth of *Candida* strains at varying concentrations (12.5–800 µg mL^{-1}). In a study on the antifungal activity of chlorogenic acid, the yeasts *Trichosporon beigelii* (KCTC 7707), *Malassezia furfur* (KCTC 7744), and *C. albicans* (TIMM 1768) were susceptible to the compound, with MIC values between 40 and 80 µg/mL [32]. Chlorogenic acid achieved a partial reduction in the mycelial growth of some pathogenic plant fungi [33]. The chlorogenic acid effect on *C. albicans* dimorphism was investigated, where this was able to inhibit and destroy hyphae. The authors attribute the action of this compound, which affects yeast cells, to it damaging their membranes and disrupting their membrane potential [32].

The use of different *Spondias monbin* L. parts in combination with fluconazole inhibited the growth of *C. albicans* ATCC 90029 and *C. krusei*, where the author associated this result with the compounds present in the plant, which may have increased the effect of the drug [34]. The *S. monbin* leaf extract inhibited the *Saccharomyces cerevisiae* NCPF 3178 yeast strain (7 to 7.5 mm) in the disk diffusion method and presented a minimum inhibitory concentration (MIC) of 250 µg/mL [35] by microdilution. The authors of [25] tested *S. monbin* ethanolic extract against *C. albicans* ATCC 10231, *C. guilliermondii* ATCC 6260 and *C. krusei* ATCC 34135 using a similar technique to that applied in this study, obtaining MIC values >1000 µg/mL^{-1}.

Other species that are also from the Anacardiaceae family have been investigated for their activity against fungi. A study with *Anacardium occidentale* L. and *Mangifera indica* L. leaves against *C. albicans* obtained an MIC of 1250 µg/mL and MFC of 1250 and 2500 µg/mL, respectively [36]. The *A. occidentale* ethanolic extract from the plant's flowers, leaves, and stem bark were investigated using the disc diffusion technique, where the flower extract inhibited different *C. albicans* and *C. tropicalis* (1 mm) strains, while the leaf and stem bark extracts inhibited *C. albicans* 37 (1 mm) and fluconazole inhibited the *C. albicans* and *C. tropicalis* strains (1 to 2 mm) [37].

The diversity of chemical classes that are present in an extract are defined as complex heterogeneous mixtures, each of which has different biological or pharmacological activities and collectively contribute to the bioactivity of the extract as a whole [38,39], thus the moderate activity demonstrated by the

S. tuberosa leaf and root hydroalcoholic extracts against species from the *Candida* genus may have occurred due to the synergistic action of the chemical constituents present in the extracts.

4. Materials and Methods

1.1. Botanical Material Collection and Identification

The botanical material was collected with consent from the Biodiversity Authorization and Information System SISBIO-number 64293-1, in the community of Lameiro (07°, 15′03.1″ S and 39°, 23′48.3″ W of Greenwich), in the municipality of Crato, southern Ceará, Brazil. Specimens from seven individuals were collected from 8:00 to 9:30 in the morning. A sample of the collected material was deposited in the Herbarium Dárdano de Andrade Lima (HCDAL) of the Regional University of Cariri-URCA with the herbarium number 13.728, identified by Professor Ana Cleide Alcantara Morais Mendonça as being the species *Spondias tuberosa* Arruda.

1.2. Extract Acquisition

For the extract preparations, healthy young roots and leaves collected from the upper part of the plant were used. The hydroalcoholic extract (tincture or alcohol) was prepared in 70% alcohol (ethanol) at 500 g of fresh leaves or 400 g of dried root for every 2.652 L of alcohol/water proportion [40] with modifications.

Extract drying was performed by spray drying using the Mini-spray dryer MSDi 1.0 (Labmaq do Brasil) using a 1.2-mm spray nozzle under the following operating conditions: (a) Flow control: 500 mL/h; (b) inlet temperature: 130 ± 2 °C; (c) outlet temperature: 74 ± 2 °C; (d) atomization air flow: 45 L/min; and (e) blower flow: 1.95 m /min. The spray drying process consists of a product that is in the liquid state changing to a solid state in powder form by passing through a heated medium under continuous operation [41].

1.3. Chemical Analysis

1.3.1. Preliminary Phytochemical Analysis

The extracts were subjected to preliminary phytochemical analysis based on qualitative methods proposed by [42]. In these experiments, characterization of the main metabolite classes was performed by chemical reactions, with the addition of specific reagents resulting in color changes and/or precipitate formations characteristic of the following metabolite classes: Alkaloids, steroids, phenols, flavonoids, tannins, and triterpenoids. All experiments were performed in triplicates and the secondary metabolite classes present in the extracts were classified as present (+) or absent (-).

1.3.2. Compound Identification by Ultra-Performance Liquid Chromatography Coupled to Quadrupole/Time of Flight System (UPLC-MS-ESI-QTOF)

Identification of the compounds present in the extracts was performed in an Acquity® UPLC system coupled to a Quadrupole/Time of Flight (QTOF) system (Waters Corporation, Milford, MA, USA), provided by the Chemical and Natural Products Laboratory, Embrapa Tropical Agroindustry (Fortaleza, Ceará). Chromatographic runs were performed with a Waters Acquity® UPLC BEH column (150 × 2.1 mm; 1.7 µm), a fixed temperature of 40 °C, mobile phases with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), gradient ranging from 2% to 95% B (15 min), 0.4 mL/min flow rate, and 5 µL injection volume. The ESI^- mode was obtained in the 110–1180 Da range with a fixed source temperature of 120 °C, 350 °C desolvation temperature, 500 L/h desolvation gas flow, 0.5 V extraction cone, and 2.6 kV capillary voltage. Leucine encephalin was used as the lock mass. The MSE (high energy mass spectrometry) acquisition mode was used. The instrument was controlled by the Masslynx® 4.1 software (Waters Corporation, Milford, USA).

1.4. Antifungal Assays

1.4.1. Microorganisms, Culture Media, Inoculum Preparation, and Drugs and Reagents Used

Two standard strains, *Candida albicans*-CA INCQS 40006 and *Candida tropicalis*-CT INCQS 40042, obtained from the Oswaldo Cruz Culture Collection of the National Institute for Quality Control in Health (INCQS) and two clinical isolates, *Candida albicans*-CA URM 5974 and *Candida tropicalis*-CT URM 4262, provided by the Recife Micoteca University (URM) from the Federal University of Pernambuco–UFPE were used to evaluate antifungal activity.

The Sabouraud Dextrose Agar (SDA) solid medium, prepared according to the manufacturer's instructions, and the doubly concentrated Sabouraud Dextrose Broth (SDB) liquid medium, purchased from HIMEDIA®, were used. The Potato Dextrose Agar (PDA) medium, purchased from Difco®, depleted by dilution and added to agar was used for the fungal morphology analysis. The media were solubilized with distilled water and autoclaved at 121 °C for 15 min.

All strains were initially kept in refrigerated (8 °C) slanted SDA at the Cariri Applied Mycology Laboratory-LMAC of the Regional University of Cariri-URCA. For microdilution assays, strains were initially cultivated on SDA media at 37 °C for 24 h. Cell suspensions were then prepared in tubes containing 4 mL of sterile saline solution (0.9% NaCl) and their turbidity was compared and adjusted according to the MacFarland 0.5 scale [43].

Dimethyl sulfoxide (DMSO-Vetec) was used for the dilution of the extracts and the antifungal fluconazole 150 mg (prati-donaduzzi), diluted in water, was used as the commercial drug reference in the tests. For the initial extract solution preparations, 0.15 g were weighed and solubilized in 1 mL of DMSO. The extracts and fluconazole were then further diluted in sterile distilled water to obtain the desired test concentration (16,384 µg/mL).

1.4.2. IC_{50} Determination and Cell Viability Curve

This assay was performed using the broth microdilution technique in 96-well plates. Each well was filled with the medium and inoculum, followed by the extracts and fluconazole, before being individually microdiluted (81,92 to 8 µg/mL). The last well was reserved as a growth control, all tests were performed in quadruplicate [44], with changes in concentration. Natural product and fluconazole dilution controls (with 0.9% sodium chloride solution instead of inoculum) and sterility controls were also performed. The plates were incubated at 37 °C for 24 h, then read on an ELISA spectrophotometer (Kasuaki-DR-200Bs-BI) at a wavelength of 450 nm. Results from the absorbance were used to construct the cell viability curve; from the mean cell viability curve, the IC_{50} values of the extracts and fluconazole were calculated [45].

1.4.3. Minimum Fungicidal Concentration (MFC) Determination

For this test, a sterile rod was introduced into each well of the microdilution test plate (except for the sterility control), where after homogenization of the medium contained in the well, a petri dish with SDA was sub-cultured by transferring a small aliquot of the test solution (medium + inoculum + natural product). The plates were incubated at 37 °C for 24 h and checked for *Candida* colony growth or lack thereof [46], with modifications. The MFC was defined as the lowest natural product concentration capable of inhibiting the growth of fungal colonies.

