Abstract. The extensive use of azoles antifungals against infections caused by Candida species has been contributing to the selection of resistant strains to this antifungal class, demonstrating that the prospection of new antifungal agents is essential and urgent. Tripodanthus acutifolius (Loranthaceae) is a plant widely used in folk medicine with reported antimicrobial activity. In this context, this study aimed to evaluate the antifungal potential and the mechanisms of action of the crude methanolic extract (CME) of T. acutifolius leaves against Candida species, as well as evaluate their toxicity parameters. As results, the phytochemical characterization of CME suggested the presence of four phenolic compounds as well as a tripodantoside compound. The CME presented minimum inhibitory concentration (MIC) between 16 to 64 μg mL⁻¹, with antifungal action possibly occurring in the fungal cell wall. In addition, the CME showed a synergic effect in combination with fluconazole (FLC). The CME demonstrated no mucosal irritation or tissue damage at all tested concentrations, as well as no cytotoxicity at the MIC values. This study is unprecedented and suggests that T. acutifolius is a new promising source for the development of anti-Candida agents on its own or as chemosensitizer associated to FLC.

Keywords: antifungal agents, Candida spp., Tripodanthus acutifolius, synergism, fluconazole.
Introduction

_Candida_ spp. are human commensal yeasts that have emerged as one of the most significant causes of opportunistic fungal infections (Martins et al., 2015; Kumar et al., 2018). These microorganisms can cause local (such as superficial candidiasis, mucocutaneous and onychomycosis) or systemic infections (Arendrup & Patterson, 2014; Badiee & Hashemizadeh, 2014), thus being associated with prolonged periods of hospitalizations, high health care costs, and even with the high mortality rates (Vallabhaneni et al., 2015; Antinori et al., 2016). More than 100 _Candida_ species are described in the literature; however, approximately 15 are reported to cause local or systemic infections in humans (Uppuluri et al., 2017). Although _Candida albicans_ is the most prevalent species, the incidence of non- _C. albicans_ species that present resistance to antifungal agents, including fluconazole (FLC), has increased dramatically (Deorukhkar et al., 2014). This phenomenon may be associated with indiscriminate and excessive use of these antifungal agents, actions that markedly limit the therapeutic arsenal (O’Neill 2014; Arendrup & Patterson 2017).

Resistance acquired by microorganisms against the actual antibiotics makes fundamental and continuous the search for and development of new therapeutic agents (Treméa 2015). In this context, plants represent an important source for obtaining bioactive compounds. Once such organism, _Tripodanthus acutifolius_ (Ruiz and Pavón) Van Tieghem, is a hemiparasite plant that belongs to the Loranthaceae family; it is widely distributed in South America and frequently studied based on its uses in folk medicine (Silva, 2014). Several studies reported the _T. acutifolius_ antioxidant and gastroprotective (Silva, 2014), anti-inflammatory (Daud et al., 2006; Soberón et al., 2010a; Soberón et al., 2010b), antipyretic and antinociceptive (Daud et al., 2006) and diuretic activities (Intersimone et al., 2005). The majority and main reports concern the antimicrobial activity of this plant extract on bacteria, including bacteria resistant to multiple current antibiotics used clinically (Daud et al., 2005; Soberón et al., 2007; Soberón et al., 2010c; Silva, 2014; Souza et al., 2014). These biological activities are related to the main chemical constituents present in this plant, such as polyphenolic compounds, flavonoids (rutin, nicotiflorina, hyperoside and isoorientin), condensed tannins and catechins (catechin, epicatechin and catechin-4-β-o), besides a rare compound identified as a tripodantoside (Silva, 2014; Soberón et al., 2010c; Coelho et al., 2018). However, studies that involve the antifungal action of this extract are rare. Only one report suggests a synergistic action between the tripodantoside and amphotericin B, since this combination reduces the minimum inhibitory concentration (MIC) of this polyenic antifungal agent against _C. albicans_ and _Candida tropicalis_ (Soberón et al., 2011).

