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LC-MS Analysis and Antimicrobial Activity of Different Extracts of *Spondias purpurea* L

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Spondias purpurea L. is widely used in folk medicine to treat various pathological conditions. One of its promising activities is antimicrobial. The present work aimed to characterize the composition and evaluate the antifungal and antibacterial effects of aqueous (AESP) and ethanolic (EESP) extracts of leaves. The technique of ultra-performance liquid chromatography coupled to a quadruple time-of-flight system (UPLC-QTOF) was used for phytochemical qualification. For the antifungal tests, two strains of Candida albicans were used, and for the antibacterial tests, strains of Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus were used. Quercetin, Kaempferol, Quinic Acid, gallo (epi)catechin dimer, dehydroascorbic acid, and quercetin derivative were detected in AESP, and quinic acid, iriflofenone 3-C-β D glycopyranoside, chlorogenic acid, and stearidonic acid in EESP. It was found that AESP potentiated the action of amikacin against bacteria, and an antifungal effect of both extracts was observed at a concentration of 8,192 µg/mL for the CA URM 4127 strain. The two evaluated extracts significantly potentiated the effect of fluconazole against CA INCQS 40006. The research showed that the extracts tested alone do not present significant antibacterial or antifungal activities at low concentrations against the tested strains. However, they demonstrated potentiating effect of already standardized drugs.

1. Introduction

Antimicrobials are revolutionary medicines in medicine, playing a fundamental role in treatment of bacterial pathologies reducing morbidity and mortality rates caused by them.[1] Although its discovery was a historic milestone for health, reducing deaths caused by infections, and improving the population's quality of life, its indiscriminate use led to the rapid triggering of microbial resistance mechanisms, since microorganisms respond to environmental stress by employing various adaptive strategies that contribute to the development of resistance and survival.[2] According to the World Health Organization,[3] this is a problem that needs to be faced in time, so that it does not cause harm to future generations, with an estimated 10 million deaths per year by 2050.[4]

To mitigate these problems, natural products are considered very important and have been used for thousands of years around the world for pharmacological purposes.[5] In developing countries, majority of the population uses traditional medicine for primary health care.[3] Furthermore, a large part of the world's

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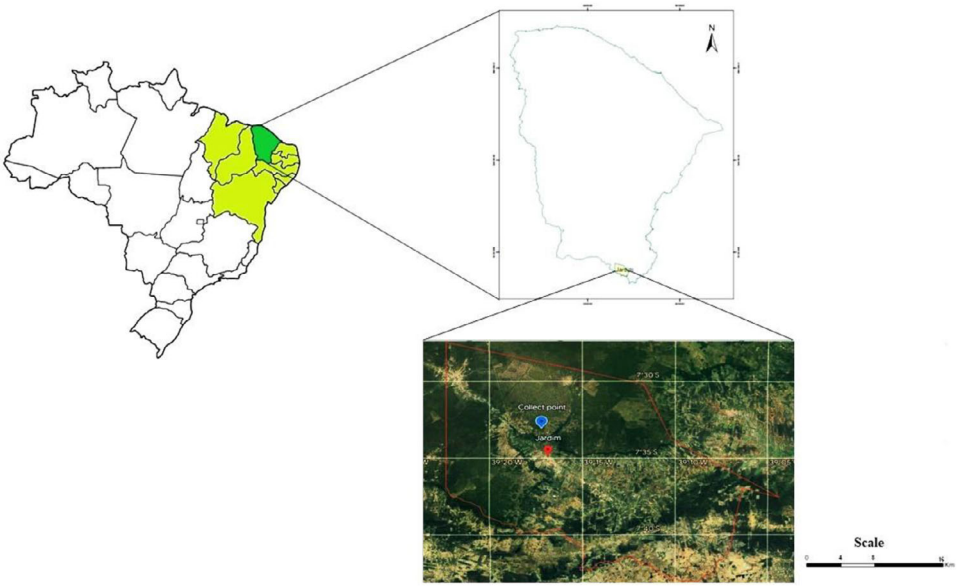


Figure 1. Cartographic representation of the collection point in the city of Jardim, Ceará, Brazil. Built from google earth.

population relies on herbal medicinal products to treat illnesses or uses traditional medicine. Some studies indicate that, when conventional medicine is not accessible, safe, and/or effective, traditional medicine can be considered as an alternative health treatment.[6]

Natural products, such as essential oils and plant extracts, are also considered important and promising because they have numerous active compounds, with varied spectrums of biological activities, where once administered in combination, they tend to act synergistically.[7] Furthermore, from the perspective of its probable use as a herbal medicine, it is relevant to evaluate the efficiency of this product in infectious conditions similar to those observed *in natura*. Extracts, as well as phytochemicals from plants, can act positively in improving the effectiveness of conventional antimicrobials, to reduce adverse effects and reduce the resistance of the most varied medicines used in the treatment of infectious diseases.[8]