1.4.4. Evaluation of the Modifying Effect of Fluconazole Action

The combination of the extracts with the reference drug (fluconazole) was performed to verify whether the antifungal action was modified by the presence of the extract in the medium. Solutions containing the extracts were tested at a sub-inhibitory concentration (MFC/16), according to the methodology used by [47], with changes in concentrations. If the extracts potentiated the action of the antifungal, the observed effect

was considered to be synergistic; however, if it impaired its action, it was considered to be an antagonistic effect. Fluconazole was microdiluted at a 1:1 proportion up to the penultimate well. Sterility controls were also performed. All tests were performed in quadruplicate. The plates were incubated at 37 °C for 24 h. The readings were performed in an ELISA spectrophotometry device (Kasuaki-DR-200Bs-BI) [45].

1.4.5. Effect of the Extracts and Fluconazole on *Candida* Morphological Transition

Microculture chambers were used to verify changes in fungal morphology by the extracts and fluconazole through the reduction or prevention of hyphae development. The extracts and fluconazole were individually evaluated at different concentrations (HCE-8192 µg/mL, HCE/4-2048 µg/mL, and HCE/16-512 µg/mL, where HCE is the highest concentration evaluated by microdilution). The tests were performed according to [48,49], with modifications: Two parallel striae were made in the solid medium (PDA), which were subsequently covered with a sterile cover slip. For comparative purposes, growth controls as well as fluconazole antifungal controls were performed. The chambers were incubated for 24 h (37 °C), inspected, and recorded by an optical microscope (AXIO IMAGER M2-3525001980-ZEISS-Germany) using a 20X objective. The Zen 2.0 software was used to measure hyphae and pseudohyphae extensions, where images were taken from each complete stria, with five random images being selected from these, according to the concentration. The values were used for statistical analysis [50].

1.5. Statistical Analysis

A two-way ANOVA was applied to each sample, comparing the values for each extract concentration, with Bonferroni's post hoc test, in which $p < 0.05$ and $p < 0.0001$ were considered significant while $p > 0.05$ was not significant. IC_{50} values were obtained by nonlinear regression with unknown interpolation of standard curves obtained from fungal growth values as a function of the extract concentration, expressed in µg/mL. Statistical analysis was performed using the Graphpad Prism software, version 6.0.

Measurement of the complete striae borders, as well as of the regions where hyphal growth was observed, was performed for virulence analysis. Then, measurements of all hyphal filaments identified in five randomly selected regions from each striae and concentration were performed. Hyphal filament length was averaged and analyzed by ANOVA followed by Bonferroni's correction for multiple comparisons according to the product concentration [50].

5. Conclusions

Preliminary phytochemical analysis of the extracts detected the presence of phenolic and flavonoid compounds in both extracts. The activity of the extracts against the tested strains was not clinically relevant since direct contact growth inhibition only began at high concentrations. In the fluconazole modulatory assay, the extracts presented synergistic effects with some concentrations. The natural product inhibited hyphal and pseudohyphal growth in the fungal morphological transition assay, depending on the tested concentrations.

Studies on the modulatory and morphological activity of *S. tuberosa* extracts are rare, with the present study being the first to report on these activities, as well as to report on the analysis of *S. tuberosa* extracts by high performance liquid chromatography. Thus, research addressing the mechanisms of action of these extracts and the bioactivity of their chemical constituents is necessary to better explore the pharmacological potential of the species.

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CAPÍTULO 3

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COMPARATIVE ANTIFUNGAL ACTIVITY OF THE *Spondias tuberosa* ARRUDA AQUEOUS LEAF AND ROOT EXTRACTS AND UPLC-MS-ESI-QTOF ANALYSIS

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ABSTRACT

Ethnopharmacological relevance: Spondias tuberosa Arruda (umbu) is an endemic Caatinga plant used in popular medicine to treat infections, inflammation, digestive disorders and female discharge, these being symptoms that may be associated with the effects of a *Candida* spp. infection. This study aimed to identify the chemical composition of the *S. tuberosa* aqueous leaf and root extracts (EALST and EARST) and to evaluate their effect, comparatively, against opportunistic pathogenic fungi.

Materials and methods: Ultra-Performance Liquid Chromatography Coupled to a Quadrupole/Time of Flight System (UPLC-MS-ESI-QTOF) was employed for chemical analysis. *Candida albicans* and *C. tropicalis* standard strains and clinical isolates were used (CA INCQS 40006, CT INCQS 40042, CA URM 5974 and CT URM 4262). The 50% Inhibitory Concentration for the fungal population (IC_{50}) was determined for both the intrinsic action of the extracts and the extract/fluconazole (FCZ) associations. The determination of the Minimum Fungicidal Concentration (MFC) and the verification of

effects over fungal morphological transitions were performed by subculture in Petri dishes and humid chambers, respectively, both based on microdilution.

Results: UPLC-MS-ESI-QTOF analysis revealed the presence of phenolic and flavonoid compounds. The intrinsic leaf extract IC_{50} ranged from 8305.3 µg/mL to 16090.9 µg/mL, while the intrinsic root extract IC_{50} varied from 1306.6 µg/mL to 7785.9 µg/mL. The association of the extracts with fluconazole, resulted in IC_{50} values from 2.62 µg/mL to 308.96 µg/mL. The MFC of the extracts was ≥ 16,384 µg/mL for all tested strains, while fluconazole obtained a MFC of 8192 µg/mL against *Candida albicans* strains. A reduction in MFC against CA URM 5974 (EALST: 2048 µg/mL and EARST: 1024 µg/mL) occurred in the extract/fluconazole association. A synergistic effect against the tested strains was observed with some concentrations in the extract/FCZ microdilution association, where in this test the leaf extract was more effective against the CA INCQS 40006 strain when compared to the root extract, however, the root extract was more effective against *C. tropicalis* strains. As for fungal morphological transitions, the leaf extract obtained better results than the root extract since it reduced fungal dimorphism of the tested strains more effectively, although the effect was dose dependent. However, the root extract at the highest concentration completely inhibited *C. tropicalis* strains. 17 compounds were identified in the chemical analysis, these mostly belonging to the flavonoid class. By combining the extracts with fluconazole, the MFC and IC_{50} values were reduced, thus increasing the efficacy of the drug.

Conclusions: In view of the results obtained, the EALST may be an alternative for the treatment of fungal infections, since it presented similar results to those of the EARST. Extracts affect fungal morphological transition, preventing the formation of pseudohyphae and hyphae, despite this being dose dependent, however, further studies are needed to identify the active compounds and prove their applicability, safety, mechanism of action and involved genes.

Keywords: Popular medicine, Umbu, Flavonoids, Fluconazole.

1. Introduction

Ethnobiology seeks to explain mankind's knowledge of the environment and the way in which surrounding natural resources are manipulated (da Silva, 2016). The use of biological diversity by populations is one of the largest sources of new bioactive

chemical molecule discoveries, among which medicinal plants stand out for their richness (Queiroz et al., 2015). It is noteworthy that although the collection and use of medicinal plants is a common practice in different world cultures, this activity can pose challenges for the conservation of the used resources (da Silva et al., 2019).

Thus, the local community uses medicinal resources is the first to realize the reduction of its availability, and for this reason, these individuals are the key holders of important knowledge which may be used to determine species with priority for conservation, as well as for the production of strategies that allow their sustainable exploration (Soldati and de Albuquerque, 2012).

The use of medicinal plants for the purpose of curing diseases has been passed down from generation to generation. This knowledge contributes significantly to the propagation of therapeutic plant virtues that are commonly prescribed, despite the lack of an evaluation of their chemical constituents (López, 2006). Thus, ethnobotanical, phytochemical and pharmacological studies significantly collaborate to increase the knowledge surrounding natural products. This is especially due to the search for new therapeutic resources for the treatment of various diseases and to reduce dissatisfaction with conventional medicines (Heinzmann and de Barros, 2007; López, 2006).

Spondias tuberosa Arruda is an endemic Caatinga tree, popularly known as umbu, which serves as medicine, food for pollinators and domestic ruminants, in addition to producing fruits that are used *in natura* and processed to produce sweets, compote, pulps and jellies. Despite its benefits, this plant faces many natural and anthropogenic threats that may lead to its extinction. Thus, scientific studies can add value and encourage its preservation (de Freitas Lins Neto et al., 2010; Mertens et al., 2017).

In popular medicine this species is used to treat infections, digestive disorders and inflammatory conditions (da Silva Siqueira et al., 2016). The leaves are used to treat diabetes, constipation, stomach and uterine pain (Jorge et al., 2007; Uchôa et al., 2015). The stem bark is used for migraines, cholesterol control and cicatrization (Júnior et al., 2012; Junior et al., 2014). The root-tubers (infusion) are used to treat female discharge, a symptom which may be associated with *Candida* spp. infections (Ribeiro et al., 2014).

The use of *S. tuberosa* roots by the human population may cause damages in the future due to anthropic pressure on the species. The roots are vital parts with important roles in plant survival since they accumulate water and energy reserves. Thus, their exploitation could cause a decrease in plant populations in their environments (Macêdo

et al., 2013). With this in mind, in order to add chemical and pharmacological information to the popular knowledge surrounding this species, the objective of this study was to comparatively identify and evaluate the chemical composition and antifungal action of the *Spondias tuberosa* (umbu) aqueous leaf and root extracts against *Candida albicans* and *Candida tropicalis* standard strains and clinical isolates.

