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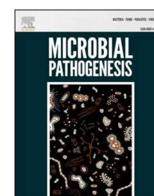

Caryocar coriaceum fruits as a potential alternative to combat fungal and bacterial infections: *In vitro* evaluation of methanolic extracts

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ABSTRACT

Caryocar coriaceum, commonly known as 'pequi', is a medicinal species used traditionally for the herbal treatment of infectious and parasitic diseases in the Brazilian Northeast region. In this study, we investigated whether the fruits of *C. coriaceum* have bioactive chemical constituents against etiological agents of infectious diseases. The methanolic extract of the internal mesocarp of the fruits of *C. coriaceum* (MECC) was chemically analyzed and evaluated for its antimicrobial and drug-enhancing activity against multidrug-resistant pathogenic bacteria (*Escherichia coli*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus*), and *Candida* spp. strains. The extract had flavones, flavonols, xanthones, catechins, and flavanones as major classes. A total of 11.26 mg GAE/g of phenolics, and 5.98 mg QE/g of flavonoids were found. No intrinsic antibacterial activity was observed; however, the extract was able to intensify the action of gentamicin and erythromycin against multi-resistant strains. The anti-*Candida* effect observed in this study was mainly due to the formation of reactive oxygen species. The extract was capable of causing damage to the plasmatic membrane of *Candida tropicalis* through pores formation. Our findings partially support the ethnopharmacological uses of the fruit pulp of *C. coriaceum* against infectious and parasitic diseases.

1. Introduction

The mortality as a result of infectious and parasitic diseases (IPDs) has grown in recent decades, mainly due to microbial resistance originating from the indiscriminate use of antibiotics. Pathogenic bacteria, fungi, and protozoa have been shown to overcome the action of commercially available drugs, and the discovery of new drugs has not kept pace with the adaptation speed of these microorganisms [1–3].

Another factor that significantly contributes to the increase in mortality due to infectious and parasitic diseases, is the lack of access to appropriate medicines by the population, especially in underdeveloped countries. On the other hand, medicinal plants have stood out as the therapeutic alternative for the treatment of IPDs, due to their easy access, low cost, and traditional and cultural usage by many communities [4,5]. Therefore, the investigation of the bioactive potential of plant species becomes important to formulate new medicines or drug enhancers [6,7].

In Brazil, medicinal plants are widely used by different communities, the country is one of the top ten consumers of *in natura* plant therapeutic resources [8–14]. Brazil has a huge diversity in terms of traditional communities which make use of herbal medicine (e.g., indigenous peoples, afro-descendant quilombos, rubber tappers, and traditional inhabitants of the coastal regions, among others). However, the use of medicinal plants is not only related to these cultural aspects but also to

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the plant diversity found in the different Brazilian phytogeographic domains [15].

Among Brazilian ecosystems, the Caatinga, a type of seasonally dry tropical forest, located in the Northeast region of Brazil, accounts for 17.2% of the total botanical taxa found in the country [15]. More than 700 spermatophytes are endemic to this region. Many of these species are used in traditional medicine to treat various diseases [6,15]. In the northern region of the Northeast, in the states of Ceará, Pernambuco and Piauí, more precisely in the area of Chapada do Araripe, a tree that stands out is *Caryocar coriaceum* Wittm. (Caryocaraceae), popularly known as "pequi" and "pequizeiro" and by the native indigenous Kariri as "*pyrantecaira*". This species produces fruits that are used in oil production, which is highly appreciated in regional cuisine. In addition to food use, this oil is highly versatile, displaying a notorious medicinal use [15–19].

In ethnopharmacology, the fruits of *C. coriaceum* and its by-products are used against infectious and parasitic diseases, also a series of digestive, genitourinary, skin, and subcutaneous diseases [19]. These medicinal properties can be attributed to the chemical composition of the fruits since they have flavonoids in their constitution [20]. In this context, considering the increasing resistance of microorganisms to the standard drugs, and the rising search for natural products with biological properties, it is pertinent to investigate the biological potential of *C. coriaceum* against microorganisms that cause infections, as well as to describe its chemical composition.

This study hypothesized that the extract of the fruit pulp of *C. coriaceum* has biological activity against microorganisms that cause infections. The objective of this research was to investigate whether the fruits of *C. coriaceum* have bioactive chemical constituents against etiological agents of infectious diseases, and also its antimicrobial and drug-enhancing activity against multidrug-resistant pathogenic fungi (*Candida* spp.) and strains bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*).

2. Methodology

2.1. Collection of botanical material and environmental licenses

Ripe and undamaged fruits of *C. coriaceum* were collected in February 2021 in the Environmental Protection Area (APA) of Chapada do Araripe, belonging to the municipality of Jardim - CE, Brazil (Fig. 1), under coordinates 07°29′269″S and 39°18′050″W, and at an altitude of 925 m. Concurrently, branches with fertile parts were collected, and herborization techniques were applied to make an exsiccate. Following, it was deposited in the Herbarium UFP - Geraldo Mariz under registration number 88,948. This study was registered in the Biodiversity Information and Authorization System (SISBio) under registration number 77450-1; and at the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under registration number A4848B1.

2.2. Extract obtaining

The internal mesocarp of *C. coriaceum* harvested from 300 fruits was subjected to dehydration at 40 °C for seven days, obtaining 760 g of dehydrated pulp. The pulp was subjected to exhaustive extraction with *n*-hexane for 72 h to remove compounds with low polarity. The residue was subjected to a new extraction with methanol for the same period. After filtration, the extract was concentrated on a rotary evaporator to obtain the crude extract (yield = 4.84%). The methanolic extract of *C. coriaceum* (MECC) was stored in an amber bottle at room temperature until chemical analysis and biological tests [21,22].

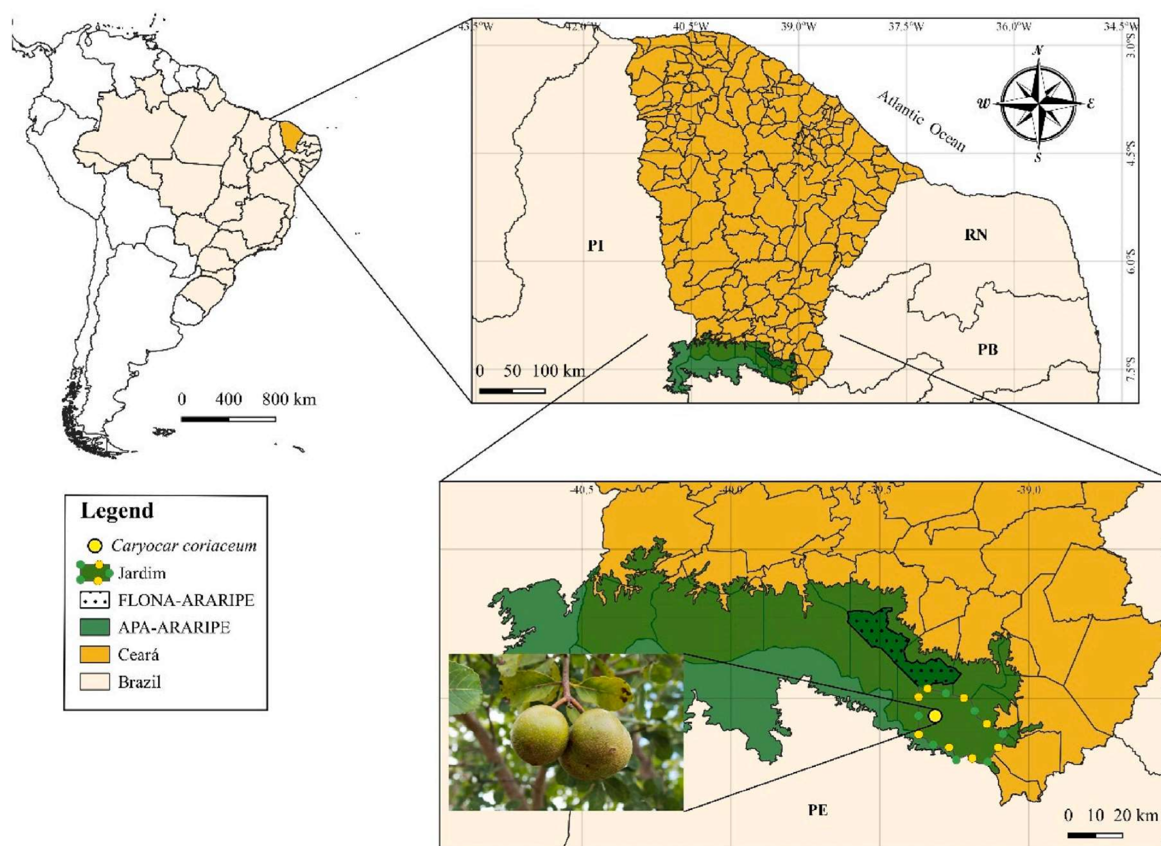

Fig. 1. *Caryocar coriaceum* Wittm. fruit collection location map in the municipality of Jardim, in the Environmental Protection Area of Chapada do Araripe, Brazil.