Among the plants used in traditional medical practices in different cultures, those of the genus *Spondias* stand out, one of which is the species *Spondias purpurea* L. (Anacardiaceae), whose leaves are used in the treatment of gastric disorders, as antidiarrheals and antibiotics.[9] *S. purpurea* is a plant native to Central America and can be found in various parts of the world, such as Mexico, Guatemala, and the Caribbean, as well as in some South American countries, especially in the Northeastern region of Brazil.[10,11] This species is popularly known as "cajá," "mexican-plum," or "ciriguela." Its fruits and other parts have been traditionally used for centuries in the treatment of various ailments, such as diabetes, inflammation, diarrhea, and gastritis. These uses are mainly associated with its antioxidant, anti-inflammatory, and photoprotective properties.[12,13]

In this context, it is important to develop new research with an emphasis on the biological potential of this plant and its intrinsic antimicrobial effects or combined with antibiotics. Therefore, the main objective of our study is to evaluate the chemical composition of *S. purpurea* leaf extracts, as well as to investigate the intrinsic and combined antimicrobial activity against strains of *Escherichia coli (E. coli)*, *Staphylococcus aureus (S. aureus)*, *Pseudomonas aeruginosa (P. aeruginosa)*, and *Candida albicans* (*C. albicans*).

2. Material and Methods

2.1. Plant Material

The collection of the leaves of *S. purpurea* was carried out in the municipality of Jardim in the state of Ceará, Brazil, under the coordinates latitude 07° 33′ 17.6′′ and longitude 039° 18′ 23.5′′ (Figure 1). A specimen of the plant species was selected and deposited in the Herbarium Caririense Dárdano de Andrade-Lima where it was also identified, under voucher 01 13.525.

2.2. Extracts Preparation

The collected leaves of *S. purpurea* were initially cut and weighed and subsequently used to prepare two different (fresh) extracts. The solvents were chosen considering the differences in solubility of the various compounds commonly found in plant extracts, in order to optimize the extraction of a broader spectrum of hydrophilic substances. To prepare the ethanolic (EESP) and aqueous (AESP) extracts, the leaves were added separately to bottles containing two liters of 90% ethanol (460 g of leaves) and two liters of water (133.3 g of leaves), respectively. Subsequently, they were stored in a place protected from light and air for 72 h, a value determined experimentally (data not shown), to minimize the chances of material contamination. After this time,

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the materials were filtered and concentrated in a rotary evaporator, and the extracts obtained were stored under refrigeration at 6 °C for later use in the tests.

2.3. Phytochemical Analysis

The extracts were subjected to phytochemical analysis using UPLC coupled to a quadruple time-of-flight (QTOF) system (Waters Corporation, Milford, USA) at the Chemistry and Natural Products Laboratory of the Brazilian Agricultural Research Corporation - EMBRAPA (Fortaleza, Ceará). The chromatographic assays were performed on a UPLC BEH column (150 x 2.1 mm, 1.7 µm), at a fixed temperature of 40 °C, aqueous mobile phases with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), gradient ranging from 2% to 95% of B (15 min), flow rate of 0.4 mL/min and injection volume of 5 µL. The electrospray ionization (ESI) interface in negative ionization mode was acquired in the 110–1180 Da range, with the source temperature set at 120 °C and the desolvation temperature at 350 °C. The ESI mode was acquired in the 110–1180 Da range from a fixed source temperature of 120 °C. Leucine enkephalin was used as the lock mass. The acquisition mode was MSE (high-energy mass spectrometry). The instrument was controlled by Masslynx 4.1 software (Waters Corporation, Milford, USA). Compound identification was based on retention time, molecular ion mass, fragmentation pattern, molecular formula, and literature data.

2.4. Antifungal and Antibacterial Tests

2.4.1. Drugs, Reagents, Solution Preparation, and Microorganisms

The following were used in the antifungal assays: the *C. albicans* strain (CA) INCQS 40006, provided by the Oswaldo Cruz Culture Collection of the National Institute for Quality Control in Health (INCQS, Brazil) and the clinical isolate CA URM 4127, provided by Micoteca from the Federal University of Pernambuco - Recife Mycology University (URM).

In this experiment, the stock solution of each extract was prepared using 0.15 g of each dry extract diluted in 1 mL of dimethylsulfoxide (DMSO) (Merck KGaA, Darmstadt, Germany). To obtain the first evaluated concentration (16384 µg/mL), the extracts and the reference medicine with antifungal activity were diluted in sterile distilled water, where the latter consisted of fluconazole (FLUCOMED Capsule, Cimed, Brazil). The following culture media were used: semi-solid Sabouraud Dextrose Agar (SDA) (Kasvi, Brazil); and doubly concentrated liquid medium, Sabouraud Dextrose Broth (SDB) (Isofar, Brazil).