2 Materials and methods

2.2 Botanical material collection and identification

The *Spondias tuberosa* Arruda botanical material was obtained under permission from the Biodiversity Authorization and Information System - SISBIO under number 64293-1, from the community of Lameiro (07º,15'03.1" South latitude and 39º, 23'48,3" longitude West of Greenwich), in the municipality of Crato, southern Ceará, Brazil. Leaves and roots were harvested from a total of seven individuals, between 8:00am and 9:30am during June 2018. An exsiccate was produced from the collected material and a specimen was deposited in the Herbarium Dárdano de Andrade Lima (HCDAL) from the Regional University of Cariri - URCA with the herbarium number 13.728, identified by Professor Ma. Ana Cleide Alcantara Morais Mendonça.

2.3 Extract acquisition

Young leaves and roots, which were cut into smaller fragments to increase their contact surface with the extractor (water), were used for the preparation of the aqueous extracts by infusion. The infusion was prepared by pouring boiling water on the leaves, at a rate of 133.2 g for every two liters of water, with the container being closed and left to rest for 15 minutes after cooling (Matos, 2002) before subsequent filtration.

The extracts were dried by spray drying with the Mini-spraydryer MSDi 1.0 (Labmaq do Brasil), using a 1.2 mm spray nozzle, under operating conditions: a) flow control: 500 mL/hr; b) inlet temperature: 130 ± 2 °C; c) outlet temperature: 74 ± 2 °C; d) atomization air flow: 45 L/min; e) blower flow rate: 1.95 m^3/min (Masters, 1991).

2.4 Chemical analysis

2.4.1 Preliminary phytochemical analysis

The extracts were submitted to preliminary phytochemical analysis based on qualitative methods proposed by Sousa et al. (2015). In these experiments, characterization of the main classes of special metabolites was performed through chemical reactions that resulted in the development of color and/or precipitates, after the addition of specific reagents, that are characteristic for the following classes of metabolites: alkaloids, steroids, phenols, flavonoids, tannins and triterpenoids. Solutions were prepared by dissolving 1.0 g of each crude extract with 100 mL of distilled water. Subsequently, 3 mL aliquots from these solutions were added to test tubes to characterize the groupings. All experiments were performed in triplicates and the metabolite classes were identified as present (+) or absent (-).

2.4.2 Compound identification by ultra-performance liquid chromatography coupled to quadrupole/time of flight system (UPLC-MS-ESI-QTOF)

The identification of compounds present in the extracts was performed in an Acquity® UPLC system coupled to a Quadrupole/Time of Flight Time (QTOF) system (Waters Corporation, Milford, USA), at the Chemical and Natural Products Laboratory, Embrapa Tropical Agroindustry (Fortaleza, Ceará, Brazil). Chromatographic runs were performed on a Waters Acquity® UPLC BEH column (150 x 2.1 mm; 1.7 µm), with a fixed temperature of 40 °C, mobile phases with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), gradient ranging from 2% to 95% B (15 min), at a flow rate of 0.4 mL/min and injection volume of 5 µl. The ESI^+ mode was acquired in the 110-1180 Da range, 120 °C fixed source temperature, 350 °C desolvation temperature, 500 L/h desolvation gas flow and 3.2 kV capillary voltage. The ESI^- mode was obtained in the 110-1180 Da range, 120 °C fixed source temperature, 350 °C desolvation temperature, 500 L/hr desolvation gas flow, 0.5 V extraction cone and 2.6 kV capillary voltage. Leucine encephalin was used as lockmass. The acquisition mode used was MSE (high energy mass spectrometry). The instrument was controlled by Masslynx® 4.1 software (Waters Corporation, Milford, USA).

2.5 Antifungal assays

2.5.1 Microorganisms

For the antifungal activity evaluation, two *Candida albicans* - CA INCQS 40006 and *Candida tropicalis* - CT INCQS 40042 standard fungal strains were obtained from the Oswaldo Cruz Culture Collection of the National Institute for Quality Control in Health (INCQS), while the two *Candida albicans* - CA URM 5974 and *Candida tropicalis* - CT URM 4262 clinical isolates were provided by the Recife Micoteca University (URM) of the Federal University of Pernambuco - UFPE.

2.5.2 Culture media

The Sabouraud Dextrose Agar (SDA) solid medium, prepared according to the manufacturer's instructions, and the double concentrated Sabouraud Dextrose Broth (SDB) liquid medium were used for the antifungal activity evaluations. The Potato Dextrose Agar (PDA) medium, purchased from Difco®, depleted by dilution and supplemented with agar was used for the fungal morphology analysis. The media were solubilized with distilled water and autoclaved at 121°C for 15 minutes.

2.5.3 Inoculum preparation

All strains were replicated and initially kept in test tubes containing inclined SDA, refrigerated at 8 ºC, at the Cariri - LMAC Applied Mycology Laboratory of the Regional University of Cariri - URCA. For the fungal inoculum preparation, these were first cultured in SDA medium poured into Petri dishes at 37 °C for 24 hours (overnight). From these, microorganism suspensions were prepared in tubes containing 4 mL of a sterile solution (0.9% NaCl). These suspensions were then shaken with the aid of a vortex apparatus and their turbidity was compared and adjusted to that shown by the McFarland scale 0.5 barium sulfate suspension, which corresponds to an inoculum of approximately 10^5 colony-forming units/mL – CFU/mL (Souza et al., 2007). The inocula were used for intrinsic and drug-combined antifungal activity tests performed by broth microdilution and Minimum Fungicide Concentration (MFC).

2.5.4 Used drugs and reagents

Dimethyl sulfoxide (DMSO - Vetec) was used for extract dilution, while the antifungal fluconazole 150 mg (prati - donaduzzi) was diluted in water and used as a reference drug for the tests, since it is the most commonly used antifungal in the Brazilian healthcare system. To prepare the initial extract solution, 0.15 g were weighed and solubilized in 1 mL of DMSO. Subsequently, the extracts and fluconazole were further diluted in sterile distilled water to obtain the desired initial concentration for the tests (16384 µg/mL).

2.5.5 IC_{50} determination and cell viability curve

This experiment was performed using the broth microdilution technique with 96 well plates. Each well was filled with 100 µL of SDB containing 10% fungal inoculum, followed by 100 µL of the natural product or fluconazole, at the same concentrations, followed by serial microdilution up to the penultimate well, starting from a concentration of 8192 to 8 µg/mL. The last well was reserved for the growth control (Javadpour et al., 1996 with changes in concentration). Dilution controls for the natural product and fluconazole (with 0.9% sodium chloride solution replacing the fungal inoculum), as well as media sterility controls, were also performed. All tests were performed in quadruplicate. The plates were incubated at 37 °C for 24 h and then read on an ELISA spectrophotometer (Kasuaki - DR - 200Bs - BI) at a wavelength of 450 nm. The results obtained from the ELISA readings were used to construct the cell viability curve and determine the IC_{50} of the extracts and fluconazole (Morais-Braga et al., 2016).

2.5.6 Determination of Minimum Fungicidal Concentration (MFC)

A sterile rod was added into each well of the plate used in the microdilution test following 24 h of incubation and the ELISA reading (except for the sterility control) to perform this test. After homogenization of the solution contained in the well, subcultures were performed in a Petri dish containing SDA and a guide card by transferring a small aliquot from the test solution (medium + inoculum + natural product). The plates were incubated at 37 °C for 24 hours and checked for growth or

non-growth of *Candida* colonies (Ernst et al., 1999 with modifications). The MFC was defined as the lowest concentration capable of inhibiting fungal colony growth.

2.5.7 Evaluation of the modifying effect over fluconazole action

The effect of combining the extracts with the reference drug (fluconazole) was performed to verify the potentiating action of the antifungal by the addition of the extracts. The solutions containing the extracts were tested at sub-inhibitory concentrations (MFC/16), according to the methodology used by Coutinho et al. (2008), with changes in concentrations. When natural extracts potentiate the action of the antifungal, this is considered a synergistic effect, when it impairs the activity of the drug, this is considered an antagonistic effect. The plates were filled with 100 µL of the medium + inoculum + extract, being then microdiluted with 100 µL of fluconazole, where this was mixed into the first well by serial microdilution at a 1:1 ratio up to the penultimate well. Medium sterility controls were also performed. All tests were performed in quadruplicates. The plates were incubated at 37 °C for 24 hours. The readings were performed in an ELISA spectrophotometry device (Kasuaki - DR - 200Bs - BI) (Morais-Braga et al., 2016).