2.3. Phytochemical analysis

2.3.1. Qualitative chemical prospection

Were evaluated the presence of secondary metabolites in the extract via colorimetric alteration and formation of precipitates [23]. Tests were carried out for the detection of pyrogallic tannins, condensed tannins, anthocyanins, anthocyanidins, flavones, flavonols, xanthones, chalcones, aurones, leucoanthocyanidins, catechins, flavanones, and alkaloids.

2.3.2. Total phenols and flavonoids

To determine the total phenol content, the Folin-Ciocalteu method was used, according to Singleton et al. [24], with some adaptations. An ethanolic solution (1 mg/mL) of *C. coriaceum* extract was added to a volumetric flask, plus 250 μL of Folin-Ciocalteu reagent, and 3 mL of distilled water. This solution was stirred for 0.5 min, and 1 mL of 15% sodium carbonate (Na_2CO_3) was added. After 2 h, the absorbance was read in a spectrophotometric device at 760 nm (SmartSpec Plus, Bio Rad, USA). The total phenolic content was expressed in μg of gallic acid equivalents per mg of *C. coriaceum* extract (GAE/mg).

For the quantification of total flavonoids, the method described by Woisky and Salatino [25] was used with some alterations. In an aqueous solution of the extract (1 mg/mL), were added 3 mL of methanol and 1 mL of 5% aluminum chloride, left resting for 30 min. After this period, the absorbance of the solution was measured at 425 nm in a spectrophotometer device (SmartSpec Plus, Bio Rad, USA). Total flavonoid content was determined using a quercetin standard curve (Sigma-Aldrich®). The total flavonoid content was expressed in μg of quercetin equivalents per mg of *C. coriaceum* extract (QE/mg).

2.4. Antifungal activity

2.4.1. Strains, culture medium, drugs, reagents, and reparation of solutions

The standard strains of *Candida albicans* CA INCQS 90028, *Candida krusei* – CK INCQS 40095, and *Candida tropicalis* CT INCQS 40042 obtained from the Microbial Collection and Reference in Health Surveillance – CMRVS of the Instituto Nacional de Controle de Qualidade em Saúde (FIOCRUZ-INCQS) were used.

The fungal strains were inoculated in Petri dishes containing *Sabouraud Dextrose Agar* (SDA, Kasvi), and then incubated in a microbial incubator at 37 °C for 24 h. After growth, aliquots were collected and transferred to test tubes containing 3 mL of 0.9% saline solution, and adjusted to 0.5 McFarland turbidity (1×10^8 CFU/mL) [26]. For the microdilution assays and determination of the average inhibitory concentration (IC_{50}), *eppendorfs* containing *Sabourand Dextrose Broth* (CSD, Himedia) were prepared using a two-fold concentration. For the virulence inhibition assays, a *Potato Dextrose Agar* (PDA, Kasvi) medium depleted of nutrients was prepared to stimulate the formation of hyphae and pseudohyphae [27].

The methanolic extract of *C. coriaceum* (20 mg) was initially diluted in dimethylsulfoxide (0.5% DMSO, Merck, Darmstadt, Germany), and later in sterile distilled water, until reaching the concentration of the stock solution (2048 μg/mL). As a positive control, fluconazole (Capsule – FLUCOMED, São Paulo, Brazil) was used, this compound acts in the synthesis of ergosterol, which was diluted in sterile water [28].

2.4.2. Determination of half maximal inhibitory concentration (IC_{50})

To determine the anti-*Candida* action of the *C. coriaceum* extract, the broth microdilution technique was used, using a 96-well plate (Kasvi). During the procedure, each well was initially filled with 90 μL of *Sabouraud Dextrose Broth* (SDB), which preceded a serial microdilution (1:1 v/v) with the natural product (MECC) until the penultimate well of the plate, in numerical order. The concentrations obtained ranged from 1 to 1024 μg/mL. After this process, 10 μL of inoculum was added. No tested products were added in the last well, it was kept for fungal growth control. Using the same method for the positive control (fluconazole). Following, the plates were kept in a microbial incubator for 24 h at 37 °C. The samples were read using an ELISA spectrophotometer model DR.-200BS-NM-BI (Kazuaki, Wuxi, China) at a wavelength of 630 nm. Dilution controls for the natural product and drug (with 0.9% sodium chloride solution instead of the inoculum), and sterility controls, were also performed [29,30].

The absorbance results were used to determine the Minimum Inhibitory Concentration (MIC) and design the cell survival curve. Based on the average cell survival curve, the IC_{50} values of the *C. coriaceum* extract and fluconazole were calculated. The MIC was considered to be the concentration responsible for completely inhibiting fungal growth. When there was no MIC, the matrix concentration (2048 μg/mL) was considered as the starting point for the sub-inhibitory concentrations.

2.4.3. Assessment of fluconazole modifier activity

After determining the MIC, the assessment of the Fluconazole modifier activity was carried out using sub-inhibitory concentrations (MIC/8) according to Morais-Braga et al. [2]. During the testes, 96-well flat-bottomed plates (Kasvi) were used, which were added with *Sabouraud Dextrose Broth* (SDB) medium (SDB, Himedia) containing the methanolic extract of *C. coriaceum* in its sub-inhibitory concentrations. Subsequently, this solution was microdiluted with Fluconazole (1:1 v/v) up to the penultimate row of the wells. The concentrations ranged from 1024 to 1 μg/mL. Finally, the inoculum of *Candida* spp. was added until obtaining a concentration of 10% in each well of the plate. This procedure followed the same methodology as described in section 2.4.2.

2.4.4. Evaluation of fungal virulence inhibition

On a sterile microscope slide, 3 mL of Potato Dextrose Agar (PDA) depleted of nutrients containing the methanolic extract of *C. coriaceum* at sub-inhibitory concentrations (MIC/2 and MIC/4) was added. After solidification and stabilization of the medium on the slide, two parallel streaks of *Candida* spp. inoculum were made, which were placed in a humid chamber (Fig. 2), and kept in a microbial incubator under the same conditions previously mentioned. After a period of 24 h, the slides were observed under an optical microscope at a magnification of 400 ×. It captured images where there was emission or inhibition of filamentous structures. As a positive control, fluconazole was used at the same sub-inhibitory concentrations mentioned previously. While the growth control consisted of the presence of PDA and fungal strains [27].

2.4.5. Induction of reactive oxygen species (ROS)

Candida spp. were initially cultivated in SDB medium, for a period of 18 h at 37 °C. Subsequently, 100 μL of the fungal inoculum (10^6 cells/mL) were incubated with 100 μL of the methanolic extract of *C. coriaceum* at the concentrations of their IC_{50} (μg/mL) against their respective strains of *Candida* spp., at 37 °C for 24 h in an environment completely absent of light. Following, the yeasts from each treatment were incubated with 2,7-dichlorofluorescein diacetate (DCFH-DA) at 37 °C for 30 min in the dark. The yeasts were then centrifuged (Mikro 200R, Hettich, Germany) at 3000×*g* for 5 min at 22 °C, and washed three times using NaCl (0.15 M). Finally, they were properly prepared for observation under a fluorescence microscope (Olympus System BX 60; excitation and emission wavelengths of 488 and 525 nm, respectively). The positive control (Fluconazole), and the negative control (NaCl 150 mM) were preceded by the same method [31].

2.4.6. Determination of cell membrane integrity

Yeast cells were incubated in SDB for 24 h at 37 °C, plus methanolic extract of *C. coriaceum* in its IC_{50} concentration, and with 150 mM NaCl (negative control), or with fluconazole (positive control). After incubation, aliquots (100 μL) of treated cells were incubated with 1 mM propidium iodide for 30 min, at 37 °C, under moderate agitation (75 rpm). Cell visualization was performed using a fluorescence microscope (Olympus BX 60 System, excitation wavelength – 490 nm; emission wavelength – 520 nm) [32].

