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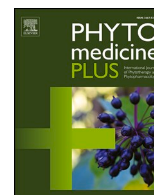

Synergistic effects of *Thymus vulgaris* essential oil in combination with antifungal agents and inhibition of virulence factors of *Candida albicans*

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ABSTRACT

Background: Combination antifungal therapy has become a prominent approach in medical practice as it takes advantage of synergistic interactions by interfering with multiple targets of the pathogen, broadening the spectrum of activity, reducing the development of resistance, and improving treatment outcomes. Therefore, combining conventional antifungal drugs with natural products can increase antifungal activity, reduce side effects, and optimize therapeutic effects.
Purpose: The present study aimed to evaluate the antifungal activity of thyme essential oil (ThyEO) in combination with amphotericin B, caspofungin, fluconazole, itraconazole, and posaconazole (PSZ), as well as its inhibitory effect on *Candida albicans* virulence factors.
Materials and Methods: The microbroth dilution assay was employed to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). Interactions were assessed using the microdilution checkerboard assay. Ergosterol and methylene blue assays were used to detect effects on fungal membrane, while the sorbitol assay was used to detect effects on fungal cell wall. Inhibition of yeast virulence factors (adherence to epithelial cells, germ tube and pseudomycelium formation, secretion of hydrolytic enzymes, and biofilm formation) was assessed with previously reported methods.
Results: Almost all combinations showed additivism against *C. albicans*, except ThyEO/PSZ (31.25/0.0039 μg/ml), which showed partial synergism. Furthermore, all mixtures were fungicidal against *C. albicans* strains. ThyEO/PSZ, its components alone, and thymol were shown to disrupt the fungal cytoplasmic membrane, increasing its permeability. ThyEO/PSZ, at sub-inhibitory concentrations, significantly decreased the ability of *C. albicans* to adhere to buccal epithelial cells. ThyEO/PSZ, ThyEO, and PSZ were able to reduce the pseudomycelium production of *C. albicans* while thymol completely inhibited its formation. ThyEO/PSZ, each combination component on its own, and thymol inhibited biofilm formation and preformed biofilm of *C. albicans*. Notably, ThyEO/PSZ showed synergistic and fungicidal activity against a resistant strain of *C. albicans*, reducing the PSZ dose by 4-fold.
Conclusion: These findings make ThyEO and ThyEO/PSZ mixture valuable candidates for the development of alternative antifungals with a lower incidence of adverse effects.

Abbreviations

AMB	Amphotericin B
ANOVA	Analysis of variance
BECs	Buccal Epithelial Cells
CCC	Center of Reference in Mycology
CFG	Caspofungin
CFU	Colony Forming Units
DMSO	Dimethyl sulfoxide
DRI	Dose Reduction Index
EOs	Essential oils
FCZ	Fluconazole
FICI	Fractional Inhibitory Concentration Index
GC–MS	Gas Chromatography coupled to Mass Spectrometry

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GT	Germ tube
ITZ	Itraconazole
MFC	Minimal Fungicidal Concentration
MIC	Minimal Inhibitory Concentration
MOPS	3-(N-morpholino)propanesulfonic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate Buffered Saline
PSZ	Posaconazole
p-value	Probability value
Pz	Precipitation or halo zone
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
ThyEO	*Thymus vulgaris* Essential oil
YPD	Yeast extract Peptone Dextrose

Introduction

Candidiasis is the main cause of systemic opportunistic fungal diseases in immunocompromised patients associated with a significant global burden, with an estimated 750,000 cases occurring annually and overall mortality ranging between 20-50%. More than 90% of candidiasis is attributed to *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis*, and *C. krusei* being the first one the main etiologic species associated with nosocomial invasive candidiasis globally (WHO, 2022).

Clinically useful antifungals are limited to azoles, echinocandins, and polyenes. Azoles, either imidazoles or triazoles, target the biosynthesis of ergosterol by inhibiting lanosterol 14-α-demethylase (Lee et al., 2020). Triazoles have a broader spectrum of action than imidazoles and are commonly used as first-line antifungal therapy for the treatment of several fungal infections. First-generation [such as fluconazole (FCZ) and itraconazole (ITZ)] and second-generation [including voriconazole, posaconazole (PSZ), isavuconazole, and ravuconazole] triazoles have been developed to widen the range of activity and combat resistant fungal infections (Lee et al., 2020). Polyenes, such as amphotericin B (AMB), are amphipathic antifungals directed against membrane ergosterol, forming channels leading to leakage of intracellular molecules, resulting in fungal death (Hoehamer et al., 2010). Echinocandins, such as caspofungin (CFG), anidulafungin, and micafungin, are acylated cyclic hexapeptides that exert fungicidal activity by non-competitive inhibition of β-1,3-glucan synthase, resulting in the absence of β-glucans and cell death (Hoehamer et al., 2010). However, these agents have significant limitations, which restrict their routine clinical use. Regarding azoles, the main limitation is their fungistatic action, which imposes strong directional selection pressure and promotes the development of resistance (Lee et al., 2020). Polyenes have adverse side effects such as nephrotoxicity, renal failure, and infusion-related toxicity. Echinocandin antifungals are somewhat expensive and have been associated with fever, thrombophlebitis, and liver toxicity (Hoehamer et al., 2010). Given these limitations, the development of new antifungals for the effective treatment of *Candida* infections is highly needed.

Studies have demonstrated that combination therapy enhances the effectiveness of antifungal treatment (Livengood et al., 2020). It offers advantages over monotherapy, including a broader spectrum of action, reduced risk of recurrence and resistance development, as well as lower toxicity and side effects (Livengood et al., 2020). Furthermore, combining two drugs with different mechanisms of action hinders resistance, enhances fungicidal efficacy, and can reverse drug resistance (Lee et al., 2020). Several studies have reported that plant essential oils (EOs) and their metabolites enhance the activity of antifungal drugs by inhibiting ergosterol synthesis, altering cell wall morphology, inhibiting enzymes involved in cell wall synthesis, modifying cell membrane permeability, producing reactive oxygen species, and interacting with the mitochondrial membrane, thereby causing cidal effects (Nazzaro et al., 2017).

Thymus vulgaris L. (Lamiaceae) is a perennial aromatic plant native to the Mediterranean region used by the world's population as food preservative, and medicinal plant. It possesses phytocompounds responsible for numerous pharmacological activities, including antibacterial and antifungal ones (de Oliveira, 2017). Several studies have demonstrated the synergistic antifungal activity of *T. vulgaris* EO (ThyEO), and its main component thymol, with FCZ against *Candida* spp. (Saad et al., 2010; Jafri and Ahmad, 2020).

Targeting pathogen-specific virulence factors is an effective strategy, as it preserves the normal commensal host microbiome and reduces the selection pressure for the evolution of drug resistance (Lee et al., 2020). Pathogenicity of *Candida* species is attributed to certain virulence factors such as adherence to host cells and tissues, transition from bud to pseudohyphae (morphogenesis), secretion of tissue-damaging hydrolytic enzymes, and biofilm formation (in host tissues and medical devices) (Butassi et al., 2019a). Biofilms are less susceptible to antimicrobial agents than planktonic cells, thus increasing resistance to antifungal drugs and host defenses, leading to failure of conventional antifungal therapy (Ramage et al., 2012). The search for substances that inhibit virulence factors may represent the basis for effective therapeutic options for the management of mycoses, including those resistant to conventional therapies.

Interestingly, many bioactive compounds alone or in combination with conventional antifungals also modulate *C. albicans* virulence (Lee et al., 2020). These agents in synergistic combinations with low-dose antifungal drugs provide a more effective alternative against fungal infections (Ayaz et al., 2019).

