Evaluación de la actividad *in vitro* de aceites esenciales destilados de *Lippia origanoides* y algunos de sus compuestos mayoritarios, contra especies de *Candida* con diferentes perfiles de sensibilidad antifúngica y búsqueda de una posible diana

Trabajo de Investigación

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1. Introducción general

Las infecciones fúngicas son causa importante de morbilidad y mortalidad [1,2]. Se calcula que de aproximadamente 2,8 - 3,8 millones de especies de hongos existentes, alrededor de 300 pueden causar infecciones en el hombre; siendo especies de los géneros *Aspergillus* spp., *Cryptococcus* spp. y *Candida* spp. las más frecuentes a nivel mundial. [3,4]

El género *Candida* comprende varias especies [5] algunas de las cuales hacen parte de la microbiota la cavidad oral, del tracto gastrointestinal, vaginal y del pene, principalmente [6–8]. No obstante, bajos determinadas condiciones del hospedero, factores demográficos o practicas clínicas pueden causar diferentes cuados clínicos, principalmente en personas con afectación del sistema inmune o con largas estancias hospitalarias, con altas tasas de morbilidad y de mortalidad [9–11].

Candia albicans fue por muchos años la especie predominante como agente causal de candidiasis; sin embargo, cada vez es más frecuente el registro de otras especies como *C. parapsilosis, C. tropicalis, C. glabrata* (*Nakaseomyces glabrata*) y más reciente *C. auris* [10,12,13]. La candidiasis oral, vulvovaginal, invasiva, la candidemia y la onicomicosis son las infecciones más frecuentes causadas por especies de este género [14].

En Colombia, aunque no se tienen datos que den cuenta de la epidemiologia de las infecciones causadas por *Candida* spp., se ha reportado a *Candida* spp. como uno de los principales agentes de onicomicosis [15][16]. Además, en un estudio realizado en el 2020 se demostró que la candidemia corresponde al 88% de las infecciones fúngicas en pacientes hospitalizados [17] Adicionalmente, Colombia es uno de los países que ha aportado datos a la epidemiologia mundial de las infecciones por *C. auris* en pacientes hospitalizados y de la resistencia a los azoles y a anfotericina B (AMB) [18–21].

La terapia para la candidiasis se basa principalmente en el uso de tres clases de antifúngicos con diferentes mecanismos de acción: los azoles que actúan inhibiendo la enzima lanosterol 14-α-demetilasa involucrada en la vía de síntesis de ergosterol, principal esterol de la membrana celular fúngica, la AMB formando complejos con los lípidos de la

membrana fúngica causando fuga de los componentes celulares e induciendo estrés oxidativo y las equinocandinas inhibiendo la síntesis de β-1,3 D-glucanos en la pared fúngica mediante la inhibición no competitiva de la subunidad catalítica de la β-(1,3) D-glucano sintasa [22]. Aunque estos medicamentos son eficaces contra especies sensibles, la hepatotoxicidad y la nefrotoxicidad han sido asociadas al uso de los azoles y de la AMB, respectivamente [23,24]. Las equinocandinas son menos tóxicas pero la baja absorción gastrointestinal y la necesidad de la aplicación intravenosa suele limitar su uso [25].

El incremento de especies de *Candida* multirresistentes o panresistentes, como el caso de *C. auris*, es una situación que actualmente está complicando el manejo terapéutico de las infecciones causadas por estas levadura y alerta sobre la disminución de alternativas terapéuticas [26,27] El CDC ha estimado que aproximadamente el 7% de los aislados clínicos de casos de candidemia son resistentes [28]. La resistencia antifúngica puede ser el producto de la presión selectiva generada por el uso prolongado de antimicóticos, por la baja adherencia a los tratamientos o por la capacidad para formar biopelículas que tienen algunas especies [29].

En los últimos años se han adelantado estudios con propuestas de nuevos antifúngicos los cuales se encuentran en diferentes fases de estudios clínicos como es el caso del triterpenoide ibrexanfungerp; este antifúngico tiene un mecanismo de acción similar al de las equinocandinas [30], el manogipex, un inhibidor de la inositol aciltransferasa Gwt1 esencial en el anclaje y tráfico de manoproteínas a la membrana y pared fúngica, el olorofim, miembro de las ortomidinas, inhibe la síntesis de pirimidinas y la rezafungina, una equinocandina de segunda generación diseñada con el fin de mejorar las propiedades farmacocinéticas y disminuir la hepatotoxicidad de sus antecesores [31]. Si bien estos fármacos son prometedores, algunos tienen mecanismos o dianas similares a los antifúngicos disponibles o actúan sobre una diana específica, características que pueden hacerlos susceptibles a desarrollar resistencia por parte de los hongos. Lo anterior sumado a la necesidad de obtener antifúngicos menos tóxicos ha motivado a buscar nuevas opciones terapéuticas. La interacción sinérgica puede ser una de ellas ya puede reducir los efectos adversos al disminuir las dosis (potencia sinérgica) y mejorar el resultado aumentando el efecto deseado (eficacia sinérgica) [32,33].

Los productos naturales tienen una amplia diversidad de estructuras químicas complejas muchas de ellas con importante actividad biológica que han servido de base para desarrollar compuestos para el tratamiento de enfermedades como es el caso de los anticancerígenos alcaloide vinca y del terpeno paclitaxel derivados de las plantas *Catharanthus roseus* y *Taxus baccata*, respectivamente, del antimalárico artemisinina (lactona sesquiterpénica) de *Misia annua L* o del reductor del colesterol sanguíneo lovastatina (estatína) obtenido de *Aspergillus terreus* [34,35].

Fármacos con actividad anti-*Candida* spp. como AMB o caspofungina (equinocandinas) fueron identificadas en productos naturales, en el primer caso en la bacteria *Streptomyces nodosus* [36] y en el segundo en hongo *Glarea lozoyensis* [37]. Sin embargo, ya se ha registrado resistencia a ambos antifúngicos. [21,38,39].

Dentro de los productos naturales de interés actualmente con actividad anti-*Candida* spp. se encuentran los aceites esenciales (AE), sus fracciones o componentes mayoritarios [40]–[46]. Los AE son metabolitos secundarios producidos por más de 17.000 especies de 60 familias de plantas, su síntesis obedece a mecanismos de adaptación e interacción entre las plantas y el ambiente biótico y cumplen funciones protectoras contra bacterias, virus y hongos patógenos [47,48]. Los AE son mezclas volátiles de diversa naturaleza química, principalmente terpenos y fenilpropanoides [47,49,50]. A los terpenos (mono y sesqui terpenos) y sus compuestos oxidados (alcoholes, óxidos, aldehídos, cetonas, fenoles, ácidos, esteres y lactonas [51] [47], se les atribuyen las propiedades biológicas y el olor característico de los AE [51]. Los monoterpenos carvacrol, timol, *p*-cimeno, eugenol y limoneno son algunos de los derivados de AE con mayor actividad biológica reportada [52–55].

Los AE se obtienen por hidrodestilación de diferentes partes de la planta como flores, hojas, frutos, semillas, raíces, rizomas, madera, corteza, bulbos y capullos de flores secas [47]; factores como las variaciones en los diferentes estados de desarrollo de la planta, la altitud, la temporada de cosecha, la parte de la planta utilizada para la destilación, las modificaciones estructurales y fisiológicas de la planta, pueden afectar la composición química y cantidad de metabolitos secundarios de los AE, [49,56].

La enorme diversidad vegetal en Colombia y el conocimiento generado a partir de esta, está siendo utilizado como fuente de recursos para identificar compuestos bioactivos y avanzar en el desarrollo sostenible del país [57–61]. El enfoque biorracional basado en la etnobotánica dirigida usando información de la planta tal como quimiotipo, similitud estructural y bioactividad informada puede ser usado como una estrategia exitosa en el descubrimiento de productos terapéuticos a partir de recursos naturales [62]. En la riqueza vegetal de Colombia pueden identificarse compuestos con potencial antifúngico con estructuras químicas o mecanismos de acción diferentes a los existentes actualmente. En ese sentido, en extractos y AE esenciales obtenidos de plantas, recolectadas en diferentes regiones en Colombia se ha encontrado actividad anti-*Candida* spp. [63–65].

Una de las plantas de interés en Colombia es *Lippia origanoides* (Verbenaceae); popularmente conocida como "orégano de monte", el uso tradicional de la infusión de sus hojas y flores para aliviar trastornos estomacales o como antiséptico de boca y garganta [66–68], da cuenta de sus propiedades curativas. Además, estudios *in vitro* han demostrado las diversas actividades biológicas de los AE de esta planta. [40,65,69–71]

El estudio de la composición de los AE destilados de *L. origanoides* ha demostrado su diversidad química en términos del contenido de monoterpenos, lo que ha permitido clasificarlos en tres diferentes quimiotipos [56]. En los quimiotipos carvacrol y timol se ha encontrado actividad antibacteriana, anti-*Candida* y antibiopelículas [65,72]. La actividad antimicrobiana de amplio espectro del timol y el carvacrol ha sido atribuida a la presencia del grupo hidroxilo en su estructura fenólica [73,74] no obstante, se ha demostrado que la interacción sinérgica entre los diferentes componentes de los AE juega un papel importante en su actividad antimicrobiana y citotóxica [75]. Investigaciones sobre los efectos de los AE y los terpenos en el fenotipo fúngico han relacionado su actividad con la naturaleza lipofílica de estos compuestos, esto facilita la interacción con las membranas lipídicas, aumenta la permeabilidad y ocasiona fuga de los componentes celulares [76–79].

El metabolismo de los lípidos además de ser clave en la biogénesis de membrana fúngica también está relacionado con la señalización celular, el metabolismo bioenergético, la biogénesis de pared fúngica y la división celular [80–83]. Se ha descrito que el timol, el carvacrol, así como otros terpenos disminuyen la cantidad de ergosterol en la membrana de

Candida spp. [77,84]. Por sus múltiples funciones, el ergosterol es considerado como una "hormona fúngica", además de ser el blanco algunos de los antifúngicos disponibles actualmente [22,81]; su disminución conduce a la falta de viabilidad celular, sin embargo, la resistencia a múltiples fármacos está asociada a los bajos niveles del esterol fúngico como consecuencia de fallas en alguna de las enzimas de la vía biosintética del ergosterol [80,85]. Los dominios proteicos transmembrana tienen funciones biosintéticas, de transporte, homeostasis lipídica, mantenimiento de la morfología fúngica o señalización celular, algunas enzimas presentes en la membrana plasmática son blanco de fármacos [31,86,87]; por lo tanto, cambios en la organización en la estructura lipídica o proteica de la membrana plasmática influye en el funcionamiento de dicha estructura llevando a perdida de la viabilidad celular.

La mitocondrial se ha propuesto como un objetivo prometedor para el desarrollo de nuevos fármacos antifúngicos [88,89]. Además de ser clave en la producción de energía, las mitocondrias también se asocian con la virulencia fúngica, homeostasis de lípidos y apoptosis [88]. El deterioro de la función mitocondrial cambia la composición lipídica de la membrana plasmática, afectando las enzimas biosintéticas que se encuentran en ella [90] se ha descrito la desregulación de la homeostasis de fosfolípidos mitocondriales como un desestabilizador de la pared fúngica [91,92]. Los estudios de los efectos en *Candida* spp. de algunos AE y sus derivados, han demostrado su capacidad para disminuir el potencial de membrana mitocondrial [93–96], sin embargo, la información sobre el mecanismo especifico aun es escasa. Debido a su importancia en la supervivencia fúngica el ciclo del glioxilato se ha estudiado como un posible objetivo mitocondrial prometedor para el desarrollo de nuevas terapias antifúngicas, esta vía no está presente en mamíferos por lo tanto podría ser una diana especifica de los hongos [97]. La integridad del ADN, la señalización del calcio, el aumento del estrés oxidativo y el arresto en diferentes fases del ciclo celular también se han propuesto como posibles dianas de los AE y los terpenos [76,98,99].

Aunque en la última década se ha avanzado en las investigaciones sobre las posibles dianas antifúngicas de los AE y terpenos, la información es escasa [98]. El enfoque basado en los efectos fenotípicos de estos compuestos da pistas sobre los posibles objetivos

fúngicos, sin embargo, no es posible establecer los blancos moleculares implicados, por lo tanto, se dificulta enforcar las investigaciones [100]; en ese sentido el análisis de la expresión génica puede ser usada para identificar las vías metabólicas o genes de importancia en la actividad antifúngica. En la actualidad se han explorado poco los efectos a nivel transcriptómico, proteómico o metabolómico de AE o terpenos con actividad antifúngica.

Con base a lo anterior, este proyecto busca determinar la actividad anti-*Candida* spp. de los AE de *L. origanoides* y algunos terpenos, así como, evaluar su toxicidad, elucidar sus efectos fenotípicos y conocer sus posibles objetivos moleculares, con el fin de aportar información relevante que pueda ser utilizada para futuros desarrollos farmacológicos.

2. Objetivos

2.1. Objetivo general

Evaluar la actividad *in vitro* de aceites esenciales destilados de *Lippia origanoides* y algunos de sus compuestos mayoritarios contra especies de *Candida* con diferentes perfiles de sensibilidad antifúngica y búsqueda de una posible diana.

2.2. Objetivos específicos

2.2.1. Evaluar la actividad antimicótica *in vitro* de aceites esenciales destilados de plantas cultivadas en Colombia y terpenos contra cepas de diferentes especies de *Candida*.

2.2.2. Evaluar la toxicidad de los aceites esenciales y terpenos más activos

2.2.3. Evaluar el efecto de aceite esenciales o terpeno con mejor actividad anti-*Candida* en combinación con el fluconazol contra cepas de *C. tropicalis* con diferentes perfiles de sensibilidad antifúngica

2.2.4. Evaluar el efecto del aceite esencial y el terpeno más activo en combinación con fluconazol sobre la función o estructuras de la célula fúngica

2.2.5. Evaluar la expresión génica en *C. tropicalis* luego de la exposición al aceite esencial o terpeno con mayor actividad antifúngica.

El objetivo específico 1 se cumple con el artículo # 1; el objetivo específico 2 se cumple con el artículo # 1 y la propuesta de artículo #3; los objetivos específicos 3 y 4 se cumplen con el artículo # 2; el objetivo específico #5 se cumplen con la propuesta de artículo #4

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3. Artículo 1.

In vitro activity of essential oils distilled from Colombian plants against *Candida auris* and other *Candida* species with different antifungal susceptibility profiles

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Abstract: Multi-drug resistant species such as *Candida auris* are a global health threat. This scenario has highlighted the need to search for antifungal alternatives. Essential oils (EOs), or some of their major compounds, could be a source of new antifungal molecules. The aim of this study was to evaluate the in vitro activity of EOs and some terpenes against *C. auris* and other *Candida* spp. The eleven EOs evaluated were obtained by hydro-distillation from different Colombian plants and the terpenes were purchased. EO chemical compositions were obtained by gas chromatography/mass spectrometry (GC/MS). Antifungal activity was evaluated following the CLSI standard M27, 4th Edition. Cytotoxicity was tested on the HaCaT cell line and fungal growth kinetics were tested by time–kill assays. *Candida* spp. showed different susceptibility to antifungals and the activity of EOs and terpenes was strain-dependent. The *Lippia origanoides* (thymol + *p*-cymene) chemotype EO, thymol, carvacrol, and limonene were the most active, mainly against drug-resistant strains. The most active EOs and terpenes were also slightly cytotoxic on the HaCaT cells. The findings of this study suggest that some EOs and commercial terpenes can be a source for the development of new anti-*Candida* products and aid the identification of new antifungal targets or action mechanisms.

Keywords: *Candida auris*; *Candida* spp; *Lippia* spp; antifungal agents; essential oils; MIC; cytotoxicity; time–kill assays

3.1. Introduction

In recent decades, there has been a notorious increase in infections caused by naturally resistant *Candida* spp. or by strains that have developed resistant phenotypes during treatment [1]. *Candida albicans, C. tropicalis, C. parapsilosis,* and *C. glabrata* are still the most frequent species causing infections. However, multi-resistant emerging *Candida* spp., such as *C. auris,* have been increasingly reported. *C. auris* is associated with high morbidity and mortality rates, almost exclusively in hospital settings [2]. The management of these infections is challenging due to the resistance of *C. auris* to several antifungals [3–5]. Additionally, these yeasts have the ability to form biofilms on medical devices and

biomaterials, such as catheters and heart valves [1]. *C. auris* can also persist in hospital environments causing outbreaks, mainly in intensive care units (ICU) [2]. The selection of resistant isolates of different *Candida* spp. and the emergence of pan-resistant or multi-resistant species threaten the future management of fungal infections due to the scant antifungal options for clinical use [5,6]. Moreover, most antifungal drugs have a narrow spectrum and cause considerable side-effects [7]. This scenario has increased the amount of interest in exploring new molecules targeting different cellular components.

Natural sources, such as plant extracts and essential oils (EOs), may be effective alternatives in the search for new antifungal agents [8,9]. EOs are volatile secondary metabolites distilled from aromatic plants and are molecules of different chemical natures, mostly terpenoids and phenylpropanoids [10]. The antifungal properties of different EOs, as well as their compounds, have been previously demonstrated [11].

Colombia is among the top ten most biodiverse countries in the world and ranks second in plant diversity. In 2020 there were 30,014 recorded plant species, of which 6499 were endemic [12]. The enormous biodiversity of Colombia becomes an invaluable source of natural bioactive compounds [13], within which it may be possible to find some active compounds against clinically relevant fungi, including multi-drug or pan-drug resistant species. *Lippia origanoides* and *L. alba* (both from the Verbenaceae family), growing in various countries of South America, including Colombia [12,13], are some of the most studied neo-tropical plants due to the different biological activities that their EOs have displayed, including antifungal properties [14].

Two chemotypes of *L. alba* growing in Colombia have been identified: the (citral + caryophyllene oxide) chemotype and the (carvone + limonene) chemotype, named by their major EO compounds [15,16]. The anti-inflammatory and antimicrobial activities of these EOs, some of their enriched fractions, and commercial terpenes have previously been studied [15,17].

This study focuses on investigating the in vitro effects of EOs distilled from Colombian plants of the Verbenaceae family, and some of their major compounds, against clinical isolates of *C. auris* and other *Candida* spp. with different antifungal susceptibility to the

most common antifungal drugs. The chemical composition, cytotoxicity, and fungal growth kinetics of the most active EOs and commercial terpenes were evaluated.

3.2. Materials and methods

3.2.1. Plant materials and essential oil distillation

All plants used in this work were cultivated under controlled agricultural conditions in the experimental plots of the garden of the National Center for Agroindustrialisation of Aromatic and Medicinal Tropical Plant Species (CENIVAM) at the Industrial University of Santander (UIS, Bucaramanga, Colombia). The taxonomic identification was performed at the Colombian National Herbarium (National University of Colombia, UN, Bogotá, Colombia) and at the UIS Herbarium. The exsiccatae and vouchers were placed at the UIS Herbarium. The EOs were distilled from different chemotypes of *L. origanoides* (Codes 2206, 0008, 0010, 0018, and 0019). The voucher numbers of these plants are shown in **Table 1**. EOs were also distilled from the following plants: the *L. alba* citral chemotype (Code 0046; 22002 UIS Herbarium), *L. micromera* (Code 0020, sample in Herbarium), *V. curassavica* (Code 0042; 20892 UIS Herbarium), *P. marginatum* Jacq (Code 0024; 21966 UIS Herbarium), *A.* cf. *popayanensis* (Hieron) R. King & H. Rob (Code 0034; 22040 UIS Herbarium), and *P. cablin* (Code 0049; 20890 UIS Herbarium). Plants were initially collected in the countryside in Barbosa, Betulia or in San Vicente de Chucurí (Santander, Colombia), propagated, and grown in the CENIVAM experimental plots. The mean environment temperature varied between 26 and 29 °C, with relative humidity of 75–80%. The plants were gathered in their flowering stages and only undamaged aerial plant parts were used for EO extraction by hydro-distillation. The hydro-distillation was carried out immediately after the vegetable material was harvested, without its previous drying or weathering. EOs were distilled (2 h) from fresh plant material (500 g) on a Clevenger apparatus. The EOs were dried using anhydrous sodium sulphate and kept under nitrogen atmosphere at 4 °C in darkness.

3.2.2. Sample preparation

Each EO was weighed (50 mg) and dissolved in 1 mL of CH_2Cl_2; an aliquot of this dilution (2 µL) was injected into a gas chromatograph (GC) coupled to a mass selective (MS) or flame ionization (FID) detection system.