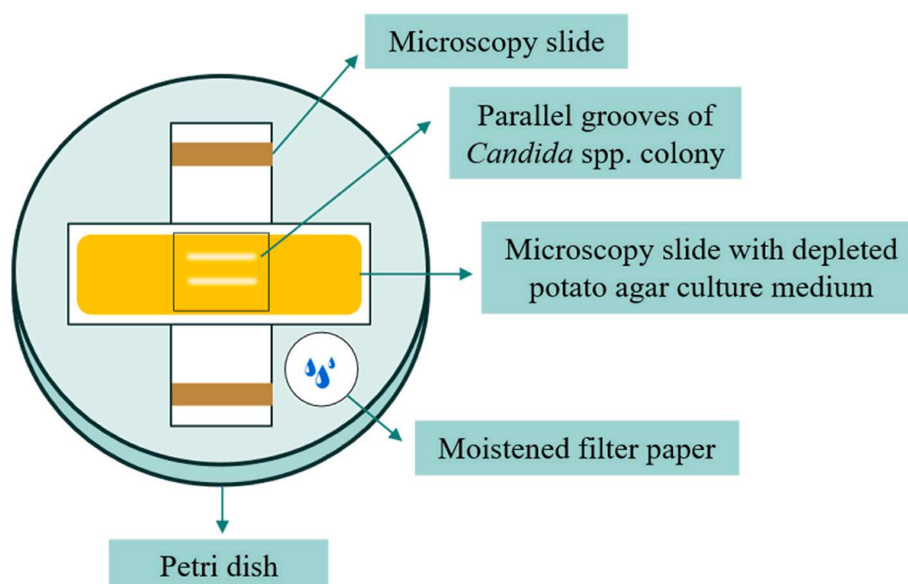

Fig. 2. Humid chamber scheme used for the induction of morphological transition in *Candida* yeasts.

2.5. Antibacterial activity

2.5.1. Strains, culture medium, drugs, reagents, and preparation of solutions

For the evaluation of the antibacterial activity, the standard bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 25853, and *Staphylococcus aureus* ATCC 22923), and the multi-resistant strains *Escherichia coli* 06, *Pseudomonas aeruginosa* 24, both urine culture derived, and *Staphylococcus aureus* 10, from rectal tissue culture (Table 1) were used. These bacteria were grown in Petri dishes containing Heart Infusion Agar (HIA), and placed in a microbial incubator at 37 °C for 24 h. After growth, samples of each bacterial culture were collected, and diluted in saline solution (0.9%), reaching a turbidity of 0.5 on the McFarland scale (1×10^8 CFU/mL). From these inoculums, 150 μL were taken and added to a 10% Brain Heart Infusion (BHI) solution, to be used in antibacterial assays.

For the determination of the minimum inhibitory concentration (MIC), the methanolic extract of *C. coriaceum* was diluted in dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany) reaching a concentration of 20,000 μg/mL, later this solution underwent a new dilution with sterile distilled water until reaching a concentration of 1024 μg/mL. The antibacterial drugs used in the intensifier activity assays were norfloxacin (fluoroquinolone class), gentamicin (aminoglycosides), and erythromycin (macrolide group).

Table 1
Antibacterial resistance profile of clinical bacterial isolates. Source: Laboratory of Microbiology and Molecular Biology – LMBM, Regional University of Cariri - URCA.

Bacteria	Resistance profile
Escherichia coli 06	Cephalotin, cephalexin, cefadroxil, ceftriaxone, cefepime, ampicillin-sulbactam, amikacin, imipenem, ciprofloxacin, levofloxacin, piperacillin-tazobactam, ceftazidime, meropenem, cefepime
Pseudomonas aeruginosa 24	Amikacin, imipenem, ciprofloxacin, levofloxacin, piperacillin-tazobactam, ceftazidime, merpenem, cefepime
Staphylococcus aureus 10	Cefadroxil, cephalexin, cephalothin, oxacillin, penicillin, ampicillin, amoxicillin, moxifloxacin, ciprofloxacin, levofloxacin, ampicillin-sulbactam, amoxicillin/ac. clavulanic, erythromycin, clarithromycin, azithromycin, clindamycin

2.5.2. Minimum inhibitory concentration - MIC

For MIC determination, 96-well flat bottom plates (Kasvi) were used, which were filled with 100 μL of BHI solution + inoculum. Following, a serial microdilution (1:1 v/v) was performed with the *C. coriaceum* extract, obtaining different concentrations (0.5–512 μg/mL). These plates were placed in a microbial incubator for 24 h at 37 °C. After this period, a solution of 20 μL resazurin (Sigma Aldrich, St. Louis, Missouri, USA) at 0.01% was added to each well of bacterial growth, targeting the occurrence of redox reactions. It was considered the highest MIC which inhibited bacterial growth [27].

2.5.3. Drug-enhancing activity

After determining the MIC, the drug-enhancing activity test was performed, in which the product was evaluated at sub-inhibitory concentrations (MIC/8) [7]. A BHI solution was prepared in association with the inoculum (10%), and the natural product at a sub-inhibitory concentration, which was distributed in the wells of the plates. Then, serial microdilution (1:1 v/v) was performed using the aforementioned antibacterial drugs in varied concentrations (0.5–512 μg/mL). After bacterial growth (24 h at 37 °C), the reading was performed with the addition of 20 μL of aqueous resazurin solution.

2.6. Statistical analysis

All assays were performed in triplicate. Data were expressed as means, and their respective standard errors (±SEM). Subsequently, they were submitted to a one-way analysis of variance (ANOVA One-way) using Tukey's test at 95% reliability. *P* values were defined as < 0.0001 (**** = extremely significant), 0.0001 to 0.001 (*** = extremely significant), 0.001 to 0.01 (** = very significant), 0, 01 to 0.05 (* = significant) and >0.05 (ns = not significant). The average inhibitory concentrations (IC_{50}) values were calculated using non-linear regression. All analyzes were performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, United States). The photomicrographs of the mechanisms of action were analyzed using the point picker tool in the ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) version 1.53.

3. Results

3.1. Chemical composition

The MECC phytochemical tests showed the presence of flavones, flavonols, xanthones, catechins, and flavanones, and negative results for the other classes investigated (Table 2). Quantitatively, the total phenols and flavonoids represented 11.26 mg GAE/g and 5.98 mg QE/g of extract, respectively.

3.2. Antifungal activity

3.2.1. Cell survival curve and average inhibitory concentration

According to Fig. 3, the MECC was not effective in reducing the fungal growth of the three tested strains. Only the highest extract concentration (1024 μg/mL) reduced the cell survival curve of C. *albicans* (96.61%), and *C. tropicalis* (85.11%). For *C. krusei*, the reduction was only 22%.

Regarding the intensifier activity of fluconazole, it was possible to notice that MECC was not able to increase the antifungal effect against *C. albicans* and *C. krusei* strains (Fig. 3). However, against *C. tropicalis*, the intensifying action of MECC was significant at concentrations from 32 to 256 μg/mL (* = $p < 0.05$, ** = $p < 0.01$, respectively).

The IC_{50} values (μg/mL) for the anti-*Candida* activity of fluconazole, MECC, and MECC + fluconazole, indicate that the IC_{50} values for the MECC were higher than those for fluconazole, demonstrating the ineffectiveness of the extracts (Table 3). Nevertheless, the IC_{50} of the MECC against *C. albicans* could be considered relevant, since the concentration of 351 μg/mL was able to inhibit the yeast growth by 50%. This value is similar to that of fluconazole (362 μg/mL) against *C. tropicalis*.

Regarding the synergistic effect, the antifungal effect of fluconazole was intensified when associated with MECC against *C. tropicalis* strains. The IC_{50} value was reduced by almost 70% after association with the extract, which is a promising result. However, MECC reduces the antifungal activity of fluconazole against *C. albicans* and *C. krusei* (Table 3).