The objective of the present study was to evaluate the synergistic effect of ThyEO combined with classical antifungal drugs against *C. albicans*. Furthermore, the effects of the most active combination on virulence factors were evaluated.

Materials and methods

Essential oil, antifungal agents and chemical compounds

The ThyEO (CAS# 8007-46-3 FEMA 3065) was purchased from EUMA SAICIYF (Buenos Aires, Argentina), a supplier of EOs and fragrances that complies with the provisions of the National Administration of Drugs, Food and Medical Technology and the international standards IFRA (International Fragrance Association). All samples were protected from light and humidity and stored at -20 °C until use. Stock solutions of each EO were prepared in dimethyl sulfoxide (DMSO) (Anedra, Buenos Aires, Argentina) to obtain a final concentration of 50 μg/ml and stored at -20 °C.

AMB, CFG, FCZ, ITZ and PSZ (Fig. 1) were purchased from Sigma-Aldrich (St. Louis, MO, USA) (purity ≥ 98% by HPLC). Stock solutions were prepared at 2 mg/ml in DMSO and stored at -20 °C. Thymol was acquired in Sigma-Aldrich (purity ≥ 98.5% by HPLC) and a stock solution was prepared at 12.5 mg/ml in DMSO and stored at -20 °C.

GC-MS analysis

Chemical composition of ThyEO was determined by Gas Chromatography coupled to Mass Spectrometry (GC-MS) on a Shimadzu GC-MS QP2010 Plus spectrometer linked on-line with an HP mass selective detector (MSD 5970 HP). A Supelco SPB-1 column (30 m x 0.25 mm, coating thickness 0.25 μm) was used at a temperature programmed to rise from 50 to 300 °C at a rate of 25 °C/min, maintaining the final temperature for 3 min. Helium was employed as carrier gas (0.9 ml/min). A solution (1 mg/ml in CH_2Cl_2) of ThyEO was prepared and injected (1 μl) into the chromatograph. Electron impact at 70 eV was employed for ionization. Full-scan analyses were conducted within the mass range of 40-650 m/z. Chromatographic parameters included a detector temperature of 270 °C and an injection temperature of 250 °C.

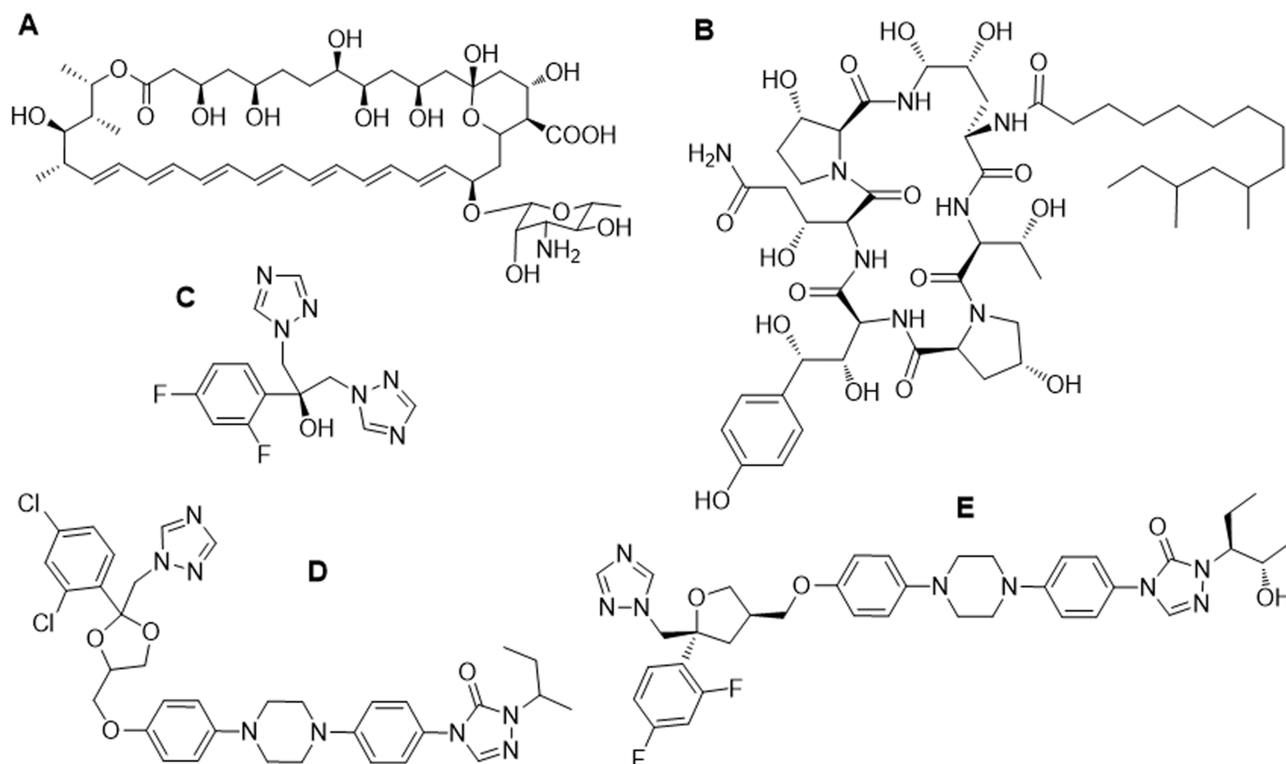

Fig. 1. Chemical structures of the antifungal drugs **A)** amphotericin B, **B)** caspofungin, **C)** fluconazole, **D)** itraconazole, and **E)** posaconazole.

The analysis was performed using GC-MS solutions software (version 4.44, Shimadzu). Qualitative analysis was carried out by assessing the percent area of each peak corresponding to the sample compounds. The mass spectrum of each compound was compared with those of the NIST14 spectra library (USA National Institute of Science and Technology software).

Microorganisms and culture conditions

Clinical isolates of *C. albicans* (CCC193-13 and CCC121-16) were obtained from the Center of Reference in Mycology (CEREMIC, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina). Additionally, *C. albicans* LMDM-526, a spontaneous mutant strain (with S645Y amino acid change in *FKS1*) belonging to the strain collection of the Laboratorio de Micología y Diagnóstico Molecular (LMDM) and kindly provided by Dr. Guillermo García-Effron [LMDM, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina], was included in this study. Strains were grown on Sabouraud Dextrose Agar (SDA) plates at 30 °C and conserved in 20% v/v glycerol at -20 °C. Inocula were obtained according to the CLSI M27-Ed4 document and adjusted to 1-5 $\times$ 10^3 Colony Forming Units (CFU)/ml (CLSI, 2017).