For antibacterial tests, three different species from the American type culture collection (ATCC) were used: *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027, and *S.s aureus* ATCC 25923; in addition to the multi-resistant clinical isolates: *E. coli* 06, *P. aeruginosa* 24, and *S. aureus* 10 were obtained from the Microbiology and Molecular Biology Laboratory of the Regional University of Cariri. The characterization of the resistance profile and description of the origins of these isolates have already been described previously.[14]

The six different strains were initially sub-cultured on Brain Heart Infusion Agar (BHIA) (Himedia, India) and incubated at 37 °C for about 24 h in a microbiological oven. After determining the growth of colonies, samples taken from these were suspended in glass tubes containing 3 mL of sterile saline solution (0.9% NaCl) with subsequent adjustment of turbidity to 0.5 McFarland scale, which is equivalent to 1.5 x 10^8 colony forming units (CFU)/mL.[15] A total of 0.02 g of the extracts (AESP and EESP) and the antibiotics amikacin, norfloxacin, and ampicillin were initially diluted in 1 mL of DMSO (Merck KGaA, Darmstadt, Germany), then added to the respective solutions: 8765 µL of sterile distilled water, reaching dilutions with a concentration between 1024 µg/mL and 2048 µg/mL for antibiotics and extracts, respectively.[16] The indicator resazurin sodium (7-hydroxy-3H-phenoxazin-3-one-10-oxide) was diluted in sterile distilled water to obtain a solution with a concentration of 0.4 mg/mL used to identify the presence of bacterial metabolism.

Antimicrobial trials should be conducted using available reference drugs, based on clinical trial evidence, medical recommendations, specific guidelines for the type of test being evaluated, and the expected prevalence of resistance to the agent(s) being compared at the study sites. This will ensure the overall validity of noninferiority trials.[17]

2.4.2. Inhibitory Concentration of 50% of Microorganisms (IC_{50}) and Cell Viability Curve

The assay was performed using the broth microdilution method in 96-well plates. Initially, each well was filled with the medium and fungal inoculum and then 100 µL of extracts (16384 µg/mL) or fluconazole at the same concentration were added to the first well, followed by serial microdilution (8192 to 8 µg/mL). The last well did not contain any product or drug, therefore, it was reserved as a growth control. Sterility controls for media and controls for product diluents were also prepared using only saline solution (sodium chloride 0.9%) without fungal inoculum. The plates were incubated at 37 °C for 24 h and subsequently read in an ELISA spectrophotometer Thermoplate (Kasuaki, China) at a wavelength of 630 nm. All assays were performed in quadruplicate, so that results obtained were used to construct the cell viability curve, determining the IC_{50}, and the minimum inhibitory concentration (MIC) of the extracts and fluconazole.[15]

The evaluation of the intrinsic antibacterial activity of the extracts (AESP and EESP) was carried out according to.[18] In Eppendorfs, 900 µL of Brain Heart Infusion Broth (BHIB) (Himedia, India) at 10% and 100 µL of bacterial inoculum were added, then, the contents of the Eppendorfs were distributed in 96-well microdilution plates (100 µL/well), in alphabetical direction. Serial microdilution (1:1) was performed with 100 µL of extracts, except for the last well, which was used for growth control. Sterility controls for media and controls for product diluents in the antibacterial assay were carried out in the same way as mentioned above. The concentration of AESP and EESP in the plates

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ranged from 1024 to 1 µg/mL, these values were determined to maximize the chances of identifying the concentration inhibiting the growth of bacterial species. The experiments were performed in triplicate and the plates were kept at 37 °C for 24 h. The results were read after adding 20 µL of resazurin to each well and after 1 h at room temperature (25 °C) the colorimetric variation of resazurin was observed (blue = inhibition/pink = bacterial growth).

2.4.3. Determination of the Minimum Fungicidal Concentration (MFC)

At the minimum fungicidal concentration, the fungi evaluated were homogenized with a sterile loop and sub-cultured in Petri dishes containing SDA culture medium (Sabouraud Dextrose Agar). Then, the plates were incubated in an oven at 37 °C for 24 h and then reading was done to check whether there was growth, or not, of *Candida* colonies.

2.4.4. Evaluation of the Modulating Activity of the Natural Product

The combination of extracts with fluconazole (reference drug) was carried out to verify whether or not there was a change in the antifungal action due to the presence of the extract in the medium. For this purpose, the extracts were tested at a subinhibitory concentration (MIC/16), following the method proposed by Coutinho et al.,[19] with changes. The plates were filled with a solution containing SDB, extract, and fungal inoculum. After this, 100 µL of fluconazole was added to the first well and serially microdiluted in a 1:1 ratio until the penultimate well. Sterility controls were also performed. The tests were performed in quadruplicates and the plates were incubated at 37 °C for 24 h. The readings were carried out in an ELISA spectrophotometry device Termoplate (Kasuaki, China). If the extracts intensified the action of the antifungal, the observed effect was considered as a potentiation, whereas if they impaired the action of the drug, it was considered as an apparent antagonist of the drug effect.[20]

In antibacterial tests, the extracts (AESP and EESP) were used in the subinhibitory concentration (MIC/8), 150 µL of the inoculum of the multi-resistant strains were added to the Eppendorfs (1.5 mL), and its total volume was completed with 10% of BHI. The solutions contained in the microtubes (100 µL/well) were numerically distributed in the 96-well plates. Microdilution took place with 100 µL of antibiotics up to the penultimate well and its concentration in the plates varied from 1024 to 1 µg/mL. These tests were also performed in triplicate and the incubation and reading processes were done as in the MIC tests.

