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Chemical composition and anti-*Candida* potencial of the extracts of *Tarenaya spinosa* (Jacq.) Raf. (Cleomaceae)

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ABSTRACT

Phytochemical prospecting was performed by HPLC-DAD. The Inhibitory Concentration of 50% of mortality the microorganisms (IC_{50}) was determined and a cell viability curve was obtained. Minimum Fungicidal Concentration (MFC) was determined by subculture in Sabourad Dextrose Agar. The effect of the combination extract/fluconazole was verified by microdilution, with the extracts in subinhibitory concentrations (MFC/16). Caffeic acid was the major compound of both extracts, representing 6.08% in the aqueous extract and 7.62% in the ethanolic extract. The extracts showed a fungistatic effect (MFC ≥ 16,384 μg/mL). The IC_{50} results demonstrated that the combination of the extracts with fluconazole were more significant than the products tested alone, with values from 4.9 to 34.8 μg/mL for the ethanolic extract/fluconazole and 5 to 84.7 μg/mL for the aqueous extract/fluconazole. The potentiating effect of fluconazole action was observed against C. albicans and C. tropicalis. In C. krusei the aqueous extract had an antagonistic effect.

1. Introduction

Resistance cases of Candida to fluconazole have been reported in several studies [1–3]. Because of this, many attempts to potentiate the effect of this drug through interactions between natural products and commercial drugs have been tested against clinical isolates and multi-resistant Candida [4]. One of the alternatives for the modulation of this drug is the use of plant extracts or the isolated substances that compose it, since it has been more effective against microbial strains than the isolated conventional drug [5]. However, it is important to report that this potentiation of the drug does not always occur, several studies have shown an antagonism between drugs and products of natural origin [6,7].

Like humans, fungi are eukaryotic, in view of that fact that commercial drugs can cause adverse effects on host cells and can lead to cytotoxicity of the kidneys and liver [8]. In addition, this similarity makes it difficult to define pharmacological targets, which limits and influences the cost for the development of new drugs [2].

Alternatively, plants have been promising sources of substances of biological and pharmacological interest because they can produce an enormous diversity of resulting substances from your secondary metabolism, among these phenolic compounds, which are quite known to be present in most of the drugs used by herbal medicine [9].

Data on phenolic compounds report several biological activities, among them the antifungal activity [10]. The antimicrobial activity of phenolic compounds can be directly attributed to action against microorganisms or suppressing factors needed for their virulence [11], for example inhibit Candida formation of filamentous forms, a factor necessary for its virulence and pathogenicity. Filamentous structures (hyphae and / or pseudohyphae) are invasive forms that can promote

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tissue penetration even in the initial phase of infection, while the yeast form is important in spreading the species into the bloodstream [12].

Tarenaya spinosa (Jacq.) Raf. popularly known in northeastern Brazil as "mussambê", is an herbaceous plant belonging to the Cleomaceae family. It is cited in other works by the synonymy Cleome spinosa Jacq. In the available literature, anti-helminthic activities [13], anti-inflammatory and antimicrobial activities [14] are cited.

In the popular medicine, species belonging to this genus are used as stimulants, antiscorbutic, anthelmintic, vesicant, carminative and analgesic [15,16]. The flowers of T. spinosa are popularly indicated for the treatment of cough and fever [17], the infusion of their leaves are used for the treatment of cough, asthma and bronchitis [18].

In studies aimed at experiments with plants as an alternative to the use of synthetic drugs it is very important that the appropriate solvent is chosen. In our study water and ethanol were the most appropriate solvents. Water is generally the solvent used by populations reporting the medicinal use of plant extracts, as in infusions and decoctions. We use ethanol as solvent for its ability to extract hydrophilic compounds, such as sugars, polysaccharides and nucleotides.

The objective of this work was to identify the chemical composition of extracts of T. spinosa, its effect against fungi of the genus Candida and to evaluate its potential modulator of the antibiotic fluconazole.