2.5.8 Effect of the extracts and fluconazole on the micromorphology of *Candida* spp.

Humid chambers were prepared with sterile microscopy slides for yeast observation to analyze changes in fungal morphology by the extract and fluconazole via the inhibition or reduction of hyphae emission. Three mL of depleted PDA medium with HCE - 8192 µg/mL, HCE/4 - 2048 µg/mL and HCE/16 - 512 µg/mL (Highest Concentration Evaluated) natural product and fluconazole concentrations were added to the chambers. Aliquots from the subcultures were taken to make two parallel striations in the solid medium (PDA), which were subsequently covered with a sterile cover slip. The chambers were incubated for 24 hours (37 °C), being inspected and recorded by an optical microscope (AXIO IMAGER M2-3525001980- ZEISS- Germany) using a 20X objective. A control for yeast growth (nutrient restriction-stimulated hyphae and pseudohyphae) was performed, as well as a control with the conventional antifungal fluconazole for comparative purposes. The tests were performed according to Sidrim and Rocha, (2010) and Mendes, (2011), with modifications. The Zen 2.0 software was used to measure hyphae and pseudohyphae extensions, where images were taken from

each complete striae and later five random images from these were used for statistical analysis (Pereira Carneiro et al., 2019).

2.6 Statistical analysis

A two-way ANOVA was applied to each sample, comparing the values for each extract concentration, with Bonferroni's posthoc test, in which $p<0.05$ and $p<0.0001$ are considered significant and $p>0.05$ is not significant. IC_{50} values were obtained by nonlinear regression with unknown interpolation of standard curves obtained from fungal growth values as a function of the extract concentration, expressed in μg/mL. The Graphpad Prism software, version 6.0, was used for statistical analysis.

The measurement of the entire striae edge and the regions where hyphae growth occurred were performed for virulence analysis. Subsequently, all identified hyphal filaments were measured from five randomly selected regions of each striae and concentration. Hyphal filament length was averaged and analyzed by an ANOVA followed by Bonferroni's correction for multiple comparisons according to the product concentration (Pereira Carneiro et al., 2019).

3 Results

The *S. tuberosa* Aqueous Leaf Extract (EALST) had a yield of 1.59% and the *S. tuberosa* Aqueous Root Extract (EARST) had a yield of 0.78%. Preliminary phytochemical analysis of the extracts revealed the presence of metabolite classes, such as: alkaloids, phenols, flavonoids (flavanones, anthocyanins, anthocyanidins, leucoanthocyanidins, catechins) and triterpenoids (Table 1). The presence of phenols, flavonoids, triterpenoids and alkaloids prevailed in both extracts, with leucoanthocyanidins and catechins being detected in the leaf extract. Among the secondary metabolites investigated, steroids, flavones, flavonols, flavonoids, flavonones, tannins and xanthones were not detected.

Table 1 – Preliminary phytochemical analysis of aqueous extracts of leaves and roots of *Spondias tuberosa*

	Special Metabolite Classes (SMC)									
	SMC 1	**SMC 2**	**SMC 3**	**SMC 4**	**SMC 5**	**SMC 6**	**SMC 7**	**SMC 8**	**SMC 9**	**SMC 10**
EALST	+	-	-	+	+	+	-	-	+	+
EARST	+	-	-	+	-	+	-	-	+	+

SMC1: Phenols; SMC2: Tannins; SMC3: Flavonoids of the flavone, flavonol and xanthone type; SMC4: Flavonoids of the anthocyanin and anthocyanidin type; SMC5: Flavanoid of leucoanthocyanidins, catechins; SMC6: Flavanone type flavanoid; SMC7: Flavanoid type flavanoid; SMC8: Steroids; SMC9: Triterpenoids; SMC10: Alkaloids; (+): positive; (-): missing

The results from the chromatographic analyzes are shown in Tables 2 and 3, where compound identification was performed based on their molecular ion mass, retention time, fragmentation pattern and available data in the literature, shown in order of elution order, molecular formula, error and major fragments (MS^2).

The inhibition of 50% of the microorganismal population (IC_{50}) for the EALST occurred at high concentrations for all tested strains, while the EARST obtained an IC_{50} of 1306.6 µg/mL against CA URM 5974 and higher values for the other strains, as shown in Table 4. Fluconazole (FCZ) presented an effect at concentrations ranging from 3.97 µg/mL to 88.08 µg/mL. In the extract/FCZ combination, a synergism was observed for the EALST + FCZ combination against both *C. albicans* strains (11.80 and 2.62 µg/mL) and an antagonism was observed against *C. tropicalis* (177.41 and 308.96 µg/mL). For the EARST + FCZ combination an antagonism was observed against CA URM 5974 (5.46 µg/mL), while a synergism was observed against the other strains (ranging from 12.68 to 35.62 µg/mL). Despite the high concentrations, the results show the root extract was more effective than the leaf extract, results which corroborate with their use by the population.

Table 4 - 50% Inhibitory Concentrations (IC50) of microorganisms (µg/mL) by aqueous extracts of *Spondias tuberosa*

	CA INCQS 40006	**CA URM 5974**	**CT INCQS 40042**	**CT URM 4262**
EALST	8305.3	11090.9	16288.03	13335.7
EARST	5344.8	1306.6	6678.9	7785.9
FLUCONAZOLE	22.74	3.97	88.08	47.30
EALST + FCZ	11.80	2.62	177.41	308.96
EARST + FCZ	12.68	5.46	30.42	35.62

CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: National Institute of Quality Control in Health; URM: University Recife Mycology; EALST: Aqueous Extract of *Spondias tuberosa* Leaves; EARST: Aqueous Extract of *Spondias tuberosa* Root; FCZ: Fluconazole

In terms of an inhibitory action, as visualized by the cell viability curve, the extracts were not as effective as fluconazole (Figure 2). The root extract, despite high concentrations, presented a greater effect than the leaf extract against all tested strains. The antifungal fluconazole obtained a more effective result with the *C. albicans* isolate.

The MFC values for the extracts were ≥ 16384 µg/mL against all tested strains with a fungistatic effect being observed (Table 5). Fluconazole was fungicidal at a concentration of 8192 µg/mL against *C. albicans* strains and ≥ 16384 µg/mL against *C. tropicalis* strains. In the EALST and EARST combinations with the drug, synergistic effects were observed against CA URM 5974 at concentrations of 2048 µg/mL and 1024 µg/mL, respectively, as well as CT URM 4262. With the EARST/FCZ combination, a fluconazole MFC antagonism was observed. With the remaining strains, the concentrations ranged from 8,192 µg/mL to ≥ 16,384 µg/mL.

Table 5 – Minimum Fungicidal Concentration (MFC) of microorganisms (µg/mL) by extracts, fluconazole and their combination

	CA INCQS 40006	**CA URM 5974**	**CT INCQS 40042**	**CT URM 4262**
EALST	≥ 16.384	≥ 16.384	≥ 16.384	≥ 16.384
EARST	≥ 16.384	≥ 16.384	≥ 16.384	≥ 16.384
FLUCONAZOLE	8.192	8.192	≥ 16.384	≥ 16.384
EALST + FCZ	8.192	2.048	≥ 16.384	8.192
EARST + FCZ	≥ 16.384	1.024	≥ 16.384	8.192

CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: National Institute of Quality Control in Health; URM: University Recife Mycology; EALST: Aqueous Extract of *Spondias tuberosa* Leaves; EARST: Aqueous Extract of *Spondias tuberosa* Root; FCZ: Fluconazole

In the extract/drug microdilution combinations, the EALST combination with fluconazole presented a synergistic effect against CA INCQS 40006 (16 µg/mL to 64 µg/mL), CA URM 5974 (8 µg/mL to 16 µg/mL), CT INCQS 40042 (16 µg/mL to 512 µg/mL) and CT URM 4262 (8 µg/mL at 512 µg/mL), with other concentrations obtaining results similar to fluconazole (Figure 3).

The EARST when combined with fluconazole presented synergism against CA INCQS 40006 (16 µg/mL to 32 µg/mL), CA URM 5974 (8 µg/mL to 16 µg/mL), CT INCQS 40042 (8 µg/mL to 256 µg/mL) and CT URM 4262 (8 µg/mL to 512 µg/mL),

obtaining and a similar curve to that of the used drug for the remainder. In this essay, the leaf extract was more effective against the CA INCQS 40006 strain when compared to the root extract. However, the root extract was more effective than the leaf extract against *C. tropicalis* strains.

As for the effect on yeast morphological transition, fluconazole inhibited the tested strains at all concentrations (Figure 4). The EALST inhibited all strains at the HCE concentration, with a 36.01% reduction in CA INCQS 40006 filament emission, 41.17% in CA URM 5974 and 22.35% in CT URM 4262 at the HCE/4 concentration, all compared to the growth control. The results were not significant at the lowest concentration.

The EARST completely inhibited morphological transition of the *C. tropicalis* standard and clinical isolate strains at the highest concentration (HCE), causing a 63.32% reduction in filamentation in CA INCQS 40006 and 63.36% in CA URM 5974. At the HCE/4 concentration, this reduction was of 18.18% for CA URM 5974 and 25.11% for CT URM 4262, with the results being insignificant for the remaining strains. In the fungal morphology analysis, the leaf extract obtained greater effects than the root extract against the tested strains.

Figure 5.A shows that in the CA INCQS 40006 growth control, filament formation with extensive hyphae is evident. Figure 5.B shows the effect of fluconazole at the lowest concentration (HCE/16), where a total filament reduction can be observed. The progressive effect of the EALST on *C. albicans* pleomorphism inhibition can be seen in Figures 5.C to 5.E.

