Chemical composition, antifungal activity and potential anti-virulence evaluation of the *Eugenia uniflora* essential oil against *Candida* spp.

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ABSTRACT

The development of fungal resistance to antifungal drugs has been worsening over the years and as a result research on new antifungal agents derived from plants has intensified. *Eugenia uniflora* L. (pitanga) has been studied for its various biological actions. In this study the chemical composition and antifungal effects of the *E. uniflora* essential oil (EULEO) were investigated against *Candida albicans* (CA), *Candida krusei* (CK) and *Candida tropicalis* (CT) standard strains. The essential oil obtained through hydro-distillation was analyzed by gas chromatography coupled to mass spectrometry (GC-MS). To determine the IC_{50} of the oil, the cellular viability curve and the inhibitory effects were measured by means of the oil's association with Fluconazole in a broth microdilution assay with spectrophotometric readings. The Minimum Fungicidal Concentration (MFC) was determined by solid medium subculture with the aid of a guide plate while the assays used to verify morphological changes emerging from the action of the fractions were performed in microculture chambers at concentrations based on the microdilution. Two major oil constituents stand out from the chemical analysis: selina-1,3,7(11)-trien-8-one (36.37%) and selina-1,3,7(11)-trien-8-one epoxide (27.32%). The concentration that reduced microorganismal growth was $\geq 8{,}192\,\mu g/mL$ while the IC_{50} varied, this being between 1892.47 and 12491.80 µg/mL (oil), 10.07 – 80.78 µg/mL (fluconazole) and 18.53 – 295.60 µg/mL (fluconazole + oil). The combined activity (fluconazole + oil) resulted in indifference and antagonism. A MFC of the oil in association with fluconazole was recorded at the concentration of 8,192 µg/mL against CA and CK. The oil caused the inhibition of CA and CT morphological transition. In view of the results obtained, additional research is needed to elucidate the activity of the *E. uniflora* oil over genetic and biochemical processes regarding its effect on *Candida* spp. virulence.

1. Introduction

Fungi from the *Candida* genus are very common as they are part of the normal human microbiota and can be isolated in the most diverse anatomical sites; in the mouth, for example, around 20 to 50% of healthy teeth are colonized by *Candida* spp. These are yeast fungi whose most well-known species, *Candida albicans*, is usually associated with

pathological conditions, however, other species such as *C. tropicalis*, *C. glabrata* and *C. krusei*, are frequently identified (Zomorodian et al., 2011).

Fungal infections caused by opportunistic pathogens have become increasingly frequent, especially as a result of the relentless use of antimicrobial therapies and an increase in the number of immunocompromised patients. While some *Candida* species form part of

the natural human microbiota, these can also become pathogenic, often in association with virulence factors from the microorganism itself, due to immunological conditions of the individual (Chaves, Santos & Colombo, 2012). Among the main virulence factors associated with the *Candida* genus, are: epithelial cell adhesion; hydrolytic enzyme secretions such as proteases, phospholipases and hemolysins; biofilm formation; phagocytic cell evasion and transitioning between yeast and hyphal states (Chaves et al., 2013).

The choice of initial antimycotic agent for the treatment of a suspected invasive *Candida* infection is also difficult. The development of resistance to antifungal agents used in *Candida* treatment is a situation which has been worsening. Prophylaxis with fluconazole in the last two decades is suggested to have led to an increase in non-*albicans* species prevalence with a reduced susceptibility to this drug (Perfect, 2004).

In this sense, by utilizing the rich Brazilian flora, research for new antifungal agents derived from plant extracts and essential oils has intensified due to the relatively small arsenal of drugs available for the treatment of mycoses, mainly due to the problem involving the resistance of strains to these (Eddouzi et al., 2013).

Eugenia uniflora L., popularly known as pitanga, is an arboreous plant from the Myrtaceae family which is widely distributed in Brazil and other South American countries. Several plant phytoconstituents of this plant have been studied, such as flavonoids, mono and triterpenoid compounds, tannins, anthraquinones, cineol and essential oils, the results of which proposed that in addition to presenting antimicrobial activity, the species presents several biological actions, such as antioxidant, antihypertensive and diuretic actions (Amorim, Lima, Hovell, Miranda, & Rezende, 2009).

The objective of this study was to investigate the chemical composition and *in vitro* effect of the *Eugenia uniflora* essential oil, both alone and in combination with fluconazole, on the growth inhibition and drug action potentiation against *Candida* spp, as well as to verify the inhibition of fungal pleomorphism, a virulence factor of yeasts.

The association of the *E. uniflora* essential oil at a subinhibitory concentration with fluconazole to verify the modulating effect is unprecedented.

