UNIVERSIDADE REGIONAL DO CARIRI – URCA

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE - CCBS

DEPARTAMENTO DE QUÍMICA BIOLÓGICA - DQB

PROGRAMA DE PÓS-GRADUAÇÃO EM QUÍMICA BIOLÓGICA - PPQB

LUCIENE FERREIRA DE LIMA

ANÁLISE DA COMPOSIÇÃO QUÍMICA E AVALIAÇÃO DO POTENCIAL ANTI-*Candida* DE EXTRATOS DE *Gossypium hirsutum* L.

Crato – CE, 2022

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Tese apresentada ao Programa de Pós-Graduação em Química Biológica, da Universidade Regional do Cariri, como requisito para obtenção do Título de Doutora em Química Biológica.

Orientadora: Prof.ª Dra. MARIA FLAVIANA BEZERRA MORAIS BRAGA

Coorientador: Prof. Dr. HENRIQUE DOUGLAS MELO COUTINHO

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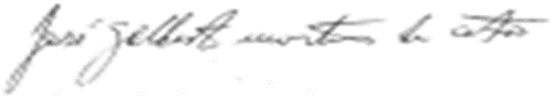

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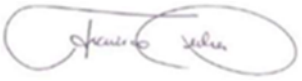

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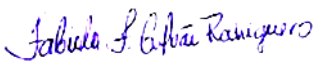

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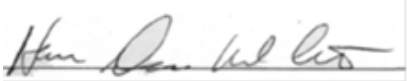

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“Não basta viver; é necessário merecê-lo. E sempre é tempo para ao menos intentar essa obra de reajuste a si mesmo, diante da tarefa que o nosso lugar no mundo nos assinala.”

Emilio Mira y López

TABELAS e FIGURAS

FUNDAMENTAÇÃO TEÓRICA

CAPÍTULO 1

CAPÍTULO 2

SÍMBOLOS, SIGLAS E ABREVIATURAS

AELG – hydroethanolic extract of leaves of *Gossypium hirsutum*

AERG – aqueous extract of roots of *Gossypium hirsutum*

CA – *Candida albicans*

CFM - Concentração Fungicida Mínima

CT - *Candida tropicalis*

CV - Crystal Violet method

DMSO - dimethyl sulfoxide

EAFG - Extrato aquoso das folhas de *G. hirsutum*

EARG - Extrato aquoso das raízes de *G. hirsutum*

EHFG - Extrato hidroalcóolico das folhas de *G. hirsutum*

EHRG - Extrato hidroalcóolico das raízes de *G. hirsutum*

HELG – hydroethanolic extract of leaves of *Gossypium hirsutum*

HERG – hydroethanolic extract of roots of *Gossypium hirsutum*

IC_{50} – The half maximal inhibitory concentration

INCQS: Collection Cultures of the National Institute of Quality Control in Heal

MC – Matriz Concentration

UPLC–QTOF-MS/MS - Ultra-efficient liquid chromatography coupled to quadrupole/time of flight system

URM: University Recife Mycology

RESUMO

ANÁLISE DA COMPOSIÇÃO QUÍMICA E AVALIAÇÃO DO POTENCIAL ANTI-*Candida* DE EXTRATOS DE *Gossypium hirsutum* L.

O gênero *Gossypium* spp. (algodão), da família Malvaceae, possui diversas espécies com atividades biológicas, por exemplo, *G. arboreum, G. barbadense, G. herbaceum* e *G. hirsutum*. Este trabalho investigou a composição química, o efeito anti-*Candida* de extratos aquosos e hidroalcóolicos das folhas e raízes da espécie *Gossypium hirsutum* L. frente a leveduras do gênero *Candida*, contra as cepas de *Candida albicans* (INCQS 40006 e URM 4387) e *Candida tropicalis* (INCQS 40042 e URM 4262). Os extratos de *G. hirsutum* foram analisados em condições optimizadas de cromatografia de ultra eficiência, permitindo a caracterização dos compostos tomando como referências banco de dados e a literatura geral. Quanto aos testes anti-*Candida* foram avaliados a viabilidade celular intrínseca e combinada, assim como a CI_{50}, e em seguida, a inibição da virulência através da formação de biofilme e dimorfismo fúngico pelos os extratos das folhas e raízes. A análise química detectou a presença de flavonoides como flavan, flavan-3-ol, flavonol, além de, terpenos, ácidos orgânicos e derivados do gossipol nos extratos. Os resultados sobre a atividade anti-*Candida* mostraram quanto à atividade intrínseca e combinada, uma resposta dependente do aumento da concentração, inibindo o crescimento de algumas cepas. Em combinação com fluconazol para avaliar a interferência do perfil de crescimento fúngico, um efeito potencializador significativo contra *C. albicans* e *C. tropicalis* cepas foi demonstrado nos ensaios com extrato hidroalcóolico das raízes do que para extrato aquoso das raízes. O extrato aquoso das folhas demonstrou melhor atividade antifúngica de fluconazol contra *C. albicans* INCQS 40006 e *C. tropicalis* URM 4262, e o extrato hidroalcóolico combinado com fluconazol mostrou efeitos inibitórios potentes no crescimento de *C. albicans* INCQS 40006. Os extratos apresentaram uma capacidade redutora da biomassa do biofilme, destacando a eficácia do extrato aquoso das raízes e das folhas contra os isolados padrão e clínicos de *C. tropicalis*, especialmente em concentrações mais baixas. Quanto a atividade anti-pleomórfica, o extrato aquoso das folhas inibiu o crescimento das hifas de *C. albicans* em cepas isoladas padrão e clínica, enquanto o extrato hidroalcóolico inibiu o crescimento das hifas apenas para o isolado clínico, *C. albicans* URM 4387. Ambos os extratos das raízes foram eficazes contra a *C. albicans* URM 4387 inibindo a formação de hifas em todas as concentrações e o extrato hidroalcóolico da raiz foi eficaz na redução da formação de hifas na cepa isolada de *C. tropicalis*. Conclui-se que o extrato aquoso das folhas e o extrato hidroalcóolico das raízes associados ao fluconazol demonstraram potencialização do efeito mais expressivo do que os outros extratos. Somente o extrato aquoso das raízes e folhas inibiu a biomassa do biofilme na levedura *C. tropicalis*. Os extratos aquoso e hidroalcóolico das folhas inibiram a formação de hifas das cepas CA e, os extratos das raízes inibiram as hifas da cepa *C. albicans* URM 4387 e *C. tropicalis* isolada.

Palavras-Chave: *Candida albicans. Candida tropicalis.* Etnobotânica. Atividade antifúngica. Fitoquímica.

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ABSTRACT

CHEMICAL COMPOSITION ANALYSIS AND EVALUATION OF ANTI-*Candida* OF EXTRACTS FROM *Gossypium hirsutum* L.

Gossypium spp. (Cotton) genus, Malvaceae family, has several species with biological activities, for instance, *G. arboreum, G. barbadense, G. herbaceum,* and *G. hirsutum*. This work investigated the chemical composition, and anti-*Candida* effect of aqueous and hydroalcoholic extracts from the leaves and roots of the species *Gossypium hirsutum* L. in front of yeasts of the genus *Candida*, against the strains of *Candida albicans* (INCQS 40006 and URM 4387) and *Candida tropicalis* (INCQS 40042 and URM 4262). The extracts of *G. hirsutum* were analyzed under optimized ultra-efficient chromatography conditions, allowing the characterization of the compounds taken as references database and the general literature. As for the anti-*Candida* tests, intrinsic and combined cell viability were evaluated, as well as the IC_{50}, and then the inhibition of virulence through the formation of biofilm and fungal dimorphism by the extracts of leaves and roots. The chemical analysis detected the presence of flavonoids such as flavan, flavan-3-ol, and flavonol, in addition, to terpenes, organic acids, and gossypol derivatives in extracts. The results on the anti-*Candida* activity showed intrinsic and combined activity, a response dependent on the increase in concentration, inhibiting the growth of some strains. In combination with fluconazole to assess the interference of fungal growth profile, a significant potentiating effect against *C. albicans* and *C. tropicalis* strains was demonstrated in the assays with hydroalcoholic extract of the roots than for aqueous extract of the roots. The aqueous extract of the leaves demonstrated better antifungal activity of fluconazole against *C. albicans* INCQS 40006 and *C. tropicalis* URM 4262, and hydroalcoholic extract combined with fluconazole showed potent inhibiting effects on the growth of *C. albicans* INCQS 40006. The extracts showed a reducing capacity of the biofilm biomass, highlighting the efficacy of the aqueous extract of the roots and leaves against the standard and clinical isolates of *C. tropicalis,* especially at lower concentrations. As for anti-pleomorphic activity, the aqueous extract of leaves inhibited the growth of hyphae *C. albicans* in standard and clinical isolated strains, while hydroalcoholic extract inhibited hyphae growth only for the clinical isolate, *C. albicans* URM 4387. Both root extracts were effective against *C. albicans* URM 4387 inhibiting hyphae formation in all concentrations and hydroalcoholic extract from the root was effective in reducing hyphae formation in the isolated strain of *C. tropicalis.* It was concluded that the aqueous extract of the leaves and the hydroalcoholic extract of the roots associated with fluconazole showed potentiation of the effect more expressive than the other extracts. Only the aqueous extract of roots and leaves inhibited biofilm biomass in yeast *C. tropicalis.* The aqueous and hydroalcoholic extracts of the leaves inhibited the formation of hyphae of the strains *C. albicans* and, root extracts inhibited hyphae from the strain *C. albicans* URM 4387 and isolated *C. tropicalis.*

Keywords: *Candida albicans, Candida tropicalis.* Ethnobotany. Antifungal activity. Phytochemicals.

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IMPORTÂNCIA/RELEVÂNCIA PARA A SOCIEDADE

Os fármacos disponíveis para o tratamento de infecções fúngicas é limitado, e seu uso constante pode provocar resistência fúngica. Essa resistência pode ser reduzida com o uso de plantas medicinais, como o algodoeiro. Os produtos naturais possuem menor toxicidade, o que os torna favoráveis ao paciente, além de possuir menor custo. Este estudo buscou verificar as bioatividades de extratos do algodão baseado em relatos de uma comunidade cratense que o utiliza para a saúde íntima feminina. Este trabalho respondeu aos seguintes questionamentos: "Os extratos aquosos e hidroalcóolicos são capazes de inibirem o crescimento de populações fúngicas e o desenvolvimento/formação de hifas e biofilmes de *C. albicans* e *C. tropicalis*?" e "A composição química dos extratos pode ter influência na possível inibição do crescimento fúngico, e das hifas e redução da biomassa do biofilme?*"*. Os extratos de *G. hirsutum* podem ser uma alternativa para o combate a resistência já que os mesmos potencializaram a ação do fluconazol, impediram a formação das hifas diante de algumas cepas de *Candida* spp., e apresentaram redução da biomassa do biofilme. Dessa forma, esta tese pode contribuir na descoberta de substâncias capazes de atuar na reversão da resistência fúngica, que poderão ser base para a formulação de novos medicamentos e inovação de tratamentos nas infecções fúngicas persistentes.

SUMÁRIO

1INTRODUÇÃO GERAL

1.1 Estratégias de Pesquisa

Para responder aos objetivos dessa pesquisa foi realizado primeiramente a produção dos extratos vegetais para que assim pudessem ser aplicados protocolos químicos e antifúngicos. Os extratos aquosos e hidroalcóolicos de *G. hirsutum* L. foram submetidos a análise química em Cromatografia Líquida de Ultra Eficiência (UPLC–QTOF-MS/MS) na Empresa Brasileira de Pesquisa Agropecuária – EMBRAPA, CE através do Dr. Paulo Riceli, Dr. Edyr Sousa de Brito e Dr. Kirley Marques Canuto. A UPLC na identificação de compostos químicos é mais rápida, possui alta resolução e sensibilidade dentro da cromatografia líquida (SWARTZ, 2005). Além disso, faz uma análise qualitativa o que atendeu a necessidade desse projeto. Através dos picos revelados nos cromatogramas pode-se verificar quais metabólitos estavam presentes nos extratos e, através da literatura foi possível identificar os compostos que possivelmente pudessem agir ou não contra microrganismos.

Os dados químicos são ferramentas importantes para correlacionar os possíveis metabólitos secundários associados à atividade anti-*Candida*, leveduras que são estudados no Laboratório de Micologia Aplicada do Cariri – LMAC, Universidade Regional do Cariri - URCA. No LMAC foram executados os protocolos de inibição do crescimento fúngico e de inibição de fatores de virulência que elucidaram a ação anti-*Candida* dos extratos vegetais do algodão.

A verificação da curva de viabilidade fúngica com o extrato sozinho ou combinado a droga padrão, o Fluconazol, foi realizada pela microdiluição em placas de 96 poços. Esses testes são capazes de nos mostrar o potencial do extrato vegetal sozinho ou se o mesmo pode modificar a ação do Fluconazol na presença das espécies de *Candida* através da microdiluição em uma escala de concentração decrescente. A partir desses testes e análise estatística também pode-se estabelecer a CI_{50} que é a capacidade que o extrato vegetal sozinho ou associado ao fluconazol pode agir eliminando 50 % da população fúngica (ERNST *et al*. 2000; COUTINHO *et al*. 2008; MORAIS-BRAGA *et al*. 2016). A investigação da Concentração Fungicida Mínima (CFM), realizada por subcultivo da microdiluição, foi feita em placas de *Petri* e possibilitou determinar se os extratos exercem efeito capaz de inviabilizar a célula (efeito fungicida) impedindo formação de colônias ou se apenas exerceram inibição de crescimento (efeito fungistático), sem causar danos comprometedores à célula fúngica.

Extratos vegetais podem agir inibindo fatores de virulência como a formação de hifas/pseudohifas, estruturas consideradas aptas a invadirem tecidos durante a infecção, ou o desenvolvimento de biofilme, outro fator de virulência capaz de ajudar as células fúngicas a escapar do sistema imunitário do hospedeiro (ANDRADE *et al*., 2019; CARNEIRO *et al*., 2019) por parte de espécies de *Candida* spp. Realizou-se dois testes para verificar se os extratos de folhas e raízes do algodoeiro podem inibir esses processos. Um deles é o teste de inibição da transição morfológica fúngica no qual aplicamos o extrato em três concentrações diferentes a partir da Concentração Matriz (CM) de 16.384 μg/mL (CM/4 – 4.096 μg/mL, CM /8 – 2.048 μg/mL, CM /16 –1.024 μg/mL), já que a CFM não foi determinada. Sobre uma lâmina com meio empobrecido de nutrientes e com o extrato, traçamos estrias com inóculos de *Candida* e após 24 horas em estufa, verificamos se o extrato vegetal foi capaz ou não de inibir a formação de estruturas filamentosas. O meio empobrecido de nutrientes provoca estresse fúngico e promove o crescimento das hifas nas leveduras. Leveduras do gênero *Candida* causam infecções sistêmicas invasivas em indivíduos saudáveis e em pacientes imunocomprometidos por intermédio dessa transição da levedura para hifas, sendo essa uma das suas capacidades de virulência e invasão tecidual e em mucosas (FRAGA-SILVA et al., 2022).

Outra forma de virulência é a capacidade que as leveduras têm de formar uma comunidade estruturada e autossustentável, o chamado biofilme (SASANI et al., 2021). Então, estabelecemos parceria com a Universidade Federal de Pernambuco (UFPE) na pessoa da prof.ª Doutora Rejane Pereira Neves e realizou-se o método de Cristal Violeta para verificar a capacidade de erradicação de biofilme e a capacidade de redução da biomassa do biofilme por parte dos extratos. Com esse ensaio finalizamos os testes que avaliaram a capacidade anti-*Candida* dos extratos aquosos e hidroalcóolicos de *G. hirsutum* L.

2 OBJETIVOS

2.1 Objetivo Geral

Investigar a composição química, o efeito anti-*Candida* de extratos aquosos e hidroalcoólicos das folhas e raízes da espécie *Gossypium hirsutum* L. frente a leveduras do gênero *Candida*.

2.2 Objetivos Específicos

- ✓ Identificar compostos químicos presentes nos extratos por Cromatografia Líquida de Alta Eficiência - UPLC-QTOF-MS/MS;
- ✓ Identificar a Concentração Inibitória de 50% dos microrganismos (CI_{50}) e a Concentração Fungicida Mínima (CFM) dos extratos e fluconazol e da combinação destes, classificando o tipo de efeito (fungistático, fungicida);
- ✓ Verificar a existência de efeito anti virulência em relação ao pleomorfismo de *Candida* spp. e a formação de biofilme;

2.3 Objetivos e questionamentos

Na década de 80, o Ceará era um grande produtor de algodão por causa das condições climáticas favoráveis e essa cultura passou a ter um valor histórico, cultural, econômico na agricultura familiar no semiárido brasileiro passando a ser chamado de “Ouro Branco” (SOUZA 2000; LIMA; OLIVEIRA, 2001; BELTRÃO 2003; GUERRA; SOUZA; LUSTOSA, 2012). Após essa década de grande produção no Ceará, a produção de algodão declinou e atualmente o estado investe em tecnologia e gestão para voltar a produzir o algodão (AUGUSTO, 2020).

Mesmo com a queda da produção de algodão em grande escala, as pessoas em comunidades continuam plantando e usando o algodão para diversos fins, como o uso medicinal. As plantas dentro da medicina tradicional são utilizadas há muito tempo e demonstram seu potencial farmacêutico através do estudo de substâncias bioativas (LEITÃO *et al.*, 2014) e extratos vegetais (MORAIS *et al*, 2017). Os estudos etnobotânicos demonstram a relação das plantas com as pessoas que fazem uso da medicina tradicional através de chás, infusões e xaropes comercializados em feiras livres ou produzidas em comunidades rurais e quilombolas (CUNHA *et al*., 2012; PAULINO *et al*., 2011).

O Brasil detém cerca de um terço da flora mundial e é rico em biodiversidade (MMA, 2021), isso inclui as plantas medicinais que são matérias-primas para a fabricação de fitoterápicos e outros medicamentos. O apoio para a pesquisa, desenvolvimento tecnológico e inovação com base na biodiversidade brasileira, de acordo com as necessidades epidemiológicas da população, constitui importante desafio para a Política Nacional de Plantas Medicinais e Fitoterápicos (BRASIL, 2006; ALVES, 2013).

Em pesquisa etnobotânica de uma integrante do grupo de pesquisa do Laboratório de Micologia Aplicada do Cariri - LMAC, Fernandes *et al*. (2020), foi relatado o uso do algodão por pessoas da comunidade Baixa do Maracujá, na Chapada do Araripe, município de Crato. Mulheres dessa comunidade relataram o uso de algodoeiro (folhas, raízes e sementes) para cuidar da saúde íntima. Essa informação embasou o planejamento e a execução do projeto que é o potencial das plantas medicinais na inibição de crescimento e da virulência das leveduras do gênero *Candida*.

Quanto às leveduras em estudo, o gênero *Candida* é o causador da candidíase ou candidose, que se expressa na forma de micoses superficiais da região superficial da pele ou das mucosas de forma universal. Pode estar associada ao biofilme em infecções relacionadas ao uso de lentes de contato, cáries dentárias, infecções por bactérias do gênero *Pseudomonas*. A principal espécie para essas micoses é a *C. albicans*, entretanto outras espécies, que é endógena da vagina humana, são também causadoras da candidíase vulvovaginal (ABU-LUBAB *et al*., 2021).

Analisando todas essas informações e associando com o que foi pesquisado, foi estabelecido o estudo de extratos aquosos e hidroalcóolicos das folhas e raízes do *Gossypium hirsutum* L. através da investigação da sua composição química e o efeito anti-*Candida.* A análise do efeito anti-*Candida* foi realizada com o intuito de verificar a inibição do crescimento e de fatores de virulência como o dimorfismo fúngico e a capacidade de formação de biofilme pelas leveduras.

3FUNDAMENTAÇÃO TEÓRICA

3.1 **Família Malvaceae**

A família Malvaceae compreende cerca de 244 gêneros e 4225 espécies, apresentando uma distribuição predominantemente pantropical, onde 72 gêneros e 779 espécies são brasileiros, sendo a região Sudeste a que possui a maior riqueza de espécies. O país ainda conta com um elevado endemismo, com 406 espécies endêmicas, o que corresponde a 53% do total de espécies. É caracterizada por ervas, subarbustos, arbustos, lianas e árvores de pequeno e grande porte (ABAT *et al*., 2017; YOSHIKAWA, DUARTE, 2017; RIGUEIRAL *et al*., 2019).

Malvaceae representa uma família valiosa quanto à obtenção de matéria para alimentos, bebidas, fármacos, madeira e paisagismo (MEIRA-NETO, ALMEIDA, 2015). Um importante gênero da família que apresenta grande potencial ornamental é o *Hibiscus*, como exemplo de espécie ornamental tem-se *Hibiscus rosa-sinensis* L. Outras espécies do gênero além de serem empregadas na ornamentação são usadas também na alimentação, como *Hibiscus sabdariffa* L., bem como utilizada na medicina popular (ESTEVES *et al*., 2014).

Outras espécies pertencentes a gêneros de Malvaceae como, *Abutilon indicum*, *Gossypium herbaceum*, *Sida acuta*, *Sida cordifolia*, *Sida rhombifolia* entre outras são relatadas pelo seu valor etnomedicinal (ABAT *et al*., 2017). *Malva verticillata* é utilizada para remover cálculos renais (SHIM *et al*., 2016). *Theobroma cacao* L. espécie importante por seu valor econômico ao fornecer matéria prima para a produção de chocolate, cosméticos e fármacos (DA SILVA *et a.l*, 2017).

3.2 **Gênero *Gossypium***

O gênero *Gossypium* é um dos mais importantes do ponto de vista econômico, constituindo uma cultura de grande relevância em todo o mundo devido ser uma fonte significativa de fibra têxtil, rações, alimentos, óleo e biocombustível, compreende cerca de 50 espécies, geralmente formados por arbustos. Quatro espécies do gênero *Gossypium* (*G. hirsutum* L, *G. barbadense* L, *G. arboretum* L. e *G. herbaceum* L.) foram domesticadas como fonte de fibra têxtil, com destaque para *G. hirsutum* e *G. herbaceum* por serem amplamente cultivados além de dominarem o comércio mundial de algodão (ADE-ADEMILUA, OKPOMA, 2018; WU *et al*., 2021).