3.2.3. Chromatographic analysis

Analysis was performed on a GC 6890 Plus gas chromatograph (Agilent Technologies, AT, Palo Alto, CA, U.S.A.) equipped with a mass selective detector MS 5973 Network (AT, Palo Alto, CA, U.S.A.) using electron ionization (EI, 70 eV). Helium (99.995%, AP gas, Messer, Bogotá, Colombia) was used as a carrier gas, with initial inlet pressure at the head of the column of 113.5 kPa; the volumetric flow rate of the carrier gas during the chromatographic run was kept constant (1 mL/min). The injection mode was split (30:1) and the injector temperature was kept at 250 °C.

Compound separation was carried out on two capillary columns, one with the polar stationary phase of poly(ethylene glycol) (PEG) (DB-WAX, J & W Scientific, Folsom, CA, U.S.A.) of 60 m (L) × 0.25 mm (I.D.) × 0.25 μm (d_f), and another with a non-polar stationary phase of 5%-phenyl-poly(methyl siloxane) (5%-Ph-PDMS) (DB-5MS, J & W Scientific, Folsom, CA, U.S.A.) of the same dimensions. In the polar column (DB-WAX), the oven temperature was programmed from 50 °C (5 min) to 150 °C (7 min), at 4 °C/min, and then up to 230 °C (50 min) at 4 °C/min. For the non-polar column (DB-5MS), the temperature of the chromatographic oven was programmed from 45 °C (5 min) to 150 °C (2 min), at 4 °C/min, then up to 300 °C (10 min) at 5 °C/min. The temperature of the GC-MS transfer line was set at 230 °C when the polar column was used and at 300 °C for the non-polar column. The temperatures of the ionization chamber and the quadrupole were 250 °C and 150 °C, respectively. The mass range for the acquisition of ion currents was m/z 45–450 u, with an acquisition speed of 3.58 scan/s. Data were processed with MSD ChemStation G1701DA software (AT, Palo Alto, CA, U.S.A.). The identification of compounds was carried out based on their linear retention indices (LRI), calculated from the retention times of the compound of interest and the C_6-C_{25} and C_8-C_{40} n-alkanes (Sigma-Aldrich, San Luis, MO, U.S.A.) on both polar and non-polar capillary columns.

For EO component identification, the experimental mass spectrum of each compound was compared to that from QUADLIB-2007, NIST (2017), and Wiley (2008) spectral databases. Confirmatory identification of some compounds was made by comparison of their LRIs and mass spectra with those of available standard substances. For the quantitative analyses (relative amounts, %), the EO samples, prepared as described above,

were injected into the GC 6890 Plus gas chromatograph (AT, Palo Alto, CA, U.S.A.) coupled to a flame ionization detection system (GC/FID) and a non-polar 5%-Ph-PDMS capillary column (DB-5MS, J & W Scientific, Folsom, CA, U.S.A.) of the same dimensions (L, I.D., d_f) as that used for the GC/MS analysis. The GC-FID oven temperature was programmed in a similar manner as for the GC-MS equipment described previously; the EO samples were injected in split mode (30:1) and the temperatures of the injection port and the FID were maintained at 250 °C.

3.2.4. Antifungals

For antifungal profile identification of *Candida* species, amphotericin B (AMB), fluconazole (FLC), itraconzole (ITC), and caspofungine (CSF) (Sigma-Aldrich, St. Louis, MO, U.S.A.) were tested.

3.2.5. Essential oils and terpenes

The antifungal activity of eleven EOs and eight commercial terpenes was evaluated *in vitro*. EOs were distilled from five plants of different *L. origanoides* chemotypes (Codes 2206, 0008, 0010, 0018, and 0019), the *L. alba* citral chemotype (Code 0046), *L. micromera* (Code 0020), *V. curassavica* (Code 0042), *P. marginatum* (Code 0024), *A.* cf. *popayanensis* (Code 0034), and *P. cablin* (Code 0049). The terpenes tested were limonene (97%), carvacrol (98%), thymol (98.5%), *p*-cymene (99%), perillyl alcohol (96%), carveol, mixture *cis* and *trans* (≥95%), verbenone (≥99%), and trans-*β*-caryophyllene (98.5%) (Sigma-Aldrich St. Louis, MO, U.S.A.). A stock solution of each sample was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich St. Louis, MO, U.S.A.).

3.2.6. Fungi

The fungal strains used in this study were *C. albicans* ATCC 10231, *C. albicans* ATCC 64550, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 750, *C. tropicalis* ATCC 200956 (resistant to azoles and AMB) [35], *C. glabrata* LMDM 34 (resistant to echinocandins) [36], *C. metapsilosis* MUM 15.12, *C. orthopsilosis* MUM 17.13, *C. lusitaniae* MUM 17.08, *C. krusei* ATCC 6258 (*Issatchenkia orientalis* ATCC 6258), and *C. auris* CDC B11903. Additionally, nine clinical isolates were included: *C. parapsilosis* Synlab 406 (FLC-resistant) and eight *C. auris* isolates identified by MALDI-TOF MS (Bruker Daltonics,

Bremen, Germany) according to Zhao *et al.* [19]. These yeasts were cultured on Sabouraud Dextrose Agar (SDA; Sigma-Aldrich, St. Louis, MO, U.S.A.) for 24 h at 35 °C.

3.2.7. Antifungal susceptibility testing (antifungals, EOs, and terpenes)

Antifungal susceptibility testing was performed according to Clinical and Laboratory Standards Institute M27, 4th Edition (CLSI standard M27, 4th Edition) [20]. Some modifications were applied for evaluation of the antifungal activity of EOs and terpenes. Initially, a screening was carried out to determine the antifungal activity of the EOs and of commercial terpenes. Stock concentrations of EOs and terpenes were prepared at 512 µg/mL and an inoculum of 2.5 × 10^3 CFU/mL of each yeast was prepared in RPMI 1640-MOPS (Sigma- Aldrich St. Louis, MO, U.S.A.). Thereafter, 100 µL of each stock solution of EOs or terpenes was dispensed in 96-well microdilution plates (Corning®, Costar ®, N.Y., U.S.A.) and 100 µL of each yeast inoculum was added. Hence, the tested concentration of the EOs and terpenes was 256 µg/mL, and the concentration of the inocula was 1.25 × 10^3 CFU/mL. Microdilution plates were incubated at 35 °C and, after 24 h, the presence or absence of growth was visually checked using a manual mirror viewer and then compared with the amount of growth in the control (no EO or terpene). Compounds able to inhibit yeast growth were tested to determine the minimal inhibitory concentration (MIC) as follows: 100 µL of 10 two-fold dilutions of the EOs or commercial terpenes was dispensed in 96-well microplates and the yeast inoculum was added. Final concentrations of the EOs or terpenes ranged from 0.5 µg/mL to 256 µg/mL. Microdilution plates were incubated at 35 °C for 24 h. MICs were visually determined at the lowest concentration that produced visual inhibition compared to the growth control. The assays were performed at least three times in duplicate on different days. The results were expressed as geometric means (GM) and ranges. As an antifungal susceptibility testing control, the activity of ITC and AMB against the reference strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 was evaluated in all experiments following the CLSI standard M27, 4th Edition [32]. It was mandatory that the MIC values remained in the accepted range.

3.2.8. Cytotoxicity of the essential oils and terpenes

The cytotoxicity of the most active EOs and commercial terpenes studied was tested on a non-tumor keratinocytes HaCaT cell line. The non-tumor keratinocytes HaCaT cell line

was derived from primary epidermal keratinocytes from normal human adult (HEKa) PCS-200-011TM and was obtained from Dr. Juan Carlos Gallego-Gómez (Molecular and Translational Medicine Group, Universidad de Antioquia). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, U.S.A.), 1% penicillin, streptomycin, and neomycin (Invitrogen, Carlsbad, CA, U.S.A.), and 1% L-glutamine (Invitrogen, Carlsbad, CA, U.S.A.) at 37 °C in a humidified atmosphere of 5% CO_2. Once the confluence of the cells reached 80%, the cells were dissociated using trypsin (Sigma-Aldrich, St. Louis, MO, U.S.A.) and subcultured in 96-well microplates at a density of 1.6×10^4 cells per well at 37 °C for 24 h with 5% CO_2. Subsequently, the cells were treated with concentrations of selected compounds in a range between 0.25 and 2000 µg/mL at 37 °C for 24 h with 5% CO_2. Afterwards, the culture medium was removed and 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, U.S.A.) at 5 mg/mL was added at 37 °C for 2 h. Subsequently, DMSO was added to dissolve the formazan crystals. Finally, the absorbances were measured with a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.) at $\lambda = 570$ nm. The CC_{50} values were obtained by linear regression analysis with concentration–response curves, which were performed with absorbance data using GraphPad Prism software (Prism 9.3.0, San Diego, CA, U.S.A.). The assays were conducted at two separate times in triplicate. The data were expressed as means. Additionally, the SI values were calculated by dividing CC_{50} values by the MIC values.

3.2.9. Time–kill assays

Time–kill assays with the *L. origanoides* (thymol + *p*-cymene) chemotype (Code 0018) EO, thymol, FLC, and AMB against *C. albicans* ATCC 10231, *C. tropicalis* ATCC 200956, and *C. auris* CDC B11903 were performed according to the protocol published by Klepser *et al.* [21] with some modifications. Strains were subcultured at 35 °C for 24 h on SDA; therefore, 100 µL of different compound concentrations (0.5, 1, and 2X MIC) and 100 µL of a suspension of 5×10^5 CFU/mL were added in 96-well microplates and were incubated at 35 °C for 24 h. Absorbance at $\lambda = 490$ nm was measured every 2 h with a

Multiskan SkyHigh Microplate Spectrophotometer. AMB and FLC were included as fungicidal and fungistatic control drugs, respectively.

3.3. Results

3.3.1. Essential oil composition

Eleven EOs distilled from different *L. origanoides* chemotypes (*L. alba*, *Varronia curassavica*, *Piper marginatum*, *Ageratina* cf. *popayanensis*, and *Pogostemon cablin*) were chemically characterized by GC/MS. Information regarding the plants from which the most active EOs were obtained, plant chemotypes, and EO chemical compositions are shown in **Table 1.**

Table 1. Plant information, chemotypes, and chemical compositions of the most active EOs.

Code	Plant Species and Chemotype	Collection Site	Voucher Number	Principal Compounds
2206	*L. origanoides* (Carvacrol + thymol) chemotype	Barbosa — Santander, Colombia	COL 587104	**Carvacrol (34.9%), thymol (23.3%)** *γ*-terpinene (11.1%), *p*-cymene (9.0%), *trans*-*β*-caryophyllene (5.0%), *α*-humulene (2.5%), *α*-terpinene (1.8%), *β*-myrcene (1.7%), thymyl methyl ether (1.6%), and carvacryl acetate (0.8%).
0008	*L. origanoides* (Carvacrol + *p*-cymene) chemotype	Bucaramanga — Santander, Colombia	22034 UIS Herbarium	**Carvacrol** (**35.0%), *p*-cymene (14.4%),** thymol (8.0%), *γ*-terpinene (5.3%), *trans*-*β*-caryophyllene (4.4%), *β*-myrcene (2.4%), carvacryl acetate (2.0%), thymyl methyl ether (1.9%), *α*-terpinene (1.7%), and *α*-thujene (1.6%).
0010	*L. origanoides* Thymol chemotype	Bucaramanga — Santander, Colombia	22035 UIS Herbarium	**Thymol (75.3%),** *trans*-*β*-caryophyllene (5.4%), carvacrol (4.9%), *α*-humulene (3.2%), *p*-cymene (2.3%), thymyl acetate (1.6%), thymyl methyl ether (1.3%), caryophyllene oxide (1.3%), and *trans*-*β*-bergamotene (1.0%).
0018	*L. origanoides* (Thymol + *p*-cymene) chemotype	Bucaramanga — Santander, Colombia	22039 UIS Herbarium	**Thymol** (**49.4%), *p*-cymene (19.1%)**, *γ*-terpinene (9.2%), *β*-myrcene (5.2%), *α*-terpinene (2.9%), carvacrol (2.7%), thymyl methyl ether (1.8%), *trans*-*β*-caryophyllene (1.6%), *cis*-*β*-ocimene (1.2%), and limonene (0.9%).
0019	*L. origanoides* Thymol chemotype	Bucaramanga —Santander, Colombia	22036 UIS Herbarium	**Thymol** (**71.7%**), *p*-cymene (10.5%), carvacrol (4.4%), *β*-myrcene (2.1%), *γ*-terpinene (2.0%), caryophyllene oxide (1.6%), thymyl methyl ether (0.9%), *trans*-*β*-caryophyllene (0.9%), humulene epoxide II (0.7%), and terpinen-4-ol (0.7%).

UIS: Industrial University of Santander (Bucaramanga, Colombia).

Candida spp. strains and clinical isolates displayed different antifungal susceptibility profiles. The minimal inhibitory concentration (MIC) values for amphotericin B (AMB),

fluconazole (FLC), itraconazole (ITC), and caspofungin (CSF) are presented in Table 2. The azole and AMB resistance of *C. tropicalis* ATCC 200956 was confirmed, as well as the resistance to CSF and FLC of *C. glabrata* LMDM 34 and *C. parapsilosis* Synlab 406, respectively. The susceptibility of *C. auris* was strain-dependent. High MIC values to FLC and AMB were obtained. According to the Centers of Disease Control and Prevention (CDC) breakpoints [18], *C. auris* Ca 41, *C. auris* Ca 45, and *C. auris* Ca 46 were considered AMB-resistant (MIC range 1–2 µg/mL) and *C. auris* Ca 17 FLC-resistant (MIC = 32 µg/mL).

Table 2. Minimal inhibitory concentration values of the most active EOs studied and of some commercial terpenes against *Candida* species.

Species	MIC (µg/mL)								Source
	AMB		ITC		FLC		CSF		
	Range	GM	Range	GM	Range	GM	Range	GM	
C. albicans ATCC 64550	0.03–0.12	0.04	0.5–1	0.7	4–8	5.6	0.25–0.5	0.35	Collection
C. albicans ATCC 10231	0.03–0.12	0.04	0.03–0.125	0.06	4	4	0.12–0.25	0.18	Collection
C. parapsilosis ATCC 22019	0.06	0.06	0.25–0.50	0.35	0.5–1	0.70	1	1	Collection
C. krusei ATCC 6258	0.12–0.25	0.15	0.12–0.50	0.28	8	8	1	1	Collection
C. tropicalis ATCC 750	0.06–0.12	0.11	0.06–0.25	0.12	1–2	1.4	0.25–0.5	0.35	Collection
C. tropicalis ATCC 200956	1–2	1.41	>16	>16	>64	>64	0.5	0.5	Collection
C. glabrata LMDM 34	0.06–0.12	0.06	1	1	2–4	2.8	8	8	Collection
C. metapsilosis MUM 15.12	<0.03	<0.03	0.06–0.12	0.09	1–2	1.4	1	1	Collection
C. orthopsilosis MUM 17.13	<0.03	<0.03	0.12	0.12	1	1	1	1	Collection
C. lusitaniae MUM 17.08	0.03	0.03	0.03–0.06	0.04	0.5	0.5	1	1	Collection
C. auris CDCB11903	0.06–0.12	0.07	0.06–0.12	0.04	1–2	1.4	0.5	0.5	Collection
C. auris Ca 1	0.12–0.25	0.20	0.03–0.12	0.06	4	4	0.5	0.5	Subcutaneous tissue
C. auris Ca 9	0.12–0.5	0.25	0.03–0.12	0.06	4	4	0.5	0.5	No data available
C. auris Ca 10	0.12–0.25	0.18	0.06–0.12	0.09	4	4	0.5–1	0.7	Pleura tissue
C. auris Ca 32	0.12–0.5	0.19	0.25–0.50	0.35	8–16	11.3	0.5	0.5	Parietal pleura
C. auris Ca 41	1	1	0.03–0.12	0.06	2	2	0.5	0.5	Groin smear
C. auris Ca 45	1–2	1.41	0.06–0.12	0.04	2	2	0.5	0.5	Axillary smear
C. auris Ca 46	1–2	1.4	0.06–0.12	0.04	2–4	2.8	0.5	0.5	Axillary smear
C. auris Ca 17	0.06–0.25	0.15	0.12–0.5	0.25	32	32	0.5	0.5	Urine culture
C. parapsilosis Synlab 406	0.06–0.25	0.15	0.5–1	0.70	8–16	11.3	2	2	Blood culture

GM: geometric mean; LMDM: Laboratorio de Micología y Diagnóstico Molecular; MUM: Micoteca da Universidade do Minho; AMB: amphotericin B; ITC: itraconazole; FLC: fluconazole; CSF: caspofungin.

3.3.2. Antifungal Activity of Essential Oils and Terpenes

The results of the screening of the eleven EOs and the eight commercial terpenes at 256 µg/mL against the twenty *Candida* spp. strains are shown in **Figure 1**. Antifungal activity was strain-dependent. Limonene, thymol, and carvacrol were active against all tested *Candida* spp. Perillyl alcohol and p-cymene were active against 90% and 100% of *C. auris*, respectively, while verbenone, carveol, and trans-β-caryophyllene were active against only some of the clinical isolates of *C. auris*. The EOs of the *L. origanoides* (thymol + *p*-cymene) chemotype (Code 0018) and of the *L. origanoides* (carvacrol + thymol) chemotype (Code 2206) were active against 80% of the tested yeasts. The EOs of the *L. origanoides* carvacrol chemotype (Code 0008) and *L. origanoides* thymol (Codes 0019 and 0010) chemotypes inhibited 75% and 70% of the yeasts evaluated, respectively. The EOs distilled from *Lippia micromera* (Code 0020), P. marginatum (Code 0024), A. cf. *popayanensis* (Code 0034), *Verronia curassavica* (Code 0042), the *L. alba* citral chemotype (Code 0046), and *P. cablin* (Code 0049) were less active (**Figure 1**).

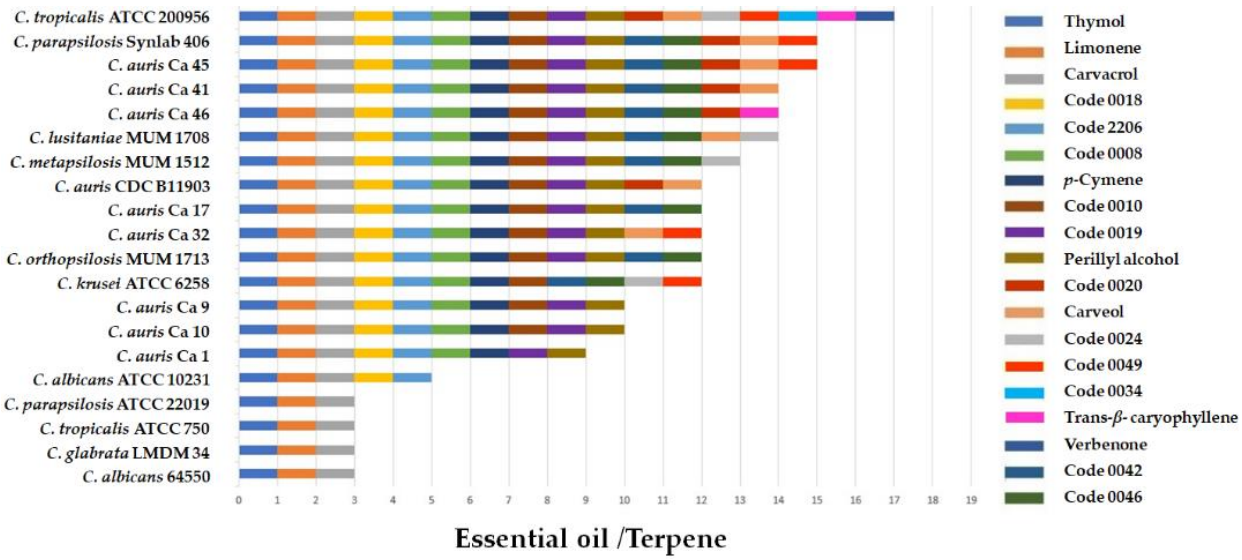

Figure 1. Results of screening the *in vitro* activity of EOs and some commercial terpenes against different *Candida* species. The presence of the bar indicates antifungal activity at 256 µg/mL.

