Antioxidant and Antifungal Activity of the *Cynophalla flexuosa* (L.) J. Presl (Capparaceae) against Opportunistic Fungal Pathogens

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Abstract: The chemical composition of the *Cynophalla flexuosa* hydroethanolic extract (CFHEE) was identified and its antioxidant, antifungal and antipleomorphic activities against *C. albicans* and *C. tropicalis* strains were evaluated. Phytochemical prospecting evidenced polyphenolic and triterpenoid compounds while UPLC-MS analysis revealed the presence of Isopropyl/n-propyl-GLS; Methylpropyl-GLS/butyl-GLS; Methylbutyl-GLS; Quercetin O-di-hexoside; Quercetin-pentosyl-hexoside; Rutin; Quercetin O-glucoside; Kaempferol O-rhamnosyl-hexoside; Kaempferol O-pentosyl-hexoside and Lariciresinol hexoside. The CFHEE inhibited the effect of the DPPH• radical, both when evaluated individually and in combination with the antifungal fluconazole. When associated with 256 µg/mL fluconazole, the extract, at concentrations from 128 µg/mL, reduced the effect of the DPPH• free radical with values ranging from 37.7% to 95.8%. The extract’s antifungal effect was considered clinically irrelevant and its combination with the antifungal triggered an antagonistic effect against the two strains, thus indicating the popular use of bravo beans in the form of teas or infusions should not be combined with the intake of the drug Fluconazole as this may lead to a reduction of its clinical effect. The extract, however, inhibited morphological changes (pleomorphism) in *Candida* species, preventing the development of hyphae. The CFHEE possesses an antioxidant effect and a potential pharmacological activity for the inhibition of one of the *Candida* spp. virulence factors.

Keywords: feijão-bravo; antioxidant activity; antagonism; antipleomorphism; *Candida*
1. Introduction

Species belonging to the *Candida* genus are responsible for causing various fungal infections that can range from a simple superficial pathology to more serious ones, such as systemic affections, especially in immunocompromised individuals, where performing different diagnostic methods and therapeutic combinations become necessary for their treatment [1,2]. *Candida albicans* is the main species associated with hospitalizations due to infections with mortality rates greater than 50% in cases of septicemia [3]. *Candida tropicalis* also causes candidemia in debilitated individuals, being one of the main pathogens associated with candidiasis dispersion in patients with severe hematological diseases, presenting high virulence and low sensitivity to commonly used antifungals such as azoles [4,5].

In recent decades, infections caused by species from this genus have been increasing; however, their treatment is still limited due to the restricted number of available antifungals and the toxicity that they present [4,6]. Therefore, the search for new therapeutic alternatives, such as the association of antifungals with bioactive molecules, as well as the evaluation of the antifungal activity of natural products, becomes very relevant [7–9].

Synergistic interactions between antimicrobial agents offer improvements in the efficacy of clinical treatment, the inhibitory concentration of antibacterial agents, the risk associated with the development of bacterial resistance and the reduction of the toxic effect of antifungals to the host. Therefore, the use of associated substances has been widely studied and used as a treatment for infections [10–12].

At the same time, natural products can have an antioxidant effect on biological organisms. This action is linked to the chemical composition of the secondary metabolism of plants, especially phenolic compounds, such as flavonoids. Such action is responsible for eliminating or reducing free radicals that are formed during metabolic reactions. If not eliminated by enzymes or antioxidant substances, they can cause degenerative diseases of aging and cell death overtime [12–14].

*Cynophallaflexuosa* (L.) J. Presl, belonging to the Capparaceae family, which has *Capparis flexuosa* (L.) L. as a synonym, is popularly known as “feijão-bravo” and is closely related to the mustard (Cruciferae) family with an abundance of glucosinolates and flavonoids. *Cynophalla flexuosa* develops in the Brazilian semiarid region as a shrub or tree reaching up to 4 m in height, with straight, glabrous branches and leaves, varying from approximately 3 to 5 cm in length; the species is used to treat fevers, as a depurative, in the restoration of the menstrual flow to normal levels and against cough, in addition to possessing great nutritional value [15–20].

The *C. flexuosa* entire plant, its leaves, bark and wood are used by the population for producing abortive venom, as a combustible, for veterinary purposes and mainly as fodder, for staying green throughout the year and possessing chemical characteristics such as good palatability, thus being of great importance for animal feeding in drought periods [18,21].