3.2.2. Inhibition of fungal virulence

In addition to the direct anti-*Candida* activity of the extracts, their ability to inhibit fungal virulence through hyphal suppress the development was also evaluated. In Figs. 4 and 6, it is possible to observe the natural formation of hyphae of *C. albicans* and *C. tropicalis* (control group), which were inhibited by MECC at a concentration of 512 μg/mL. Although MECC at a concentration of 256 μg/mL was less effective in inhibiting hyphal formation compared to fluconazole, the inhibition was noticeably greater compared to the control group. Thus, MECC can inhibit one of the virulence mechanisms present in these pathogens.

On the other hand, MECC was not able to inhibit the formation of hyphae of *C. krusei* at the tested concentrations. Interestingly, the lowest concentration (512 μg/mL) considerably increased the formation of hyphae and pseudohyphae in *C. krusei*. While fluconazole (256 and 512 μg/mL) completely inhibited the formation of these filamentous structures (Fig. 5).

3.2.3. Mechanisms of action

As observed in Figs. 7–9, the main mechanism to reduce yeast growth was the formation of reactive oxygen species (ROS). In all *Candida* species, the verification of fluorescent fungal cells labeled with 2 ′,7′-dichlorofluorescein was higher than the negative control (150 mM NaCl). Among the three strains evaluated, *C. krusei* was the most susceptible to the effect of MECC, which was able to produce a significantly higher amount of ROS compared to fluconazole (Fig. 8). In addition to ROS formation, it was observed during fluorescence microscopy that MECC can act by permeabilizing the cell membrane. *C. tropicalis* strains showed to be susceptible to this mechanism of action (Fig. 9). On the other hand, there was no change in cell membrane permeabilization in *C. albicans* (Fig. 7), and *C. krusei* strains (Fig. 8).

Table 2
Total phenolic and flavonoid content of the methanolic extract of *Caryocar coriaceum* (MECC).

Sample	Total Phenolics (mg gallic acid/g)	Total Flavonoids (mg quercetin/g)
MECC	11.26 ± 1.01	5.98 ± 1.16

3.3. Antibacterial activity

The MECC did not show direct activity against the standard multiresistant strains of *Pseudomonas aeruginosa*, *Escherichia coli,* and *Staphylococcus aureus*. The MIC found was >512 μg/mL, indicating that MECC was not able to inhibit the growth of these multi-resistant bacteria at concentrations of clinical interest. However, when combining the MECC with standard drugs it was possible to verify that there was a modifying action (Fig. 10). MECC combined with gentamicin significantly increased the antibacterial activity against the three multidrug-resistant strains evaluated (** = $p < 0.01$, **** = $p < 0.0001$). In addition, the extract was able to significantly reduce (**** = $p < 0.0001$) the MIC of erythromycin against strains of *P. aeruginosa* and *E. coli*, potentializing drug activity. Interestingly, the extract of *C. coriaceum* reduced the antibacterial activity of norfloxacin, as it significantly increased (**** = $p < 0.0001$) its MIC in all multi-resistant bacteria evaluated.

4. Discussion

Located in Northeast Brazil, Chapada do Araripe has a diversity of flora throughout its territory [33]. This botanical richness associated with the cultural plurality of the region, resulted in a diverse and important ethnopharmacological knowledge of many plant species, in which *C. coriaceum* has a prominent place [34,35]. Among which stands out *C. coriaceum*, a species used in the popular pharmacopoeia for centuries in the aforementioned region [19]. Among the ethnopharmacological indications, the use of fruits for the treatment of infectious and parasitic diseases stands out [17–19].

Such medicinal indications may be directly linked to the chemical composition of the fruits of *C. coriaceum*. Previous studies identified the flavonoids rutin, isoquercitrin, and quercetin in extracts from the peel and pulp of *C. coriaceum* fruits [22]. The occurrence of flavonoids in other species of the genus *Caryocar* [36], demonstrates that in addition to lipids, fruits are also sources of compounds with a phenolic nature. Our findings showed that MECC contains flavones, flavonols, xanthones, catechins, and flavanones, reinforcing the occurrence of phenolic compounds in *C. coriaceum* fruits.

In our study, it was evidenced the absence of intrinsic antibacterial activity of the fruit extract of *C. coriaceum* at clinically relevant concentrations [37]. This lack of activity may be related to the low content of total flavonoids present in the internal mesocarp. It is known that extracts with high levels of flavonoids tend to have antibacterial activity, and several flavonoid mechanisms of action have already been described [38–40]. However, Lacerda Neto et al. [41] demonstrated that the ethanolic extract of the leaves of *C. coriaceum*, which is rich in flavonoids, was able to inhibit bacterial growth at low concentrations against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 12692, and *Pseudomonas aeruginosa* ATCC 15442. Thus, the antimicrobial activity also depends on the qualitative composition of the extract.

Due to its known ethnopharmacological use, the hypothesis that the internal mesocarp of the fruits of *C. coriaceum* would display antibacterial activity was not verified. However, according to Costa et al. [42], the fixed oil of the internal mesocarp of *C. coriaceum* showed to reduce the growth of *Salmonella choleraesuis* ATCC 13314, *Staphylococcus aureus* ATCC 12692, *Pseudomonas aeruginosa* ATCC 15442, and *Streptococcus pneumoniae* (ATCC6314). Thus, the antimicrobial potential of the mesocarp of *C. coriaceum* is more directly related to its lipidic composition, and not to the phenolic constituents of the internal mesocarp.

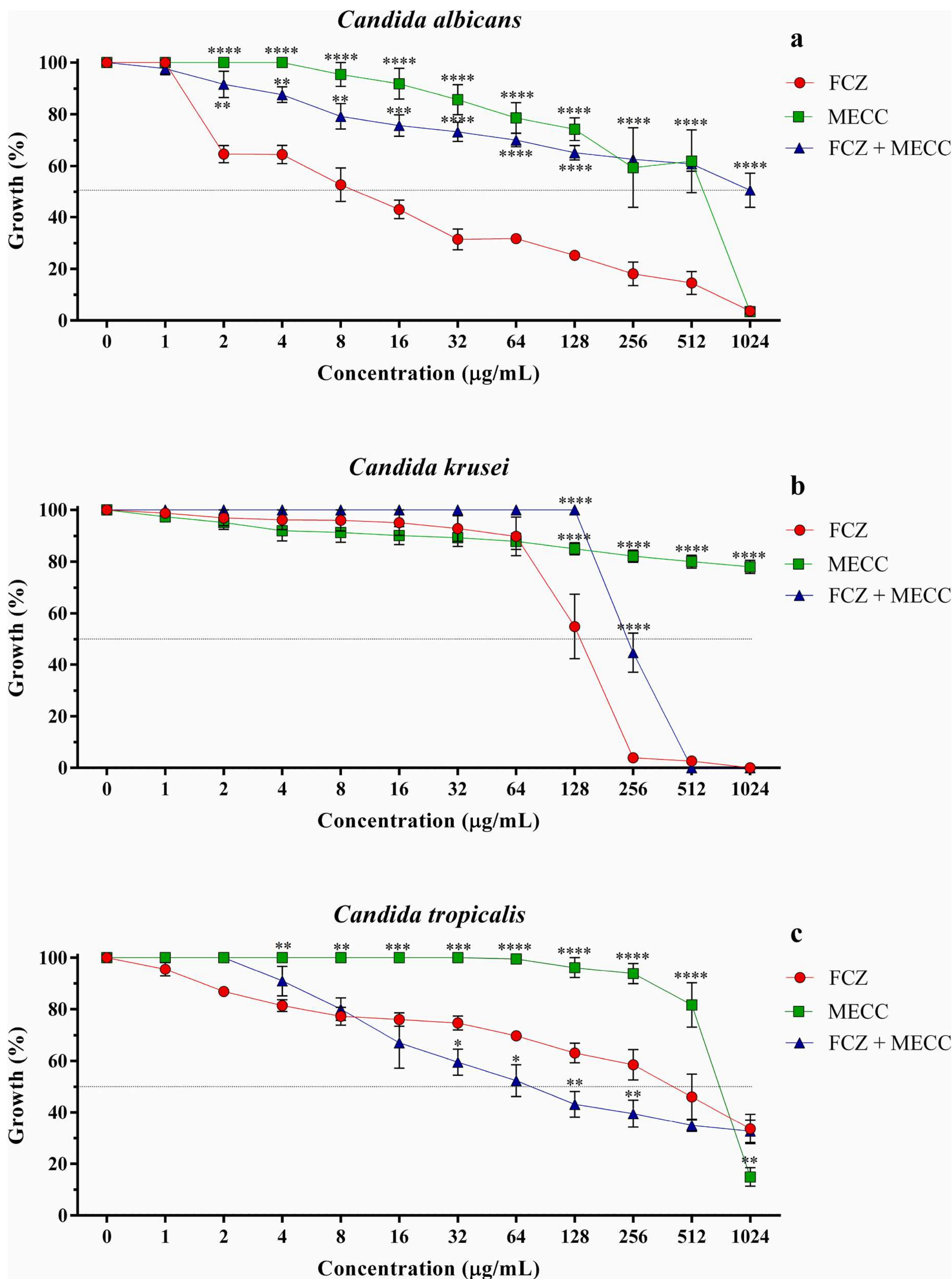

Fig. 3. Cell viability curve and IC_{50} value (dotted line) of different concentrations of methanolic extract of *Caryocar coriaceum* (MECC), fluconazole (FCZ) and its combination (FCZ + MECC) against *Candida albicans* (INCQS 90028) (3a), *Candida krusei* (INCQS 40095) (3b) and *Candida tropicalis* (INCQS 40042) (3c). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. The bars represent the standard error of the mean (n = 3).