2.5. Statistical Analysis

Data were analyzed using the *GraphPad Prism* software, v. 6.0. A two-way analysis of variance (ANOVA) was applied to each sample, comparing the mean values for each concentration of extracts with the values referring to the control groups using the Bonferroni test and considered significant when $p < 0.05$. IC_{50} values were obtained by non-linear regression with interpolation of unknowns from standard curves obtained from fungal growth values as a function of extract concentration and expressed in µg/mL.

For antibacterial tests, results were expressed as the geometric mean. A one-way analysis of variance (ANOVA-One-Way) followed by the post-hoc Bonferroni test (where $p < 0.05$ and $p < 0.0001$ are considered significant and $p > 0.05$ not significant) was applied using the software *GraphPad Prism* 6.0.

3. Results

3.1. Phytochemical Analysis–UPLC-ESI-QTOF-MS

The final weight of the ethanolic and aqueous extracts after the concentration process was between 0.2602 and 0.214 g, obtaining an approximate final yield of 0.056% and 0.16% respectively. From the analysis of the AESP chromatograms, it was possible to notice the presence of six compounds, namely, two flavonoids (Quercetin and Kaempferol), a phenolic acid (Quinic Acid), a gallo(epi)catechin dimer, dehydroascorbic acid, and a derivative of quercetin (Table 1). From the analysis of EESP chromatograms, the presence of four compounds was observed, namely quinic acid, iriflophenone 3-C-β D glycopyranoside, chlorogenic acid, and stearidonic acid (Table 2).

3.2. Antibacterial Activity

3.2.1. Minimum Inhibitory Concentration (MIC)

S. purpurea extracts (AESP and EESP) did not show intrinsic antibacterial activity against any of the standard and multidrug-resistant (MDR) strains tested that have already been considered clinically relevant in the specialized literature[28] (Table 3).

3.2.2. Effect of the Activity Combined with Antibiotics

For *P. aeruginosa* 24, AESP potentiated the effect of amikacin, thus the concentration of antibiotic needed to inhibit bacterial growth was reduced, obtaining a MIC of 32 µg/mL. However, the combined action of the extract and antibiotics ampicillin and norfloxacin caused an apparently antagonistic effect since a higher concentration of antibiotics was necessary to inhibit the growth of *P. aeruginosa*. This effect was also observed for EESP in combination with amikacin, ampicillin, and norfloxacin (Figure 2a).

Both extracts of *S. purpurea* potentiated the action of the antibiotic amikacin when evaluated against strains of *E. coli* 06 ($p < 0.001$). On the other hand, AESP when associated with norfloxacin showed an apparently antagonistic effect. For ampicillin and norfloxacin, no specific action of antibiotics was observed when associated with EESP against *E. coli* 06, which was also observed when the antibiotic norfloxacin was combined with EESP (Figure 2b). Finally, when evaluated in strains of *S. aureus* 10, both extracts were able to potentiate the action of the antibi-

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Table 1. Chemical composition of AESP where six substances were found.

Peak no.	Rt min	[M-H]⁻ Observed	[M-H]⁻ Calculated	Ions Products (MS/MS)	Empirical Formula	Ppm (Error)	Putative Name	Reference
1	2.73	191.0557	191.0556	191.0529 (100); 707.1838 (27)	C7H11O6	0.5	Quinic Acid	[21]
2	3.51	173.0081	173.0086	191.0477 (100); 353.0653 (27)	C6H5O6	−2.9	Dehydroascorbic	[22]
3	3.86	609.1281	609.1244	609.1332 (100); 300.0204 (31%)	C30H25O14	6.1	Gallo(epi)catechin dimer	[23]
4	4.11	593.1370	593.1354	593.1437 (100); 285.0351 (66); 594.1537 (27)	C23H29O18	2.7	Quercetin derivative	[24]
5	5.32	301.0336	301.0348	301.0345 (100); 151.0017 (89)	C15H9O7	−4.0	Quercetin	[21]
6	5.92	285.0372	285.0399	285.0363 (100)	C15H9O6	−9.5	Kaempferol	[24]

Table 2. Chemical composition of EESP where four substances were found.