Determination of antifungal activity of each combination partner on their own

The determination of minimal inhibitory concentrations (MICs) was conducted following the broth microdilution method recommended by CLSI (2017), with slight modifications as described in a previous study by Butassi et al. (2019b). Briefly, 100 µl of fungal inoculum was added to 96-well microplates (Greiner Bio-One GmbH, Frickenhausen, Germany) containing two-fold serial dilutions of samples in Sabouraud Dextrose Broth (SDB; Laboratorios Britania, Buenos Aires, Argentina) prepared from DMSO stock solutions. ThyEO and thymol were assessed at concentrations range 15.63-1000 µg/ml and 3.9-250 µg/ml, respectively. Commercial antifungals were evaluated within the CLSI-recommended ranges: AMB, ITZ, and CFG 0.0156-16 µg/ml; PSZ 0.0019-2 µg/ml; and FCZ 0.125-64 µg/ml (CLSI, 2017). A growth control well (medium and inoculum), a sterility control well (medium, DMSO 2%, and sterile water), and a sample control well (sample, medium, and sterile water) were included for each yeast strain. Tests were performed in duplicate. Microtiter plates were incubated in a moist dark chamber at 30 °C for 24 h and then read on a VERSA Max microplate reader (Molecular Devices, Sunnyvale, CA, USA). MIC value was assigned as the lowest concentration of tested samples that completely inhibited growth of microorganisms after incubation. Samples with MICs $>$ 1000 µg/ml were considered inactive. MIC for AMB was defined as the lowest concentration that resulted in $\geq$ 90% growth inhibition, whereas for azoles and echinocandins, it was defined as the lowest concentration that resulted in 50% growth inhibition compared to the growth control. The interpretive breakpoints suggested by CLSI (2017) were used to classify strains as resistant or susceptible. To determine the Minimal Fungicidal Concentration (MFC), aliquots of 10 µl from wells with no visible growth were transferred to SDA plates (Butassi et al., 2019b). MFC was defined as the lowest concentration of samples capable of killing at least 99.9% of the initial inoculum. AMB was used as standard fungicidal drug.

Determination of antifungal activity in combination

The interaction between ThyEO and antifungals was tested using the broth microdilution checkerboard method. To create a checkerboard concentration pattern, serial two-fold dilutions of the components were performed, resulting in final concentrations above and below their respective MICs. The experimental conditions were identical to those used for the determination of antifungal activity alone (as described previously). MFC of combinations was determined as described above but testing fixed proportions equal to the MIC and at double-serial concentrations in a ray design.

The combination effect was evaluated by calculating the Fractional Inhibitory Concentration Index (FICI) for each combination that

achieved 100% inhibition of fungal growth as follows: FICI=$\sum$($MIC_{in\ combination}/MIC_{alone}$). The interpretation of FICI values was as follows: FICI≤0.5, synergism; 0.5<FICI≤0.75, partial synergism; 0.75<FICI≤1, additivism; 1<FICI≤4, no interaction; and FICI>4, antagonism (Fadil et al., 2018).

Dose Reduction Index (DRI) was calculated as the ratio of MIC_{alone} to $MIC_{in\ combination}$. DRI quantifies the extent to which the combination dose can be reduced, compared to the dose of an individual component alone, while achieving a specific level of effect.

Exogenous ergosterol effect assay

MICs of the mixture, its constituents, and thymol, either in the absence or presence of different concentrations (50, 100, and 200 µg/ml) of ergosterol (Sigma-Aldrich) added to the assay medium, were determined according to Butassi et al. (2019b), against *C. albicans* CCC193-13 and CCC121-16. AMB was used as standard positive control. MIC was determined after 24 h of incubation in a moist dark chamber at 30 °C.

Methylene blue dye exclusion assay

The effect of each sample on membrane permeability was studied using the methodology reported by Makarasen et al. (2018). First, an overnight SDB culture of each yeast was harvested by centrifugation (10, 000 g, 5 min) at 4 ºC and washed twice in Phosphate Buffered Saline (PBS)+0.01% v/v Tween80 (Sigma-Aldrich). Yeasts were resuspended in sterile distilled water and adjusted to 1-5 × 10^6 CFU/ml. Control (0.5 ml inoculum and 0.5 ml distilled water) and treatment (0.5 ml inoculum and 0.5 ml sample) tubes were prepared, incubated at 30 °C with shaking, collected at 0, 90, 180, 270, and 360 min, and concentrated by centrifugation. Each sample pellet (20 µl) was stained with 80 µl of 0.05% w/v methylene blue and observed microscopically. AMB was used as positive control. Stained cells indicated cell damage, and the % of damaged yeast cells was calculated and plotted. Determinations were performed in triplicate and expressed as mean ± SD.

Sorbitol assay

The assay was performed using SDB medium with and without sorbitol (control), to evaluate possible mechanisms involved in antifungal activity against *C. albicans* CCC193-13 and CCC121-16 cell walls. Sorbitol (Sigma-Aldrich) was added to the culture medium in a final concentration of 0.8 M (Butassi et al., 2019b). MICs were determined for both conditions at 2 and 7 days. CFG was used as standard positive drug.

Virulence factor inhibition assays

Prior to conducting these assays, MICs were obtained under the specified conditions (culture medium and inoculum), then tests were performed at sub-inhibitory concentrations (MIC/2) obtained from these values. The mixtures were evaluated maintaining the proportion of the partners previously found.

Adherence to Buccal Epithelial Cells (BECs) inhibition assay

The effect of ThyEO/PSZ, ThyEO, PSZ, and thymol on the adherence of *C. albicans* to BECs was performed using a previously described methodology (Butassi et al., 2019a). BECs were obtained from healthy human subjects by gently swabbing the inside of their cheeks using sterile swabs that were then washed twice in PBS and resuspended in the same buffer to achieve a final concentration of 2.5 × 10^5 cells/ml. *C. albicans* was cultured on SDB at 30 °C for 24 h, washed twice in PBS, and adjusted to 2.5 × 10^7 CFU/ml. For the experiment, a mixture containing 0.5 ml of BECs, 0.5 ml of fungal inoculum, and either 1 ml of SDB (control) or 1 ml of SDB with each sample at sub-inhibitory concentrations was prepared. The mixture was then incubated at 37 °C on a 150 g shaker for 1 h. Hydrophilic polyvinylidene fluoride filters with a pore size of 0.47 µm (Merck Millipore, Billerica, MA, USA) were utilized to collect the BECs, which were subsequently washed with PBS to eliminate any unattached fungi. Subsequently, the filter was cautiously removed and firmly positioned on a glass slide with the BECs in contact with the glass surface. The preparations were air-dried, heat-fixed, and stained by Gram-Nicolle technique. AMB (at sub-inhibitory concentration) was used as standard positive drug. At least 100 BECs were counted, and the number of yeasts adhered per BEC was determined using an optical microscope (Eclipse E100, Nikon Corp., Tokyo, Japan). Determinations were performed in triplicate, and the results were expressed as mean ± SD and plotted.

Germ tube (GT) inhibition assay

This assay was performed according to Butassi et al. (2019a). Cell suspensions from *C. albicans* overnight cultures in SDB were adjusted to a concentration of 1×10^6 CFU/ml and added to tubes containing fetal bovine serum and SDB (control) or samples. The tubes were then incubated at 37 °C for 3 h and 100 cells were counted using an optical microscope. AMB was used as standard positive drug. GTs were counted when they were as long as the diameter of the blastospore. Exclusions were made for protuberances exhibiting a constriction at the point of connection to the mother cell, which is a characteristic feature of pseudohyphae. The % of cells with GT in the presence of each sample was determined by the following equation: $\%\,\text{GT formation} = \frac{\text{number of GT in treatment}}{\text{number of GT in control}} \times 100$. Results were presented as means ± SD of three separate experiments.

Pseudomycelium formation inhibition assay

Induction of pseudomycelium formation on solid media of *C. albicans* in presence of each sample was performed according to Silva-Rocha et al. (2015). Briefly, cell suspensions from overnight SDB cultures adjusted to 1 × 10^6 CFU/ml, were spotted on the surface of Spider medium in the presence and absence of each sample at sub-inhibitory concentrations. Plates were incubated in darkness at 30 °C for 8 days for the subsequent macro- and microscopic observation of colonies. FCZ was used as standard positive drug. The assay was performed in triplicate and results were expressed as mean ± SD.