Despite the absence of intrinsic antibacterial activity, the combination of the *C. coriaceum* extract with gentamicin and erythromycin showed an intensifying action of these antibiotics against the strains, especially when associated with gentamicin, which belongs to the class of aminoglycosides. Drug potentiating activity in microbiological assays refers to the assessment of the ability of a substance or compound to enhance or enhance the effects of a given drug. This type of assay is important in pharmaceutical research and drug development, as it

Table 3
Median inhibitory concentration (IC_{50}) values in μg/mL of methanolic extract of *Caryocar coriaceum* (MECC), fluconazole (FCZ), as well as in combination (FCZ + MECC), against strains of *Candida albicans* (INCQS 90028), *Candida krusei* (INCQS 40095) and *Candida tropicalis* (INCQS 40042).

Treatment	IC_{50} (μg/mL)		
	Candida albicans	Candida krusei	*Candida tropicalis*
MECC	351	>1024	703
FCZ	12.33	131.6	362
FCZ + MECC	1144	254.3	109.9

allows identifying substances that can increase the therapeutic efficacy of a drug or reduce the dose required to obtain a certain effect [2,7]. The fundamentals of this assay involve performing experiments in biological models or in vitro systems to assess the synergistic or additive effects of two or more substances. The substance or compound being tested is added to the parent drug in varying concentrations, and the resulting effect is compared with the effect produced by the parent drug alone [27,28].

Among the mechanisms of bacterial resistance to antibiotics, the most common against the class of aminoglycosides is the enzymatic destruction or inactivation of the drug. This can occur through the production of acetyltransferases, nucleotidyltransferases., and phosphotransferases [43]. Górniak, Bartoszewski, and Króliczewski [38]

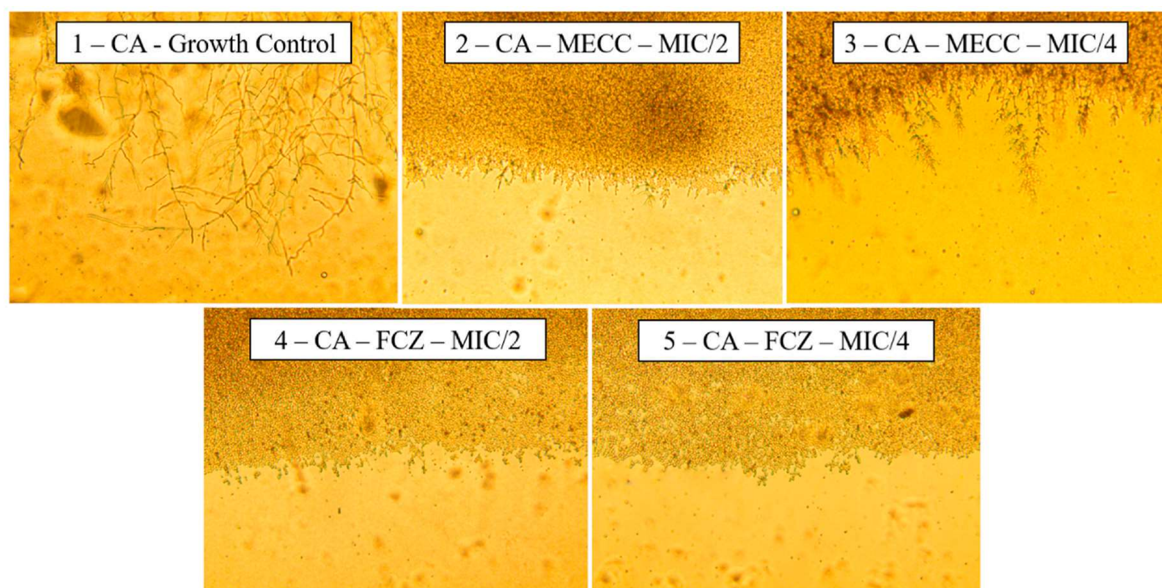

Fig. 4. Effects of methanolic extract of *Caryocar coriaceum* (MECC) on the dimorphism of *Candida albicans* INCQS 90028. Slide (S1): Growth control; S2-3: Effect of MECC on the concentration of 512 μg/mL (S2) and 256 μg/mL (S3). S4-5: Effect of fluconazole at the concentration of 512 μg/mL (S4) and 256 μg/mL (S5). 400 × magnification.

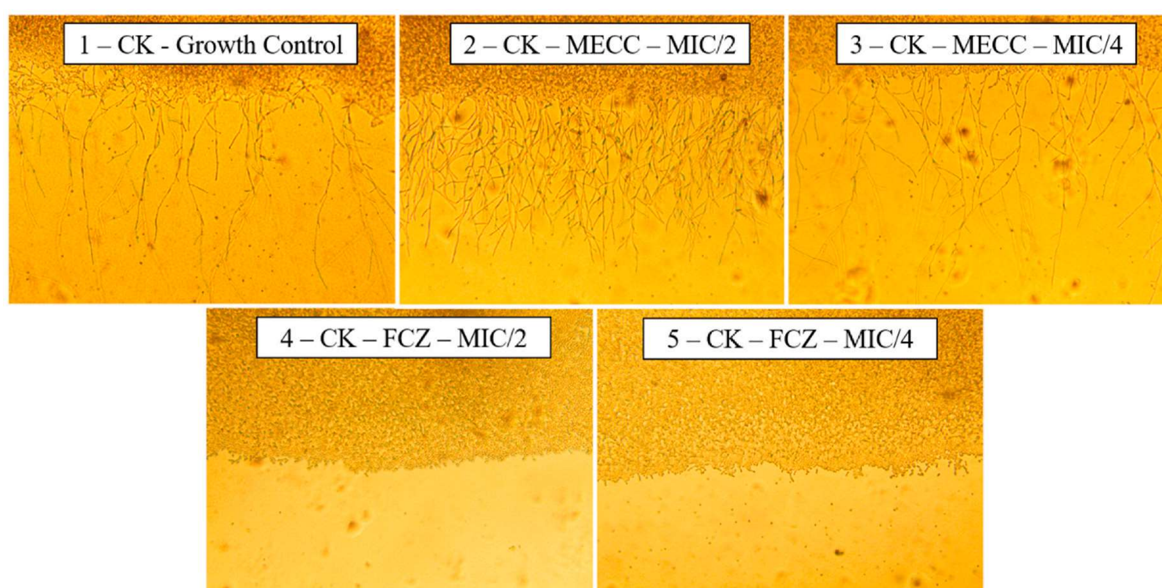

Fig. 5. Effects of methanolic extract of *Caryocar coriaceum* (MECC) on the dimorphism of *Candida krusei* INCQS 40095. Slide (S1): Growth control; S2-3: Effect of MECC on the concentration of 512 μg/mL (S2) and 256 μg/mL (S3). S4-5: Effect of fluconazole at the concentration of 512 μg/mL (S4) and 256 μg/mL (S5). 400 × magnification.