Due to the food and medicinal use of *C. flexuosa* in the Brazilian semi-arid region, the objective of this study was to carry out a phytochemical prospecting, the comprehensive identification of its phytochemicals and to evaluate the in vitro antioxidant and antifungal potential of the *C. flexuosa* hydroethanolic extract against *Candida* spp. strains, as well as to evaluate the ability of the extract to reduce the virulence of these fungi by affecting their morphological transition.

2. Materials and Methods

2.1. Plant Material

The *C. flexuosa* plant material was randomly collected from the municipality of Milagres—CE, Brazil, in October 2017. Botanical identification was carried out by the Herbarium of the Regional University of Cariri and identified by Dr Karina Vieira Alves Linhares. A voucher specimen of the species was deposited in the Herbarium Dárdano deAndrade Lima—HCDAL—URCA under number #13257.
2.2. Preparation of the Cynophalla flexuosa Hydroethanolic Extract (CFHEE)

A total of 414.0 g of freshly picked leaves of *C. flexuosa* were chopped and extracted at room temperature in a mixture (2000 mL) of co-solvents containing 30% of water and 70% of ethanol (v/v) [22]. The plant material remained in contact with the extractive mixture at room temperature, protected from air and light and being shaken every 12 h, for 72 h, time in which the leaves reached approximately 75% depigmentation. The resulting solution was concentrated under reduced pressure in a rota-evaporator (Q-344B—Quimis—Brazil—40 rpm, 60 °C), and was subsequently filtered and dried in a 65 °C water bath, after which, it was obtained the extract in the form of amorphous paste.

2.3. Chemical Analyses

2.3.1. Qualitative Chemical Prospection

Chemical assays were used for the qualitative analysis for the presence of secondary metabolites. In the evaluation for the presence of phenols, tannins, flavonoids and alkaloids, the method described by Matos [22] was adopted based on the visualization of color changes and the formation of precipitates after the addition of specific reagents. The Liebermann–Burchard test was used for the detection of phytosteroids and/or triterpenoids, using the method adapted from Campbell and Shawn [23]. According to this method, colorimetric changes or precipitate formation in the fraction solution after the addition of specific reagents are indicative of the presence of the corresponding class of secondary metabolites. The screening of phenolic compounds was performed using FeCl₃, 1% methanolic solution, and 1 N HCl, 10% (w/v) NaOH, while the screening of alkaloids was performed using 5% (v/v) acetic acid, 10% (v/v) NH₄OH, chloroform p.a., and Dragendorff’s reagent. For the screening of phytosteroids and/or triterpenoids the acetic anhydride and sulfuric acid were used as reagents of detection.

2.3.2. Total Phenol Quantification

Evaluation of the phenolic compounds present in the CFHEE was performed using the method developed by Singleton and Rossi [24], modified by Nuutila et al. [25], using the Folin–Ciocalteu reagent colorimetric method, based on the reduction of phosphomolybdic and phosphotungstic acids in alkaline solution.

A total of 200 µL of the extract, 600 µL of ethanol 70% (v/v), 400 µL of 10% (v/v) Folin–Ciocalteu reagent and 2000 µL of 7.5% (w/v) sodium carbonate solution were added to test tubes. The mixture was homogenized and another 800 µL of 7.5% (w/v) sodium carbonate solution was then added to the test tubes and they were incubated for 20 min at 45 °C in the dark. Subsequently, the sample was centrifuged in a Donner CD100 centrifuge at 3500 rpm for 5 min and left to stand for 15 min at room temperature.

The absorbance reading of the blue color produced by the reduction of the Folin–Ciocalteu reagent due to phenolic compounds was performed at 735 nm using the UV-visible T80 spectrophotometer PG Instruments LTD. Calculation of the phenolic content was performed through the preparation of a gallic acid standard curve using triplicates at concentrations ranging from 0.5 to 5 µg/mL. The extract was analyzed in triplicates, and the results obtained were expressed in milligrams of gallic acid per gram of dry extract.