2. Materials and methods

2.1. Collection area

The plant material was collected from an urban area in the municipality of Crato, south of Ceará, Brazil (317 m, 07°,14′,08.4″ South latitude and 39°,24′,57″ West of Greenwich). The collection period included January, February, March and April. The samples were collected between 08:30 and 10:30 in the morning. From the collected material, an exsiccate was prepared which was later identified and deposited in the Caririense Dárdano de Andrade-Lima Herbarium (HCDAL) of the Regional University of Cariri – URCA, registered under the number 13099.

2.2. Plant material and essential oil preparation

The study was carried out using young and healthy *E. uniflora* leaves, locally known as pitanga, without any indication of parasitic contamination which were later washed and cut into small pieces. To obtain the oils, the methodology by Gottlieb (1960) was followed where the leaves were separated and placed in a 5 L glass flask, this being used to extract 221.7 kg of foliar mass, followed by the addition of approximately 3 L of distilled water, which was heated for 2 h in a Clevenger-type equipment. The water/oil mixture obtained was separated, treated with anhydrous sodium sulfate and subsequently filtered. The *Eugenia uniflora* Leaf Essential Oil (EULEO) obtained by distillation was kept under refrigeration and protected from light until the time of microbiological assays.

2.3. Gas chromatography coupled to mass spectrometry (GC-MS) – determination of volatile essential oils

GC-MS analysis was performed on an Agilent instrument, model GC-7890B/MSD-5977A (quadrupole), with a 70 eV electron impact, HP-5MS methylpolysiloxane column (30 m × 0.25 mm × 0.25 μm, Agilent), helium carrier gas with a 1.00 mL min^{-1} (8.8 psi) flow rate and 36.8 cm s^{-1} constant linear velocity, 250 °C injector temperature, 150 °C detector temperature, 280 °C transfer line temperature. Chromatographic oven programming included: 70 °C initial temperature with a 4 °C min^{-1} heating ramp up to 180 °C and an additional 10 °C/min up to 250 °C at the end of the run (34.5 min). Compound identification was performed by analyzing the fragmentation patterns exhibited in the mass spectra with those present in the database provided by the equipment (NIST version 2.0 of 2012-243893 compounds) and literature data.

2.4. Antifungal assays

2.4.1. Strains and culture media

The strains used were of a standard type and obtained from the Oswaldo Cruz Culture Collection (FIOCRUZ) of the Instituto Nacional de Controle de Qualidade em Saúde (INCQS), namely CA INCQS 40006, CT INCQS 40042 and CK INCQS 40095. The strains were inoculated in Sabouraud Dextrose Agar (SDA, KASVI) and incubated for 24 h at 37 °C. Subsequently, small aliquots of the yeast were transferred to test tubes, each containing 3 mL of sterile saline (0.9%). The inoculum concentration was standardized by comparison to the 0.5 McFarland scale (NCCLS, 2002). Sabouraud Dextrose broth (SDB, HIMEDIA), doubly concentrated, was used in the microdilution assay. In the micromorphological analysis, Potato Dextrose Agar (PDA) depleted with bacteriological agar was used.

2.4.2. Drugs, reagents and solution preparation

Dimethyl sulfoxide (DMSO, Merck, Germany) was used to dilute the oil, while the antifungal fluconazole (Capsule; Prati donaduzzi, Brazil) was diluted in water and used as a reference drug. The product solution was prepared by weighing 0.15 g of the oil and diluting this in 1 mL of DMSO. To obtain the desired concentration for the test, the oil was further diluted in sterile distilled water (16,384 μg/mL) such that the DMSO concentration did not exert any activity on the tested cells (Stoppa et al., 2009).

2.4.3. IC$_{50}$ determination and cellular viability curve

This assay was performed using the broth microdilution method in 96-well plates. Each well was filled with 100 μL of CSD containing 10% fungal inoculum and either 100 μL of the natural product (16,384 μg/mL) or fluconazole (antifungal reference), in the same concentration to the first well, followed by serial dilution. Concentrations in the wells ranged from 8 to 8192 μg/mL. The last well contained no product or drug and served as a normal growth control (Javadpour et al., 1996). Controls were also prepared for the product diluents (with 0.9% Sodium Chloride solution instead of the inoculum) and the sterile medium. All the tests were performed in quadruplicates. The plates were incubated at 37 °C for 24 h and subsequently read on an ELISA spectrophotometer (Thermoplate®) with a 630 nm wavelength. The results obtained in the ELISA reading were used to construct the cell viability curve and to determine the IC$_{50}$ of the *E. uniflora* essential oil.

2.4.4. Determination of the minimum fungicide concentration (MFC)

For this assay the tip of a sterile rod was inserted into each well of the previously tested plate (except for the sterility control). After mixing the medium in each well, the rod was taken to a Petri dish containing SDA with the aid of a guide plate fixed at the bottom of the plate for yeast subculture and cell viability checks. After 24 h of incubation, the plates were inspected for any formation of *Candida*

colonies (Ernst Klepser, Ernst, Messer, & Pfaller, 1999, with modifications). The concentration in which there was no fungal colony growth was considered the essential oil MFC.