Lytic enzymes inhibition assay

C. albicans CCC193-13 and CCC121-16 were first tested for their ability to produce phospholipases, proteinases, hemolysins, and esterases by plating a 10 µl aliquot of yeast suspension (1×10^6 CFU/ml) on specific test medium (enzyme producer controls). Then, the inhibition of the production of each enzyme was evaluated in the presence of each sample as described in Butassi et al. (2019a). For this purpose, samples were added to each specific test medium at sub-inhibitory concentrations and incubated at 35–37 °C for 4-7 days. The level of enzymatic activity, termed $Pz = \frac{\text{colony diameter}}{\text{colony diameter}+\text{halo zone}}$, was classified as: absence of enzymatic activity (Pz = 1.0); positive activity (1.0 > Pz ≥ 0.64); or strongly positive activity (Pz < 0.64). Assays were performed in triplicate. A significant increase in Pz index, for treated cells compared to control ones, was indicative of lytic-enzyme inhibition.

Inhibition of biofilm formation and preformed biofilms

Tests were performed according to Pierce et al. (2008) with the biofilm-forming strain *C. albicans* CCC121-16 in 96-well flat-bottomed microtiter plates. The yeast was grown in Yeast extract Peptone Dextrose (YPD) broth for 24 h on an orbital shaker (150 g) at 30 °C. Planktonic cells were harvested by centrifugation (3,000 g, 5 min), washed twice with PBS and adjusted to 1 × 10^6 CFU/ml in RPMI-1640 medium with L-glutamine, supplemented with 1.8% glucose and buffered with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich) to pH 7.

Biofilm formation inhibition assay was performed in the presence of

serially double-diluted sample concentrations and incubated statically for 48 h at 37 °C. Then, the liquid content was discarded and each well was gently washed thrice with PBS to remove non-adherent cells.

Biofilm quantification was performed using the colorimetric tetrazolium reduction assay with modifications (Pires et al., 2011), and viability was expressed in terms of % of metabolic activity. Briefly, 100 µl of prewarmed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) solution (0.5 mg/ml) in PBS was added to each well. Plates were incubated at 37 °C for 4 h, and MTT solution was then removed. Biofilms were washed twice with PBS, and the formazan product was resuspended in DMSO. Finally, the absorbance was measured at 540 nm.

Preformed biofilm inhibition assays were performed against mature biofilms previously formed by dispensing 100 µl of cell suspension into the wells of microtiter plates and incubating statically for 48 h at 37 °C. After biofilm formation, the medium was aspirated and non-adherent cells were removed by washing three times with PBS. Then, 100 µl of serially double-diluted sample concentrations were added to pre-washed biofilm wells and incubated for 24 h at 37 °C. Subsequently, metabolic activity was determined by the colorimetric MTT reduction assay.

Determinations were made in triplicate and results were represented as % inhibition of biofilm formation or % reduction of preformed biofilm for each sample expressed as mean ± SD. A biofilm formation control (yeast cell suspension and culture medium), a standard positive control with AMB, and a sterility control (culture medium and water instead of inoculum) were included in both assays.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 2.01 software (GraphPad Prism Software Inc., San Diego, CA, USA). Means and SD were determined. Differences between treatment groups were analyzed using ANOVA, Kruskall-Wallis, and Dunn tests; p-values < 0.05 were considered significant.

Results

ThyEO chemical composition analysis

Chemical composition of ThyEO was evaluated by GC-MS (Table 1). Eleven components were identified, mainly mono-terpenes and sesquiterpenes, representing 94.88% of the total constituents detected. The main components were *p*-cymene (36.78%), thymol (30.87%), α-terpineol (7.50%), linalool (7.11%), α-pinene (3.14%), limonene (1.69%), β-caryophyllene (1.30%) and β-myrcene (1.23%), as reported in the literature (Schmidt et al., 2012).

Overall, the main constituents are responsible for the biological activity of any EO. In many cases, the greater effect could be attributed to the synergistic combination of the major and minor constituents of the EO. In the present context, *p*-cymene and thymol were the major constituents of ThyEO, however, to ascertain the effect of the major constituents, we decided to include only thymol in the following assays because, according to the literature, *p*-cymene showed significantly less antifungal activity than thymol against *C. albicans* (Dutta et al., 2020).

Table 1
Chemical composition of Thyme essential oil (ThyEO) determined by GC-MS.

Nº	Rt (min)	Total Area %	EM	Constituents*
1	4.38	3.14	136.24	α-pinene
2	4.49	0.99	136.24	(+)-camphene
3	4.69	1.23	136.24	β-myrcene
4	4.95	36.78	134.21	*p*-cymene
5	5.03	1.69	136.24	limonene
6	5.43	7.11	154.25	linalool
7	5.72	0.67	154.25	fenchol
8	5.98	0.85	154.25	isoborneol
9	6.11	7.50	154.25	α-terpineol
10	6.67	30.87	150.22	thymol
11	7.56	1.30	204.36	β-caryophyllene

Rt: Retention time; EM: Exact masses. *Constituents presented in order of elution from the SPB-1 Supelco column

Antifungal activity of the partners alone and in combination

MIC and MFC values of ThyEO and antifungal agents alone and in combination along with FICI and DRI for each combination against *C. albicans* CCC193-13 and CCC121-16 are detailed in Table 2.

As can be seen, ThyEO/PSZ mixture (31.25/0.0039 µg/ml) showed partial synergism (FICI = 0.53) along with a 32- and 2-fold reduction in the doses of ThyEO ($DRI_{ThyEO} = 32$) and PSZ ($DRI_{PSZ} = 2$) respectively against both strains. All other combinations were additive. Based on this result, we decided to evaluate the activity of thymol alone and in combination with PSZ. Table 2 shows that thymol/PSZ (15.6/0.0039 µg/ml) showed partial synergism (FICI = 0.625) along with an 8- and 2-fold reduction in doses of thymol ($DRI_{thymol} = 8$) and PSZ ($DRI_{PSZ} = 2$) respectively, against both strains.

MFC determination showed that the combinations were fungicidal against both strains (Table 2); however, in all cases, the concentrations of both ThyEO and thymol were the same at which they were fungicidal alone (1000 and 250 µg/ml respectively), indicating that the fungicidal effect in each mixture would be attributed to them. This is a relevant result, particularly for azoles, since they are fungistatic, and the use of these antifungals at lower doses in mixtures could be interesting in treatments requiring its action with the advantage of also being fungicidal.

ThyEO/PSZ was selected instead of thymol/PSZ to further study its antifungal action because although both mixtures have a partially synergistic effect against *C. albicans*, the former showed a lower FICI.

Studies of mechanisms of antifungal action

Binding to fungal membrane ergosterol

MICs of ThyEO/PSZ, the partners alone, and thymol with or without the addition of exogenous ergosterol against *C. albicans* CCC193-13 and CCC121-16 were determined to evaluate whether their antifungal mechanism of action was through binding to fungal membrane ergosterol. Results showed that MIC values of none of the samples changed in the presence of exogenous ergosterol, indicating that they would not exert their antifungal action through this mechanism, while the MIC of AMB increased more than 16-fold (Supplementary Material, Section 1).

Alterations in membrane permeability

Organic dyes are known to be unable to pass through the membrane of intact cells, but can enter the cell and stain the cytoplasm if damage or increased membrane permeability occurs. To evaluate whether the samples produce alterations in fungal membrane permeability, the methylene blue dye exclusion assay was used (Makarasen et al., 2018). As can be seen in Fig. 2, there is a significant increase in the number of *C. albicans* CCC193-13 and CCC121-16 cells stained after 90 min of incubation in the presence of ThyEO/PSZ (31.25/0.0039 µg/ml), ThyEO (1000 µg/ml), PSZ (0.0078 µg/ml) and thymol (125 µg/ml) compared to untreated (control) cells. These results suggest that both the mixture and the partners alone would damage the integrity of the cell membrane. In the combination, this damage occurs at lower concentrations of both partners than when used alone.