2. Material and methods

2.1. Plant material

The leaves of Tarenaya spinosa (Jacq.) Raf. were collected in the city of Quixelô - CE, under the coordinates: Latitude 6° 14′34.62″S Longitude 39° 16′11.80″ W. A specimen was deposited in the Caririense Dárdano de Andrade Lima Herbarium - HCDAL of the Regional University of Cariri - URCA, under number 12.627.

2.2. Preparation of the aqueous and ethanolic extracts of T. spinosa

The leaves collected from T. spinosa were placed to dry in the sun and then ground to increase the contact surface with the solvent used. To obtain the aqueous extract, 250 g of the leaves were placed in glass bottles, properly autoclaved, and 2 L of boiling distilled water was added. After a period of 72 h the infusion received a double filtration with cotton and was frozen. Subsequently the vegetable material was taken to the lyophilizer to obtain the extract. The solvent used to obtain the ethanolic extract was ethanol 96%, 250 g of the leaves were placed in glass bottles, after 72 h of exposure to the solvent, the material was filtered and then taken to the rotoevaporator to liberate the ethanol [19].

2.3. Chemical, apparatus and general procedures

All chemical were of analytical grade. Methanol, phosphoric acid, gallic acid, chlorogenic acid, ellagic acid and caffeic acid purchased from Merck (Darmstadt, Germany). Quercetin, rutin and kaempferol were acquired from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography (HPLC-DAD) was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominence Auto Sampler (SIL-20 A), equipped with Shimadzu LC-20 AT reciprocating pumps connected to the degasser DGU 20 A5 with integrator CBM 20 A, UV-VIS detector DAD (diode) SPD-M20 A and Software LC solution 1.22 SP1.

2.4. Quantification of compounds by HPLC-DAD

Reverse phase chromatographic analyses were carried out under gradient conditions using C18 column (4.6 mm × 250 mm) packed with 5 µm diameter particles. The mobile phase was water containing 1% phosphoric acid (A) and methanol (B), and the composition gradient was: 13% of B until 10 min and changed to obtain 15%, 30%, 50%, 60%, 70%, 20% and 10% B at 20, 30, 40, 50, 60, 70 and 80 min, respectively [20] with slight modifications. Extracts of T. spinosa were analyzed dissolved in methanol at a concentration of 10 mg/mL. The flow rate was 0.6 ml/min, injection volume 40 µl and the wavelength were 254 nm for gallic acid, 327 nm for caffeic, ellagic and chlorogenic acids, and 366 nm for quercetin, rutin and kaempferol. All the samples and mobile phase were filtered through 0.45 µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.030 – 0.500 mg/ml. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200–600 nm). Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. All chromatography operations were carried out at ambient temperature and in triplicate.

2.5. Fungal strains and culture media used

The strains used were obtained from the National Institute of Quality Control in Health (INCQS), being Candida albicans (CA INCQS 40,006), C. tropicalis (CT INCQS 40,042) and C. krusei (CK INCQS 40,095). These strains were inoculated into Sabouraud Dextrose Agar (SDA, KASVI) and incubated for 24 h at 37 °C. Subsequently, aliquots were removed and transferred to test tubes with 3 mL sterile saline solution (0.9%). Inoculum concentration was standardized by the McFarland scale, giving a standard yeast suspension of 1×10^5 cells/mL. The inocula prepared were used for microbial assays by double microdilution in Sabouraud Dextrose Broth (CSD, HIMEDIA) double concentrated [4].

2.6. Drugs, reagents and solution preparation

Dimethylsulfoxide (DMSO Merck, Darmstadt, Germany) was used to dilute the natural product and fluconazole (Capsule - FLUCOMED), diluted in water to obtain a concentration of 16.384 µg / mL, was used as a reference antifungal. The stock solutions of the extracts were prepared by weighing 0.15 g of the extract and diluting in 1 mL of DMSO. To obtain the desired concentration of 16.384 µg / mL, the extracts were diluted in sterile distilled water, so that the concentration of DMSO in the natural product did not exert any activity on the cells tested [4].