For the most active EOs and commercial terpenes, minimal inhibitory concentrations (MICs) were determined. Important results were observed with the resistant strains *C.*

tropicalis ATCC 200956 and *C. parapsilosis* Synlab 406. MIC values for non-*C. auris* species are shown in **Table 3**

Table 3. Minimal inhibitory concentration values of the most active EOs studied and of

EO Code/Terpene	GM—Range MIC (µg/mL)					
	C. krusei ATCC 6258	*C. tropicalis* ATCC 200956	*C. parapsilosis* Synlab 406	*C. metapsilosis* MUM 17.13	*C. orthopsilosis* MUM 15.12	*C. lusitaniae* MUM 17.08
2206	256	181 (128–256)	16	128	128	181 (128–256)
0008	256	181 (128–256)	16	128	128	181 (128–256)
0010	256	128	22.6 (16–32)	90.5 (64–128)	128	128
0018	256	128	22.6 (16–32)	90.5 (64–128)	128	90.5 (128–64)
0019	NA	181 (128–256)	64	128	128	181 (128–256)
Thymol	181 (128–256)	90.5 (64–128)	64	128	128	90.5 (64–128)
Carvacrol	256	128	45.3 (32–64)	128	128	181 (128–256)
Perillyl alcohol	NA	181 (128–256)	90.5 (64–128)	256	256	181 (128–256)
p-Cymene	181 (128–256)	181 (128–256)	256	256	256	256
Limonene	32	64	64	64	16	22.6 (16–32)

GM: geometric mean; NA: non active.

We separately analysed results obtained with the emergent yeast *C. auris*. MIC values for EOs, and some commercial terpenes, are shown in **Table 4**. As for the other species, the antifungal activity of EOs and some commercial terpenes was strain-dependent. The best activity was observed with limonene (MIC range 16–64 µg/mL).

Table 4. Minimal inhibitory concentration values of the most active EOs studied and of some commercial terpenes against *C. auris.*

EO Code/Terpene	**GM—Range MIC (µg/mL)**								
	***C. auris* CDC B11903**	***C. auris* Ca 1**	***C. auris* Ca 9**	***C. auris* Ca 13**	***C. auris* Ca 32**	***C. auris* Ca 17**	***C. auris* Ca 41**	***C. auris* Ca 45**	***C. auris* Ca 46**
2206	128	256	256	256	64	128	128	128	90.5 (64–128)
0008	128	256	256	256	181 (128–256)	128	128	128	90.5 (64–128)
0010	64	NA	256	256	64	90.5 (64–128)	64	64	64
0018	64	256	181 (128–256)	256	128	64	64	64	64
0019	128	256	181 (128–256)	256	90.5 (64–128)	128	128	128	128
Thymol	64	181 (128–256)	128	128	64	64	64	64	64
Carvacrol	90.5 (64–128)	181 (128–256)	256	181 (128–256)	64	128	90.5 (64–128)	90.5 (64–128)	64
Perillyl alcohol	256	256	128	256	128	256	128	256	256
p-Cymene	256	NA	256	256	128	256	256	256	256
Limonene	64	64	22.6 (16–32)	22.6 (16–32)	22.6 (16–32)	64	16	16	16

GM: geometric mean; NA: non active.

3.3.3. Cytotoxic Activity

The cytotoxicity of the EOs and commercial terpenes that showed the highest antifungal activity was evaluated by MTT assay on the immortalized human keratinocytes cell line (HaCaT). The 50% cytotoxic concentrations (CC_{50}) and the selectivity index (SI) values are shown in Table 5. The less cytotoxic EOs corresponded to the *L. origanoides* thymol chemotype (Code 0010), the *L. origanoides* (carvacrol + thymol) chemotype (Code 2206), the *L. origanoides* (carvacrol + *p*-cymene) chemotype (Code 0008), and the *L. origanoides* (thymol + *p*-cymene) chemotype (Code 0018). CC_{50} values were 903.6, 788.0, 877.9, and 665.9 µg/mL, respectively. The SI values were strain-dependent, and the highest SI values were observed with resistant strains or strains that had high MICs towards antifungals but were sensitive to EOs or commercial terpenes.

Table 5. The 50% cytotoxic concentration (CC_{50}) and SI values of the most active EOs and commercial terpenes.

EO Code/Terpene	HaCaT Cells Mean CC_{50} (µg/mL)	SI Range (CC_{50}/MIC)
2206	788.0	3.0–49.2
0008	877.9	3.4–54.8
0010	903.6	3.5–56
0018	665.9	2.6–41
0019	354.7	1.4–5.5
Thymol	427.5	3.3–6.7
p-Cymene	831.2	3.2–5.5
Carvacrol	410.7	1.6–12.8
Limonene	400.5	3.1–50
Perillyl alcohol	400.7	1.6–6.25

3.3.4. Time–Kill Assays

Plots of the activity of the *L. origanoides* (thymol + *p*-cymene) chemotype (Code 0018) EO, thymol, and the antifungals AMB and FLC against *C. albicans* ATCC 10231, *C. tropicalis* ATCC 200956, and *C. auris* CDC B11903 are shown in Figure 2.

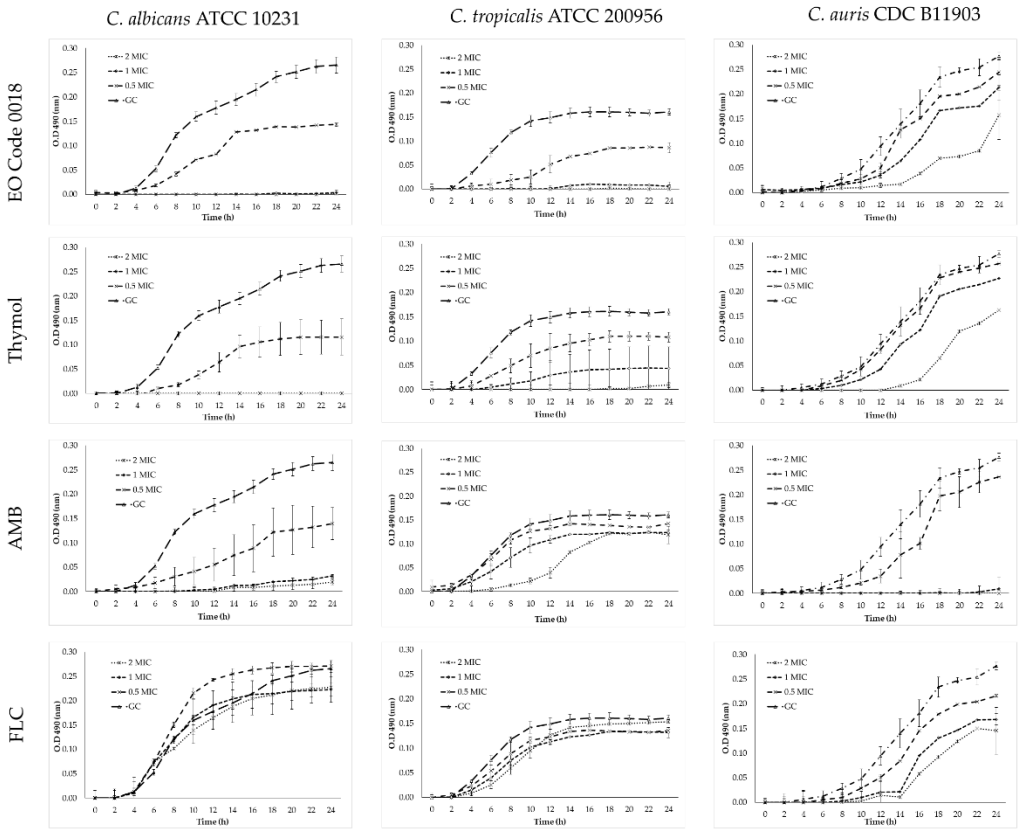

Figure 2. Time–kill curve plots for *C. albicans* ATCC 10231, *C. tropicalis* ATCC 200956, and *C. auris* CDC B11903 in the presence of the *L. origanoides* (thymol + *p*-cymene) chemotype (Code 0018) EO, thymol, AMB, and FLC.

Both the *L. origanoides* (thymol + *p*-cymene) chemotype (Code 0018) EO and the commercial terpene thymol showed fungicidal effects at 1X and 2X MIC against *C. albicans* ATCC 10231 and *C. tropicalis* ATCC 200956. On the other hand, *C. auris* CDC B11903 growth was not affected, and an extended lag-phase was observed at 2X MIC. Additionally, the expected fungistatic and fungicidal activities of FLC and AMB, respectively, were demonstrated.

3.4. Discussion

Fungal infections are increasing at an alarming rate in parallel with the occurrence of infections caused by antifungal-resistant strains. The morbidity and mortality of these infections have led researchers to seek options for the development of new, less-toxic antifungal agents with new targets or mechanisms of action [7,22].

EOs have been considered as promising agents for their antimicrobial activity [10]. In recent decades, several studies have demonstrated the antifungal activity of EOs, and some of their compounds, against fungi of clinical and agricultural importance (*Candida* spp. and filamentous fungi) [9,20–31]. These antifungal activities have been attributed to either the synergy between the multiple compounds that constitute EOs, or the major components of EOs [10,32].

This study showed that the EOs distilled from the *L. origanoides* thymol (Codes 0010 and 0019) chemotype and from *L. origanoides (*thymol + *p*-cymene) chemotype (Code 0018) were the most active against different *Candida* spp. (**Tables 3** and **4**). The EO activity can be attributed to the major compounds (thymol, carvacrol or *p*-cymene).

Currently, there are no reference protocols for evaluating the *in vitro* antifungal activity of natural compounds. Therefore, there is a great variability in the scientific literature about the techniques used and concentrations tested [9,25,28,33,34]. The evaluation of anti-*Candida* activity for the EOs and commercial terpenes in this study was performed with the standard CLSI M27 technique, which was designed for the evaluation of antifungals for clinical use, with some adjustments [20].

To define the antifungal activity of the different samples, we categorized the activity according to Holetz *et al.* [35] as follows: MIC values of ≤100 μg/mL were classified as having good activity, values of >100 and ≤500 μg/mL were classified as moderate, and values of >500 μg/mL were classified as weak activity. In agreement with these criteria, it was possible to identify good activity for some EOs and commercial terpenes; the highest activity was observed for the monoterpene limonene (MIC range 16–64 μg/mL) (**Tables** 3 and **4**). **¡Error! No se encuentra el origen de la referencia.**We found that the strains resistant to the main antifungal agents in clinical use (*C. tropicalis* ATCC 200956, *C. parapsilosis* Synlab 406, and *C. auris*) were the most susceptible to the studied EOs and commercial terpenes (**Tables 3** and **4**). These findings suggest that these compounds have different targets and/or mechanisms of action to those described for conventional antifungals in clinical use [36].

In addition, considering that the cross-resistant *C. tropicalis* ATCC 200956 strain harbors a deletion of 132 bp in the *ERG11* gene, a mutation in the *ERG3* gene, and the lack

of ergosterol in the membrane [37], it is possible that the activity of the EOs and terpenes was not related to the main azoles or AMB targets. However, the fact that few EOs and terpenes showed activity against *C. glabrata* LMDM 34 (echinocandin-resistant strain harbouring a substitution at the Fks2p subunit of the *β*-D-1,3-glucan synthase catalytic complex, the target of these lipopeptides) [38,39] suggests that the aforementioned compounds may act on fungal cell wall synthesis or its structure. This assumption can be supported by the results obtained by Brennan *et al*. [40]. They demonstrated that limonene inducted the expression of *Saccharomyces cerevisiae* genes linked to the organization and biogenesis of the cell wall. Additionally, there were no changes in the compounds and characteristics of the lipid membrane (fluidity, fatty acids, ergosterol, and saturated or unsaturated fatty biosynthesis pathways had not changed). Further, bearing in mind that *FKS2* expression is dependent upon the calcium/calcineurin/Hsp90 signaling pathway [39,31], the activity of the EOs and commercial terpenes studied could be associated with the same pathway as well [42]. Further work is needed to elucidate the targets or mechanisms of action of these EOs and terpenes.

Concerning *C. auris*, few studies have examined the *in vitro* activity of the EOs and terpenes studied here against this species. Recently, Baldim *et al.* [43] reported the anti-*C. auris* activity of *L. sidoides* EO (MIC range 140–563 μg/mL). Shaban *et al.* [4] reported a moderate activity of carvacrol (MIC range 63–250 μg/mL) and thymol (MIC range: 156–625 μg/mL). These results were similar to those obtained in this study. It is worth mentioning that this is the first study which describes the anti-*C. auris* activity of limonene, *p*-cymene, and of the *L. origanoides* EOs. *C. auris* can produce biofilms on hospital surfaces and medical devices or colonize healthcare personnel [2,6]; therefore, the EOs and commercial terpenes studied could be promising options for disinfection and/or decontamination of hospital surfaces and environments [32].

Other studies have also evaluated the cytotoxicity of the EOs distilled from plants not belonging to the genus *Lippia* and from the terpenes studied here on the HaCaT cell line, with CC_{50} values ranging between 33.93 and 1701.97 μg/mL [44–46]. The CC_{50} values obtained in our study with the *Lippia* spp. EOs and commercial terpenes ranged between 354.7 and 903.6 μg/mL (**Table 5**). In general, the EOs were less cytotoxic than the

commercial terpenes, presumably due to the interactions among the EO compounds, which can decrease their cytotoxicity. The CC_{50} values obtained in this study for the EOs of *L. origanoides*, limonene, carvacrol, and thymol were higher compared to those published by other authors [47–49], indicating that the EOs studied were less cytotoxic.

The low toxicity and preference of the EOs for fungal cells are ideal characteristics for the development of new antifungals. Interestingly, both the *L. origanoides* (thymol + *p*-cymene) chemotype (Code 0018) EO and thymol could be adjusted to those characteristics. These samples were selected to perform time–kill assays to define whether the effect was fungicidal or fungistatic. **Figure 2** shows that the *L. origanoides* (thymol + *p*-cymene) chemotype (Code 0018) EO and thymol were fungicidal against *C. albicans* ATCC 10231 and *C. tropicalis* ATCC 200956 at 1X and 2X MIC. Oppositely, these compounds did not show fungicidal activity at any concentration against *C. auris* CDC B11903, even when MIC values were low. This *C. auris* behavior could possibly be explained by a quorum sensing effect promoted by the higher inoculum size used in the time–kill assays (5×10^5 CFU/mL) compared to that used for the MICs ($0.5–5 \times 10^3$ CFU/mL).

The data obtained in this study could be the starting point for further research aimed at the development of topical or antiseptic products against resistant *Candida* spp. based on aromatic and medicinal Colombian plants. In the future, models such as the one proposed by Rayan *et al.* and Masalha *et al.* [50,51] could be applied to confirm whether the results obtained in this *in vitro* study could be confirmed with this predictive model. It would also be of interest to carry out an analysis via molecular docking in order to obtain information on the bioactivity mechanism of the most active terpenes or of those components of the active EOs identified in this study.

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4. Artículo 2:

Lippia origanoides essential oil or thymol in combination with fluconazole produces damage to cells and reverses the azole-resistant phenotype of a *Candida tropicalis* strain

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Abstract: *Candida tropicalis* is one of the most pathogenic species within the genus. Increased antifungal resistance has been reported, which is in part due to the organism's ability to form biofilms. In natural products derived from plants, such as essential oils (EOs) or their major components, there is significant potential to develop new antifungals or to both enhance the efficacy and reduce the toxicity of conventional antifungals. This study aimed to evaluate the effect of combining an EO of *Lippia origanoides* or thymol with fluconazole on an azole-resistant *C. tropicalis* strain. Synergism was observed in the combination of fluconazole with the EO and with thymol, and minimal inhibitory concentrations for fluconazole decreased at least 32-fold. As a consequence of the synergistic interactions, mitochondrial membrane potential was reduced, and mitochondrial superoxide production increased. Alteration in nuclear morphology, cell surface, and ultrastructure was also observed. In conclusion, the synergistic interaction between *L. origanoides* EO or thymol with fluconazole reverted the azole-resistant *C. tropicalis* phenotype. These findings suggest that *L. origanoides* EO or thymol alone, or in combination with fluconazole, have the potential for development as antifungal therapies for this yeast, including resistant strains.

Keywords: Essential oil, *Lippia origanoides*, antifungal activity, synergism, *Candida tropicalis*

4.1. Introduction

Candida species are opportunistic pathogens that cause infections with a wide range of clinical presentations. These infections have increased in recent years, particularly in immunocompromised patients [1]. Although *Candida albicans* remains the most prevalent species worldwide, non-*albicans* species such as *C. tropicalis*, C. *parapsilosis*, *C. glabrata*, *C. krusei*, and *C. auris* are now recognized as relevant pathogens [2].

Candida tropicalis is a constituent of the human microbiome and is the cause of a number of common infections, including onychomycosis and oral, genital, and skin candidiasis [3,4]. More seriously it can cause invasive infections associated with high morbidity and mortality [5,6]. As a consequence, this yeast is included in the list of fungal priority pathogens by the World Health Organization (WHO) [7].

Studies carried out in Latin America have shown that *C. tropicalis* is one of the more common species identified in cases of candidemia [2,8]. In Colombia, *C. tropicalis* is the second-most isolated species from patients with bloodstream infections [9].

Current therapies for *Candida* species infections are based mainly on azoles, amphotericin B (AMB), and echinocandins [10]. However, the resistance in different species of *Candida* to these antifungals has been documented in the scientific literature. Although, in general, *C. tropicalis* is susceptible to antifungals, several studies have demonstrated the emergence of clinically resistant isolates of *C. tropicalis* to one or various classic antifungals [11–13]. Furthermore, the use of these antifungals is limited by their toxicity and low selectivity [14–16].

Natural products are a well-documented source of molecules with biological activities [17]. As a result, different bioactive molecules have been developed, such as the anti-cancer vinca alkaloids and the paclitaxel terpene derived from the *Catharanthus roseus* and *Taxus brevifolia* plants, respectively. The antimalarial artemisinin (sesquiterpene lactone) from *Misia annua* L and the blood cholesterol reducer lovastatin (statin) obtained from *Aspergillus terreus* are further examples [18,19].

Currently, essential oils (EOs) distilled from different plants around the world are of interest in the search for new antifungal compounds with targets or mechanisms of action different from those of conventional antifungals for clinical use, or that have a synergistic effect in combination with known antifungals [20,21]. Relevant activity against different *Candida* species has been identified in EOs from aromatic plants such as *Melaleuca alternifolia* L., *Thymus vulgaris* L., *Mentha piperita* L., *Rosmarinus office*, *Juniperus oxycedrus* L., *Cinnamomum zeylanicum*, and *Ruta graveolens* [22–27]. The anti-*Candida* spp. activity of EOs and terpenes has been related to the alteration in cell membrane permeability, DNA integrity, the calcium signaling pathway, cell cycles, mitochondrial functions, and an increase in the level of intracellular reactive oxygen species (ROS) [28–30].

Colombia is a megadiverse country that ranks in fourth place internationally in abundance and diversity of plant species [31,32]. This may provide an opportunity to discover EOs with antifungal activity. The aromatic species of plants of the *Lippia*

(Verbenaceae) genus, have been studied in Colombia for their biological activities [33–35]. *Lippia origanoides* is a species of aromatic plant commonly known as "mountain oregano" and has been used by native communities for its medicinal properties [36,37]. Phytochemical analysis of *L. origanoides* EOs indicates that they are composed mainly of terpenes, terpenoids, and phenylpropanoids. According to this analysis, five *L. origanoides* chemotypes have been described: *p*-cymene, *α*- and *β*-phellandrene, and limonene (chemotype A); carvacrol (chemotype B); thymol (chemotype C); 1,8-cineole (chemotype D); and (E)-methyl cinnamate and (E)-nerolidol (chemotype E) [38]. In a previous study carried out by our group, the activity of EOs derived from different chemotypes of *L. origanoides* against *Candida* spp. with different antifungal sensitivity profiles was demonstrated [39].

Combination therapy is a well-recognized approach to overcoming fungal resistance and maximizing the therapeutic efficacy and selectivity of antifungals [40,41]. The synergistic effect of fluconazole (FLC) has been demonstrated with other antifungals of clinical use and other agents with specific activity against *Candida* spp. [42–44]. EO and terpenes combined with FLC have been shown to be effective against FLC-resistant *Candida* spp. [45].

In this study, we focused on evaluating the effect of the interaction of an EO distilled from the *L. origanoides* (thymol + *p*-cymene) chemotype or of thymol in combination with FLC against an azole-resistant *C. tropicalis* strain on specific cell functions and structures.