Peak no.	Rt min	[M-H]⁻ Observed	[M-H]⁻ Calculated	Íons Products (MS/MS)	Empirical Formula	Ppm (Error)	Putative Name	Reference
1	3.27	191.0529	191.0556	127.0005 (100); 188.9989 (79); 83.0138 (50)	C7H11O6	−14.1	Quinic Acid	[21]
2	5.50	409.1096	409.1135	161.0196 (100); 409.1099 (64); 243.0605 (56)	C19H21O10	−9.5	Iriflofenone 3-C-β D Glucopyranoside	[25]
3	5.97	353.0858	353.0873	443.0587 (100); 127.0038 (91); 111.0448 (88)	C16H17O9	−4.2	Chlorogenic Acid	[26]
4	6.51	277.2156	277.2168	277.2147 (100); 397.1324 (74); 675.3602 (52)	C18H29O2	−4.3	Stearidonic acid	[27]

Table 3. Minimum Inhibitory Concentration (MIC) of aqueous and ethanolic extracts of *S. purpurea* against standard (ATCC) and MDR bacterial strains.

Substances	Bacterial Strains					
	Escherichia coli ATCC 25922	*Pseudomonas aeruginosa* ATCC 9027	*Staphylococcus aureus* ATCC 25923	*Escherichia coli* 06	*Pseudomonas aeruginosa* 24	*Staphylococcus aureus* 10
AESP	≥ 2048 µg/mL	≥ 2048 µg/mL	≥ 2048 µg/mL	≥ 2048 µg/mL	≥ 2048 µg/mL	≥ 2048 µg/mL
EESP	≥ 2048 µg/mL	≥ 2048 µg/mL	1024 µg/mL	≥ 2048 µg/mL	≥ 2048 µg/mL	≥ 2048 µg/mL

AESP: *Spondias purpurea* aqueous extract; EESP: Ethanol extract of *Spondias purpurea*; ATCC: Standard bacterial strains; EC: *Escherichia coli*; PA: *Pseudomonas aeruginosa*; SA: *Staphylococcus aureus*.

otic amikacin, while EESP together with the antibiotics ampicillin and norfloxacin caused apparent antagonism, an effect also observed in the combination of AESP and ampicillin (Figure 2c).

3.3. Antifungal Activity

3.3.1. Inhibitory Determination of 50% of Microorganisms (IC_{50}) and Cell Viability Curve

Table 4 shows the IC_{50} values of the extracts, fluconazole, and the combined action of the drug with the natural products. It was found that the extracts did not have a clinically relevant antifungal effect against the strains of *C. albicans*, since the IC_{50} values are high, which are 10740 (AESP) and 1553 µg/mL (EESP) for CA INCQS 40006 and 2050 (AESP) and 4687 µg/mL (EESP) for CA URM 4127 (Table 4), in addition to CFM test values that gave ≥16384.

It was observed that for the CA INCQS 40006 strain, both extracts potentiated the effect of fluconazole, as the drug had an IC_{50} of 19.29 µg/mL alone and in combination with AESP and EESP the values decreased significantly ($p < 0.0001$) for 11.86 and 8.97 µg/mL, respectively. For the CA URM 4127 strain, no potentiating effect was verified, on the contrary, an antagonistic effect is suggested, since there was an increase in IC_{50} from the asso-

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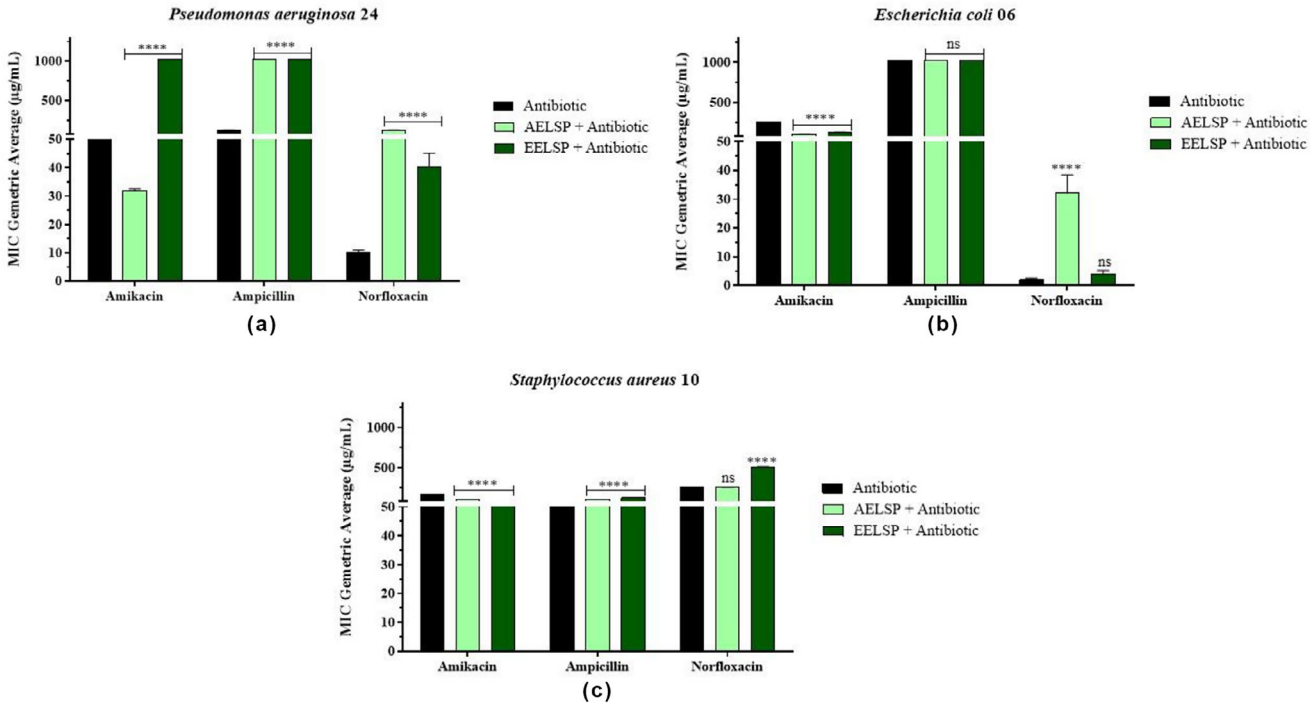


