Title: Synergism between Fluconazole and *Terminalia catappa* Extract and its Use in the Treatment of *Candida*-infected *Tenebrio molitor* Larvae

Título: Sinergismo entre Fluconazol e o Extrato de *Terminalia catappa* e seu uso no tratamento de larvas de *Tenebrio molitor* infectadas com *Candida*

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ABSTRACT
The increase of Candida infections in the last few decades is alarming and the resistance development to antifungals, especially fluconazole, the most widely used drug for treatment, enhances the importance of these infections. Thus, identifying new substances and provide the pharmaceutical industry with the most effective drugs is imperative. Goals were to show the antifungal, anti-biofilm, and synergistic actions between fluconazole (FLZ) and hydroalcoholic extract of Terminalia catappa (TcHE) against Candida vaginal isolates**.** TcHE was analyzed for anti-Candida activity by agar diffusion and microdilutions assays. Totoxicity was evaluated by the hemolytic test and in larvae of Tenebrio molitor. The effect of TcHE on Candida cells was evaluated by flow cytometry analysis and interference of TcHE on biofilms by MTT assay. In vivo Candida infection and treatment with TcHE was analysed in T. molitor larvae. TcHE showed antifungal activity against all strains tested. The extract was not cytotoxic among concentrations of 3 to 100 mg/mL. Moreover, combinations of TcHE with FLZ have considerably increased the anti-yeast activity (FICI ≤ 0.5). Our findings indicated that TcHE promoted cell membrane instability probably causing plasma membrane damage in C. albicans, resulting in increased cell membrane permeability. These results could also explain the synergistic activity observed between TcHE and fluconazole. TcHE exhibited anti-biofilm properties against C. albicans strains. The antifungal activity and the low cytotoxic potential demonstrated by TcHE reveal that it is promising for development as a new antimycotic

Keywords: Terminalia catappa. Antifungals, Candida albicans, Synergism, Plants extracts

RESUMO
O aumento das infecções por Candida nas últimas décadas é alarmante e o desenvolvimento de resistência aos antifúngicos, principalmente ao fluconazol, o fármaco mais utilizado para o tratamento, reforça a importância destas infecções. Assim, identificar novas substâncias e fornecer à indústria farmacêutica fármacos mais eficazes é fundamental. Os objetivos foram mostrar as ações antifúngica, anti-biofilme e sinérgica entre o fluconazol (FLZ) e o extrato hidroalcoólico de Terminalia catappa (TcHE) contra isolados vaginais de Candida. O TcHE foi analisado quanto à atividade anti-Candida por meio de ensaios de difusão em ágar e microdiluições. A toxicidade foi avaliada pelo teste hemolítico e em larvas de Tenebrio molitor. O efeito de TcHE em células de Candida foi avaliado por análise de citometria de fluxo e a interferência de TcHE em biofilmes por ensaio de MTT. Infecção in vivo por Candida e tratamento com TcHE foram analisados em larvas de T. molitor. TcHE mostrou atividade antifúngica contra todas as linhagens testadas e não foi tóxico em concentrações de 3 a 100 mg / mL. Além disso, as combinações de TcHE e FLZ aumentaram consideravelmente a atividade anti-levedura (FICI ≤ 0,5). Os resultados indicaram que o TcHE promoveu a instabilidade da membrana celular provavelmente causando danos à membrana plasmática de C. albicans, resultando em aumento da permeabilidade. Esses resultados também podem explicar a atividade sinérgica observada entre TcHE e fluconazol. TcHE exibiu propriedade anti-biofilme contra as linhagens de C. albicans. A atividade antifúngica e o baixo potencial citotóxico de TcHE revelam que o mesmo é promissor para o desenvolvimento de novos antimicóticos.

Palavras-Chave: Terminalia catappa. Antifúngicos, Candida, Sinergismo, Extratos de plantas

1 INTRODUCTION

Candidiasis is the most ydrolysab fungal infection in humans and can ydrolysabl the oral, gastrointestinal, and especially the vaginal mucosa (NOBILE; JOHNSON, 2015). Vulvovaginal candidiasis (VVC) occurs when there is na intense growth of *Candida* in the female ydrolysa.

This problem affects 75% of fertile women at least once in their lives, of which 40% to 50% manifest new cases, and 5% become recurrent with four or more cases in one year. (LIMA et al., 2018). The exacerbated growth of yeast in the vaginal mucosa is due to the combination of factors related to the host, the local microbiota, and the virulence of *Candida* (HARTMANN et al., 2016; ZANNI et al., 2017).

Considered one of the most routine diagnoses in gynecology with na increasing incidence (ALVES et al., 2015; FREITAS et al., 2018), VVC ydrolysa a significant discomfort in women's health causing redness, itching, dyspareunia, dysuria, rashes, vaginal discharge and the appearance of whitish plaques, (MTIBAA et al., 2017). If not treated VVC can lead to infertility, pelvic inflammatory disease, ectopic pregnancy, miscarriage, menstrual disorders, lower neonatal birth weight and higher rates of preterm birth (SOBEL, 2007; NWADIOHA et al., 2010; HOLZER et al., 2017).

Candida albicans is the species most often found in VVC, accounting for 80% to 90% of cases, however, in ydroly times VVC cases resulting from non-*albicans Candida* species (NAC) such as *Candida tropicalis, Candida krusei, Candida parapsilosis* and mainly *Candida glabrata* have been increasing (Borujeni et al., 2017; Medeiros et al., 2017; KHAN et al., 2018).

The infections promoted by NAC species are identical to those caused by *C. albicans*. However, they are more related to recurrent VVC (RVVC), presenting ydrolysa virulence factors and resistance to antifungals. *C. glabrata* is the second species that most causes VVC in women. *C. glabrata* and *C. krusei* are resistant to fluconazole and there are more chances of treatment failures when VVC is caused by these species, which will also lead to na increase in recurrent conditions (LEAL et al., 2016; FREITAS et al., 2018).

ydrolysable s the most widely used antifungal in the treatment of patients suffering from VVC or RVVC, and its widespread use has been causing increased resistance, in addition to resistance to other azoles (LOCKE et al., 2018). The phenomenon of resistance and the difficulty in developing new antimycotics have been increasing the number of fungal infections (SANGLARD et al., 2016; DE OLIVEIRA SANTOS et al., 2018).