Protective effect of sorbitol on fungal cell wall

The MIC of each sample was determined against *C. albicans* CCC193-13 and CCC121-16 in the absence and presence of the osmotic protectant sorbitol. Sorbitol in the culture medium can stabilize cells with a weakened cell wall. Consequently, the effects caused by cell wall antifungals may be reversed and thus exhibit a higher MIC (Butassi et al., 2019b). Results showed that when both yeasts were treated with

Table 2
MIC and MFC values (μg/ml) of ThyEO, thymol, and antifungal agents alone and in combination along with FICI and DRI values against *Candida albicans*.

	Samples	MIC	MFC	FICI	Interaction type	$DRI_{ThyEO/thymol}$	DRI_{AA}
Candida albicans CCC193-13	ThyEO	1000	1000				
	AMB	0.5	0.5				
	CFG	0.0625	0.5				
	FCZ	2	>64				
	ITZ	0.0625	>16				
	PSZ	0.0078	>16				
	ThyEO/AMB	500/0.25	1000/0.5	1	Additivism	2	2
	ThyEO/CFG	500/0.0313	1000/0.0625	1	Additivism	2	2
	ThyEO/FCZ	500/1	1000/2	1	Additivism	2	2
	ThyEO/ITZ	500/0.0313	1000/0.0625	1	Additivism	2	2
	ThyEO/PSZ	31.25/0.0039	1000/0.125	0.53	Partial synergism	32	2
	Thymol	125	250				
	Thymol/PSZ	15.6/0.0039	250/0.0625	0.625	Partial synergism	8	2
Candida albicans CCC121-16	ThyEO	1000	1000				
	AMB	0.5	0.5				
	CFG	0.125	1				
	FCZ	4	>64				
	ITZ	0.0625	>16				
	PSZ	0.0078	>16				
	ThyEO/AMB	500/0.25	1000/0.5	1	Additivism	2	2
	ThyEO/CFG	500/0.0625	1000/0.125	1	Additivism	2	2
	ThyEO/FCZ	500/2	1000/4	1	Additivism	2	2
	ThyEO/ITZ	500/0.0313	1000/0.0625	1	Additivism	2	2
	ThyEO/PSZ	31.25/0.0039	1000/0.125	0.53	Partial synergism	32	2
	Thymol	125	250				
	Thymol/PSZ	15.6/0.0039	250/0.0625	0.625	Partial synergism	8	2

MIC: Minimum Inhibitory Concentration; MFC: Minimum Fungicidal Concentration; FICI: Fractional Inhibitory Concentration Index; DRI: Dose Reduction Index; AA: Antifungal agent; AMB: Amphotericin B; CFG: Caspofungin; FCZ: Fluconazole; ITZ: Itraconazole; PSZ: Posaconazole; ThyEO: Thyme essential oil

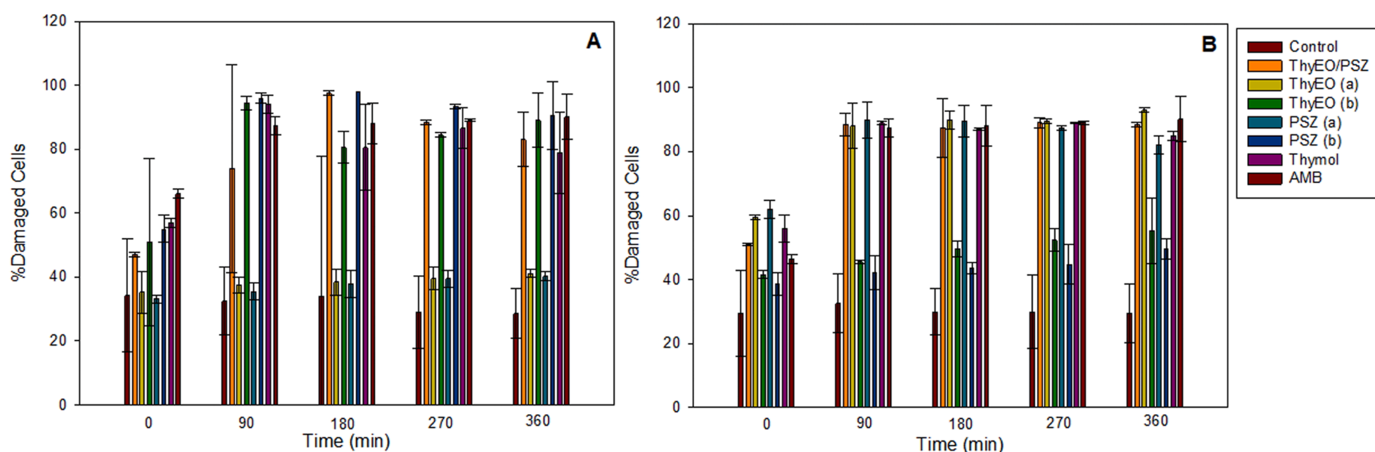

Fig. 2. Percentage (%) of damaged cell membranes of *Candida albicans* **A)** CCC193-13 and **B)** CCC121-16 in the absence (control) and in the presence of Thyme essential oil/Posaconazole mixture [ThyEO/PSZ (31.25/0.0039 μg/ml)], ThyEO (31.25 and 1000 μg/ml), PSZ (0.0039 and 0.0078 μg/ml), and thymol (125 μg/ml) as a function of incubation time. Amphotericin B (AMB, 0.5 μg/ml) was used as a positive control for cell membrane damage. (a) Concentration in mixture; (b) MIC. Results are expressed as mean ± SD (n = 3).

ThyEO/PSZ, ThyEO, PSZ and thymol in the presence of sorbitol, their MICs did not shift to a higher value compared to those without sorbitol, at either of the two incubation times, suggesting that they would not act directly through inhibition of cell wall synthesis or assembly. The MIC of CFG increased 32-fold in the presence of sorbitol (Supplementary Material, Section 2).

Inhibition of C. albicans virulence factors

Inhibitory effect of yeast adherence to BECs

Adherence to an epithelial surface is the first step by which a microorganism can initiate an infection, and is clearly associated with its virulence (Butassi et al., 2019a).

For both strains, a significant decrease ($p < 0.05$) in the number of yeast cells attached to BECs was observed with all samples at sub-inhibitory concentrations (MIC/2) compared to the control (Fig. 3). ThyEO and PSZ alone at the mixture concentration slightly inhibited adhesion to BECs. Interestingly, the inhibition of adherence by ThyEO/PSZ was greater than that of each component alone.