4.2. Materials and Methods

Candida tropicalis ATCC 200956 and *C. tropicalis* ATCC 750 strains, resistant and susceptible to azoles, respectively, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured on Sabouraud Dextrose Agar (SDA; Sigma-Aldrich, St. Louis, MO, USA). The *Candida tropicalis* ATCC 200956 strain carries a 132 bp deletion in the *ERG11* gene (44 amino acids) and a C773T substitution in the *ERG3* gene, leading to the S258F mutation. The aforementioned changes provide this strain with cross-resistance to both azoles and AMB [11].

4.2.1. Compounds

The EO of the *L. origanoides* (thymol + *p*-cymene) chemotype was obtained via hydrodistillation and characterized by gas chromatography-mass spectrometry (GC-MS), as previously described by Zapata-Zapata et al. [39] and as shown in Table 1.

Plant Species and Chemotype	**Collection Site**	**Voucher Number**	**Principal Compounds (Relative Amount, %)**
Lippia origanoides (Thymol + *p*-cymene) chemotype	Bucaramanga—Santander, Colombia	UIS * herbarium 22039	Thymol (49.4), *p*-cymene (19.1), *γ*-terpinene (9.2), *β*-myrcene (5.2), *α*-terpinene (2.9), carvacrol (2.7), thymyl methyl ether (1.8), trans-*β*-caryophyllene (1.6), cis-*β*-ocimene (1.2), and limonene (0.9).

* UIS: Industrial University of Santander. The major compounds are indicated in bold.

Thymol (2-isopropyl-5-methyl phenol) and FLC were obtained commercially (Sigma-Aldrich, St. Louis, Mo, USA). Stock solutions of compounds were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). Following this, work solutions were prepared using RPMI (Roswell Park Memorial Institute 1640) medium (Sigma-Aldrich St. Louis, MO, USA) and buffered with 3-(N-morpholino) propane-sulfonic acid (MOPS) (Sigma-Aldrich St. Louis, MO, USA).

4.2.2. Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of FLC was determined according to the Clinical and Laboratory Standards Institute M27, 4th edition [46]. The antifungal activity of EO and thymol was also evaluated using the same protocol, with some modifications. In brief, work solutions were prepared at 512 µg/mL 2X (two-fold final concentration); then, 100 µL of five serial two-fold dilutions were dispensed in 96-well plates (Corning®, Costar®, New York, NY, USA), thus achieving final concentrations between 16 and 256 µg/mL after the addition of the same volume of inoculum at a concentration of 2.5×10^3 CFU/mL (2X). Microdilution plates were incubated at 35 °C for 24 h. MICs were visually determined at the lowest concentration that produced visual inhibition compared to the

growth control (inoculum without compounds). The assays were performed at least three times in duplicate on different days. The data were expressed as geometric media (GM) of the MIC values (GM-MICs). As antifungal susceptibility testing control, the activity of itraconazole and AMB (Sigma-Aldrich, St. Louis, MO, USA) against the reference strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 was evaluated.

4.2.3. Interaction Assay

Interaction between the EO or thymol with FLC was evaluated against a *C. tropicalis*-resistant strain as well as in the susceptible one, following the fixed ratio method described by Fivelman et al. [47]. Initially, concentrations equivalent to eight-fold (8X) MIC of the FLC, EO, or thymol were prepared as indicated in Table 2.

Table 2. Combinations of the compounds evaluated

Combination	FLC MIC (X)	Thymol or EO MIC (X)
1	0	8
2	0.5	4
3	1	2
4	2	1
5	4	0.5
6	8	0

Note: X = fold-number MIC values.

A total of 100 µL was taken from each combination and dispensed in 96-well plates, and six serial two-fold dilutions were prepared from each combination. Subsequently, 100 µL of the previously prepared suspension of *C. tropicalis* adjusted to 2.5×10^3 CFU/mL (2X) in RPMI + MOPS was added to each well, for a final volume of 200 µL per well. Then, the plates were incubated at 35 °C for 24 h in a humid chamber. MIC values of EO/FLC or thymol/FLC mixes were determined as described above. The assays were performed in triplicate in three independent experiments. MIC values were used to calculate the fractional inhibitory concentration (FIC) using the following formula [48]:

$$\text{FIC compound A} = \frac{\text{MIC (A) in combination}}{\text{MIC (A) alone}}$$

$$\text{FIC compound B} = \frac{\text{MIC (B) in combination}}{\text{MIC (B) alone}}$$

The results of the combinations were interpreted by calculating the FIC index (FICI = ΣFIC). Values of FICI $\leq$ 0.5, 0.5 < FICI $\leq$ 4, and > 4 allowed us to categorize the interactions as synergism, indifference, and antagonism, respectively [49]. Additionally, the FIC values were used to construct isobolograms. The FIC of FLC was plotted on the X axis and the FIC of EO or thymol on the Y axis. An additivity line that joins the theoretical points of the MIC values for each compound (FIC = 1) was drawn. Results were interpreted according to Huang et al. [50]. Experimental points below the additivity line indicated a synergistic effect.

4.2.4. Assessment of Plasmatic Membrane Integrity

The plasma membrane integrity was tested using propidium iodide (PI) (BD™ DNA QC Particles, San José, CA, USA). *Candida tropicalis* ATCC 200956 cells (4×10^5 CFU/mL (2X)) were treated with EO/FLC or thymol/FLC MICs. After incubation at 35 °C for 4 h, cells were stained with PI (2 ng) and incubated at room temperature for 30 min. The percentage of PI-positive cells was measured by a flow cytometer (CitoFlexS™ Beckman Coulter, Indianapolis, IN, USA). The results were analyzed with the FlowJo software, version 7.6 (BD, Franklin Lakes, NJ, USA). Cells heated to 56 °C for 1 h were used as a positive control.

4.2.5. Evaluation of Mitochondrial Membrane Potential (Δψm)

Candida tropicalis ATCC 200956 cells [4×10^5 CFU/mL (2X)] were treated with the previously established MICs for EO/FLC or thymol/FLC and incubated at 35 °C for 4 h to evaluate changes in Δψm. Subsequently, cells were stained with 2 µM of 5,5,6,6-tetrachloro-1,1′,3′,3′-tetraethyl benzimidazole carbocyanine iodide MitoProbe™ dye (JC-1) (Invitrogen, Carlsbad, CA, USA) and with 2 ng of 3,3’-Dihexyloxacarbocyanine Iodide [$DiOC_6(3)$] (Invitrogen, Carlsbad, CA, USA) and then incubated at 37 °C and at room temperature, respectively, for 30 min in the dark. The cells stained with JC-1 were visualized by fluorescence microscopy (Nikon Eclipse, Tokyo, Japan). Furthermore, the

fluorescence of cells stained with JC-1 and $DiOC_6(3)$ was analyzed using a CitoFlexS™. The data obtained were analyzed by FlowJo 7.6. The red/green ratio was calculated with the data of median fluorescence intensity of cells stained with JC-1, as described by Sivandzade et al. [51]. The percentage of high and low cells for $DiOC_6(3)$ uptake was also calculated. Both cells untreated and treated with AMB at 4 µg/mL were included as controls.

4.2.6. Mitochondrial Superoxide Indicator

The production of superoxide anion (O_2-) by *C. tropicalis* ATCC 200956 cells treated with EO/FLC or thymol/FLC was evaluated using MitoSOX™ Red (Invitrogen, Carlsbad, CA, USA). An inoculum of 4×10^5 CFU/mL (2X) prepared using RPMI + MOPS was exposed to MICs at 35 °C for 4 h. Following this, the cells were collected by low-speed centrifugation, washed twice in 1X phosphate-buffered saline (PBS), and stained with MitoSOX™ Red 2 µM at 37 °C for 1 h in the dark. At least 15 cells from each tested treatment were visualized by fluorescence microscopy, and fluorescence intensity was quantified by NIS-Element 4.0.0. software (Tokyo, Japan) according to the manufacturer's instructions. For comparisons between treatments, the Kruskal–Wallis test was performed using the Prism 6.0 statistical program (GraphPad, San Diego, CA, USA), with $p < 0.05$ regarded as statistically significant.

4.2.7. Nuclear Effect Assessment

The effect of EO/FLC or thymol/FLC on *C. tropicalis* ATCC 200956 cells was assessed by staining with diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA). Cells at 4×10^5 CFU/mL (2X) were treated with MICs and incubated at 35 °C for 4 h; then, yeasts were washed with PBS (1X), fixed in paraformaldehyde (4%) for 20 min, and permeabilized with Triton™ X-100 (0.25%) (Sigma-Aldrich, St. Louis, MO, USA) for 60 min. Finally, cells were stained with 1 µg/mL of DAPI and incubated at room temperature in the dark for 30 min. The samples were collected by low-speed centrifugation, washed twice, and re-suspended in PBS (1X). Yeasts were visually analyzed under a fluorescence microscope at an emission/excitation wavelength (λ) of 341/452 nm, respectively. Untreated cells were used as a control.

4.2.8. Cell Cycle Assessment

To evaluate the effect of the EO/FLC and thymol/FLC on the cell cycle, *C. tropicalis* ATCC 200956 was grown on SDA at 35 °C for 24 h. Following this, a concentrated yeast solution was prepared in distilled water and kept at 25 °C for 2 h. The inoculum was adjusted at 4×10^5 CFU/mL (2X) in RPMI + MOPS, treated with the MICs, and incubated at 35 °C for 4 h. Then, cells were fixed overnight with 70% cold ethanol and washed twice with 50 mM sodium citrate buffer before being treated with 20 µg/mL of RNAse A (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 45 min. Two washes were done with a citrate buffer; the yeasts were resuspended in 200 µL of the same buffer. Finally, 100 µL of PI at 50 µg/mL was added to 30 µL of the yeast solution and incubated at 25 °C for 20 min in the dark. All experiments were performed in triplicate, and readings were carried out using an LSR Fortessa™ flow cytometer (BD, Franklin Lakes, NJ, USA) and analyzed using FlowJo software, version 7.6. DNA content was estimated by measuring PI median fluorescence intensity (MFI); cell percentages with 1, 1.5, and 2 MFI as DNA content indicators were determined. Statistical analysis was performed by a one-way ANOVA test using the Prism 6.0 statistical program, with $p < 0.05$ regarded as statistically significant.

4.2.9. Morphology Assessment by Scanning Electron Microscopy

Candida tropicalis ATCC 200956 cells at 4×10^5 UFC/mL (2X) obtained from an SDA culture at 35 °C for 24 h were incubated at 35 °C for 4 h with EO/FLC or thymol/FLC MICs. Cells treated with FLC, AMB, and caspofungin (CSF; Sigma-Aldrich, St. Louis, MO, USA) at 64, 8, and 4 µg/mL, respectively, were included as the positive control. Cells without treatment were used as the negative control. After treatments, the yeasts were fixed in glutaraldehyde (2.5%) overnight and then submerged in Sorensen's phosphate buffer pH 7.0 for 12 h. Three washes were carried out with the same buffer, and one wash with distilled water at the end. The samples were fixed and dehydrated with increasing ethanol concentrations (50, 75, 95, and 100%). The samples were deposited on graphite tape and thinly coated with gold (Au) using a cold sputter coater (Denton Vacuum Desk IV, Beijing, China) and then observed using a High Vacuum Scanning Electron Microscope (SEM) (JEOL JSM 6490 LV Tokyo, Japan). A secondary electron detector was used to evaluate

their morphology. Elemental analysis was determined using an INCA PentaFETx3 X-ray microprobe EDS (Oxford Instruments, Abingdon, UK).

4.2.10. Ultrastructure Assessment by Transmission Electron Microscopy

Ultrastructural evaluation by transmission electron microscopy (TEM) was performed on *C. tropicalis* ATCC 200956 cells in the exponential growth phase [4×10^5 UFC/mL (2X)]. Cells were treated with EO/FLC or thymol/FLC MICs and incubated in agitation at 35 °C for 4 h, fixed in a sodium hydrogen phosphate buffer 0.1 M (pH 7.4), glutaraldehyde (2%), and paraformaldehyde (4%) at room temperature for 2 h, then washed three times with sodium hydrogen phosphate buffer and fixed with potassium permanganate (1%) at 4 °C for 1 h and tannic acid (0.15%) and uranyl acetate (2%) for 1 min and 1 h, respectively, at room temperature. The cells were dehydrated by incremental concentrations of ethanol (50, 75, 90, 95, and three times with 100%) at 4 °C for 10 min each. Infiltration was performed at room temperature by agitation using increasing concentrations of epoxy-resin (25, 50, 75, and 100%). Polymerization was performed at 60 °C for 48 h. Ultrathin sections of the samples (50 to 70 nm) were obtained with a Leica EM UC6 ultramicrotome and harvested on 100-mesh Formvar-coated copper grids and stained following standard procedures with 4% uranyl acetate and 2% lead citrate. Images were recorded at nominal magnifications of 15,000 × to 67,000 × with a CCD (Charged Coupled Device) FEI Ceta camera on a Tecnai 12 electron microscope (FEI) operated at 120 kV. The cell wall thickness was determined by measuring, in one or two points, at least 20 cells of each treatment with ImageJ software (version 1.52p). One-way analysis of variance (ANOVA) was carried out using the Prism 6.0 statistical program to identify significant differences between treated and control cells ($p < 0.05$ was regarded as statistically significant).

4.3. Results

Table 3 shows the GM-MIC values of FLC, EO, and thymol alone or in combination (EO/FLC and thymol/FLC) evaluated with two strains of *C. tropicalis* (azole-resistant and azole-susceptible). The susceptible and resistant FLC phenotypes were confirmed with the following MIC values: 2 and > 64 µg/mL, respectively.

Additionally, the synergistic interaction between EO/FLC and thymol/FLC (FICI = 0.28) when the azole-resistant strain was tested resulted in a significant reduction in the

individual MIC values: from > 64 to 2 µg/mL (approximately 32-fold) in FLC and from 128 to 32 µg/mL (4-fold) in the EO and thymol. The thymol/FLC combination was synergistic with the azole-susceptible strain (FICI = 0.47). In this case, both FLC and thymol MIC values decreased from 2 to 0.60 µg/mL (3.3-fold) and from 256 to 42 µg/mL (6-fold), respectively. In contrast, the EO/FLC interaction was additive (FICI = 0.53). However, the reduction of the FLC MIC from 2 to 0.25 µg/mL was observed (8-fold).

Table 2. Essential oil or thymol interaction with FLC on azole-resistant and azole-susceptible *C. tropicalis* strains.

Strain	Compound/ Combination	GM-MIC (µg/mL)	FICI	Interaction type	N° times ↓MIC		
					FLC	EO	Thymol
C. tropicalis ATCC 200956*	FLC	>64	-	-	-	-	-
	EO	128	-	-	-	-	-
	Thymol	128	-	-	-	-	-
	EO/FLC	2/32	**0.28**	Synergism	32	4	-
	Thymol/FLC	2/32	**0.28**	Synergism	32	-	4
C. tropicalis ATCC 750**	FLC	2	-	-	-	-	-
	EO	512	-	-	-	-	-
	Thymol	256	-	-	-	-	-
	EO/FLC	0.25/181	0.53	Additive	8.0	2.8	-
	Thymol/FLC	0.60/42	**0.47**	Synergism	3.3	-	6

GM-MIC: Geometric mean values of the minimal inhibitory concentration (MIC); FLC: Fluconazole; EO: essential oil; FIC: Fractional Inhibitory Concentration; FICI: FIC Index. Values in bold indicate a synergistic interaction (FICI ≤ 0.5); *azole-resistant, **azole-susceptible. The arrow (↓) indicates a MIC decrease.

As mentioned in the experimental procedure, interactions between EO/FLC and thymol/FLC were represented by isobolograms (Figure 1). The straight line (additivity) indicates an indifferent effect, which means no interaction between the tested combinations was observed, while the concave lines below the additivity line indicate a synergistic effect of the combinations.

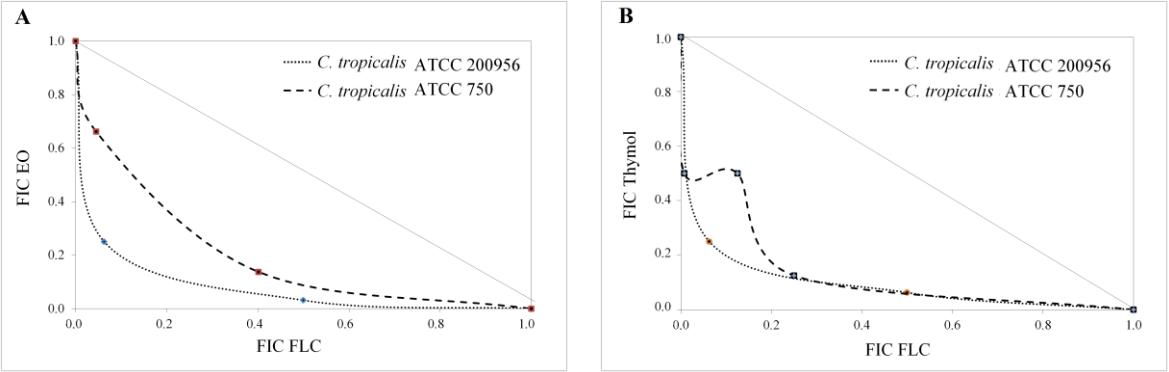

Figure 1. Isobologram of EO/FLC (A) and thymol/FLC (B) interaction against *C. tropicalis* strains. Points below the additivity line indicate a synergistic effect.

4.3.1. Lippia origanoides EO or Thymol in Combination With FLC Reduce Mitochondrial Membrane Potential (ΔΨm) But Do Not Affect the Plasmatic Membrane

The effect on the mitochondrial membrane potential of EO, thymol, EO/FLC, or thymol/FLC was tested with JC-1 and $DiOC_6(3)$ fluorescent stains. In Figure 2A,C, the images show cells stained with JC-1 under fluorescence microscopy. Green fluorescence indicates the loss of mitochondrial membrane potential. Additionally, the JC-1 MFI was measured by cytometry, and the ratio of red/green was calculated. The results were as follows: EO (1.9), thymol (1.5), EO/FLC (2.4), thymol/FLC (2.0), FLC (2.0), negative control (3.9), and positive control (1.7). Lower values in the ratio of red/green indicate a greater effect in the mitochondrial membrane potential. Our results indicate that thymol was the treatment that caused the greatest loss of mitochondrial membrane potential, even above that observed in the positive control treated with AMB.

In Figure 2B,D, the percentages of high (H) and low (L) $DiOC_6(3)$ uptake cells are shown. Both JC-1 and $DiOC_6(3)$ results indicate mitochondrial depolarization. In addition, the percentages of positive cells for PI are indicated at the top of the panels of Figure 2B,D. The low percentage of cells that took up the PI evidenced that the evaluated treatments did not affect the integrity of the plasmatic membrane; 98% of the positive control cells captured the PI.