Figure 2. Antibacterial activity of aqueous and ethanolic extracts of *S. purpurea* leaves combined with antibiotics (amikacin, ampicillin and norfloxacin) against the MDR strains (*P. aeruginosa* 24, *E. coli* 06 and *S. aureus* 10). **** means $p < 0.0001$ compared to control (antibiotic group), ($n = 4$).

Table 4. IC_{50} values of *S. purpurea* extracts against *Candida albicans*.

Tested Products	Yeasts	
	CA INCQS 40006	CA URM 4127
Fluconazole (FCZ)	19.29 µg/mL	1.69 µg/mL
AESP	10740 µg/mL	2050 µg/mL
AESP + FCZ	11.86 µg/mL	3.76 µg/mL
EESP	1553 µg/mL	4687 µg/mL
EESP + FCZ	8.97 µg/mL	13.75 µg/mL

AESP: *Spondias purpurea* aqueous extract; EESP: Ethanol extract of *Spondias purpurea*; FCZ: Fluconazole; CA INCQS 40006: *Candida albicans* strain 40006 from the Culture Collection of the National Institute for Quality Control in Health; CA URM 4127: *Candida albicans* clinical isolate 4127 from the University of Mycology of Recife.

ciation of fluconazole and extracts. Thus, based on the high IC_{50} values and the behavior of the inhibition curve, it appears that the extracts of *S. purpurea* do not demonstrate clinically relevant antifungal activity when evaluated alone (Figure 3).

4. Discussion

Most of the compounds detected from the chromatographic analyses of *S. purpurea* extracts were categorically identified. In the case of the presence of unknown substances, these are usually detected in low concentrations, which indicate low chances of having detectable biological properties.[29] Kpomah and Odokkwo[30] when analyzing the composition of *S. mombin* leaves, it was possible to identify the presence of alkaloids, flavonoids, tannins, cyanogenic glycosides, saponins, phenols, oxalates, and phytates in variable amounts. These alkaloids have therapeutic as well as antibacterial properties.[31]

Similar to the present study, dos Santos et al.[32] analyzed the ethanolic and aqueous extracts of *S. tuberosa*, which when tested alone with the MDR bacteria species *S. aureus* 10, *P. aeruginosa* 24, and *E. coli* 06, it was possible to observe the absence of antibacterial activity considered clinically relevant. When tested in combination with different antibiotics, they caused an intensification of their effects in all species tested, the same was observed here for all antibiotics, in different bacteria, especially for amikacin, which was significantly enhanced in all tests. The average MIC of amikacin for *E. coli* normally ranges from 87 to 91 µg/mL, however here this parameter presented a much higher value, indicating the resistance pattern, which was significantly attenuated by both antibiotics (Figure 2).[33] Similar to the present study, recent research has also reported that amikacin, when combined with isolated or commercial compounds, can exert a positive synergistic effect, promoting the destruction of the cell wall of some *E. coli* strains, causing K + and protein leakage.[34]

Recent research has shown, through the disk diffusion method, that quinic acid, in addition to presenting antifungal activity, also presents antibacterial activity for gram-positive bacteria such as *S. aureus* (Inhibition zone diameter or IZD = 17.57 ± 0.9 mm), and also for gram-negative bacteria such as *E. coli* and *P. aeruginosa*, with IZDs of 15.10 ± 0.18 mm and 13.20 ± 1.2 mm, respectively.[35] Susceptibility tests involving kaempferol against strains of *S. aureus* and *E. coli* showed that the substance tested in isolation showed little or no antimicrobial activity, with MIC ≥ 512 µg/mL.[36]