Inhibitory effect of GT and pseudomycelium formation

C. albicans complex has the ability to produce GT or filamentation (hypha) and is associated with virulence and pathogenicity, although all forms may be involved in disease progression (Butassi et al., 2019a). GT and pseudomycelium inhibition were tested to verify whether exposure to sub-lethal concentrations of ThyEO/PSZ reduced these virulence factors. Results clearly indicated that ThyEO/PSZ, ThyEO, PSZ, and thymol (at MIC/2) had no significant effect on the inhibition of GT formation, as there was no significant difference in the % of treated cells that formed GT compared to untreated control cells ($p > 0.05$), in

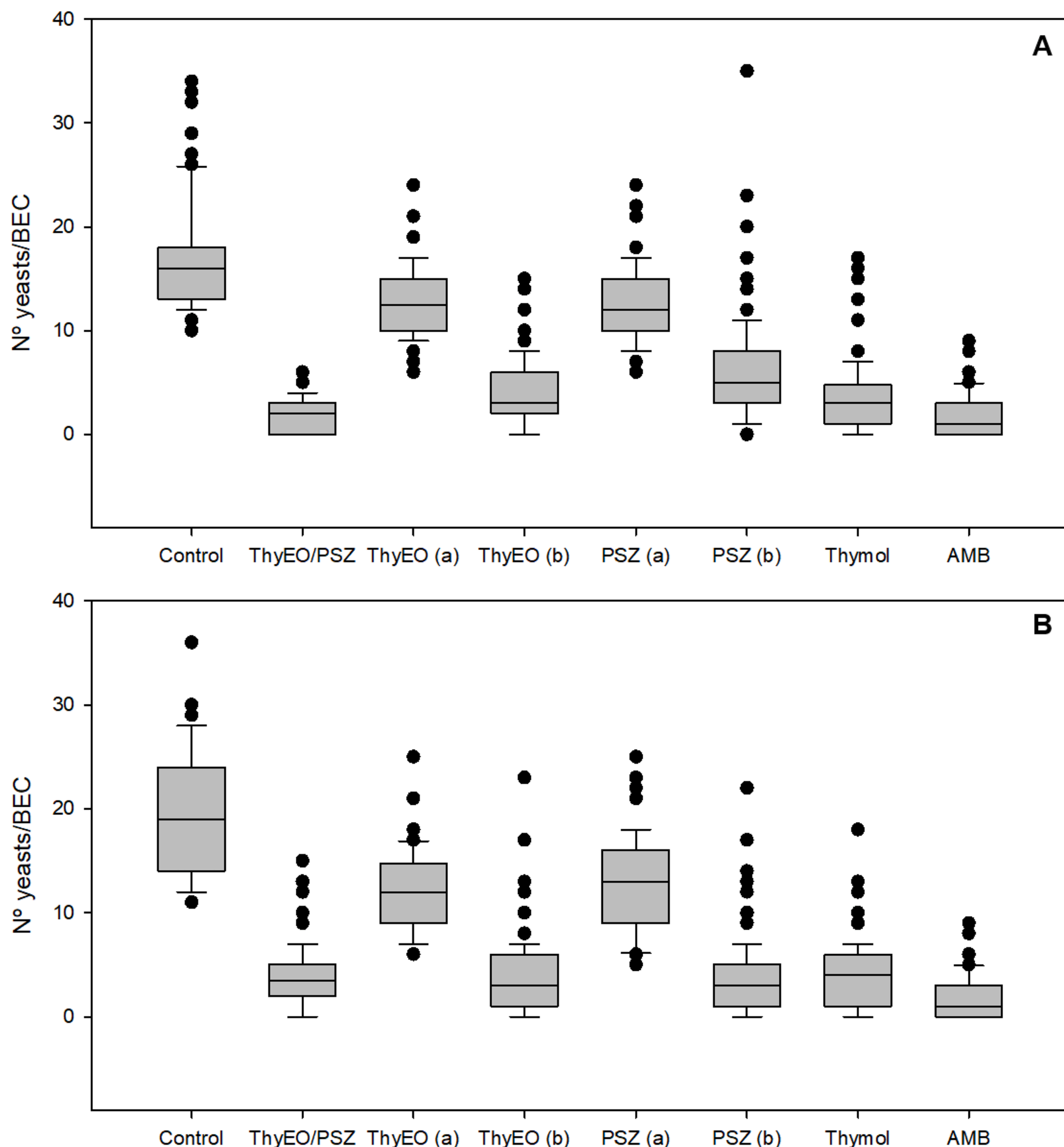

Fig. 3. Adherence values of *Candida albicans* **A)** CCC193-13 and **B)** CCC121-16 to human Buccal Epithelial Cells (BECs) after incubation of the yeast cells in media containing Thyme essential oil/Posaconazole mixture [ThyEO/PSZ (31.25/0.0039 μg/ml)], ThyEO (31.25 and 1000 μg/ml), PSZ (0.0039 and 0.0078 μg/ml), and thymol (125 μg/ml). Amphotericin B (AMB, 0.5 μg/ml) was used as standard positive drug. BECs adherence is expressed as number of yeasts/BEC (N = 100). (a) Concentration in mixture; (b) MIC/2.

contrast to the almost complete inhibition of GT formation by the standard drug AMB (Supplementary Material, Section 3).

On nutrient-poor Spider medium, both *C. albicans* strains formed wrinkled colonies and typical filamentation was observed at their edges (Fig. 4). ThyEO/PSZ (31.25/0.0039 μg/ml), ThyEO (1000 μg/ml) and PSZ (0.0078 μg/ml) reduced the level of filamentation, while thymol produced its complete inhibition.

Inhibitory effect of lytic enzyme secretion

The ability of each sample to inhibit lytic enzyme production by enzyme-producing strains was evaluated. Results showed that only *C. albicans* CCC193-13 secreted phospholipases, hemolysins, and esterases. There was no evidence of proteinase secretion in any of the tested strains.