Este gênero ainda se destaca por seus valores medicinais (ADE-ADEMILUA, OKPOMA, 2018). Havendo relatos de sua utilização no tratamento de miomas uterinos, alguns

tipos de cânceres e de leishmaniose tegumentar, apresentando ainda propriedades citotóxica, antioxidante, gastroprotetora (SHARIFI *et al.*, 2014; EGBUTA *et al*., 2017), além de antimicrobiana, onde sua semente é considerada um antifúngico e antibacteriano por natureza (KRISHNAVENI *et al*., 2014). Através do conhecimento dessas pesquisas e relatos na região do Cariri foram realizados testes antimicrobianos, mais especificamente antifúngicos, para verificar se os extratos do algodão possuem atividade anti-*Candida*.

3.2.1 ***Gossypium hirsutum* L.**

Gossypium hirsutum L. (heterotípico *Gossypium pubescens* Splitg. Ex de Vriese e *Gossypium religiosum* L.) é conhecida popularmente como algodão (Figura 1) com forma de vida arbustiva, naturalizada e, portanto, não endêmica do Brasil. No país, possui distribuição geográfica confirmada no Norte (Amazonas), Nordeste (Alagoas, Bahia Ceará, Maranhão, Paraíba, Pernambuco, Rio grande do Norte), Centro-Oeste (Goiás), Sudeste (Espírito Santo, Minas Gerais, Rio de Janeiro, São Paulo) e Sul (Paraná), tendo Amazônia, Caatinga, Cerrado e Mata Atlântica como domínios fitogeográficos (FERNANDES-JÚNIOR, 2021).

Classificação Botânica (JANEIRO, 2020):
Reino Plantae
Divisão Magnoliophyta
Classe Magnoliopsida
Ordem Malvales
Família Malvaceae
Gênero *Gossypium*
Espécie *Gossypium hirsutum* L.

O algodão, *G. hirsutum* também chamado de algodão herbáceo, mexicano ou americano produz fibra vegetal e é cultivado em mais de 50 países, correspondendo a quase 97% da produção mundial de algodão com 87% da área de cultivo no mundo em países em desenvolvimento (NIX *et al*.,2017; EGBUTA *et al*., 2017). O Brasil é o quinto maior produtor do algodão. Essa planta é empregada também na produção de óleo e até mesmo na alimentação do gado (RODRIGUES *et al*., 2017).

Além disso, *G. hirsutum* é utilizado para fins medicinais, sendo relatada para o tratamento de diarreia (MUSSARAT *et al*., 2014; RODRIGUES *et al*., 2017), hemorragia uterina pelo preparo de chá a partir das folhas e como cicatrizante, também pelo emprego das folhas, assim como bioinseticida, antitripanocidas (DELGADO *et al*., 2018), além de ser detentora de potencial antimicrobiano (ADE-ADEMILUA, OKPOMA, 2018).

Figura 1. Espécime de *Gossypium hirsutum* L. **A**. Espécime jovem. **B.** Flor. **C**. Flor e gazula

Fonte: Lima, 2022.

3.3 Gênero *Candida* e a resistência fúngica

3.3.1 Gênero *Candida* spp.

O gênero *Candida* é o causador da candidíase ou candidose, se expressa na forma de micoses superficiais da região superficial da pele ou das mucosas de forma universal. As micoses superficiais são definidas como infecções fúngicas limitadas a superfície da pele, pelos e as unhas e são ocasionadas por leveduras e por dermatófitos, as leveduras também podem causar micoses cutâneas e estão relacionadas principalmente ao gênero *Candida* (BAUER *et al*., 2021). As leveduras são fungos unicelulares, não filamentosos e esféricos ou ovais, podem formar hifas e pseudo-hifas, como é o caso da *C. albicans* (CZECHOWICZ *et al*., 2022; ROCHA *et al*., 2021).

Pode estar associada ao biofilme em infecções relacionadas ao uso de lentes de contato, cáries dentárias, infecções por bactérias do gênero *Pseudomonas*. A principal espécie para essas micoses é a *C. albicans, C. tropicalis* entre outras, como *C. glabrata* (BOHNER, F.; GACSER, A.; TOTH, R., 2021); são endógenas da vagina, causadores da candidíase vulvovaginal (ABU-LUBAB *et al*., 2021). As espécies desse gênero são comensais do ser humano colonizando o aparelho digestivo (ALONSO-MONGE *et al*., 2021; SUN *et al*., 2021), respiratório, tegumento

cutâneo e, sua patogenia está totalmente ligada às condições de saúde, alimentação e meio socioeconômico do paciente como recém-nascidos, pacientes com AIDS e indivíduos com tratamento com antibióticos de amplo espectro (DA ROCHA *et al*., 2021).

3.3.2 ***Candida albicans***

Considerada uma das causas mais comuns de infecções fúngicas em humanos, *Candida albicans* é um patógeno oportunista que reside como comensal em diferentes partes do corpo especialmente no trato gastrointestinal e geniturinário. No entanto, quando há um comprometimento do sistema imunológico do hospedeiro *C. albicans* pode causar desde infecções superficiais a sistêmicas (PRISTOV, GHANNOUM, 2019; NAGLIK *et al*., 2019).

Candida albicans está relacionada a cerca de 50% de todas as infecções ocasionadas por *Candida* (WHALEY *et al*., 2017; KADOSH, MUNDODI, 2020), é um dos patógenos identificados com maior frequência em infecções nosocomiais (TSUI *et al*., 2016), e um dos agentes etiológicos mais comumente isolados em infecções da corrente sanguínea, tendo como resultados elevadas taxas de morbidade e mortalidade (LARA *et al*., 2015).

A espécie possui variados fatores de virulência que favorecem de forma significativa a patogenicidade incluindo adesão, formação de biofilmes, secreção de enzimas e troca de fenótipos. Sendo importante para sua virulência a capacidade deste microrganismo de alternar entre a forma de levedura e a filamentosa produzindo hifas verdadeiras capacidade exclusiva de *C. albicans* e as espécies de *Candida* não-*albicans* (KADOSH, 2016; ZUZA-ALVES *et al*., 2017; PAIS *et al*., 2019).

3.3.3 ***Candida tropicalis***

Candida tropicalis é um importante agente causador de candidíase invasiva, colonizando 60 a 80% dos imunocomprometidos, sendo a espécie *C. tropicalis* mais comumente isolada de pacientes com leucemia e neutropênicos, (NAVARRO-ARIAS *et al*., 2019; YAPAR, 2014) com mortalidade elevada para esses pacientes, com variação de 30 a 70%, sendo as taxas dos idosos as mais altas (WHALEY *et al*., 2017).

Assim como *C. albicans*, é capaz de produzir hifas verdadeiras bem como de produzir biofilme com forte aderência a células epiteliais e endoteliais assim como também é uma das espécies mais importantes em termos de virulência perdendo apenas para *C. albicans* (ZUZA-ALVES *et al*., 2017; DE BARROS *et al*., 2018).

Além disso tem apresentado resistência ao fluconazol e outros azóis como voriconazole e isavuconazole (CHEN et al, 2021) devido a ocorrência de substituições de aminoácidos específicos em ERG11 e pela superexpressão de bombas de efluxo, mecanismos de resistência semelhante ao de outras espécies de *Candida*, ocasionando um grande problema de saúde pública (PRISTOV, GRANNOUM, 2019; MEGRI *et al*., 2020).

3.3.4 **Virulência fúngica por pleomorfismo fúngico e formação de biofilme**

Recentemente, em uma revisão foi discutido a terapia combinada com fármacos de referência, demonstrando que há vantagens como a diminuição da toxicidade, melhora do prognóstico do paciente com infecção difícil de tratar, porém são necessários mais estudos para comprovar essa prática clínica (VITALE, 2021). Assim como, estudos para verificar a ocorrência de cepas resistentes a um, ou mais, dos antifúngicos. A anfotericina B e os azóis (cetoconazol, fluconazol e itraconazol, tioconazol, voriconazol, clotrimazol, terconazol) e nistatina têm sido os fármacos de primeira escolha na terapia antifúngica (RAIMUNDO; TOLEDO, 2017).

Esses fármacos disponíveis para infecções fúngicas superficiais e invasivas são limitados e precisa ser prescrito após confirmação laboratorial, para evitar o uso generalizado e aumento da resistência antifúngica, já que cerca de 20 a 25% da população global é afetada por micoses superficiais, o que pode causar uma morbidade considerável, e há relatos de aumento dessa incidência (GUPTA *et al*., 2021).

Segundo Moreira *et al.* (2017) em seu estudo foi demonstrado que as cepas isoladas de um hospital apresentaram alto índice de resistência aos antifúngicos azólicos utilizados no controle dessas leveduras. Esse fato demonstra a grande importância de uma terapêutica adequada para os diferentes pacientes que são expostos a esses agentes. O autor ressalta a necessidade de uma vigilância rígida da susceptibilidade das espécies de *Candida*, já que o uso prolongado e indiscriminado de alguns fármacos tem aumentado a taxa de resistência dessas espécies frente aos antifúngicos convencionais.

Além disso, fatores de transcrição mutantes contribuem para a formação da resistência, mutações em ERG11, que tem como resultado alteração no sítio de ligação da enzima com os azóis, assim como, a bomba de efluxo, mecanismo de resistência mais comum (VIEIRA, SANTOS, 2017; PRIVOST, GHANNOUM, 2019).

Soluções mais efetivas estão sendo estudadas para o problema da resistência antimicrobiana (bacteriana e fúngica), com estudos promissores de novas drogas através de

ferramentas da genômica, e a bioprospecção de produtos naturais, continua sendo uma poderosa aliada nesse processo de descoberta de novos antibióticos e antifúngicos (KHAN *et al*., 2021).

Fatores que contribuem para a sobrevivência, crescimento, adesão e, infecção em um hospedeiro devido a interrupção da homeostase, é definida como fatores de virulência. A transição morfológica (emissão de hifas) é um dos fatores de virulência mais importante e é promovida em resposta às condições alteradas que lhes são oferecidas como, temperatura corporal, pH, aminoácidos, *N*-acetilglucosamina e nutrientes (ZUZA-ALVES *et al*., 2017; JIANG *et al*., 2016; KADOSH, 2019).

Esta transição é um fator de fundamental importância para a virulência dos fungos já que estas estruturas filamentosas utilizam enzimas hidrolíticas facilitando a invasão de células do epitélio, rompimento de células endoteliais e de macrófagos, penetração nos tecidos do hospedeiro e ainda a entrada de fungos não pleomórfico. A formação dos filamentos também é de grande relevância para o estabelecimento de biofilmes no processo de adesão (SZERENCSÉS *et al*., 2020; KADOSH, 2020).

Biofilmes são comunidades microbianas encerradas em uma matriz extracelular aderidos em superfícies bióticas ou abióticas, como em superfícies de dispositivos médicos — cateteres venosos, urinários, marca-passos, válvulas cardíacas e dentaduras. Essas comunidades podem se formar em superfícies do hospedeiro, incluindo mucosas, células epiteliais e órgãos parenquimatosos, estabelecendo uma barreira aos antifúngicos. As estimativas são de que até 65% das infecções que ocorrem em humanos relacionadas a biomateriais implantados e na superfície do hospedeiro são por causa da formação de biofilmes (LOHSE *et al*., 2018; PEREIRA *et al*., 2020).

Essa formação de biofilmes confere muitas vantagens aos microrganismos como, adesão, proteção aos ataques imunológicos do hospedeiro, nutrientes, resistência a estresses químicos e físicos, regulação da expressão gênica, cooperação metabólica, comunicação celular e resistência a drogas (EL-HOUSSAINI *et al*., 2019; SANTOS *et al*., 2018).

As células do biofilme se dispersam durante toda sua formação — adesão, maturação, dispersão — dessa maneira contribuem para a disseminação da infecção uma vez que podem formar novos biofilmes, ocasionando infecções sistêmicas na corrente sanguínea e nos tecidos do hospedeiro, além disso as células que se dispersam do biofilme são mais virulentas que as células planctônicas (LOHSE *et al*., 2018). O biofilme evita a penetração de antifúngicos através da matriz, provocando à seleção de mutantes resistentes e tolerantes a drogas. Foi comprovado que o biofilme de *C. albicans* contribui para o desenvolvimento de resistência a polienos e azóis (ATRIWAL, 2021; MROCZYŃSKA; BRILLOWSKA-DĄBROWSKA, 2021).

3.4 Metabólitos secundários

3.4.1 Metabólitos secundários com atividade antimicrobiana

As plantas apresentam dois tipos de metabolismo, o primário e o secundário, este último é definido como moléculas não essenciais para o crescimento e desenvolvimento do organismo o que difere do metabolismo primário. Os metabólitos secundários são associados a defesa da planta, de insetos, fungos, vírus e bactérias, assim como do ataque de outras plantas, além de atuarem como atrativos para polinizadores e dispersores de sementes (JACOBY *et al*., 2021; DE SOUSA, DE SOUSA, 2017). No entanto, é em razão de suas atividades biológicas para o ser humano que os metabólitos secundários despertam grande interesse, seja por seu valor comercial ou farmacológico (NETO *et al*., 2015).

As plantas produzem uma vasta diversidade de metabólitos secundários (JACOBY *et al*., 2021), que podem ser distinguidos basicamente em três classes principais de compostos, os compostos nitrogenados, compostos fenólicos e terpenos. Essas classes, são ainda constituídas por diversos compostos individuais (CHOMEL *et al*., 2016; DE SOUSA, 2017).

Terpenos, os compostos fenólicos, como os flavonoides, são conhecidos por terem atividades antibacterianas (SHEHADI *et al*., 2014), bem como triterpenos pentacíclicos e seus derivados (DO NASCIMENTO *et al*., 2014), quinonas, taninos e alcaloides (OTHMAN *et al*., 2019), os polifenóis possuem tanto atividade fungicida quanto bactericida e antiviral (MARIÑO *et al*., 2019). Os flavonoides constituem um dos principais grupos de fitoquímicos que tem sido bastante estudado devido as suas propriedades antimicrobianas, sendo inclusive relatadas propriedades antifúngicas e antivirais, gerando assim um interesse sobre a aplicação dessas propriedades (GÓRNIAK *et al*., 2019).

3.4.2 Metabólitos secundários do gênero *Gossypium* spp.

A distribuição dos compostos presentes no algodoeiro diversifica-se em diferentes partes da planta, tendo alguns compostos concentrados em locais específicos, essa distribuição deve-se as suas propriedades e funcionalidades na planta (EGBUTA *et al*., 2017).

O gênero *Gossypium* é conhecido por produzir um grupo especializado de terpenos, incluindo o gossipol, um sesquiterpeno, isolado das sementes do algodão, estando relacionado a defesa das plantas contra pragas e patógenos. Além disso, várias outras propriedades biológicas são atribuídas ao gossipol, como antifertilidade/anticoncepcional, antioxidante,

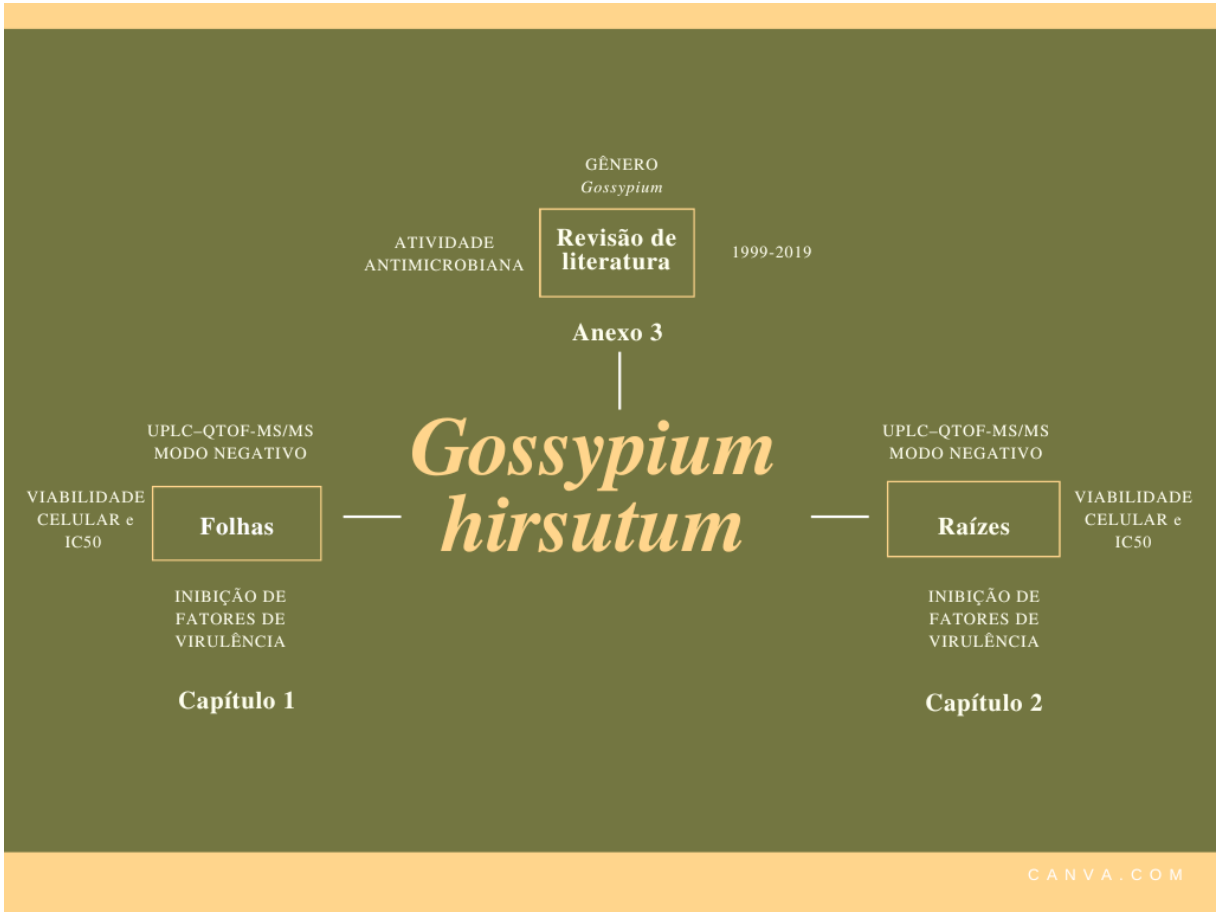
antiparasitárias, anticâncer, antiviral antimicrobianas entre outras (LIU *et al*., 2015; LIU *et al*., 2018; KESHMIRI-NEGHAB, GOLIAEI, 2013; NTIE-KANG, 2014).

Além do gossipol, podem ser citados outros sesquiterpenos como, α-bergamoteno, bisaboleno, cariofileno, farneseno, humuleno e copaneno e produzidos também monoterpenos, como α-pineno, β-pineno e limoneno e derivados de triterpenos (β-sitosterol e β-amirina), que consistem em classes de terpenos, estes por sua vez são indispensáveis à sobrevivência as mudanças do ambiente. No gênero há também a produção de fenóis (EGBUTA *et al*., 2017; LIU *et al*., 2018), bem como a presença de alcalóides (CHANDRASHEKHAR *et al*., 2019).

4PRODUÇÃO CIENTÍFICA

A produção científica está dividida em dois capítulos (Figura 2) e em um anexo. O primeiro artigo publicado foi uma revisão de literatura, que enfatiza o estudo de atividades antimicrobianas com o gênero *Gossypium,* a primeira página da publicação está anexada no final da tese (Anexo 3). A análise química dos extratos aquosos e hidroalcóolicos e testes que avaliam a atividade anti-*Candida* de *G. hirsutum* L. estão em um artigo publicado contendo os extratos das folhas (Capítulo 1), e o outro submetido com os dados obtidos dos extratos das raízes (Capítulo 2). Resumindo, serão três produções científicas a partir dessa tese.

Figura 2. Fluxograma com a produção científica.



Fonte: Lima, 2022.

Artigo 1 – **Ethnobotanical and antimicrobial activities of the *Gossypium* (Cotton) genus: a review**

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Situação: **Publicado**

RESUMO

A família Malvaceae, um importante grupo de plantas que têm o gênero *Gossypium* (algodão) tem sido usada na medicina popular para tratar doenças e sintomas microbianos. Este artigo tem como objetivo compreender sua expressão etnobotânica nas comunidades e elucidação científica das atividades antimicrobianas desse gênero por meio da revisão da literatura. O levantamento bibliográfico foi realizado de 1999 a 2019 com combinações de palavras-chave como "*Gossypium* + etnobotânico", " *Gossypium* + medicinal", "*Gossypium* + a atividade biológica" em bases de dados científicas como Pubmed, Scopus, Web of Science, DOAJ, Scielo, Bireme. Após a análise dos dados, descobrimos que o gênero *Gossypium*, especificamente *Gossypium hirsutum*, *G. barbadense*, *G. herbaceum*, *G. arréeum* são as espécies mais citadas no tratamento de doenças microbianas e sintomas em comunidades em todo o mundo. À luz da elucidação científica das atividades biológicas, o gênero *Gossypium* tem sido usado para tratar doenças protozoárias, bacterianas, fúngicas e virais. A revisão demonstrou que o gênero *Gossypium* é uma fonte promissora de atividades biológicas contra doenças microbianas, especialmente no tratamento de doenças protozoárias como a malária.

Palavras-chave: Família Malvaceae, Medicina popular, Doenças Microbianas

Artigo 2 – **Anti-*Candida* properties of *Gossypium hirsutum* L.: enhancement of fungal growth, biofilm production and antifungal resistance**

Luciene Ferreira de Lima, Jacqueline Cosmo Andrade Pinheiro, Maria Audilene Freitas, Adriely Idalina da Silva, Victor Juno Alencar Fonseca, Taís Gusmão da Silva, Josefa Carolaine Pereira da Silva, Rosilaine Honorato de Lima, Isaac Moura Araujo, Marta Regina Kerntopf, Débora Lima Sales, Rejane Pereira Neves, Edy Sousa de Brito, Paulo Riceli Vasconcelos Ribeiro, Kirley Marques Canuto, Henrique Douglas Melo Coutinho, Abolghasem Siyadatpanah, Polrat Wilairatana, Maria Flaviana Bezerra Morais-Braga

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RESUMO

Candida é um gênero de leveduras com notáveis patogenicidade e capacidade significativa de desenvolver resistência antimicrobiana. *Gossypium hirsutum* L., uma planta medicinal que é tradicionalmente usada devido às suas propriedades antimicrobianas, demonstrou atividade antifúngica significativa. Por isso, este estudo investigou a composição química e os efeitos anti-*Candida* dos extratos aquosos (AELG) e hidroalcoólicos (HELG) obtidos a partir das folhas desta planta. Os extratos foram quimicamente caracterizados por UPLC-QTOF-MS/MS, e suas atividades anti-*Candida* foram investigadas pela análise da viabilidade celular, produção de biofilme, transição morfológica e aumento da resistência antifúngica. A análise UPLC-QTOF-MS/MS revelou a presença de vinte e um compostos tanto no AELG quanto no HELG, destacando a predominância de flavonoides. A combinação dos extratos com fluconazol reduziu significativamente seus valores IC_{50} contra *Candida albicans* INCQS 40006, *Candida tropicalis* INCQS 40042 e *C. tropicalis* URM 4262 cepas, indicando atividade antifúngica aprimorada. Quanto à produção de biofilmes, observou-se inibição significativa apenas para a cepa *C. tropicalis* URM 4262 tratada pelo AELG em comparação com o controle não tratado. Assim, este extrato mostrou efeitos inibitórios mais significativos na transição morfológica das cepas INCQS 40006 e URM 4387 de *C. albicans*. *Gossypium hirsutum* L. apresenta efeitos antifúngicos promissores, que podem estar potencialmente ligados à atividade combinada de constituintes químicos identificados em seus extratos.

Palavras-chave: *Malvaceae*; UPLC-QTOF-MS/MS; Erradicação de biofilmes; resistência antimicrobiana.

Manuscrito 1 – **UPLC–QTOF-MS/MS method of Upland cotton extracts and anti-*Candida* activities**

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RESUMO

Gossypium hirsutum L. é uma espécie com muitas atividades biológicas, usada na medicina popular em todo o mundo. O objetivo foi analisar quimicamente extratos aquosos e hidroalcóolicos de raízes *G. hirsutum* L. (AERG, HERG) e avaliá-los para inibição de fatores de crescimento e virulência como atividade contra *candida albicans* (INCQS 40006; URM 4387) e *Candida tropicalis* (INCQS 40042; URM 4262). Os extratos foram investigados quimicamente pela UPLC-QTOF-MS/MS, a curva de viabilidade celular e 50% do efeito inibidor sobre o fúngico (IC_{50}). O efeito dos compostos foi determinado pela atividade intrínseca e combinada. Para avaliar os fatores de virulência, foi executado o ensaio Cristal Violeta (CV). A análise química revelou flavonoides. A atividade intrínseca e combinada dos extratos tem uma resposta dependente de dose com maiores concentrações. O extrato de HERG mostrou resultados que podem indicar um efeito potencializado do fluconazol. Os extratos não mostraram ação efetiva na redução do biofilme. Na atividade anti-pleomórfica, os extratos foram eficazes contra o CA URM 4387. Os extratos *G. hirsutum* mostraram ação antifúngica em altas concentrações e potencializaram o efeito do fluconazol e interferiram na expressão do fator virulência, porém, esses efeitos não se aplicavam a todas as cepas.

Palavras-chave: Família Malvaceae; *Gossypium hirsutum* L.; análise química; erradicação de biofilmes; anti-pleomorfismo.

4.1 CAPÍTULO 1 - Artigo 2 – Anti-*Candida* properties of *Gossypium hirsutum* L.: enhancement of fungal growth, biofilm production and antifungal resistance

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Abstract: (1) Background: Candida is a genus of yeasts with notable pathogenicity and significant ability to develop antimicrobial resistance. Gossypium hirsutum L., a medicinal plant that is traditionally used due to its antimicrobial properties, has demonstrated significant antifungal activity. Therefore, this study investigated the chemical composition and anti-Candida effects of aqueous (AELG) and hydroethanolic (HELG) extracts obtained from the leaves of this plant. (2) Methods: The extracts were chemically characterized by UPLC–QTOF-MS/MS, and their anti-Candida activities were investigated by analyzing cell viability, biofilm production, morphological transition, and enhancement of antifungal resistance. (3) Results: The UPLC–QTOF-MS/MS analysis revealed the presence of twenty-one compounds in both AELG and HELG, highlighting the predominance of flavonoids. The combination of the extracts with fluconazole significantly reduced its IC_{50} values against Candida albicans INCQS 40006, Candida tropicalis INCQS 40042, and C. tropicalis URM 4262 strains, indicating enhanced antifungal activity. About biofilm production, significant inhibition was observed only for the AELG-treated *C. tropicalis* URM 4262 strain in comparison with the untreated control. Accordingly, this extract showed more significant inhibitory effects on the morphological transition of the INCQS 40006 and URM 4387 strains of *C. albicans* (4) Conclusions: *Gossypium hirsutum* L. presents promising antifungal effects, that may be potentially linked to the combined activity of chemical constituents identified in its extracts.

Keywords: *Malvaceae*; UPLC–QTOF-MS/MS; Biofilm eradication; antimicrobial resistance.

1. Introduction

Candida spp. is a commensal yeast normally found in the oral cavity, urogenital system, gastrointestinal tract, as well as in mucous and cutaneous surfaces of healthy individuals [1]. Despite their importance as constituents of the human microbiota, this genus of yeasts is associated with a wide spectrum of human infections with high morbidity and mortality rates, which is at least partially due to the development of resistance to azoles [1, 2].

Candida albicans and *Candida tropicalis* are commonly isolated from hospitalized patients with a variety of clinical manifestations, ranging from candidiasis to septicemia [3]. As opportunistic pathogens, these species usually cause severe infection in immunocompromised patients, such as those with neutropenia and malignancy [4]. The remarkable pathogenicity of *Candida* species can be attributed to their virulence arsenal, including biofilm production, morphological transition, efflux pump expression [5], epithelial/endothelial adhesion, degrading enzyme secretion, and phenotypic switching [6].

The development of resistance to the most effective antifungals has stimulated the search for new antifungal compounds, both of synthetic and natural origin. In this context, natural product research has identified several compounds with antimicrobial activity, including against clinically important strains of *Candida*, placing medicinal plants as important weapons in the fight against antifungal resistance [7].

The genus *Gossypium* (Malvaceae) is characterized by the presence of chemical compounds such as terpenes, flavonoids, phenols [8, 9], and alkaloids [10], which are potentially linked to the significant number of biological activities demonstrated by the genus, including antifungal, antibacterial, antiparasitic, and antiviral, among others [8, 11–13]. Ethnobotanical studies have demonstrated that the species *Gossypium hirsutum* is traditionally used in the preparation of antiseptic remedies for a vaginal wash in the *Chapada do Araripe* region (Ceará, Brazil) [14]. Accordingly, evidence has indicated that this species presents antifungal activity [15-17], especially due to the presence of phenolic compounds such as flavonoids and terpenes [9,18,19]. However, the potential of this species to combat pathogenic *Candida* strains remains to be investigated.

Thus, considering the phytochemical profile of the genus, as well as the traditional use of the species for the treatment of candidiasis, the present study aimed to characterize the chemical profile of *G. hirsutum* aqueous and hydroethanolic extracts and investigate in vitro their effectiveness against the standard and clinical isolates of *Candida albicans* and *Candida tropicalis.*

2. Results

2.1. UPLC–QTOF-MS/MS Profile of Gossypium hirsutum L.

The UPLC–QTOF-MS/MS analysis of *G. hirsutum* extracts allowed the identification of fifteen constituents, mostly phenolic compounds, as summarized in Figures 1a, b, and Table 1. While the same peaks appeared on both AELG and HELG chromatogram, the latter showed higher relative intensity for the compounds coumaroylglucaric acid, catechin, chlorogenic acid, epi-catechin, while the former showed higher relative intensity for quercetin *O*-hexoside-pentoside.

The compounds **1**, **7**–**11**, **16**, **17**, which showed $[M-H]^-$ ion at *m*/*z* 191, 289, 353, 163, 179, 289, and 463, respectively, were identified as citric acid, catechin, chlorogenic acid, *p*-coumaric acid, caffeic acid, epi-catechin, rutin, and quercetin 3-O-glucoside, respectively.

The compounds **2**–**5** showed the same $[M-H]^-$ ion at *m*/*z* 371 and exhibited, in MS2, loss of a caffeoyl unit generating glucaric acid (*m*/*z* 209) and glucaric acid (*m*/*z* 191), less H_2O as product ions. Based on their fragmentation patterns, the compounds are identified as caffeoyl-glucaric acid isomers [18]. Likewise, compound **6** exhibited a $[M-H]^-$ ion at *m*/*z* 355 with the same fragmentation pattern at *m*/*z* 209 and *m*/*z* 191, being thus identified as coumaryl-glucaric acid, less one hydroxyl group of caffeoyl-glucaric acid [18].

Compound **15** exhibited a predominant $[M-H]^-$ ion at *m*/*z* 595 with a product ion at *m*/*z* 301 and 300, indicating a quercetin aglycone. According to the literature, it was tentatively identified as quercetin O-hexoside-pentoside [18]. Compound **18** showed a predominant $[M-H]^-$ ion at *m*/*z* 593 with loss of rutinoside and typical product ions of

kaempferol aglycone at *m*/*z* 285 and 255 (loss of HCOH). Based on their fragmentation pattern and literature data, it was identified as kaempferol O-rutinoside.

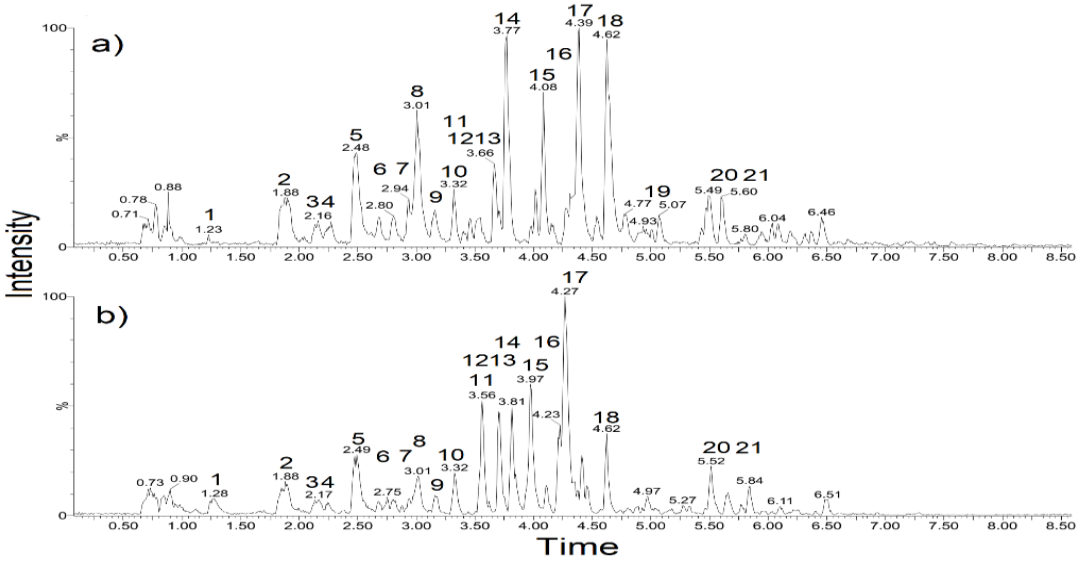


Figure 1. Chemical chromatogram of the (a) hydroethanolic (HELG) and (b) aqueous (AELG) extracts obtained from *G. hirsutum* leaves.

Table 1. Chemical profile of the aqueous and hydroethanolic extracts obtained from the leaves of *Gossypium hirsutum* L.

Peak	Rt min	[M-H]⁻ Observed	[M-H]⁻ Calculated	Product Ions (MS/MS)	Empirical Formula	Ppm Error	Putative Name	Ref.	A[a]	H[a]
1	1.28	191.0190	191.0192	111	$C_6H_7O_7$	−1.0	Citric acid *	[19]	+	+
2	1.88	371.0623	371.0614	209, 191	$C_{15}H_{15}O_{11}$	2.4	Caffeoylglucaric acid	[18]	+	+
3	2.17	371.0624	371.0614	209, 191	$C_{15}H_{15}O_{11}$	2.7	Caffeoylglucaric acid	[18]	+	+
4	2.25	371.0613	371.0614	209, 191	$C_{15}H_{15}O_{11}$	−0.3	Caffeoyglucaric acid	[18]	+	+
5	2.48	371.0606	371.0614	209, 191	$C_{15}H_{15}O_{11}$	−2.2	Caffeoylglucaric acid	[18]	+	+
6	2.67	355.0653	355.0665	209, 191	$C_{15}H_{15}O_{10}$	−3.4	Coumaroylglucaric acid	[18]	+	++
7	3.02	289.0722	289.0712	-	$C_{15}H_{13}O_6$	3.5	Catechin *	[20]	+	+++
8	3.04	353.0874	353.0873	191	$C_{16}H_{17}O_9$	0.3	Chlorogenic acid *	[21]	+	+++
9	3.16	163.0399	163.0395	119	$C_9H_7O_3$	2.5	*p*-coumaric acid *	[21]	+	+
10	3.32	179.0336	179.0344	135	$C_9H_7O_4$	4.5	Caffeic acid	[20]	+	+
11	3.52	289.0718	289.0712	-	$C_{15}H_{13}O_6$	2.1	Epi-catechin *	Std	+	+++
12	3.56	469.1721	469.1710	359	$C_{22}H_{29}O_{11}$	2.3	Unknown	-	+	+
13	3.70	467.1564	467.1553	359	$C_{22}H_{27}O_{11}$	2.4	Unknown	-	+	+
14	3.81	517.1037	517.1041	241	$C_{17}H_{25}O_{18}$	−0.8	Unknown	-	+	+
15	4.11	595.1310	595.1299	301, 300	$C_{26}H_{27}O_{16}$	1.8	Quercetin *O*-hexoside-pentoside	[22]	++	+
16	4.27	609.1444	609.1456	301, 300	$C_{27}H_{29}O_{16}$	−2.0	Rutin *	[20]	+	+
17	4.41	463.0869	463.0877	301, 300	$C_{21}H_{19}O_{12}$	−1.7	Quercetin 3-*O*-glucoside *	[13,23]	+	+
18	4.62	593.1512	593.1506	285, 255	$C_{27}H_{29}O_{15}$	1.0	Kaempferol *O*-rutinoside	[24]	+	+
19	4.97	451.1646	451.1663	96.9579	$C_{15}H_{31}O_{15}$	−3.8	Unknown	-	+	+
20	5.66	501.1093	501.1092	96.9587	$C_{17}H_{25}O_{17}$	0.2	Unknown	-	+	+
21	5.84	453.1818	453.1819	96.9605	$C_{15}H_{33}O_{15}$	−0.2	Unknown	-	+	+

Rt: retention time; A [a]: aqueous extract; H [a]: hydroethanolic extract. +, ++, +++ Relative intensity ratio shows the higher compound concentration in each extract. * STD, comparison with an authentic standard.

2.2. Anti-Candida Activities

2.2.1. Cell Viability Curve and IC_{50}

The cell viability curves, which show the intrinsic activity of the aqueous (Figure 2) and hydroethanolic (Figure 3) extracts, demonstrated that both extracts presented weak antifungal activity, inhibiting only the growth of *C. albicans* URM 4387 and *C. tropicalis* INCQS 40042 at concentrations above 8.192 μg/mL. The pharmacological control fluconazole (FCZ) inhibited the growth of almost all strains from 8.192 μg/mL, except for the *C. albicans* URM 4387 strains, which showed higher sensitivity to the antifungal drug (64 μg/mL).

The minimum fungicidal concentration (MFC) was defined as the lowest concentration capable of fully preventing fungal growth. However, none of the tested substances (extracts and fluconazole) presented an MFC value comprehended in the concentration range adopted in this study (MFC ≥ 16.384 μg/mL). While the isolated extracts presented fungistatic effects, the combination of the hydroethanolic extract with fluconazole resulted in a fungicide effect against *C. albicans* URM 4387 (4096 μg/mL) and both *C. tropicalis* strains (2048 μg/mL).

To evaluate the ability of the extracts to potentiate the antifungal activity of fluconazole, the antifungal drug was serially diluted in the presence of a subinhibitory concentration of the extracts (MC/16 = 1024 μg/mL), considering the matrix concentration as better detailed below. It was demonstrated that extracts potentiated the effects of fluconazole in the standard strain of *C. albicans* and the clinical isolate of *C. tropicalis*. The tests with the URM 4387 strain of *C. albicans* showed that the aqueous extract did not change the inhibitory activity of fluconazole, while the hydroethanolic extract antagonized its effects.

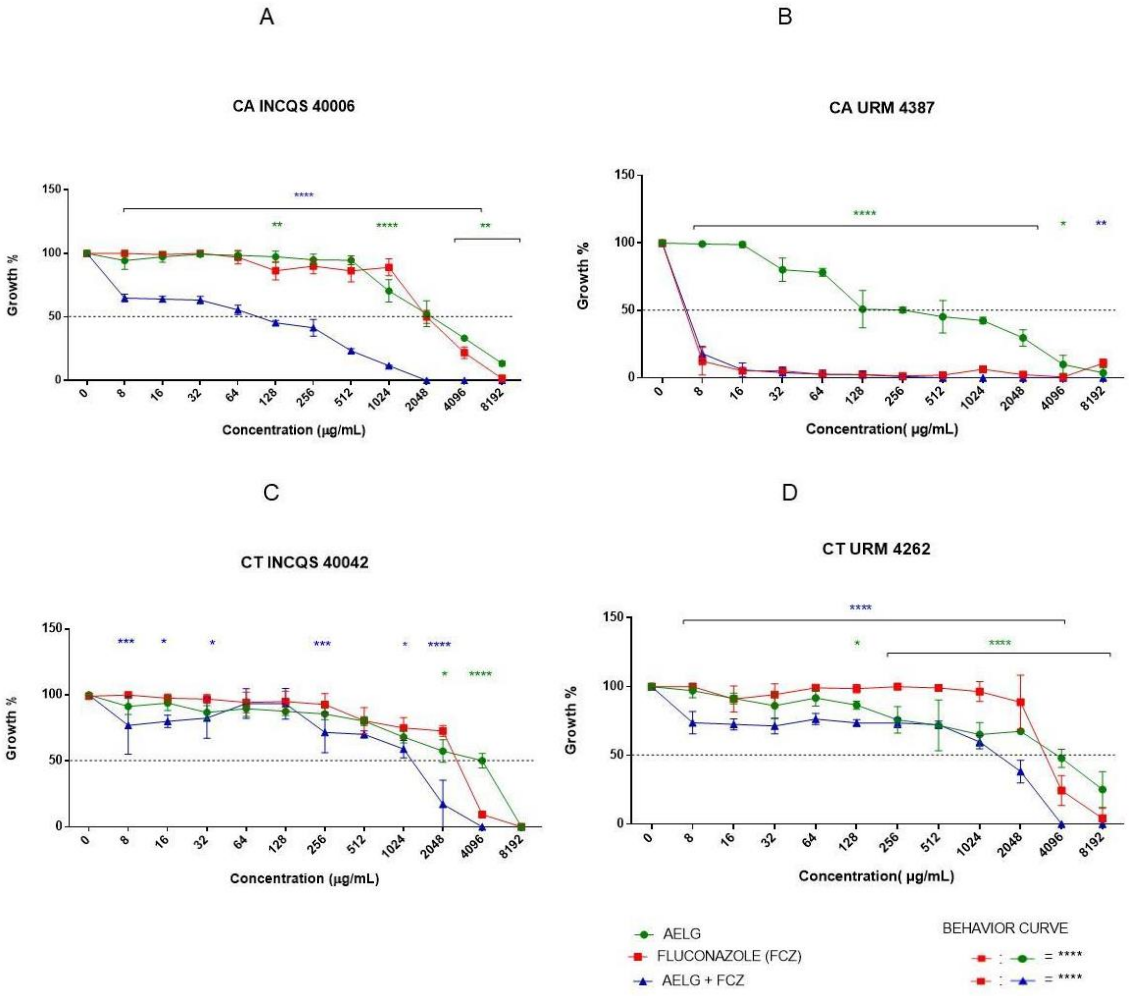


Figure 2. Cell viability curve of AELG alone and in combination with Fluconazole. *Candida albicans INCQS 40006 (**A**), Candida albicans URM 4387 (**B**), Candida tropicalis INCQS 40042 (**C**) and Candida tropicalis URM 4262 (**D**). Statistical significance was determined considering the difference in relation to fluconazole, **** = p < 0.0001; *** = p < 0.001; ** = p < 0.01; * = p < 0.1.*

The antifungal activity of the extracts and fluconazole was also represented in terms of their half-maximal inhibitory concentrations (IC_{50}), as shown in Table 2. As observed, both extracts presented the most promising antifungal effects (lowest IC_{50} values) when tested against the URM 4387 strain of *C. albicans*. Curiously, the extracts potentiated (reduced the IC_{50}) the antifungal activity of fluconazole against the other strain, but not against this clinical isolate.

Table 2. IC_{50} of the AELG and HELG against different strains of *Candida*.

	CA INCQS 40006	CA URM 4387	CT INCQS 40042	CT URM 4262
AELG	2257	317.2	2214	2986
AELG + FCZ	62.76	2.344	819.8	628.5
HELG	8865	1165	NI	8567
HELG + FCZ	29.12	2476	701.1	788.0

FLUCONAZOLE	2125	0.036	2476	3196

NI: Not identified.

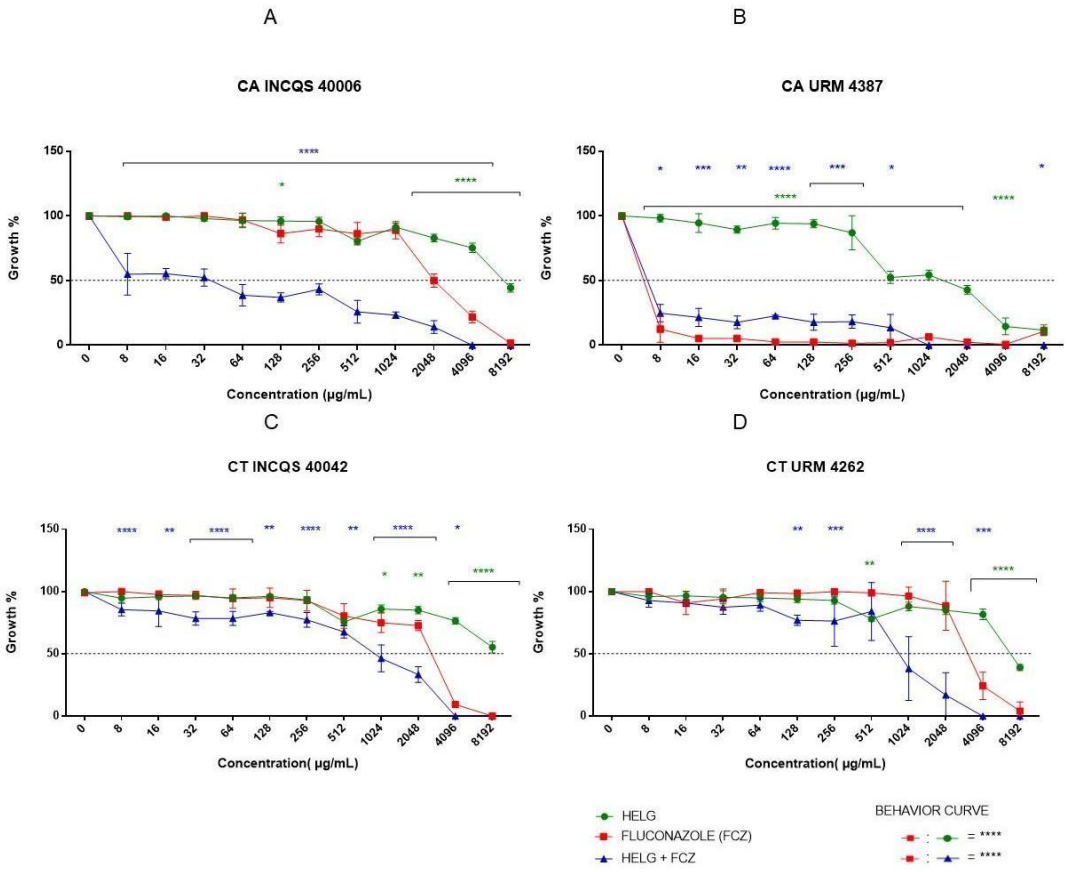


Figure 3. Cell viability curve of HELG alone and in combination with Fluconazole. *Candida albicans INCQS 40006 (**A**), Candida albicans URM 4387 (**B**), Candida tropicalis INCQS 40042 (**C**) and Candida tropicalis URM 4262 (**D**). Statistical significance was determined considering the difference in relation to fluconazole, **** = p < 0.0001; *** = p < 0.001; ** = p < 0.01; * = p < 0.1.*

2.2.2. Effects of *G. hirsutum* Extracts on Biofilm Production by *Candida* Strains

Figure 4 shows the biofilm production capacity of the strains under study, revealing that biofilm production varies among the strains, with *C. tropicalis* URM 4262 demonstrating the higher production capacity. An analysis of the effects of the different in vitro treatments (Figure 5) revealed that the HELG stimulated biofilm production by standard strains of *C. albicans* and *C. tropicalis* and the clinical isolate of *C. albicans* at all concentrations analyzed. On the other hand, the aqueous extract significantly inhibited biofilm production by the *C. tropicalis* isolate at 512 and 128 µg/mL. However, the higher concentrations stimulated biofilm production, probably due to the higher concentration of nutrients such as proteins and polysaccharides, which could contribute to biomass growth [25]. However, the mechanisms underlying this concentration-influenced phenomenon remain to be further investigated.

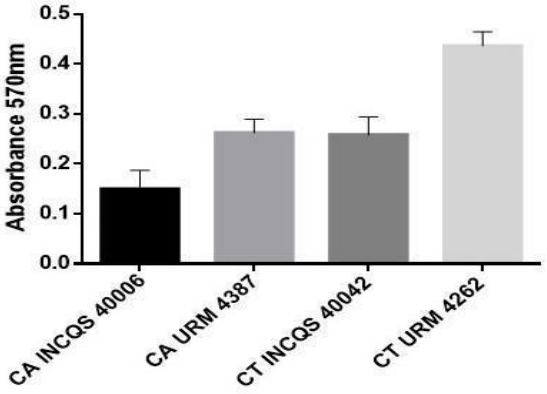
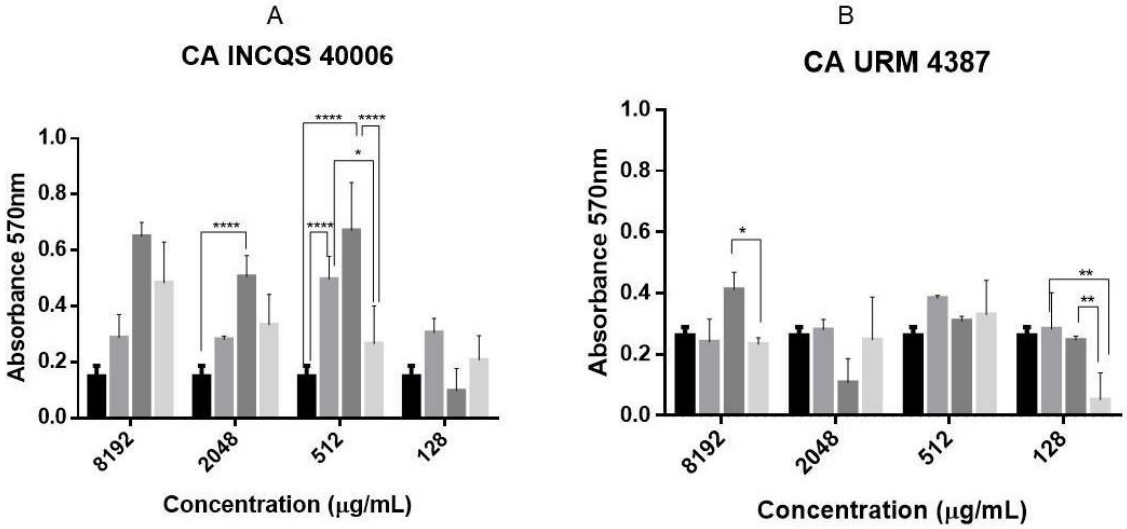


Figure 4. Biofilm formation by different *Candida* strains. *The results are expressed as biofilm staining intensity read at 595 nm C. tropicalis INCQS 40042—moderate; C. albicans INCQS 40006; C. tropicalis URM 4262; C. albicans URM 4387. INCQS—National Institute for Quality Control in Health; URM—University Recife Mycology.*



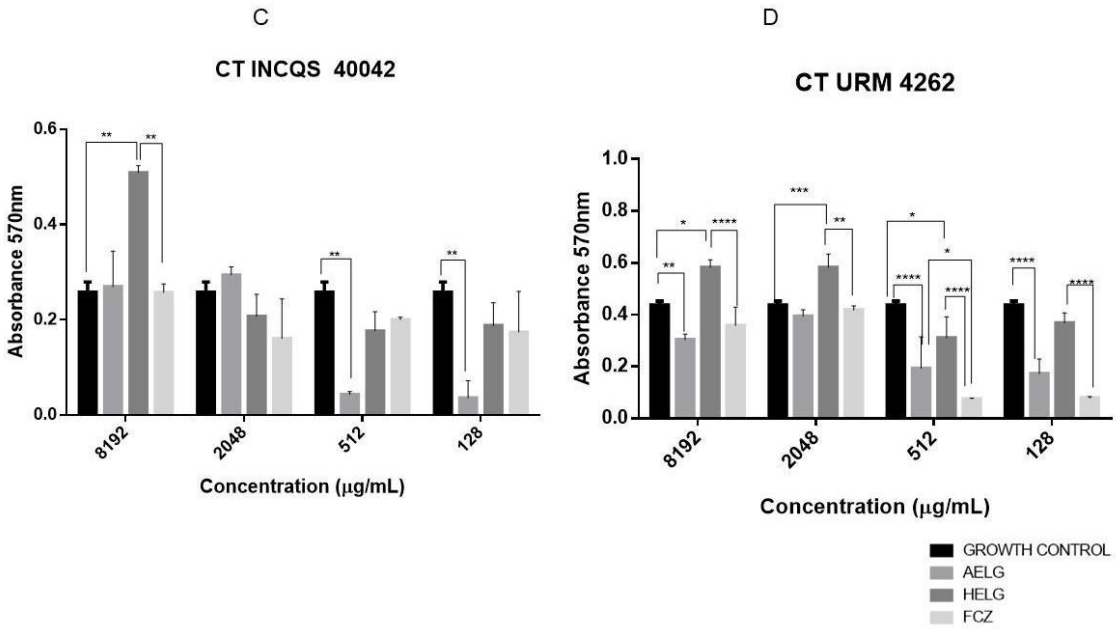


Figure 5. Effects of *G. hirsutum* extracts and fluconazole against *Candida albicans* INCQS 40006 (A), *Candida albicans* URM 4387 (B), *Candida tropicalis* INCQS 40042 (C) and *Candida tropicalis* URM 4262 (D. *Statistical significance was determined considering the difference in relation to growth control/fluconazole. **** = p < 0.0001; *** = p < 0.001; ** = p < 0.01; * = p < 0.1. AELG—Aqueous extract of the leaves of G. hirsutum. HELG—Hydroethanolic extract of the leaves of G. hirsutum.*

2.2.3. *G. hirsutum* Extracts Inhibit *Candida* spp. Morphological Changes

To analyze the anti-pleomorphic effect of the extracts, we investigated their ability to inhibit the emission of hyphal formation by the fungal strains (Figures 6 and 7). About the URM 4387 strain of *C. albicans*, it was found that both extracts fully inhibited hyphal growth at the relative concentrations of MC/4 and MC/8, while filament growth was stimulated at MC/16. The aqueous extract also showed significant inhibitory effects against the standard strain of *C. albicans* but had little impact on the morphological transition of *C. tropicalis clinical isolate.* Curiously, the standard strain of this species has its morphological transition significantly stimulated by all AELG concentrations. Concerning the hydroethanolic extract, except for the URM 4387 strain of *C. albicans*, significant hyphal growth was observed in all treatment conditions. Importantly, the morphological transition was fully inhibited by fluconazole, highlighting the effectiveness of this standard antifungal drug.

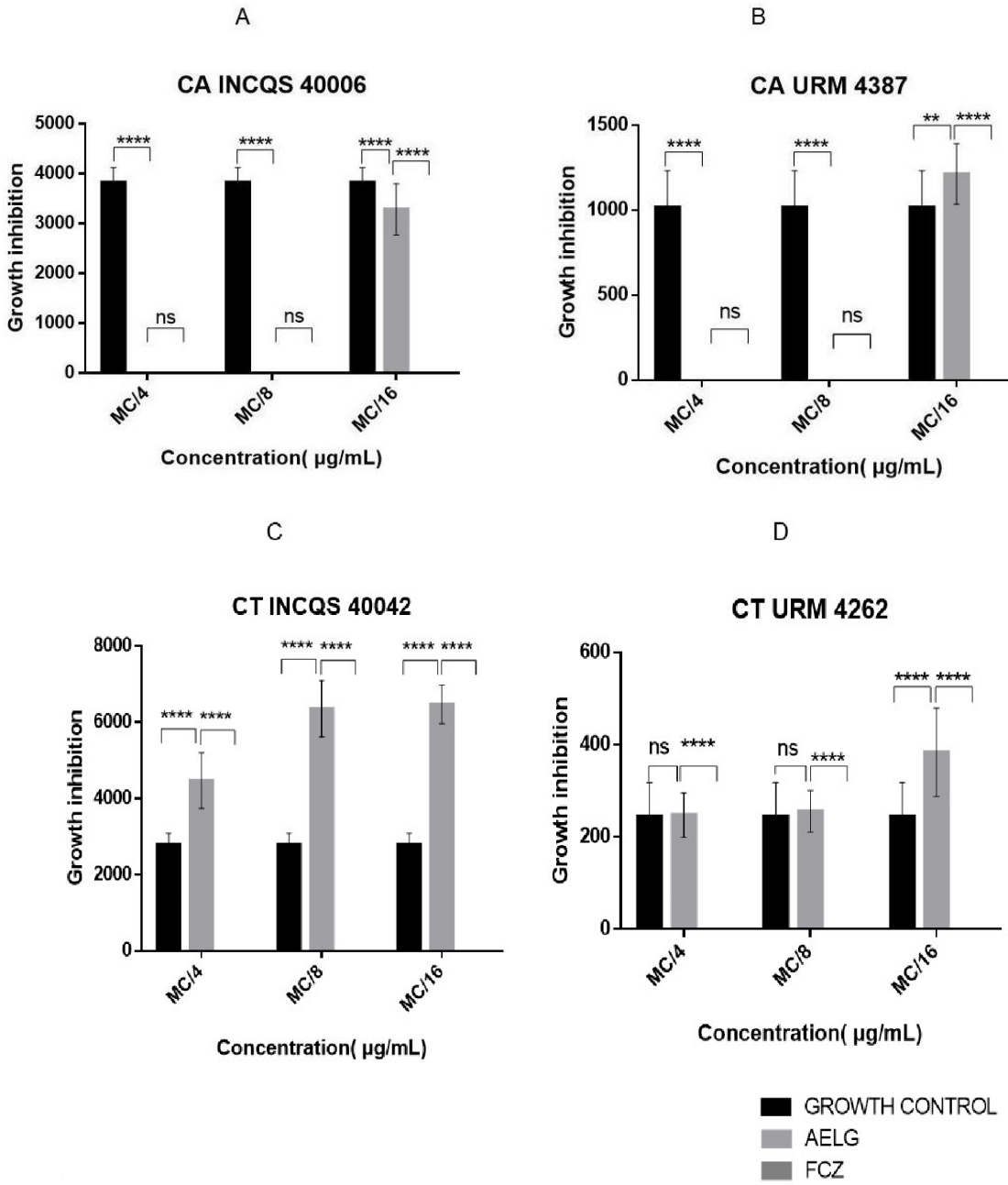


Figure 6. Effects of *G. hirsutum* aqueous extract and fluconazole on the morphological transition of *Candida albicans* INCQS 40006 (A), *Candida albicans* URM 4387 (B), *Candida tropicalis* INCQS 40042 (C) and *Candida tropicalis* URM 4262 (D). *MC: Matrix Concentration (16.384 µg/mL); MC/4: 4.096 µg/mL, MC/8: 2.048 µg/mL, MC/16: 1.024 µg/mL; Growth inhibition—measured by micrometer-sized hyphae (µm); No bar—no hyphae growth. **** = $p < 0.0001$.*

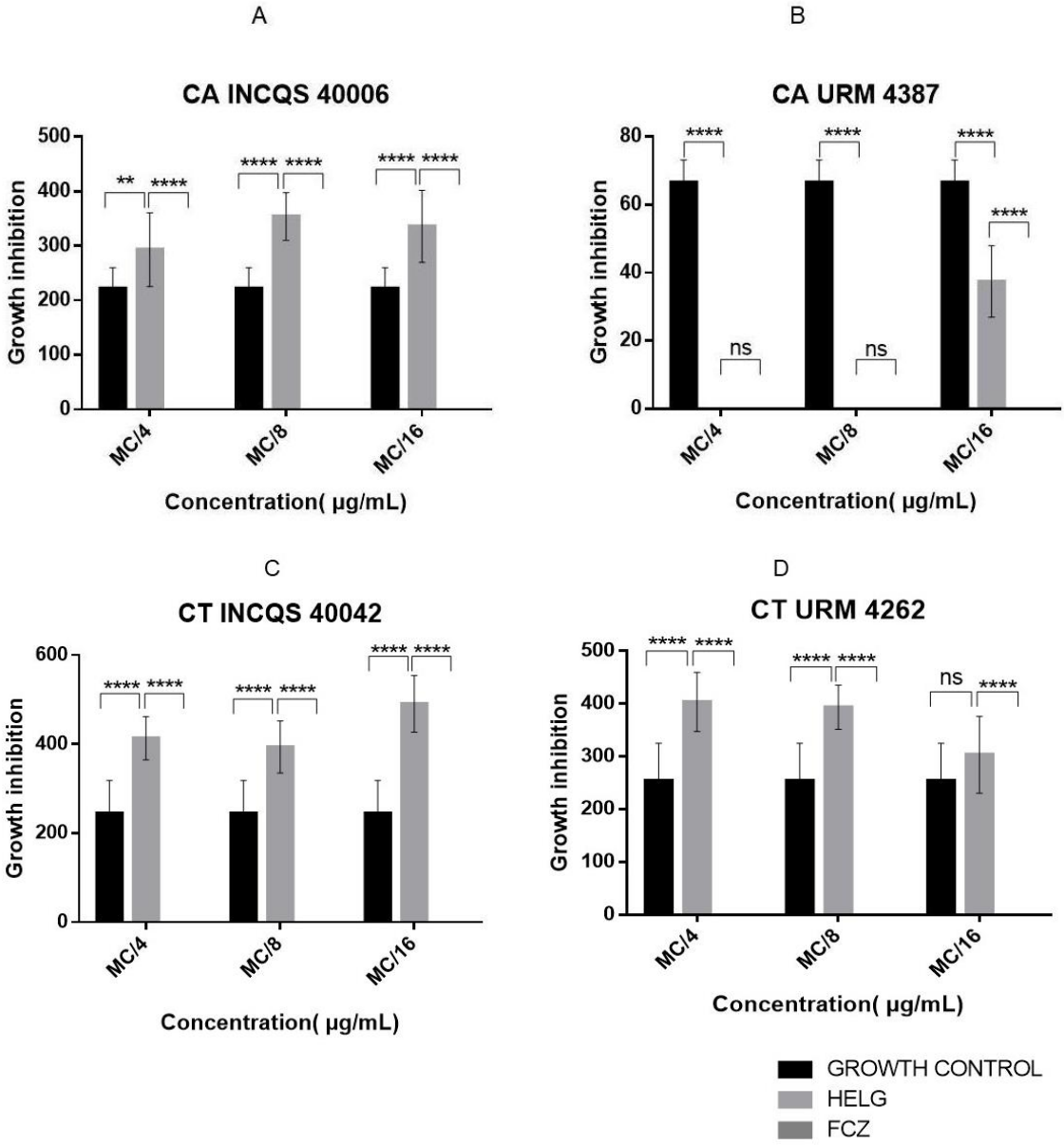


Figure 7. Effects of *G. hirsutum* hydroethanolic extract and fluconazole on the morphological transition of *Candida albicans* INCQS 40006 (A), *Candida albicans* URM 4387 (B), *Candida tropicalis* INCQS 40042 (C) and *Candida tropicalis* URM 4262 (D). *MC: Matrix Concentration (16.384 µg/mL); MC/4: 4.096 µg/mL, MC/8: 2.048 µg/mL, MC/16: 1.024 µg/mL; Growth inhibition—measured by micrometer-sized hyphae (µm); No bar—no hyphae growth. **** = p < 0.0001.*

3. Discussion

The present research characterized the UPLC–QTOF-MS/MS profile and in vitro antifungal activity of the aqueous and hydroethanolic extracts of *G. hirsutum* L. (Malvaceae), a botanical species that is used in the treatment of urogenital tract infections in Brazilian folk medicine. The phytochemical analysis identified 15 of a total of 21 compounds of the samples, revealing that both extracts are predominantly

constituted of phenolic compounds, such as epi-catechin, catechin, quercetin *O*-hexoside-pentoside, quercetin 3-*O*-glucoside, kaempferol *O*-rutinoside

Both extracts demonstrated moderate-to-weak intrinsic antifungal activity. Nevertheless, significant inhibition of fungal growth was observed against the standard strain of *C. albicans* and both *C. tropicalis* strains, which may be due to the presence of antimicrobial secondary metabolites such as flavonoids. Shetti et al. [26] reported that these compounds can accumulate in *G. hirsutum* specimens, causing their color to change from green to brown due to the expression of flavonoid-related genes. This class of compounds acts mainly as phytoalexins and photoprotectors, modulating the transport of the phytohormone auxin, which influences plant structure and function. In this context, it has been demonstrated that catechin is responsible for increasing the antifungal resistance of *G. hirsutum* to the fungus *Verticilium dahlia* [27], which may indicate a role of *G. hirsutum* flavonoids as antifungal agents.

Research conducted with herbalists from popular markets in the South of the Republic of Benin cited the use of decoction prepared from the leaves of *G. hirsutum* for the topical treatment of Candidiasis [28]. Studies have demonstrated the ethanolic extracts of the Malvaceae species *Guazuma ulmifolia* Lam. and *Luehea paniculata* Mart. & Zucc. demonstrated a potentiating effect when combined with the antifungal drug fluconazole against *C. tropicalis* and *C. albicans*. The authors have identified the presence of catechin, chlorogenic acid, rutin, and quercetin [29,30] compounds also identified in the extracts of *G. hirsutum* L., indicating a possible relation between the chemical content and biological activity of these species. Accordingly, a chloroformic extract obtained from *Sida cordifolia* (Malvaceae) showed antifungal activity against *C. albicans*, possibly due to the presence of flavonoids [31, 32].

In addition to showing some intrinsic antifungal activity, *G. hirsutum* L. extracts were found to significantly enhance the activity of fluconazole against the standard and clinical isolates of *Candida* yeasts, which was observed through the reduction of the inhibitory concentrations of the standard antifungal drug when combined with a subinhibitory concentration of the extracts. Importantly, combined therapy has the

benefit to improve the effectiveness of the treatment allied to lower doses, which can significantly reduce toxicity [33].

Candida albicans, followed by *C. tropicalis*, is the most important causative agent of opportunistic infections [34] in immunocompromised patients, which are more susceptible to the virulence arsenal of *Candida* yeasts, among which biofilm production and morphological change (emission of pseudohyphae and hyphae) are highlighted. In addition, these virulence mechanisms are associated with increased resistance to antifungal drugs such as azoles, placing *Candida* infections as an important public health concern [2].

Among the yeasts included in this study, the URM 4262 strain of *C. tropicalis* demonstrated the highest (strong) biofilm-producing potential, while the other strains showed moderate activity. These findings differ from those observed by [35], who found that *C. albicans* are better biofilm producers than non-albicans strains. On the other hand, previous research has corroborated the here demonstrated evidence that *C. tropicalis* are among the main biofilm producers of its genus [36].

Biofilm production by *Candida* strains contributes to antifungal resistance through different mechanisms, including by affecting cell density, changing the matrix constitution, promoting nutrient limitation, inducing the expression of the efflux pump and other resistance genes, as well by producing molecules that regulate cell morphology and biofilm maturation [37,38].

Understanding the resistance mechanisms associated with biofilms production may significantly contribute to the development of novel antifungal therapies. In this context, some compounds of natural products have been identified as promising drug candidates in an antimicrobial resistance context. Additionally, due to the novelty of many of these structures, secondary metabolites may represent promising alternatives for the treatment of fungal infections in immunocompromised patients [2,39].

Importantly, the present research demonstrated that *G. hirsutum* extracts showed a strain-selective and extract type-dependent capacity of inducing biofilm eradication, and therefore, further studies investigating the mechanisms underlying

this phenomenon may significantly contribute to the targeted development of antibiofilm agents from this species [40].

Yeasts of the genus *Candida* can produce mycelium, a virulence factor that contributes to the maintenance of biofilms. In addition, these microorganisms can undergo a morphological transition from yeast to pseudohyphae and hyphae, which also represents an important virulence factor for opportunistic fungi [41]. Previous studies have demonstrated that flavonoids such as quercetin, quercitrin, isoquercetin, and rutin are capable of downregulating the expression of the CDR1 and ERG11 genes, which are significantly involved in the resistance of *C. albicans* by regulating biofilm production, hyphae growth, and many other virulence mechanisms [42]. From a clinical point of view, hyphae growth is an important mechanism used by the yeasts to penetrate the host tissues. Additionally, dimorphism is accompanied by changes in cell surface antigens, which represents a significant immune evasion mechanism. Thus, inhibiting fungal pleomorphism can significantly reduce the pathogenicity of yeasts [43].

While the UPLC–QTOF-MS/MS analysis allowed the identification of most secondary metabolites, the identification of some constituents was not possible. Nevertheless, in light of the specialized literature, it is suggested that the biological effects of the extracts can be attributed to the presence of phenolic compounds (especially flavonoids) in the extracts. Flavonoids are an extensive group of plant secondary metabolites with proven antifungal effects against *C. albicans*, *C. tropicalis*, and other fungal strains [44]. Compounds such as catechin, epi-catechin, quercetin, rutin, kaempferol, which were identified in the extracts, showed antifungal activity against *C. albicans* and *C. tropicalis* [45]. Of note, catechin, quercetin, and kaempferol derivatives were found to inhibit morphological transition, in addition to potentiating fluconazole activity against *Candida* yeasts [45,46]. Therefore, the development of further studies investigating the effectiveness of isolated phenolic compounds identified in the extracts would significantly contribute to understanding the anti-*Candida* potential of *G. hirsutum.*

4. Materials and Methods

4.1. Botanical Material

Gossypium hirsutum specimens were collected in a rural area of the municipality of Araripe, Ceará, Brazil (coordinates: 7°12′12.5″ S 40°01′10.2″ W; 7°12′23.1″ S 40°00′50.7″ W; 7°13′38.5″ S 39°59′44.5″ W). The collection took place in April 2019, from 7:30 to 9:00 in the morning. The plant material was sent to the laboratory, cleaned, and weighed. A voucher specimen was registered in the Herbarium of the Regional University of Cariri—URCA under registry number 65.2019.

4.2. Extract Preparation

For the preparation of the extracts, a total of 1172.3 g of fresh leaves were cut and dried at room temperature. Amounts of 512.8 g and 654.5 g were used in the preparation of the aqueous and hydroethanolic extracts, respectively. Then, distilled water or 70% ethanol was added to the corresponding sample, which was kept protected from light and air [47]. After 72 h, the extracts were filtered and taken to a spray-drying (130 °C, flow rate 0.5 L/h, blower control 1.95, outlet temperature 84.6, flow meter 40 L/min air, particles with one millimeter in diameter), producing crude extracts yielding 8380 and 5486 g, respectively.

4.3. Candida Strains

The clinical isolates (*Candida albicans* URM 4387 and *Candida tropicalis* URM 4262) were obtained from the Federal University of Pernambuco by the University Recife Mycologia (URM). The standard strains of *Candida albicans* INCQS 40006 (ATCC 10231), *Candida tropicalis* INCQS 40042 (ATCC 13803) were obtained from the National Institute for Quality Control in Health (INCQS, FIOCRUZ).

4.4. Chemical Characterization by UPLC–QTOF-MS/MS

The samples were filtered through PTFE syringe filters (0.2 μm pore, Millipore Millex, Sigma- Aldrich®, Darmstadt, Germany) and 5 μL of each sample were injected into the ultra-performance liquid chromatography coupled to quadrupole/time of

flight system (UPLC-QTOF-MS/MS) (Waters Co., Milford, MA, USA) equipped with an ACQUITY UPLC BEH column (150 × 2.1 mm, 1.7 μm, Waters Co.) set to 40 °C. The binary gradient elution system consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), with a linear gradient from 2 to 95% B (0–15 min), 100% (15.01–17 min), 2% (17.01–21 min) and a flow rate of 0.4 $mL.min^{-1}$. The samples were analyzed through an electrospray ionization interface (ESI) in the negative ionization mode. The desolvation gas used was nitrogen and set to 350 °C with a flow rate of 500 L/h. The capillary and cone voltages were adjusted to 2.6 kV and 0.5 V, respectively. Mass accuracy and reproducibility were maintained by infusing lock mass (leucine-enkephalin, 0.2 ng/uL; $[M-H]^-$ ion at *m*/*z* 556.2771). MS data were collected for *m*/*z* values in the range of 110–1180 Da with a scan time of 0.1 over an analysis time of 19 min. The accurate mass and molecular formula assignments were obtained with the MassLynx 4.1 software (Waters MS Technologies).

4.5. Anti-Candida Activities

4.5.1. Cell Viability Curve, IC_{50} and Minimum Fungal Concentration (MFC)

The strains were maintained in Sabouraud Dextrose Agar (SDA, KASVI) medium and incubated for 24 h at 37 °C. An initial suspension was subsequently prepared in 5 mL of sterile saline solution (NaCl, 0.85% saline) and its turbidity was visually adjusted according to the 0.5 value of the MacFarland scale, providing a standard concentration of yeasts ranging from 1×10^6 to 5×10^6 cells per mL.

An initial solution was prepared by adding 1350 μL of doubly concentrated SDA and 150 μL of the fungal strain inoculum (corresponding to 10% of the total solution). Then, 150 μL of this solution was removed and added to each well on a microdilution plate. After this, the wells were filled with 100 μL of extract solution at concentrations ranging from 8192 to 8 μg/mL, and the plates were incubated at 37 °C. After 24 h, the readings were performed at 630 nm in a spectrophotometer (Thermoplate®). The optical density values were used to build the cell viability curve and calculate the IC_{50}. Diluent (0.9% NaCl) sterility and growth controls were also used [48]. All tests were performed in quadruplicate.

For MFC determination, a sterile rod was inserted into each well of the microdilution plate, which was homogenized and subcultured in a Petri dish containing SDA. The plates were incubated at 37 °C, and readings were taken after 24 h, by observing the growth of *Candida* colonies [49]. The MFC was defined as the lowest concentration capable of fully preventing fungal colony growth. Then, the combined activity and morphological transition were evaluated by using MFC-based subinhibitory concentrations. For the treatments whose MFC could not be determined, the Matrix Concentration (MC) was used.

4.5.2. Enhancement of Fluconazole Antifungal Activity

To evaluate the ability of the extracts to potentiate the antifungal activity of fluconazole, the antifungal drug was serially diluted in the presence of a subinhibitory concentration of the extracts (MC/16 = 1024 μg/mL) [33]. Briefly, the plates were numerically filled by adding 100 μL of a solution containing the extract, culture medium (SDB), and 150 μL of the microbial suspension (corresponding to 10% of the solution). Growth and dilution controls were prepared. Thereafter, serial dilutions of fluconazole were performed as previously described, and 100 μL of the drug were added to each well on the plate. The plates were incubated at 37 °C for 24 h, and the readings were carried out as previously described.

4.6. Evaluation of Biofilm Eradication by Candida spp.

To prepare the treatment solutions, 0.15 g of each extract was dissolved in 1 mL of dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) and diluted to concentrations ranging from 16.384 μg/mL to 128 μg/mL. Fluconazole (Pfizer) was used as a reference antifungal drug at concentrations varying between 64 μg/mL and 0.5 μg/mL.

The qualitative evaluation of biofilm formation capacity was performed using the visual method adapted from [50]. After seeding the isolates in the Sabouraud agar medium and preparing the suspensions as described above, 20 μL of inoculum and 180 μL of liquid Sabouraud medium were added to the wells on the microplate and

kept at 35 °C for 72 h without shaking. The contents were then aspirated and the wells were washed with distilled sterile water and a Fuchsin dye (QEEL-Specialized Chemistry Erich Ltd. São Paulo, Brazil).

The staining intensity was evaluated visually and classified as follows: (1) Strongly stained: when the biofilm was intensely stained allowing the correct determination of the contour of the areas containing the biofilm; (2) Average staining: when the biofilm was stained more weakly but still allowed the determination of the contour of the areas containing the biofilm; (3) Poor staining: when the biofilm was not stained, making it impossible to distinguish the color of the plate and the characteristic color of the dye. According to the staining intensity, the biofilm production activity was classified as strong, moderate, and weak.

The induction of biofilm formation and the in vitro treatments were performed according to previous reports [36,51]. Briefly, 20 μL of the suspensions and 180 μL of YPD (Yeast Extract-Peptone-Dextrose) medium were transferred to the wells on a 96-well plate. The plates were incubated at 37 °C for 72 h. Following the incubation time, the wells were carefully aspirated and washed twice with 200 μL of PBS buffer. The prewashed wells were stained with 110 μL of 0.4% aqueous violet crystal solution for 45 min. Thereafter, they were washed three times with 200 μL of ultrapure sterile water (Milli-Q) and the biofilm was discolored using 200 μL ethanol for 45 min. Later, 100 μL of the well content was transferred to a new microplate and biofilm formation was evaluated using the optical density difference between the biofilm formed and the control well, by reading the absorbance in a spectrophotometer adjusted with a wavelength of 595 nm. Each strain was tested three times, and the absorbance values of the control wells were subtracted from the tested wells to minimize interference [36].

To analyze the impact of the extracts, following 48 h of biofilm production, the wells were filled with 200 μL of either the *G. hirsutum* L. or fluconazole following the serial dilution using RPMI 1640 medium to achieve each concentration as previously reported. Untreated wells and biofilm-free wells were included as positive and negative controls, respectively. The microdilution plates were incubated for 72 h at 35 °C. After this period, the biofilm was quantified as previously described [51].

4.7. Effects of G. hirsutum Extracts on the Morphological Transition of Candida Yeasts

A sterile microscope slide was filled with 3 μL of depleted Potato Dextrose Agar (PDA), to which the extracts were added at MC-based concentrations (MC/4, MC/8, and MC/16) and mounted in humid chambers. A yeast loop was removed from a previously inoculated plate and two parallel grooves were made in the solidified medium, over which a sterile coverslip was placed. The chambers were incubated and visualized under an optical microscope (AXIO IMAGER M2-3525001980—ZEISS—Jena, Germany) using a 20× objective after an incubation period of 24 h at 37 °C. The emission of filament was verified, and the images were captured. Growth and treatment (fluconazole) controls were also applied. Thereafter, the images were analyzed by measuring the total length of the inoculum striae and the length of hyphal or pseudohyphal extensions in μm using the Zen 2.0 software, ZEISS—Jena, Germany [52].

4.8. Statistical Analysis

Data derived from biofilm production and the cell viability curves were analyzed using the arithmetic mean from triplicates or quadruplicates, respectively, for each tested concentration. The differences were analyzed using two-way ANOVA with Bonferroni's post hoc test. The average hyphal length was calculated and analyzed by one-way ANOVA followed by Bonferroni's correction for multiple comparisons. These analyses were performed using the GraphPad Prism software version 5.0. Statistical significance was considered when $p < 0.05$.

5. Conclusions

Both aqueous and hydroethanolic extracts of *G. hirsutum* are characterized by the known bioactive secondary metabolites such as catechin and epicatechin, showing a predominance of phenolic compounds. Nevertheless, the extracts had quantitative differences in their constituents as the hydroethanolic extract demonstrated higher relative intensity for coumaroylglucaric acid, catechin, chlorogenic acid, and

epicatechin, while the aqueous extract showed higher relative intensity for quercetin *O*-hexoside-pentoside.

While both extracts showed weak-to-moderate intrinsic antifungal activity, they were found to downregulate important virulence factors of both standard and clinical isolates of *Candida* spp. The aqueous was found to enhance the antifungal activity of fluconazole against *C. albicans* INCQS 40006 and *C. tropicalis* URM 4262, and the hydroethanolic extract combined with fluconazole showed potentiated inhibitory effects on the growth of *C. albicans* INCQS 40006. *G. hirsutum* extracts showed a strain-selective and extract type-dependent capacity of inducing biofilm eradication, highlighting the effectiveness of the aqueous extract against the standard and clinical isolates of *C. tropicalis*, especially at lower concentrations. However, a significant increase in biofilm content was observed for most experimental conditions; further studies investigating the mechanisms underlying this phenomenon may significantly contribute to the targeted development of antibiofilm agents from this species. Finally, the aqueous extract inhibited *C. albicans* hyphal growth on both standard and clinical isolate strains, while the hydroethanolic extract inhibited hyphal growth only for this clinical isolate.

In conclusion, the present research found that *G. hirsutum* has the potential to enhance the virulence of standard and clinical isolates of *Candida albicans* and *Candida tropicalis* in vitro. However, such phenomenon was found to be highly affected by experimental variables including type and concentration of extracts as well as the species and strain of *Candida*. Therefore, further research is strongly recommended to better characterize the antifungal properties of this species about its chemical constituents and their mechanisms of action.

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analysis; H.D.M.C. and B.K.: project administration.; J.R.-F.: writing, review, and critical analysis; M.F.B.M.-B.: supervision, project administration. All authors have read and agreed to the published version of the manuscript.

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4.2 CAPÍTULO 2 – Manuscrito 1 – UPLC-QTOF-MS/MS analysis of Upland cotton extracts and anti-*Candida* activities

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Abstract: *Gossypium hirsutum* L. (Upland cotton) is a species with many biological activities, used in folk medicine around the world. (1) Background: The aim was to chemically analyze aqueous and hydroethanolic extracts of *G. hirsutum* L. roots (AERG, HERG) and evaluate them for inhibition of growth factors and virulence as activity against *Candida albicans* (INCQS 40006; URM 4387) and *Candida tropicalis* (INCQS 40042; URM 4262). (2) Methods: The extracts were investigated chemically by UPLC-QTOF-MS/MS, the cell viability curve, and 50% of the inhibitory effect over the fungal (IC_{50}). The effect of the compounds was determined by intrinsic and combined activity. To assess virulence factors the Crystal Violet assay (CV) was executed. (3) Results: Chemistry analysis revealed anthraquinones, phenolic acid and flavonoids. The intrinsic and combined activity of the extracts has a dose-dependent response with increased concentrations. HERG extract showed results that may indicate a potentiated effect of fluconazole. The extracts did not show effective action in reducing the biofilm. In the anti-pleomorphic activity, the extracts were effective against CA URM 4387. (2) Conclusions: The extracts *G. hirsutum* showed antifungal action at high concentrations and has potentiated the effect of fluconazole and interfered in the expression of the virulence factor, however, these effects did not apply to all strains.

Keywords: Malvaceae family; *Gossypium hirsutum* L.; chemistry analysis; biofilm eradication; anti-pleomorphism.

1. Introduction

Upland cotton is included in the *Gossypium* genus, Malvaceae family. It is among the most economically important staple crops in the textile industry worldwide [1], [2]. Usually, it is used by people and communities in folk medicine around the world against respiratory problems, reproductive health and genitourinary infections. It is also used for kidney and dermal diseases, the treatment of malaria, hepatitis, fever, pneumonia, flu, coughing with secretions, sore throat, nose disorders, bloody diarrhea, and as a purgative [3], [4], [13] – [15], [5] – [12].

Cotton can be used in the forms of a decoction, infusion, mixtures, maceration, crushed and juice. The most used parts are the leaves, the roots and the seeds, followed by the flowers [3], [4], [9]. This plant is differentiated for its phytochemical contents, antioxidant activities, antimicrobial activities against bacteria, protozoa, and fungal strains, like the *Candida* spp., which is an opportunistic fungus [16] – [19].

Candida spp. is a dimorphic fungus with the ability to adapt and proliferate easily, having a series of virulence factors that allow it to survive mainly in mucous tissues, causing infections [20], [21]. *Candida* can be fatal with significant rates of morbidity and mortality. Polyene, azoles, fluoropyrimidines, and echinocandins are used indiscriminately, and it is believed that it has led to the development of molecular resistance mechanisms avoiding the fungicidal or fungistatic effect of these drugs. The development of new formulations can be promising with bioactive compounds for the treatment of *Candida* diseases using medicinal plants such as *G. hirsutum* [22] – [24].

Upland cotton has been tested against *Candida albicans* which advances skin infections, mucosal infections, or systemic infections and showed inhibitory activity against them [25] – [27]. In this research, we used upland cotton based on previous ethnobotanical research that has reported the use of roots to care for women's intimate health [28]. Therefore, we investigated the phytochemical compounds of the aqueous

and the hydroethanolic extracts of *G. hirsutum* L. roots (AERG, HERG) and evaluated them for the inhibition of growth factors and virulent activity against *Candida albicans* (INCQS 40006; URM 4387) and *Candida tropicalis* (INCQS 40042; URM 4262).

2. Materials and Methods

2.1 Collection area and plant material

The roots were collected from three upland cotton specimens located in Brejinho, in the rural area of the Araripe municipality, south of the state of Ceará, Brazil. The geographical coordinates, south latitude and west longitude of Greenwich are: 7°12'12.5"S 40°01'10.2"W; 7°12'23.1"S 40°00'50.7"W; 7°13'38.5"S 39°59'44.5"W. The collection took place in April 2019, from 7:30 to 9:00 in the morning. The plant material was sent to the laboratory, cleaned, and weighed. The exsiccate specimen produced can be found deposited in the Herbarium Dárdano de Andrade Lima of the Regional University of Cariri – URCA under n° 65.2019 and identified as *Gossypium hirsutum* L.

2. 2 Extract preparation

The *G. hirsutum* L. aqueous and hydroethanolic extracts from the roots (AERG and HERG) were prepared according to Matos [29]. The 1. 265,4 g of roots were cut to increase their surface area, were dried at room temperature, and ground in a mechanical mill. Subsequently, both were added to distilled sterile water or alcohol 70% and maintained in a container protected from light and air. After 72 h the extracts were filtered and taken to a Spray drying (130 °C, flow rate 0,5 L/H, blower control 1.95, outlet temperature 84.6, flowmeter 40L/min air), producing a crude extract of 2,273 and 0,624 g, respectively.

2.3 Compound identification through ultra-performance liquid chromatography coupled to quadrupole/time of flight system (UPLC–QTOF-MS/MS)

The samples were filtered through Syringe filters (PTFE, 0.2 μm pore and 13 mm diameter, Millipore Millex) and 5 μL of each sample was injected into the UPLC system (Waters Co., Milford, MA, USA). The instrumental UPLC analysis was

performed in an ACQUITY UPLC BEH column (150 × 2.1 mm, 1.7 μm, Waters Co.) on a Waters Acquity UPLC system. The column temperature was set to 40 °C. The binary gradient elution system consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), with a linear gradient from 2 to 95% B (0–15 min), 100% (15.01-17 min), 2% (17.01-21 min) and a flow rate of 0.4 $ml.min^{-1}$. Profiling was performed by coupling the Waters ACQUITY UPLC system to the Q-TOF Premier mass spectrometer (Waters MS Technologies, Manchester, UK) with electrospray ionization interface (ESI) in the negative ionization mode. The desolvation gas used was nitrogen and set to 350 °C with a flow rate of 500 L/h. The capillary and cone voltages were adjusted to 2.6 kV and 0.5 V, respectively. Mass accuracy and reproducibility were maintained by infusing lock mass (leucine-enkephalin, 0.2 ng/uL; [M-H] - ion at *m*/*z* 556.2771). MS data were collected for *m*/*z* values in the range of 110–1180 Da with a scan time of 0.1 over an analysis time of 19 min. The accurate mass and molecular formula assignments were obtained with the MassLynx 4.1 software (Waters MS Technologies).

2.4 Cell viability curve and IC_{50} determination

One-hundred microliter of a solution containing 1350 μL of doubly concentrated SDB (Sabouraud Dextrose Broth) plus 150 μL of the fungal strain inoculum (corresponding to 10% of the total solution), respectively, were added to each well of a microdilution plate. Thereafter a serial dilution of the 100 μL extracts solution was performed (concentrations varied from 8192 μg/ml to 8 μg/ml for fungal tests). Microdilutions were performed in quadruplicates and were taken to an incubator for 24 h at 37 °C. As for the fungal plates, spectrophotometric readings were performed in an ELISA apparatus (Termoplate®), using a 630 nm wavelength with the results being used to obtain a cell viability curve and IC_{50}. Dilution (with 0.9% sodium chloride instead of the inoculum), medium sterility, and growth controls were also performed [30].

2.5 Fluconazole action modulation test

To verify the combined action of the natural product with commercial drugs, the method proposed by Coutinho and collaborators [31] was used, in which the product is tested at a sub-inhibitory concentration (MC/16 – fungi). The plates were filled in the numerical sense by adding 100 μL of a solution containing the natural substance, culture medium (SDB), and 150 μL of the microbial suspension (corresponding to 10% of the solution). Growth and dilution controls were prepared. Thereafter, serial microdilution was performed with 100 μL of the drug. The plates were incubated at 37 °C for 24 h. Reading was performed as described in the previous session. If a positive action was observed for the extracts in combination with the drug, the effect was termed enhancer drug potentiating effect.

2.6 Minimal fungal concentration – MFC

A sterilized rod was introduced into each well of the microdilution plate from the MC test, which was homogenized and sub-cultured in a petri dish containing SDA (Sabouraud Dextrose Agar) and a tested concentrations guide, except for the sterility control. The plates were incubated at 37 °C and readings were performed after 24 h by observing the growth or suppression of *Candida* colonies [32]. The concentration in which there was no fungal colony growth was considered the MFC of natural product. Subsequently, the tests of the combined activity and the morphological transition were performed from the sub-inhibition concentrations of MFC. For those whose MFC cannot be visualized, these were based on the Matriz Concentration (CM) of dilution of the products (16.384 μg/ml).

2.7 Biofilm production evaluation and Candida genus eradication

2.7.1 Used strains

Standard lineages were obtained from the Oswaldo Cruz Culture Collection (FIOCRUZ) of the Brazilian Institute of Quality Control in Health (INCQS). *C. albicans* INCQS 40006, *C. tropicalis* INCQS 40042, and isolated strains were obtained from the Culture Collection of the Federal University of Pernambuco - URM (Recife Mycology

University) *C. albicans* URM 4387, *C. tropicalis* URM 4262. The strains were inoculated in Sabouraud Dextrose Agar (SDA, KASVI) and incubated for 24 h at 37 °C. An initial suspension was subsequently prepared in 5 ml of sterile saline solution (NaCl, 0.85% saline) and its density was adjusted according to the 0.5 MacFarland scale with 90% transmittance determined by spectrophotometry, using a wavelength of 530 nm. This provides a standard yeast concentration containing from 1×10^6 to 5×10^6 cells per mL.

2.7.2 Preparation of solutions for biofilm assays

Products solutions were prepared through dilution of 0.15 g of each extract followed by further dilution in 1 mL of dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany). The tested concentrations varied from 16.384 μg/mL to 128 μg/mL. Fluconazole (Pfizer) was used as a reference antifungal drug with concentrations varying from 64 μg/mL to 0.5 μg/mL.

2.7.3 Qualitative evaluation of Candida spp. biofilm development

Qualitative evaluation of biofilm formation capacity was performed using the visual method adapted from Shin and collaborators [33]. After seeding the isolates in the Sabouraud agar medium and preparing the suspensions as described above, 20 μL were inoculated into 180 μL of liquid Sabouraud contained in the microplate wells, which were maintained at 35 °C for 72 h without shaking. The contents were then aspirated and the wells were washed with distilled sterile water and a Fuchsin dye (QEEL-Specialized Chemistry Erich Ltda.) was added to evaluate according to the intensity of the staining. Staining was evaluated visually and classified as follows: 1) Strongly stained: when the biofilm was intensely stained allowing the correct determination of the contour of the areas containing the biofilm; 2) Average staining: when the biofilm was stained more weakly but still allowed the determination of the contour of the areas containing the biofilm; 3) Poor staining: when the biofilm was not stained making it impossible to distinguish the color of the plate and the characteristic color of the stain. The interpretation represented a strong, moderate, and weak biofilm formation activity, respectively.

2.7.4 Biofilm formation induction

Ninety-six well microdilution plates were used for biofilm production where 20 μL of the suspensions were transferred to microplate wells containing 180 μL of YPD (*Yeast Extract-Peptone-Dextrose*). The plates were incubated at 37 °C for 72 h. Following the incubation time, the wells were carefully aspirated and washed twice with 200 μL of PBS buffer. The prewashed wells were stained with 110 μL of 0.4% aqueous violet crystal solution for 45 min. Thereafter, they were washed three times with 200 μL of ultrapure sterile water (Milli-Q) and the biofilm was discolored using 200 μL ethanol for 45 min. In the end, 100 μL from each well were transferred to a new microplate and biofilm formation was evaluated using the optical density difference between the biofilm formed and the control well, by reading the absorbance in a spectrophotometer adjusted with a wavelength of 595 nm. Each strain was tested three times and the absorbance values of the control wells were subtracted from the tested wells to minimize interference [34].

2.7.5 Biofilm treatment

Biofilms were formed onto microdilution plates, as aforementioned. After a 48h period, wells with biofilms were filled with 200 μL of the *G. hirsutum* L. (AERG and HERG) aqueous and hydroethanolic extracts, and Fluconazole serial dilutions. The extracts and standard drugs were diluted in RPMI 1640 to achieve each concentration. Untreated biofilm wells and biofilm-free wells were included as positive and negative controls respectively. The microdilution plates were incubated for 72 h at 35 °C. After each period, the biofilm was quantified as previously described [35].

2.8 Evaluation of the fungal virulence effect

Three milliliters of depleted PDA (Potato Dextrose Agar) mixed with the extracts at a concentration defined by the MC (MC/4, MC/8, and MC/16) were poured on a microscopy slide (sterile) and mounted in sterile humid chambers. The inoculum was removed from the previously inoculated Petri dishes and two parallel grooves

were made on an already solidified medium, over which a sterile plate was then placed. The chambers were incubated and visualized under an optical microscope (AXIO IMAGER M2-3525001980 – ZEISS - Germany) using a 20X objective after a period of 24 h (37 °C).

Filament emissions or inhibitions were verified and the images were captured. Antifungal and growth controls were also performed. Subsequently, the photos were analyzed by measuring the total length of the inoculum striae and the length of hyphal extensions using the Zen 2.0 software. Data were then taken for statistical analysis [36].

2.9 Statistical analysis

Measurements to biofilm test, *GraphPad Prism* v. 5.0. (Free version) the software was used. Data were analyzed using the arithmetical mean from triplicates for each tested concentration and posteriorly analyzed using a two-way ANOVA ($P < 0.05$; *$P < 0.1$; ****$P < 0.0001$), comparing values for each extract concentration, point by point, using Bonferroni's post hoc test. A general behavior comparison for each substance against the tested strains was inferred through the already cited analysis.

Measurements of all hyphal filaments were taken from five randomly selected areas in each stria for each concentration to assess virulence. The average hyphal filament length was calculated and analyzed by a one-way ANOVA followed by Bonferroni's correction for multiple comparisons, verifying the values according to the concentration of the product.

3. Results

3.1. Compound identification through UPLC–QTOF-MS/MS

The identification using the molecular formula, fragment pattern, error, and references from literature data resulted in eighteen peaks with fourteen compounds tentatively identified. The chromatogram of the aqueous and hydroalcoholic extract is shown in figure 1 by elution order.

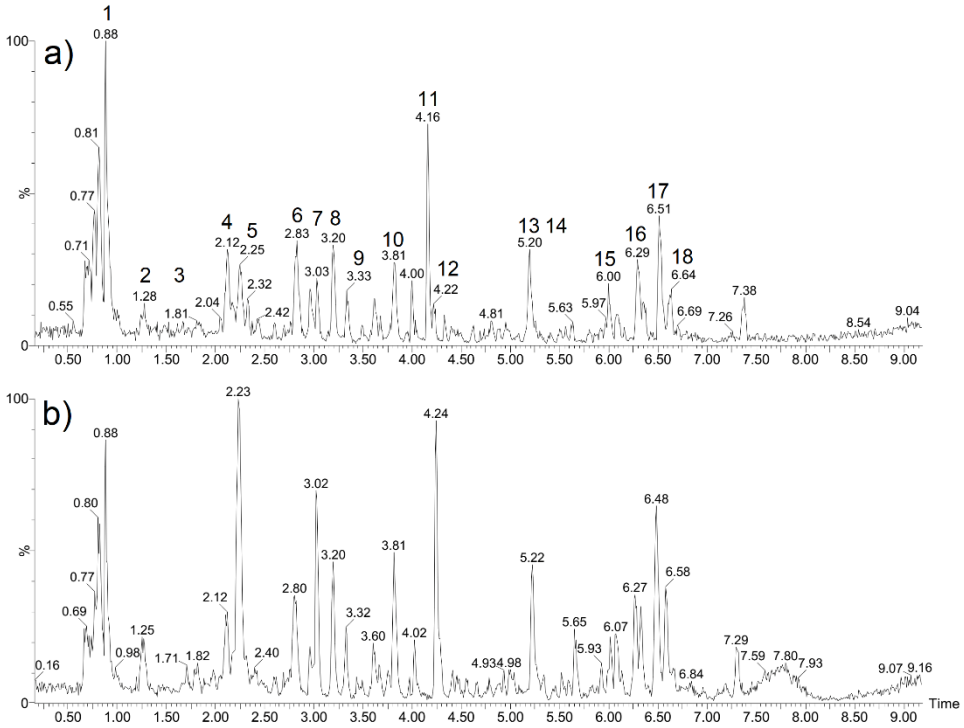

Figure 1. UPLC-QTOF-MS/MS chromatogram of the aqueous (a) and hydroethanolic (b) extract from the roots of *G. hirsutum* in negative mode.

Table 1. UPLC-QTOF-MS/MS identification of compounds from extract roots of *Gossypium hirsutum* L.

Peak	Rt min	[M-H]- Observed	[M-H]- Calculated	Product Ions (MS/MS)	Empirical Formula	Ppm error	Putative Name	Ref.	A[a]	H[a]
1	0.88	133.0138	133.0137	-	$C_4H_5O_5$	0.8	Malic acid	STD *	++	+
2	1.28	191.0190	191.0192	111 [M-H-CO_2-2 H_2O]-	$C_6H_7O_7$	-1.0	Citric acid	STD *	+	+
3	1.82	169.0502	169.0501	125 [M-H-CO_2]-	$C_7H_6O_5$	0.6	Gallic acid	STD *	+	++
4	2.12	593.1282	593.1295	425, 407, 305 [M-H-$C_{15}H_{12}O_6$]-, 289 [M-H-$C_{15}H_{12}O_7$]-	$C_{30}H_{25}O_{13}$	-2.2	(epi)-Catechin-(epi)-gallocatechin	37	+	++
5	2.25	305.0656	305.0661	179 [M-H-$C_6H_6O_3$]-	$C_{15}H_{13}O_7$	-1.6	Gallocatechin	STD *	+	++ +
6	2.83	353.0580	353.0567	191 [M-H-$C_9H_6O_3$]-, 179 [M-H-$C_7H_{10}O_5$]-	$C_{16}H_{17}O_9$	3.7	3-*O*-Caffeoylquinic acid	STD *	+	+
7	3.03	289.0711	289.0712	-	$C_{15}H_{13}O_6$	-0.3	Catechin	STD *	+	++
8	3.20	425.0859	425.0873	-	$C_{22}H_{17}O_9$	-3.3	Unknown	-	+	+

9	3.33	349.0562	349.0560	-	$C_{16}H_{13}O_9$	0.6	Unknown	-	+	+
10	3.81	517.1067	517.1067	437 [M-H-OSO_3H]$^-$, 275 [M-H-OSO_3H-Glu]$^-$	$C_{21}H_{25}O_{13}S$	0.0	13,15-Dihydroxy-7-O-(6′-O-sulfate-β-D-glucopyranosyl)-Desoxyhemigossypol	[38]	+	++
11	4.16	441.1761	441.1761	279 [M-H-Glu]$^-$	$C_{21}H_{29}O_{10}$	0.0	Phaseic acid glucoside	[39]	+	++
12	4.22	441.1763	441.1761	279 [M-H-Glu]$^-$	$C_{21}H_{29}O_{10}$	0.5	Phaseic acid glucoside	[39]	+	++
13	5.22	409.2029	409.2015	381 [M-H-CO]$^-$	$C_{25}H_{29}O_5$	3.4	Heliocide H isomer	[40]	+	++
14	5.46	409.2020	409.2015	381 [M-H-CO]$^-$	$C_{25}H_{29}O_5$	1.2	Heliocide H isomer	[40]	+	+
15	6.00	501.1077	501.1067	421 [M-H-OSO_3H]$^-$, 259 [M-H-OSO_3H-Glu]$^-$	$C_{21}H_{25}O_{12}S$	2.0	13-Hydroxy-7-O-(6′-O-sulfate-glucopyranosyl)-desoxyhemigossypol	[38]	+	++
16	6.29	479.2091	479.2070	317 [M-H-Glu]$^-$	$C_{21}H_{19}O_{13}$	4.4	Gossypetin glucoside (Gossytrin)	[41]	+	++
17	6.51	395.1371	395.1371	-	$C_{12}H_{27}O_{14}$	-1.3	Unknown	-	+	+
18	6.69	453.1927	453.1913	-	$C_{15}H_{33}O_{15}$	-0.2	Unknown	-	+	+

Rt: retention time; A [a]: aqueous extract; H [a]: hydroethanolic extract. +, ++, +++ Relative intensity ratio shows the higher compound concentration in each extract. * STD, comparison with an authentic standard.

The peaks **1**, **2**, **3**, **5**, **6**, and **7** were identified as malic, citric, gallic, 3-O-caffeoylquinic acid, catechin, and gallocatechin, respectively, using standard comparison. The peak **4** was identified as (epi)-Catechin-(epi)-gallocatechin dimeric by the fragments at *m*/*z* 305, gallocatechin, and *m*/*z* 289, catechin [37]. The peaks **10** and **15** showed gossypol derivatives and were identified previously as 13,15-Dihydroxy-7-O-(6′-O-sulfate-β-D-glucopyranosyl)-desoxyhemigossypol and 13-Hydroxy-7-O-(6′-O-sulfate-glucopyranosyl)-desoxyhemigossypol, respectively. Derivatives of abscisic acid are common in the *Gossypium* genus [38]. Peak **11** and **12** were identified as phaseic acid glucoside using the loss of glucose (162 Da) and phaseic acid ion at *m*/*z* 279. The peaks **13** and **14** were tentatively identified as hydroxyanthraquinones

derivatives isomers, heliocide H. However, the exact elution order was not established [39, 40]. Peak **16** was identified as similar to peak 11 using the loss of glucose (162 Da) and the gossypetin ion at *m*/*z* 317 [41].

3.2 Candida Activity

3.2.1 Cell viability and IC_{50} using *Gossypium hirsutum* L. extracts

Cellular viability curves are shown in graphs in Figures 2 and 3. The intrinsic activity of the extracts showed a reduction of microorganisms at the 8192 μg/mL concentration for almost all tested strains to AERG and HERG, except to *C. albicans* INCQS 40006 and *C. tropicalis* INCQS 40042. Where this effect is considered clinically insignificant due to a high effective concentration in the in vitro test. Fluconazole affected similar to that of the extract for all microorganisms, except for the isolate of *C. albicans* where it obtained an inhibiting effect at low concentrations (64 μg/mL).

The extracts' modulatory potential evaluation over Fluconazole against *Candida* strains activity demonstrated at sub-inhibitory concentrations (MC/16: 1024 μg/mL) caused microorganismal growth inhibition in the viability curve in some *Candida* strains to the drug in isolation. The modulator effect demonstrated potentiating effect of AERG/FCZ against the standard strain of *C. albicans* and the isolate *C. tropicalis*, and HERG/FCZ against the standard strain of *C. albicans* (1024 μg/mL) and *C. tropicalis* strains (512 μg/mL).

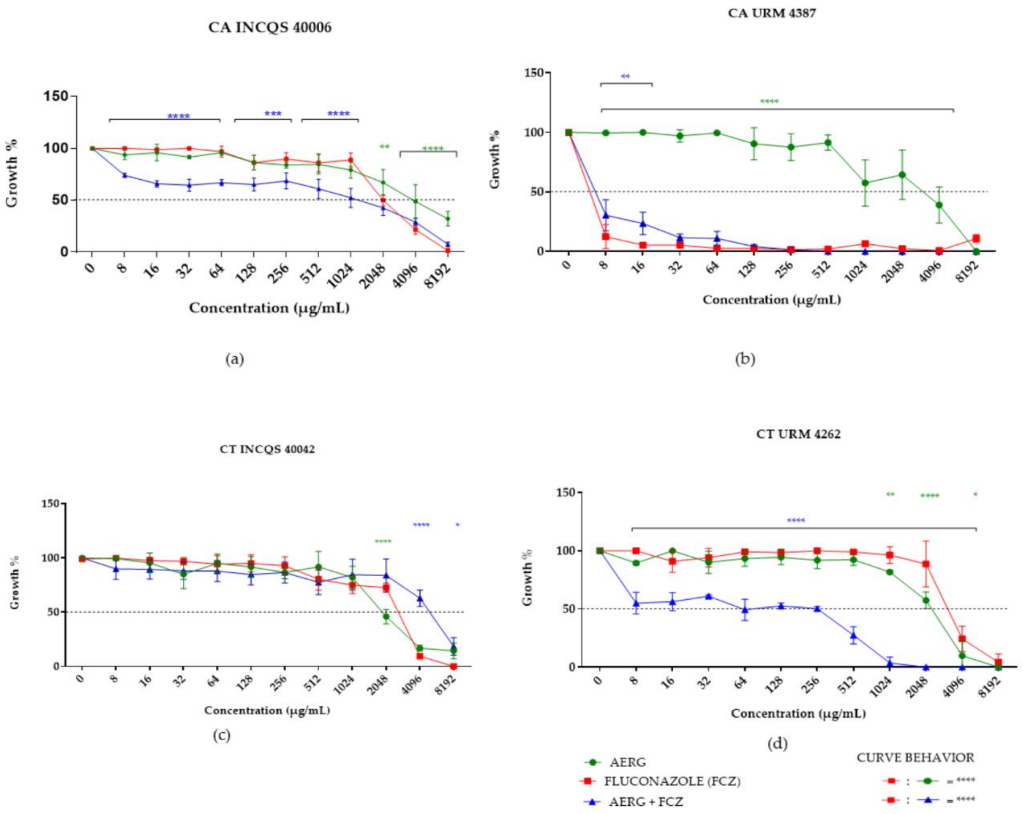

Figure 2. *Antifungal actions of Fluconazole alone and associated with AERG in the sub-inhibitory concentration MC/16: 1024 µg/mL against: (a) CA INCQS 40006; (b) CA URM 4387; (c) CT INCQS 40042; (d) CT URM 4262*. CA: *Candida albicans*; CT: *Candida tropicalis*; FCZ: FCZ: Fluconazole; HERG: hydroethanolic extract of root *G. hirsutum*; INCQS: Collection Cultures of the National Institute of Quality Control in Health; URM: University Recife Mycology.

When analyzing the 50% inhibitory effect over the fungal population (Table 2) AERG showed a potentiating effect against *C. albicans* INCQS 40006 (IC_{50} of 516.9 µg / mL) compared to the standard drug (IC_{50}: 2125 µg / ml). The *C. tropicalis* URM 4262 strain showed a potentiating effect, obtaining an IC_{50} of 52.43 µg / mL about the isolated standard drug IC_{50}: 3196 µg / mL. As for the potentiating effect of HERG, it stood out against the standard strain of C. *albicans* (20.74 µg / mL) and the standard and isolated strains of *C. tropicalis* (7.535 µg / mL and 3.671 µg / mL), showing a possible relevant antifungal activity when associated with fluconazole.

Table 2. IC_{50} (50% inhibitory concentration of the fungal population – μg/mL) of the AERG and HERG against different strains of *Candida.*

	CA INCQS 40006	CA URM 4387	CT INCQS 40042	CT URM 4262
AERG	4083	2156	1939	2083
FLUCONAZOLE	2125	0,036	2476	3196
AERG + FCZ	516,9	3,044	5335	52,43
HERG	2467	591,0	2752	1773
FLUCONAZOLE	2125	0,036	2476	3196
HERG + FCZ	20,75	7,963	7,535	3,671

Legend: AERG (aqueous extract of root *G. hirsutum*), HERG (hydroethanolic extract of root *G. hirsutum*); CA: *Candida albicans*; CT: *Candida tropicalis*; FCZ: Fluconazole; INCQS: Collection Cultures of the National Institute of Quality Control in Health; URM: University Recife Mycology.

The extracts-antifungal combination showed lower inhibitory concentrations when compared to the individual action of HERG, besides, it is worth highlighting the better-combined effect also obtained by HERG against *C. albicans* INCQS 40006, *C. tropicalis* INCQS 40042, and *C. tropicalis* URM 4262 strains. AERG was effective only against *C. albicans* INCQS 40006 and *C. tropicalis* URM 4262. The MC was defined as the concentration which caused the fungicidal effect of colony growth. The extract alone AERG, HERG, and FCZ demonstrated fungistatic effect (MC ≥ 16,384 μg / mL) in front of all strains. While for AERG in combination with FCZ showed a fungicide effect in all concentrations with MC – 4096 μg / mL, except for *C. albicans* URM 4387 which was MC -1024 μg / mL. HERG combined with FCZ showed a fungistatic effect in the presence of *C. albicans* INCQS 40006 (MC ≥ 16,384 μg / mL) and fungicide effect for the other strains MC = 1024 μg / mL for *C. albicans* URM 4387 and *C. tropicalis* MC = 8192 μg / mL.

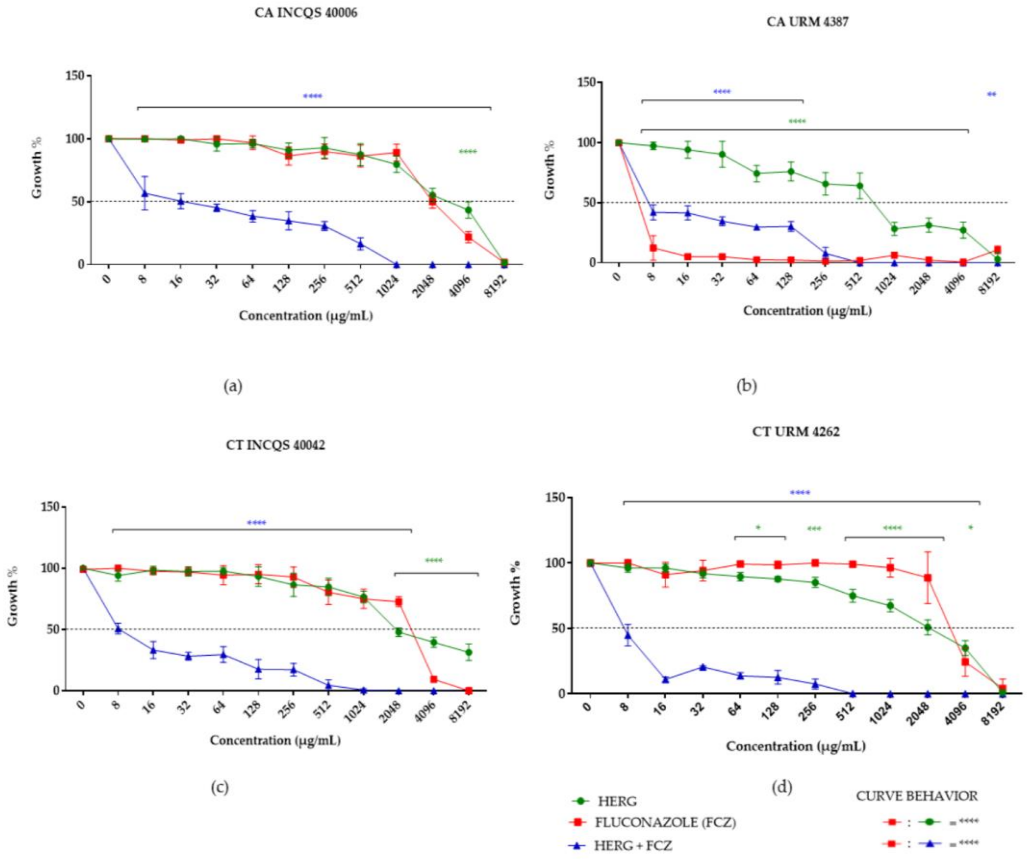

Figure 3. Antifungal actions of Fluconazole alone and associated with HERG in the sub-inhibitory concentration MC/16: 1024 µg/mL against: (a) CA INCQS 40006; (b) CA URM 4387; (c) CT INCQS 40042; (d) CT URM 4262. CA*: Candida albicans; CT: Candida tropicalis; FCZ: Fluconazole; HERG: hydroethanolic extract of root* G. hirsutum*; INCQS: Collection Cultures of the National Institute of Quality Control in Health; URM: University Recife Mycology.*

3.2.2 Biofilm Assay

The biofilm was stained using the Crystal Violet (CV) method to quantify bulk biofilm production. Fig. 4 shows the biofilm formation capacity of the tested fungal strains with the interpretation being performed taking into account color intensity. *C. tropicalis* URM 4262 (0,433 nm) which presented maximal biofilm production, followed by URM 4387 (0,262 nm), INCQS 40042 (0,238 nm), and *C. albicans* INCQS 40006 (0,149 nm).

Figures 5 and 6 present the biofilm induction results obtained based on optical density values, with all yeast isolates being capable of forming biofilms within 72 h, with different intensities. Analyzing the graphs, the extracts AERG and HERG were

not effective in reducing the production of the biofilm against any of the strains. On the contrary, the strains showed adaptation in the presence of extracts since there was an increase in biofilm when we analyzed the graphs. Despite that, *C. tropicalis* 4262 at the concentration of 128 μg/mL treated with HERG and when treated with AERG at concentrations of 2048 μg/ml and 512 μg/mL demonstrated inhibition when compared to GC, but not when compared to FCZ. Figure 6 shows the *C. albicans* biofilm formation results for the INCQS 40006 and URM 4387, with no inhibition effect in the presence of the extracts.

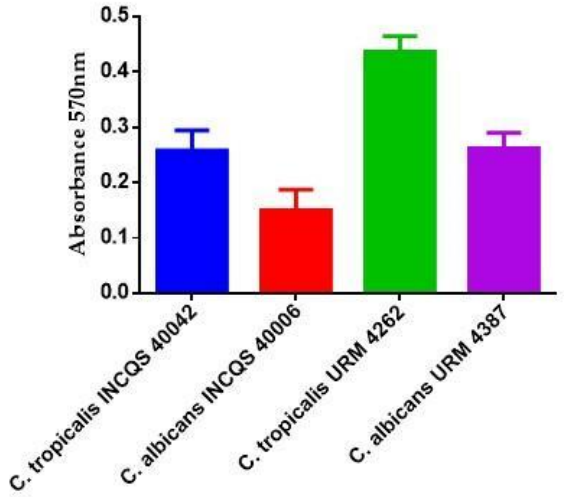

Figure 4. The capacity of biofilm formation by fungal strains by (%) growth (GC- Growth Control). *C. tropicalis INCQS 40042 - moderate; C. albicans INCQS 40006 – moderate; C. tropicalis URM 4262 – strong; C. albicans URM 4387 – moderate. Legend: INCQS - Brazilian Institute of Quality Control in Health.; URM – University Recife Mycology.*

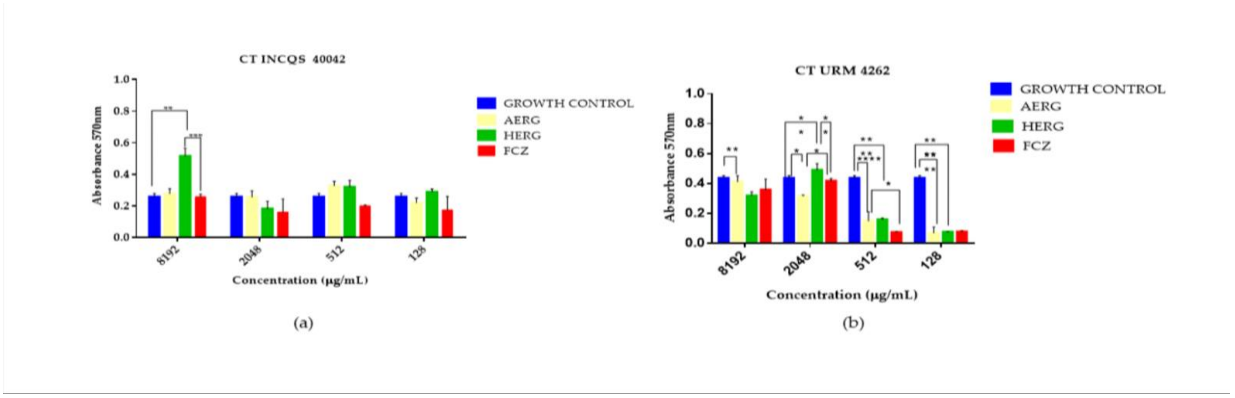

Figure 5. Eradication of *C. tropicalis* INCQS 40042 (a) and *C. tropicalis* URM 4262 (b) biofilm by hydroethanolic, and aqueous extracts of *G. hirsutum*, compared to fluconazole. On the columns is the statistical relevance of the substance in relation: growth control/fluconazole. $p < 0.05$; * ($p > 0.01$); **** ($p < 0.0001$). GC - Growth control. AERG - Aqueous extract of the roots of *G. hirsutum*. HERG - Hydroethanolic extract of the roots of *G. hirsutum.*

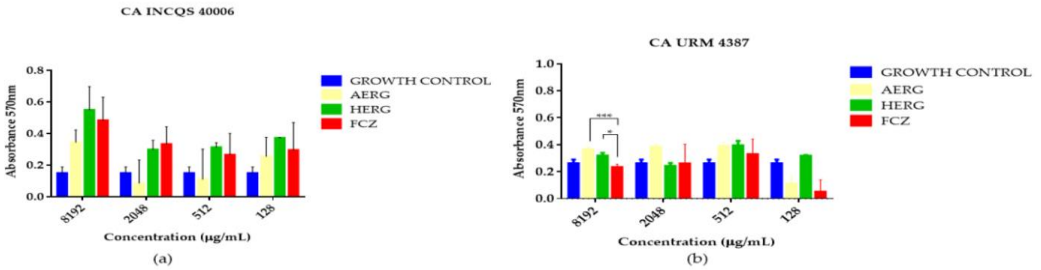

Figure 6. Eradication of *C. albicans* INCQS 40006 (a) and *C. albicans* URM 4387 (b) biofilm by hydroethanolic, and aqueous extracts of *G. hirsutum*, compared to fluconazole. On the columns is the statistical relevance of the substance in relation: growth control/fluconazole. $p < 0.05$; * ($p > 0.01$); **** ($p < 0.0001$). GC. Growth control. AERG - Aqueous extract of the roots of *G. hirsutum*. HERG - Hydroethanolic extract of the roots of *G. hirsutum*.

3.2.3 Extract *Gossypium hirsutum* action over a fungal virulence factor

The AERG and HERG were tested at the MC/4 – 4.096 µg/mL, MC/8 – 2.048 µg/mL, MC/16 – 1.024 µg/mL concentrations in the fungal morphology analysis (Figure 9). Figure 7 displays graphics from the antifungal effect, where it demonstrates the AERG results. This extract inhibited the hyphae formation in the MC/4 concentration to *C. albicans* INCQS 40006 and *C. tropicalis* URM 4262 as well as was effective against *C. albicans* URM 4387 in all concentrations to AERG. HERG against *C. albicans* URM 4387 and *C. tropicalis* URM 4262 inhibited the hyphae formation. However, HERG indicates that virulence stimulation occurred of the highest to the lowest concentration in the presence of other strains (Fig. 8). For *C. albicans* URM 4387, the extracts of this study showed potential in inhibiting the morphological transition of yeasts, avoiding the development of the morphological transition of hyphae, which could consequently reduce infection by *Candida*.

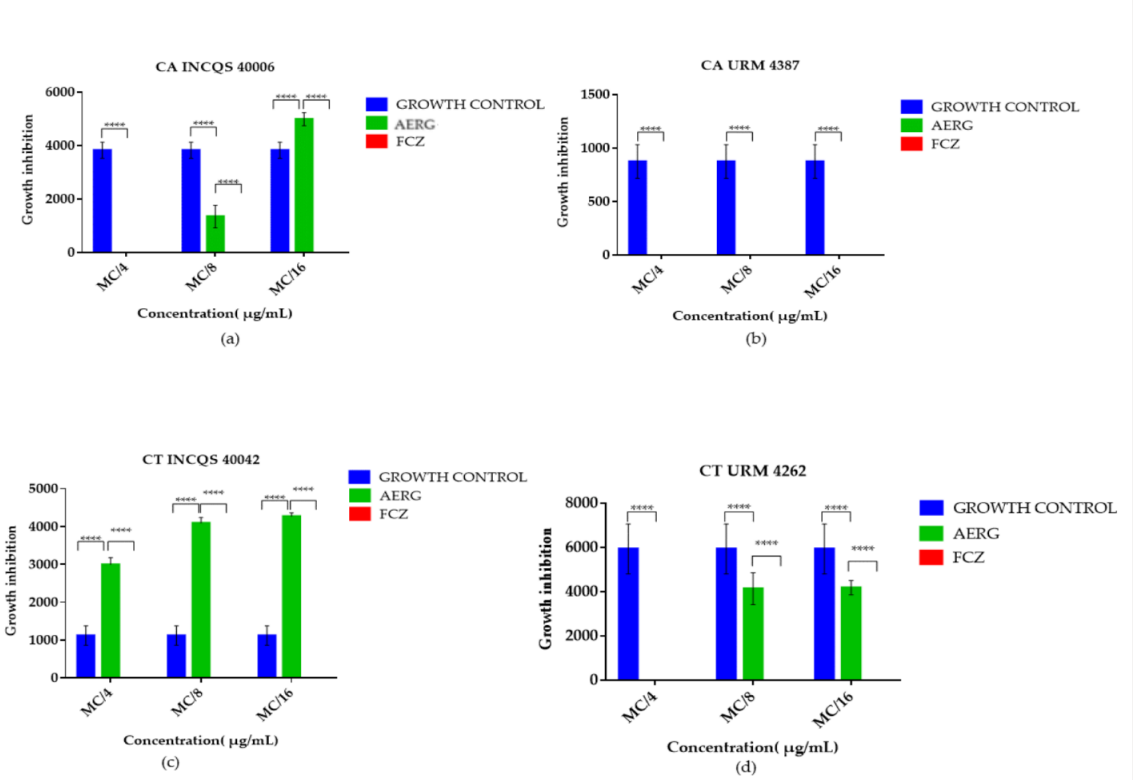

Figure 7. Antifungal effect of Fluconazole and AERG (aqueous extract of the root *of G. hirsutum*) on the polymorphism of *Candida* spp., from strains*:* (a) CA INCQS 40006; (b) CA URM 4387; (c) CT INCQS 40042; (d) CT URM 4262. CA: *C. albicans*; CT: *C. tropicalis*; CG: Growth control; FCZ: Fluconazole; INCQS: Culture Collection of the National Institute for Quality Control in Health; URM: Recife University of Mycology. MC / 4 - 4,096 µg/mL, MC / 8 - 2,048 µg/mL, MC / 16 – 1,024 µg/mL

4. Discussion

In this study, the roots extracts showed flavonoids (peak 4, 5, 7), terpenes (peak 10 and 15), anthraquinones (peak 13 and 14), and phenolic acid (peak 3). In the chemical analysis of the S. tuberculata (Malvaceae) root infusions, the presence of flavonoids with antimicrobial activity, especially active against non-albicans Candida (NAC) was demonstrated, with a minimum inhibitory concentration (MIC) of 3.9–62.5 µg/mL and 1.95–31.25 µg/ml, respectively [40] like this study. According to Zacchino and collaborators [46], combinations of terpenes with antifungals result in increased potentiation capacity and activity of antifungals, as well as the presence of gossypol isomer, which has antimicrobial activity. In this study, terpene and gossypol

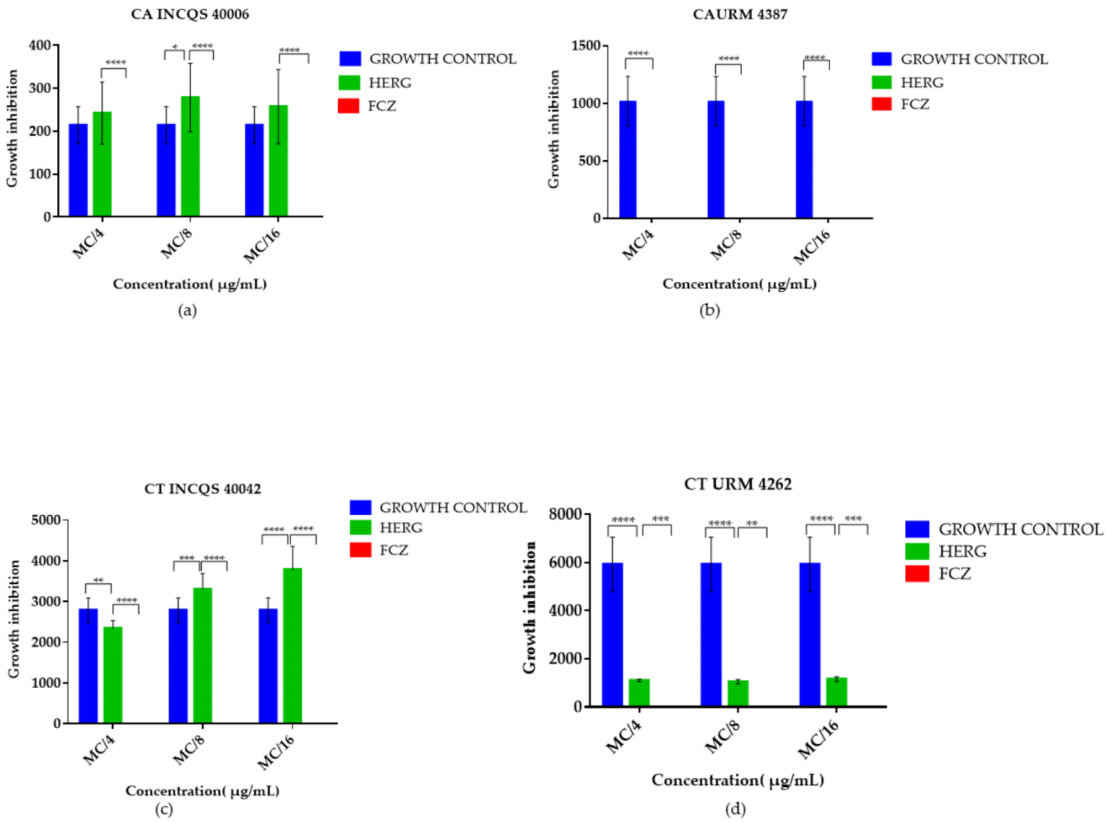

Figure 8. Antifungal effect of Fluconazole and HERG (hydroethanolic extract of root *G. hirsutum*) on the polymorphism of *Candida* spp., from strains: (A) CA INCQS 40006; (B) CA URM 4387; (C) CT INCQS 40042; (D) CT URM 4262. CA: *C. albicans*; CT: *C. tropicalis*; Growth Control; FCZ: Fluconazole; INCQS: Collection Cultures of the National Institute of Quality Control in Health; URM: University Recife Mycology. MC/4 – 4.096 µg/mL, MC/8 – 2.048 µg/mL, MC/16 –1.024 µg/mL.

derivatives were identified in the extracts, which can be related to the results in this intrinsic activity. Anthraquinone derivatives have been extensively studied *in vitro* on pure compounds or in crude plant extracts. Most of them exhibit positive activity against the most common pathogens, including the *Candida albicans* [43].

The combination of Fluconazole with natural products can be an alternative way of minimizing the side effects of the antibiotic since it leads to a significant enhancer effect [47] as shown in these studies with Malvaceae plants. *Guazuma ulmifolia* Lam. and *Luehea paniculata* Mart. & Zucc. have flavonoids in its composition and the antifungal against strains of *C. tropicalis* and *C. albicans* showed potentiating effect when combined with FCZ [45], [46].

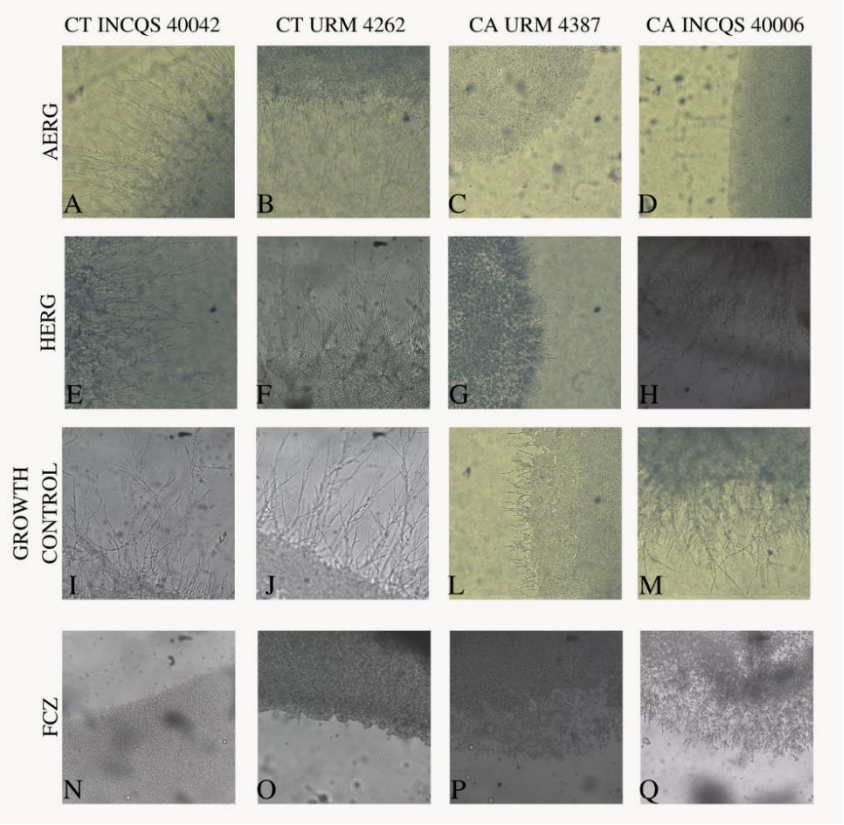

Figure 9. Antifungal effect of Fluconazole and HERG (hydroethanolic extract of root *G. hirsutum*) on the polymorphism of *Candida* spp., from strains: (A) CA INCQS 40006; (B) CA URM 4387; (C) CT INCQS 40042; (D) CT URM 4262. MC/4 (4.096 μg/mL), MC/8 (2.048 μg/mL) and MC/16 (1.024 μg/mL) concentrations.

These results may be of clinical interest in the use of natural products and anti-*Candida* agents in combination to improve the drug's spectrum of activity. Nonetheless, selecting an appropriate combination requires an understanding of the potential interactions between the antimicrobial agents present in the extracts that may have a therapeutic benefit by reducing the required antifungal dosage, mainly HERG which showed the highest relative intensity, the highest concentration of compound than EARG [49], [50].

In the studies of Melo and collaborators and Zuza-Alves and collaborators [21], [34] *C. tropicalis* produced significantly the highest biofilm followed by *C. albicans* determined through the CV, and this species has been recognized as a very strong biofilm producer. *Candida* biofilm can be grown in 96-well microliter plates and this method was devised with high efficiency on the testing of biofilm susceptibility to antifungal agents. Currently, CVs are used to assess the biofilm-forming ability and it

is more appropriate to discriminate strong, moderate, and modest biofilm-producing strains [51].

Biofilm characteristics of the *C. albicans* are: It has a basal blastospore layer with a dense overlying matrix composed of exopolysaccharides, yeast-form cells, pseudohyphal, hyphae, extra-cellular DNA (eDNA) that contributes to the stability of the biofilm and the high biofilm-forming capacity [52], [53], while the *C. tropicalis* biofilm contains chains of cells with thin, but large amounts of extracellular matrix material and normal biofilm-forming capacity, which was different in this study with strong biofilm production of the *C. albicans* URM 4262 ([52].

Different from this study, the infusions obtained from leaves of the *Sida tuberculata* (Malvaceae) could be used to eradicate non-albicans *Candida* (NAC) biofilm formed in CV after 90 min of contact [40]. The phytoconstituents isolated from the Malvaceae family included Upland cotton, which contains tannin, phenols, glycosides, polysaccharides, flavonoids, flavan-3-ol, catechin, phenolic acids, gallic and caffeic acid as part of a role in plant defense mechanisms and presents therapeutic activities. Especially the flavonoids, which aid in resistance to fungal pathogens [54], [55]. These components are present in the extracts that could be responsible for the reduction of formation in *C. tropicalis* URM 4262 biofilms in the 512 and 128 concentrations.

The drug test was used as a control positive but it could not act strongly against *C. albicans* and *C. tropicalis* biofilm. Antifungal azoles like fluconazole, possess a fungistatic effect, blocking ergosterol synthesis, targeting the enzyme lanosterol 14α-demethylase, and leading to an accumulation of toxic sterol pathway intermediates. The planktonic isolates are more susceptible to this drug. However, fluconazole has decreased activity against *Candida* in biofilms because isolates of the community of microorganisms were resistant [34], [53].

Resistance mechanisms to azoles present in biofilms such as cell density, differential regulation of drug targets, increased expression of drug efflux pumps with genes Cdr1 and Mdr1, protective features of the extracellular matrix, the existence of "persister" cells, upregulation of genes linked in the ergosterol biosynthesis pathway

as Erg3, positive regulation of different pathways develop resistance to a maximum level [52], [53].

The genus *Candida* can present in three morphotypes, yeasts, pseudohyphae, and hyphae that differ in cell morphology, function, and specific growth conditions that contribute and are closely linked to pathogenesis. The formation of pseudohyphae and hyphae can occur in several ways such as Cek MAPK (mitogen-activated protein kinase), cAMP-PKA (cyclic adenosine monophosphate— protein kinase A), pH-response, Hog MAPK, Tup1-mediated negative regulatory pathway regulating the virulence process [56], [57]. The ideal is that the therapeutic alternatives act on the signaling pathways that promote morphogenesis and stretching, mycelium formation, and hyphae adherence [68] to inhibit the virulent capacity of yeasts.

In upland cotton extracts showed terpenes and in a study by Raut and collaborators [59] these compounds were associated with inhibition of yeast morphogenesis for hyphae as a therapeutic strategy. According to this literature, there is a possibility that terpenes target specific cellular components or signaling pathways involved in hyphae formation. Even as, flavonoids are effective antifungal agents against *C. albicans, C. tropicalis*, and other fungal strains [47]. Caffeic acid derivatives molecule has strong fungicidal activity and potentiated the effects of fluconazole against *C. albicans* strains [48]. All extract demonstrates action effective against CA URM 4387 that inhibited in all concentrations the hyphae formation with $P<0,0001$ when compared to growth control.

After evaluating several studies Zuza-Alves and collaborators [21] reported that trials with natural products with biological activity against the genus *Candida* could be an alternative for the treatment of fungal infections because these products have less resistance ability than the standard drugs. This study demonstrated that *G. hirsutum* extracts have hyphae growth inhibition capacity and may be an alternative to be investigated by verifying the pathways of action.

5. Conclusions

The UPLC-QTOF-MS/MS analysis presented in this study detected the presence of flavonoids, hydroxyanthroquinone, gossypol derivatives, terpenes in the Upland cotton extracts. The results about anti-*Candida* activity also imply that root extracts Upland cotton has no clinically relevant. As for intrinsic and combined activity, the extracts demonstrated a dose-dependent response related to increased concentrations. Nevertheless, in combination with a standard drug for evaluating the interference of the fungal growth profile, a significant potentiating effect against *C. albicans* and *C. tropicalis* strains was demonstrate in the assays better to HERG than to AERG. The treatment to the reduction of the biofilm in CV assay demonstrates stimulation to production biofilm by the *Candida* strains when compared with GC and FCZ. Both extracts were effective against the CA URM 4387 strain by inhibiting hyphae formation in all concentrations and HERG was effective in reducing hyphae formation in the isolated strain of *C. tropicalis.* Although the extracts have been investigated for their anti-*Candida* action, they need in-depth research that represents interesting alternative efforts in combating candidiasis as to the results that demonstrated potentiation of the standard drug and the inhibition of hyphae formation.

Author contributions section

L.F.L. – Methodology of the biofilm, fungal virulence, modulatory effect, and writer; J. C. A. – Methodology of Compound identification UPLC–QTOF-MS/MS; M. A. F. – Methodology of Biofilm Test; A. I. S. - Methodology of Biofilm Test; V. J. A. F.; T. G. S.; J. C. P. S. - Methodology of the fungal virulence, and modulatory effect; R. H. L. - Methodology of the fungal virulence, and modulatory effect; R. P. N.- Methodology of Biofilm Test; M.R.K.; I.M.A - Material extraction; D. L. S. – Statistical Analysis; E. S. B.; P. R. V. R.; G. J. Z. - Methodology of Compound identification through UPLC–QTOF-MS/MS; H. D. M. C. and M.I. - Project administration.; M. F. B. M. B. – Supervision; Project administration.

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5 CONSIDERAÇÕES FINAIS

5.1 Aspectos Gerais da Produção Científica

O “Ouro Branco” como foi conhecido o algodão no Ceará, é usado para diversos fins, inclusive medicinal. O seu uso demonstrado em pesquisa etnobotânica (FERNANDES, 2019) por pessoas da comunidade Baixa do Maracujá, na Chapada do Araripe, município de Crato embasou o planejamento e a execução desse projeto. Mulheres dessa comunidade relataram o uso de algodão (folhas, raízes e sementes) para cuidar da saúde íntima. Essa informação foi um ponto direcional à proposta de investigação, que é o potencial das plantas medicinais na inibição de crescimento e da virulência das leveduras do gênero *Candida* spp. (TSANG *et al*., 2012; ZUZA-ALVES *et al.*, 2017; RODRÍGUEZ, 2020).

O gênero *Candida* spp. é um fungo dimórfico com capacidade de se adaptar e proliferar facilmente, tendo uma série de fatores de virulência que permitem que ele sobreviva principalmente em tecidos mucosos, causando infecções como a candidíase. Antifúngicos são usados indiscriminadamente, e acredita-se que isso levou ao desenvolvimento de mecanismos de resistência. O desenvolvimento de novas formulações pode ser promissor com compostos bioativos para o tratamento de candidíase utilizando plantas medicinais como *G. hirsutum* L. O algodão foi testado contra o gênero *Candida* spp. e demonstrou atividade inibitória (ROJAS *et al*., 2001; BALLIANA *et al*., 2015; SHARMA *et al*., 2017).

O gênero *Gossypium* inclui espécies bioativas e grande potencial contra doenças bacterianas, fúngicas, virais e com grande expressão no tratamento de doenças protozoárias como a malária (HADI; BREMNER, 2001; KADIR *et al*., 2013; TEKLEHAYMANOT, 2009; AL-FATIMI *et al*., 2007). Em consideração às atividades biológicas, a espécie mais citada no anexo 3 foi a *Gossypium barbadense*, que é usada para infecções virais, bacterianas, fúngicas e protozoárias. *Gossypium hirsutum* é usada para infecções fúngicas, bacterianas e protozoárias. *Gossypium herbaceum* e *G. arboreum* são usadas para infecções protozoárias (AJIBESIN *et al*., 2007; KRISHNAVENI *et al*., 2014; SHARMA *et al*., 2017; SEMENYA, MAROYI, 2019). Na medicina popular, os estudos etnobotânicos mostraram a utilização das folhas, raízes, sementes como as partes mais utilizadas em comparação com a forma de pó, suco, infusão e maceração. Essa revisão mostrou a importância de estudos etnodirigidos para compreender o uso de plantas medicinais por comunidades e, a partir disso, desenvolver experimentos científicos para comprovação do potencial das espécies do gênero *Gossypium*.

Este conhecimento empírico direcionou a execução de experimentos para verificar a atividade anti-*Candida* de extratos do *Gossypium hirsutum* L. Para isso, inicialmente foi realizada a análise química dos extratos pelo método UPLC-QTOF-MS/MS apresentada nos capítulos 1 e 2. Nesses capítulos está expressa a identificação química de flavonoides e outros compostos nos extratos das raízes e folhas do algodão. O ácido cítrico e a catequina foram identificados nos extratos das folhas e raízes. Nos extratos das folhas foram encontrados os ácidos orgânicos e outros compostos, tais como ácidos cafeoil glucárico, ácido coumaroil glucárico, ácido clorogênico, ácido p-cumarico, ácido cafeíco, epi-catequina, quercetina O-hexoside-pentoside, rutina, quercetina 3-O-glucosideo, kaempferol O-rutinosídeo. Destes, os que apresentaram grau de intensidade relativa maior foram os compostos ácido cumaroil glucárico, ácido clorogênico, catequina e epi-catequina no extrato hidroalcóolico das folhas.

Nos extratos das raízes do algodão foram identificados derivados de flavonoides e terpenos como (epi)-catequina, (epi)-gallocatequina dimérico, gallocatequina gossipetina glucosídeo (Gossytrin), 13,15-Dihidroxi-7-O-(6'-O-sulfato-β-D-glucopiranosil) - desoxihemigossipol, 13-Hidroxi-7-O-(6'-O-sulfato-glucopiranosil) – desoxihemigossipol e outros compostos como ácido cumaril-glucárico, ácido 3-*O*-cafeoil-quinico, ácido málico, ácido gálico, heliocide H isômero, ácido faséico glucosídeo. Assim como nos extratos das folhas, o extrato hidroalcóolico das raízes apresentou grau de intensidade relativa maior para os compostos ácido gálico, (epi)-catequina, (epi)-gallocatequina, gallocatequina, catequina, 13,15-Dihidroxi-7-O-(6'-O-sulfato-β-D-glucopiranosil) - Desoxihemigossipol, ácido faséico glucosídeo, isômero Heliocide H, 13-Hidroxi-7-O-(6'-O-sulfato-glucopiranosil) - desoxihemigossipol e gossipetina glucosideo (Gossytrina).

Os resultados sobre a atividade anti-*Candida* mostram que os extratos das raízes e folhas do algodão não possuem ação clinicamente relevante quanto a atividade intrínseca. Tanto para a atividade intrínseca quanto para a combinada, os extratos demonstraram uma resposta dependente de dose relacionada ao aumento das concentrações. No entanto, em combinação com o antifúngico fluconazol, na avaliação da interferência no perfil de crescimento fúngico, um efeito potencializador contra as cepas *de C. albicans* e *C. tropicalis* foi demonstrado nos ensaios, com maior expressão para o extrato hidroalcóolico em comparação com o aquoso. O extrato aquoso das folhas de *G. hirsutum* demonstrou efeito potencializador no teste de viabilidade celular frente as leveduras *C. albicans* e *C. tropicalis* padrão e contra *C. albicans* isolada quando associado ao fluconazol.

Mediante o ensaio com Cristal Violeta, os extratos aquosos e hidroalcóolico do algodão promoveram, na maioria das concentrações, estimulação a produção de biofilme nas cepas de *Candida* quando comparada com grupo controle e fluconazol, exceto o extrato aquoso das

folhas que reduziu a formação de biofilme na levedura *C. tropicalis* URM 4262 nas concentrações de 512 e 128 μg/mL.

Em relação à inibição da transição morfológica, os extratos das raízes foram eficazes contra a cepa *C. albicans* URM 4387, inibindo a formação de hifas em todas as concentrações e o extrato hidroetanólico das folhas de *G. hirsutum* foi eficiente na redução da formação de hifas na cepa isolada de *C. tropicalis.* O extrato aquoso das folhas de *G. hirsutum* inibiu a formação de hifas nas concentrações CM/4 e CM/8 das cepas *C. albicans* padrão e isolada, enquanto o extrato hidroalcóolico das folhas de *G. hirsutum* inibiu o crescimento em *C. albicans* isolada nas MC/4 e MC/8.

No entanto, esses resultados pontuais, como o extrato hidroalcóolico das raízes contra as cepas *de C. albicans* e *C. tropicalis,* o extrato aquoso das folhas frente as leveduras *C. albicans* e *C. tropicalis* padrão e na *C. albicans* isolada – ambos demonstraram efeito potencializador do fluconazol. O extrato aquoso das folhas que reduziu a formação de biofilme na levedura *C. tropicalis* URM 4262 nas concentrações de 512 e 128 μg/mL e os extratos das raízes foram eficazes contra a cepa *C. albicans* URM 4387 na inibição da transição morfológica poderiam ser mais explorados, assim como, testar isoladamente os flavan-3-ols e terpenos que possuem capacidade antioxidante frente as leveduras estudadas.

5.2 **Conclusões Gerais**

Analisando os objetivos que direcionaram esse estudo determinou-se as seguintes conclusões gerais:

O gênero *Gossypium* spp. é usado para tratar doenças bacterianas, fúngicas, virais e antiprotozoárias identificadas através das pesquisas etnobotânicas e ensaios laboratoriais demonstradas na revisão de literatura (anexo 3).

A análise química dos extratos das folhas e raízes de *G. hirsutum* detectou a presença de flavonoides como flavan, flavan-3-ol, terpenos, flavonol e ácidos orgânicos e derivados do gossipol nos extratos, sendo identificados mais compostos da classe dos flavonoides.

Quanto à atividade intrínseca e combinada, os extratos demonstraram uma resposta dependente de dose relacionada ao aumento das concentrações, inibindo o crescimento de algumas cepas. Em combinação com a droga padrão para avaliar a interferência do perfil de crescimento fúngico, um efeito potencializador significativo contra *C. albicans* e *C. tropicalis* cepas foi demonstrado nos ensaios com extrato hidroalcóolico da raiz do que para extrato aquoso da raiz. O extrato aquoso das folhas demonstrou melhor atividade antifúngica contra

C. albicans INCQS 40006 e *C. tropicalis* URM 4262, e o extrato hidroalcóolico combinado com fluconazol mostrou efeitos inibitórios no crescimento de *C. albicans* INCQS 40006.

Os extratos apresentaram uma capacidade redutora da biomassa do biofilme, destacando a eficácia do extrato aquoso contra os isolados padrão e clínicos de *C. tropicalis,* especialmente em concentrações mais baixas. Diante de *C. albicans* e concentrações mais altas, os extratos estimularam a produção de biofilme quando comparado com grupo controle e o fluconazol. Quanto a atividade anti-pleomórfica, o extrato aquoso das folhas inibiu o crescimento das hifas de *C. albicans* em cepas isoladas padrão e clínica, enquanto o extrato hidroalcóolico inibiu o crescimento das hifas apenas para o isolado clínico, *C. albicans* URM 4387. Ambos os extratos das raízes foram eficazes contra a *C. albicans* URM 4387 inibindo a formação de hifas em todas as concentrações e o extrato hidroalcóolico da raiz foi eficaz na redução da formação de hifas na cepa isolada de *C. tropicalis.*

5.3 **Perspectivas de Investigações Futuras**

O algodão continua a ser uma cultura de grande importância econômica em muitos países em desenvolvimento e em alguns países desenvolvidos. Agregado a isso, pudemos avaliar nesse estudo outras potencialidades do *G. hirsutum* L., o seu valor etnobotânico, etnomedicinal e a suas bioatividades mediante os microrganismos, principalmente em relação as leveduras do gênero *Candida* spp.

Em uma perspectiva etnobiológica e a partir desses ensaios anti-*Candida,* os extratos das folhas e das raízes se mostraram ativos contra uma ou outra levedura específica. Seria necessário, ensaios para elucidar os mecanismos de ação das atividades abordadas através do ensaio de *checkerboard,* por exemplo. Testes *in vivo* com *zebrafish* estimulando infecção por espécies de *Candida* spp. para avaliar a evolução de uma infecção na presença de extratos do algodão. Explorar outros tipos de extratos do *G. hirsutum* L. com polaridades diferentes para a extração de outros metabólitos secundários também seria pertinente, assim como ampliar os testes utilizando outras cepas de *Candida* spp. como *C. parapsilosis, C. krusei* entre outras que possuem caráter clínico.

Para complementar os dados de erradicação de biofilme, propõe-se ensaio de anti-formação para comparar a ação dos extratos, com o tratamento que foi realizado e ensaio por Microscopia Eletrônica de Transmissão dos biofilmes para verificar a interação ou o dano na membrana fúngica.

Avaliar a citotoxicidade dos extratos de *G. hirsutum* seria relevante e complementar. Ao utilizarmos células da medula de camundongos *Swiss*, por exemplo, e avaliar a formação de micronúcleos (ADLER; ATTIA, 2003) na presença dos extratos ou usar linhagem celular HeLa para determinar a citotoxicidade dos extratos usando o método de Keusch *et al*. (1972) (LARAYETAN *et al*., 2019).

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Anexo 1 – Comprovante do SISGEN

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 A853006

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

Número do cadastro:	**A853006**
Usuário:	**LUCIENE FERREIRA DE LIMA**
CPF/CNPJ:	**014.024.963-03**
Objeto do Acesso:	**Patrimônio Genético/CTA**
Finalidade do Acesso:	**Pesquisa**

Espécie

Gossypium hirsutum

Gossypium hirsutum

Fonte do CTA

CTA de origem não identificável

Título da Atividade:	**ANÁLISE DA COMPOSIÇÃO QUÍMICA E AVALIAÇÃO DOS POTENCIAIS ANTIFÚNGICO, ANTIOXIDANTE E TÓXICO DE EXTRATOS DE Gossypium hirsutum L..**

Equipe

LUCIENE FERREIRA DE LIMA	**INDEPENDENTE**
Maria Flaviana Bezerra Morais Braga	**Universidade Regional do Cariri - URCA**

Anexo 2 – Número de herbário e Identificação botânica

Herbário Caririense Dárdano de Andrade – Lima
Universidade Regional do Cariri - URCA

HCDAL / URCA

Número de Herbário

Remetente: N°65.2019

HERBÁRIO CARIRIENSE DÁRDANO DE ANDRADE-LIMA (HCDAL/URCA)
Contato: Dra. Maria Arlene Pessoa da Silva (herbario@urca.br)
Universidade Regional do Cariri - URCA
Departamento de Ciências Biológicas
Rua: Cel. Antônio Luiz, 1161
Campus Pimenta
Crato – Ceará - Brasil
CEP: 63.105-100

Destinatário: Laboratório de Microbiologia Aplicada do Cariri **Data:** 29.11.2019

Contato: Lucilene Ferreira de Lima
Universidade Regional do Cariri - URCA

N° Amostras: 04 Tipo de Operação: **Número de Herbário e Identificação Botânica**

	N° HERBÁRIO	NOME POPULAR	FAMÍLIA	NOME CIENTÍFICO	DETERMINADOR
01	14.210		Malvaceae	*Gossypium hirsutum* L.	Luciana Silva Cordeiro
02	14.211		Malvaceae	*Gossypium hirsutum* L.	Luciana Silva Cordeiro
03	14.212		Malvaceae	*Gossypium hirsutum* L.	Luciana Silva Cordeiro
04	14.213		Malvaceae	*Gossypium hirsutum* L.	Luciana Silva Cordeiro

Dra. Maria Arlene Pessoa Silva
Curadora do HCDAL

Herbário Caririense Dárdano de Andrade - Lima / Universidade Regional do Cariri - URCA
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Anexo 3 – Artigo 1 no ***Journal of Ethnopharmacology - Ethnobotanical and antimicrobial activities of the Gossypium (Cotton) genus: a review***

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Ethnobotanical and antimicrobial activities of the *Gossypium* (Cotton) genus: A review

Luciene Ferreira de Lima [a,*], José Oreste de Oliveira [b], Joara Nályda Pereira Carneiro [a], Cícera Norma Fernandes Lima [a], Henrique Douglas Melo Coutinho [a], Maria Flaviana Bezerra Morais-Braga [a]

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Malvaceae family
Folk medicine
Microbial diseases

ABSTRACT

Ethnopharmacological relevance: The Malvaceae family, an important group of plants that have the *Gossypium* (cotton) genus has been used in folk medicine to treat microbial diseases and symptoms.
Aims of the study: This article aims to understand its ethnobotany expression in communities and scientific elucidation of antimicrobial activities of this genus through literature review.
Materials and methods: The bibliographic survey was carried out from 1999 to 2019 with keywords combinations such as "*Gossypium* + ethnobotanical", " *Gossypium* + medicinal ", "*Gossypium* + the biological activity" in scientific databases as Pubmed, Scopus, Web of Science, DOAJ, Scielo, Bireme.
Results: After data analysis, we found that the *Gossypium* genus, specifically *Gossypium hirsutum, G. barbadense, G. herbaceum, G. arboreum* are the species most cited in the treatment of microbial diseases and symptoms in communities all over the world. In light of scientific elucidation of biological activities, the *Gossypium* genus has been used to treat protozoal, bacterial, fungal, and viral diseases.
Conclusions: The review demonstrated that the *Gossypium* genus is a promising source of biological activities against microbial diseases, especially in the treatment of protozoal diseases like malaria.

1. Introduction

The Angiosperm Phylogeny Group (2016) shows the Malvaceae family containing 243 genera with 4225 known species. According to The Plant List (2013), the family Malvaceae is in the major Angiosperms group and it is known all over the world. This data bank includes 14,539 scientific plant names of species rank for this family. Being that these 4465 are accepted species names and belong to 245 plant genera. Generally, the life form is a shrub, tree, herb, liana/fickle/creeper, subshrub. It is present in aquatic, hemiepiphyte, rupiculture, terriculture substrate.

The Malvaceae family includes cotton (*Gossypium* spp.), which is an important natural fiber source used by the textile industry worldwide (Thompson et al., 2017), cacao (*Theobroma* spp.), durian (*Durio* spp.), a major Southeast Asian food known as the 'king of fruits; and okra (*Abelmoschus* spp.), which is a nutritious vegetable (Wang et al., 2020). In Brazil, it is a native family, but it is not endemic (Esteves, 2015). The 18th and 19th centuries were prosperous eras for cotton growing in the Brazil northeast because of the propitious climatic conditions. The state of Ceará was the largest cotton producer until the early 1980s (Guerra et al., 2012; Lima and Oliveira, 2001). The cotton plantation had a historic, cultural, economic value in the familiar agriculture in the Brazilian semiarid. Therefore, it was called an "*ouro branco*" or white gold (Beltrão, 2003; Souza, 2000). Then, this was important to the regional people, however, the cotton production went through crises and came to decline in the state of Ceará. Currently, Ceará invests in technology and management expecting to return to being one of the main cotton producers in Brazil, in the hope for better days (Augusto, 2020).

The phytoconstituents present in the plant extracts of the Malvaceae family (*Abutilum indicum, Hibiscus sabdariffa, Sida acuta,* and *Sida rhombifolia*) belong to categories such as flavonoids, phenolics, acids, and polysaccharides to which many of the ethnopharmacological activities can be attributed. Phytoconstituents are naturally occurring

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