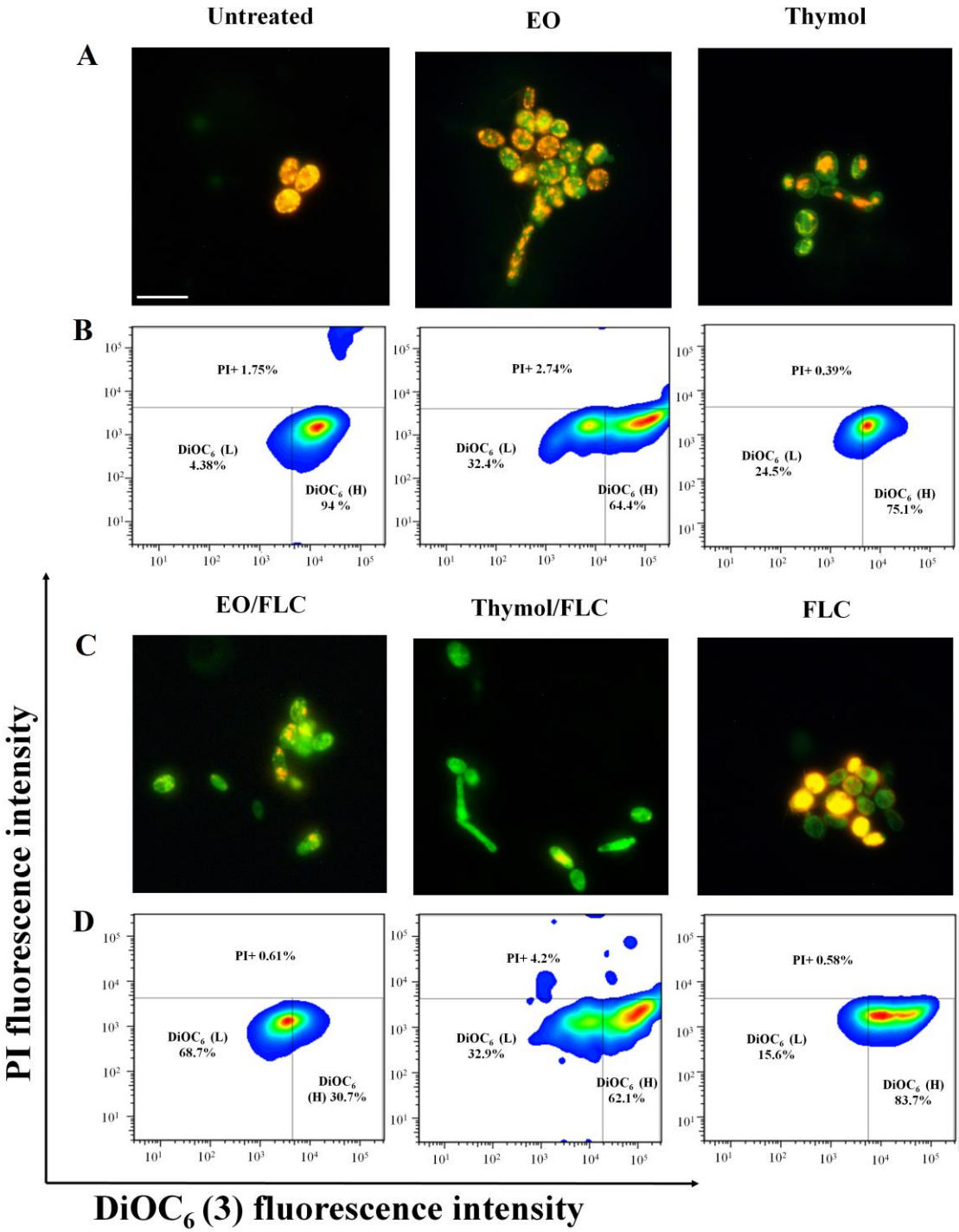

Figure 2. Effect of the different treatments on *C. tropicalis* cell mitochondrial membrane potential (Δψm) and plasmatic membrane. (**A**,**C**) Photos of cells stained with JC-1 obtained by fluorescence microscopy. Green indicates monomers, and yellow indicates aggregates. (**B**,**D**) Representative plots of percentages of $DiOC_6(3)$ uptake cells (high (H) and low (L)). PI-positive cell percentages are shown above. Scale bar: 25 µm.

4.3.2. Thymol Used Alone and in Combination with FLC Increased Mitochondrial Superoxide Production

In Figure 3, microscopy fluorescence images and the quantification of MitoSOX^{TM} Red fluorescence intensity are shown. Superoxide anion (O_2-) production was significant when thymol (p = 0.0008) and the thymol/FLC combination were added (p < 0.0001) (Figure 3B).

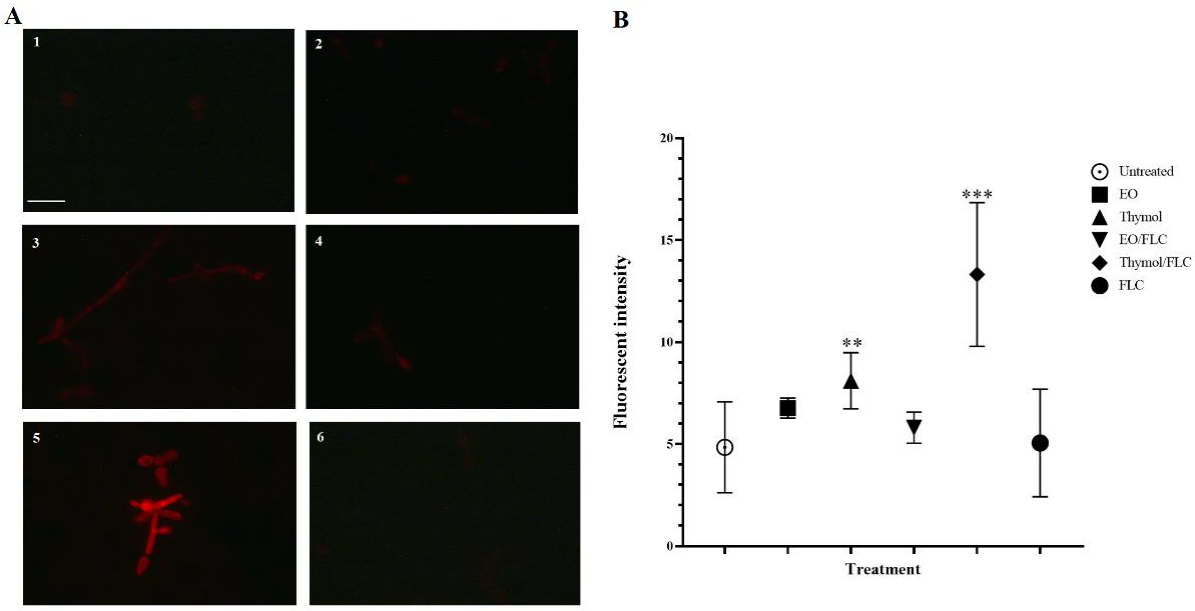

Figure 3. *Candida tropicalis* ATCC 200956 cells stained with MitoSOX^{TM} Red were observed under a fluorescence microscope. (**A1**) Untreated cells; (**A2**) cells treated with EO, (**A3**) thymol, (**A4**) EO/FLC, (**A5**) thymol/FLC, and (**A6**) FLC. (**B**) Mean fluorescence intensity values of cells with different treatments. ** p = 0.0008; *** p < 0.0001. Scale bar: 25 µm.

4.3.3. Lippia origanoides EO or Thymol in Combination with FLC Causes Nuclear Alterations.

Morphological changes in *C. tropicalis* ATCC 200956 nucleus were visualized in yeasts stained with DAPI and observed under a fluorescence microscope after EO, thymol, EO/FLC, thymol/FLC, or FLC treatments (Figure 4). Normal characteristics of the nuclei were observed in untreated cells (Figure 4A), while EO-treated yeast showed typical

karyopyknosis, with condensed shrinkage cell nuclei (Figure 4B), which were more evident in the thymol-treated ones (Figure 4C). An unorganized and dispersed DNA-stained pattern was observed with FLC treatment (Figure 4D). Interestingly, in yeast treated with EO/FLC (Figure 4E) or thymol/FLC (Figure 4F), typical karyorrhexis, with small and compacted chromatin granules spreading into the cytoplasm, was observed.

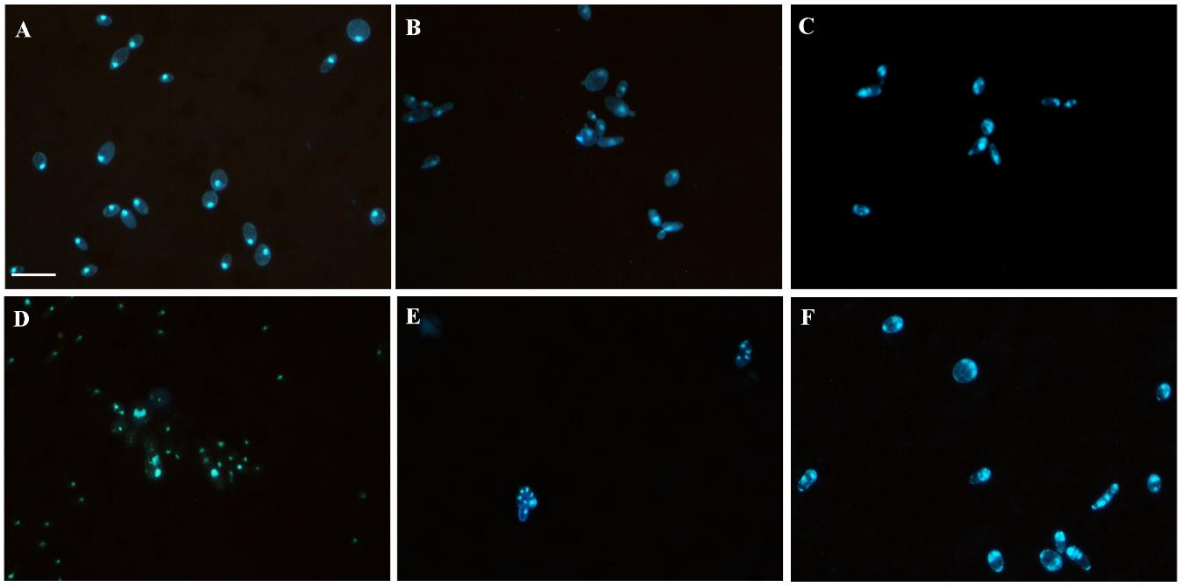

Figure 4. Fluorescence microscopy pictures depicting the changes on the nucleus of *C. tropicalis* ATCC 200956. (**A**) Untreated and treated cells with (**B**) EO, (**C**) thymol, (**D**) EO/FLC, (**E**) thymol/FLC, and (**F**) FLC. Scale bar: 25 µm.

4.3.4. Lippia Origanoides EO or Thymol in Combination with FLC Causes Alterations in The Cell Cycle

The effect of EO, thymol, EO/FLC, and thymol/FLC on the *C. tropicalis* ATCC 200956 strain cell cycle was evaluated. Analysis of the DNA content estimated by the MFI of the PI showed a significant decrease in the percentage of 1 MFI cells after treatment with EO/FLC (48.5%; $p = 0.039$) and thymol/FLC (47.7%; $p = 0.030$) when compared to untreated cells (63.3%). In addition, a significant increase ($p < 0.05$) in polyploidy (1.5 MFI + 2 MFI) was observed after treatment with EO (41.4%), EO/FLC (36.5%), thymol/FLC (37.4%), and FLC (38.2%). DNA content in thymol-treated cells was similar to the control (25.9% and 25.1%, respectively). Moreover, all of the treatments except thymol increased the 1.5 MFI or 2 MFI cell percentage in comparison with untreated cells (Figure 5B).

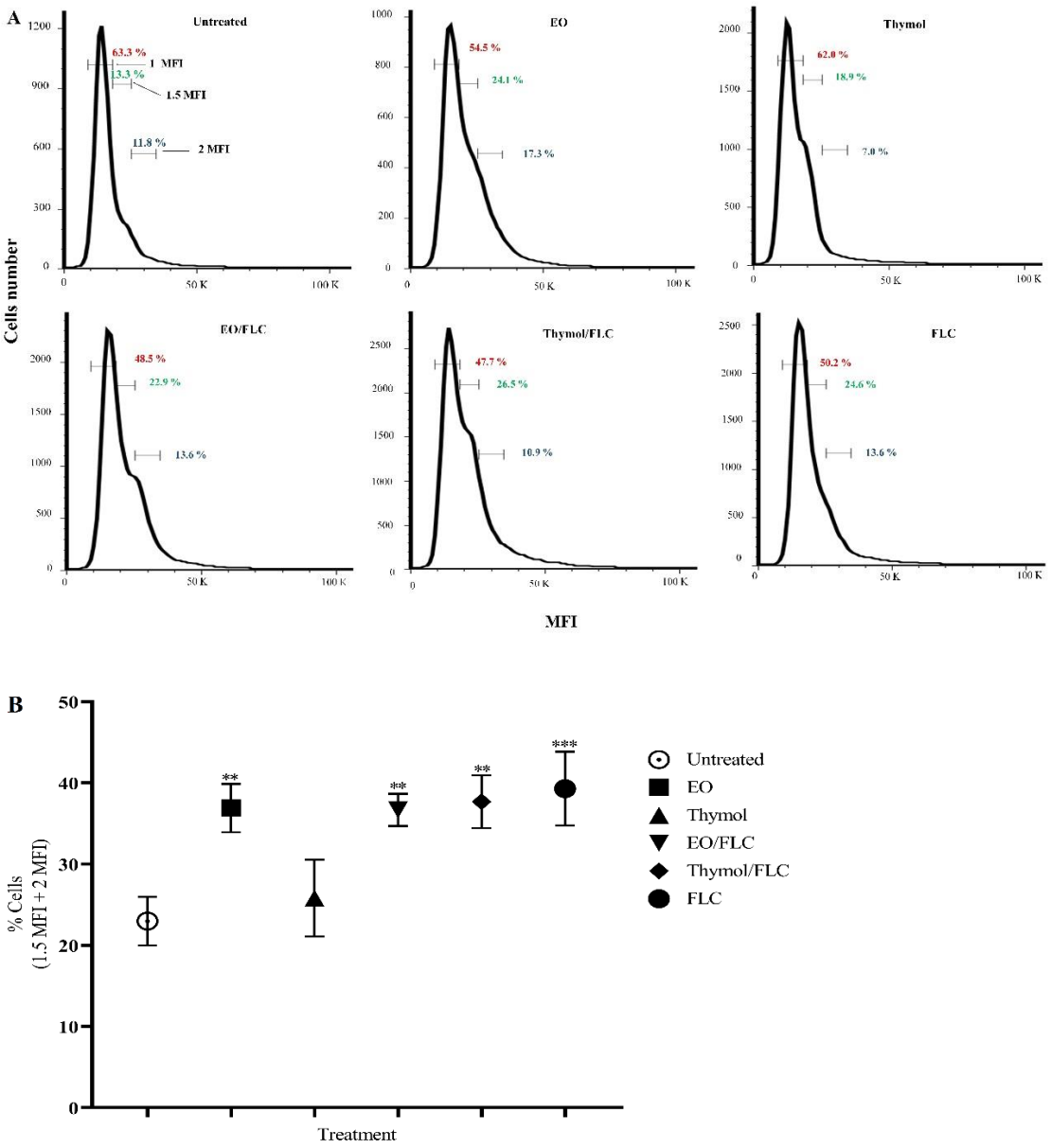

Figure 5. Effect of different treatments on the *C. tropicalis* ATCC 200956 cell cycle. A) Representative plots of data indicating 1 MFI (red), 1.5 MFI (green), and 2 MFI (blue) cell percentage. B) Average 1.5 MFI + 2 MFI cell percentage. MFI: Median fluorescence intensity. *P*-values: EO = 0.006; EO/FLC = 0.003; thymol/FLC = 0.002; FLC = 0.001. (** $p < 0.01$; ***$p < 0.001$).

4.3.5. Lippia origanoides EO or Thymol in Combination with FLC Cause Both Superficial and Ultrastructural Alterations

SEM observations of untreated cells revealed a normal superficial appearance (Figure 6A). After exposure to the EO, thymol, EO/FLC, or thymol/FLC, aberrant morphologies, including wrinkles, shrinkages, and depression of the cell surface, were observed (Figures 6B–E respectively). Furthermore, a similar effect was observed in yeasts treated with FLC, AMB, and CSF (Figures 6F–H, respectively).

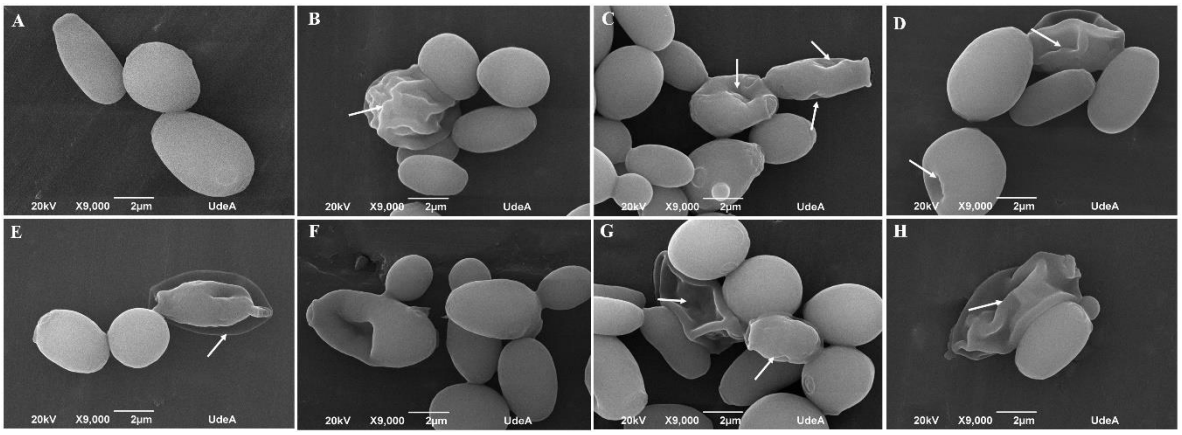

Figure 6. SEM images of *C. tropicalis* ATCC 200956: (A) Untreated cells; (B) treated with EO, (C) thymol, (D) EO/FLC, (E) thymol/FLC, (F) FLC, (G) AMB and (H) CSF. Arrows indicate alterations on the cell surface. AMB: amphotericin B; FLC: fluconazole; CSF: caspofungin; EO: essential oil.

In Figure 7, micrographs obtained by TEM show the ultrastructural effect of treatments on *C. tropicalis* ATCC 200956 cells**.** Untreated or FLC-treated cells demonstrated small and round mitochondria with well-contrasted cristae and a homogeneously light matrix (Figure 7A,F). In contrast, yeasts treated with the EO or EO/FLC showed diffused cristae but meaningless changes in mitochondrial shape and size (Figure 7B,D). Conversely, morphology changes and loss of cristae in cells treated with thymol or thymol /FLC were observed (Figure 7C,E).

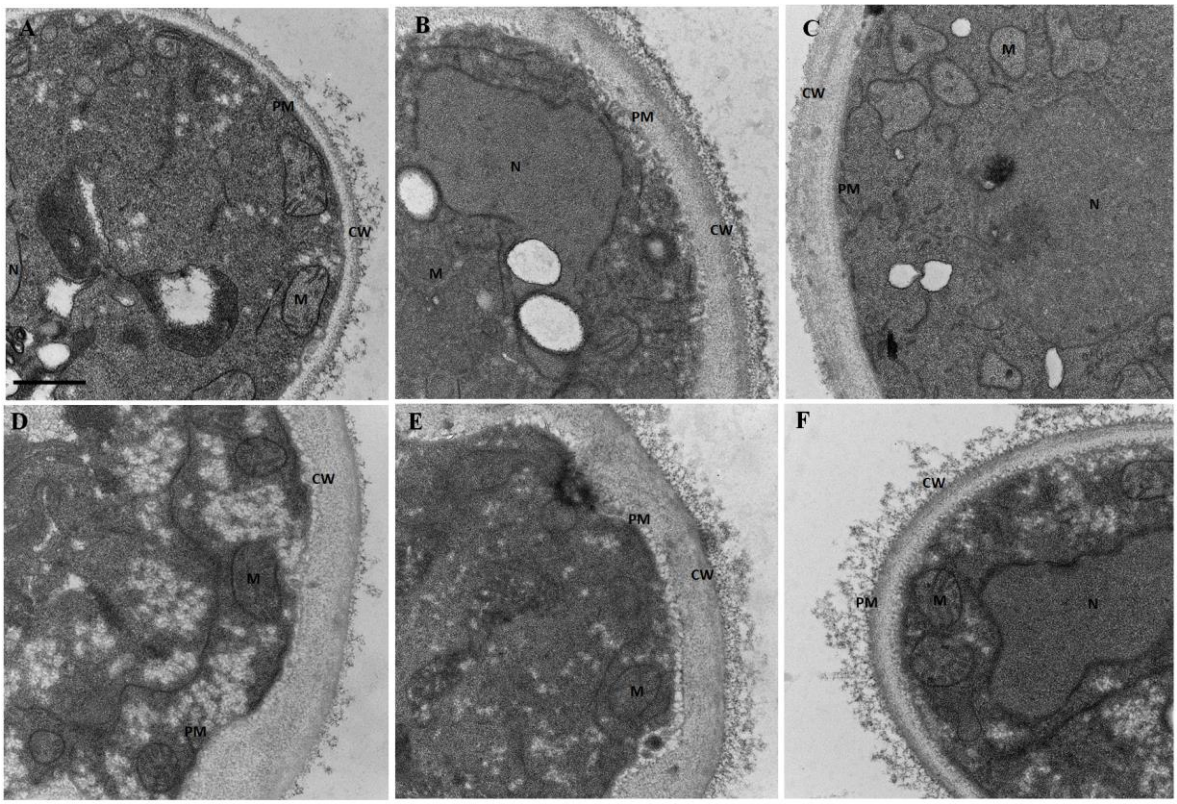

Figure 7. TEM micrographs of *C. tropicalis* ATCC 200956. (A) Untreated cells or cells treated with: (B) EO; (C) thymol; (D) EO/FLC (E) thymol/FLC; (F) FLC. PM: Plasmatic membrane; CW: Cell wall; M: Mitochondria; N: Nucleus. Scale bar: 500 nm.