2.7. Determination of IC_{50} and cell viability

Eppendorfs containing 1350 µL of the CSD medium and 150 µL of the inoculum were prepared to be distributed on the microdilution plate. The plate was filled by adding 100 µL of this solution into each well, and then serial dilution was performed from 100 µL of the natural product (aqueous and ethanolic extract), where concentrations ranged from 8.192 to 8 µg/mL. The last well of the plate was reserved for growth control. Dilution control was performed, in which the inoculum was replaced with saline and, in addition, the sterility control of the culture medium was also prepared. The test was performed in triplicate. Plates were placed into the incubator for a period of 24 h at 37 °C [21, with modifications to concentrations and controls]. The reading was performed using the ELISA spectrophotometer (Thermoplate) with a wavelength of 630 nm nm and reading data were used to determine the IC_{50} and obtain the cell viability curve [22].

2.8. Evaluation of modulating activity

In this assay the effect of the combination of the extracts and the fluconazole was evaluated to determine whether or not the antifungal action was potentiated by the oil. According to the methodology used

by Coutinho et al. [23, with modifications in relation to concentrations and controls], in which every extract was tested in sub-inhibitory concentrations (MFC/16). Eppendorfs containing 1.5 ml containing CSD culture medium, 10% inoculum and the natural product at MFC/16 concentration were prepared. The plate was completed by adding 100 µL of this solution to each well. Then, 100 µL of the reference drug fluconazole was mixed with the first well in the alphabetical direction of the plate, serially diluted to the penultimate cavity. Fluconazole concentration ranged from 8192 to 8 µL. For the test, dilution control of fluconazole, dilution control of modulation and control of sterility of the culture medium were performed. The plates were read in a spectrophotometer with ELISA reader (630 nm) [22].

2.9. Determination of minimum fungicidal concentration – MFC

In each well of the test plates, a sterile rod was added, which after homogenizing the medium contained in the well was subcultured into Petri dishes containing ASD, by transferring a small aliquot of the test solution (medium + inoculum + natural product) to verification of cell viability. The plates were incubated at 37 °C for 24 h and checked for fungistatic or fungicidal effect of the Candida colonies. MFC was defined as the lowest concentration capable of inhibiting the growth of fungal colony [24, with modifications].

2.10. Statistical analysis

The obtained data were checked for their normal distribution and then analyzed by a two-way ANOVA test with Bonferroni post hoc test, comparing the values for each concentration of the treatments, point by point. Values of $p < 0.05$ were considered significant. The IC_{50} values were obtained by non-linear regression with interpolation of standard curve unknowns obtained from fungal growth as a function of extracts concentration and expressed in µg/mL. For the statistical analysis, the software Graphpad Prism, v. 5.0.

3. Results

3.1. HPLC-DAD analysis

The yield obtained was 3.06% and 2.35% for the aqueous and ethanolic extracts, respectively. The HPLC-DAD analysis of the extracts of T. spinosa allowed the identification of their phenolic compounds (Fig. 4). Both samples contained gallic acid (retention time -tR = 10.27 min), catechin (tR = 16.08 min), caffeic acid (tR = 25.17 min), ellagic acid (tR = 34.09 min), rutin (t R = 40.15 min) and quercetin (t R = 52.36 min). Chlorogenic acid (tR = 19.43 min), p-coumaric acid (tR = 37.62 min) and apigenin (tR = 67.01 min) were detected only in T. spinosa ethanolic extract (Fig. 1A and B). As seen in Table 1, caffeic acid was the major compound in both the aqueous extract (6.08%) and the ethanolic extract (7.62%) (Figs. 2 and 3).

Table 1
Components of Tarenaya spinosa extracts.

Compounds	T. spinosa ethanolic mg/g	T. spinosa aqueous mg/g
Gallic acid	1.42 ± 0.03 a	3.11 ± 0.05 a
Catechin	3.09 ± 0.01 b	1.39 ± 0.02 b
Chlorogenic acid	3.04 ± 0.01 b	–
Caffeic acid	7.62 ± 0.04 c	6.08 ± 0.01 c
Ellagic acid	1.59 ± 0.02 a	2.75 ± 0.03 d
p-Coumaric acid	4.85 ± 0.03 d	–
Rutin	0.28 ± 0.05 e	0.96 ± 0.02 e
Quercetin	5.32 ± 0.01 f	2.84 ± 0.01 d
Apigenin	1.49 ± 0.02 a	–

Results are expressed as mean ± standard deviations (SD) of three determinations.
Averages followed by different letters differ by Tukey test at $p < 0.05$.