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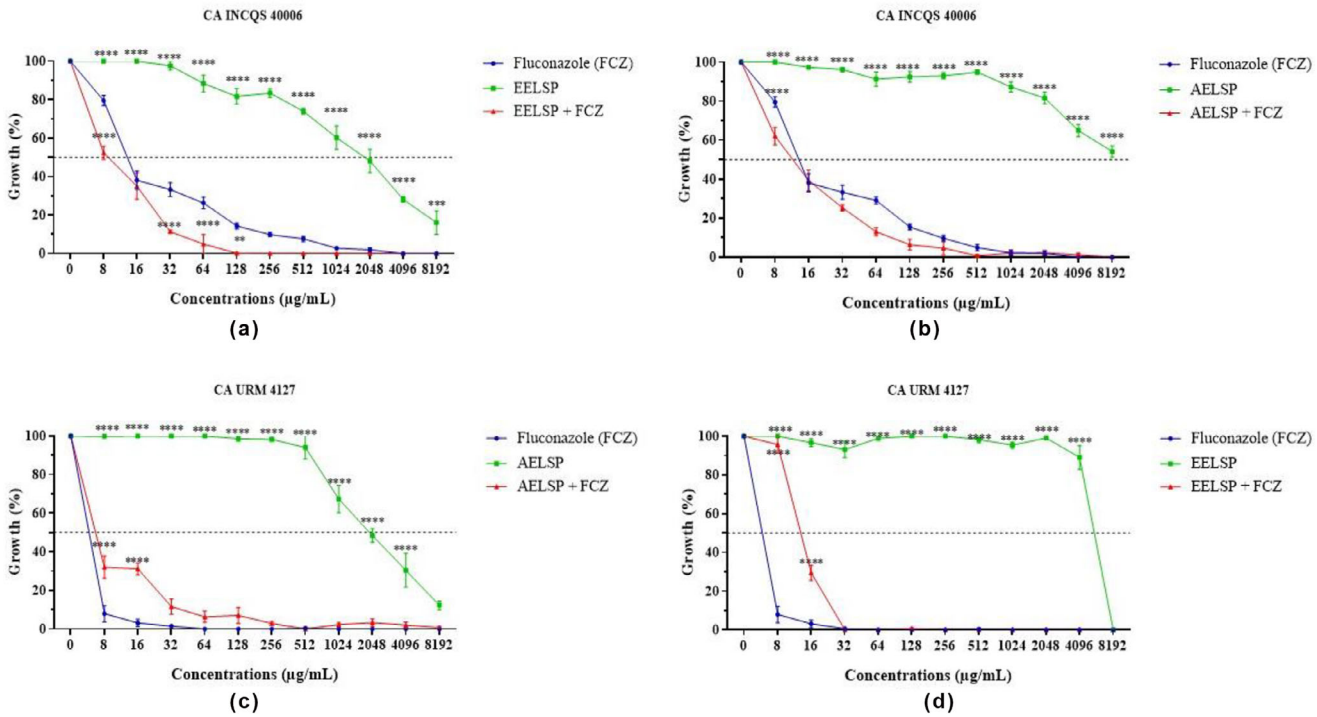


Figure 3. Potential of *S. purpurea* extracts against *Candida albicans* strains. a,b) Candida albicans – CA INCQS 40006; c,d) *Candida albicans* – CA URM 4127. Statistical significance was determined considering the difference in relation to fluconazole, **** = $p < 0.0001$; *** = $p < 0.001$ ($n = 4$).

In recent studies, it was demonstrated that gram-positive bacteria such as *S. aureus* showed sensitivity to several plant extracts when tested in isolation, resulting in low MIC values (21.5 and 25.0 µg/mL), suggesting the potential of natural compounds for the development of new antibacterial agents.[37,38] Gram-negative bacterial isolates resistant to beta-lactams, such as ampicillin, usually present similar resistance patterns, especially regarding their response to other classes of antibiotics.[39] For fluoroquinolone-resistant *S. aureus* strains, considerably variable MIC values are observed; however, in these cases there also appears to be some phenotypic similarity, with a higher MIC concentration = 32 µg/mL.[40]

Species of the genus *Spondias* are described in the literature as sources of phenolic compounds that have great medicinal importance, such as tannins, phenolic acids, and flavonoids. Among these species, some stand out for their biological and pharmacological properties, due to their secondary metabolites acting as the antibacterial activity of *S. mombin* and *S. pinnata*.[41] and the antioxidant and antifungal activity of *S. tuberosa* and *S. purpurea*.[42] One aspect that may corroborate the modulating and antimicrobial properties exhibited by both extracts is the fact that they present a similar pattern when used against two gram-negative bacteria, which in theory should have similar physiologies. A potential limitation of the antibacterial testing methodology applied here is the use of only one strain of each bacterial species, as multiple microbial strains may respond differently to treatments, as visualized here for *C. albicans* (Figure 3).