Changes in cell wall thickness were observed following each treatment. The cell wall width of at least of 20 cells was measured and is represented in Figure 8. All the treatments, except FLC, increased the cell wall width, but this increment was especially noteworthy with EO and thymol.

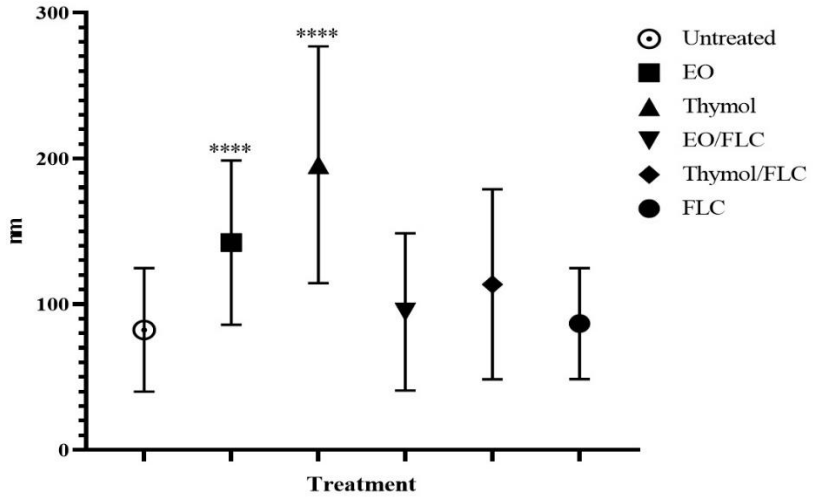

Figure 8. *Candida tropicalis* ATCC 200956 cell wall thickness measuring. **** p <0.0001.

4.4. Discussion

The use of antifungal combinations and their amalgamation with other molecules is considered an effective strategy in reducing antifungal doses required for treatment, avoiding the development of resistance as well as reversing existing drug resistance, and inhibiting the formation or disintegration of biofilms [23,42]. The synergistic effect of EOs or terpenes in combination with FLC against both planktonic and biofilm growth of *C. albicans*, *C. tropicalis*, as well as the multidrug-resistant *C. auris* has already been demonstrated [23,25,52]. Our group previously demonstrated the anti-*Candia* activity of *L. origanoides* EOs and terpenes, both on planktonic and biofilm cultures [39,53]. Of note was the marked effect of the EOs and terpenes on resistant strains, including the *C. tropicalis* ATCC 200956 strain. This effect was greater than on the more susceptible strains [39]. In this current study, we investigated whether the interaction of *L. origanoides* EO or thymol potentiated FLC activity against the azole- and AMB-susceptible *C. tropicalis* ATCC 750 strain and the resistant *C. tropicalis* ATCC 200956 strain. For the latter strain, the resistance mechanisms, due to *ERG 11* and *ERG 3* gene mutations, the absence of ergosterol in the cell membrane, changes in the wall structure, and an activated antioxidant mechanism, were identified previously [11,54].

Table 2 and Figure 2 confirm the synergistic effect of EO/FLC and thymol/FLC combinations against *C. tropicalis* strains. The synergistic interaction with *C. tropicalis*

ATCC 200956 led to a phenotypic shift (from resistant to susceptible), resulting in EO/FLC and thymol/FLC MIC values similar to those of the FLC-susceptible strain. The above results (Table 2 and Figure 2) show that the EO and thymol potentiated the FLC activity against the two strains, but this effect was more noticeable with the resistant strain.

Applying the Holetz et al. criteria for defining the activity of natural products [55], the activity of the EO/FLC and thymol/FLC mixtures against the resistant strain changed from moderate (GM-MIC = 128/64 µg/mL) to good (GM-MIC = 32/2 µg/mL), while in the susceptible strain, the EO/FLC changed from low (GM-MIC = 512/2 µg/mL) to moderate activity (GM-MIC = 181/0.25 µg/mL), and thymol/FLC from moderate (GM-MIC = 256/2 µg/mL) to good activity (GM-MIC = 42/0.60 µg/mL). These results strongly suggest that the EO and thymol potentiated the action of the FLC in the two strains, but more so with the resistant strain.

The use of strains with a known resistance mechanism to evaluate the antifungal activity of new molecules or phytocompounds can offer some insights not only to identify compounds with activity against resistant strains, but also to discover new targets or mechanisms of action.

A review by Bhattacharya et al. [21] described different mechanisms of synergistic action in EO–antifungal combinations. These comprise disruption of the cell wall structure and ergosterol biosynthesis pathway, pump/transporter activation in the cell membrane, cell membrane permeability alterations, intracellular leakage of cellular contents, inhibition of germ tube formation, fungal biofilm formation, and competition for a primary target. Furthermore, Zhang et al. proposed that the synergistic effect of *Melaleuca leucadendra* (L) EO and four antifungals from the azoles family against *C. albicans* was the result of mesosome-like structures forming around the cell membrane and in the cytoplasm. The EO altered the membrane permeability, thus facilitating antifungal cell penetration [45].

In this study, using TEM and SEM, we observed changes in external structure continuity and pores and an increase in the wall thickness after exposing cells to EO/FLC or thymol/FLC mixtures. Similar effects were observed with the antifungals (Figures 6 and 7). The fungal plasmatic membrane and the cell wall have important cell functions as a stress response and allow the selective entry and exit of molecules. Damage to its structure

affects normal physiological processes and leads to cell death [56]. Although we did not investigate the targets nor mechanisms of action that would allow us to explain the FLC-resistance phenotype reversal, it is reasonable to suggest that the *C. tropicalis* ATCC 200956 membrane composition changes (without ergosterol but with 14α-methylated accumulated sterols, as previously confirmed) [11], making the cell membrane more permeable to EO compounds and thymol. In addition to superficial morphological changes, the mitochondrial membrane potential of *C. tropicalis* ATCC 200956 cells was altered by all the treatments (Figure 2).

Several studies have demonstrated that changes in mitochondrial functions have an important role in the susceptibility to different antifungal molecules [28,57,58]. On the other hand, an increase in O_{2-} production was detected when cells were treated with thymol and thymol/FLC (Figure 3). Experiments carried out with different fungal models allowed for the detection of ROS production in mitochondria in response to antifungal compounds or to phytocompound mixtures. Reactive Oxygen Species affect cell viability due to cell membrane oxidation, affect proteins, lipids, and nucleic acids, and also activate proapoptotic signals [59–61]. For these reasons, mitochondria are considered a possible target for the development of new antifungal drugs [62].

Nuclear morphology and the cell cycle were affected by EO, thymol, FLC, and by their combinations (EO/FLC and thymol/FLC) (Figures 4 and 5). The cell cycle analysis showed an increase in the amount of DNA (MFI 1.5 + MFI 2) (Figure 5). This may correspond to an increase in polypoidal cell states [63,64]. In yeasts, under optimal conditions, haploid and diploid cells as well as the number of chromosomes are usually stable; however, under stress conditions, it is possible to reach polypoidal states. It is known that tetraploid cells are arrested in the stationary phase, have unstable genomics, and lose chromosomes [63,65]. Additionally, polyploidy in yeasts is related to greater sensitivity to antifungal treatments, whose target is microtubule depolymerization or inhibition of DNA replication [63].

In a recent analysis of *C. tropicalis* ATCC 200956 and *C. tropicalis* ATCC 750 carried out by our group, we observed significant changes in gene expression in response to *L. origanoides* EO and thymol. The gene regulation implicated in energy metabolism, sterol

synthesis, nucleosome assembly, mitotic spindle and microtubule organization, and transmembrane domains involved in wall lipid homeostasis fungal function were altered). These results concur with the findings in this study.

4.5. Conclusion

From the results shown in this paper, we are able to conclude firstly that *L. origanoides* EO or thymol interaction with FLC results in a synergistic antifungal effect in both azole- and AMB-resistant or -susceptible *C. tropicalis* strains. The effect was more notable with the resistant *C. tropicalis,* where the phenotype was reverted. Secondly, this synergistic effect also applied to their impact on fungal cell structures and important cell viability functions.

Further research is required to understand the mechanisms by which the synergism reverses the resistant *C. tropicalis* strain phenotype. This could shed light on new antifungal targets and mechanisms of action. Additionally, in vivo studies could be undertaken to confirm the findings of this work and evaluate the potential for the development of future antifungals.

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5. Propuesta de artículo # 3

De los modelos *in vitro* a *Galleria mellonella* para la evaluación de la toxicidad de un aceite esencial de *Lippia origanoides* y del timol

Resumen

Introducción. Actualmente para la evaluación de la toxicidad *in vivo* de fármacos o de productos naturales con actividad biológica, se prefieren modelos que cumplan la regla de las "3Rs" (reducir, reemplazar y refinar), condiciones con las que cumple el lepidóptero *Galleria mellonella.* Dada las diversas actividades biológicas del aceite esencial de *L. origanoides* y el timol, es importante evaluar su toxicidad. ***Objetivo.*** Evaluar la toxicidad del aceite esencial (AE) de *Lippia origanoides* quimiotipo (timol + *p*-cimeno) y del timol en *Galleria mellonella.* ***Metodología***. Inicialmente, se evaluó la genotoxicidad por medio del ensayo cometa alcalino en línea celular HaCaT. Para la evaluación de la toxicidad *in vivo* se inocularon grupos de larvas de *G. mellonella* con 128 y 256 µg/mL del aceite esencial o del timol. La diferencia en la supervivencia de las larvas entre los tratamientos se determinó con la prueba de Mantel-Cox. Los experimentos se realizaron por triplicado en momentos diferentes. ***Resultados.*** El AE y el timol no fueron genotóxicos (95,5 y 97,5 % de las células sin daño, respectivamente). Un bajo porcentaje de células presentaron daño tipo uno (4,5 y 2,5 % de las células tratadas con el AE o el timol, respectivamente). La evaluación de la toxicidad en *G. mellonella* mostró que el AE y el timol no fueron tóxicos ($p > 0.05$). ***Conclusión.*** Los resultados sugieren que el AE y del timol no son tóxicos en los modelos evaluados y que *G. mellonella* es un modelo que podría considerarse para la evaluación de la toxicidad *in vitro* de AE y terpenos.

5.1. Introducción

El estudio de la toxicidad de compuestos químicos, con potencial uso en humanos o animales es esencial para reducir el riesgo de exposición a materiales que puedan afectar la salud o impactar en la economía de un país [1]. Si bien la evaluación de la toxicidad en diferentes líneas celulares evidencia el efecto de determinado compuesto en la proliferación celular (citotoxicidad) o daños en el ADN (genotoxicidad) [2,3], los factores asociados al metabolismo del compuesto no se pueden simular en cultivos celulares [3]; además, factores como el uso de diferentes líneas celulares, los métodos de conservación de estas, condiciones de cultivo y el número de pases, pueden generar diferencias en los resultados entre laboratorios [4].

Actualmente para la evaluación de la toxicidad *in vivo* de fármacos o de productos naturales con actividad biológica, se prefieren modelos que cumplan la regla de las "3Rs" (reducir, reemplazar y refinar), condiciones con las que cumple el lepidóptero *Galleria mellonella*. Las larvas *G. mellonella* han sido utilizadas para evaluar la toxicidad relativa a diferentes agentes antimicrobianos, la toxicidad de aditivos alimentarios, la virulencia microbiana y eficacia antimicrobiana, con resultados comparables a estudios realizados en mamíferos [5]. *Galleria mellonella* es un modelo que actualmente no supone restricciones éticas para su uso; además, la estructura y función de su sistema inmunitario innato, así como enzimas implicadas en la desintoxicación de drogas son similares a las de los mamíferos [6,7]. Otra ventaja que ofrece este modelo es la posibilidad de usar grandes grupos de larvas y así aumentar el poder estadístico de los ensayos [8].

Las diferentes actividades biológicas de los AE de *L. origanoides* y algunos de los componentes se ha demostrado previamente (antifúngica, antibacteriana, antibiopelícula, antiviral y anti-*Trypanosoma* spp). [9–13], además han mostrado baja toxicidad en líneas celulares [14–16]. Sin embargo, es necesario escalonar los estudios a modelos *in vivo* y así tener datos más cercanos a los posibles efectos de estos en el hombre o en mamíferos más complejos

El objetivo de este apartado del trabajo fue evaluar la genotoxicidad y la toxicidad de un AE de *L. origanoides* quimiotipo (timol + *p*-cimeno) y del timol en *G. mellonella.*

5.2. Materiales y métodos

5.2.1. Compuestos

Se utilizó un AE destilado de *L. origanoides* quimiotipo (timol + *p*-cimeno). El AE se obtuvo por hidrodestilación y caracterizó por cromatografía de gases (GC-MS), como se describió anteriormente [14]. El timol (2-isopropil-5-metil fenol) y el FLC (fluconazol) se obtuvieron comercialmente (Sigma-Aldrich, St. Louis, Mo, USA). Las soluciones Stock de los compuestos se prepararon en dimetil sulfoxido, (DMSO; Sigma-Aldrich, St. Louis, MO, USA); las soluciones de trabajo se prepararon en Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, U.S.A.), para el ensayo de genotoxicidad y en PBS 1X para el ensayo de toxicidad *in vivo*. Para ambos ensayos se tomó como referencia

los valores de la concentración mínima inhibitoria (CMI) de cada compuesto, obtenidos con la cepa *Candida tropicalis* ATCC 200956 resistente a FLC [14].

5.2.2. Línea celular

Se usó la línea celular HaCaT derivada de queratinocitos epidermales de adultos normales (HEKa) PCS-200-011TM, fueron donadas por el Dr. Juan Carlos Gallego-Gómez (Molecular and Translational Medicine Group, Universidad de Antioquia). Las células se cultivaron en DMEM y se suplementaron con 10 % de suero fetal bovino (Invitrogen, Carlsbad, CA, USA.), 1% de penicilina, estreptomicina y neomicina (Invitrogen, Carlsbad, CA, USA) y 1% L-glutamina (Invitrogen, Carlsbad, CA, U.S.A); posteriormente, las células se incubaron a 37 °C en ambiente humidificado con 5% de CO_2. Después de alcanzar una confluencia del 80%, las células se disociaron usando tripsina (Sigma-Aldrich, St. Louis, MO, U.S.A.), se lavaron con PBS 1X y se mantuvieron en crecimiento hasta su uso.

5.2.3. Modelo in vivo

*S*e seleccionaron larvas de *G. mellonella* en la sexta etapa de desarrollo suministradas por Mous, R.J. - Live Bait v.o.f. (Netherlands); para cada tratamiento se usaron 2 grupos de 10 larvas con peso entre 0,25 – 0,40 g (para un total de 20 larvas por grupo).

5.2.4. Ensayo cometa alcalino

El efecto genotóxico del AE, el timol y el FLC en células HaCaT se evaluó por medio del ensayo cometa alcalino como lo describen Quintero y col. [17]. En resumen, el cultivo inicial se mantuvo bajo las condiciones descritas anteriormente, luego de 24 h de crecimiento, las células se cultivaron en microplacas de 96 pozos (Corning®, Costar®, NY, USA) a una densidad de 20.000 cel/pozo, se incubaron a 37 °C, durante 24 h con 5% de CO2 y 95 % de humedad. Posteriormente, se trataron con el AE o el timol a 128 µg/mL y se incubaron bajo las mismas condiciones de tiempo y temperatura. Como control negativo de daño se usaron células sin tratamiento y como control positivo genotoxicidad las células se trataron con DMSO al 4%. Adicionalmente, se incluyeron células tratadas con 121 µg/mL de FLC. Después del tratamiento, las células se disociaron con 50 µL de tripsina + DMEM, durante 10 min a 37 °C; luego, se centrifugaron a 1000 g y se resuspendieron en DMEM. Posterior a ello, las células se mezclaron con 50 µL de agarosa de bajo punto de

fusión (Promega, Madison, WI, USA) al 0,5 % y se pusieron sobre láminas tratadas previamente con agarosa (PanReac AppliCem, Barcelona, España) al 1.5%, las láminas se incubaron 20 min a 4 °C en la oscuridad; luego se sumergieron durante 2 h en la solución de lisis (NaCl 2,5 M; EDTA 100 mM; Tris 10 mM). La electroforesis en tampón alcalino se realizó a 25 V por 40 min. Finalmente, las láminas se sumergieron en la solución de neutralización (tris 0.4 M, pH 7,5) durante 5 min y se tiñeron con 1 µg/mL de diamidino-2-fenilindol (DAPI) (Sigma-Aldrich, St. Louis, MO, USA).

Las células teñidas se observaron con un microscopio de fluorescencia (Nikon Eclipse, Tokyo, Japan). La determinación del daño causado al genoma celular, se realizó de forma visual de acuerdo a la intensidad de la cola en relación con la cabeza del cometa, la cual está dada por la migración del ADN de cadena sencilla. Se analizaron 100 células y a cada una se le asignó un puntaje desde cero (sin daño evidente del ADN) hasta 4 (daño mayor del ADN) de acuerdo a Collins y cols. [18], los experimentos se realizaron por duplicado y los resultados se expresaron como porcentaje de células en cada nivel de daño ± DS.

5.2.5. Evaluación de la toxicidad de un aceite esencial de L. origanoides y de timol en G. mellonella

El AE y el timol se inocularon directamente en el hemocele de las larvas en la última propata izquierda mediante inyección con una jeringa Hamilton. Cada larva se inoculó con 10 µL del AE o timol a 128 µg/mL y 256 µg/mL (44,8 y 89,6 µg por larva, respectivamente). Como controles se usaron grupos de larvas no inoculadas o inoculadas con PBS 1X o FLC a una concentración de 8 µg/mL. Posterior a la inoculación, las larvas se incubaron a 37 °C y la supervivencia se verificó cada 24 h, durante 7 días. Se consideró que las larvas habían muerto cuando no respondían al estímulo físico. Los ensayos se realizaron por triplicado en experimentos diferentes. Los datos se graficaron en curvas de muerte y el análisis de las diferencias en la supervivencia entre los grupos con la prueba de Mantel-Cox, utilizando Prisma 6.0 (GraphPad, San Diego, California, USA). Un valor de $p < 0,05$ se consideró significativo.

5.3. Resultados

5.3.1. El aceite esencial de L. origanoides y el timol no tienen efecto genotóxico en células HaCaT

La migración del ADN de las células HaCaT, se usó como indicador de daño genómico. En la **Figura 1.** se observa la fluorescencia emitida por las células HaCaT después de los tratamientos. En el control positivo de daño genotóxico (DMSO al 4%) se observó migración del ADN formando el fenómeno conocido como "cola del cometa" con niveles de daño desde 1 hasta 4 mientras que la en las células tratadas con el AE, el timol y el FLC la migración del ADN fue menor mostrando únicamente daño tipo 1.