3.2. Evaluation of anti-Candida activity and modulating effect

The IC50 values are shown in Table 2, where the IC50 of fluconazole ranged from 9.0 to 80.7 µg/mL. The IC50 values for the ethanolic extract (EETS) and aqueous extract (EATS) were shown to be clinically irrelevant, and it was not possible to determine the IC50 of the EATS with the yeast CK INCQS 40,095 in view that the cell viability curve was increasing. The results of MFC indicate that the products have fungistatic effect rather than fungicide as only reduced the formation of colonies (MFC ≥ 16,384 µg/mL). In the combination test there was a variation between 5.0 and 34.8 µg/mL in the IC50 of the EETS + FCZ, whereas for the EATS + FCZ the values ranged from 4.9 to 84.7 µg/mL. The MFC results of the products combined with fluconazole also indicate fungistatic effect (MFC ≥ 16,384 µg/mL). The results showed that the combination of the aqueous extract with fluconazole showed a presumed synergism against C. albicans (Fig. 5), whereas the ethanolic extract in combination showed similar results against yeast C. krusei (Fig. 4).

4. Discussion

In the present study the extracts of T. spinosa were tested against fungal strains of Candida isolated and in combination with the antifungal fluconazole, in addition the phenolic profile of both extracts was determined.

Caffeic acid (3,4-dihidroxicinamic acid), the major compound of both extracts, is the main subgroup of secondary metabolites, available literature reports several biological and pharmacological activities [25], including antimicrobial activity [7]. Sun, Liao and Hang [26] have demonstrated that caffeic acid may be an important ally in the treatment of fluconazole-resistant Candida albicans (Figs. 6 and 7).

It should be noted that this is the first time that the chemical composition and antifungal activity of ethanolic and aqueous extracts of T. spinosa is reported. In the study by Silva et al. [27] in different types of extracts (cyclohexane, chloroform, ethyl acetate) of Tarenaya spinosa phytochemicals constituents were identified, like saponins,

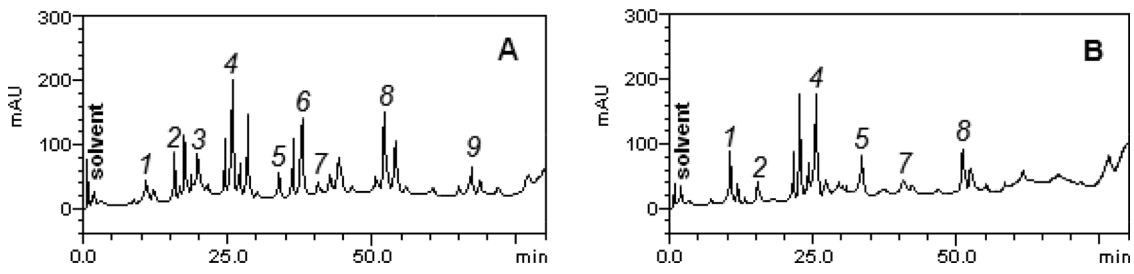

Fig. 1. Representative high performance liquid chromatography profile of Tarenaya spinosa. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), p-coumaric acid (peak 6), rutin (peak 7), quercetin (peak 8) and apigenin (peak 9).