2.4.5. Evaluation of the essential oil's modifying effect over fluconazole action

Initially, the intrinsic action of the oil and fluconazole on yeast growth was verified, whereby, in this assay the effect of the combination of the oil and the reference drug was evaluated to determine whether or not the antifungal action was potentiated by the oil. For this, the oil was used at a sub-inhibitory concentration (MFC/16), according to the methodology used by Coutinho, Costa, and Siqueira-Júnior (2008) with minor modifications. If the oil potentiated the action of the antifungal drug, the verified effect would be considered of a synergistic type, if the effect hindered the performance of the drug, an antagonistic effect would be verified. The plates were filled with 100 μL of medium + inoculum + oil followed by microdilution with 100 μL fluconazole at the concentration of 16,384 μg/mL. The mixture was added to the first well of each column to undergo serial dilutions at concentrations ranging from 8192 to 8 μg/mL. The last well was intended as a fungal growth control. Dilution controls for the essential oil (where saline substituted the inoculum) and medium sterility controls were performed. The IC_{50} of fluconazole was also determined for comparative purposes while a dilution control was also required. The plates were incubated at 37 °C for 24 h. The reading was performed on an ELISA spectrophotometric apparatus (Thermoplate®).

2.4.6. Effect of natural products on fungal morphology

To determine if the natural product caused any changes in fungal morphology through the inhibition of hyphal development, yeast microculture chambers were prepared. Three milliliters of PDA medium depleted by dilution were added to the chambers containing the following essential oil concentrations: Superior Concentration Assayed – SCA (8192 μg/mL), SCA/4 and SCA/16. Subculture aliquots were removed from the Petri dishes to make two parallel streaks on the solid medium (PDA) which were then covered with a sterile cover slip. The chambers were placed in an incubator for 24 h (37 °C), inspected and the results recorded using an optical light microscope (AXIO IMAGER M2-3525001980-ZEISS-Germany) using a 40× objective. A control for yeast growth (hyphae stimulated by means of exhaustion) was carried out, as well as a control with the conventional antifungal fluconazole for comparative purposes. The assays were performed according to Sidrin and Rocha (2010) and Mendes (2011), with some modifications.

2.5. Statistical analysis

The data obtained were checked for their normal distribution and analyzed using a *Two-Way* ANOVA ($P < 0.05$; $^*P < 0.1$; $^{****}P < 0.0001$) comparing the values for each concentration of the extract, point by point, with Bonferroni's *post hoc* test. The IC_{50} values were obtained by non-linear regression analysis with interpolation of the standard curve unknowns obtained from fungal growth assays as a function of the extract concentration and expressed in μg/mL.

For statistical analysis, the software GraphPad Prism, v. 5.0 was used.

3. Results and discussion

3.1. Chemical analysis

Table 1 shows the composition of the essential oil evaluated, determined by gas chromatography (GC/MS). In this study, we identified 09 compounds, representing 87.71% of the total composition. Of the components identified, two stand out: selina-1,3,7(11)-trien-8-one (36.37%) and selina-1,3,7(11)-trien-8-one epoxide (27.32%). The chemical composition identified partially corroborates with other studies

Table 1
Chemical composition of the *Eugenia uniflora* essential oil.

Compounds	KI calc	KI lit	Relative area %
ß-Elemene	1395	1390	3.18
Caryophyllene	1424	1419	3.16
ᴅ-Germacrene	1485	1481	2.12
Bicyclogermacrene	1499	1500	4.76
α-Bulnesene	1508	1509	1.34
Germacrene B	1563	1561	7.95
Selina-1,3,7(11)-trien-8-one	1642	1634	36.37
Germacrone	1699	1693	1.51
selina-1,3,7(11)-trien-8-one epoxide	1755	1748	27.32

Relative area: compound percentages were obtained electronically from the GC-MS percent area data. KI calc (Kovats Index calculated) was calculated from the GC-MS chromatograms, KI lit (Kovats Index literature) calculated using Adams data.

that describe the chemical composition of the *E. uniflora* essential oil (Weyerstahl, Marschall-Weyerstahl, Christiansen, Oguntimein, & Adeoye, 1988; Kanazawa & Greene, 2000; Amorim, Lima, Hovell, Miranda, & Rezende, 2009; Costa et al., 2010; Oliveira, Lopes, Cabral, & Eberlin, 2006; Victoria et al., 2012).

The existence of germacrene and selina-1,3,7-trien-8-one components have been reported as the cause of the *E. uniflora* essential oil antifungal action (Costa et al., 2010). In the study by Victoria et al. (2012) on the *E. uniflora* essential oil antifungal activity against *Candida* spp. and its chemical composition, the study presented germacrene and seline-1,3,7-(11)-trien-8-one oxide as the oil's main constituents, constituents also found in our work.

3.2. Antifungal activity

In the assays evaluating the cellular viability of *C. albicans* when exposed to the *Eugenia uniflora* essential oil, the natural product was only able to reduce the fungal cell numbers at high concentrations, higher than 4096 μg/mL. The Table 2 shows IC_{50} values the evaluated product. This result contradicts the study by Victoria et al. (2012) where their results using the same fungus and essential oil were 208.3 ± 72.1 μg/mL. It is noteworthy that in the present study, for greater result reliability, the assay results relied on the sensitivity of the ELISA spectrophotometric reading method.

In another study with the same species, however using the leaf hydroalcoholic extract, an effective concentration of 500 μg/mL was obtained for the cellular viability evaluation (Auricchio, Bugno, Barros, & Bacchi, 2007). The results obtained from the combination of the essential oil with fluconazole showed a similar behavior to the isolated use of fluconazole, which can be observed by the decay of the curve in the graph. Costa et al. (2015) also evaluated the interaction of this species with the antifungal fluconazole and the hydroalcoholic extract where they verified a synergistic effect demonstrated through the FIC index (Fractional Inhibitory Concentration). In this same study, Minimum Inhibitory Concentration (MIC) results of 125 μg/mL for *C. albicans* and 31.2 μg/mL for *C. tropicalis* were obtained.

Table 2
IC_{50} (μg/mL) of the *Eugenia uniflora* Essential Oil (EOLEU) against species from the *Candida* genus.

Products Tested	Strains		
	CA INCQS 40006	CT INCQS 40042	CK INCQS 40095
Fluconazole (FCZ)	10.07	30.05	80.78
EOLEU	1892.47	4511.82	12491.80
EOLEU + FCZ	18.53	24.20	295.60

CA: *Candida albicans*; CT: *Candida tropicalis*; CK: *Candida krusei*; INCQS: Instituto Nacional de Controle de Qualidade em Saúde.
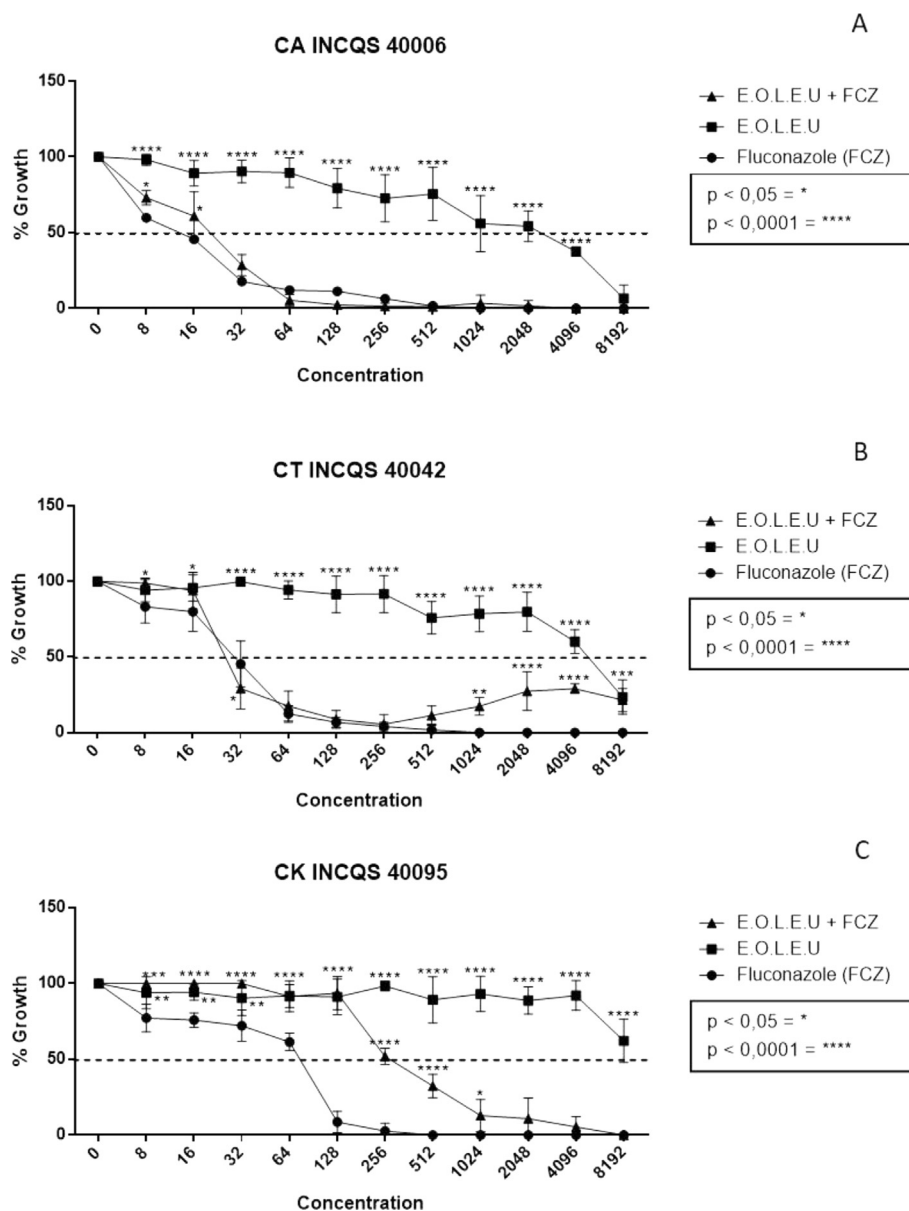
Fig. 1. Cell viability curve of the *Eugenia uniflora* essential oil (μg/mL) and Fluconazole versus *Candida* spp. A. *Candida albicans* (CA); B. *Candida tropicalis* (CT); *Candida krusei* (CK); *Eugenia uniflora* Leaf Essential Oil (E.O.L.E.U) and fluconazole (FCZ).

The results against *C. tropicalis* (Fig. 1B) were similar to those obtained with *C. albicans* where the essential oil under study also showed inhibition only at high concentrations, achieving a 50% reduction in fungal growth only at the 4,096 μg/mL concentration. The decay of the curve was similar for fluconazole as well as for its association with the essential oil, only distancing themselves at 2,048 μg/mL where fluconazole showed a more significant effect.

In the study by Castro and Lima (2011), the *E. uniflora* essential oil antifungal potential against five *C. albicans* and six *C. tropicalis* strains was also evaluated. The results showed a weak antifungal action as the formation of inhibition halos was observed for only one *C. albicans* strain using the disc diffusion method, results which corroborates with the data obtained in this work. Santos et al. (2013) evaluated the antifungal and modulatory activity of the *E. uniflora* ethanolic extract against *C. tropicalis* observing a MIC of 1,024 μg/mL, which is also a clinically irrelevant result. However, in the same study, when the natural product was combined with the antifungal metronidazole, a decrease in the MIC of this drug was observed, a result which differs from the present study since the association of the essential oil with

fluconazole did not present statistically significant data.

In the experiments against *C. krusei* (Fig. 1C), the essential oil presented high inhibition values, demonstrating that approximately 50% fungal growth reduction was observed only at 8,192 μg/mL concentrations. The use of fluconazole alone demonstrated a considerable effect at the 128 μg/mL concentration. On the other hand, the modulating effect was similar to that of fluconazole at a concentration of 512 μg/mL. Thus, the use of the *Eugenia uniflora* essential oil in combination with the commercial antifungal fluconazole, requires a higher concentration of this drug to reach similar values to its use in isolation.

When comparing the literature results to those obtained in this study, the essential oil analyzed in this study did not show promising antifungal activity and resistance modulation effects as demonstrated by other authors. The oil composition may have directly interfered with this species' biological action, in view of the contradictory results obtained. However, such results are important to scientifically support the notion that natural product effects in popular medicine can suffer several changes due to several factors, thus presenting different actions.

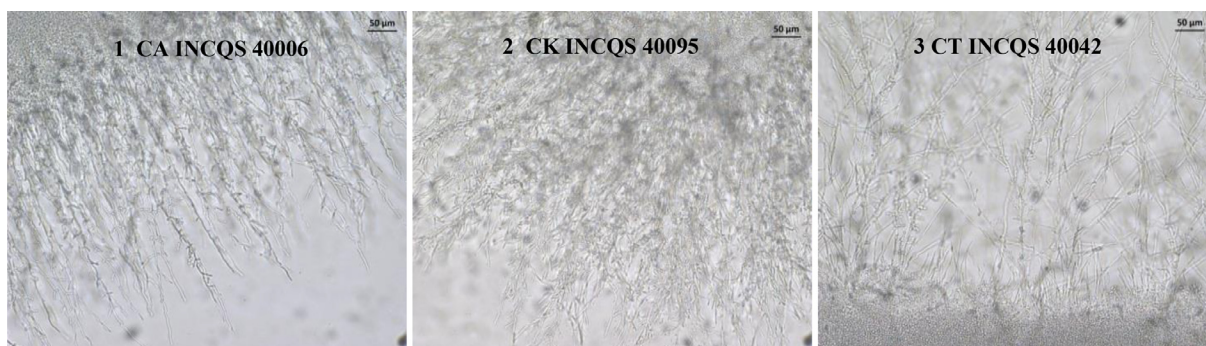In order to verify the effect of the oil on the morphology of yeasts,

Fig. 2. Controls used in the micromorphological *Candida* yeast assays. CA: *Candida albicans*, CK: *Candida krusei*, CT: *Candida tropicalis*; INCQS: Instituto Nacional de Controle de Qualidade em Saúde. In 1, 2 and 3 - Lineage growth control. Visualisation using a 40× objective view.
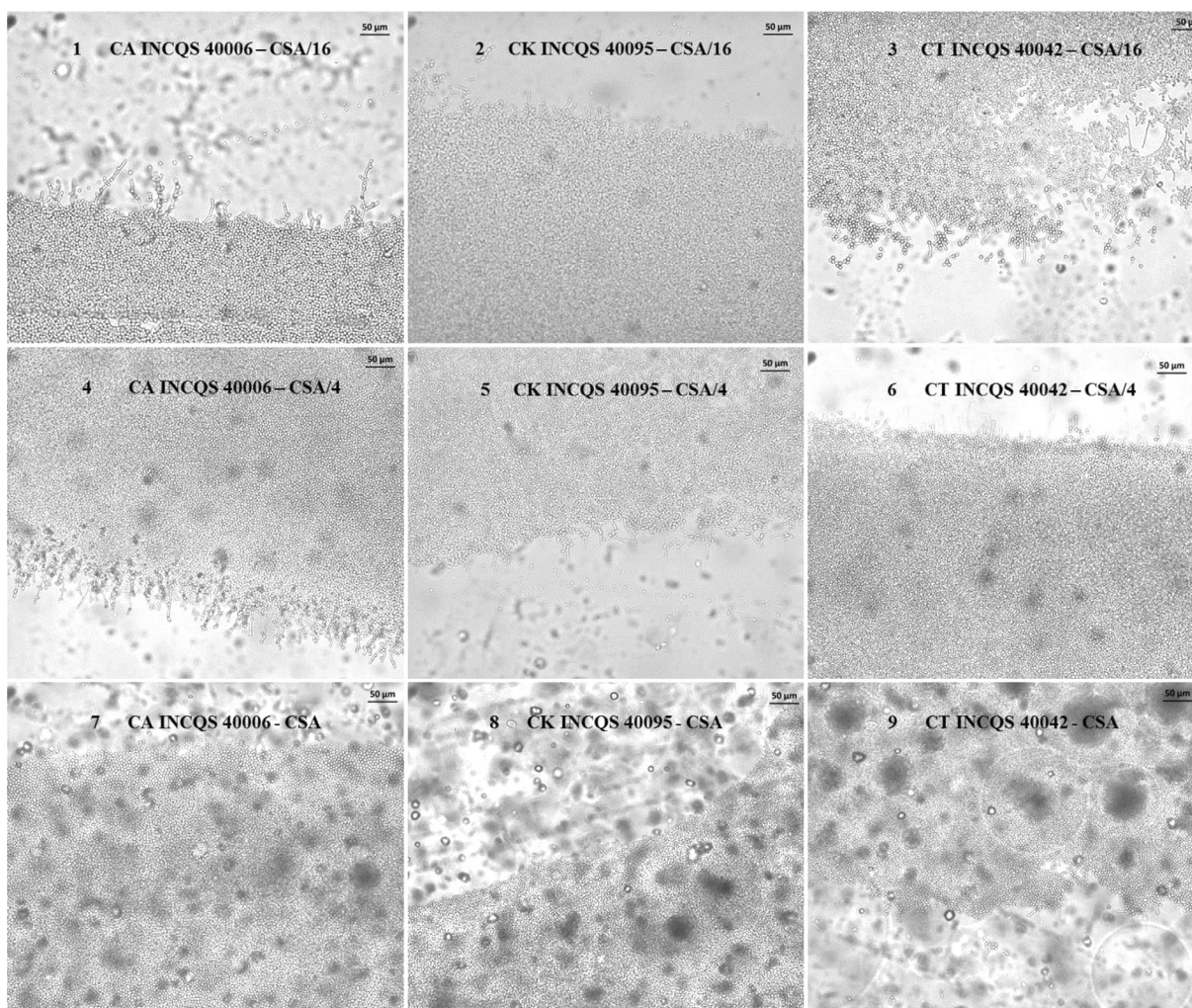

Fig. 3. Controls used in the micromorphological *Candida* yeast assays. CA: *Candida albicans*, CK: *Candida krusei*, CT: *Candida tropicalis*; INCQS: Instituto Nacional de Controle de Qualidade em Saúde. SCA: Superior Concentration Assayed. In 1, 2 and 3 – Effect of fluconazole at a concentration of 512 µg/mL (SCA/16); 4, 5 and 6 at the concentration of 2048 µg/mL (SCA/4); 7, 8 and 9 at 8192 µg/mL (SCA). Visualisation using a 40× objective view.

fungal stress was provoked using a depleted medium facilitating the formation of hyphae and pseudohyphae that act in the search of nutrients, demonstrating the pleomorphic characteristic of yeasts (Fig. 2). Regarding fluconazole, filamentous structure inhibition occurred effectively at the highest concentration (Fig. 3), whereas when compared to the growth control, inhibition was already observed from lower concentrations.

As for the essential oil effect, it was observed that this inhibited the growth of *C. albicans* filamentous structures at all concentrations. In *C.*

tropicalis, this same effect was indicated only at the highest concentration (SCA). The oil, however, was inefficient against *C. krusei* where no inhibitory action was observed (Fig. 4).

Virulence inhibition of *Candida* species has already been observed both by the action of extracts from other species belonging to the Myrtaceae family as well as by the action of plant products derived from other families. Morais-Braga et al. (2016; 2017) reported in their study that the aqueous and hydroethanolic extracts from *Psidium guajava* and *Psidium brownianum* (Myrtaceae) leaves affected the fungal
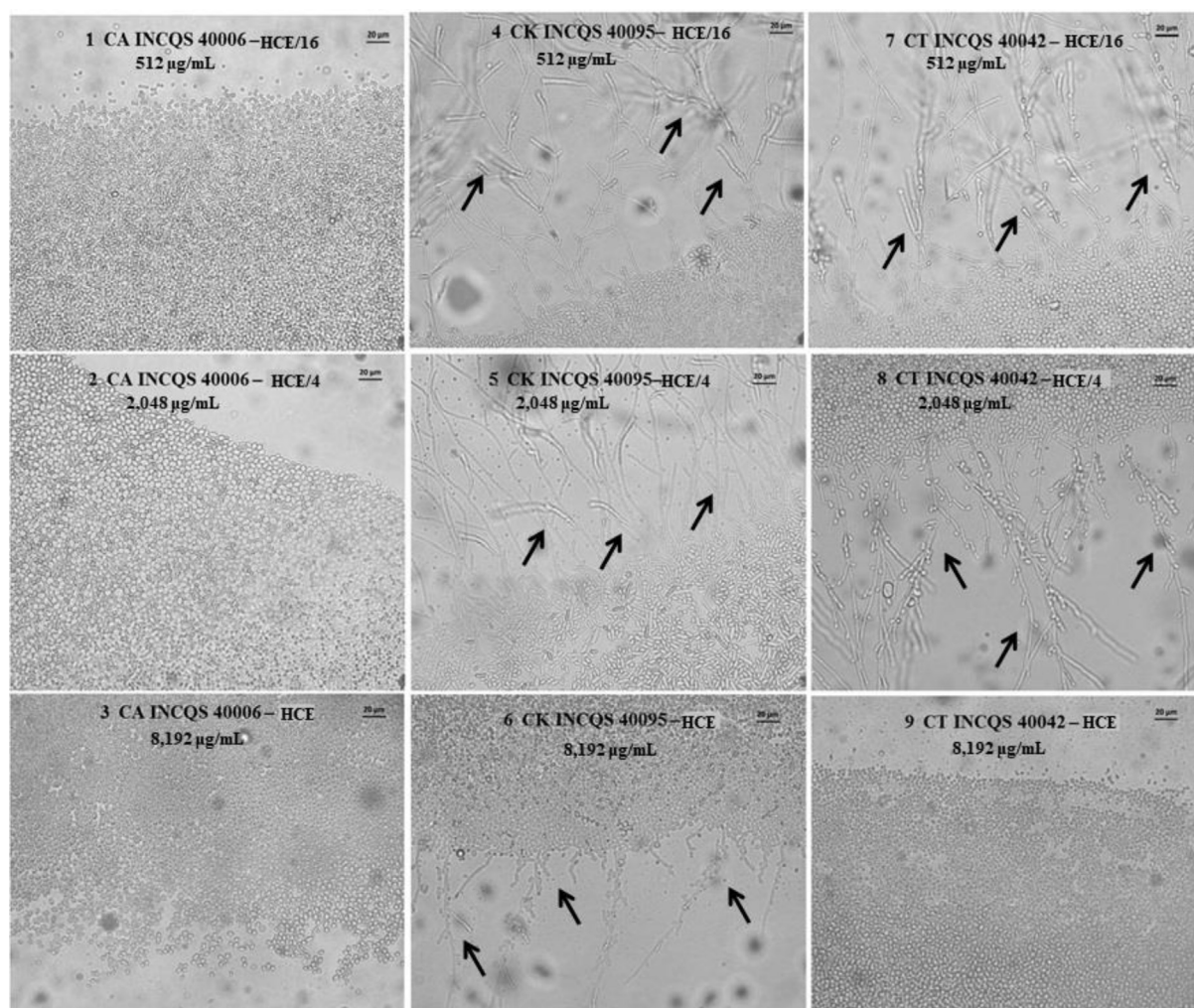
Fig. 4. CA: *Candida albicans*, CK: *Candida krusei*, CT: *Candida tropicalis*; INCQS: National Institute of Quality Control in Health. SCA: Superior Concentration Assayed. Effect of the *Eugenia uniflora* essential oil on *C. albicans*, *C. krusei* and *C. tropicalis* dimorphism. Concentrations of 512 μg/mL (SCA/16); 2048 μg/mL (SCA/4); 8192 μg/mL (SCA). In 1, 2 and 3 with *C. albicans*; 4, 5 and 6 with *C. krusei* and 7, 8 and 9 with *C. tropicalis*. Visualisation using a 40× objective view. Arrows indicate the presence of hyphae.

virulence of *C. albicans* and *C. tropicalis* at the 16.384 μg/mL concentration. Brito et al. (2015), showed that the *C. krusei* and *C. tropicalis* strains cultivated in culture medium in the presence of the *Lippia sidoides* (Verbenaceae) essential oil did not present pseudohyphae and hyphae at the 256 μg/mL concentration. Meanwhile, Oliveira, Batista, Fernandes, Sales, and Nogueira (2016) showed a reduction in *C. albicans* hyphae at the 1.25 mg/mL and 2.5 mg/mL concentrations, as well as *C. albicans* (0.625 mg/mL and 2.5 mg/mL) and *C. parapsilosis* (1.25 mg/mL and 2.5 mg/mL) treated with the *Ocimum gratissimum* (Lamiaceae) essential oil.

In the case of *C. albicans*, the hyphae and pseudohyphae inhibitory action can be observed from the lowest (512 μg/mL) to the highest concentration (8.192 μg/mL). This effect may be directly related to the inhibition of fungal cell growth and to the formation of inhibition halos. This may suggest dose-dependency, justifying the action observed in the micromorphology assays.

O efeito de inibição de hifas visto em todas as concentrações em *C. albicans* e na maior concentração em *C. tropicalis* pelo óleo essencial de *E. uniflora,* deve estar relacionado a sua composição química. Visto que os compostos encontrados em maior quantidade foram a selina-1,3,7(11)-trien-8-one e selina-1,3,7(11)-trien-8-one epoxide, respectivamente. A ação inibitória de crescimento pode estar associada a estes compostos ou com a combinação de outros também identificados, mas em menor quantidade. Mais estudos são necessários visando

compreender a ação isolada destas substâncias quando se tratando da micromorfologia, se as mesmas são responsáveis pela inibição de hifas e pseudo-hifas isoladamente ou em associação com os outros compostos, pois na literatura esta informação ainda não foi elucidada.

The hyphael inhibitory effect seen by the *E. uniflora* essential oil at all concentrations against *C. albicans,* and at the highest concentration against *C. tropicalis,* could be related to its chemical composition since the compounds found in greatest quantities were selena-1,3,7(11)-trien-8-one and selina-1,3,7(11)-trien-8-one epoxide, respectively. The inhibitory growth action may be associated with these compounds or with the combination of other compounds also identified, however in lower quantities. Further studies are needed to understand the isolated action of these substances with regards to micromorphology and to determine if they are responsible for the inhibition of hyphae and pseudohyphae alone or in association with the other compounds as this information has not been previously elucidated in the literature.

4. Conclusion

High concentrations of the *E. uniflora* essential oil were required to obtain an antifungal effect. The combination of the oil and fluconazole did not show synergism, however good results were obtained especially in relation to inhibition of the *C. albicans* and *C. tropicalis* morphological transition process, which in human infections can cause tissue invasion.

Further research is needed to understand the oil activity over the fungi biochemical and genetic processes related to fungal virulence inhibition.

The *E. uniflora* essential oil had in this study a reduced antifungal potential in the experiments performed and it presented in its composition the predominance of mono- and sesquiterpenes, constituents possessing antifungal effect already evidenced in the literature which may have contributed to the fungistatic effect observed in the assays. Inhibition of *Candida* spp. colonies using the oil was evidenced in the MFC assay revealing a fungistatic action, however, this had an antagonistic effect when it was associated with Fluconazole and it is thus not advisable to use them in conjunction due to the reduced the performance of the drug. However, this association regressed and affected morphological transition through the emission of filamentous structures, an important fungal virulence factor, especially against *C. albicans* and *C. tropicalis*. These results show that this compound has active antifungal constituents, however, further studies are needed to prove its effectiveness and safety.

Further studies using animal models are required to verify the *in vivo* efficacy of the oil.

Declaration of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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