2.3.3. Ultra-Performance Liquid Chromatography—ElectroSpray Ionization—Quadrupole-Time-of-Flight—Tandem Mass Spectrometry (UPLC-ESI-qTOF-MS/MS)

An Acquity UPLC system (Waters) coupled to a Quadrupole/Time of Flight system (QtoF, Waters) was performed. Chromatographic runs were performed on a Waters Acquity UPLC BEH column C18 (150 mm × 2.1 mm, 1.7 µm) with a fixed temperature of 40 °C, mobile water phases with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), a gradient ranging from 2% to 95% B (15 min), a flow rate of 0.4 mL/min and injection volume of 5 µL. The ESI⁻ mode was acquired in the 110–1180 Da range, with a fixed source temperature at 120 °C, desolvation temperature at 350 °C, desolvation gas flow of 500 L/h,
an extraction cone of 0.5 V and capillary voltage of 2.6 kV. Leucine enkephalin was used as a lock mass. The acquisition mode used was MSE. The instrument was controlled by the Masslynx 4.1 Software (Waters Corporation, Taunton, MA, USA) [26].

2.4. Antioxidant Activity

The DPPH• sequestering activity of Fluconazole and the CFHEE in isolation or in combination with fluconazole was determined using the DPPH• (1,1-diphenyl-2-picrylhydrazyl) photocolorimetric method, according to Choi et al. [27], with modifications, using ascorbic acid as the standard antioxidant. DPPH• inhibition was accompanied by the disappearance of the DPPH• absorption [28], where the degree of discoloration is proportional to the concentration and potency of the present antioxidants. The tests were performed in triplicates.

A total of 20 µL of ascorbic acid, antifungal and extract solutions were resuspended separately in 80 µL of 95% ethanol and 100 µL of a 0.4 mM ethanolic DPPH• radical solution in 96-well ELISA plates; generating the final concentrations of ascorbic acid and the extract (2048, 1024, 512, 256, 128, 64, 32, 16, 8, 4 and 2 µg/mL) as well as DPPH• (0.2 mM).

A protocol was also established, for the first time, to evaluate the association of the extract with a fixed antifungal concentration. A total of 20 µL of the antifungal solution and 20 µL of the extract were resuspended in 60 µL of 95% ethanol and 100 µL of the 0.4 mM ethanolic DPPH• radical solution; generating the final antifungal (256 µg/mL), extract (2048 up to 2 µg/mL) and DPPH• (0.2 mM) concentrations. After 30 min of incubation at room temperature in the dark, absorbance measurements were taken at 518 nm using a UV-Visible TR Reader Shimadzu spectrophotometer. Under the same conditions and separately, absorbance readings for the antifungal, extract, antifungal plus the extract and ascorbic acid blanks were performed by adding 20 µL of each substance’s dissolution plus 95% ethanol up to the volume of 200 µL, generating the same concentrations for each protocol.

DPPH• free radical inhibition percentages (%) were expressed as the percentage (%) compared to the control, consisting of 100 µL of the 0.4 mM ethanolic DPPH• solution and 100 µL of 95% ethanol, calculated using the formula:

\[
\text{DPPH \% inhibition} = \left[ \text{Abs.}_\text{Control} - (\text{Abs.}_\text{Extract} - \text{Abs.}_\text{Blank}) \right] \times 100\%
\]  

where Abs.\text{Control} is the absorbance of the DPPH• solution without the extract; Abs.\text{Extract} is the absorbance of the extract with DPPH•; Abs.\text{Blank} is the absorbance of the extract without DPPH•.

2.5. Antifungal Activity

2.5.1. Strains, Culture Media Used and Solution Preparation

Standard strains (Candida albicans—CA INCQS 40006 and Candida tropicalis—CT INCQS 40042 yeasts) were obtained from the Reference Micro-organisms Collection in Sanitary Surveillance-CMRVS, FIOCRUZ-INCQS National Institute of Quality Control in Health, Rio de Janeiro, RJ, Brazil. The microorganisms were inoculated into Sabouraud Dextrose Agar (SDA, KASVI) and incubated for 24 h at 37 °C. Small yeast aliquots were then transferred to test tubes, each containing 3 mL of sterile saline (0.9%). The concentration of the inoculum was standardized by comparing its turbidity with the 0.5 McFarland scale standard [29].

The prepared inoculums were thus used to determine the Minimum Inhibitory Concentration (MIC) in Dextrose Sabouraud double concentrate (SDB, HIMEDIA) broth. Potato Dextrose Agar (PDA, DIFCO), used for the micromorphology analysis, was prepared by diluting it more than the manufacturer’s recommendation to make it an impoverished medium capable of stimulating the production of hyphae. The diluted agar was added to obtain a solid medium.

Dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) was used for the dilution of the extract while Fluconazole (Capsule-Flucomed) was diluted in water and used as reference. The matrix solution of the extract was prepared by weighing 0.05 g of the extract
and diluting it in 1 mL of DMSO. To obtain the desired concentration for the assays, the extract was further diluted in sterile distilled water such that the DMSO concentration would not exert an effect on the test cells [30].

2.5.2. Determination of the 50% Fungal Inhibitory Concentration IC₅₀

The broth microdilution method in 96-well plates was performed to determine the 50% Fungal Inhibitory Concentration. Each well was filled with 100 µL of SDB containing 10% fungal inoculum, followed by the addition of 100 µL of the CFHEE (4096 µg/mL) or Fluconazole (antifungal reference, at the same concentration) to the first well followed by serial double dilution. The concentrations in the wells ranged from 2 to 2048 µg/mL. The last well, without the extract or antifungal, served as normal growth control [31].

Controls were prepared for the product’s diluents (using saline instead of the inoculum) as well as the sterile medium. All tests were performed in triplicate. The plates were incubated at 37 °C for 24 h and subsequently read at 630 nm. The reading results were used to construct the cell viability curve and determine the CFHEE IC₅₀ [32].

2.5.3. Determination of the Minimum Fungicidal Concentration (MFC)

A small sterile rod was placed in each well of the MIC test plate (except for the sterility control), which after mixing the medium in each well, was taken to a petri dish containing SDA, transferring the solution (medium + inoculum + CFHEE) for subculture and cell viability verification. After 24 h of incubation, the plates were inspected for any Candida colony formations [33], with modifications. The concentration in which no fungal colony growth was observed was considered the MFC of the CFHEE.

2.5.4. Extract Effect on Fungal Morphology

To determine if the CFHEE caused alterations in fungal morphology by inhibiting the development of hyphae, microculture chambers were prepared. A total of 3 mL of PDA medium depleted by dilution were added to the chambers, containing concentrations of the CFHEE at HCA/4, HCA and HCA × 2, where HCA is the Highest Concentration Assayed (HCA) in microdilution.

Inoculum aliquots were removed from the Petri dish culture to make two parallel striae in the solid medium, which were covered with a sterile coverslip. The chambers were placed in an incubator for 24 h (37 °C) and inspected under a light microscope at 400× magnification. A chamber was attached to the microscope to randomly capture images at 4× zoom. Controls for yeast growth (hyphae stimulated by exhaustion) and Fluconazole, for comparative purposes, were used [34] with modifications.

2.6. Statistical Analysis

The data obtained from each sample and concentration were checked for their normal distribution and analyzed by a one-way ANOVA followed by Bonferroni’s post-hoc test. The IC₅₀ values were obtained by non-linear regression with the objective of interpolating standard curve values (using the Graphpad Prism 6.0 Software, Graphpad Software Inc., San Diego, CA, USA) of the percentage growth as a function of the concentrations, and the IC₅₀ values in µg/mL.

3. Results

3.1. Extraction of Plant Material, Chemical Prospection and Total Phenols

The hydroethanolic extract of Cynophalla flexuosa (CFHEE) was obtained as a dark brown amorphous paste. The extraction showed a yield of 6.01% (w/w) which corresponds to the initial mass of plant material. The chemical characterization revealed that the medicinal species presents chemical heterogeneity arising from its secondary metabolism. In the reaction with FeCl₃, the formation of a red color indicated the presence of phenols, and the formation of a green precipitate revealed the presence of condensed tannins. Color changes among red, lilac, blue, yellow and orange of the alcoholic sample, when reacting with
HCl and NaOH, revealed the presence of anthocyanidins, flavones, flavonols, xanthones, chalcones, aurones, and flavononols; the same conditions reaction when subjected to heat, the sample exhibited colors ranging from red, orange and yellow, indicating the presence of leucoanthocyanidins, flavonones and catechins. The formation of a deep green color with acetic anhydride, after a few minutes, confirmed the presence of phytosteroids. The non-formation of precipitate with Dragendorff’s reagent indicated the absence of alkaloids. In addition, it was possible to note that the extract does not have pyrogallates tannin in its constitution as shown in (Table 1).