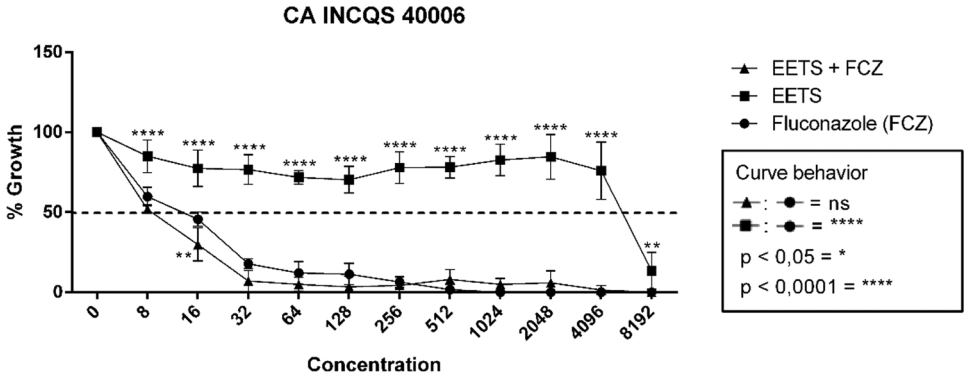

Fig. 2. IC_{50} and modulatory effect of EETS against Candida albicans. EETS - Ethanolic extract of Tarenaya spinosa; FCZ - Fluconazole; CA - Candida albicans; INCQS - National Institute of Health Control; ns - statistically insignificant value.

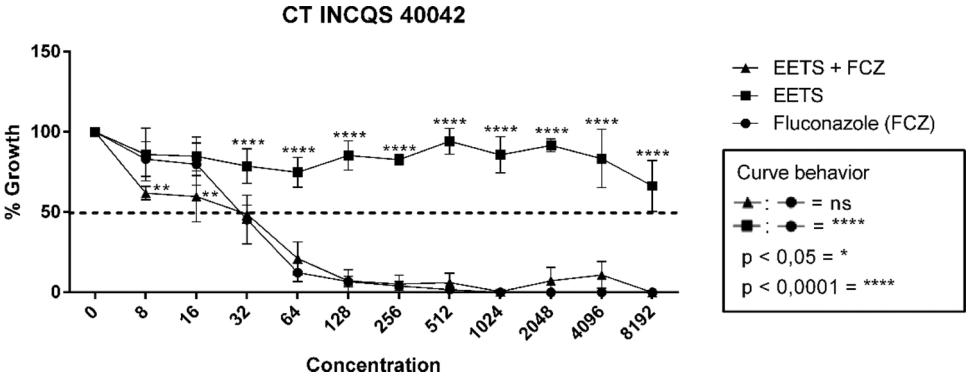

Fig. 3. IC_{50} and modulatory effect of EETS against Candida tropicalis. EETS - Ethanolic extract of Tarenaya spinosa; FCZ - Fluconazole; CT - Candida tropicalis; INCQS - National Institute of Health Control; ns - statistically insignificant value.

Table 2
IC_{50} (μg/mL) values of the products against Candida strains.

Products tested	CA INCQS 40,006	CK INCQS 40,095	CT INCQS 40,042
FCZ	9.0	80.7	26.7
EETS	4,999.0	10,267.7	12,534.2
EATS	10,508.6	ND	12,066.0
EETS + FCZ	4.9	34.8	21.3
EATS + FCZ	5.0	84.7	19.1

CA - C. albicans; CK - C. Krusei; CT - C. tropicalis; EETS - Ethanolic extract of Tarenaya spinosa; EATS - Aqueous extract of Tarenaya spinosa; FCZ – Fluconazole; ND - Not determined.

flavonoids, tannins, coumarins and terpenoids, compounds known for their inhibitory activity against bacteria and fungi. A similar result to that found by Bose et al. [15], with a plant phylogenetically close to T. spinosa, where saponins, tannins and flavonoids were identified.

While in the study by Santos et al. [19], the phytochemical results of the extracts show that all the phenolic compounds identified in this study are present in the extracts of T. spinosa, however there are some quantitative variations in these constituents, and this is justified by internal and external factors. Within the internal factors, these may be genetic, while the external factors may be the collection site, the preparation mode of the extracts and mainly geographical origin [28].