Regarding the inhibition of phospholipase, hemolysin, and esterase

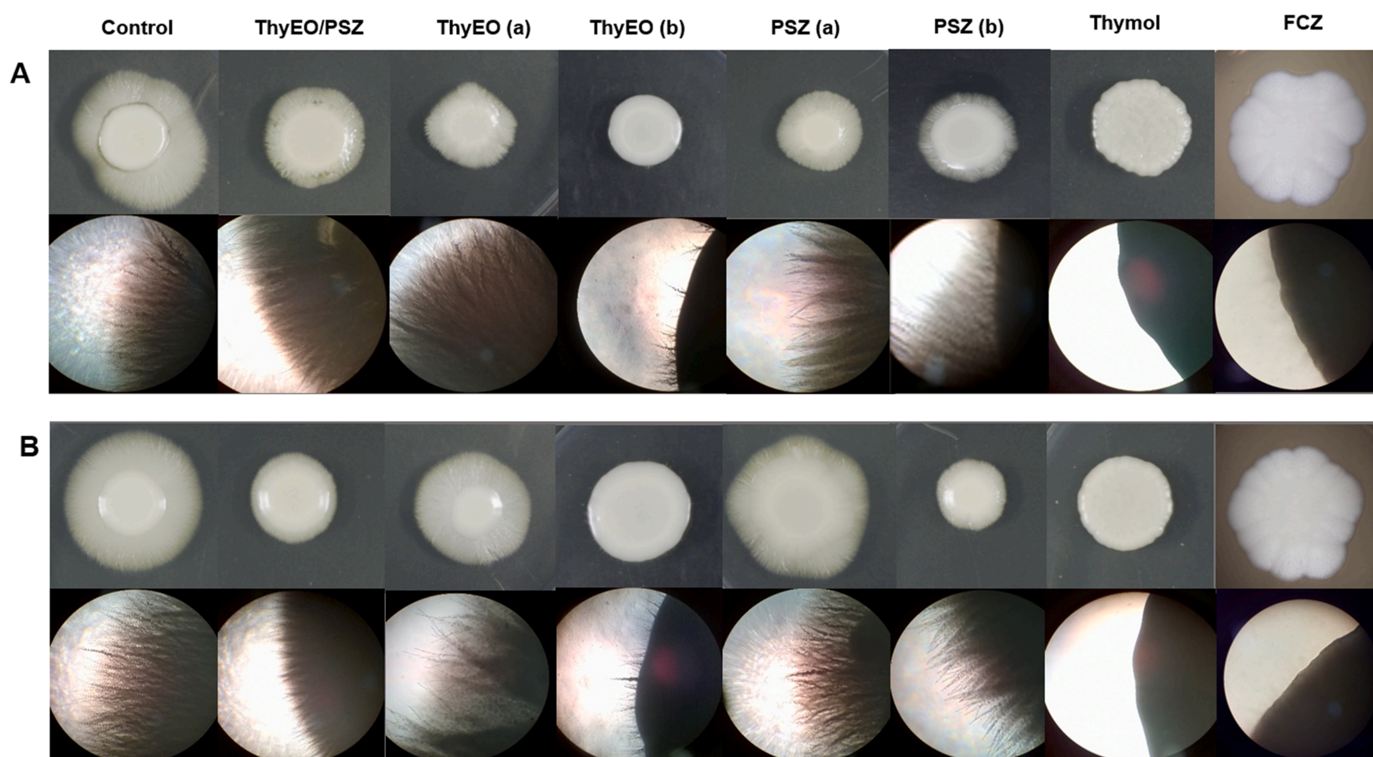

Fig. 4. Different phenotypes of hyphae formation on Spider medium of *Candida albicans* clinical isolates [CCC193-13 (**A**) and CCC121-16 (**B**)] treated with ThyEO/PSZ (31.25/0.0039 µg/ml), ThyEO (31.25 and 1000 µg/ml), PSZ (0.0039 and 0.0078 µg/ml), thymol (125 µg/ml), and FCZ (1 µg/ml). (a) Concentration in mixture; (b) MIC/2. Above: macroscopic observation of agar plates. Bottom: microscopic observation (100x) of colony edges. ThyEO/PSZ: Thyme essential oil/Posaconazole.

production, results showed that ThyEO/PSZ, ThyEO and thymol (at sub-lethal concentrations) did not inhibit their production in the tested strain, since Pz index value remained the same with respect to untreated control. However, PSZ inhibited phospholipase and hemolysin secretion, while production of esterase was strongly positive (Supplementary Material, Section 4).

Inhibitory effect of biofilm formation

C. albicans CCC193-13 was a low biofilm-producing strain and for this reason was excluded from these assays. *C. albicans* CCC121-16, classified as a high biofilm producer, was then used. Prior to conducting the assay, the MIC of ThyEO/PSZ was determined under biofilm-forming conditions (2000/0.25 µg/ml). Then, biofilm-forming capacity was assessed over a range of sub-inhibitory concentrations obtained by two-fold serial dilutions of the mixture and the partners alone. The % biofilm formation of the mixture was compared with ThyEO and PSZ at the same concentration, showing that no significant differences were obtained between ThyEO/PSZ (1000/0.125 µg/ml) and ThyEO (1000 µg/ml) ($p > 0.05$) or PSZ (0.125 µg/ml), suggesting that both partners were responsible for the observed activity (Table 3). Furthermore, the capacity of ThyEO to inhibit *C. albicans* biofilm formation could be attributed to the presence of thymol, since thymol and ThyEO alone achieved almost the same % inhibition.

Inhibitory effect of preformed biofilms

Table 4 shows the % reduction values of preformed *C. albicans* biofilms in the presence of different sample concentrations. The % biofilm reduction of the mixture was compared to that of the individual partners at the same concentration. Results showed no significant difference between ThyEO/PSZ (1000/0.125 µg/ml) and ThyEO (1000 µg/ml) ($p > 0.05$). Significant differences ($p < 0.05$) were observed with respect to PSZ (0.125 µg/ml), suggesting that the ability of the mixture to inhibit the preformed biofilm could be due to the presence of ThyEO. The effects of ThyEO/PSZ and ThyEO were concentration-dependent, as reflected in the progressive reduction in cell viability with increasing concentrations of both. Therefore, the ability of ThyEO to reduce *C. albicans* biofilm could be attributed to the presence of thymol, since thymol and ThyEO alone achieved almost the same % reduction.

Table 3
Percentage inhibition (% inh) of *Candida albicans* CCC121-16 biofilm formation at different concentrations (Conc, µg/ml) of ThyEO/PSZ, ThyEO, PSZ, and thymol.

ThyEO/PSZ		ThyEO		PSZ		Thymol	
Conc	% inh	Conc	% inh	Conc	% inh	Conc	% inh
1000/0.125	81.89 ± 1.17 [a]	1000	81.48 ± 0.31 [a]	0.125	72.19 ± 1.92 [a]	250	81.08 ± 0.75 [a]
500/0.0625	73.07 ± 3.92 [a]	500	70.73 ± 4.92 [a]	0.0625	72.52 ± 3.09 [a]	125	80.48 ± 4.56 [a]
250/0.031	73.09 ± 3.93 [a]	250	79.61 ± 4.72 [a]	0.031	68.98 ± 3.31 [a]	62.5	82.45 ± 0.78 [a]
125/0.0156	63.83 ± 12.18 [a]	125	74.26 ± 3.63 [a]	0.0156	72.44 ± 1.79 [a]	31.25	68.91 ± 4.85 [a]
62.25/0.0078	42.79 ± 8.43 [a]	62.5	58.28 ± 7.24 [a]	0.0078	69.40 ± 2.32 [a]	15.625	37.37 ± 2.70 [a]
31.25/0.0039	43.32 ± 9.89 [a]	31.25	53.90 ± 3.95 [a]	0.0039	68.19 ± 1.34 [a]	7.812	47.31 ± 10.91 [a]
15.63/0.0019	47.53 ± 17.36 [a]	15.63	52.27 ± 2.37 [a]	0.0019	49.99 ± 9.58 [a]	3.91	47.00 ± 5.26 [a]

ThyEO: Thyme essential oil; PSZ: Posaconazole. The control drug AMB (Amphotericin B) produced 92.39% of biofilm formation inhibition at 2 µg/ml. The differences in the percentages of biofilm formation inhibition were compared for the combination and components at the same concentration with a 95% confidence interval (CI). Equal letters indicate that there are no statistically significant differences

Table 4
Percentage of reduction (% red) of preformed biofilm by *Candida albicans* CCC121-16 at different concentrations (Conc, μg/ml) of ThyEO/PSZ, ThyEO, PSZ, and thymol.

ThyEO/PSZ		ThyEO		PSZ		Thymol	
Conc	% red	Conc	% red	Conc	% red	Conc	% red
1000/0.125	75.55 ± 5.00 [a]	1000	71.80 ± 4.95 [a]	0.125	0.88 ± 0.36 [b]	250	60.06 ± 4.95 [a]
500/0.0625	49.60 ± 9.71 [a]	500	57.72 ± 6.89 [a]	0.0625	0.75 ± 0.18 [b]	125	56.51 ± 6.89 [a]
250/0.031	44.62 ± 3.14 [a]	250	53.91 ± 5.16 [a]	0.031	0.63 ± 0.24 [b]	62.5	51.06 ± 5.17 [a]
125/0.0156	42.20 ± 1.10 [a]	125	50.86 ± 7.72 [a]	0.0156	1.02 ± 0.69 [b]	31.25	51.49 ± 7.72 [a]
62.25/0.0078	34.80 ± 0.27 [a]	62.5	33.03 ± 7.63 [a]	0.0078	0.97 ± 0.14 [b]	15.625	39.40 ± 7.63 [a]
31.25/0.0039	19.20 ± 1.20 [a]	31.25	21.54 ± 3.08 [a]	0.0039	0.55 ± 0.33 [b]	7.812	21.09 ± 3.06 [a]
15.63/0.0019	10.23 ± 1.16 [a]	15.63	14.57 ± 4.97 [a]	0.0019	0.73 ± 0.23 [b]	3.91	20.73 ± 4.98 [a]

ThyEO: Thyme essential oil; PSZ: Posaconazole. The control drug AMB (Amphotericin B) produced 82.34% of biofilm preformed inhibition at 4 μg/ml. The differences in the percentages of reduction of preformed biofilm were compared for the combination and components at the same concentration with a 95% confidence interval. Equal letters indicate that there are no statistically significant differences