Table 1. Phytochemical prospection of the *Cynophalla flexuosa* hydroethanolic extract (CFHEE).

| Metabolites |
|------------|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| + | - | + | + | + | + | + | + | + | + | + | + | - | + |

1—Phenols; 2—Pyrogallatestannin; 3—Condensed tannins; 4—Anthocyanidins; 5—Leucoanthocyanidins; 6—Flavones; 7—Flavonols; 8—Flavononols; 9—Flavonones; 10—Xantones; 11—Aurones; 12—Chalcones; 13—Catechins; 14—Alkaloids; 15—Phytosteroids and/or triterpenoids; (+) presence; (−) absence.

Regarding the total of phenols, it was shown that *C. flexuosa* hydroethanolic extract (CFHEE) has a total of 1.53 ± 0.042 mg/g of gallic acid.

3.2. Chemical Composition by UPLC-ESI-qTOF-MS/MS

The CFHEE was analyzed using the UPLC-ESI-qTOF-MS/MS in the ESI− mode. The constituents were identified based on their exact masses, fragmentation patterns and comparison with data reported in the literature (Table 2). The chromatogram with the results from the analysis, in which the components are represented by peaks, can be visualized in Figure 1.

![Figure 1. Peaks attributed to metabolites in the *Cynophalla flexuosa* hydroethanolic extract (CFHEE) using UPLC-ESI-qTOF-MS/MS in the negative mode.](image-url)
Table 2. Peak assignment of metabolites in the Cynophalla flexuosa hydroethanolic extract (CFHEE) identified as intact deprotonated molecules, [M-H]−, using UPLC-ESI-qTOF-MS/MS in the negative mode. GLS are glucosinolates.

| No | Peak | Rt Min | [M-H]− Observed | [M-H]− Calculated | Product Ions (MS/MS) m/z | Empirical Formula | Mass Error (ppm) | Putative Name | Reference |
|----|------|--------|-----------------|-------------------|-------------------------|-------------------|-----------------|--------------|-----------|
| 1  | 1.19 | 341.1091| 341.1084        | 179               | C_{12}H_{21}O_{11}     | 2.1              | Sucrose         | [35]        |
| 2  | 1.36 | 360.0249| 360.0245        | 96, 74            | C_{18}H_{18}NO_{3}S_{2} | 1.1              | Isopropyl/ν-Propyl-GLS isomer | [36]        |
| 3  | 2.05 | 360.0252| 360.0410        | 96, 74            | C_{18}H_{18}NO_{3}S_{2} | 1.9              | Isopropyl/ν-Propyl-GLS isomer | [36]        |
| 4  | 2.44 | 374.0619| 374.0600        | 259, 195          | C_{3}H_{20}N_{2}O_{3}S_{3} | 2.1              | Methylpropyl-GLS/ butyl-GLS | [37]        |
| 5  | 2.96 | 388.0750| 388.0736        | 96, 74            | C_{20}H_{22}NO_{3}S_{2} | 3.6              | Methylbutyl-GLS | [38]        |
| 6  | 3.45 | 451.2181| 451.2179        | 405, 193, 161     | C_{20}H_{35}O_{11}     | 0.4              | Unknown Quercetin | -          |
| 7  | 3.58 | 625.1401| 625.1405        | 301, 300          | C_{27}H_{29}O_{17}     | 0.6              | O-di-hexoside Quercetin-pentosyl-hexoside | [39]        |
| 8  | 3.72 | 595.1258| 595.1240        | 301, 300          | C_{26}H_{25}O_{16}     | −2.4             | -              |             |
| 9  | 3.78 | 609.1382| 609.1362        | 301, 300          | C_{27}H_{29}O_{16}     | 3.3              | Quercetin        | [40,41]     |
| 10 | 3.89 | 463.0888| 463.0877        | 301, 300          | C_{21}H_{20}O_{12}     | 2.4              | O-glucoside Kaempferol | [39]        |
| 11 | 3.98 | 593.1509| 593.1506        | 447, 285          | C_{27}H_{29}O_{15}     | 0.5              | O-hamnosyl-hexoside Kaempferol | [42]        |
| 12 | 4.01 | 579.1343| 579.1350        | 447, 285          | C_{25}H_{27}O_{15}     | 1.7              | O-pentosyl-hexoside Kaempferol | [42]        |
| 13 | 4.17 | 581.2229| 581.2234        | 401               | C_{26}H_{25}O_{13}     | −0.5             | Unknown        | -           |
| 14 | 4.27 | 521.2040| 521.2023        | 359, 329          | C_{26}H_{35}O_{11}     | 2.1              | Lariresinol hexoside | [43]        |
| 15 | 4.41 | 331.1226| 331.1232        | 179               | C_{20}H_{19}O_{8}      | −1.8             | Unknown        | -           |
| 16 | 4.60 | 745.2650| 745.2649        | 195, 179          | C_{14}H_{10}O_{11}     | 0.1              | Unknown        | -           |