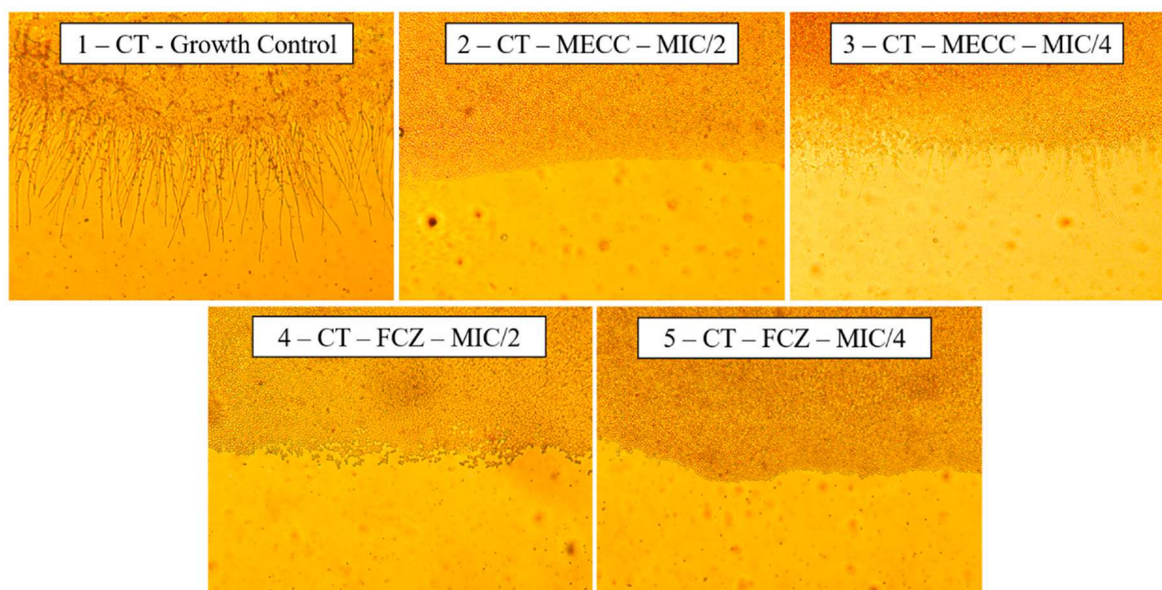

Fig. 6. Effects of methanolic extract of *Caryocar coriaceum* (MECC) on the dimorphism of *Candida tropicalis* INCQS 40042. Slide (S1): Growth control; S2-3: Effect of MECC on the concentration of 512 μg/mL (S2) and 256 μg/mL (S3). S4-5: Effect of fluconazole at the concentration of 512 μg/mL (S4) and 256 μg/mL (S5). 400 × magnification.

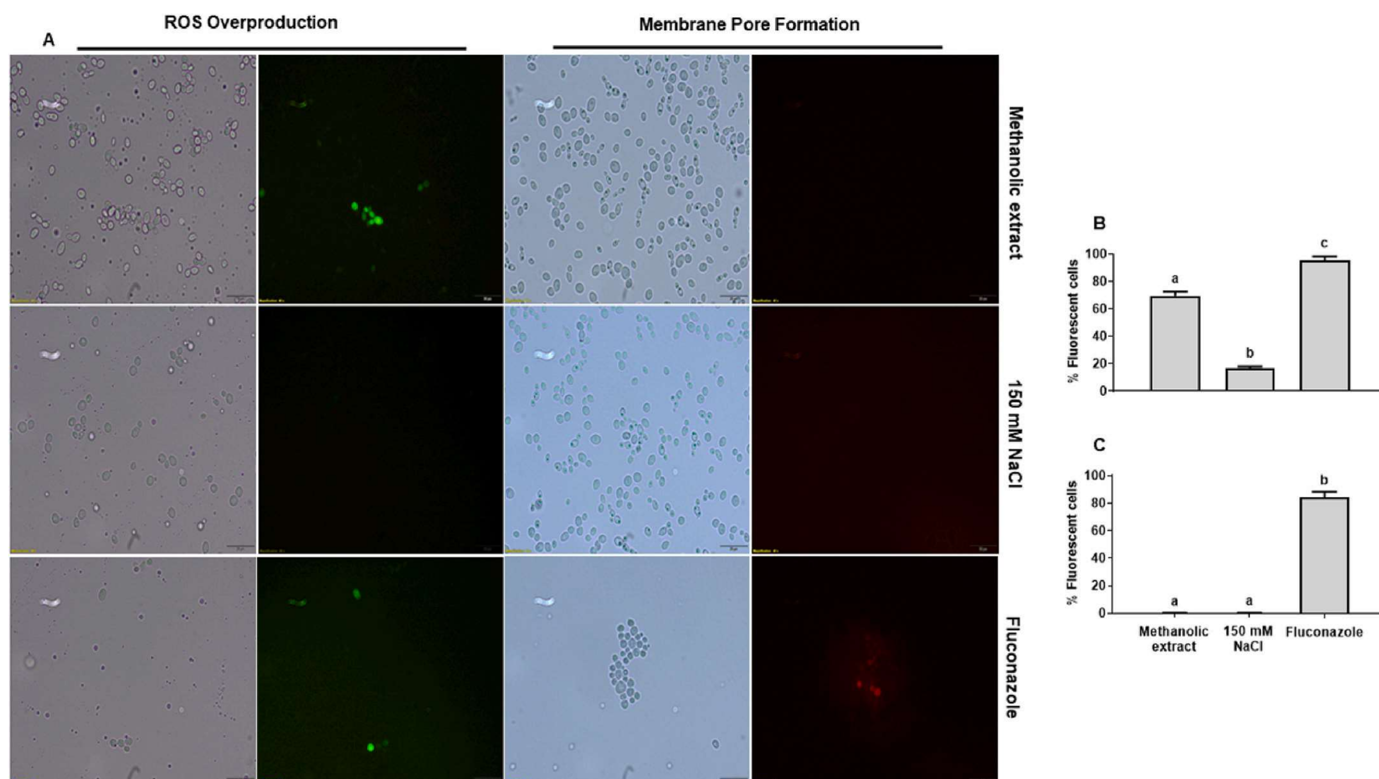

Fig. 7. Effect of methanolic extract of *Caryocar coriaceum* on *Candida albicans* (INCQS 90028). (A - Left) Reactive Oxygen Species. (A - Right) Cell membrane permeabilization. (B) Percentage of fungal cells labeled with 2′,7′-dichlorofluorescein. (C) Percentage of fungal cells stained with propidium iodide. Different letters represent statistical difference between means ($p < 0.05$). Results are presented as mean ± standard deviation.

reported that some flavonoids, even at low concentrations, can act as inhibitors of these enzymes. Thus, the intensifying action found in the present study may be due to the activity of the extract in combination with gentamicin which could cause the inactivation of these bacterial enzymes.

As mentioned, the fruits of *C. coriaceum* have been used in traditional medicine to treat infections associated with the genitourinary tract [19], which are often caused by yeasts of the genus *Candida* [44,45]. Thus, the symptoms related to candidiasis are popularly treated with derivatives of *C. coriaceum* fruits, such as fixed oil [19]. Our findings corroborate the ethnopharmacological use of *C. coriaceum* since the MECC was able to reduce the growth of *Candida* spp. yeasts, as well as inhibit one of its