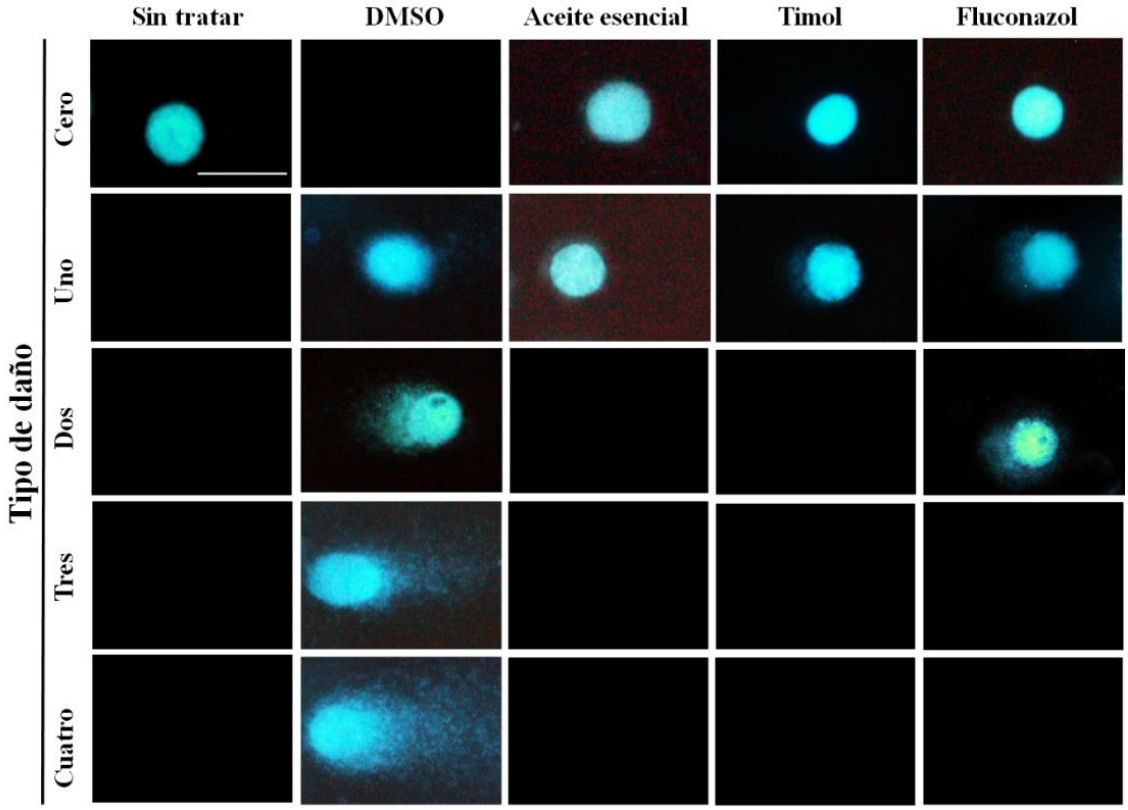

Figura 1. Imágenes obtenidas por microscopía de fluorescencia de células HaCaT teñidas con DAPI posterior al tratamiento con el AE, timol, fluconazol, sin tratamiento o expuestas al DMSO (controles). La barra de escala corresponde a 20 µm.

En el 95,5 % de las células tratadas con el AE y el 97,5 % con el timol no se observó daño (daño tipo cero). Aunque con FLC hubo daño tipo cero, uno y dos, el porcentaje de células sin daño fue similar al observado con el AE y el timol (**Tabla 1)**.

Tabla 1. Tipo de daño genómico observado en células HaCaT

Compuestos	**Tipo de daño (% ±D.S)**		
	cero	**uno**	**dos**
AE	95,5 (±0,7)	4,5 (±0,7)	0
Timol	97,5 (±0,7)	2,5(±0,7)	0
FLC	92 (±2,8)	5 (±1,4)	3 (±1,4)

5.3.2. El aceite esencial de L. origanoides y el timol no afectaron la supervivencia de G. mellonella

La **Figura 2** corresponde a las curvas de supervivencia de las larvas de *G. mellonella* tras el tratamiento con dos concentraciones del AE y el timol. A una concentración de 128 µg/mL, en ambos casos, las larvas mostraron una supervivencia similar a las inoculadas con PBS, después de siete días la supervivencia fue del 82 %. Las larvas tratadas con 256 µg/mL del AE y del timol mostraron resultados similares el día seis de seguimiento (86,5 y 89 % de supervivencia, respectivamente); no obstante, el día siete la supervivencia fue del 43 % (AE) y el 77 % (timol). Luego del análisis estadístico no se evidenciaron diferencias significativas en la supervivencia de las larvas tratadas con ambas concentraciones del AE y el timol comparado con el PBS. La supervivencia de las larvas tratadas con el FLC (62,8 %) fue significativamente menor $p = 0,03$).

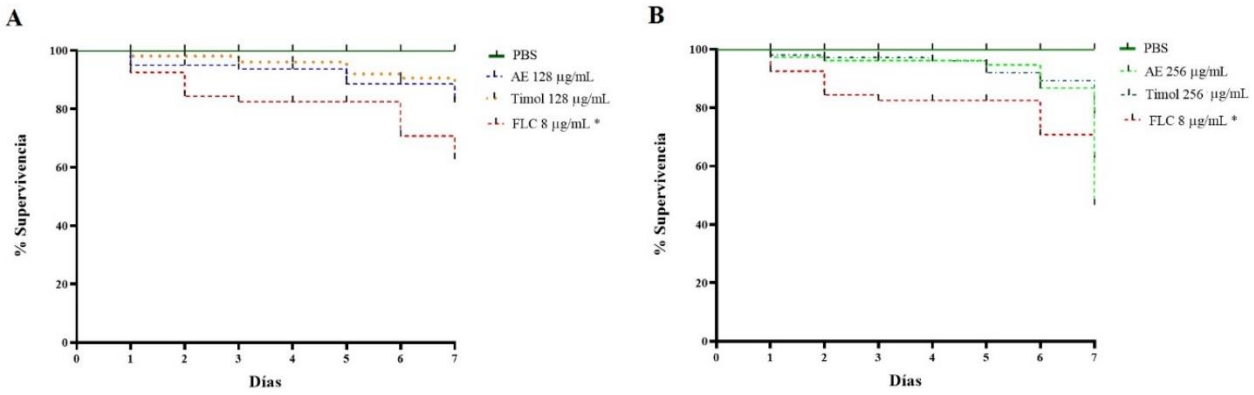

Figura 2. Supervivencia de larvas de *G. mellonella* tratadas con diferentes concentraciones del AE o el timol. A) 128 µg/mL; B) 256 µg/mL. En cada gráfica se incluyó el grupo control (PBS) y larvas inoculadas con FCL.

5.4. Discusión

El uso de las plantas aromáticas con fines curativos tiene orígenes ancestrales, sin embargo, las propiedades benéficas atribuidas a estas no las hace inofensivas [19]. En ese sentido, la diversidad química de los componentes de los AE puede ser fuente de compuestos bioactivos, pero también pueden ser potencialmente tóxicos [3,20,21]. Los ensayos de citotoxicidad y la genotoxicidad *in vitro* son un acercamiento válido para la identificar la toxicidad de estos compuestos; sin embargo, el uso de modelos animales es necesario para determinar el riesgo toxicológico de un compuesto y evaluar la seguridad para su uso especifico [22].

Estudios previos, demostraron que los aceites de AE de *L. origanoides* y algunos de sus componentes mayoritarios (timol y carvacrol) son poco tóxicos en líneas celulares HaCaT, Vero y fibroblastos de ligamento periodontal [14–16]. En el mismo sentido, los resultados del presente estudio indican que el AE de *L. origanoides* quimiotipo (timol + *p*-cimeno) y el timol no son genotóxicos en células HaCaT. Vicuña y cols., reportaron el efecto antigenotóxico del AE de *L. origanoides*, el timol y el carvacrol [23]; además, se ha reportado el efecto protector de estos terpenos frente a la citotoxicidad inducida por el H_2O_2 [24].

En la actualidad diferentes modelos como el gusano *Caenorhabditis elegans*, el pez zebra y varios insectos incluyendo *G. mellonella* se están utilizando para la evaluación de la toxicidad de compuestos antimicrobianos [6,25–27]. *Galleria mellonella* es un modelo

versátil, ampliamente utilizado por ser una opción poco costosa, de fácil manipulación, y de momento, sin restricciones éticas [5,28,29]. Los resultados derivados de este trabajo, permitieron demostrar que el AE de *L. origanoides* quimiotipo (timol + *p*-cimeno) y el timol no son tóxicos para *G. mellonella* a la concentración equivalente a la CMI obtenida en varias especies de *Candida;* estos hallazgos están en concordancia con lo encontrado por Hernandes y cols. quienes demostraron que el AE de *L. origanoides* no es tóxico en ratones ni induce cambios mutagénicos [30]; otros investigadores han publicado resultados similares con los AE de *Origanum majorana* también rico en carvacrol, *Syagrus coronata* rico en ácidos grasos y *Cinnamomum verum* rico en eugenol [31–33].

Finalmente, se ha encontrado correlación positiva entre la dosis letal 50 ($DL5_{50}$) de diversos compuestos evaluados en *G. mellonella* y en modelos murinos [34]. Lo anterior sugiere que los ensayos realizados para la evaluación de la toxicidad de AE en *G. mellonella* pueden ser comparables con los realizados en mamíferos.

5.5. Conclusión

El aceite esencial de *L. origanoides* y el timol, no son tóxicos, por lo que pueden ser potencialmente seguros para su uso, sin embargo, se requieren más estudios. Adicionalmente, se puede concluir que *G. mellonella* es un sistema adecuado para la evaluación de la toxicidad *in vivo* como una primera aproximación en el desarrollo de productos comercializables.

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6. Propuesta de articulo # 4

La expresión génica en *Candida tropicalis* sugiere un efecto multidiana de un aceite esencial de *Lippia origanoides* y del timol

Resumen

Introducción. En los aceites esenciales de *Lippia origanoides* y el timol, se ha identificado actividad anti-*Candida* spp.; sin embargo, no es clara la diana (s) involucrada o el mecanismo (s) de acción. **Objetivo.** Evaluar la expresión de genes en *C. tropicalis* tratada con un AE y con timol. **Metodología.** El efecto del AE de *L. origanoides* quimiotipo timol + *p*-cimeno y del timol sobre la expresión génica con *C. tropicalis* (azol-sensible y azol-resistente) se evaluó usando la plataforma RNAseq con un Log_2 FC ±2 y un *p*-valor ajustado <0,05. El enriquecimiento de los genes expresados diferencialmente se realizó con la plataforma FungiFun. **Resultados**. El AE y el timol afectaron la expresión de genes en ambas cepas; se observó una regulación negativa de 91 y 313 genes con el timol y 493 y 233 genes con el AE en la cepa resistente y sensible, respectivamente. La regulación al alza fue de 265 y 16 genes con timol y 229 y 13 genes con el AE con la cepa resistente y sensible, respectivamente. Las categorías GO más significativas estuvieron relacionadas con transporte transmembrana, síntesis de ergosterol, metabolismo energético, respuesta a estrés, procesos ribosomales entre otros. **Conclusión.** El AE de *L. origanoides* y el timol tienen un efecto multi-diana, alteran varios procesos celulares, diferente a lo causado por los antifúngicos de uso clínico actuales.

6.1. Introducción

Candida tropicalis es un patógeno fúngico emergente, de manejo prioritario por su potencial para causar enfermedades invasivas en pacientes inmunosuprimidos o en unidades de cuidados intensivos (UCI) [1,2], en *C. tropicalis* se ha descrito la resistencia cruzada a los azoles y la anfotericina B (AMB) debido a mutaciones en el *ERG11* y el *ERG3*, genes involucrados en la biosíntesis de ergosterol, el principal esterol de la membrana fúngica [3,4]; también se ha descrito la presencia de bombas de eflujo, formación de biopelículas y aumento en la síntesis de ergosterol como mecanismo de resistencia a estos antifúngicos [5]. La resistencia antimicótica, la toxicidad de las terapias actuales y el lento desarrollo de nuevos antifúngicos supone un desafío que ha llevado a la búsqueda de compuestos con potencial antifúngico que superen o mejoren las terapias actuales [6,7]. En ese sentido, la riqueza en la naturaleza química de los aceites esenciales y sus componentes mayoritarios (terpenos) los hace fuertes candidatos [8,9].

La diversidad de plantas en Colombia ha propiciado las investigaciones sobre el potencial biológico de aceites esenciales destilados de plantas aromáticas de diferentes familias, la familia Verbenaceae es de gran interés, dentro de ella las plantas del género *Lippia*, son usadas en la medicina tradicional para aliviar dolencias gastrointestinales y respiratorias [10], además se ha demostrado su actividad biológica [11–13].

Lippia origanoides es una planta aromática nativa de Centroamérica, Suramérica y África [10] las propiedades anti-*Candida* de los AE destilados de esta planta y sus componentes mayoritarios han sido han sido evaluadas anteriormente [14,15], sin embargo, se conoce poco sobre los mecanismos de dicha actividad.

Por las características lipofílicas de los AE y al pequeño tamaño de los terpenos Algunos autores han propuesto la membrana plasmática (MP) y la membrana mitocondrial fúngica como posibles dianas de estos compuestos [16,17]. La membrana plasmática es una estructura fluida compuesta por fosfolípidos, glucolípidos y esteroles; esta estructura cumple funciones de absorción de nutrientes, transducción de señales, morfogénesis y síntesis de pared celular [18]; su versatilidad funcional se debe a la compartimentalización lateral en diferentes dominios los cuales están formados por lípidos y proteínas [19]. Dada la importancia de la MP en la virulencia, la señalización celular y respuesta a estrés, la desregulación en la síntesis de sus componentes de la afectan la funcionalidad y viabilidad fúngica. [19,20]

Por otro lado, la producción de energía es un determinante en la viabilidad celular, la mitocondria interviene en el crecimiento, la virulencia y homeostasis lipídica de la célula fúngica, debido su importancia a nivel celular es un blanco interesante para nuevas terapias antimicóticas [21]. Estudios han demostrado la asociación de la actividad antimicótica de AE y terpenos con su efecto en la mitocondrial [17,22].

Además del efecto en la MP fúngica y en la mitocondria, también se ha descrito el aumento en la producción de especies reactivas del oxígeno, el arresto en diferentes etapas del ciclo celular y la fragmentación del ADN como resultado del tratamiento con AE o terpenos [16,23,24]. A pesar de las evidencias a nivel fenotípico, pocos estudios han evaluado el efecto de los AE o terpenos a nivel transcripcional en *Candida* spp.

El objetivo de esta parte del trabajo fue evaluar la expresión génica de dos cepas *C. tropicalis* en respuesta al tratamiento con un AE de *L. origanoides* y el timol.

6.2. Materiales and Métodos

6.2.1. Cepas

Las cepas *Candida tropicalis* ATCC 200956 y *C. tropicalis* ATCC 750, resistente y sensible a los azoles, respectivamente, se obtuvieron de la colección American Type Culture Collection (Manassas, VA, USA) y se mantuvieron en agar Sabouraud Dextrosa (SDA; Sigma-Aldrich, St. Louis, MO, USA), a 4 °C. Antes del tratamiento con los compuestos, se propagaron masivamente en caldo Sabouraud (SB; Sigma-Aldrich, St. Louis, MO, USA) 24 horas a 35 °C.

6.2.2. Compuestos

El AE de *L. origanoides* quimiotipo (timol + *p*-cimeno) fue obtenido por hidrodestilación y caracterizado por cromatografía de gases (GC-MS), como se describió anteriormente [25]. El timol (2-isopropil-5-metil fenol) se obtuvo de forma comercial (Sigma-Aldrich, St. Louis, Mo, USA). Las soluciones de trabajo se prepararon en RPMI + MOPS como se describió en el apartado 6.2.2.

6.2.3. Condiciones de tratamiento

La sensibilidad de *C. tropicalis* ATCC 200956 y *C. tropicalis* ATCC 750 al AE de *L. origanoides* quimiotipo (timol + *p*-cimeno) y al timol se evaluó como se describe en el apartado 6.2.7. Para la evaluación de la expresión diferencial, *C. tropicalis* se propagó masivamente en SB durante 24 h a 35 °C en agitación continua, posteriormente se realizaron 2 lavados en PBS 1X y se ajustó el inóculo a 4 x 10^6 células/mL. Luego las levaduras se trataron con 128 µg/mL del AE o el timol durante 4 h bajo las mismas condiciones de temperatura y agitación descritas. Posteriormente se retiró el medio de cultivo por medio de centrifugación y se realizaron dos lavados con PBS 1X, el pellet se almacenó a -20 °C durante toda la noche. Ambas cepas de *C. tropicalis* sin tratamiento se incluyeron como controles de expresión basal.

6.2.4. Extracción, preparación de librerías y secuenciación del ARN

La extracción del ARN se realizó con el Trizol (TRI Reagent®, Merck, Sigma-Aldrich, St. Louis, Mo, USA), con algunas modificaciones, las levaduras se rompieron usando perlas de zirconio/silica de 0,5 mm de diámetro usando FastPrep-24 (MPTM, CA, USA), mediante 6 ciclos de 20 seg alternando con 1 minuto de hielo. Los extractos se resuspendieron en 20 µL de agua ultrapura y se almacenaron a -20 °C hasta su uso. La concentración y calidad de las muestras se estimó usando Agilent 2100 Bioanalyzer. Las librerías se prepararon usando 1 ng de ARN total, usando TruSeq Stranded mRNA kit (Illumina, 20019792). El ARN poliadenilado se purificó usando perlas de oligodT, el ARN se fragmentó y convirtió en ADN copia de doble cadena (ds cDNA), luego se adicionaron los adaptadores índices y se obtuvieron las librerías por medio de PCR. La calidad y concentración de las librerías, se estimó usando Agilent 2100 Bioanalyzer y QuantiFluor® RNA System (Promega, E3310). La secuenciación se realizó usando en kit NextSeq 500/550 Mid Output v2.5 en la plataforma NextSeq 550 Illumina (Illumina, 20024904).

6.2.5. Análisis de la expresión génica

Los genes expresados diferencialmente en respuesta a el AE o el timol se definieron usando un fold change (FC) Log_2 ±2, donde el FC es el cambio de veces en fragmentos por kilobase por millón de valores de lecturas mapeadas (FPKM). Para el enriquecimiento génico se usó la plataforma Fungi Fun 2.2.8. (Instituto Hans Knöll, Jena, Alemania), la función de las proteínas de los genes anotados se obtuvo con UniProt.

6.3. Resultados

Los cambios transcriptómicos asociados al tratamiento con el AE o el timol se determinaron por medio de la secuenciación del ARN (RNAseq) de *C. tropicalis* sensible y resistente a los azoles. En el perfil génico de la cepa azol-resistente tratada con el AE o el timol se observó la expresión diferencial de 722 y 584 genes, respectivamente. En la cepa azol-sensible el AE indujo la expresión de 246 genes y el timol 546 genes.

En ambas cepas de *C. tropicalis* se observó una regulación global a la baja luego del tratamiento con el AE, en comparación con las levaduras sin tratar. El timol causó regulación negativa en la cepa sensible, mientras que en la cepa resistente la mayoría de genes expresados diferencialmente estuvieron al alza en respuesta a este tratamiento.

La herramienta Gene Ontology permite identificar el papel biológico de un conjunto de genes desde tres aspectos diferentes: procesos biológicos, función molecular y componente celular [26]. El análisis de la ontología génica (GO), reveló 70 categorías enriquecidas en la cepa resistente y 78 categorías enriquecidas en la cepa sensible. En la **Tabla 1** se muestran los resultados de la sobreexpresión y subexpresión génica en ambas cepas con cada tratamiento.

Tabla 1. Número de genes expresados diferencialmente y enriquecimiento génico

	Cepa	Compuesto	# de genes	Genes sin anotación	# Genes en las categorías p <0,05	# Catrgorías p <0,05
Sobrexpresión	*C. tropicalis* ATCC 200956	AE	229	110	42	6
		Timol	265	124	14	11
	C. tropicalis ATCC 750	AE	13	5	7	18
		Timol	16	7	7	14
Subexpresión	*C. tropicalis* ATCC 200956	AE	493	154	238	36
		Timol	91	34	34	17
	C. tropicalis ATCC 750	AE	233	115	59	15
		Timol	313	149	94	31

Las categorías GO más significativas asociadas al tratamiento con el AE o el timol en ambas cepas, se muestran en la **Figura 1A.** En la cepa resistente los términos GO se relacionaron con procesos mitocondriales (sobre) y la síntesis de proteínas (sub); mientras que en la cepa sensible con procesos de respuesta a estrés (sobre) y transporte transmembrana (sub). El enriquecimiento génico en la cepa resistente en respuesta al timol se relacionó con la producción de energía celular (sobre) y síntesis de esteroles (sub); en la cepa sensible con respuesta a estrés (sobre) y transporte transmembrana (sub).