N° peak: Number used to identify each compound in the chromatograms in Figure 1. Rt min: Retention time of the eluted compounds under the experimental conditions described in the UPLC-MS section. [M-H]−: Exact mass value of the deprotonated molecule. (MS/MS) m/z: mass—charge ratio of the major ions produced by the fragmentation of deprotonated molecules. Mass error (ppm): in parts per million, ppm = 10^6 × (accurate mass exact). The molecular structure of the compounds were designed with the program Chem Draw Ultra 12.0.2.1076.lnk. Licensed to: Mulder X-Filer 875-317589-4732. 1986–2010 CambridgeSoft.

3.3. Antioxidant Activity

The evaluation of the CFHEE antioxidant activity and the positive control of ascorbic acid in the different reaction systems are represented in Figure 2. The extract alone possessed a DPPH* free radical sequestering activity with an IC_{50} of 124 μg/mL and when in combination with the antifungal Fluconazole it presented an IC_{50} of 196.6 μg/mL (Table 3). On the other hand, the antifungal Fluconazole did not exhibit significant antioxidant activity compared to the extract and the ascorbic acid control. AA obtained a superior antioxidant activity, obtaining an IC_{50} value lower than 2 μg/mL. At the two highest concentrations, the extract alone and in association with the antifungal showed a greater inhibitory potential than AA.

The extract in combination with Fluconazole exhibited a significantly higher antioxidant activity from the concentration of 64 μg/mL compared to the antifungal alone. This may have occurred because the antifungal possesses a pro-oxidant effect at the tested concentration (256 μg/mL), which was more expressive than the antioxidant effect of the extract up until the 64 μg/mL concentration. From this concentration onwards until reaching the 2048 μg/mL concentration, the extract exhibited an antioxidant activity that significantly reversed the antifungal effect, thus inhibiting the DPPH* radical.
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The effect of Fluconazole on reducing the antioxidant potential of the extract is confirmed by observing that, in the absence of an association, Fluconazole showed a significantly higher antioxidant activity up to the 64 μg/mL concentration. At the 128 μg/mL concentration, the extract associated with Fluconazole inhibited DPPH• by 37.79% and when evaluated in isolation it inhibited DPPH• by 42%, with no significant differences in percentage inhibition between these two systems being observed until the 1024 μg/mL concentration. This demonstrates that the extract reversed the effect of Fluconazole and still managed to significantly inhibit the DPPH• free radical. When associated with the antifungal at a concentration of 2048 μg/mL, the extract inhibited DPPH• by 95.84%, compared to 89.62% DPPH• inhibition with the isolated extract.

3.4. Antifungal Activity

The CFHEE showed an inhibitory effect on the growth of resistant Candida strains when used at high concentrations with a MIC value equal to or greater than 4096 μg/mL. The evaluation of the potential modulatory activity of the extract on the Fluconazole antifungal effect against different Candida strains revealed the combination of this drug with the hydroethanolic extract, at subinhibitory concentrations, causes the inhibition of microorganismal growth at concentrations greater than when it was tested in isolation. This effect was also verified in the cell viability curve (Figure 3).

The IC50 values (Table 4) obtained in the evaluation showed that CFHEE possesses a high inhibitory concentration compared to Fluconazole and, when in combination with this drug, the extract significantly increased its IC50 against C. albicans, revealing an antagonism. A small reduction in the IC50 of Fluconazole, however without significance, was observed when this was associated with the extract against C. tropicalis.

**Table 3.** Inhibitory concentration 50% (IC50) of several products against the DPPH• free radical.

| IC50 DPPH* (μg/mL) | FCZ  | AA   | CFHEE | CFHEE + FCZ |
|--------------------|------|------|-------|-------------|
|                    | >2048| <2   | 124   | 196.6       |

CFHEE: *Cynophalla flexuosa* hydroethanolic extract; FCZ: Fluconazole; AA: Ascorbic acid.