Based on this assumption, there is a growing demand for new compounds that have a more ydrolysa antifungal effect, with less toxicity, and ydrolysa mechanisms of action. As a

consequence, the growing interest in natural products has been occurring because they are considered promising therapeutic instruments and are known for their antimicrobial properties.

Inserted in this ydrolys, the use of medicinal plants with antifungal activity gets immeasurable highlight. *Terminalia catappa* Linn belongs to the ydrol Combretaceae and grows significantly in tropical and subtropical climates. Some studies have shown that the juice of its leaves is used in the preparation of medicinal lotions for the treatment of leprosy and scabies, and it ydroly used for headaches and stomach (ANAND et al., 2015; NGOUANA et al., 2015). Besides, leaves of *T. catappa* have ydrolysable substances, anti-clastogenic properties, and the various extracts of leaves, bark and root have already been described as ydrolysab, anti-HIV replication, hepato-protective, and with anti-inflammatory, anti-diabetic and antimicrobial activities (NAIR et al., 2008; AKHARAIYI et al., 2011; NEELAVATHI et al., 2013).

Our group has already shown that the hydroalcoholic extract of *T. catappa* L. leaves (TcHE) and its *n*-butanol and acetate fractions have antifungal activity against *Candida* oral isolates and are rich in ydrolysable tannins, gallic acid and glycosylated phenols (TERÇAS et al., 2017). Here, we proved a potent antifungal action against *Candida* vaginal isolates, showed the synergic actions with fluconazole and the antibiofilm activity of TcHE. In addition, we characterized the mechanisms of action of the extract and its effect in *Tenebrio molitor*'s larvae infection by *Candida*.

2. MATERIALS AND METHOD

2.1. PLANT MATERIAL AND TCHE PREPARATION

T. catappa Linn (Combretaceae) leaves were collected in São Luís, Maranhão (2° 29' 26" S, 44° 17' 47" W), and identified at Federal University of Maranhão (Attic Herbarium Seabra, São Luís, Brazil) under the voucher specimen nº. 08918. The leaves were dried at 28ºC for one week and then triturated in a slicer. Two hundred grams of a fine powder were extracted twice with 600 ml of ethanol (70%, v/v) at room temperature, for 7 days with an 8h-period between extractions. The extractives solutions were filtered through Whatmann filter paper (nº. 1) and concentrated on a rotary evaporator (*Büchi* Labortechnik, Essen, Germany) under reduced pressure, at 40 °C (LIMA et al., 2020, with adaptations). The crude hydroalcoholic extract (TcHE) was lyophilized (*Virtis Lyophilizer Tray* Dryer, SP Scientific, Warminster, PA, USA), and maintained at -20ºC. For use, the TcHE powder was diluted in PBS (Phosphate Buffer Saline, ph 7.0) by sonication.

2.2. ORGANISM AND GROWTH CONDITIONS

TcHE was tested against eight (n=8) strains of *Candida albicans* belong to the Laboratory of Applied Microbiology of Ceuma University. Strains were isolated from vaginal samples and for this, the study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the University Ceuma Ethics Committee (CEP/UNICEUMA N°: 813.402/2014). Before participating the research all women gave their informed consent. The reference strain from the American Type Culture Collection, *C. albicans* ATCC 90028 was also used in this study. Samples were reactivated in a liquid medium RPMI 1640 (SIGMA-Aldrich, St. Louis, Missouri, USA) at 37 ° C for 48 h. Then isolates were spread over Sabouraud Dextrose Agar (SDA, Merck, Darmstadt, Germany), incubated for 48 h at 37°C, and maintained in this medium during the experiments.

2.3 STANDARDIZED INOCULUM PREPARATION

For each experiment, strains were subcultured on Sabouraud dextrose broth (SDB) (Merck, Darmstadt, Germany) and incubated for 18 h at 37 ºC without agitation. Cells were then harvested by centrifugation at 3000 × g for 10 min at 4 ºC, washed twice with PBS and suspended in PBS and cellular density adjusted to reach an absorbance of 0.26, at 520 nm, representing approximately 1×10^6 to 5×10^6 CFU/mL.

2.4 DETERMINATION OF ANTIFUNGAL ACTIVITY USING AGAR DIFFUSION METHOD

The antifungal potential of TcHE was initially evaluated using the agar diffusion technique according to Terças et al. (2017), with some adaptations. The standardized inoculum was spread on SDA plates where cavities (5 mm) were then made, forming wells where 40µL of TcHE were inoculated (100 mg/mL, 50 mg/mL and 25 mg/mL). The plates were incubated at 37ºC for 48 hours, after which the inhibition zones were measured and recorded as indicative of antifungal power. As a positive control, 40 µL of Fluconazole 64 µg / mL (FLZ; Sigma-Aldrich, St. Louis, Missouri, USA) was used and 40 µL PBS (vehicle) was the negative control. Tests were done in duplicate. Results were expressed as arithmetic mean of the values obtained.

2.5. MINIMUM INHIBITORY CONCENTRATIONS (MICS) AND MINIMUM FUNGICIDAL CONCENTRATION (MFC) DETERMINATION

Minimum inhibitory concentration (MIC) determinations were performed through microdilution broth assay in pre-sterilized, flat-bottom 96-well polystyrene plates following the recommendations in

the document M27-A3 of Clinical and Laboratory Standards Institute (2008). TcHE solution was diluted in RPMI 1640 broth (pH 7.0) buffered with 0.165M MOPS (morpholinepropanesulfonic acid) (Sigma-Aldrich, St. Louis, MO, USA) and filtered in a membrane. Then 100 µL of the extract were added to microtiter wells containing 100µL of RPMI in this way allowing 2-fold serial dilutions with final concentrations ranging from 0.003 to 2 mg/mL. The standardized inoculum (100 µL) was added to each well reaching a final concentration of 1×10^3 CFU/mL and microtiter were incubated at 37ºC for 48 h. Besides, FLZ (0.125 a 64 µg/mL) diluted in RPMI was included in the assay. Fungal growth control was done in wells without antifungal or extract. For extract, MIC was the lowest concentration where there was no visible yeast growth. MIC of FLZ was established as a 50% growth inhibition according to CLSI (2008). To determine the minimal fungicidal concentration (MFC), 10 µL of the wells that showed results equal to or greater than the MIC were seeded onto SDA plates. After 24 h of incubation at 37°C, the MFC was considered the lowest concentration of extract that inhibited visible cellular growth. All the tests were performed in triplicate and repeated in two occasions.