In both extracts it was possible to identify the presence of flavonoids, although in the aqueous extract in a lower percentage. Some studies show that degradation of flavonoids dependent on temperature increase may occur [29]. In this sense the prolonged exposure to high

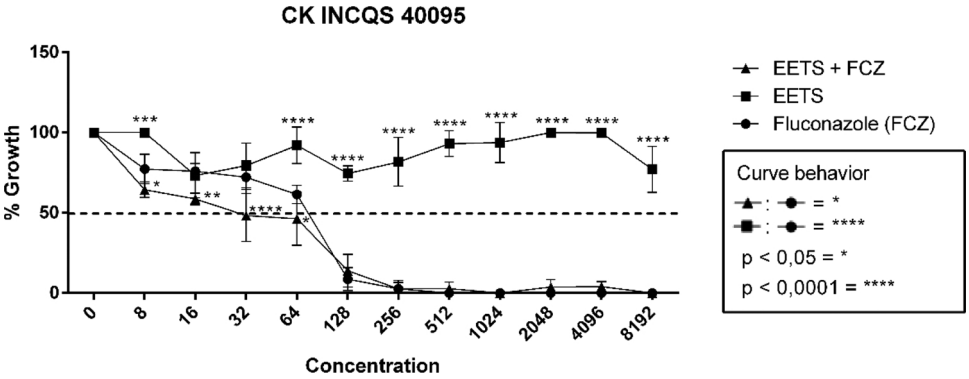

Fig. 4. IC_{50} and modulatory effect of EETS against Candida krusei. EETS - Ethanolic extract of Tarenaya spinosa; FCZ - Fluconazole; CK - Candida krusei; INCQS - National Institute of Health Control.

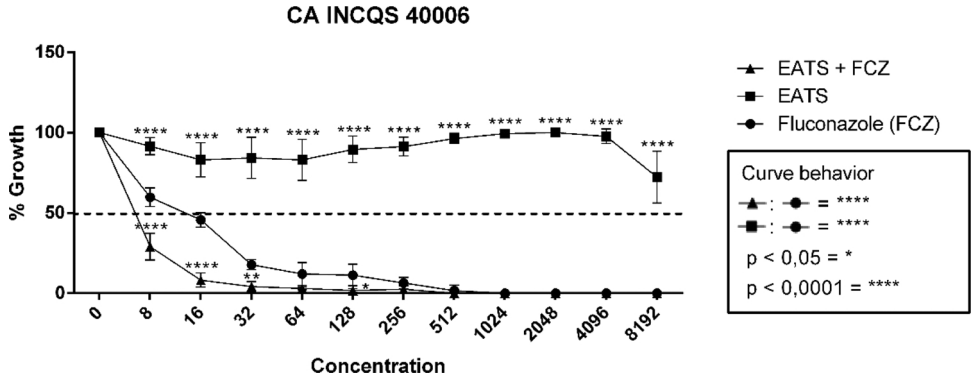

Fig. 5. IC_{50} and modulatory effect of EATS against Candida albicans. EATS - Aqueous extract of Tarenaya spinosa; FCZ - Fluconazole; CA - Candida albicans; INCQS - National Institute of Health Control.

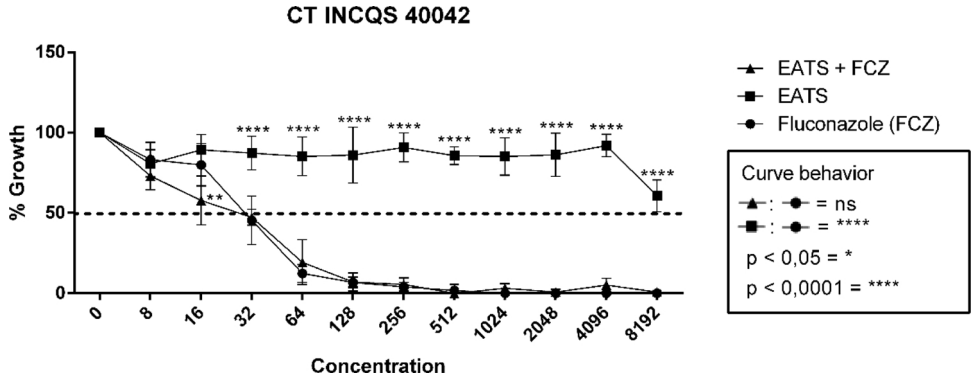

Fig. 6. IC_{50} and modulatory effect of EATS against Candida tropicalis. EATS - Aqueous extract of Tarenaya spinosa; FCZ - Fluconazole; CT - Candida tropicalis; INCQS - National Institute of Health Control; ns - statistically insignificant value.