In the prospect of new antifungal agents, the association between plant extracts and commercial antifungals is outstanding and considered a promising strategy for antifungal therapy (Anand & Rai 2017). This association has been widely studied and suggested due to its improved effectiveness, since the different substances, when they reach distinct targets within the yeast cell, can potentiate the antifungal action and thus reverse resistance, optimise the therapy and minimise possible side effects (Wagner & Ulrich-Merzenich 2009; Pfaller et al., 2014; Pippi et al., 2015; Silva et al., 2020). Thus, the exposed context demonstrates the need for identifying new antifungal substances, as well as their interaction with antifungals used as therapeutics. Thus, the objective of this study was to evaluate in vitro antifungal activity of a crude methanolic extract (CME) obtained from _T. acutifolius_ leaves, evaluate its interaction with FLC, as well as to evaluate its interaction with FLC, suggesting their mechanism of action and toxicological parameters.

Methods

Plant material

_T. acutifolius_ leaves, with _Ligustrum lucidum_ (Oleaceae) as host, were collected in the city of Santa Cruz do Sul, Rio Grande do Sul, Brazil. The identified species is deposited in the herbarium of the botanic department of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil, under number ICN 167796. Access to the Brazilian genetic patrimony was informed by the National System for Management of Genetic Heritage and Associated Traditional Knowledge (SisGen), under number AAFC1F0. Only intact and healthy leaves were considered for collection. These leaves were washed in tap water and dried in a circulating air oven at 40°C for 48 h. They were then ground in a Willye-type knife mill, stored at room temperature in closed vials and protected from light for 48 h. The crude vegetable extract was obtained by utilising Soxhlet, an extraction technique that uses a solvent under high temperature to isolate and concentrate compounds from a solid vegetable sample (Castro & Ayuso, 2000), using methanol as the solvent at approximately 60°C for 4 h. Subsequently, the extracted liquid was concentrated in a rotary evaporator and then lyophilised to obtain the _T. acutifolius_ leaf CME.

Phytochemical characterisation

The phytochemical characterisation of _T. acutifolius_ CME was determined using high-performance liquid chromatography with detection by a diode arrangement (HPLC-DAD) according to Coelho et al. (2018), with modifications. The mobile phase consisted of a linear gradient system that contained ultrapure water and 1% phosphoric acid (v/v) (solution A; Fmaia, Minas Gerais, Brazil) as the organic phase. This gradient

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composition was 13% (v/v) of solution B up to 10 min, and changed to 20, 30, 50, 60, 70, 20 and 13% of solution B at 20, 30, 40, 50, 60, 70 and 80 min, respectively. The mobile phase flow rate was 0.7 mL min⁻¹, injection volume was 10 µL and the column furnace was set at 40°C. Gallic acid, catechin, rutin and quercetin (Sigma-Aldrich, Missouri, USA) were used as reference substances (RS). Concentrations used for reference RS and CME were 1 and 10 mg mL⁻¹, respectively. The spectra of each RS and CME were analysed by ultraviolet (UV) scanning, and a specific wavelength was subsequently selected for each RS: gallic acid (271 nm), catechin (280 nm), rutin (255 nm) and quercetin (371 nm). RS and CME were solubilised in methanol, filtered through a 0.45 μm pore size membrane and sonicated. They were then transferred to vials and inserted into the chromatograph. In order to verify the presence of the tripodantoside, the CME was fractionated by normal phase column chromatography using 230-400 mesh ASTM silica gel (Merck, Darmstadt, Germany) and a polarity gradient between the solvents hexane, chloroform and ethyl acetate (Neon, São Paulo, Brazil) and methanol. The obtained fractions were concentrated in a rotary evaporator and solubilized in methanol for further HPLC reading at a concentration of 1 mg mL⁻¹. After HPLC, only the best isolated fraction (with retention times different from the RS) were selected for infrared (IR) reading, since they potentially represented the compound of interest. This fraction was then analysed with an attenuated total reflection (ATR) accessory on a Perkin Elmer Spectrum 400 FTIR FT-NIR, in the range of 4000-650 cm⁻¹, eight scan pulses and 30 psi gauge pressure.