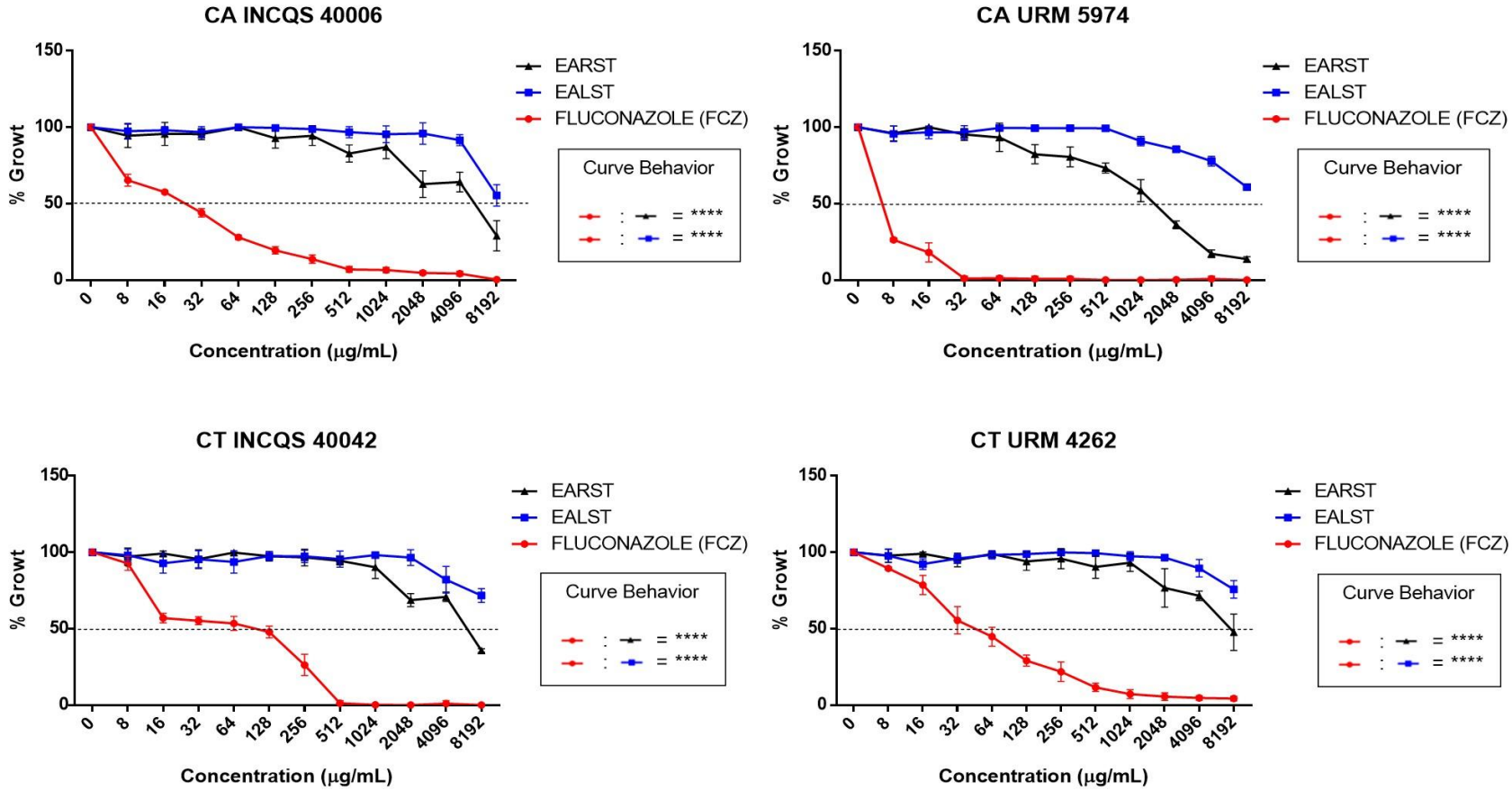
Table 2 - UPLC-MS-ESI-QTOF Water Extract Compound Identification by *Spondias tuberora* (Positive Mode)

Peak no.	Rt min	$[M-H]^-$ Observed	$[M-H]^-$ Calculated	Product Ions (MS/MS)	Empirical Formula	Ppm (error)	Putative Name	Referências
1	2.52	214.9169	214.9151	344.8752, 214.9161, 198.9392	$C_4H_7S_5$	8.4	No identified	-
2	2.67	443.8156	443.1888	381.1805, 281.1358, 119.0361	$C_{21}H_{32}O_{10}$	2.5	Dehydrophaseic acid hexose	Cunha et al. (2017)
3	2.74	477.0002	447.0927	301.0336, 300.0258, 255.0332	$C_{21}H_{20}O_{11}$	0.4	Quercetin 3- O rhamnoside	Cunha et al. (2017)
4	4.19	166.0861	166.0868	166.0867, 163.0409, 120.0829	$C_9H_{12}NO_2$	-4.2	No identified	-
5	4.35	461.1872	461.1870	285.04, 257.04, 151.00	$C_{21}H_{18}O_{12}$	0.4	Kaempferol glucuronide	Sayed et al.(2010)
6	4.49	447.1198	447.1197	284.03	$C_{21}H_{20}O_{11}$	0.2	Kaempferol hexoside	Sayed et al. (2010)
7	4.52	475.1795	475.1816	284.03	$C_{22}H_{20}O_{12}$	-4.4	Kaempferol-6″-methyl-	Sayed et al. (2010)
8	4.55	285.1272	285.1279	-	$C_{15}H_{10}O_6$	-2.5	glucuronide	Sayed et al. (2010)
9	4.80	679.2955	679.2966	679.2952, 303.0507, 127.0370	$C_{34}H_{47}O_{14}$	-1.6	Kaempferol	-
10	5.49	335.2591	335.2586	183.0415	$C_{15}H_{12}O_9$	1.5	No identified Methyl digallate	da Rocha et al. (2019)

Table 3 - UPLC-MS-ESI-QTOF Aqueous Extract Compound from Spondias tuberora Roots (Negative Mode)