Investigations of biological and pharmacological activities of *Spondias* species mainly reflect their popular use as medicinal plants, being used for the treatment of various infectious diseases.[43] The therapeutic knowledge and use of *Spondias* spp. led to interest in exploring the phytochemical profile of these species, which reveals the presence of a variety of compounds of great scientific importance for acting as substances with antimicrobial potential. Compounds such as quercetin, chlorogenic acid, and kaempferol were identified in *S. tuberosa* and also in our study in *S. purpurea* extracts and that may be related to the observed activities.[44]

Research carried out to determine the antimicrobial activity of kaempferol and quercetin compounds revealed that they showed a significant capacity to inhibit proliferation in planktonic culture of some *Candida* spp., with MICs ranging from 32 µg/mL to 64 µg/mL for kaempferol and 0.5 µg/mL to 16 µg/mL for quercetin, compounds found in the AESP of the present study and which may explain its potentiating effect.[45]

Similarly, Muthamil et al.[46] observed that quinic acid, another compound present in AESP and EESP has a potential antibiofilm against *Candida* at concentrations ranging from 50 to 800 µg/mL. The same study showed that the chlorogenic acid identified in the EESP showed antifungal action against yeasts of *Trichosporon beigelii* (KCTC 7707) and *Malassezia furfur* (KCTC 7744), in addition to inhibiting and destroying the hyphae of *C. albicans* TIMM 1768. In a different investigation into the antimicrobial activity of chlorogenic acid, it was reported that this compound can interfere with the antifungal activity of some azole drugs, against *Candida* spp, enhancing the effect of drugs as also seen here for CA INCQS 40006 (Figure 3).[47] According to,[48] when investigating 2029 isolates of *C. albicans*, they observed that at least 98% of these yeasts showed sensitivity to

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fluconazole, that is there is phenotypic similarity between them and it is not necessary to use a large number of clinical strains to determine susceptibility patterns.

Santos et al.,[49] evaluating the antifungal activity of the hydroalcoholic extract of the leaves of *S. tuberosa*, observed that the extract, when tested in isolation, did not show a clinically relevant inhibitory action against *C. albicans*, but associated with the antifungal fluconazole, the extract showed a synergistic effect and potentiated the drug's action, results similar to those found here with extracts prepared from *S. purpurea* leaves (Table 4 and Figure 3). Both extracts presented quinic acid in their composition, which is likely responsible for the modulating effect, given that this organic acid can disturb the oxidative phosphorylation pathway and alter the levels of glycerophospholipids and fatty acids and can interfere with the fluidity of the cell membrane.[50]

Therefore, although *S. purpurea* extracts (AESP and EESP) did not show clinically relevant antifungal activity, they did show an apparent modulatory effect for fluconazole against CA INCQS 40006. This finding is relevant, considering that since the introduction of antifungal azoles (ketoconazole, miconazole, and fluconazole) for the treatment of infections caused by *Candida* spp., the emergence of species of this genus resistant to these drugs has been observed.[51] Clinical isolates of *C. albicans* may exhibit variable responses when exposed to the same plant extract, with considerably different MIC values.[52] Aliaghazadeh et al.[53] demonstrated in their studies that the flavonoid icariin significantly potentiated the effect of fluconazole in a strain of *C. albicans* (CECT 11821). This same activity was also observed in other azoles, such as ketoconazole and itraconazole, probably due to increased membrane instability causing cell permeability. It is also possible to observe that both AESP and EESP caused similar inhibition patterns of fungal growth, although not always significant when associated with the same microorganism, such similarities contribute to ensuring the reproducibility of the observed results.

5. Conclusion

Despite the lack of results that demonstrate the proportions of each compound identified here in the ethanolic and aqueous extracts of *S. purpurea*, such as quercetin, kaempferol, gallic acid, and rutin, the indication of their presence can help in formulating hypotheses that explain the antimicrobial properties observed. Also, the present research revealed that the extracts of *S. purpurea* studied, separately, do not present significant antibacterial or antifungal activity against the strains tested. However, alcoholic extract potentiated the action of the amikacin when evaluated against different MDR bacteria. For fungal strains, the two extracts together with the fluconazole proved to be relevant, enhancing their effect against CA INCQS 40006 at low concentrations. Therefore, new studies must be carried out to elucidate and improve the application of *S. purpurea* in antibiotic therapies, for example in the development of new drugs that can act in combination with conventional medicines. Finally, we emphasize the importance of fully elucidating the factors and conditions that affect microbial resistance to take comprehensive measures to control the development of this condition even before starting to use natural antimicrobial products in clinical routine.

Author Contributions

M.E.P.S., C.V.B.O., A.T.L.S., and V.B.S. contributed to the conception and design of the study. **J.N.P.C., E.S.B., J.C.A.P., G.A.D., and A.R.C**. carried out material preparation and data collection. **J.W.A.B., P.R.V.R., R.P.C., and D.L.S**. performed data analysis. **M.F.B.M.B. and H.D.M.C**. reviewed and submitted the manuscript, and all authors commented on its previous versions. All authors read and approved the final version.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Anacardiaceae · Antibacterial · Anti-Candida · Apotenciated effect · *Spondias purpurea*

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