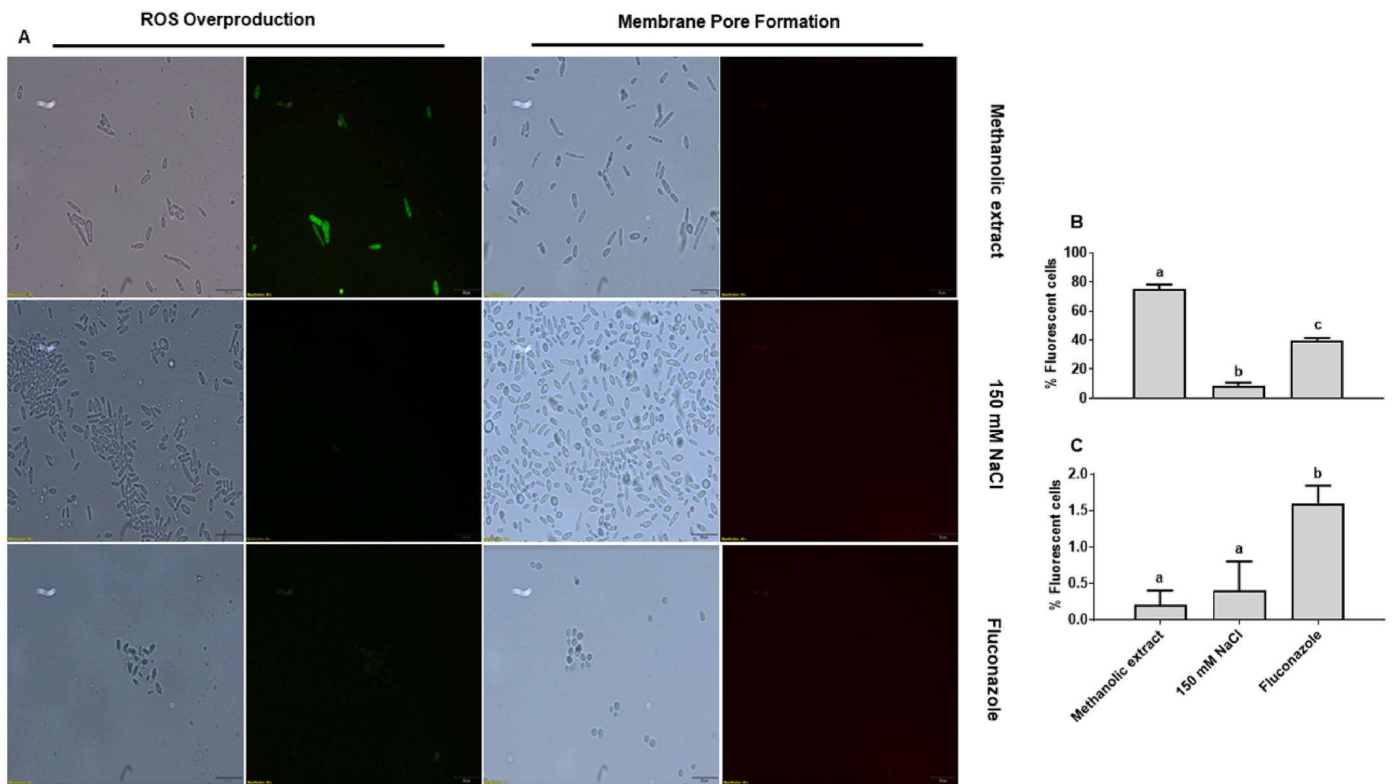

Fig. 8. Effect of methanolic extract of *Caryocar coriaceum* on *Candida krusei* (INCQS 40095) (A - Left) Reactive Oxygen Species. (A - Right) Cell membrane permeabilization. (B) Percentage of fungal cells labeled with 2′,7′-dichlorofluorescein. (C) Percentage of fungal cells stained with propidium iodide. Different letters represent statistical difference between means ($p < 0.05$). Results are presented as mean ± standard deviation.

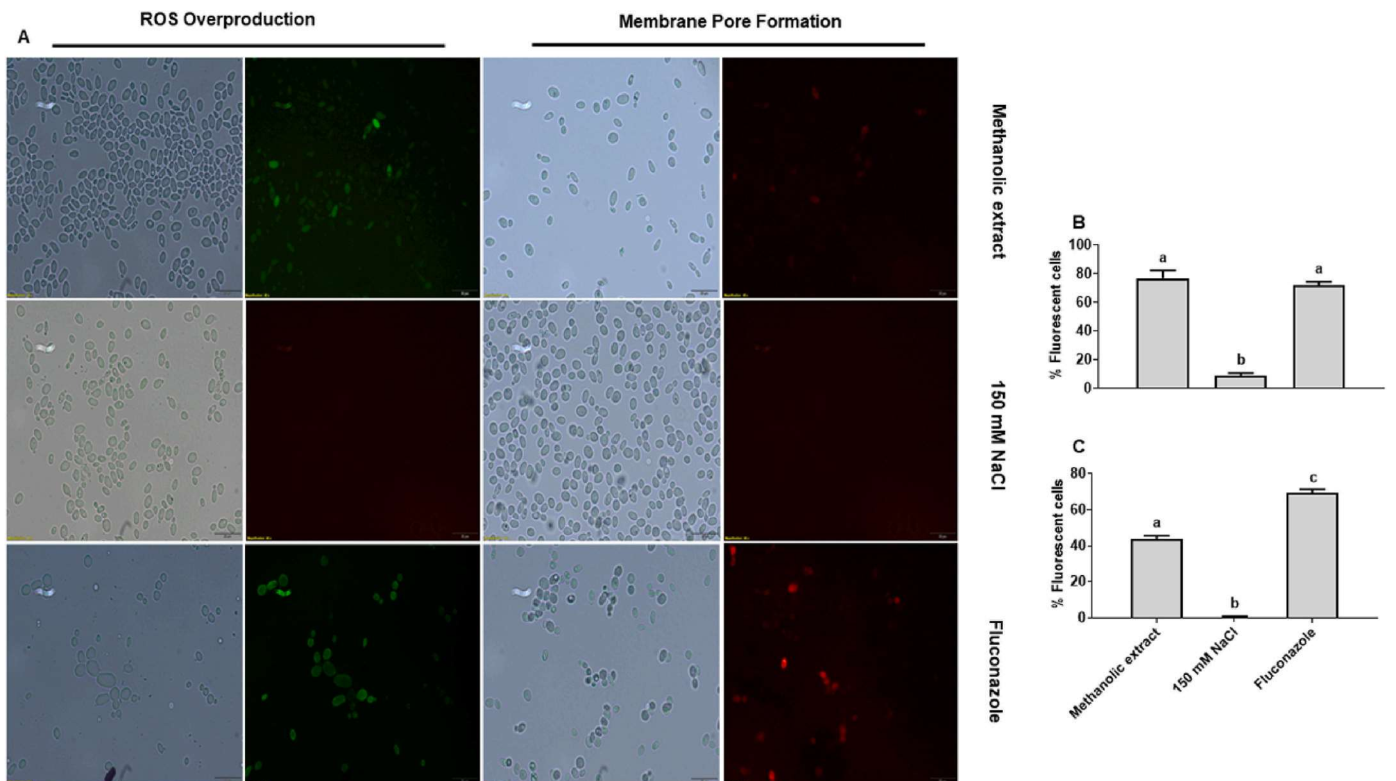

Fig. 9. Effect of methanolic extract of *Caryocar coriaceum* on *Candida tropicalis* (INCQS 40042) (A - Left) Reactive Oxygen Species. (A - Right) Cell membrane permeabilization. (B) Percentage of fungal cells labeled with 2′,7′-dichlorofluorescein. (C) Percentage of fungal cells stained with propidium iodide. Different letters represent statistical difference between means ($p < 0.05$). Results are presented as mean ± standard deviation.

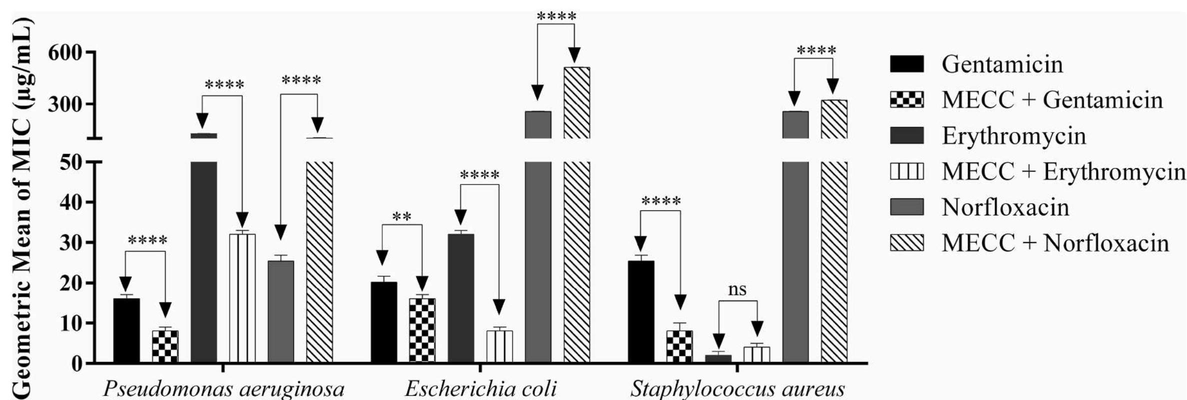

Fig. 10. Geometric mean minimum inhibitory concentration (MIC) in μg/mL of methanolic extract of *Caryocar coriaceum* (MECC) in association with different antibiotics against different multidrug-resistant bacterial strains. ns = $p > 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$. The bars represent the standard error of the mean (n = 3).

virulence factors.