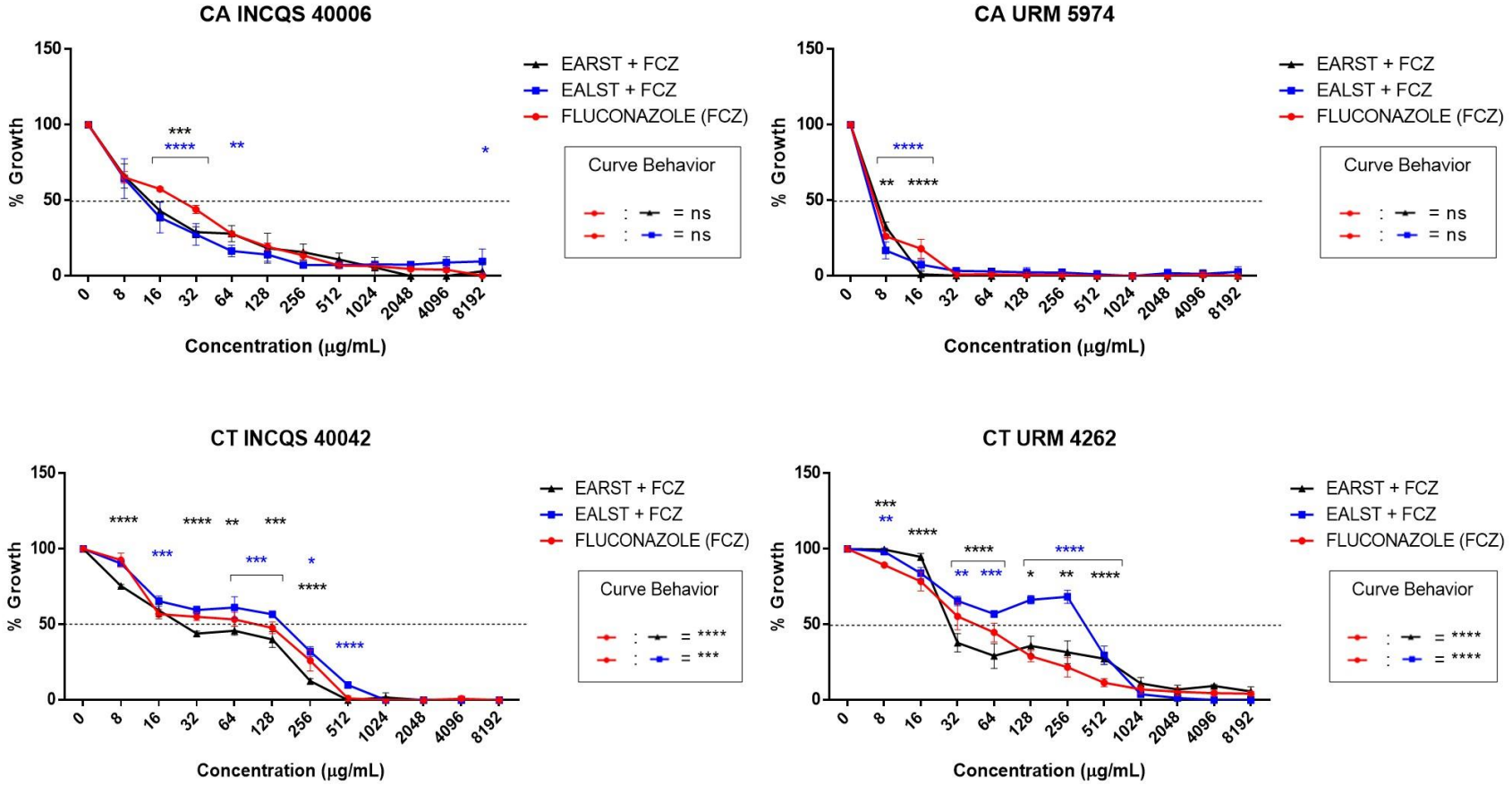
Peak no.	Rt min	$[M-H]^-$ Observed	$[M-H]^-$ Calculated	Product Ions (MS/MS)	Empirical Formula	Ppm (error)	Putative Name	Referências
1	2.89	172.9540	172.9519	274.9536, 273.9563, 158.9753	$C_{15}H_{12}O_5$	7.7	(±)-Naringenin	Lasano et al. (2019)
2	3.16	377.0822	377.0812	237.1489, 189.1246, 179.0478	C25H13O	2.1	No identified	-
3	3.20	341.1040	341.1025	297.2246, 295.2048, 119.0465	4	4.4	Anacardic acid 1	Cunha et al. (2017)
4	3.51	343.1224	343.1240	299.2344	$C_{22}H_{30}O_3$	-4.7	Anacardic acid 2	Cunha et al. (2017)
5	4.55	461.1209	461.1236	257.0848, 229.8624, 151.0033	$C_{22}H_{21}O_3$	-5.9	Kaempferol-7-Oglucuronide	Cunha et al. (2017)
6	4.66	197.0406	197.0391	199.06, 198.05, 182.0187	$C_{22}H_{21}O_{11}$	7.6	2-Hydroxy-3,4-	Lazano et al. (2019)
7	4.89	433.0420	433.0427	300.9944, 271.0716	$C_9H_{10}O_5$	3.0	dimethoxybenzoic acid	Da Rocha et al.
8	5.07	315.0095	315.0082	394.9633, 315.0095, 299.9882	$C_{20}H_{18}O_{11}$	4.1	Quercetin O-pentoside	(2019)
9	5.37	345.2204	345.2218	301.2471	$C_{16}H_{12}O_7$	-4.1	Rhamnetin	Cádiz-Gurrea et
10	5.55	331.2397	331.2426	271.0428, 241.0313, 125.0252	$C_{22}H_{34}O_3$	-8.8	Anacardic acid 3	al.(2019)
11	5.92	339.2084	339.2113	-	$C_9H_{16}O_{13}$	-8.5	Monogalloyl-glucose	Cunha et al. (2017)
12	7.00	361.1890	361.1862	317.2411, 255.2323, 133.8362	$C_{22}H_{28}O_3$	7.8	Caffeoyl-D-glucose	Cunha et al. (2017)
13	7.59	537.1879	537.1855	479.2250, 375.048, 211.107	$C_{18}H_{34}O_7$	4.5	Giberellin GA19	Cunha et al. (2017)
					$C_{30}H_{17}O_{10}$		Agathisflavone	Cunha et al. (2017)
								Galvão et al. (2018)

Figure 2 - Cell viability curve of fluconazole and aqueous extracts of *Spondias tuberosa* against *Candida* strains



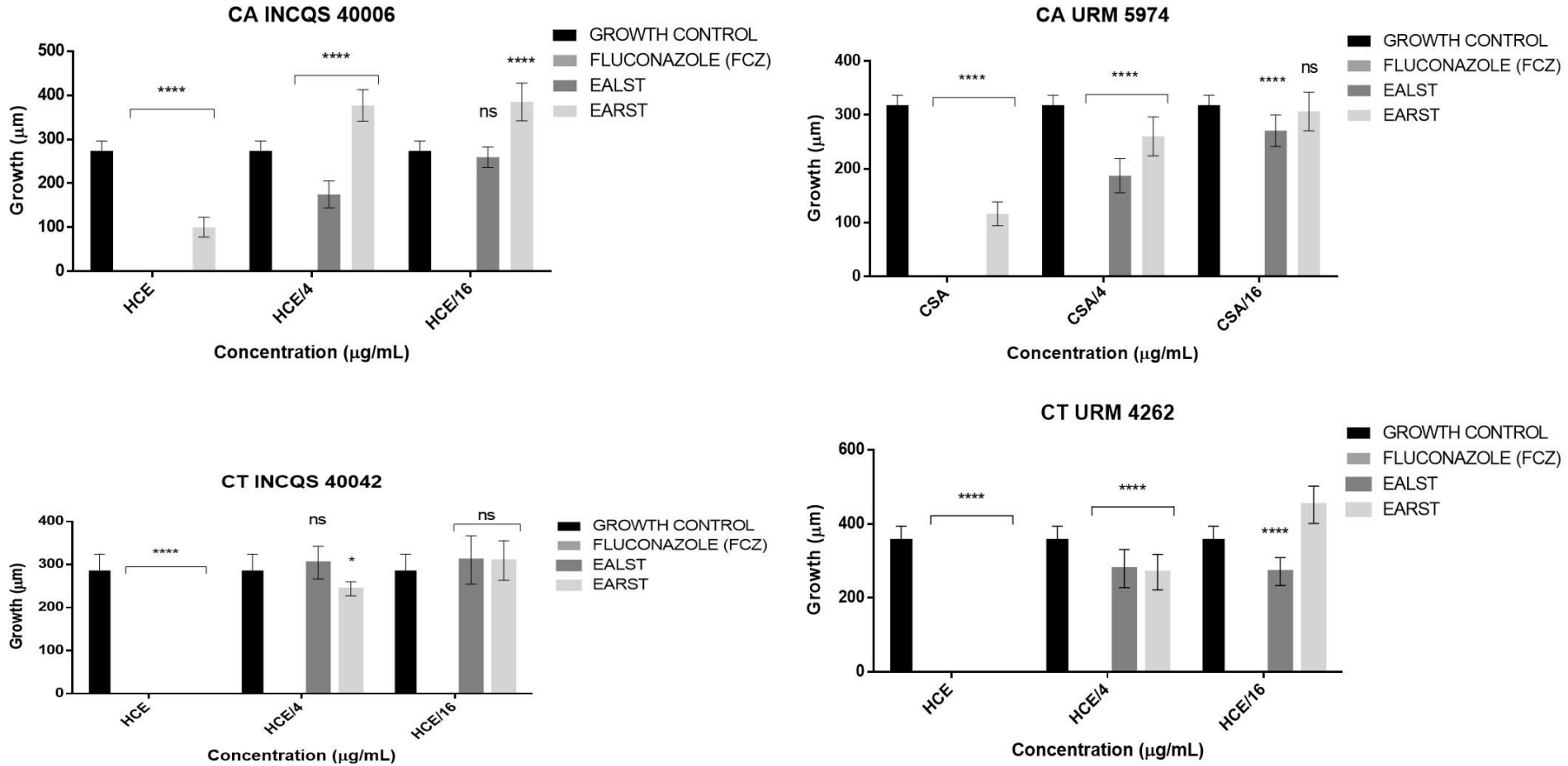
CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: National Institute of Quality Control in Health; URM: University Recife Mycology; EALST: Extract Aqueous of *Spondias tuberosa* Leaves; EARST: Extract Aqueous of *Spondias tuberosa* Root; FCZ: Fluconazole