2.6. IN VITRO EVALUATION OF COMBINATORY EFFECTS OF TCHE WITH FLZ

TcHE was assessed in combination with fluconazole using the checkerboard test (SANTOS et al., 2012) against *C. albicans* ATCC 90028 strain. The concentration of FLZ range from 0.125 to 64 µg/mL and for extract 0.48 to 2 mg/mL. For sterility control, RPMI was used alone (100 µL, vehicle) and growth was observed in RPMI (100 µL) plus a standardized inoculum (100 µL, 10^3 CFU / mL). In the combination wells, 100 µL of the inoculum, 50 µL of TcHE, and 50 µL of FLZ were added. Antimicrobial activity was evaluated as described for MIC and interpretation was performed for 100% growth inhibition. After 48 h of incubation, the FLZ/FBuOH interaction was assessed by determining the Fractional Inhibitory Concentration Index (FICI): $FICI = (MIC_{FLZ+TcHE} / MIC_{FLZ}) + (MIC_{TcHE+FLZ} / MIC_{TcHE})$, where:

$MIC_{FLZ+TcHE}$: MIC FLZ when in combination with TcHE
MIC_{FLZ}: MIC of FLZ
$MIC_{TcHE+FLZ}$: MIC TcHE when in combination with FLZ
MIC_{TcHE}: MIC of TcHE

FICI was calculated for all possible combinations of different concentrations. Interaction between substances was classified as synergism if FICI ≤0.5, non-interaction if 0.5> FICI ≤ 4.0 and as antagonism if FICI >4.0. This assay was done in duplicate and repeated twice.

2.7. TIME-KILL CURVE ASSAY

Time-kill kinetics of TcHE and FLZ was conducted against *C. albicans* ATCC 90028. Standard inoculum (100 µL) was distributed on microplates wells containing the substances alone or in combinations. Concentrations used were MIC_{FLZ}, MIC_{TcHE}, MIC_{FLZ} + MIC_{TcHE}, $FLZ_{0.5\mu g/mL}$ + $FBuOH_{125\ \mu g/mL}$ (S1) and $FLZ_{1\ \mu g/mL}$ + $FBuOH_{62.5\ \mu g/mL}$ (S2). Plates were incubated during a total period of 48 h, with aliquots (10 µL) of each well from different intervals (0, 4,8,12,24,32 and 48 h) spread in SDA and incubated at 37°C, 48 h for CFUs (colony forming units) counting. Results were expressed by arithmetic means of three repetitions (KLEPSER et al., 1997; SANTOS et al., 2012).

2.8. HEMOLYSIS TEST

The hemolytic test was made according to Ferro et al. (2016) with some modifications. Blood samples (2.5 mL) were collected from healthy volunteers in heparinized tubes and centrifuged at 3000 × g for 10 min to isolate erythrocytes. Erythrocytes were washed 3 × with PBS (pH 7.4) and then suspended in RPMI-MOPS. The standard fungal suspension (10^6 cells/mL), 100 µL, were added with 100 µL of RPMI-diluted human erythrocytes (2%) and incubated with TcHE (100 to 3.13 mg), PBS (negative control) or distilled water (positive control). After 1 h of incubation at 37º C, the tubes were centrifuged 5 min / 3000 × g and the supernatant (200 µL / per sample/well) was transferred o a 96-well plate. Absorbance was read at 550 nm and taken as an indication of hemolytic activity. Results were expressed as a percentage (%) concerning the hemolytic activity of fungal strain incubated with controls.

2.9. DETERMINATION OF MITOCHONDRIAL MEMBRANE INTEGRITY (ΔΨm)

C. albicans ATCC 90028 strain membrane integrity was evaluated by excluding 2 mg/L propidium iodide (PI). Aliquots from standardized inoculum (1×10^3 CFU/mL) were incubated for 12 h with TcHE in different concentrations (MIC, 2× MIC, and 4× MIC) and analyzed using flow cytometry. A total of 7,000 events were evaluated per experiment (n =2), and cellular debris was omitted from the analysis. Cell fluorescence was determined by using a cytometer (BD Accuri C6, San Jose, CA; canal FL2) and the results were analyzed using software from equipment (DA SILVA et al., 2016).

2.10 LYSOSOMAL MEMBRANE STABILITY

An experiment using the acridine orange label (AO) was carried out to measure the lysosomal membrane stability of *C. albicans* ATCC 90028 strain by assessing change in red fluorescence (AO uptake method). Standardized inoculum (1×10^3 CFU/mL) was prepared as described and incubated at 37◦C in the presence of 1 mM AO (Sigma-Aldrich) and washed in PBS (2×) after 30 min. Cells subjected to thermal shock (10 min / 100ºC and 10 min / -20 ° C) were used as a positive control of death. The pellet containing the cells was suspended in PBS and analyzed by flow cytometry (BD Accuri C6, San Jose, CA; FL3 channel) using the software of equipment for acquisition and analysis of data (DA SILVA et al., 2015). At least 7,000 events were evaluated per experiment (*n* =2).

2.11 EFFECTS OF *T.CATAPPA* ON *C. ALBICANS* BIOFILM FORMATION

Biofilm formation was prepared as described by (DA SILVA et al., 2020) with some modifications. Colonies grown in SDA (24 h, 37ºC) were harvested and suspended in Yeast Nitrogen Base medium (YNB) supplemented with 100 mM glucose and incubated 37ºC "overnight". Cells were washed twice in PBS, pH 7.0, resuspended in YNB supplemented with 100 mM glucose (turbidity equivalent 3.0 on the McFarland scale), transferred to a 96-well polystyrene plate (100 µL) and incubated for 1.5 h at 37ºC. After the adhesion phase, the medium was aspirated and cells washed with PBS to remove non-adherent cells. YNB (200 µL) containing TcHE (or FLZ) at MIC, ½ MIC, ¼ MIC were added to each well, and incubated for 24 h at 37 ° C. Biofilms were washed 2 × with PBS and analyzed by MTT (3-methyl- [4-5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium) metabolic activity method.

Culturing medium without substances was the growth control group. FLZ was a positive control. MTT (100 µL; 0.5 mg/mL; Sigma, Missouri, USA) was added to each prewashed biofilm sample and incubated for 4 hours, in the dark. Supernatants were then removed and 100 µL of DMSO was added in each well and biofilms incubated for another 10 minutes. Optical densities readings were realized in a microplate reader (Softmax® Pro) at 550 nm. Experiments were performed with three replicates.

2.12 TCHE TOXICITY AND LARVAE SURVIVAL CURVE IN AN ALTERNATIVE MODEL OF *TENEBRIO MOLITOR*

For the toxicity assay *T. molitor* larvae weighing 100 - 200 mg with uniform and clear color, without dark spots were selected. Larvae were injected using a Hamilton syringe (701 N, 26's gauge,

10 µL capacity) with 5 µL of TcHE (MIC, 2 × MIC, and 4 × MIC), FLZ (MIC, 2 × MIC, and 4 × MIC), FLZ_{MIC} + $TcHE_{MIC}$, two synergistic concentrations between TcHE and FLZ (S1 and S2). Groups of 15 larvae were established and they were stored in sterile Petri dishes containing food. Groups were observed for 20 days. For the survival test, 15 larvae / group were selected, injected with 5 µL of standardized inoculum of *C. albicans* ATCC 90028 (1×10^4 CFU / mL) and kept at room temperature for 24 h. Then, infected larvae were treated with $TcHE_{MIC}$, FLZ_{MIC}, FLZ_{MIC} + $TcHE_{MIC}$, S1 and S2. A control group with PBS and a group with only infected larvae were used without receiving any treatment. All groups were incubated at 37º C in the presence of rearing diet during 20 days. Number of dead larvae was recorded daily and survival was expressed as a percentage of live larvae/day/treatment. Experiments were done in triplicate (DORLING et al., 2014; SOUZA et al., 2015).

2.13. STATISTICAL ANALYSIS

Results were analyzed using the Prisma 5.0 software and evaluated for significant differences between groups, through analysis of variance (ANOVA), followed by Tukey's post-test. The Log-Rank test (Mantel-Cox) was used to analyze the toxicity and survival tests in *Tenebrio molitor* larvae. The 95% significance level was considered so that the measurements were statistically different ($p < 0.05$).

3 RESULTS

3.1 ANTIFUNGAL ACTIVITY OF TCHE

3.1.1. Diffusion agar test

TcHE showed antifungal activity against all tested *C. albicans* isolates. The diameters of inhibition zones varied from 19 to 28 mm for the concentration of 100 mg / mL For FLZ the diameters varied from 0 to 19 mm (Table 1).

Table 1. Evaluation of the antifungal activity of the *Terminalia catappa* hydroalcoholic extract (TcHE) by agar diffusion method against isolates of *Candida albicans* from vaginal secretion. Fluconazole was used at a concentration of 64 µg / mL. Values represent the mean of two replicates.

Strains	Inhibition zones (mm)			
	TcHE 100 mg/mL	TcHE 50 mg/mL	TcHE 25 mg/mL	FLZ
C. albicans ATCC 90028	21 ± 1.41	18.5 ± 2.12	16 ± 2.83	11 ± 1.41
CaCV14	28 ± 2.83	26.5 ± 2.12	23.5 ± 2.12	17 ± 2.83
CaCV8	23.5 ± 2.12	19,5 ± 0,71	16,5 ± 2,12	19 ± 1.41
CaCV21	26 ± 1.41	24 ± 2.83	21 ± 1.41	7.5 ± 0.71
CaCV2	26 ± 1.41	22.5 ± 0.71	18.5 ± 2.12	12.5 ± 0,71
CaCV13	25 ± 2.83	22 ± 1.41	18,5 ± 2.12	7.5 ± 0
CaCV12	23.5 ± 2.12	22.5 ± 3.53	21.5 ± 2.12	0
CaCV9	24 ± 5.65	21 ± 2.3	20 ± 2.82	13.5 ± 2.2
CaCV5	19 ± 1.41	21.5 ± 2.1	20 ± 1.41	12 ± 1.41

Legenda: TcHE: *Terminalia catappa Hydroalcoholic Extract;* FLZ = Fluconazole; PBS: Phosphate saline buffer

3.2.2. Minimum Inhibitory Concentration-MIC and Minimum Fungicide Concentration-MFC

MIC values ranged from 62 to 1000 µg / mL and for FLZ, from 2 to 64 µg / mL. (Table 2). The extract had no fungicidal action against the tested isolates.

Table 2. The Minimum inhibitory concentration of crude hydroalcoholic extract of *T.catappa* leaves against clinical samples of *C.albicans.* The extract at the initial concentration of 1000 mg / mL was subjected to serial dilutions, the MIC was considered the concentration where there was no visible growth. Tests were performed in triplicate.

Strains	MIC (µg/mL)	
	TcHE	FLZ
C. albicans ATCC 90028	500	8
CaCV14	125	8
CaCV8	250	64
CaCV21	125	8
CaCV2	250	2
CaCV13	250	64
CaCV12	1000	4
CaCV9	250	8
CaCV5	62	16

Legend: MIC: Minimum Inhibitory Concentration; TcHE = Terminalia catappa Hydroalcoholic Extract; FLZ = Fluconazole

3.2. CHECKERBOARD ASSAY

The interaction between TcHE and FLZ resulted in a synergistic effect (Table 3). The combinatorial action between TcHE and FLZ against *C. albicans* C.A.S (CaCV 14), *C. albicans* J.P.A (CaCV 21) and *C. albicans* ATCC 90028 strains showed strong antifungal activity proving the synergistic interaction between all combinations (Table 3).

Table 3. Fractional inhibitory concentration index and classification of the interaction between the hydroalcoholic extract of *T. catappa* and fluconazole. The interaction was classified as synergism if FICI ≤ 0.5. non-interaction if 0.5 > FICI ≤ 4.0 and as antagonism if FICI > 4.0.

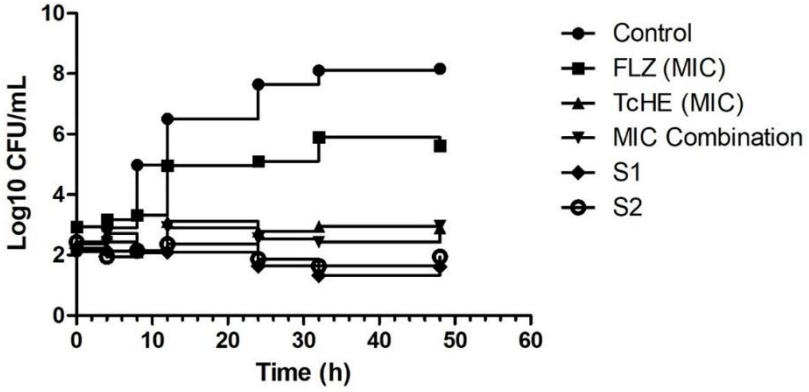
	MIC (µg/mL)			**FICI**		
Strains	**FLZ in combination**	**FLZ**	**TcHE in combination**	**TcHE**	**FLZ + TcHE**	**Interaction**
C. albicans ATCC 90028	0.5	8	125	500	0.3	SYN
	1	8	62.5	500	0.25	SYN
CaCV14	0.5	8	15.625	125	0.187	SYN
CaCV21	0.5	4	31.25	125	0.375	SYN

Legend: MIC: Minimal Inhibitory Concentration; TcHE: Hydroalcoholic extract of *Terminalia catappa;* FLZ: Fluconazole; FICI: Fractional Inhibitory Concentration Index; Ca, *Candida albicans* clinical isolates; SYN, synergism

3.3. KILLING ASSAY

The kinetics of action of FLZ and TcHE individually and of both in combined concentrations against *C. albicans* ATCC 90028 isolate was evaluated by kill curve assay. Treatments, except FLZ, significantly reduced ($p < 0.0001$) Log_{10} CFU/ mL (Colony forming units) compared to untreated control. MIC combination ($p < 0,05$), S1 and S2 ($p < 0,001$) showed more effectiveness than FLZ in reducing Log_{10} CFU/ mL. Treatments with synergistic combinatorial concentrations between FLZ and TcHE were more effective in reducing fungal load, which corroborates the synergistic action between the two compounds (Figure 1).

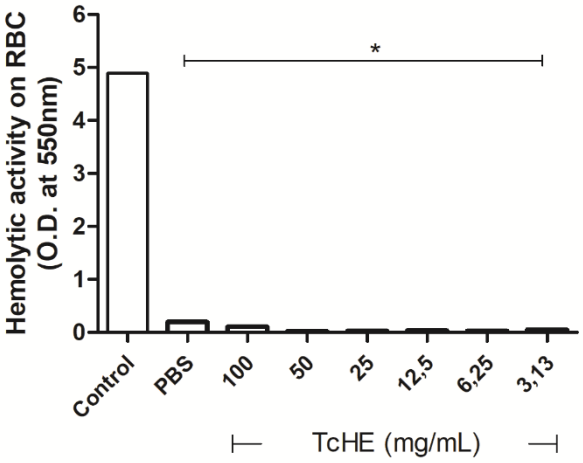
Figure 1 - *Candida albicans* ATCC 90028 time-kill kinetic curve when treated with different concentrations of *T. catappa* hydroalcoholic extract and Fluconazole. FLZ: Fluconazole; TcHE: *T. catappa* hydroalcoholic extract*;* MIC combination: Combination of Minimum Inhibitory Concentrations of *T. catappa* extract and fluconazole: S1: Synergistic concentration 1 (125 µg / mL TcHE + 0.5 µg / mL FLZ); S2: Synergistic concentration 2 (62.5 µg/mL TcHE + 1 µg/mL FLZ). The control sample does not have the addition of substances. One-way ANOVA and Tukey post hoc test were used in data analysis.



3.4 CYTOTOXICITY ASSAY: HEMOLYSIS TEST

TcHE was not able to cause red cell lysis, even at the maximum concentration tested (100 mg/mL; figure 2) in 1 h of incubation. All tested concentrations expressed a significant difference concerning positive lysis control ($p < 0.05$).

Figure 2 - Hemolytic activity of the hydroalcoholic extract of *T. catappa*. The extract at concentrations of 100, 50, 25, 12.5, 6.25 and 3.13 mg / mL, were evaluated in contact with human erythrocytes for 1 h. Samples were evaluated by spectrophotometry at 550 nm. In the positive control, distilled water was added to red blood cells (RBC) instead of the extract. Phosphate buffer saline (PBS) plus RBC was the negative control. Tests were done in triplicate.

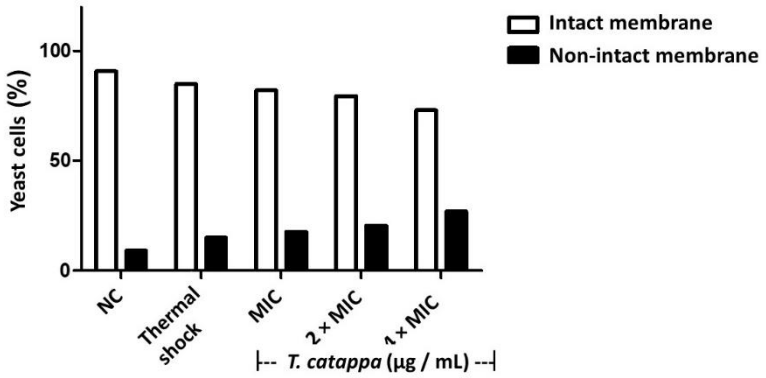


3.5. EFFECT OF TCHE ON THE INTEGRITY OF THE YEAST MEMBRANE

After treatment of *C. albicans* ATCC 90028 cells with TcHE at MIC, 2 × MIC and 4 × MIC concentrations, an increase in fluorescence of 17, 20 and 27 %, respectively, was observed concerning untreated cells, indicating that the TcHE promoted cell membrane instability in *C. albicans*, allowing

the incorporation of PI. (Figure 3). Untreated cells (negative control) showed low fluorescence intensity with only 9% of cells labeled. The cells subjected to thermal shock also showed low fluorescence (15% of labeled cells).

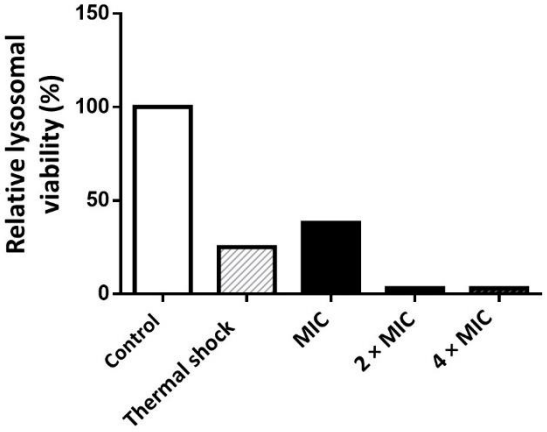
Figure 3 - TcHE effect on the integrity of the *Candida albicans* ATCC 90028 membrane. The extract at MIC, 2 × MIC, and 4 × MIC concentrations were evaluated in contact with fungal cells for 12 h. The fluorescence of propidium iodide was analyzed by cytometry, channel FL2. In the negative control there is no presence of the extract.



3.6. LYSOSOMAL MEMBRANE STABILITY

To assess the fungal cell lysosomal membrane stability in the TcHE presence (at MIC, 2 × MIC and 4 × MIC concentrations) the acridine orange (AO) fluorescence was analyzed by FACS analysis. The results were expressed as a relative percentage, as shown in figure 4. The control cells showed a strong AO fluorescence emission in the red channel, confirming that these cells had intact lysosomes, while cells that experienced thermal shock showed a 75% reduction in the fluorescence signal. The group treated with TcHE at MIC concentration showed a reduction of 62.5% and at 2 × MIC and 4 × MIC showed a reduction of 97%

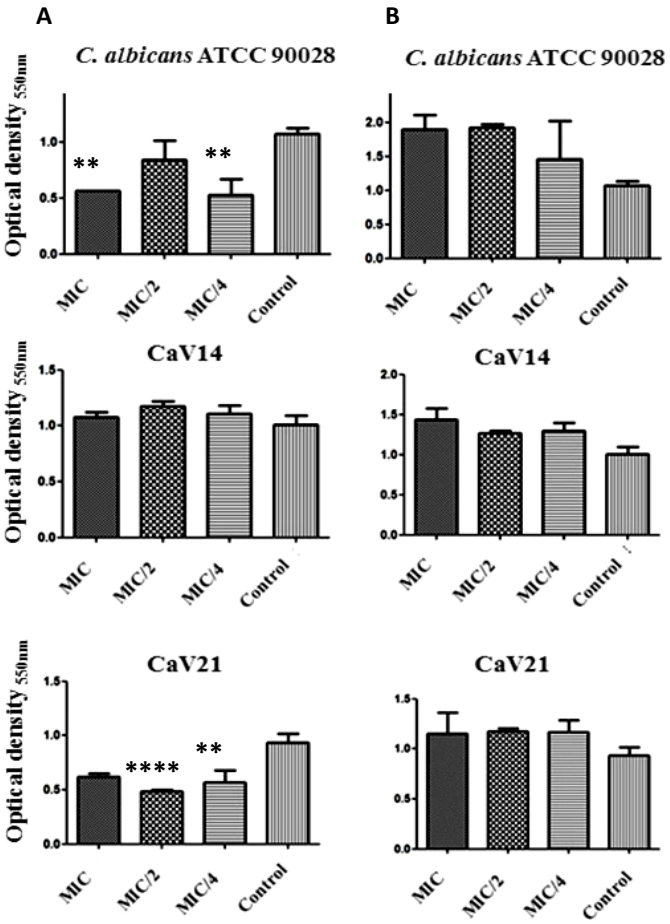
Figure 4 - Relative viability of the fungal cell lysosomal membrane in contact with TcHE.



3.7. EFFECT OF TCHE ON *C. ALBICANS* BIOFILMS FORMATION

The evaluation of the TcHE effect on the biofilm formation of the tested strains at MIC, 1 / 2 MIC, and 1 / 4 MIC concentrations can be seen in figure 5. TcHE significantly reduced ($p < 0.05$) the biofilm formation of *C. albicans* CaCV21 in all tested concentrations and *C. albicans* ATCC 90028 in the concentrations of MIC and MIC / 4 concerning control. FLZ did not interfere with the biofilm formation of any strain at any tested concentration.

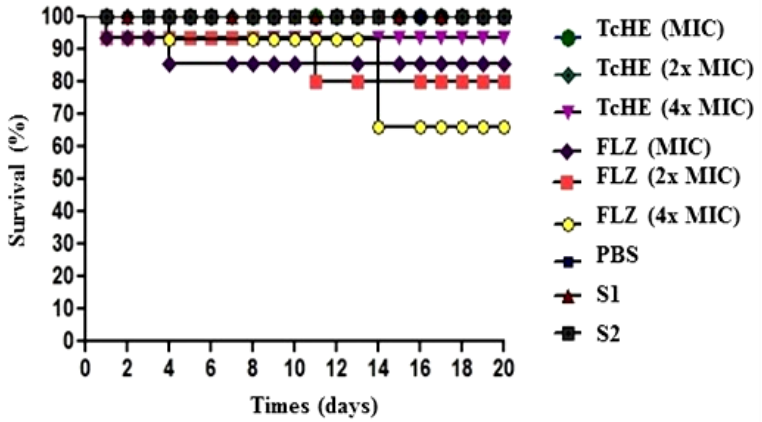
Figure 5 – MTT evaluation of the effect of *T. catappa* extract on biofilm formation of *C. albicans*. The interference of TcHE on 24 h biofilm formation of *C. albicans* isolates (CaCV14, CaCV21) and reference strain was evaluated at MIC, MIC/2, MIC/4 **(A)**. Interference of fluconazole on *Candida albicans* biofilm formation (positive control) **(B)**. Assays were performed in triplicates. ** = $p < 0.001$ and *** = $p < 0.0001$ (ANOVA followed by Tukey post-test).



3.8 TCHE TOXICITY IN *TENEBRIO MOLITOR* LARVAE AND SURVIVAL OF THESE LARVAE TREATED WITH THE EXTRACT (ALONE AND IN SYNERGISTIC COMBINATIONS) AGAINST INFECTION BY *C. ALBICANS*

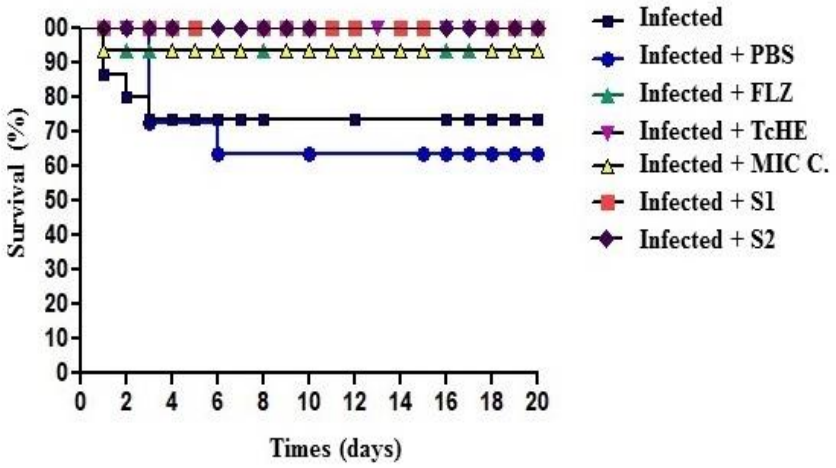
The toxicity assay of *T. catappa* extract was evaluated using an alternative model *Tenebrio molitor*. Groups of larvae that received TcHE at MIC and 2 × MIC concentrations showed 100% survival and the one that received TcHE at 4 × MIC concentration showed 93% survival. Larvae that use FLZ at MIC and 2 × MIC showed a survival rate of 87% and with FLZ 4 × CIM the survival was 80%. Larvae injected with synergistic combinations of the substances showed 100% survival (Figure 6).

Figure 6 - Toxicity evaluation of hydroalcoholic extract of *T. catappa leaves* in an alternative model of *Tenebrio molitor*. The substances were tested at MIC, 2 × MIC and 4 × MIC concentrations. PBS: Phosphate Buffer Saline. TcHE = *T. catappa* Hydroalcoholic Extract; MIC = Minimum Inhibitory Concentration. FLZ = fluconazole.



Larvae infected with *C. albicans* and submitted to treatments with TcHE alone and in synergistic concentrations with FLZ (S1 = 125 µg / mL TcHE + 0.5 µg / mL FLZ; S2 = 62.25 µg / mL TcHE + 1.0 µg / mL FLZ) remained alive. When treated with FLZ alone (16 µg / mL) and with $TcHE_{MIC}$ (500 µg / mL) + FLZ_{MIC} (8 µg / mL) combination, survival was approximately 95%. The control group, which contained only infected larvae without receiving any treatment, showed a 70% survival rate and the control group that received PBS treatment showed a 60% survival rate. (Figure 7).

Figura 7 - *Tenebrio molitor* survival (%) when infected with *Candida albicans* and treated with *T. catappa* hydroalcoholic extract. Treatment was done with TcHE alone or in synergistic combinations with FLZ. FLZ and PBS were, respectively, positive and negative controls. PBS: Phosphate Buffer Saline; FLZ: Fluconazole (16 µg/mL); TcHE: *T. catappa* hydroalcoholic extract (500 µg/mL); MIC.C: FLZ_{MIC} + $TcHE_{MIC}$ combination; S1: Synergistic concentration 1; S2: Synergistic concentration.



4 DISCUSSION

In addition to VVC remains one of the most frequent infections in women, (SOBEL, 2016) some reports have shown an association between excessive fluconazole exposure in unconfirmed episodes of acute *Candida* vaginitis as well as in recurrent VVC prolonged treatment with the resistance of isolates (EL-HOUSSAINI et al., 2018).

Thus, the development of alternative therapies to face up these infections is extremely relevant. Recently we have shown that the hydroalcoholic extract of *Terminalia catappa* and its fractions have excellent antifungal activity against oral *Candida* isolates (TERÇAS et al.; 2017).

Here, we describe not only the antifungal but also the antibiofilm activity of hydroalcoholic crude extract of *Terminalia catappa* leaves and its synergistic action with fluconazole against *Candida albicans* vaginal isolates. Besides, we also showed its use in the treatment of *Candida*-infected *Tenebrio molitor* larvae. Although some studies report the antibiofilm activities of other species of the genus this is the first one investigating the interference of *T. catappa* on *Candida* biofilm formation. Also, as far as we know, this is the first description of the effect of TcHE on the treatment of an alternative model of infection.

The crude extract has shown excellent antifungal activity against all strains at both agar diffusion and microdilution assays with MIC concentrations ranging between 62 and 1000 µg/mL. Among the strains tested, 22.2% were resistant to fluconazole, a drug of choice for candidiasis treatment in AIDS patients (SANTOS JR; 2005; SIIKALA et al; 2010; RAUTEMAA; RAMAGE, 2011; LOCKE, 2018). The increase in the empirical use of this drug in cases of VVC has increasingly promoted the emergence

of resistant isolates. The study by Marchaim et al. (2012), for instance, described 25 *C. albicans* vulvovaginitis cases with isolates showing acquired fluconazole resistance.

The antifungal effect shown by the crude extract is probably related to its chemical composition. In our previous work (TERÇAS et al, 2017) we showed that TcHE is rich in phenols, flavonoids, flavones and hydrosoluble tannins. Mininel et al. (2014) also verified that in the hydroalcoholic extract of *T. catappa*, tannins were the most abundant component observed, and the anomers α- and β-punicalagin were the major compounds.

Because fluconazole is fungistatic and sometimes not effective to candidiasis, besides the resistance showed by strains, this study had checked the combination effect between different concentrations of *T. catappa* extracts and FLZ. Most tested combinations had a synergistic antifungal effect against the isolates, which has considerably increased the anti-yeast activity in addition to decreasing the concentrations of both FLZ and the extract. This is relevant given the possibility of reducing the side effect of the drug. To the best of our knowledge, this is the first work to relate optimizing activities by coupling hydroalcoholic leaves extracts of *T. catappa* and fluconazole in combinations. This optimized action of the combinations is more likely due to components of the extract and FLZ acting synergistically against the pathogens (ONYEWU, 2003; NCUBE; FINNIE, 2012). Accordling, the combined action of compounds on specific targets could intensify their biological effect and, consequently, would reduce the expression of resistance by the isolates. Chanda et al. (2013) reported that methanolic extract of *Terminalia catappa* leaves showed synergistic action with amphotericin-B and nystatin against some fungi.

The kinetics of yeast time-kill in the presence of TcHE, alone or in synergistic concentrations was significant concerning the curve established in the control group in the absence of the extract ($p < 0.0001$). The differences observed among the time kill curves of the $TcHE_{MIC}$ ($p < 0.05$), S1 and S2 ($p < 0.001$) treatments were significant, confirming the effectiveness of the extract in relation to that of fluconazole. The study by Santos et al. (2012) somewhat corroborates our results because fluconazole, when used alone, did not show significant efficacy on *Candida* growth.

The effect of the extract alone or in combination appears to be associated with the destabilization of the fungal cell membrane as demonstrated by the uptake of propidium iodide (PI) in cells treated with various concentrations of TcHE. Thus, this finding indicates that the extract promoted cell membrane instability probably causing plasma membrane damage in *C. albicans* and the possible impairment of cell function. *T catappa* extract could cause a loss of membrane integrity, resulting in increased cell membrane permeability. Increased PI absorption in *Candida* cells is an indication that

extract compounds promote cell death, considering that this marker can bind only to the nuclear DNA of dead cells (XU et al., 2010).

The analysis of the integrity of the fungal lysosomal membrane also confirmed these data because TcHE induced permeabilization of the lysosomal membrane, which may have led to the release of enzymes and deregulation of cellular homeostasis causing the death of the fungus (KLIONSKY et al.; 1990; MESSINA JR.; HALABY; 2011).

We could assume that in synergistic combinations with FLZ, the extract would be able to increase cell permeability by facilitating the action of fluconazole in the fungal cell.

The toxicity of the extract was evaluated using the *Tenebrio molitor* alternative model and also through the hemolysis test. The results obtained here are pioneering in using *T. molitor* larvae as a model to define TcHE toxicity. The evaluated extract showed no toxicity for larvae with a survival rate greater than 90% ($p = 0.05$). In contact with human erythrocytes, TcHE did not cause hemolysis to the maximum tested concentration of 100 mg / mL, which also demonstrates the non-toxicity of the extract. In our first research with *T. catappa* (TERÇAS et al.; 2017) TcHE also showed no toxicity when tested in peripheral blood mononuclear cells, which corroborates the data from the present study.

T. molitor larvae infected with *C. albicans* and treated with TcHE in all tested concentrations showed 100% survival, which corroborates the efficiency of TcHE in combating the pathogen, even when in synergistic combinations with FLZ. This data was similar to that obtained with the group treated with FLZ (90% survival). Souza et al. (2015), when using *T. molitor* as an alternative model to study fungal infections, obtained similar results, however, the survival rate was 70%. Johnston et al. (2013) stated that *T. molitor* produces several antimicrobial peptides capable of acting against *Candida*. Thus, we suggest that *T. catappa* extract probably reinforces the larva's immunological action helping to eliminate the infection with its antifungal potential.

TcHE exhibited excellent anti-biofilm properties against *C. albicans* strains, significantly reducing the biofilm formation of the reference strain *C. albicans* 90028 and of the CaCV21 isolate at all concentrations. This reduction reaches more than 50% in sub-inhibitory concentrations (MIC / 4 for ATCC and MIC / 2 for CaCV21). FLZ did not affect biofilms.

Those findings are relevant because biofilms are frequently associated with reduced sensitivity to conventional antifungal agents and with the majority of *Candida* infections (TAFF et al.; 2013). Studies of antibiofilm activity of extracts or their compounds derived from plants of the Combretaceae family are scarce (TAGANNAA et al; 2011; DE ARAUJO et al.; 2015; SHUKLA; BHATHENA; 2015). Rare studies have documented the anti-biofilm activity of *T catappa* leaves extract

(MACHADO-GONÇALVES et al; 2018). Only some with methanol extracts or their constituents. Thus, TcHE may represent a valuable source for the development of novel anti-biofilm agents. In view of the results obtained here, one of the possibilities would be the prophylactic use of the extract against biofilms in early stages of formation.

5 CONCLUSION

In conclusion, we have demonstrated the antifungal and anti-biofilm properties of the *T. catappa* crude extract besides its effective action on *T. molitor* larvae infected with *Candida,* both alone or in synergistic combinations with fluconazole. We also observed that effective concentrations of TcHE were not cytotoxicity to human erythrocytes or *T. molitor* larvae. TcHE exhibited potential as a new therapeutic strategy to inhibit biofilm formation and we even believe that it can be used as a base for a protective film of surfaces with antibiofilm action. Within this context, and despite the limitations of this work, the antimycotic activity and the low cytotoxic potential demonstrated by TcHE reveal that it is a promising substance for development as a new antifungal.

CONFLICT OF INTEREST

Authors declare no competing and non-financial interest.

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