![Figure 2](image-url) Free radical scavenging activity of the *Cynophalla flexuosa* hydroethanolic extract (CFHEE) as measured by the DPPH• 1,1-Diphenyl-2-picrylhydrazyl (0.2 Mm) free radical assay, with ascorbic acid as a positive control. The values from the free radical scavenging activity were expressed as mean ± S.E.M. (n = 8). Non-linear regression of the curve transformations, and analyzed by one-way ANOVA followed by Tukey’s multiple comparison test, with a simple pooled variance. * Statistical significance with p < 0.05; ** Statistical significance with p < 0.01; *** Statistical significance with p < 0.001; **** Statistical significance with p < 0.0.
Figure 3. Antifungal effect of fluconazole and its association with the Cynophalla flexuosa hydroethanolic extract (CFHEE) (MIC/16 µg/mL). FCZ: Fluconazole. (A) CA: Candida albicans; (B) CT: Candida tropicalis. These values were expressed as the means ± SEM (n = 3) and analyzed by a one-way ANOVA followed by Bonferroni post-hoc test multiple comparisons. * Significant difference (p < 0.05) at equal concentrations. * Statistical significance with p < 0.05; ** Statistical significance with p < 0.01; *** Statistical significance with p < 0.001; **** Statistical significance with p < 0.0001.

Table 4. 50% Candida spp. Inhibitory Concentration (IC<sub>50</sub>—µg/mL) by the Cynophalla flexuosa hydroethanolic extract (CFHEE).

| Tested Product | CA INCQS 40006 | CT INCQS 40042 |
|----------------|----------------|----------------|
| FCZ            | 83.121         | 1544.197       |
| CFHEE          | >2048          | >2408          |
| CFHEE + FCZ    | 764.03         | 1339.706       |

FCZ: Fluconazole; CA: Candida albicans; CT: Candida tropicalis; INCQS: National Institute of Health Quality Control.

Concentrations ranging from 2 to 2048 µg/mL were used to determine the fungicidal effect of the CFHEE. After 24 h of incubation, fungal growth was observed at all concentrations, showing the MFC was greater than 4096 µg/mL.

3.5. Control of Virulence of Candida Strains

In experiments evaluating the action of the extract on fungal morphology, two controls were used: microorganismal growth, in order to evaluate if the medium is ideal for the induction of pleomorphism; and the control for comparative verification of the effect of the antifungal Fluconazole, as shown in Figure 4. The growth controls for both strains were shown to be effective.
Figure 4. Controls used for the *Candida albicans* and *Candida tropicalis* strains in fungal pleomorphism assays with the *Cynophalla flexuosa* hydroethanolic extract. HCA: Highest Concentration Assayed (2048 μg/mL); HCAx2 (4096 μg/mL) and HCA/4 (512 μg/mL); CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: National Institute of Health Quality Control.

In the Fluconazole and Extract Assays, the Highest Concentration Assayed—HCA of the microdilution (2048 μg/mL), HCAx2 (4096 μg/mL) and HCA/4 (512 μg/mL) were tested. Fluconazole significantly reduced the formation of hyphae and pseudohyphae as the concentration increased, especially against the *C. albicans* strain (INCQS 40006).

The images from the microcultures using the extract are shown in Figure 5, where the effective action of the CFHEE on *C. albicans* and *C. tropicalis* strains can be observed as the concentration increases. For *C. tropicalis*, the action can be observed from lower concentrations.
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Figure 5. Effect of the *Cynophalla flexuosa* hydroethanolic extract (CFHEE) on *C. albicans* and *C. tropicalis* fungal dimorphism. HCA: Highest Concentration Assayed (2048 μg/mL); HCAx2 (4096 μg/mL) and HCA/4 (512 μg/mL); CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: National Institute of Health Quality Control.

4. Discussion

The presence of isopropyl/propyl-GLS in the CFHEE, established a chemical relationship with the *Cynophalla ovala* and *Cynophalla spinosa* species, in which glucosinolates (GLS) were also reported [36,44,45]. These compounds demonstrated high potent antifungal effect [46]; however, the CFHEE did not exhibit antifungal activity; on the contrary, it was antagonistic in association with fluconazole, which could be due to the low isopropyl/propyl-GLS concentration. However, the antifungal effect of these compounds will be further evaluated.