Figure 3 - Evaluation of the modifying effect of fluconazole action by extracts



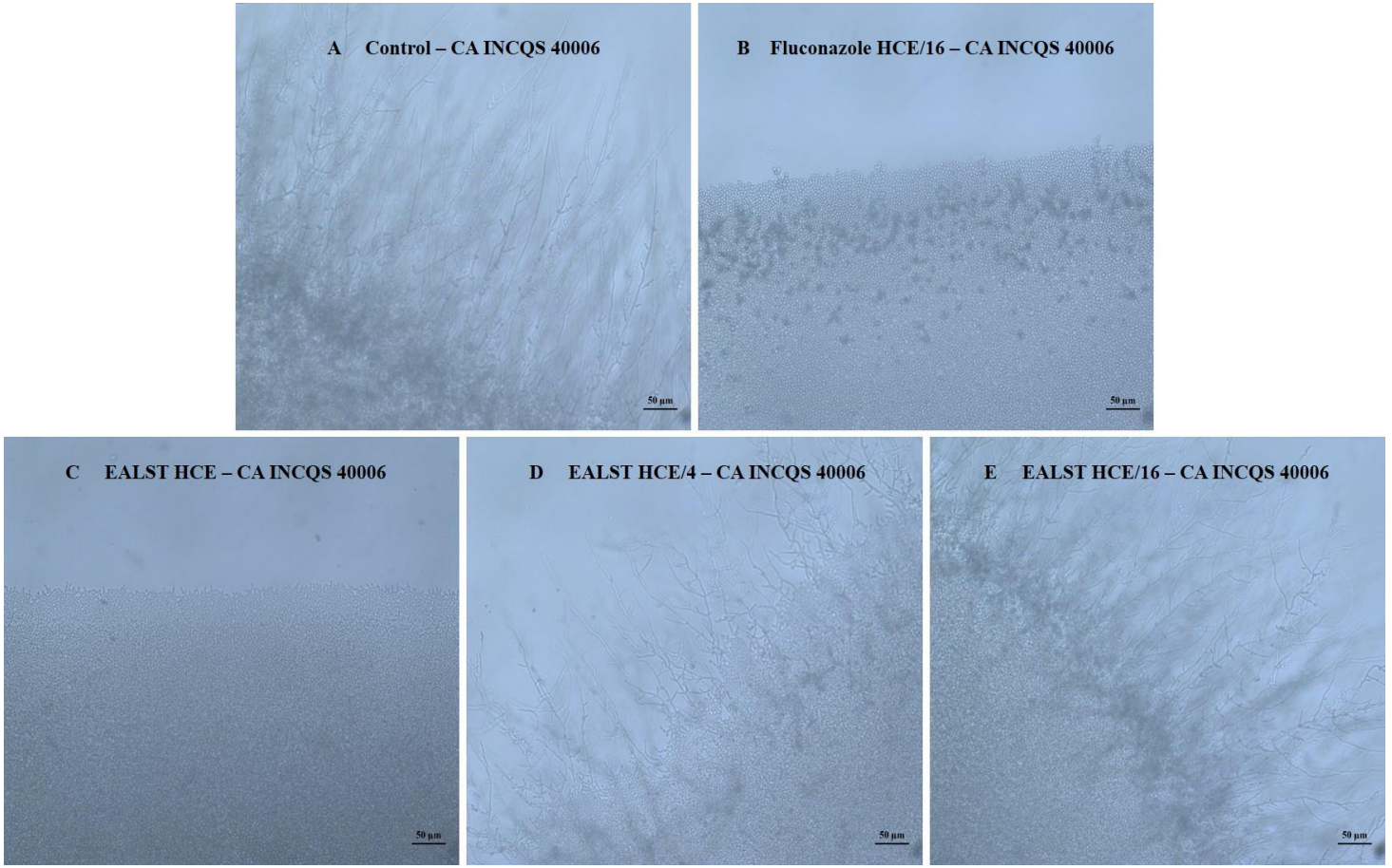
CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: National Institute of Quality Control in Health; URM: University Recife Mycology; EALST: Extract Aqueous of *Spondias tuberosa* Leaves; EARST: Extract Aqueous of *Spondias tuberosa* Root; FCZ: Fluconazole; NS: não significativo

Figure 4 – Effect of extracts and fluconazole on the morphological transition of *Candida* spp.



CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: National Institute of Quality Control in Health; URM: University Recife Mycology; EALST: Extract Aqueous of *Spondias tuberosa* Leaves; EARST: Extract Aqueous of *Spondias tuberosa* Root; FCZ: Fluconazole; HCE: Higher Concentration Evaluated; ns: not significant.

Figure 5 - Demonstrative effect of EASTL and fluconazole on the morphological transition of *Candida albicans*



CT: *Candida tropicalis*; INCQS: National Institute of Quality Control in Health; URM: University Recife Mycology; EALST: Extract Aqueous of *Spondias tuberosa* Leaves; FCZ: Fluconazole; HCE: Higher Concentration Evaluate

4 Discussion

Qualitative phytochemical studies previously performed with the crude ethanolic leaf and bark extracts from *Spondias* sp. and *S. tuberosa* revealed the presence of flavonoids, triterpenes and alkaloids, with these classes being found in both extracts in the present study. In addition, steroid, tannins, terpenoids, saponins, coumarins, monoterpenes, sesquiterpenes and diterpene compounds were also detected (Barbosa et al., 2016; de Lima et al., 2017). da Silva Siqueira et al. (2016), also revealed the presence of phenolic compounds and flavonoids in the *S. tuberosa* leaf hydroethanolic extract using the thin layer chromatography technique.

In the study by Santos et al. (2019), the authors analyzed the chemical composition of the *S. tuberosa* leaf and root hydroalcoholic extracts by UPLC-MS-ESI-QTOF, and reported the presence of dehydrophasic hexose acid, caffeine-D-glucose, (±)-naringenin, anacardic acid, kaempferol-7-Oglucuronide, 2-hydroxy-3,4-dimethoxybenzoic acid, quercetin O-pentoside, ramnetin and monogaloyl glucose.

The aqueous *Spondias mangifera* Willd. root extract was investigated for its phytochemistry and revealed the presence of flavonoids, saponins, tannins and phenolic compounds (Acharyya et al., 2010). Saha, (2019) identified the presence of alkaloids and flavonoids, similar to those found in the present study, in addition to detecting tannins, steroids and saponins in the crude *S. mombin* leaf and fruit extracts.

Santhirasegaram et al. (2015), identified the compounds kaempferol and monogalloyl-glucose, which were also found in the present analysis, in the LC-MS/MS analysis of the *Mangifera indica* L. juice, a species belonging to the same family as *S. tuberosa*. In a study using the flavonoid kaempferol, a compound identified in the leaf extract, its minimum inhibitory concentration (MIC) was determined using the broth microdilution technique against different species from the *Candida* genus. The MIC against *C. parapsilosis* ranged from 32-128 $\mu g/ml^{-1}$, from 32-64 $\mu g/ml^{-1}$ for *C. metapsilosis*, and 64 $\mu g/ml^{-1}$ for *C. orthopsilosis* and *C. krusei*, respectively (dos Santos da Rocha et al., 2019). Shao et al. (2016), obtained a MIC_{90} ranging from 256 µg/mL to 512 µg/mL in a susceptibility test against different *C. albicans* strains using kaempferol, where fluconazole ranged from 0.5 µg/mL to 2048 µg/mL.

In popular medicine, *S. tuberosa* leaves are used for uterine pain (Uchôa et al., 2015) and the root-tubers (infusion) are used to treat female discharge, symptoms that may be associated with *Candida* spp. infections (Ribeiro et al., 2014). Based on these

ethnobotany studies, it is assumed that the *S. tuberosa* therapeutic form against fungi is its use in sitz baths and tea administration, where the ingestion of the natural product may reduce an infection caused by the microorganism.

Santos et al. (2019), observed similar results to those of the aqueous *S. tuberosa* extracts when evaluating the *S. tuberosa* hydroalcoholic leaf and root extracts using the same techniques and strains in the present study, the results of which corroborate with the present results showing the *S. tuberosa* extract presented no fungicidal effect against *Candida* strains at different extractions.

De Brito Costa et al. (2013), carried out a study with the *S. tuberosa* bark ethanolic extract against different species from the *Candida* genus using the disk diffusion technique and found the extract was unable to inhibit the growth of any tested species.

Species from the same genus as *S. tuberosa* have been investigated for their antifungal activity. The aqueous fraction from the *Spondias pinnata* (L. f.) Kurz. fruit exocarp was ineffective against *C. albicans* and *Saccharomyces cerevisiae* yeasts using the disk diffusion method (Manik et al., 2013). Islam et al. (2013), obtained an inhibition of 10 mm and 4 mm, respectively, against *C. albicans* with the chloroform and dichloromethane extracts from *Spondias dulcis* leaves using the same technique. A study showed a minimum inhibitory concentration (MIC) of 0.0156 mg, 0.2500 mg and 0.2500 mg, respectively, against *C. albicans* using the *Spondias mombin* L. leaf, bark and root aqueous extracts (Umeh et al., 2009).

Some of the mechanisms attributed to fungal pathogenicity are associated with dimorphism, which is the ability of yeast to grow hyphae or pseudohyphae. Other characteristics, in addition to this, also contribute to intensify the colonization and infectious ability of fungal species (Alves et al., 2013; Jacobsen et al., 2012; Ribeiro, 2008).

In a study by Arif et al. (2009), flavonoids present in extracts were capable of forming complexes with soluble proteins present in the fungal cell walls, as well as being capable of rupturing the fungal membrane due to their lipophilic nature (Salas et al., 2011), these also being able to inhibit the budding process and decrease Ca^{+} and H^{+} homeostasis (Ansari et al., 2013). Thus, the fungal morphology action observed in the present study was probably due to the presence of these compounds in the extracts. The difference in results obtained for the different *Candida* species tested may be associated

with variations in pathogenicity and virulence between species (Inci et al., 2012; Muthamil and Pandian, 2016).

Candiracci et al. (2012) found that flavonoids may be responsible for the reduction in hyphal growth, with the compound kaempferol, which was also detected in the leaves of the present study, being identified in its honey flavonoid extract.

Yeast virulence inhibition by *S. tuberosa* extracts may be associated with its metabolites acting synergistically. However, further studies are required to ascertain this, since other compounds present in the extracts, in addition to flavonoids, may also be involved in the process.