The anti-*Candida* effect of the MECC may be related to the presence of flavonoids in the chemical composition of the mesocarp of *C. coriaceum* fruits. Flavonoids are known to have significant antifungal effects [46,47]. These displayed effects against *Candida* spp. by various mechanisms, including induced disruption of the plasma membrane, inhibition of cell wall formation, induced mitochondrial dysfunction, inhibition of cell division, inhibition of efflux pumps, inhibition of the RNA/DNA, and inhibition of protein synthesis [48–50]. Alves et al. [20] reported that the internal mesocarp of *C. coriaceum* fruits showed an antifungal effect against *Malassezia* spp. (MIC: 19.53 μg/ml), and *Microsporum canis* (MIC: 4.88 μg/ml). These authors associated the biological effect with the presence of flavonoids in *C. coriaceum*, such as quercetin, rutin, and isoquercetin.

The antifungal effect of MECC was not only restricted to reducing yeast growth. Also, was observed inhibition in the morphological transition of the yeast, one of the virulence factors of *Candida* spp [50]. This mechanism inherent to the pathogen, consists of the transition from yeast forms to filamentous forms, characterizing them as polymorphic. Such change is associated with tissue penetration and invasion, as well as escape macrophage killing. If a natural or synthetic product inhibits the filamentous form of the fungi, a certain infected body via the immune system may be able to fight the infection itself [49]. Among the mechanisms of action caused by natural products, the inhibition of the expression of proteins responsible for phenotypical transformation stands out [51].

In our findings, the MECC probably acted in the formation of reactive oxygen species (ROS). The extract tested may have induced the overproduction of ROS in all evaluated strains (Fig. 11). These ROS act in the intracellular environment, causing damage to important molecules, such as proteins, nucleic proteins (DNA and RNA), and membrane lipids, therefore, threatening cell integrity (Fig. 11). The increased production

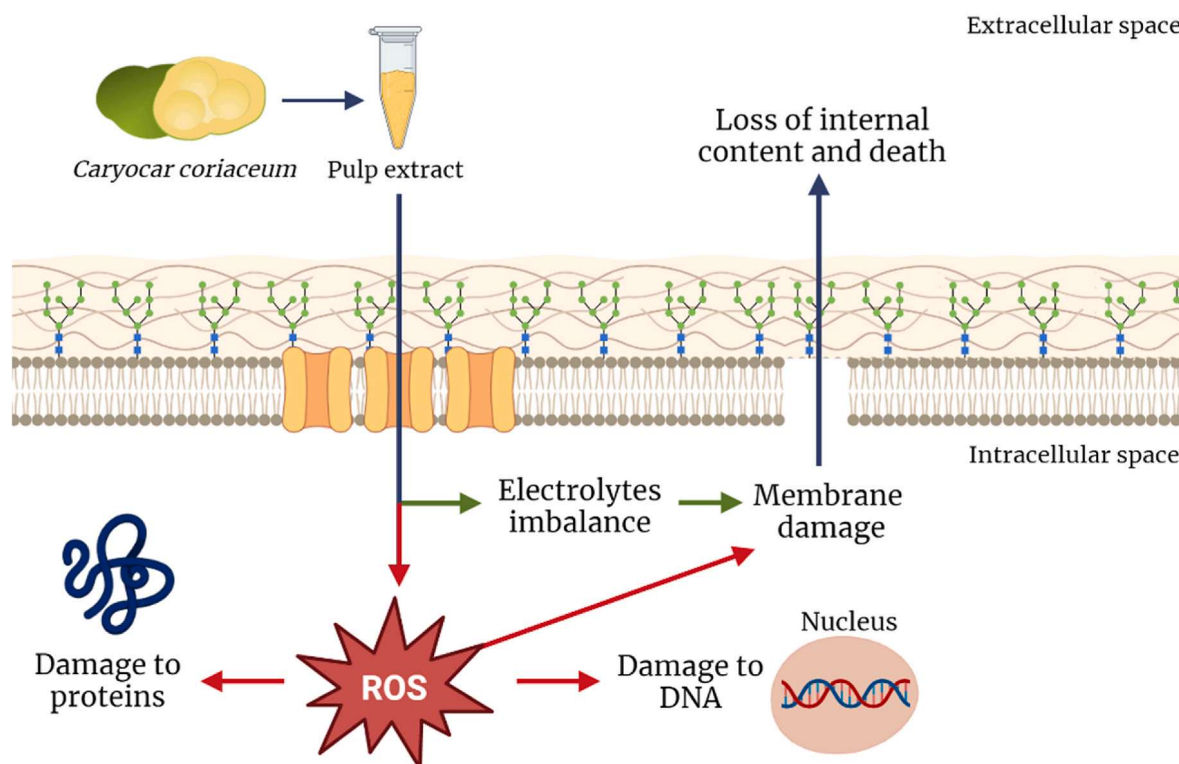

Fig. 11. Diagram indicating the possible mechanisms of action for the anti-*Candida* activity of the methanolic extract of *Caryocar coriaceum*.

of ROS can lead to oxidative stress in *Candida* spp. yeasts, leading them to apoptosis [52,53]. It was also observed the formation of pores in the membrane when tested against the strains of *C. tropicalis*. Pore formation is due to electrolyte imbalance, causing damage to the plasmatic membrane. Thus, the yeast will lose its cytoplasmic content, which may lead to cell death [54].

In addition to the intrinsic antifungal activity, an intensifying effect of fluconazole activity against *C. tropicalis* strains was verified. One of the possibilities of this synergism, is the change in membrane permeability of the yeast, favoring the entry of fluconazole, and consequently increasing its intracellular concentration. Fluconazole is known to inhibit the biosynthesis of ergosterol, one of the main constituents of the fungal membrane [48,55]. This association of products of natural origin with standard drugs is promising, as it becomes an alternative in antibiotic therapy, as the synergistic effect reduces the dosage needed during the use of commercial drugs [56–58].

Os resultados anti-*Candida* do MECC demonstrado neste estudo são promissores para as indústrias farmacêuticas a fim de desenvolver novos produtos. Pois as atividades antifúngicas ocorreram em concentrações de relevância clínica, tornando-se um candidato promissor para aplicações terapêuticas [37]. No entanto, devem ser realizados testes adicionais para avaliar o perfil farmacológico dos extratos dos frutos de *C. coriaceum*, tal como avaliação da toxicidade aguda, subaguda, crônica e a avaliação da farmacocinética e farmacodinâmica, para garantir a segurança da utilização destes extratos em medicamentos. Além disso, é essencial realizar ensaios de estabilidade química nos extratos para avaliar a degradação dos princípios ativos, a fim de garantir a eficácia dos possíveis medicamentos. Esses testes são fundamentais para comprovar a eficácia e segurança dos medicamentos que serão produzidos utilizando os extratos [59,60].

5. Conclusion

Our study demonstrated that the ethnopharmacological use of fruit pulp of *C. coriaceum* by communities in Chapada do Araripe (Brazil) for the treatment of infectious and parasitic diseases was partially supported by our findings. The *C. coriaceum* extract did not show a direct effect against pathogenic bacteria, but it was able to intensify the action of antibiotics against multi-resistant microorganisms. Regarding the antifungal activity, the extract reduced the growth of *Candida* spp., acting through the formation of reactive oxygen species. Furthermore, it was able to inhibit the morphological transition of the yeasts, one of their virulence mechanisms. *C. coriaceum* extract even intensified the activity of fluconazole.

CRediT authorship contribution statement

José Weverton Almeida-Bezerra: Conceptualization. **Rafael Pereira da Cruz:** Formal analysis. **Raimundo Luiz Silva Pereira:** Investigation. **Viviane Bezerra da Silva:** Formal analysis. **Daniele de Oliveira Bezerra de Sousa:** Investigation. **João Xavier Da Silva Neto:** Data curation. **Larissa Alves Lopes de Souza:** Methodology. **Nadine Monteiro Salgueiro Araújo:** Writing – original draft. **Rafael Guimarães Gomes Silva:** Software. **Daniel Luna Lucetti:** Software. **Henrique Douglas Melo Coutinho:** Project administration. **Maria Flaviana Bezerra Morais-Braga:** Supervision. **Antônio Fernando Morais de Oliveira:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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