The total polyphenolic content of the CFHEE was relatively low at 1.53 mg GA/g. DE, however, when associated with fluconazole, values of up to 95.8% of DPPH* radical scavenging were observed. Such values may mitigate the possible pro-oxidant effect of fluconazole against fungal strains, which could also be a component of the antibiotic properties of the antibiotic, giving rise to its antagonistic behavior in the antifungal action modulation, therefore, such result could be associated with the presence of antioxidant compounds such as the flavonoids, which possess antioxidant activities widely reported in the literature and associated with the dihydroxy (catechol) structure in ring B, which acts to
form o-quinones when reacting with free radicals, the 3-hydroxyl group on the C-ring and the 2,3-double bond in conjunction with the oxo ring C function [46–48]. Such a structural arrangement makes them labile in transferring electrons and donating protons, which characterizes them as standard antioxidants through their radical elimination activities in innumerable in vitro and in vivo studies [49–51].

Previous studies have reported the resistance of Candida species to fluconazole [5,6,52,53]. The resistance of this genus to azoles is shown by the decrease in expression of the enzyme lanosterol-14α-azol-demethylase or the presence of specific mutations that affect its structure and function, preventing the destabilization of the fungal membrane [54]. In another study, β-sitosterol is reported to exhibit a rapid activity against fungal cells, indicating that it may be a good antimicrobial agent [55]. Cowan, [56] reported the antimicrobial potency of β-sitosterol may be associated with the membrane rupture of susceptible organisms. However, the presence of β-sitosterol in the CFHEE did not contribute to an antifungal activity, leading to the assumption that it did not modify the cell membrane structure to favor the entry of fluconazole, flavonoids and GLS or antifungal constituents to the interior of fungal cells, and thus can be possible to trigger effective antifungal effects.

A high concentration of flavonoids in the C. spinosa hydroethanolic extract were found [57], and it has been considered a very important source of antifungal medications [58]. The aforementioned species presents some secondary metabolites in common with C. flexuosa. However, despite the similarities, the CFHEE presented no direct or synergistic antifungal effect with fluconazole. One reason for these results may be that flavonoid levels in the extract were not elevated (1.53 mg GA/g. DE).

In Figure 3, the graphs present the antagonistic effect observed by the union of the antifungal with the extract against C. albicans (color: red). The combination of the substances caused a decrease in the effect of the drug, this being an indication that the popular use of bravo beans, in the form of teas or infusions, should not be combined with the intake of the drug Fluconazole, due to the reduction of its clinical effect.

One of the virulence factors observed in species from the Candida genus are their capacity for morphological transition or pleomorphism. This feature endows the microorganism with the ability to change its shape from a yeast-like state to a filamentous state (hyphae and pseudohyphae) when subjected to environmental stress conditions with the possibility of becoming a tissue invasive organism in the host [59]. This change in state favors the formation of systemic diseases termed candidemias that affect people who present compromises in their immunological responses [60] and the search for less toxic natural substances to control or prevent morphological transition without the death of the commensal fungal population, which is essential for balance, has increased. The CFHEE inhibited pleomorphic transition of the tested strains with C. tropicalis obtaining the best effect when compared to C. albicans and the antifungal Fluconazole. Although C. albicans is responsible for more than 50% of superficial and systemic infections caused by yeasts [61], infections by non-albicans species have increased and C. tropicalis stands out for being characterized as an agent capable of causing invasive infections [62].

The CFHEE did not present significant intrinsic antifungal activity; however, it inhibited morphological alterations in Candida species, indicating it possesses potential pharmacological activity against fungal biology, potentially revealing an alternative for the treatment of fungal infections [63].

5. Conclusions

The medicinal species C. flexuosa presents chemical heterogeneity in its composition, presenting mainly phenolic compounds. These being the possible responsible for the antioxidant activity. Despite the lack of antifungal activity, the leaves are sources of phytochemicals capable of inhibiting the formation of hyphae of Candida strains. To advance the improvement of its use as an antifungal medicinal plant, complete elucidation of the mechanisms of action require more in-depth study. We emphasize that the species under study possesses potential for future research associated with antioxidant and antifungal
activity, using new methodologies, different microorganisms in new assays and in combination with other drugs, in order to evaluate their use for the treatment of infections and reversal of fungal resistance.

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