Antifungal activity against a C. albicans mutant strain

We explored the antifungal effect of the combination and of each component on their own against the *C. albicans* LMDM-526 mutant strain with resistance or reduced susceptibility to commercial antifungals. This clinical strain has an amino acid change in *FKS1* gene, one of the three genes encoding the Fksp subunit of the 1-3-β-D-glucan synthase enzyme complex. First, the MIC and MFC of each commercial antifungal against this mutant strain was determined to corroborate its susceptibility and resistance to all antifungals tested was observed: AMB (MIC/MFC = 2/4 μg/ml), CFG (MIC/MFC = 1/16 μg/ml), FCZ (MIC/MFC = 64/>64 μg/ml), ITZ (MIC/MFC = 0.25/16 μg/ml) and PSZ (MIC/MFC = 0.5/>64 μg/ml) (Supplementary Material, Section 5). Antifungal activity of ThyEO/PSZ, of each ThyEO and PSZ alone, and of the main ThyEO component thymol, was assessed against the mutant strain *C. albicans* LMDM-526. Results showed that all the samples inhibited the growth of this strain (Table 5). As can be seen from FICI and DRI values, ThyEO/PSZ (250/0.125 μg/ml) showed synergism (FICI = 0.5) along with a 4-fold reduction in ThyEO and PSZ doses ($DRI_{ThyEO} = DRI_{PSZ} = 4$) against *C. albicans* LMDM-526. An increase in the antifungal effect of PSZ in the mixture was observed. Notably, the mixture was fungicidal against this strain.

Discussion

Many publications have reported that several human pathogenic fungi, including yeasts, are sensitive to EOs and their chemical components (Raut and Karuppayil, 2014; Swamy et al., 2016). The combination between EOs and antifungal drugs has been considered a strategy to combat fungal development due to the production of synergistic effects (Wagner and Ulrich-Merzenich, 2009). In such combinations, EOs are expected to act as "enhancers" of antifungal activity of commercial drugs, reducing the effective antifungal dose (compared to the dose used alone). This would lead to lower toxicity of antifungals and less chance of fungal resistance developing (Wagner and Ulrich-Merzenich, 2009; Hammer and Carson, 2011; Silva et al., 2011; Rajkowska et al., 2019).

Table 5
MIC and MFC values (μg/ml) of ThyEO/PSZ, its components, and thymol, FICI and DRI values against *Candida albicans* LMDM-526.

Samples	*Candida albicans* LMDM-526					
	MIC	MFC	FICI	Interaction type	DRI_{ThyEO}	DRI_{PSZ}
ThyEO/PSZ	250/0.125	1000/1	0.5	Synergism	4	4
ThyEO	1000	1000				
PSZ	0.5	>64				
Thymol	125	250				

MIC: Minimum Inhibitory Concentration; MFC: Minimum Fungicidal Concentration; FICI: Fractional Inhibitory Concentration Index; DRI: Dose Reduction Index; ThyEO: Thyme essential oil; PSZ: Posaconazole

Based on these previous studies, we selected ThyEO for the evaluation against two *C. albicans* clinical isolates (CCC193-13 and CCC121-16), both alone and in combination with commercial antifungals. Results showed that ThyEO was marginally active against both strains (MIC/MFC = 1000/1000 μg/ml) and displayed additivism when combined with AMB, CFG, FCZ, and ITZ. The most promising mixture was ThyEO/PSZ which showed partial synergism (MIC = 31.25/0.0039 μg/ml) against both strains. All combinations were able to enhance the activity of commercial antifungals by reducing their effective dose (DRI = 2), which would reduce their adverse effects and treatment costs. Furthermore, all mixtures were fungicidal against *C. albicans* strains, which is of great importance since it kills the fungus, preventing recurrence of infection, one of the main problems, especially with azoles as they are fungistatic (Yu et al., 2019). Membrane permeability assay indicated that ThyEO/PSZ, ThyEO, PSZ, and thymol modify the fungal membrane, increasing permeability and compromising integrity due to their lipophilic nature (Nazzaro et al., 2017).

Regarding studies targeting fungal virulence factors, ThyEO/PSZ markedly reduced *C. albicans* adherence to BECs. This is important not only to avoid binding to host tissues but also to prevent biofilm formation (highly resistant to the host immune system and antifungal therapy).

Concerning inhibition of pseudomycelium production, although the mixture and the partners alone achieved partial inhibition, ThyEO showed a more pronounced pseudomycelium inhibition compared to ThyEO/PSZ and PSZ. Notably, thymol completely inhibited pseudohyphae formation, suggesting that the inhibition observed with ThyEO and the mixture could be attributed to its presence. It might be expected that this compound would prevent yeast penetration into host tissues, thus reducing their virulence, although no *in vivo* assays have yet been performed to confirm this.

Biofilm tests indicated that ThyEO/PSZ inhibited the formation and reduced preformed biofilms of *C. albicans*. In both cases, biofilm inhibition could be attributed to the presence of thymol in the EO, since thymol and ThyEO alone achieved almost the same % inhibition. These results emerge as a promising approach, as they target attributes essential for pathogen virulence, rather than affecting viability.

Since several clinical isolates of azole-resistant *C. albicans* have been described (Whaley et al., 2017), it was important to investigate the effectiveness of the mixture against one of them. For this reason, we decided to explore the antifungal effect of each sample against *C. albicans* LMDM-526 mutant strain with resistance to AMB, CFG, FCZ, ITZ, and PSZ. Susceptibility testing of this strain to the individual partners of the mixture and to thymol revealed similar results to those obtained with CCC193-13 and CCC121-16 clinical isolates. Notably, ThyEO/PSZ displayed synergism (MIC = 250/0.125 μg/ml) against this strain, along with a 4-fold reduction in PSZ dose (DRI = 4) and fungicidal activity. These results may be important for future applications of combination therapy, particularly involving azoles, since in addition to being fungistatic and causing recurrence of infection, azoles are also

capable of inducing toxicity and severe adverse reactions, especially when administered simultaneously with other therapeutic drugs in patients with compromised immune system (Martínez-Matías and Rodríguez-Medina, 2018).

PSZ is a systemic triazole antifungal mainly used for prophylaxis of invasive fungal diseases in patients receiving chemotherapy, allogeneic transplantation, and for the treatment of oropharyngeal candidiasis intolerant to first-line therapy. The most frequently reported adverse events during treatment include gastrointestinal disorders, hypokalaemia, hepatotoxicity, and cardiotoxicity (Chen et al., 2020). Therefore, the search for combinations of PSZ and natural products could overcome these problems.

Conclusions

Among the combinations evaluated in this work, ThyEO/PSZ showed partial synergism and fungicidal activity against *C. albicans* strains. This combination altered the fungal cytoplasmic membrane increasing its permeability, decreased the ability of *C. albicans* to adhere to BECs, and reduced the pseudomycelium production. In addition, it inhibited the formation of biofilms and preformed biofilms of *C. albicans* and showed synergistic and fungicidal activity against a resistant strain of *C. albicans*. The findings of the present study reinforce the suggestion that the development of a phytomedicine containing a mixture of an EO and a commercial antifungal agent at suitably low concentrations could be a promising alternative to improve treatments, replace or reduce the use of synthetic antifungals, and lead to further research on natural products, thus taking advantage of their antifungal properties against human pathogens.

CRediT authorship contribution statement

Alan Roy Blanc: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Maximiliano Andrés Sortino:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Estefanía Butassi:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Laura Andrea Svetaz:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data generated or analyzed during this study are included in this published article (and it Supplementary Information file).

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phyplu.2023.100481.

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