Preparation of T. acutifolius CME

Initially, a stock T. acutifolius CME solution was prepared at 51.2 mg mL⁻¹ and dissolved in dimethylsulfoxide (DMSO; LGC Biotechnology, São Paulo, Brazil). From this stock, a working solution at 1024 µg mL⁻¹ was prepared in RPMI 1640 broth (Gibco, Grand Island, NY, USA), at a maximum 2% DMSO. In parallel, a 1024 µg mL⁻¹ FLC (Sigma-Aldrich, St. Louis, MO, USA) solution was used as drug control.

Microorganisms

Ten yeast strains were used in this study: C. albicans (ATCC 18804 and CA 02), Candida glabrata (CG 09 and CG RL12m), Candida krusei (CK RL102 and CK RL52), Candida parapsilosis (CP RL38 and CP RL13m) and C. tropicalis (CT 57A and CT 94P), all of them from the mycology collection of yeasts of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. The strains were phenotypically identified through the Vitek Yeast Biochemical Card (BioMerieux Vitex, Missouri, USA).

Antifungal susceptibility testing.

Minimal Inhibitory Concentration (MIC) determination for the T. acutifolius CME against ten Candida spp. strains was performed through microdilution technique in broth, following the protocol M27-A3 (CLSI, 2008). The CME was evaluated from 1 to 512 µg mL⁻¹ and the reading of MIC values considered 100% inhibition of fungal growth. As a control, MIC values for FLC were determined, with the interpretation of breakpoints according to protocol M27-S4 (CLSI, 2012).

The in vitro interaction between the antifungal agents was determined through the Checkerboard Assay considering the following concentrations: MICx4, MICx2, MIC, MIC/2, MIC/4 and MIC/8. The assay was performed in quadruplicate, using each fungal inoculum at approximately 0.5 - 2.5 x 10⁻³ colony forming units (CFU) mL⁻¹. The microplates were incubated at 35°C for 48 h, with subsequent reading and determination of the Fraction Inhibitory Concentration Index (FICI). Interactions were considered synergistic when FICI < 0.5, indifferent when 0.5 > FICI < 4.0, and antagonistic when FICI ≥ 4.0. The assay followed Johnson et al. (2004), with modifications.

Mechanism of action assays.

The ergosterol binding assay consists of detecting whether the antifungal activity of an agent is attributed to its ability to bind to the ergosterol of fungal membrane; however, by providing exogenous ergosterol (Escalante et al., 2008). Thus, the MIC values for T. acutifolius CME were simultaneous determinate against five strains (ATCC 18804, C. albicans, C. tropicalis, C. parapsilosis and C. krusei), however in the absence and in the presence of exogenous ergosterol (Sigma-Aldrich, St. Louis, MO, USA), at concentrations between 50 to 250 µg mL⁻¹. Amphotericin B (AMB), an antifungal that is known to complex with the ergosterol of the fungal cell membranes, was used as a drug control. The microplates were incubated at 35°C for 48 hours. The assay was carried out according to Escalante et al. (2008), with modifications.

To evaluate the mechanism of action on the cell wall, the MIC values for T. acutifolius CME were simultaneous determinate against the same five strains (ATCC 18804, C. albicans, C. tropicalis, C. parapsilosis and C. krusei), however in the absence and in the presence of 0.8 M sorbitol (Sigma-Aldrich, St. Louis, MO, USA). As sorbitol acts as an osmotic protector of the fungal cell wall, if the CME possibly acts on the fungal cell wall, its MIC values will increase in the presence of this agent. Anidulafungin (AFG) was used as drug control, once it is an antifungal that is known to act on the fungal cell wall and, in the presence of sorbitol, its MIC values increase when compared to the MIC values without sorbitol. The microplates were incubated at 35°C and read at 48 h and after seven days. The assay was performed according to Escalante et al. (2008).