5 Conlusions

The *S. tuberosa* aqueous leaf and root extracts presented in their composition mainly phenolic and flavonoid compounds, where these may have contributed to the isolated and synergistic activity observed in the tests. In this study, the leaves and roots had their bioactive potential scientifically evaluated by tests, where the extracts together with fluconazole were able to reduce its concentration, increasing the effectiveness of the drug. The EALST was shown to be more effective than the EARST in fungal morphology inhibition, an important mechanism of *Candida* virulence, which may be important from a plant conservation point of view since the leaves may be a less harmful usage option and may lower the impact caused by using the species' roots. However, further in-depth studies are needed to identify the active compounds and prove their applicability, safety, mechanism of action and genes involved.

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CAPÍTULO 4: CONSIDERAÇÕES FINAIS

4.1 PRINCIPAIS CONCLUSÕES

Na análise química preliminar e na identificação por cromatografia líquida de ultra performance (UPLC-QTOF), os extratos apresentaram uma semelhança nas suas composições, onde na maioria dos compostos identificados são das classes dos flavonoides e compostos fenólicos. Sendo este trabalho o primeiro a relatar a identificação dos extratos de *Spondias tuberosa* por UPLC-QTOF.

Em relação aos ensaios microbiológicos todos os extratos tiveram efeito fungistático frente a cepas testadas. Na IC_{50} o EARST e EHFST obtiveram resultados melhores que EAFST e EHRST apesar dos valores elevados. Na combinação com o fluconazol os extratos foram capaz de potencializar a ação da droga em algumas concentrações. No dimorfismo fúngicos os extratos foram eficaz com as linhagens testadas apesar do efeito progressivo, onde quem se destacou foi o EAFST e EHFST. Dessa maneira, os extratos das folhas são uma alternativa de uso popular, pois obtiveram resultados melhores ou semelhantes aos extratos das raízes.

Contudo pesquisas futuras com outras técnicas que possibilitem a identificação de novos constituintes e o isolamentos dos compostos são necessários para um melhor entendimento e quantitativo de compostos presentes destes extratos, bem como sua aplicabilidade e mecanismos de ação.

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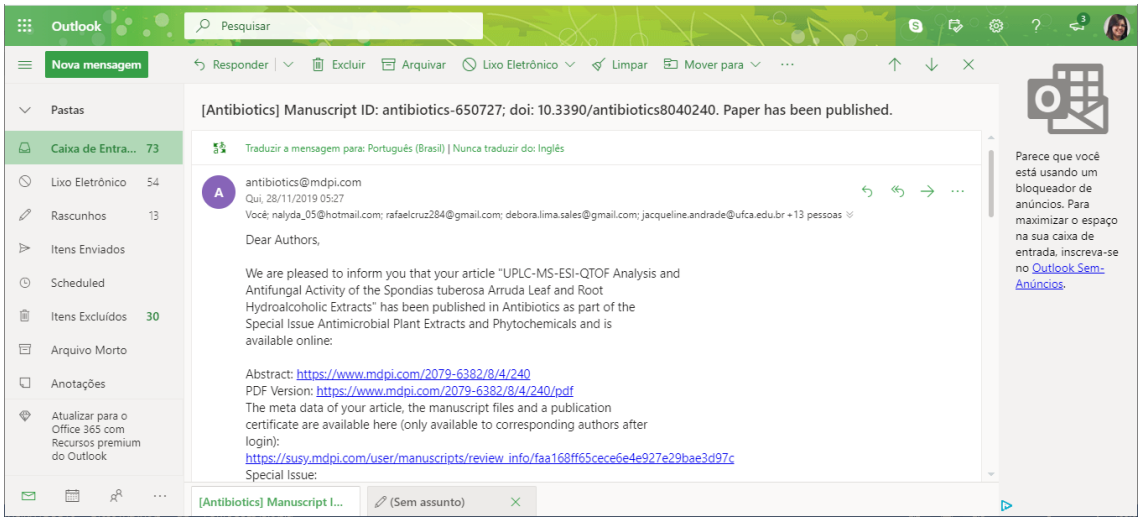
Anexos

ANEXO 1: Comprovante da publicação do artigo na revista antibiotics



ANEXO 2: Comprovante da submissão do artigo na revista Journal os etnopharmacology

Fwd: Submission JEP_2020_207 received by Journal of Ethnopharmacology

Ref: JEP_2020_207
Title: COMPARATIVE ANTIFUNGAL ACTIVITY OF THE Spondias tuberosa (ARRUDA) AQUEOUS LEAF AND ROOT EXTRACTS AND UPLC-MS-ESI-QTOF ANALYSIS
Journal: Journal of Ethnopharmacology

Dear Dr. Coutinho,

Thank you for submitting your manuscript for consideration for publication in Journal of Ethnopharmacology. Your submission was received in good order.

What happens next: Your paper will undergo a screening process by the managing editors of the journal. During this stage the manuscript is rigorously checked for alignment with aims and scope of the journal, plagiarism, checking the completeness of the general submission, adherence to authors guidelines etc. Due to the high influx of papers, please allow 2-3 weeks for this process to be completed. Once the pre-screen is completed the paper will either be returned to you or be assigned to an Associate Editor.

To track the status of your manuscript, please log into EVISE® at:
http://www.evise.com/evise/faces/pages/navigation/NavController.jspx?JRNL_ACR=JEP and locate your submission under the header 'My Submissions with Journal' on your 'My Author Tasks' view.

Thank you for submitting your work to this journal.

Kind regards,

ANEXO 3: Parecer com autorização da pesquisa pelo Sistema de Autorização e Informação em Biodiversidade (SISBIO)

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 64293-1	Data da Emissão: 11/07/2018 15:03	Data para Revalidação*: 10/08/2019

* De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: Antonia Thassya Lucas dos Santos	CPF: 033.936.893-47
Título do Projeto: Efeito antifúngico de Spondias tuberosa Arr. Cam. (umbu) frente a leveduras do gênero Candida	
Nome da Instituição : Universidade Regional do Cariri	CNPJ: 06.740.864/0001-26

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Coleta de material botânico	08/2018	09/2018

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
2	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
3	Este documento somente poderá ser utilizado para os fins previstos na Instrução Normativa ICMBio n° 03/2014 ou na Instrução Normativa ICMBio n° 10/2010, no que especifica esta Autorização, não podendo ser utilizado para fins comerciais, industriais ou esportivos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
4	A autorização para envio ao exterior de material biológico não consignado deverá ser requerida por meio do endereço eletrônico www.ibama.gov.br (Serviços on-line - Licença para importação ou exportação de flora e fauna - CITES e não CITES).
5	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
6	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.
7	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen.
8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Outras ressalvas

1	APA Chapada do Araripe: solicitar permissão dos proprietários das áreas onde ocorrerão a pesquisa. Comunicar a unidade o inicio dos trabalhos de campo.

Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	RAFAEL PEREIRA DA CRUZ	Iniciação Científica	065.630.123-61	20080760494 SSP-CE	Brasileira
2	Maria Flaviana Bezerra de Morais Braga	Coorientadora	403.805.213-34	2003034087422 ssp-CE	Brasileira

Locais onde as atividades de campo serão executadas

#	Município	UF	Descrição do local	Tipo
1		CE	AREA DE PROTEÇÃO AMBIENTAL CHAPADA DO ARARIPE	UC Federal

Este documento (Autorização para atividades com finalidade científica) foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

Código de autenticação: 97559552

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

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Dados do titular

Nome: Antonia Thassya Lucas dos Santos	CPF: 033.936.893-47
Título do Projeto: Efeito antifúngico de Spondias tuberosa Arr. Cam. (umbu) frente a leveduras do gênero Candida	
Nome da Instituição : Universidade Regional do Cariri	CNPJ: 06.740.864/0001-26

Atividades X Táxons

#	Atividade	Táxons
1	Coleta/transporte de material botânico, fúngico ou microbiológico	Spondias tuberosa

Material e métodos

1	Amostras biológicas (Plantas)	Folhas, Raízes
2	Método de captura/coleta (Plantas)	Coleta manual

Destino do material biológico coletado

#	Nome local destino	Tipo Destino
1	Universidade Regional do Cariri	coleção

Este documento (Autorização para atividades com finalidade científica) foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

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Nome da Instituição : Universidade Regional do Cariri	CNPJ: 06.740.864/0001-26

Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº 03/2014, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de amostra	Qtde.	Data

* Identificar o espécime no nível taxonômico possível.

Este documento (Autorização para atividades com finalidade científica) foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

Código de autenticação: 97559552

ANEXO 4: Parecer com autorização da pesquisa pelo Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SISGEN) do Ministério do Meio Ambiente

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº A341D06

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**A341D06**
Usuário:	**Antonia Thassya Lucas dos Santos**
CPF/CNPJ:	**033.936.893-47**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Spondias tuberosa

Título da Atividade:	**Efeito antifúngico de Spondias tuberosa Arr. Cam. (umbu) frente a leveduras do gênero Candida**

Equipe

Antonia Thassya Lucas dos Santos	**URCA**
Maria Flaviana Bezerra Morais Braga	**Universidade Regional do Cariri - URCA**
Henrique Douglas Melo Coutinho	**Universidade Regional do Cariri - URCA**

Parceiras Nacionais

06.740.864/0001-26 / Universidade Regional do Cariri - URCA

Data do Cadastro:	**10/07/2018 11:30:31**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **11:32** de **10/07/2018**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN