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HPLC–DAD analysis and antimicrobial activities of *Spondias mombin* L. (Anacardiaceae)

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Abstract

Spondias mombin is used in the folk medicine for the treatment of diarrhea and dysentery, indicating that extracts obtained from this species may present pharmacological activities against pathogenic microorganisms. The purpose of this work was to investigate the chemical composition and evaluate the antimicrobial activity of extracts obtained from the leaves (aqueous) and bark (hydroethanolic) of *S. mombin* both as single treatments and in combination with conventional drugs. Following a qualitative chemical prospection, the extracts were analyzed by HPLC–DAD. The antimicrobial activities were evaluated by microdilution. The combined activity of drugs and extracts was verified by adding a subinhibitory concentration of the extract in the presence of variable drug concentrations. The Minimum Fungicidal Concentration (MFC) was determined by a subculture of the microdilution test, while the effect of the in vitro treatments on morphological transition was analyzed by subculture in moist chambers. While the qualitative analysis detected the presence of phenols and flavonoids, the HPLC analysis identified quercetin, caffeic acid, and catechin as major components in the leaf extract, whereas kaempferol and quercetin were found as major compounds in the bark extract. The extracts showed effective antibacterial activities only against the Gram-negative strains. With regard to the combined activity, the leaf extract potentiated the action of gentamicin and imipenem (against *Staphylococcus aureus*), while the bark extract potentiated the effect of norfloxacin (against *S. aureus*), imipenem (against *Escherichia coli*), and norfloxacin (against *Pseudomonas aeruginosa*). A more significant antifungal (fungistatic) effect was achieved with the bark extract (even though at high concentrations), which further enhanced the activity of fluconazole. The extracts also inhibited the emission of filaments by *Candida albicans* and *Candida tropicalis*. Together, these findings suggest that the extract constituents may act by favoring the permeability of microbial cells to conventional drugs, as well as by affecting virulence mechanisms in *Candida* strains.

Keywords Phytochemicals · Antibacterial · Antifungal · Antimicrobial resistance

Abbreviations

IPDs	Infectious and parasitic diseases
URCA	Universidade Regional do Cariri
HPLC-DAD	High Performance Liquid Chromatography-Diode Array Detector
ATCC	American type culture collection
HIA	Heart infusion agar
BHI	Brain heart infusion
DMSO	Dimethyl sulfoxide
MIC	Minimum inhibitory concentration

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INCQS	Instituto Nacional de Controle de Qualidade em Saúde—National Institute of Quality Control in Health
FIOCRUZ	Fundação Instituto Osvaldo Cruz
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
MFC	Minimum fungicidal concentration
IC ₅₀	Concentration that inhibits 50% of the microbial growth
PDA	Potato dextrose agar
HCA	Higher concentration assessed
one-way ANOVA	One-way analysis of variance
CDRI	Central Drug Research Institute
AELSM	Aqueous extract of the leaves of <i>Spondias mombin</i>
HEBSM	Hydroethanolic extract of the bark of <i>Spondias mombin</i>
MDR	Multidrug-resistant
BAM complex	β-Barrel assembly machine complex
SD	Standard deviations
FCZ	Fluconazole

Introduction

Infectious and parasitic diseases (IPDs) have high mortality rates worldwide. Consistent evidence has demonstrated that the emergence of antimicrobial resistance is associated with the indiscriminate use of antibiotics and synthetic drugs (Aslam et al. 2018; Abadi et al. 2019; Santos et al. 2019a, b, c). Worryingly, the rising of multidrug-resistant microorganisms has overcome the development of new drugs, reflecting the efficient adaptive and evolutionary capacity of infectious agents such as bacteria and fungi (Cassini et al. 2019; Tyers and Wright 2019).

Nosocomial pathogens (i.e., those acquired in a hospital environment), including bacteria, fungi, and protozoa, have a significant impact on public health. Bacteria such as *Escherichia coli* (Enterobacteriaceae), *Staphylococcus aureus* (Staphylococcaceae), and *Pseudomonas aeruginosa* (Pseudomonadaceae), stand out for being opportunistic microorganisms that have demonstrated resistance to standard antibiotics (Asgeirsson et al. 2018; Hoang et al. 2018; Horn et al. 2018; Khalil et al. 2018).

With regard to eukaryotic microorganisms, fungi are notable nosocomial pathogens with significant mechanisms of resistance to antifungal drugs. In this context, yeasts of the genus *Candida* stand out for causing invasive infections with high incidence rates (Romo and Kumamoto 2020). Species of *Candida* have notable pathogenic potential due to a series of virulence mechanisms, which include adherence to human tissues, biofilm production, hydrolytic enzyme secretion, resistance to high temperatures, and evasion from the

immune system. In addition, these microorganisms undergo morphological changes characterized by the growth of filamentous structures in adaptation to environmental changes, and nutrient limitation (Morais-Braga et al. 2016a; Pristov and Ghannoum 2019; Bezerra et al. 2020; Eix and Nett 2020).

A large body of research has been carried out to identify novel substances with therapeutic activity against infections caused by pathogenic microorganisms, as well as to identify chemical compounds capable of increasing the effectiveness of commercial drugs to which pathogens have developed resistance mechanisms (Bezerra et al. 2019). In this context, natural products such as extracts, fractions, essential oils, fixed oils, or isolated constituents (Calixto-Júnior et al. 2015; Rodrigues et al. 2019; Santos et al. 2019a, b, c; Costa et al. 2020; Cruz et al. 2020), which can be found in medicinal plants, constitute promising sources of new molecules for the treatment of infectious and parasitic diseases (Albergaria et al. 2019).

Spondias mombin L. (Anacardiaceae), popularly known as “cajá”, is traditionally used by Brazilian communities to treat diarrhea and dysentery (Hajdu and Hohmann 2012; Castillo 2015; Delmondes et al. 2016; Roumy et al. 2020). Studies have demonstrated that both the barks and leaves of this species are rich in alkaloids, saponins, flavonoids, terpenes, and polyphenols, supporting the evidence that decoctions prepared from the leaves of this species in popular medicine have therapeutic properties (Okwuosa et al. 2012; Chaves et al. 2018; Nwidi et al. 2018).

Thus, considering the need to develop new products to combat antimicrobial resistance, as well as the ethnopharmacological evidence that *S. mombin* has antimicrobial properties, this study aims to characterize the chemical composition and investigate the antibacterial and antifungal effects of *S. mombin* extracts.

A significant number of plant extracts had their antifungal and antibacterial activities demonstrated by previous research (Chaves et al. 2018; De Freitas et al. 2020; Roumy et al. 2020; Samuggam et al. 2021). However, this study is the first to report the effectiveness of *S. mombin* in enhancing the antibacterial activity of conventional antibiotics against resistant bacteria. In addition, this pioneering work demonstrated that this species is capable of inhibiting virulence-associated morphological changes in opportunistic fungi.

Materials and methods

Botanical material

Leaves and barks of *S. mombin* were collected in the municipality of Crato, Ceará, Brazil (Coordinates 07° 13' 0.52" S

39° 23' 0.56.3" W, Fig. 1). The botanical identification was confirmed by prof. Ana Cleide Alcântara Morais Mendonça and a voucher specimen registered at the Herbarium of the Universidade Regional do Cariri—URCA (registry number 12,622).

Preparation of extracts

To prepare the extracts, 300 g botanical material (leaves or barks) were washed in running water. Subsequently, the leaves were submerged in boiling water (100 °C) and smothered for a period of 72 h. After this period, the solution was filtered, subjected to freezing temperatures, and lyophilized (Lyophilizer model K105—Liotop) (Matos 2009).

For bark extraction, 300 g of botanical material was submerged in a mixture of co-solvents containing 30% water and 70% ethanol (v:v) for a period of 72 h. After extraction, the material was concentrated in a rotary evaporator and lyophilized as previously described. After lyophilization, they were placed in amber glass and stored in a freezer (Matos 2009).

Preceding the antimicrobial tests, the extracts were verified for the presence of contaminants. To this end, 100 µL of each extract was added to plates containing culture medium

(HIA—bacteria and ASD—fungi) and taken to the oven at 37 °C for 48 h, after which no microbial growth was observed.

Phytochemical prospecting

A qualitative chemical prospecting of the *S. mombin* extracts was carried out to assess the presence of secondary metabolites according to the methodology described by Matos (2009). The readings were based on the visual observation of changes in the color of the solution or precipitation after the addition of specific reagents. The presence or absence of the following classes of compounds were evaluated: 1—Phenols; 2—Pyrogenic tannins; 3—Flabobenic tannins; 4—Anthocyanins; 5—Anthocyanidins; 6—Flavones; 7—Flavonols; 8—Xanthones; 9—Chalcones; 10—Aurones; 11—Flavonols; 12—leucoanthocyanidin; 13—Catechins; 14—Flavonones; 15—Alkaloids.

The phytochemical prospecting was carried out as follows: In the preliminary phase, 4 mL aliquots were transferred to test tubes and two 10 mL portions were transferred to numbered and tared beakers. The beakers were kept in water bath until complete solvent evaporation evaporated and kept in a Drying House until use. The remaining extract

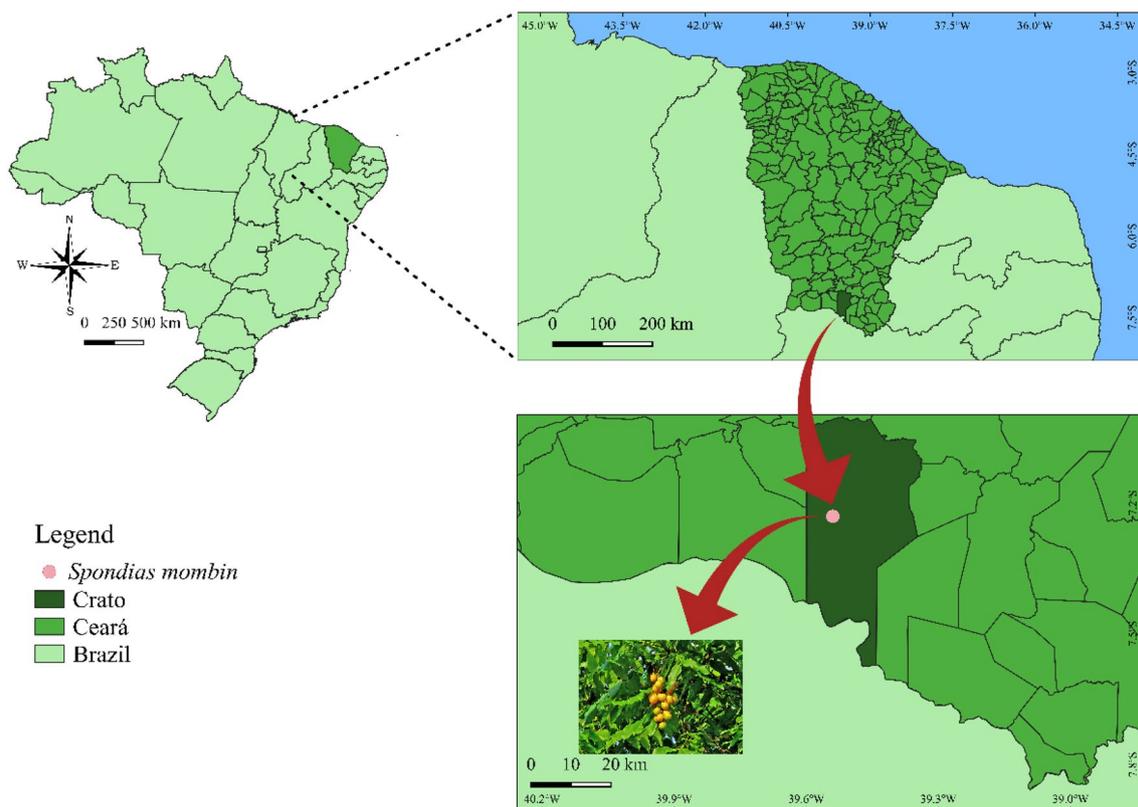


Fig. 1 Location map indicating the site of collection of *Spondias mombin*

was concentrated under water bath to half its volume and the liquid was acidified to pH 4. Both the solution and the insoluble residue were reserved for further tests. The detection of each metabolite class was carried out following specific protocols, as widely reported in the literature (Morais-Braga et al. 2016b; Barbosa et al. 2019; Carvalho et al. 2020).

High Performance Liquid Chromatography (HPLC)

The HPLC analysis was performed as previously described (Bezerra et al. 2017). Briefly, each extract was injected into a Phenomenex Synergi Hydro RP18 C18 reverse-phase column (250 mm × 4.6 mm i.d.; 5 µm) (Phenomenex, Torrance, CA, USA). The mobile phase consisted of Milli-Q water (pH 2.0), 1% phosphoric acid, and methanol. The extract (10 mg/mL) was injected at a flow rate of 0.6 mL/min, with an injection volume of 50 µL. The sample and the mobile phase were filtered through a 0.45 µm membrane and then degassed by ultrasound bath before use. Standard stock solutions were prepared in 1:1 methanol: water (v/v) at final concentrations ranging from 0.030 to 0.500 mg/mL. The chromatographic peaks were confirmed by comparing their retention times with those of reference standards and by DAD spectra (200–600 nm). All chromatography operations were performed at room temperature and in triplicate.

Antibacterial activity analysis

Strains

Staphylococcus aureus ATCC 25923, *Pseudomonas aeruginosa* ATCC 15442, and *Escherichia coli* ATCC 25922 were used as standard bacterial strains, while *Escherichia coli* 06 (urine), *Pseudomonas aeruginosa* 24 (nasal discharge), and *Staphylococcus aureus* 10 (rectum swab) were used as multidrug-resistant bacterial strains. The resistance profile of these strains is described in Almeida et al. (2020).

Bacterial cultures

All bacterial strains were maintained in Heart Infusion Agar (HIA) and cultured in 10% Brain Heart Infusion (BHI) during the experiments (at aerobic conditions, 37 °C, 24 h). For the antibacterial activity analysis, 10 mg of each extract was weighed, dissolved in DMSO, and diluted in distilled water to a final concentration of 1024 µg/mL. The antibiotics gentamicin, imipenem, and norfloxacin were prepared at the same concentration.

Minimum inhibitory concentration (MIC) determination

The sensitivity of the strains to extracts or antibiotics was evaluated through the broth microdilution method,

as described by Javadpour et al. (1996) with adaptations regarding controls and concentrations. Each inoculum was prepared in saline and turbidity was visually adjusted according to the McFarland scale (0.5).

Then, the inoculum was diluted in 10% BHI (1:9) and distributed in the wells of a 96-well plate. After this procedure, the wells were filled with the extracts serially diluted at concentrations ranging from 512 to 8 µg/mL. Controls for the microbial growth and culture medium sterility were carried out. The plates were incubated at 37 °C, and 24 h later, the readings were performed using the resazurin colorimetric method and the minimum inhibitory concentration (MIC) was determined.

Analysis of antibiotic activity modulation by *Spondias mombin* extracts

The antibiotic-modulating activity of the extracts was evaluated following the methodology described by Coutinho et al. (2008). To this end, all bacterial cultures were performed as previously described and the extracts were added to the wells at a concentration equivalent to their MIC/8 (subinhibitory concentration). Then, the antibiotics were added to the wells at concentrations ranging from 512 to 0.5 µg/mL, and the readings were performed as previously described.

Antifungal activity analysis

Strains

Standard strains of *Candida albicans* INCQS 40006, *Candida tropicalis* INCQS 40042 and *Candida krusei* INCQS 40095 were provided by the National Institute of Quality Control in Health (INCQS)—FIOCRUZ.

Fungal cultures

All strains were cultured in Sabouraud Dextrose Agar (SDA) and doubled concentrated Sabouraud Dextrose Broth (SDB) for antifungal activity analysis. Before each microdilution protocol, each inoculum was prepared in saline and the turbidity was visually adjusted according to the McFarland scale (0.5). Depleted Potato Dextrose Agar (PDA) was used to induce the emission of hyphae and pseudohyphae (morphological change analysis). Both extracts and fluconazole (reference antifungal drug) were dissolved in distilled water and diluted to a final concentration of 16,384 µg/mL.

Minimum fungicidal concentration (MFC) determination

The intrinsic antifungal activity was evaluated using the microdilution method (using a concentration range of 8–8192 µg/mL) as described by Javadpour et al. (1996)

with adaptations regarding controls and concentrations. After the colorimetric reaction, the readings were performed in a spectrophotometer (model DR-200 BS (N), Kasuaki®) at a wavelength of 630 nm, from which the cell viability curve was obtained and used to calculate the IC₅₀ of the extracts. Diluent control test was performed by replacing the inoculum with 0.9% sodium chloride. Microbial growth and sterility controls were also used (Morais-Braga et al. 2016b). The MFC of the extracts was determined by sub-cultivation in Petri dishes, using sterile rods. The absence of colony growth was interpreted as a fungicidal effect and the lowest concentration of the extracts capable of inhibiting fungal growth was defined as the MFC (Ernst et al. 1999).

Analysis of antifungal resistance modulation

The combined activity of the extracts and fluconazole was determined using the extract at a subinhibitory concentration based on the Matrix Concentration (MC/16). For this purpose, wells on a 96-well plate were filled with 100 µL of a solution containing the inoculum and the extracted diluted in the medium. Then, 100 µL of fluconazole was added to the wells at concentrations ranging from 8192 to 8 µg/mL. The last well, filled only with medium and the inoculum, was used as growth control (Coutinho et al. 2008—with adaptations). In addition, diluent and sterility controls were used to ensure the absence of contamination interference. The plates were incubated at 37 °C for 24 h and the readings were performed at 630 nm (Morais-Braga et al. 2016b).

Analysis of fungal virulence modulation

The analysis of fungal virulence was carried out following the protocols described by Sidrim and Rocha (2010) and Morais-Braga et al. (2016a). A yeast microculture was carried out in humid chambers filled with 3 mL of the extract diluted in PDA medium at three different concentrations, based on the Higher Concentration Assessed (HCA), as follows: HCA (8192 µg/mL), HCA/4, and HCA/16. The

morphological transition was observed in chambers containing *Candida* strains cultured in the depleted medium. Microcultures treated with fluconazole were used as the pharmacological control. Streaks of the fungal subcultures were traced in slides with medium, and the chambers were incubated at 37 °C for 24 h. After incubation, the morphological analysis was performed by optical microscopy.

Statistical analysis

The data were analyzed for normal distribution, expressed as the means ± standard deviations, and analyzed by one-way analysis of variance (one-way ANOVA) followed by Bonferroni's post hoc test. Statistical significance was considered when $p < 0.05$. The IC₅₀ values were obtained by non-linear regression and the results were calculated from a standard curve, expressed as a function of the concentration of the extracts (µg/mL). All analyzes were performed using the Graphpad Prism® software, version 6.0.

Results and discussion

Chemical composition

Qualitative chemical prospecting

The qualitative prospecting revealed the presence of phenols, flavones, flavonols, xanthonones, chalcones, auronones and flavononols in both *S. mombin* extracts. However, anthocyanins, anthocyanidins and alkaloids were not identified in none of the extracts (Table 1).

Chemical analysis by High Performance Liquid Chromatography (HPLC)

The HPLC fingerprint (CDRI and fruit version) analysis of *S. mombin* extracts identified six compounds in the aqueous extract of the leaves (AELSM) and five compounds in the hydroethanolic extract of the bark (HEBSM) (Table 2). All chromatogram assays were monitored up

Table 1 Chemical prospecting of the extracts obtained from *Spondias mombin*

Extracts	Metabolite class														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
AELSM	+	-	+	-	-	+	+	+	+	+	+	-	-	+	-
HEBSM	+	+	-	-	-	+	+	+	+	+	+	+	+	+	-

1—Phenols; 2—Pyrogenic tannins; 3—Flabobenic tannins; 4—Anthocyanins; 5—Anthocyanidins; 6—Flavones; 7—Flavonols; 8—Xanthonones; 9—Chalcones; 10—Auronones; 11—Flavonols; 12—Leucoanthocodines; 13—Catechins; 14—Flavonones; 15—Alkaloids; (+) Presence; (—) Absence

AELSM aqueous extract of the leaves of *Spondias mombin*, HEBSM hydroethanolic extract of the bark of *Spondias mombin*

Table 2 Phenolic composition of *Spondias mombin*

Compounds	<i>Spondias mombin</i>	
	AELSM (mg/g)	HEBSM (mg/g)
Catechin	5.83 ± 0.01 a	1.87 ± 0.03 a
Chlorogenic acid	0.27 ± 0.05 b	–
Caffeic acid	9.91 ± 0.03 c	1.95 ± 0.01 a
Ellagic acid	2.34 ± 0.02 d	1.03 ± 0.02 b
Quercetin	10.16 ± 0.01 c	5.26 ± 0.04 c
Kaempferol	5.02 ± 0.01 a	4.08 ± 0.01 d

These results are expressed as means ± standard deviations (SD) of 3 determinations. Different letters after the values indicate statistical difference by Tukey's test ($p < 0.01$)

AELSM aqueous extract of the leaves of *Spondias mombin*, HEBSM hydroethanolic extract of the bark of *Spondias mombin*

to 280 nm. The wavelengths used were 254 nm for gallic acid; 280 nm for catechin; 327 for ellagic acid, chlorogenic acid and caffeic acid; and 356 nm rutin, quercetin and apigenin (Bezerra et al. 2017). With the exception of chlorogenic acid ($t_R = 18.73$ min, peak 2), which was identified only in the AELSM, both extracts presented the following constituents: catechin ($t_R = 10.25$ min, peak 1), caffeic acid ($t_R = 26.09$ min, peak 3), ellagic acid ($t_R = 37.11$ min, peak 4), quercetin ($t_R = 49.51$ min, peak 5) and kaempferol ($t_R = 54.98$ min, peak 6) (Fig. 2). While kaempferol and quercetin were the constituents with the highest concentrations in the bark extract, quercetin, caffeic acid, and catechin were identified as major constituents in the leaf extract.

The present research characterized the chemical profile and antimicrobial properties of *S. mombin* extracts. Using an ethnopharmacological approach, Albuquerque and Hanazaki (2006) demonstrated that these extracts have constituents with biological activity against pathogenic microorganisms, which is in line with their ethnomedicinal use (Silva et al. 2020a, b).

Previous research has demonstrated that the secondary metabolites of this species can vary according to the organ analyzed, especially concerning the presence of catechins, which are found only in the bark. Such differences may explain the stronger activity of bark extracts when compared with those obtained from the leaves, since catechins present significant antifungal properties, in addition to having the ability to enhance the activity of conventional antimicrobials such as amphotericin B and fluconazole (Anand and Rai 2017).

Using a fractionated extract of the leaves (water, *n*-butanol, ethyl acetate, and dichloromethane), Akinmoladun et al. (2015), identified a similar pattern of metabolites to those found in this work, including tannins, anthraquinones, and flavonoids. However, the differences in the chemical composition observed in the studies can be justified due to a series of experimental factors such as the polarity of the solvent, as well as due to intrinsic (genetic) and extrinsic (environmental) factors (Bezerra et al. 2017). Importantly, quercetin and caffeic acid, identified as major compounds in the extract of the leaves, are flavonoids with several biological activities, including antimicrobial activity (Pessoa et al. 2018; Rocha et al. 2019; Santos et al. 2019a, b, c; Kwun and Lee 2020), corroborating the findings of the present research.

A phytochemical analysis by HPLC conducted by Rey-Blanes et al. (2020), identified phenolic compounds such as quercetin, rutin, rhamnetin, and kaempferide in *S. mombin* leaves. Additionally, HPLC analyses performed by Cristofoli et al. (2019) demonstrated that *S. mombin* leaves present caffeic acid, ellagic acid, gallic acid, vanillic acid, catechin, and rutin as significant constituents, corroborating the findings of the present research. The same work demonstrated the leaf extract has antimicrobial effects against different bacterial species, including, *Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 43888, *Listeria innocua* ATCC 33090, *Pseudomonas aeruginosa*

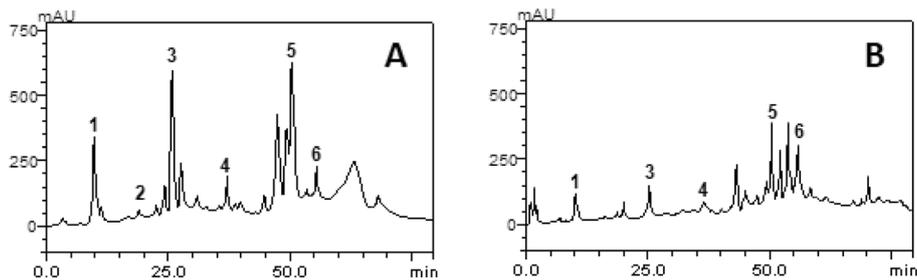


Fig. 2 Representative high performance liquid chromatography profile of *Spondias mombin* (CDRI and fruit version) at 280 nm. **A** Aqueous Extract of the Leaves from *Spondias mombin*—AELSM; **B**

Hydroethanolic Extract of the Bark of *Spondias mombin*—HEBSM. Catechin (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), ellagic acid (peak 4), quercetin (peak 5) and kaempferol (peak 6)

NCTC 12903, *Staphylococcus aureus* NCTC 12981, as well as against the yeast strain *Saccharomyces cerevisiae* NCPF 3178.

Antibacterial effect and antibiotic-modulating activity

The extract obtained from the leaves of *S. mombin* leaves presented clinically effective activity against all standard Gram-negative strains evaluated by the present research. On the other hand, the bark extract significantly inhibited the growth of the multidrug-resistant (MDR) strain of *P. aeruginosa* and the standard strain of *E. coli*. However, none of the extracts presented clinically effective effects against *S. aureus* 10, *P. aeruginosa* ATCC 15442, and *S. aureus* ATCC 25923 (Table 3).

Following the evaluation of the antibacterial activity, this study investigated the ability of the *S. mombin* extracts to modulate bacterial resistance to conventional

Table 3 Minimum inhibitory concentration (MIC) of *Spondias mombin* extracts against bacterial strains

Strain	AELSM MIC (µg/mL)	HEBSM MIC (µg/mL)
<i>Escherichia coli</i> 06	≥ 1024	512
<i>Pseudomonas aeruginosa</i> 24	512	512
<i>Staphylococcus aureus</i> 10	≥ 1024	≥ 1024
<i>Escherichia coli</i> ATCC 25922	512	≥ 1024
<i>Pseudomonas aeruginosa</i> ATCC 15442	≥ 1024	≥ 1024
<i>Staphylococcus aureus</i> ATCC 25923	≥ 1024	≥ 1024

MIC minimum inhibitory concentration, AELSM aqueous extract of the leaves of *Spondias mombin*, HEBSM hydroethanolic extract of the bark of *Spondias mombin*

antibiotics. It was demonstrated that the aqueous extract of the leaves potentiated the activity of gentamicin and norfloxacin against *S. aureus* (Fig. 3). On the other hand, the same extract antagonized the effect of norfloxacin against *P. aeruginosa*. However, no significant antibiotic-modulating effect was observed for the other associations.

The combination of the bark extract with norfloxacin resulted in a synergistic antibacterial effect against *S. aureus* (Fig. 4). Interestingly, heterogeneous results were obtained in *E. coli* cultures, as the extract potentiated the activity of imipenem, but antagonized the activity of norfloxacin. In addition, this extract potentiated the activity of norfloxacin against *P. aeruginosa* but did not affect the activity of gentamicin and imipenem against these strains.

Vipin et al. (2020) showed that the flavonoid quercetin is effective against several biofilm-producing clinical isolates of *P. aeruginosa* (at 500 µg/mL). Furthermore, it has been demonstrated that its potential to promote synergistic effects with gentamicin and other antibiotics against several strains is possible due to the ability of this compound to penetrating the biofilm matrix, causing cell death or depletion. Additionally, an in vitro toxicity assay (infection of epithelial cells), demonstrated that the combination of this compound with antibiotics reduced the infection burden and increased the viability of epithelial cells.

The use of silver nanoparticles (AgNPs) has revolutionized research into antimicrobial agents. AgNPs conjugates containing kaempferol and hydrocortisone inhibited the growth of *E. coli* exhibiting bacteriostatic (62.5 µg/mL) and bactericidal (125 µg/mL) effects. In terms of mechanisms, it was observed an increase in the production of reactive oxygen species (ROS) and lipid oxidation, affecting the integrity of the bacterial membrane, in addition to reducing biofilm production (Kannanoor et al. 2021).

Fig. 3 Antibacterial activity of the Aqueous Extract of the Leaves of *Spondias mombin* (AELSM) combined with standard antibiotics against MDR bacteria. ***Statistically significant ($p < 0.001$), ns not significant value ($p > 0.05$)

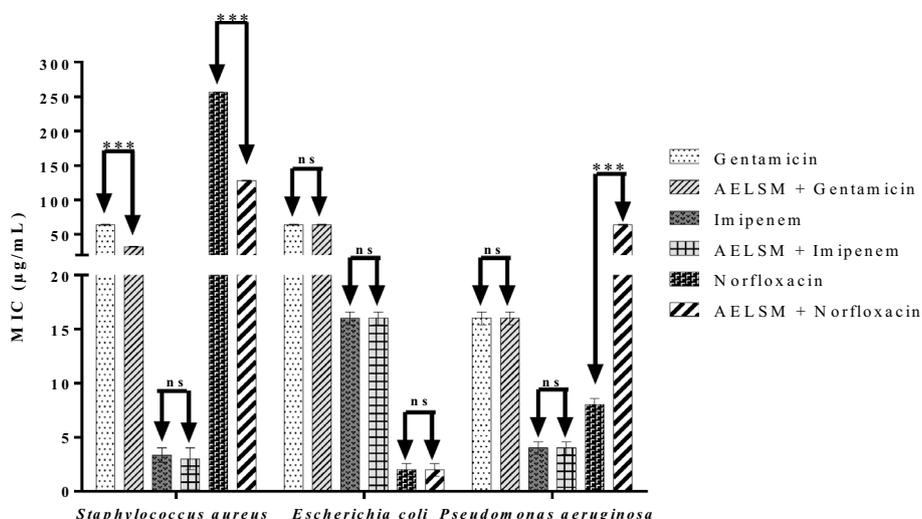
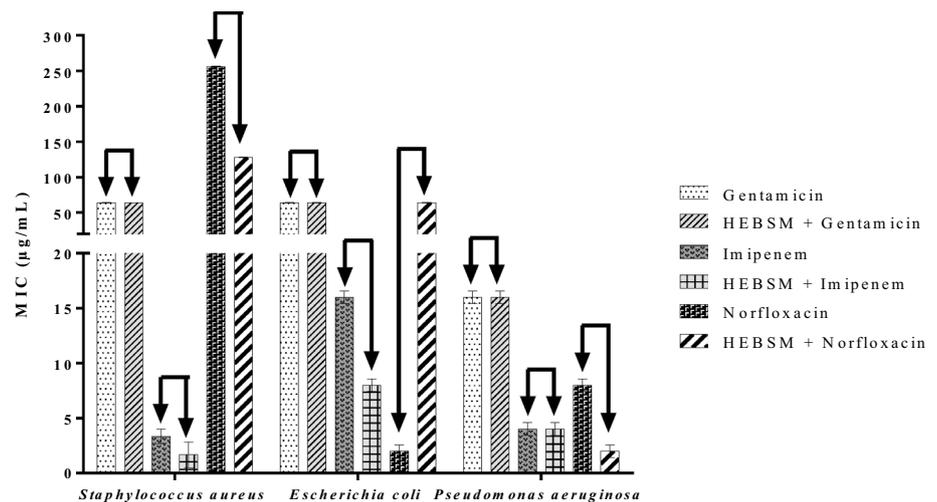


Fig. 4 Antibacterial activity of the Hydroethanolic Extract of the Bark of *Spondias mombin* (HEBSM) combined with standard antibiotics against MDR bacteria. ***statistically significant ($p < 0.001$), ns not significant ($p > 0.05$)



Samuggam et al. (2021) observed that the antibacterial effect of *S. mombin* leaf extract against biofilm-producing bacteria, including *S. aureus* and *E. coli*, was improved by the synthesis of plant nanoparticles (extract + AgNPs). When interacting with bacterial cells, the nanoparticles promoted structural changes in the cells, which were associated with increased oxidative stress, altered protein synthesis, and resulting in toxicity and cell death.

Here, it is hypothesized that the antibacterial effects of both extracts may be due, at least partially, to the presence of catechin, whose antibacterial activity has been evidenced by several studies compiled by Wu and Brown (2021). Most of these studies suggest that the mechanisms underlying the antibacterial effects of this flavonoid involves the free radical generation with consequent damage to membrane lipids, DNA and proteins. It has been also proposed that these extracts can potentiate the activity of antibiotics by mediating the release of lipoteichoic acid, weakening the cell wall of Gram-positive bacteria and by disrupting the outer membrane packaging, facilitating the entry of antibiotics into Gram-negative bacteria. This compound was shown to keep antibiotics inside the cells by inhibiting efflux pumps. Importantly, significant effort has been made to improve the stability, specificity and bioavailability of quercetin as a therapeutic agent.

The results presented in this work indicate that *S. mombin* extracts are more effective against Gram-negative bacterial strains, demonstrating little antibacterial activity against Gram-positive strains. The impact of this finding is highlighted by the evidence that Gram-negative bacteria are usually less sensitive to both antibiotics and natural products, due to the existence of limiting factors such as the BAM complex (Gu et al. 2016). Thus, it is suggested that the effects of the extracts against Gram-negative strains occur due to the presence of phytochemicals that increase the permeability of the bacterial membrane, interrupting

their defense mechanism. However, this hypothesis needs further investigation.

The antibacterial activity of the methanolic extract obtained from the *S. mombin* bark was reported by Roumy et al. (2020). In addition to inhibiting the growth of *P. aeruginosa*, the extract was active against *S. aureus*, corroborating data obtained in the present research, although different solvents have been used in the extraction. In addition, earlier reports demonstrated that the aqueous extract obtained from the leaves of *S. mombin* has antibacterial effects against *E. coli* and *P. aeruginosa* (Agbaje et al. 2020). However, these effects were observed using significantly higher concentrations of the extract.

According to Alhadrami et al. (2020), chemical compounds of both natural and synthetic origin can increase the activity of specific antibiotics, reversing the resistance of some bacteria to certain antibiotics. Although the *S. mombin* extracts have failed to present effective antibacterial effects (Houghton et al. 2007) against Gram-positive strains, both extracts enhanced the activity of conventional antibiotics against the MDR strain of *S. aureus*.

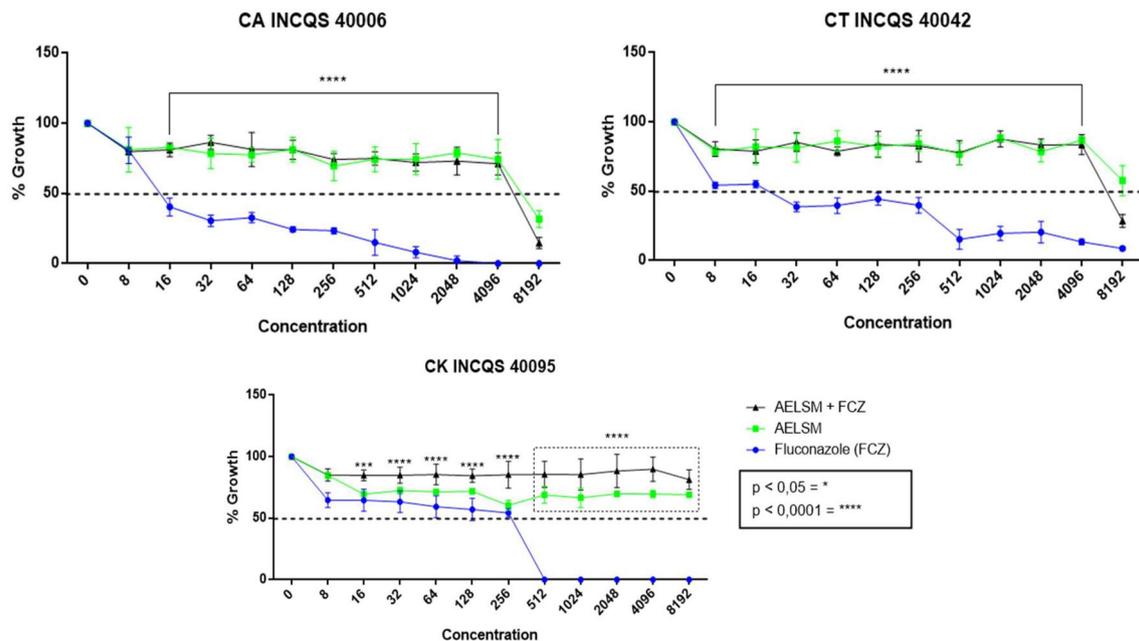
Antifungal activity of *Spondias mombin*

Table 4 shows the concentrations of the *S. mombin* extracts that inhibited fungal growth by 50% (IC_{50}). Since the leaf extract presented an IC_{50} above 1000 $\mu\text{g/mL}$, its anti-*Candida* activity is considered weak. On the other hand, the bark extract presented clinically relevant IC_{50} against *Candida albicans* (294.3 $\mu\text{g/mL}$) and *Candida krusei* (89.1 $\mu\text{g/mL}$), demonstrating stronger antifungal activity in comparison with the standard drug. In addition to directly inhibiting fungal growth, the bark extract was found to potentiate the antifungal activity of fluconazole reducing its IC_{50} against all *Candida* strains, especially *Candida krusei* (IC_{50} change: 168.7–5.3 $\mu\text{g/mL}$).

Table 4 Concentration that Inhibits 50% of the microbial growth (IC₅₀) of *S. mombin* extracts against *Candida* strains

Treatment	CA INCQS 40006 IC ₅₀ (µg/mL)	CT INCQS 40042 IC ₅₀ (µg/mL)	CK INCQS 40095 IC ₅₀ (µg/mL)
FCZ	18.3	11.4	168.7
AELSM	7720	8197.5	14,540.3
AELSM + FCZ	5288.1	6181.1	16,461.5
HEBSM	294.3	1452.6	89.1
HEBSM + FCZ	3.3	3.3	5.3

HEBSM hydroethanolic extract of the bark of *S. mombin*, *AELSM* aqueous extract of leaves of *S. mombin*, *FCZ* Fluconazol, *INCQS* National Institute for Quality Control in Health, *CA* *Candida albicans*, *CT* *Candida tropicalis*, *CK* *Candida krusei*

**Fig. 5** Antifungal effect of the Aqueous Extract of the Leaves of *Spondias mombin* (AELSM) alone associated with fluconazole (FCZ) against *Candida* strains. *Candida albicans* INCQS 40006, *Can-*

didia tropicalis INCQS 40042 and *Candida krusei* INCQS 40,095. ****Statistically significant ($p < 0.001$) when compared to the control group (Fluconazole)

As shown in the fungal growth curve (Fig. 5), the leaf extract only inhibited the growth of *C. albicans* at concentrations above 8192 µg/mL, while fluconazole caused significant inhibition of fungal growth at concentrations above 16 µg/mL. On the other hand, the association between the antifungal drug and the extract resulted in antagonism. Similar pharmacological outcomes were observed in the growth curve of *C. tropicalis* considering the same treatment conditions. However, this extract showed no significant antifungal effect against *C. krusei*, either as a single treatment or in association with fluconazole.

The analysis of the cell viability curve (Fig. 6) indicates that the anti-*Candida* effect of the bark extract is weaker than that of the standard antifungal drug. However, the association of the extract with fluconazole resulted in synergistic

effects from the concentration range of 8–64 µg/mL. Comparable outcomes were observed in the growth curve of *C. tropicalis*, where the association with the extract resulted in synergism from the concentration range of 8–256 µg/mL.

Additionally, this extract demonstrated remarkable antifungal effects against *C. krusei*. In addition to presenting strain intrinsic antifungal effect, the extract potentiated the activity of fluconazole, indicating a significant inhibition of antifungal resistance. Of note, the results expressed through the cell viability curve are in line with the IC₅₀ values described previously.

The antifungal effects of an ethanolic extract obtained from the bark of *S. mombin* were reported by Chaves et al. (2018). These authors demonstrated that this extract inhibited the growth of *C. albicans* and *C. krusei*, when used at

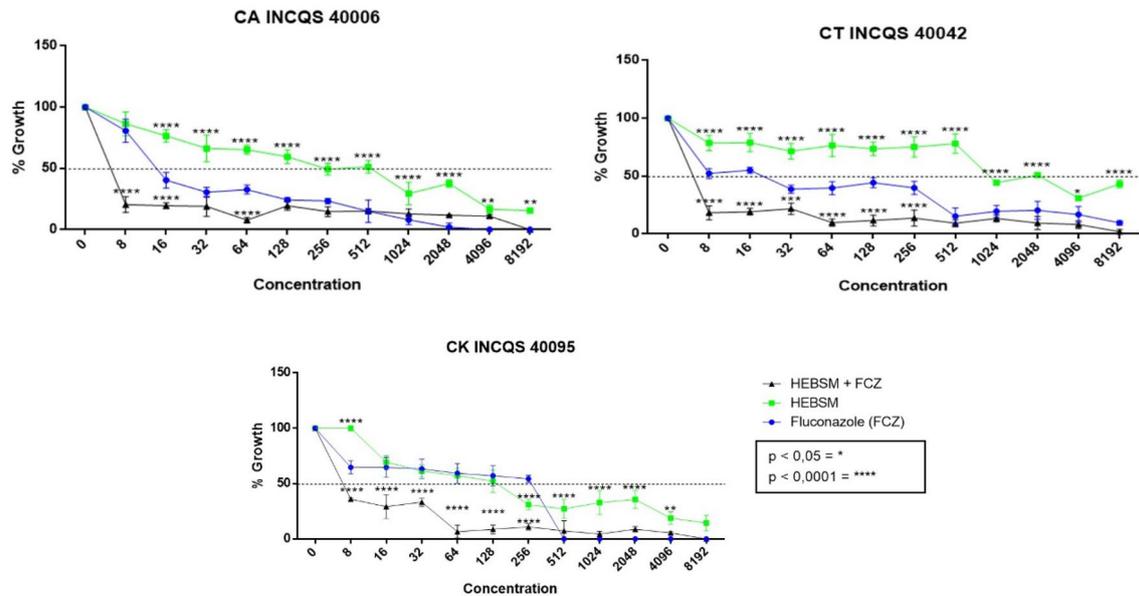


Fig. 6 Antifungal effect of the Hydroethanolic Extract of the Bark of *Spondias mombin* (HEBSM) isolated or associated with fluconazole (FCZ) against *Candida* strains. *Candida albicans* INCQS

40006, *Candida tropicalis* INCQS 40042 and *Candida krusei* INCQS 40,095. ****Statistically significant ($p < 0.001$) when compared to the control group (Fluconazole)

concentrations above 1000 µg/mL. Here, we demonstrated the antifungal effect of *S. mombin* also at high concentrations. Additionally, Temitope et al. (2017) demonstrated that different extracts of this species potentiated the activity of fluconazole against *Candida krusei*, corroborating the data obtained in our study.

The hydroethanolic extracts of the bark and fruits of *S. mombin* were evaluated for their antifungal potential against oral microorganisms, including *C. albicans*, *Candida dubliniensis* and *C. tropicalis*, demonstrating a fungistatic effect at a concentration of 8000 µg/mL. However, unlike the findings of the present research, no fungicidal effect was observed (De Freitas et al. 2020).

With regard to the potential contribution of isolated constituents of the extracts, the polyphenol quercetin has been reported to induce apoptosis in *Candida albicans* strains by decreasing redox potentials in the mitochondria, leading to DNA fragmentation (Kwun and Lee 2020). Salazar-Aranda et al. (2015) also attested the antifungal effects of kaempferol and quercetin against several species of *Candida*. Okwuosa et al. (2012) identified the presence of resins, saponins, tannins, glycosides, flavonoids, alkaloids, and lipids in the methanolic extract of the bark and leaves of *S. mombin*, demonstrating that they have remarkable antifungal activities. Phenolic compounds contain hydroxylated groups that inhibit enzymes through reaction with sulfhydryl groups or by non-specific reactions with proteins, contributing to the antifungal effect (Martins et al. 2015).

The effects of quercetin and other flavonoids against four *C. albicans* strains was evaluated by Ivanov et al. (2021). The authors obtained MIC values ranging from 37.5 to 75 µg/mL in addition to demonstrating that quercetin inhibited biofilm formation.

Effects of *Spondias mombin* extracts on fungal virulence

Figure 7 shows the effects of fluconazole on the emission of pseudohyphae, a morphological change that significantly contributes to *Candida* virulence. At concentrations equivalent to the HCA/4 or higher, the drug inhibited the emission of pseudohyphae by *C. albicans* and *C. tropicalis* but had a little inhibitory effect in *C. krusei* cultures.

The treatment with the extract obtained from the leaves (Fig. 8) and bark (Fig. 9) of *S. mombin* inhibited the emission of pseudohyphae by *C. albicans* and *C. tropicalis* at the highest concentration evaluated (8,192 µg/mL). However, none of these extracts were capable of inhibiting the formation of pseudohyphae by *C. krusei*. These findings indicate that both extracts inhibit the morphological transition of some *Candida* strains.

The emission of filamentous structures (hyphae and pseudohyphae) represents an important virulence factor associated with the invasion of superficial and systemic tissues during fungal infections (Henriques and Williams 2020). Hence, scientists have been looking for compounds capable of inhibiting these morphological

Fig. 7 Micromorphological aspects of *Candida* strains in the control groups. Growth controls (1, 2, 3); Fluconazole at 512 $\mu\text{g}/\text{mL}$ (4, 5, 6); Fluconazole at 2048 $\mu\text{g}/\text{mL}$ (7, 8, 9); Fluconazole at 8192 $\mu\text{g}/\text{mL}$ (10, 11, 12). *CA* *Candida albicans*, *CK* *Candida krusei*, *CT* *Candida tropicalis*, *INCQS* National Institute for Quality Control in Health, *HCA* Higher Concentration Assessed. Visualization under optical microscopy with 400 \times magnification

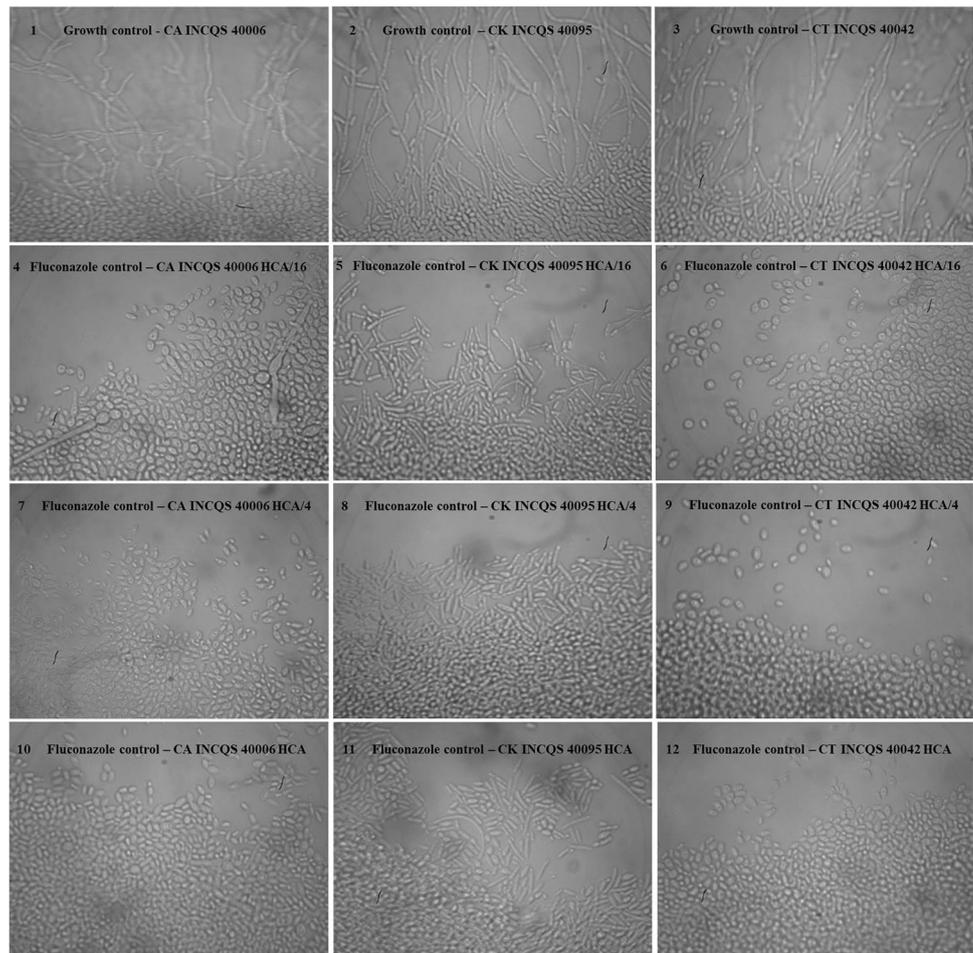


Fig. 8 Effect of the Aqueous Extract of the Leaves of *Spondias mombin* on *Candida* dimorphism. *CA* *Candida albicans*, *CK* *Candida krusei*, *CT* *Candida tropicalis*, *INCQS* National Institute for Quality Control in Health, *HCA* Higher Concentration Assessed. *HCA/16* (512 $\mu\text{g}/\text{mL}$); *HCA/4* (2048 $\mu\text{g}/\text{mL}$); *HCA* (8192 $\mu\text{g}/\text{mL}$). Visualization under optical microscopy with 400 \times magnification

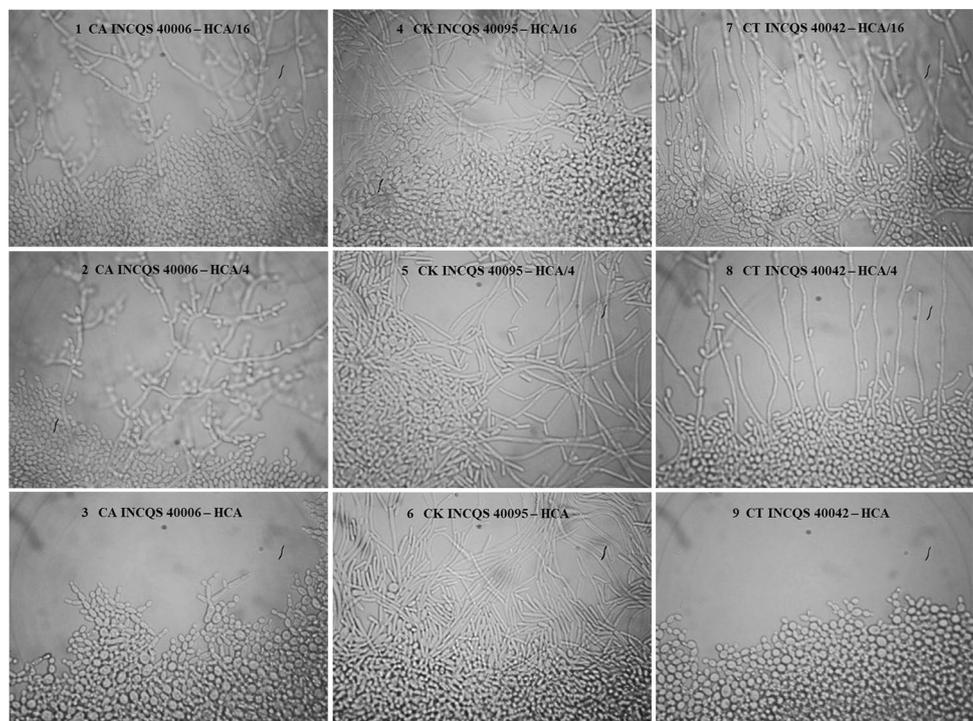
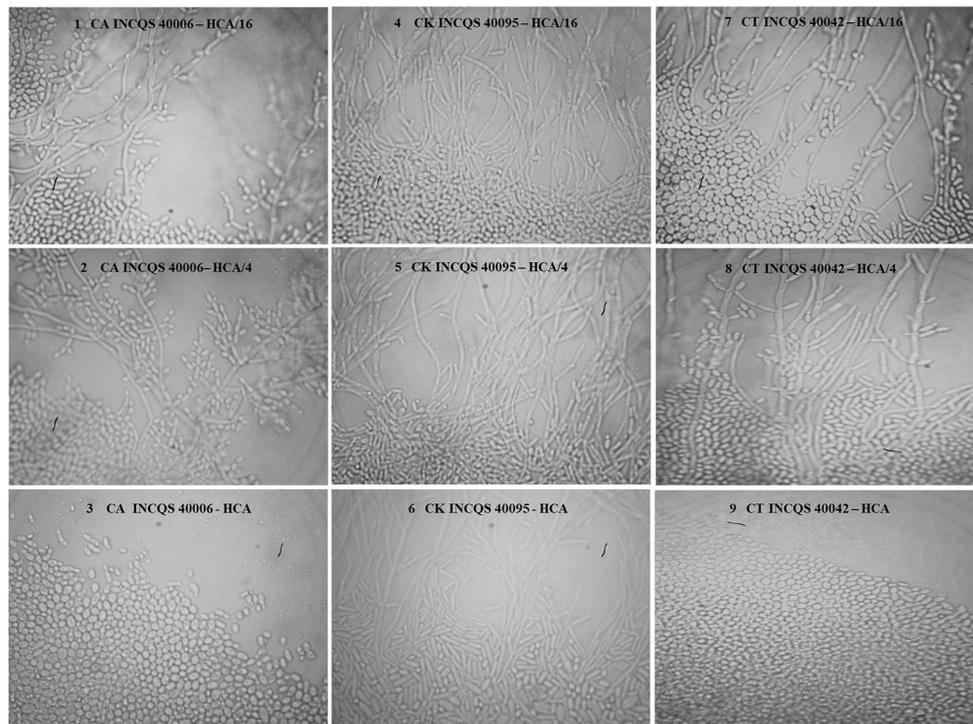


Fig. 9 Effect of the Hydro-ethanolic Extract of the Bark of *Spondias mombin* on *Candida* dimorphism. CA *Candida albicans*, CK *Candida krusei*, CT *Candida tropicalis*, INCQS National Institute for Quality Control in Health, HCA Higher Concentration Assessed. HCA/16 (512 $\mu\text{g/mL}$); HCA/4 (2048 $\mu\text{g/mL}$); HCA (8192 $\mu\text{g/mL}$). Visualization under optical microscopy with 400 \times magnification



changes, and evidence has indicated that natural products such as extracts, fractions, essential oils, fixed oils, waxes, and latex are promising sources of bioactive molecules (Morais-Braga et al. 2016a; Freitas et al. 2017; Santos et al. 2019a, b, c; Bezerra et al. 2020).

In this study, we showed that *S. mombin* extracts are capable of inhibiting these morphological changes and as such could be used as a weapon against the virulence of different *Candida* species. Accordingly, evidence indicates that flavonoids can inhibit both the growth and morphological transitions in yeasts (Canonico et al. 2014). The work carried out by Candiracci et al. (2012) suggests that secondary metabolites such as quercetin and kaempferol act synergistically inhibiting the emission of pseudohyphae, providing a link between the phytochemical profile of the extracts and their inhibitory effects on fungal virulence. Research by Ivanov et al. (2021) found that quercetin and other flavonoids moderately inhibited the emission of filamentous structures by *Candida* species, proving that this class of secondary metabolites may be acting by inhibiting the expression of genes responsible by the morphological transition of these microorganisms.

The anti-*Candida* action demonstrated by *S. mombin* extracts corroborates the evidence that the Anacardiaceae is a family with significant biological potential against *Candida* species since a similar activity was demonstrated by species such as *Myracrodruon urundeuva* Allemão, *Anacardium occidentale* L. and *Spondias tuberosa* Arruda. Importantly, all these species are native to the Caatinga, a seasonally dry

tropical forest located in Northeast Brazil (Cordeiro et al. 2018; Oliveira et al. 2017; Santos et al. 2019a, b, c; Silva et al. 2020a, b). This pioneering study shows for the first time the interference of *S. mombin* extracts on *Candida* spp. fungal pleomorphism, a significant virulence factor and aggravating mechanism of infection by these opportunistic pathogens.

Despite the qualitative chemical analysis and HPLC–DAD characterization, the chemical constitution of the *S. mombin* extracts under investigation remain to be fully elucidated. Thus, considering that extracts are complex chemical mixtures, their compounds can act either synergistically or antagonistically to promote biological effects. Therefore, further investigation is required to fully understand the effects of these extracts, as well as the antimicrobial potential of their isolated constituents, whether combined with each other or with other drugs, encapsulated or not by nanoparticles, in the search for new active compounds, so that they can be used in antimicrobial therapy against pathogenic and opportunistic bacteria and fungi.

Conclusion

Extracts obtained from both leaves and bark of *S. mombin* are constituted by phenolic compounds with recognized antimicrobial activity, which can be contributing to the biological effects demonstrated in this work.

In addition to presenting antibacterial and antifungal properties, *S. mombin* extracts can potentiate the activity of antibiotics and antifungals, suggesting that they can be used in the development of new drugs against antimicrobial resistance. The extracts also inhibited morphological changes that significantly contribute to the virulence of *C. albicans* and *C. tropicalis*.

In conclusion, the extracts obtained from *S. mombin* present antibacterial, antifungal, and antibiotic-potentiating activities, demonstrating that they are promising sources of bioactive compounds that could be useful in the combat of infections caused by MDR bacteria and invasive yeasts.

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Author contributions Methodology—antimicrobial assays (MAF, RPC and ATLS); methodology—chemical analysis (AAB, JWAB and AJTM); methodology—statistical analysis (JFSS and TSF); supervision of work—(FABC and MFBMB); coordination of the project (HDMC, JGMC and MFBMB); resources (JER, CFB and MKNS); final draft of the manuscript (ACAMM and JRF).

Declarations

Ethical statements This article is according with to the international, national and institutional rules considering biodiversity rights.

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