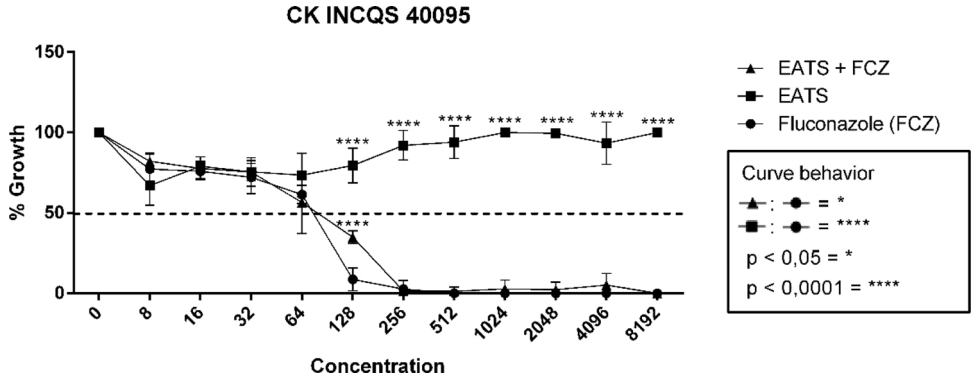

Fig. 7. IC_{50} and modulatory effect of EATS against Candida krusei. EATS – Aqueous extract of Tarenaya spinosa; FCZ - Fluconazole; CK - Candida krusei; INCQS - National Institute of Health Control.

temperature of the solvent may have been the cause of reduction in the level of flavonoids [22].

In the study by Lima et al. [30], caffeic and gallic acids, both identified in extracts of T. spinosa, were tested against strains of C. albicans and C. tropicalis, showing no antifungal activity at the concentrations evaluated, MIC ≥ 1024 μg/mL. However, when associated with fluconazole, caffeic acid showed potentiating activity against the C. albicans strain, while gallic acid showed a possible synergism with the two tested strains, C. albicans and C. tropicalis. This is a strong evidence that plant-derived substances can play a role in the modulation of commercial drugs, making it possible to reduce MIC and, consequently, to reduce the adverse effects of antibiotics [8].

In addition, the antifungal activity of medicinal plants and their derivatives has been scientifically proven in tests using essential oils, extracts, phytoalexins, coumarins, terpenes, flavonoids, amides, imides and alkaloids [19,31]. And in many cases the antimicrobial activities of plant extracts have been attributed mainly to flavonoids [32].

Silva et al. [27] demonstrated the antibacterial activity of T. spinosa. However, when tested against pathogenic fungi, MIC values proved to be irrelevant, as in our study, thus evidencing the low antifungal activity of extracts of this species when tested alone. The presumed synergistic effect of EETS + fluconazole on yeast C. krusei can be explained by its higher percentage of flavonoids. Although the association between EATS + fluconazole had a supposed synergism against C. albicans, it showed an antagonistic effect against C. krusei, a result that may be associated with a low percentage of flavonoids in the extract and the intrinsic resistance of C. krusei to fluconazole.

Plant extracts are composed of a complex mixture of several substances and although some of these have a great potential for modulation of commercial drugs such as fluconazole when isolated, they

may have their effect masked due to antagonistic interactions with the other compounds that form the extract [4].

However, plant extracts continue to be effective alternatives against growth and microbial resistance. Moreover, the probability of resistance to extracts is low due to the complex mixture of substances that compose them, making difficult the microbial adaptation [8].

The mechanisms by which extracts interfere with microbial growth are varied and may be related to the chemical composition of the extracts thus they can interfere with the lipid bilayer of the cell membrane affecting respiratory chain and energy production or affect the permeability of the membrane to antibiotics thus leading to cell death [8].

5. Conclusion

This study shows that although T. spinosa does not have a fungal potential at low concentrations, the species has a high modulatory potential of the drug fluconazole, so that this plant, a very common species in Brazil, is a new source of secondary compounds with potential modulator of drugs.

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