Toxicity parameters

The Hen’s Egg Chorioallantoic Membrane (HET-CAM) test was performed to evaluate the possible degree of T. acutifolius CME irritation on
mucous membranes since this test already showed an excellent correlation with in vivo test results. Fertile and fresh Lohmann (Lohmann Selected Leghorn, LSL) eggs were used and kept under optimised conditions of temperature (38-39°C) and humidity (55-60%) for 10 days. On the tenth day, the eggshell around the air space was carefully removed with the aid of a rotating tool (Dremel, WI) to expose the egg chorioallantoic membrane. After, 300 μL of CME at 8, 16, 32, 64 or 128 μg mL⁻¹ were added in each egg. A 0.1 mol⁻¹NaOH solution and 0.9% NaCl solution were used as a positive and negative control, respectively. Visual analysis of the possible effects caused by the extract was observed at 0.5, 2 and 5 min after its application. CME was considered non-irritant (or practically non-irritant) when the irritation score (IS) ranged from 0 to 4.9 and irritant when IS ranged from 5.0 to 21 (ICCCVMA 2010).

T. acutifolius CME dermal toxicity was evaluated by observing possible tissue damage. In this assay, pigs were slaughtered according to the rules of the Brazilian Ministry of Agriculture (Brasil, 2000), respecting animal welfare. Thus, skin samples from ears of adult male pigs, recently slaughtered, were added to a Franz cell diffuser (Logan Instrument Corp., NJ), with a diffusion area of approximately 1.75 cm². The epidermal side of the skin was exposed for 6 h to different CME concentrations (8, 16, 32, 65 or 128 μg mL⁻¹). A 0.1 mol⁻¹NaOH solution and PBS buffer (pH 7.0) were used as a positive and negative control, respectively. Tissue fragments were then collected, fixed with 10% neutral buffered formalin, routinely processed, stained with hematoxylin and eosin and examined with light microscopy. The experiments were performed in triplicate.

Finally, the cell viability was assessed by MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, MO, USA) assay (Mosmann, 1983) and carried out using a non-tumoral lineage of immortalised human keratinocytes (HaCaT), purchased from American Type Culture Collections (ATCC; Rockville, USA). Firstly, the cells were cultured in DMEM (Dulbecco’s Modified Eagle Medium; Gibco, California, USA) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C and 5% CO₂ (Freshney, 2005). When the cells reached approximately 80% confluency, an aliquot of cell suspension (5 x 10⁴ cells mL⁻¹) was seeded on the microplates and different T. acutifolius CME concentrations (8, 16, 32, 64 or 128 μg mL⁻¹) were added. After 24 h, 0.5 mg mL⁻¹ of MTT was added and the reading was performed at spectrophotometer (Molecular Devices, SpectraMax M-3) at 492 nm. The cytotoxic profile was classified as non-cytotoxic or potentially cytotoxic, according to ISO (2009), from comparison with the positive growth control (supplemented DMEM). A reduction of up to 30% in cell viability characterizes to CME as non-cytotoxic.

Statistical analysis. One-way analysis of variance (One-way ANOVA; Origin® 6.0 Working Model, Microcal™, Northampton, MA, USA) was used to evaluate the statistical differences between the antifungal activity of single agents and after CME-FLC combination. The differences were considered statistically significant when p < 0.05.

Results and discussion

The identification of compounds present in the T. acutifolius CME was performed by comparison between retention times (RT) and ultraviolet (UV) absorption maxima with the reference substances (RS), as well as spectra overlapping with these substances. Thus, through the high-performance liquid chromatography with detection by a diode arrangement (HPLC-DAD) analysis was possible to identify and prove the presence of four phenolic compounds: (a) gallic acid, 271 nm; (b) catechin, 280 nm; (c) rutin, 255 nm; and, (d) quercetin, 371 nm, as showed in Figure 1.

Besides the spectra overlapping and comparison of UV absorption maxima with the available reference substances, the presence of these metabolites are confirmed by the literature, once the same range of absorption was already described for the same compounds (Neilson et al., 2006; Pawar & Salunkhe 2013; Chaudhari et al., 2014). In addition, the presence of these compounds is in accordance with findings by Ricco et al. (2008), Wagner et al. (2009), Soberón et al. (2007; 2010a; 2010b; 2010c), Grüner et al. (2012), Silva (2014) and Coelho et al. (2018), all of whom also identify these four constituents in T. acutifolius extracts. Although the presence of other compounds was not shown in this study, the peak presented in the chromatogram with a 43.399 min RT suggests the presence of another flavonoid, since the UV absorption maxima for this peak were 255 and 354 nm. According to Gaitén et al. (2010), flavonoids, especially flavonols and flavones, have two UV/VIS absorption peaks: one band in the range of 250-285 nm and another in the 320-385 nm range.

Adding to the phytochemical analysis, the infrared (IR) spectroscopy, applied in the isolated fraction, identified the following IR bands: 3314.62 cm⁻¹ (assigned to νO-H); 2924.63 and 2854.87 cm⁻¹ (assigned to the C-H of CH₂ and CH₃); 1729.07 cm⁻¹ (assigned to νC=O); 1648.96 cm⁻¹ (assigned δC=C); 1603.55 cm⁻¹ (assigned to νC-C aromatic); 1409.70 and 1376.58 cm⁻¹ (assigned to δC=O); 1283.95 and 1260.27 cm⁻¹ (assigned to δO-H); 1154.72 cm⁻¹ (assigned to νH-C=O); 1083.28 cm⁻¹ (assigned to νO-C-H); 1051.00 cm⁻¹ (assigned to δC-OH) (Pretsch et al., 1989; Silverstein et al., 2007). Note that u means stretch or axial deformation and δ denotes angular deformation. These findings suggest that the methanolic fraction, obtained by normal phase column chromatography, corresponded to the tripodantoside, since it presented absorption bands that are compatible with
the bonds found in the isolated molecule (Soberón et al., 2010c) (Figure 2).

According to Seleem et al. (2017), vegetal compounds present a significant structural diversity and particularities in their mechanisms of action, which makes them attractive candidates to combating the fungal resistance in species of *Candida*. Several studies suggest that the antimicrobial activity of vegetal extracts is due to the flavonoids. These components are secondary metabolism of these plants and are considered constitutive antimicrobial ingredients (de Oliveira Filho et al., 2016).

![Figure 1. Chromatograms showing retention times, overlap with RS (pink) and UV absorption maximum for each compound identified in the CME of *T. acutifolius*: (a) gallic acid - retention time 7.027 min - absorption maximum UV 271 nm; (b) catechin - retention time 20.621 min - UV absorption maximum 280 nm; (c) rutin - retention time 46.597 min - UV absorption maximum 255 nm; and (d) quercetin - retention time 52.693 min - UV absorption maximum 371 nm. Reference substances at 1 mg mL$^{-1}$ and CME at 10 mg mL$^{-1}$. Analyzed by High-Performance Liquid Chromatography (HPLC) coupled to Diode Array Detector (DAD) SPD-M20A DAD. 5 μm C-18 Gemini column (250 x 4.66 mm). The mobile phase consisting of a linear gradient system with 1% phosphoric acid (solution A) and chromatographic grade methanol (solution B). Oven: 40 °C. Flow rate: 0.7 mL/min. Injection volume of 10 μL.]

![Figure 2. IR spectrum of methanolic fraction isolated from normal phase column chromatography, with respective absorption bands. In particular, the molecule corresponding to the tripodantoside.](image-url)
The antifungal susceptibility evaluation revealed that the T. acutifolius CME showed MIC values ranged from 16 to 64 μg mL⁻¹ for all tested Candida strains, with a geometric mean (GM) of 25.99 μg mL⁻¹, demonstrating more effectiveness against Candida glabrata (CG RL12m), Candida krusei (CK RL52) and Candida tropicalis (CT 94P), with MIC values of 16 μg mL⁻¹ when compared to FLC, which presented MIC values of 32, 32 and 64 μg mL⁻¹, respectively (Table 1).

There is no consensus on the acceptable level that represents significant antimicrobial activity for plant substances (Mesa-Arango et al., 2009). However, Zida et al. (2016) report that phytochemical in vitro biological activity can be considered significant if MIC values are below 100 μg mL⁻¹ for crude extract, or moderate when MIC values range from 100 to 625 μg mL⁻¹. Thus, since FLC is the first drug of choice for the treatment of several types of candidiasis (Eschenauer et al., 2015), the CME becomes a promising compound for the development of a new antifungal. It is important to note, that the effectiveness of the antifungal action of crude plant extracts can be attributed to the synergism between their constituents, since minor bioactive components can contribute to the antifungal effect, due to the sum of mechanisms (Simonetti et al., 2016).

The CME-FLC combination, evaluated through the checkerboard assay, demonstrated an indifferent interaction against C. albicans, C. krusei, and C. parapsilosis (≥ FICI < 4.0); on the other hand, the combination produced a synergistic action (FICI ≤ 0.5) against the two species most commonly reported in cases of resistance toazole derivatives, C. glabrata, and C. tropicalis (Table 1); thus, representing an important alternative for reversion of this scenario. These results suggest some CME-FLC combined mechanisms of action.

Generally, flavonoids display a relevant anti-Candida activity (Seleem et al., 2017) and a synergistic effect with FLC (Lu et al., 2016). Flavonoid and FLC interaction occur in FLC-resistant C. tropicalis strains, actions that promote DNA damage and cellular apoptosis (Silva et al., 2014). Machado et al. (2016) reported a synergistic interaction between FLC and an Acca sellowiana (Myrtaceae) crude extract fraction, which contained catechin and flavonoids, against C. glabrata; they also observed reversion of FLC resistance. Wang et al. (2016) demonstrated a synergistic interaction between a group of synthesized flavonoids and fluconazole against fluconazole-resistant C. albicans.

The checkerboard assay also yielded intriguing results, since this association reduced the MIC values required by CME when compared to its isolated assessment for most strains; and the CME-FLC combination also reduced the MIC values previously required for FLC separately for all tested strain (Table 1). Moreover, CA 02, CT 57A and CT 94P strains, described as FLC resistant, were susceptible to the CME-FLC combination, thus reversing their resistance profiles. Through one-way ANOVA, with 95% confidence interval (p ≤ 0.05), there was a significant difference between the MIC values obtained only by FLC and after combination with CME (p = 0.02744); likewise, there was a significant difference between MIC values obtained only by CME and after combination with FLC (p = 0.01212). There was no significant difference in MIC values among substances tested separately (p = 0.30). These findings suggest that this vegetal extract confers better anti-Candida efficacy to FLC.

Pfaller et al. (2014) point out that the interaction occurs when two compounds are able to reach microbial cells on two or more different targets, improving the effectiveness of the therapy. In this way, it can be suggested that the FLC-CME combination led to a more efficient antifungal response due to the fact that the substances reached different targets in the tested strains; where fluconazole acts on the biosynthesis of ergosterol present in the cell membrane of cells (Whaley et al., 2017), and the T. acutifolius CME, as demonstrated by the sorbitol assay, acts at the cell wall level. The association between conventional antifungal agents and natural products is an alternative approach to circumvent the limitations of antifungal monotherapy; and it is justified by reducing the doses previously used by both agents, where the different mechanisms of action complement each other, thus obtaining better results against strains with a resistance profile (Wagner & Ulrich-Merzenich, 2009; Pfaller et al., 2014; Pippi et al., 2015; Silva et al., 2020; Meirelles et al., 2017).

Regarding the mechanisms of actions, the MIC values of T. acutifolius CME did not demonstrate differences in the presence of increasing concentrations of Exogenous Ergosterol when compared to the evaluation of MIC values in the absence of this agent against all Candida strains. This fact suggests that the CME does not form a complex with ergosterol present in the fungal cells and, therefore, the mechanism of action is not over the yeast membrane. The Sorbitol Assay, on the other hand, showing an increase in the MICs values for all tested strains, as well as for Anidulafungin (AFG), the drug control. As showed in Table 2, after 7 days of incubation, the MIC values for T. acutifolius CME, in the presence of Sorbitol, ranged from 16 to 512 μg mL⁻¹, thus suggesting that the CME possibly presents a strong action on the yeast cell wall.
This antimicrobial activity can be related to the several mechanisms of action of the metabolic constituents also identified on the *T. acutifolius* CME, like flavonoids, which include damage on the plasmatic membrane, induction of dysfunctions on mitochondria and on efflux-mediated pumping, interferences in cell division and in the synthesis of RNA and protein as well as an inhibition on the formation of the cell wall (Aboody & Hickmynaray, 2020). Machado et al. (2016) suggest that the acetone-ethanol fraction of an aqueous extract of *Acca sellowiana* leaves which contains flavonoid compounds possibly acts on the *Candida* cell wall. Lee & Kim (2017) suggest that the ethanolic extract of *Paenia lactiflora* containing flavonoids induces sustained damage to the cell wall of *C. albicans*, with extensive changes in osmotic pressure inside the cell. A flavonoid, extracted from *Praxelis clamatidea*, demonstrated significant antifungal activity against *C. albicans*, with a probable mechanism of action involving interactions on the cell wall of this microorganism (de Oliveira Filho et al., 2016). Glabridin, a flavonoid isolated from *Glycyrrhiza glabra*, had significant antifungal activity against *C. albicans*, *C. tropicalis*, *C. neoformans*, and *C. glabrata* demonstrating deformation of the cell wall and a decrease in cell size (Liu et al., 2014). In this context, we suggested that the anti-*Candida* activity shown by *T. acutifolius* CME could be attributed to the secondary metabolism constituents of this plant species, which possibly act on the yeast cell wall, as demonstrated by the sorbitol assay.

Fungi are eukaryotic organisms and present many biochemical and physiological similarities as well as extensive gene and protein homology to humans, features that limit the antifungal therapeutic arsenal and hinder the development of low-toxicity drugs (Areal, 2015). Thus, in order to evaluate whether CME possesses limited toxicity, as well as its promising character as an anti-*Candida* agent, this study evaluated different toxicity parameters for this extract.

### Table 1. MIC values obtained for *T. acutifolius* CME and FLC, when used separately and in combination, against strains of *Candida* spp.

| Species | Strain          | MIC (µg mL⁻¹) | MIC combination (µg mL⁻¹) | FICI | Type of interaction |
|---------|-----------------|---------------|----------------------------|------|---------------------|
|         |                 | FLC | CME | FLC | CME |               |
| *C. albicans* | ATCC 18804 | 2  | 32 | 1  | 16 | 1 | Ind |
|          | CA 02 | 8  | 32 | 1  | 32 | 1.125 | Ind |
| *C. glabrata* | CG 05 | 32 | 64 | 8  | 4  | 0.3125 | Syn |
|          | CG RL12m | 32 | 16 | 8  | 4  | 0.5 | Syn |
| *C. krusei* | CK RL102 | 2  | 32 | 1  | 16 | 0.5625 | Ind |
|          | CK RL52 | 32 | 16 | 16 | 4  | 0.75 | Ind |
| *C. parapsilosis* | CP RL38 | 2  | 32 | 0.5 | 32 | 1.25 | Ind |
|          | CP RL13m | 16 | 16 | 8  | 8  | 1  | Ind |
| *C. tropicalis* | CT 57A | 16 | 32 | 4  | 4  | 0.375 | Syn |
|          | CT 94P | 64 | 16 | 4  | 4  | 0.3125 | Syn |

Geometric Mean: 11.31, 25.99, 3.70, 8.57

Abbreviations - S: sensitive; SDD: susceptible dose-dependent; R: resistant; MIC: minimum inhibitory concentration; FLC: fluconazole; CME: crude methanolic extract of *T. acutifolius*; FICI: fractional inhibitory concentration index; Ind: indifferent; Syn: synergism.

### Table 2. MICs values (µg mL⁻¹) obtained for *T. acutifolius* CME and AFG, in the presence and absence of sorbitol.

| Time | Strain          | CME | AFG | CME | AFG |
|------|-----------------|-----|-----|-----|-----|
|      |                 | 48 h| 7 days |      |      |
|      |                 | S(−) | S(+) | S(−) | S(+) | S(−) | S(+) | S(−) | S(+) |
| ATCC 18804 | 32 | 0.25 | 0.25 | 32 | 512 | 0.25 | 2 |
| CA 02 | 32 | 0.5 | 0.5 | 32 | >512 | 0.5 | 4 |
| CG RL12m | 16 | 0.125 | 0.125 | 16 | 256 | 0.125 | 0.5 |
| CK RL52 | 16 | 0.25 | 0.25 | 16 | 256 | 0.25 | 1 |
| CT 57A | 32 | 0.125 | 0.125 | 32 | 512 | 0.125 | 0.5 |

Abbreviations - MIC: minimal inhibitory concentration; CME: crude methanolic extract of *T. acutifolius*; AFG: anidulafungin (positive control); S(−): absence of sorbitol in the test; S(+): presence of sorbitol in the test; CA: *Candida albicans* (ATCC 18804 e CA02); CG: *Candida glabrata* (CG RL12m); CK: *Candida krusei* (CK RL52); CT: *Candida tropicalis* (CT 57A).
Membrane (HET-CAM) assay, performed to evaluate the degree of irritation on mucous membranes, demonstrated that the irritation score (IS) was lower than 5.0 for all evaluated concentrations. These results characterized the *T. acutifolius* CME as non-irritant (or practically non-irritant). Figure 3 shows the relationship between IS (y-axis) and the logarithm of the plant extract and control concentrations (x-axis). In histopathological test for the assessment of dermal toxicity, the *T. acutifolius* CME did not cause microscopic tissue lesions on swine ear skin cells compared to skin treated with phosphate-buffered saline (PBS; pH 7.0, negative control) at all tested concentrations (Figure 4). And, finally, the evaluation of cytotoxicity for each *T. acutifolius* CME concentration, performed through the MTT assay and quantified through cell viability percentage which related absorbance values with the metabolic activity of cells, showed cell viability percentages were less than 70% when CME was evaluated at 32, 64 and 128 μg mL\(^{-1}\), indicating a cytotoxic potential. On the other hand, CME presented above 70% cell viability at concentrations between 8 to 16 μg mL\(^{-1}\), equivalent to their MIC values, indicating the absence of potential cytotoxicity (Figure 5). According to Silva (2014), only concentrations above 250 μg mL\(^{-1}\) of a *T. acutifolius* aqueous extract are cytotoxic to fibroblasts. Such results were expected, since *T. acutifolius* is widely used in folk medicine, where the low toxicological profile can be explained by the presence of flavonoids, considered highly bioactive and low-toxicity compounds, features that make them good therapeutic candidates (Prasain & Barnes, 2014).

**Figure 3.** Dose-response relationship of *T. acutifolius* CME for mucosal irritation (HET-CAM) test: (■) CME at 8 μg mL\(^{-1}\); (♦) CME at 16 μg mL\(^{-1}\); (+) CME at 32 μg mL\(^{-1}\); (Δ) CME at 64 μg mL\(^{-1}\); (◊) CME at 128 μg mL\(^{-1}\); (●) negative control (0.9% NaCl); and, (○) positive control (0.1 mol \(^{-1}\)NaOH). Each point represents one experiment (n = 3 eggs).
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Figure 4. Histopathological evaluation of swine cells treated with T. acutifolius CME and negative control at 100 and 400 times magnification.

Figure 5. Dose-response relationship of T. acutifolius CME in the evaluation of HaCaT cell viability for 24 h: (■) CME at 8 μg mL⁻¹; (♦) CME at 16 μg mL⁻¹; (+) CME at 32 μg mL⁻¹; (Δ) CME at 64 μg mL⁻¹; (◊) CME at 128 μg mL⁻¹; (●) diluent control (DMSO); (○) positive control (DMEM). Each point represents the mean of the triplicates of two independent assays.

Conclusion
The strong antifungal potential and the synergistic interaction with FCL, together with low toxicity, makes the plant extract of T. acutifolius potential candidate for the development of a new antifungal agent, on its own or as chemosensitiser for a FLC antifungal. In addition, CME-FCL combination may be used as alternative treatment against fungal infections caused by Candida species already resistant to FCL.

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