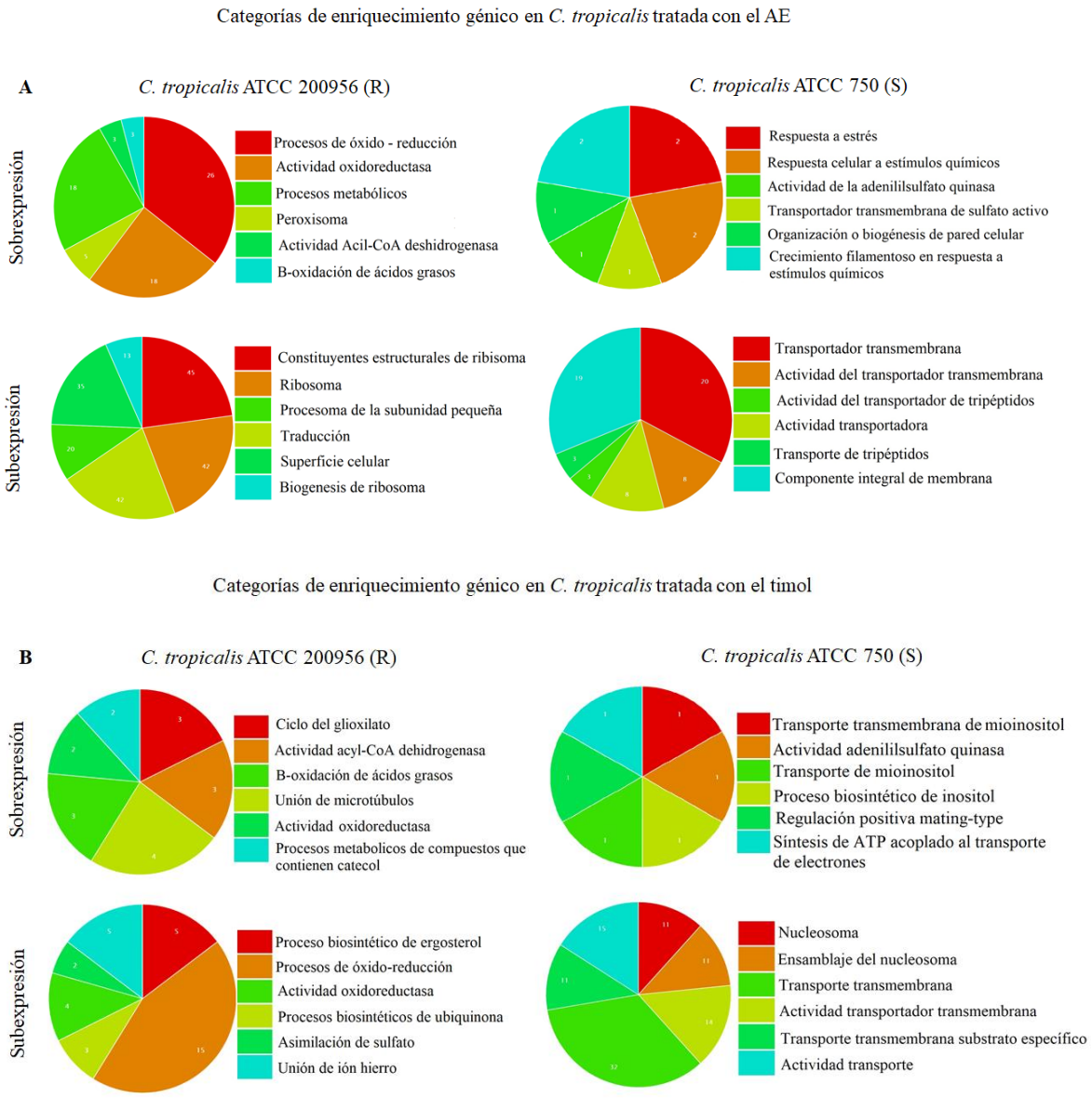

Figura 1. Categorías de enriquecimiento génico en *C. tropicalis* tratada con el AE de *L. origanoides* quimiotipo (timol + *p*-cimeno) (A); timol (B). Resistente a los azoles (R); Sensible a los azoles (S).

Algunos genes expresados diferencialmente fueron comunes en ambas levaduras, sin embargo, se observó que la expresión diferencial de varios de ellos en la cepa resistente era ocasionada por el AE, mientras que en la cepa sensible era el timol quien ocasionaba los cambios. Genes involucrados en el metabolismo de los fosfoinosítidos (**Tabla 2)**, el ensamblaje del nucleosoma (**Tabla 3**) y la formación del microdominio MCC/eisoma, siguieron este patrón. Estas diferencias pueden estar relacionadas con el contenido del timol

en el AE de *L. origanoides* (49,4%) y la sensibilidad de ambas levaduras a estos compuestos (sección 3.3.2.). En la **Tabla 2 y 3** se muestran algunos de los genes expresados en ambas levaduras como respuesta al AE de *L. origanoides* y el timol.

Tabla 2. Sobrexpresión de genes en *C. tropicalis* tratada con el AE de *L. origanoides*

	AE *L. origanoides*	
	Proteína	**Proceso biológico/vía de señalización**
Resistente	Inositol-3-fosfato sintasa	Metabolismo de los Glicerofosfolípidos /Respuesta a estrés
	Acil-coenzima A oxidasa	Vía de oxidación beta de ácidos grasos
	Proteína similar a la cinesina	Ciclo celular en levadura, regulación de la organización del huso mitótico
	Malato sintasa	Ciclo de citrato (ciclo TCA), metabolismo del piruvato
Sensible	Sulfato permeasa 1	Metabolismo del azufre
	Proteína que contiene el dominio N-terminal de ciclina	Ciclo celular – levadura
	Adenilil-sulfato quinasa	Metabolismo del azufre / Metabolismo de purinas
	Transportador de fosfato	Actividad del transportador transmembrana de fosfato inorgánico

	Timol	
	Proteína	**Proceso biológico/vía de señalización**
Resistente	Proteína que contiene el dominio motor cinesina	Regulación de la organización del huso mitótico
	Acil-coenzima A oxidasa	Actividad acetato-CoA ligasa, Unión de AMP
	Proteína similar a la cinesina	Ciclo celular en levadura, regulación de la organización del huso mitótico
	Malato sintasa	Ciclo de citrato (ciclo TCA), metabolismo del piruvato
Sensible	Inositol-3-fosfato sintasa	Metabolismo de los glicerofosfolípidos /Respuesta a estrés
	Transportador de fosfato	Regulación del transporte transmembrana de fosfatos
	Adenilil-sulfato quinasa	Metabolismo de las purinas
	Mio-inositol transportador 1	Metabolismo de los glicerofosfolípidos
	Manosiltransferasa	Biosíntesis de anclaje GPI
	NADH-ubiquinona oxidorreductasa	Fosforilación oxidativa

Tabla 3. Subexpresión de genes en *C. tropicalis* tratada con el AE de *L. origanoides*

	AE L. origanoides	
	Proteína	**Proceso biológico/vía de señalización**
Resistente	Histona H3	Organización del nucleosoma
	Proteína de tabicación SUN4	Actividad hidrolasa, que actúa sobre los enlaces glucosídicos
	Histona H4	Componente central del nucleosoma
	Escualeno monooxigenasa (Erg1)	Biosíntesis de ergosterol
	Proteína que contiene el dominio de unión de superóxido	Pared celular de tipo fúngico
	Enolasa 1	Vía pentosa fosfato, gluconeogénesis, glicolisis
	Proteína NCA3, mitocondrial	autofagia mitocondrial
	Proteína del complejo nucleolar 2	Biogénesis del ribosoma
	C-8 esterol isomerasa (Erg2)	Proceso biosintético de ergosterol
	Msb2	vía de señalización MAPK
Sensible	Proteína que contiene el dominio MFS	Importación de glucosa, Transporte de sustancias orgánicas
	Transportador de hexosa 2	Biosíntesis de ancla de glicosilfosfatidilinositol (GPI)
	Transportador de péptidos PTR2	Regulación del proceso de biosíntesis de lípidos
	Oxidasa alternativa	Ciclo de citrato (ciclo TCA), Fosforilación oxidativa
	Proteína que contiene el dominio gamma de la proteína G	Vía de señalización MAPK

	Timol	
	Proteína	**Proceso biológico/vía de señalización**
Resistente	Escualeno monooxigenasa (Erg1)	Biosíntesis de ergosterol
	C-4 metilesterol oxidasa (Erg25)	Proceso biosintético de ergosterol
	C-8 esterol isomerasa (ERG2)	Proceso biosintético de ergosterol
	Esterol 14-α-desmetilasa (Erg11)	Proceso biosintético de ergosterol
	3-keto-esteroide reductasa (Erg27)	Biosintesis de esteroles, proceso biosintético de ergosterol
	Proteína ras-1	Vía de señalización MAPK
	Proteína de unión a la subunidad beta de la proteína G	Vía de señalización MAPK
Sensible	Proteína que contiene el dominio MFS	Importación de glucosa, Transporte de sustancias orgánicas
	Transportador de péptidos PTR2	Regulación del proceso de biosíntesis de lípidos
	Transportador de hexosa 2	Importación de carbohidratos a través de la membrana plasmática
	Histona H4	Ensamblaje de nucleosomas
	Proteína de tabicación SUN4	Actividad hidrolasa, que actúa sobre los enlaces glucosídicos
	Oxidasa alternativa	Ciclo de citrato (ciclo TCA), Fosforilación oxidativa

6.4. Discusión

En la actualidad existe gran interés en los AE y terpenos debido su potencial antifúngico, sin embargo, aún no son claros los mecanismos moleculares que subyacen dicha actividad. Se ha descrito la actividad de los AE y terpenos en la membrana plasmática, en la membrana mitocondrial, en la señalización del calcio, en el ciclo celular y en la producción de ROS [16,17,23,24,27]. La membrana fúngica, se ha postulado como un posible blanco de acción. Muchos de los antimicóticos utilizados actualmente tienen como diana la membrana fúngica, debido a la especificidad del esterol fúngico [28,29].

Los esteroles están presentes en todos los eucariotas, intervienen en la señalización celular y organización de las membranas; en los hongos, el ergosterol es el componente principal de membrana celular, determina la fluidez y permeabilidad de la misma, demás promueve la proliferación celular, la adaptación al estrés y el mantenimiento de la morfología y función mitocondrial [4,30–32]. Las enzimas de la vía biosintética del ergosterol escualeno monooxigenasa (Erg1), C-8 esterol isomerasa (Erg2) y esterol 14-α-demetilasa (Erg11), son los objetivos de las alilaminas, las morfolinas y los azoles, respectivamente, [28,29].

El tratamiento con el AE de *L. origanoides* y el timol, en la cepa *C. tropicalis* ATCC 200956, indujo regulación a la baja de los genes *ERG1, ERG11, ERG25 y ERG27* esenciales en la vía de síntesis del ergosterol; los genes *ERG2, ERG3* y *ERG25*, también estuvieron subexpresados. Los genes *ERG25* y *ERG27* actúan en la vía alterna de síntesis de ergosterol y en conjunto con las mutaciones del *ERG3* y el *ERG11* contribuyen a la sensibilidad disminuida a los azoles, mientras que la falta de *ERG2* aumenta la sensibilidad al fluconazol (FLC) [4]. *Candida tropicalis* ATCC 200956 tiene una deleción en los genes *ERG11* y *ERG3*, se ha demostrado que mutaciones en ambos genes confiere resistencia cruzada a los azoles y AMB en *Candida* spp. [3,4]. La regulación negativa en los genes de la vía biosintética del ergosterol puede explicar el cambio fenotípico (de resistente a sensible a FLC) observado en la cepa azol-resistente, luego del tratamiento con el AE o el timol combinado con FLC (sección 4.3.1). Estos hallazgos sugieren que el AE y el timol afectan la biosíntesis de ergosterol en diferentes etapas la vía metabólica, no obstante, el

efecto en otras estructuras celulares también puede estar contribuyendo al cambio fenotípico observado.

Los fosfolípidos, esfingolípidos y esteroles participan en la formación de los microdominios de membrana, previamente, se han descrito el papel de la interacción entre el fosfatidilinositol y las proteinas estructurales del microdominio MCC/eisoma, un sensor de la homeostasis de lípidos altamente enriquecido en ergosterol y esfingolípidos [20,33]. *PIL1*, *LSP1* y *SUR7*, son los genes codificantes para las proteínas pil1, lsp1 y sur7, componentes estructurales y funcionales de este dominio. [34,35]; Fröhlich y cols., demostraron que pil1 y lsp1 regulan los niveles de fosfatidilinositol 4,5 bifosfato [PI (4,5) P_2], la ausencia de pil1 y lsp1 conduce a un aumento de PI (4,5) P_2, afectan la morfogénesis y la integridad de la pared fúngica [36,37]. El análisis del perfil transcriptómico de *C. tropicalis* mostró que genes involucrados en la síntesis del fosfatidilinositol (*INO1 y PSI1*) estaban regulados al alza luego del tratamiento con el AE y el timol (**Tabla 2**). En condiciones fisiológicas, *INO1 y PSI1* son genes altamente regulados y su sobrexpresión impacta el metabolismo de los lípidos derivados del fosfatidilinositol, esfingolípidos y anclajes GPI, activando vías de señalización de respuesta a estrés [38,39].

Por otro lado, se ha demostrado que las mutaciones en *PIL1*, *LSP1* y *SUR7* ocasionan invaginaciones, cambios en el grosor y composición química de la pared fúngica de *C. albicans*, además aumenta la sensibilidad al estrés oxidativo y a los antifúngicos que alteran los lípidos de la membrana plasmática [20,35,40,41]. El tratamiento con el AE y el timol causó regulación negativa de los genes *PIL1*, *LSP1* y *SUR7* (FC = -2,7; -2,6 y -7). Mediante las imágenes de SEM y TEM se pudo evidenciar los defectos en la morfología y el aumento en el grosor de la pared de la cepa *C. tropicalis* ATCC 200956, luego del tratamiento con el AE y el timol (sección 4.3.6.); Martinez y cols, observaron un efecto similar luego del tratamiento con el terpeno isoespintanol, en *C. tropicalis* [27]; lo anterior sugiere que los cambios observados pueden estar asociados con la desregulación en los genes que intervienen en la vía y funcionamiento de este microdominio. Sin embrago, se requieren más estudios.

Los eiosomas también cumple funciones en el transporte de nutrientes, este dominio es un regulador del número de transportadores ABC (del inglés ATP-binding cassette) según

el estado metabólico de la célula, además, sirve como almacenamiento de los transportadores ABC inactivos, esto contribuye a la estabilización de estas proteínas y evita su degradación prematura, la desestabilización de este dominio puede alterar el transporte transmembrana [42]. El análisis GO en ambas levaduras mostró una regulación negativa de las categorías de transporte transmembrana.

Por otro lado, los polifosfatos de inositol interactúan con los complejos remodeladores de la cromatina y regulan la transcripción [38]. La remodelación de la cromatina juega un papel importante en la virulencia y la adaptación fúngica [43], los cambios en el ensamblaje de los nucleosomas, lleva a una menor condensación de la cromatina y mayor accesibilidad del ADN, dejándolo más vulnerable a los diferentes tipos de daño [44]. Actualmente existe gran interés en el papel de las histonas en la resistencia antifúngica y el descubrimiento de nuevos fármacos antimicóticos [45].

La desregulación en la síntesis de histonas, afecta la replicación, la transcripción, la reparación del ADN [46]. El tratamiento con el AE y el timol causó regulación a la baja de los genes asociados al ensamblaje de nucleosoma (**Figura 1B),** genes codificantes para varias histonas, entre ellas la histona 4 componente central del nucleosoma [47], se expresaron negativamente en promedio -7 veces con valor *p* aj. = 0,003 en ambas levaduras (**Tabla 3**).

La síntesis de nuevas histonas es necesaria para la progresión adecuada del ciclo celular [48] el correcto ensamblaje de los nucleosomas propicia la condensación cromosómica durante la mitosis para la posterior citocinesis [49]. Resultados previos mostraron que el AE y el timol inducen cambios en la morfología nuclear y afectan el ciclo celular de *C. tropicalis*, (secciones 4.3.4. y 4.3.5.). No obstante, la progresión del ciclo celular también depende de las proteínas del citoesqueleto, las cuales influyen en la reorganización de microtúbulos para formación del huso mitótico; Andalis y cols., demostraron que las duplicaciones del genoma en *Saccharomyces cerevisiae* genera células tetraploides las cuales presentan aumento en el huso mitótico e inestabilidad genómica en comparación con las células haploides [50]. El AE y el timol regularon de forma positiva en ambas levaduras varios genes asociados a la organización del huso mitótico y los microtúbulos (**Tabla 2** y

3). Adicionalmente, en experimentos anteriores se evidenció un aumento en la poliploidía en *C. tropicalis* ATCC 200956 luego del tratamiento con el AE y el timol (sección 4.3.5.).

El balance del metabolismo energético es importante en procesos celulares como la síntesis de moléculas estructurales y funcionales, la respiración celular y senescencia. Además, las mitocondrias juegan un papel importante en la virulencia, morfogénesis y resistencia antifúngica y muerte celular programada [21,51]. La mitocondria también está asociada con homeostasis lipídica, se ha descrito que la desregulación de genes involucrados en la biogénesis y función mitocondrial, afectan la síntesis de esteroles [21,32]. Además, disfunciones mitocondriales estimulan la sobrexpresión de genes codificantes para esfingolípidos, alterando dicho equilibrio, así mismo, algunas de las enzimas de la vía biosintética del ergosterol requieren el hemo sintetizado en las mitocondrias como cofactor [52], por lo tanto, las levaduras con disfunción mitocondrial presentan una composición alterada de esteroles y alteraciones en la pared celular fúngica. [53,54].

Continuando con los efectos a nivel mitocondrial, luego del tratamiento con el AE y el timol se observó aumento en genes implicados en vía del glioxilato, necesario para la utilización alterna de carbono, esta vía ha sido propuesta como un objetivo terapéutico prometedor contra *Candida* spp., debido a su especificidad por la célula fúngica y su función en la supervivencia del hongo dentro del macrófago [55–58]. Los procesos de oxido reducción, traducción, asimilación de sulfatos y unión a hierro también fueron enriquecidos significativamente (**Figura 1**), procesos relacionados con respuestas adaptativas en *Candida* spp. y disminución en la síntesis de esteroles [21,58,59].

6.5. Conclusión

El efecto en la regulación de genes involucrados en el componente celular “membrana plasmática”, pude explicar el efecto inhibitorio del AE de *L. origanoides* y el timol observado en ambas levaduras, no obstante, la desregulación en genes que codifican para proteínas de membrana con funciones de respuestas a estrés y mantenimiento de la morfología celular sugiere que dicha actividad va más allá de solo la interacción con los lípidos o la disminución en la síntesis de ergosterol. En conjunto los resultados sugieren vías esenciales en la supervivencia del hongo como síntesis de histonas, ensamblaje del uso

mitótico, producción de energía, transporte transmembrana y mantenimiento de la pared celular están vinculados entre sí y aportan al efecto antifúngico observado. Adicionalmente con el análisis de la expresión génica se puede explicar en parte los hallazgos nivel fenotípico, observados luego del tratamiento con ambos compuestos, sin embargo, es necesario realizar más estudios.

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7. Conclusiones generales

La actividad anti-*Candida* spp., de los aceites esenciales de *L. origanoides* y sus componentes mayoritarios fue cepa dependiente; las levaduras resistentes a la AMB o los azoles mostraron mayor sensibilidad a la inhibición por los compuestos evaluados, esto sugiere que las dianas de acción pueden ser diferentes a las usadas por los antifúngicos de uso clínico. Así mismo, los compuestos evaluados fueron poco tóxicos *in vivo* e *in vitro*, su selectividad por las células fúngicas indica que pueden ser seguros para el posterior desarrollo de compuestos contra *Candida* spp.

La combinación del AE de *L. origanoides* quimiotipo (timol + *p*-cimeno) o el timol con el FLC tuvo un efecto sinérgico en *C. tropicalis* sensible y resistente a los azoles, ambos compuestos potenciaron el efecto del FLC evidenciándose un cambio fenotípico de resistente a sensible; lo anterior muestra el potencial de las combinaciones entre antifúngicos y productos naturales y abre la posibilidad de explorarlo como una alternativa para aumentar el arsenal de compuestos contra *Candida* spp.

El efecto del AE de *L. origanoides* quimiotipo (timol + *p*-cimeno) o del timol solos y en combinación con el FLC en varios procesos claves en la viabilidad fúngica, demuestra su actividad multi-diana y evidencia la necesidad de entender más a fondo las vías involucradas en dicha actividad. La disminución en el potencial de membrana mitocondrial, el aumento en la producción de ROS, el aumento de la poliploídia, los cambios en la morfología y ultraestructura de *C. tropicalis*, fueron algunos de los efectos observados que a su vez se confirmaron por medio del análisis de la expresión génica. En conjunto, estos resultados sugieren que el AE de *L. oroganoides* quimiotipo (timol + *p*-cimeno) y el timol causan un desbalance en la homeostasis lipídica en la membrana plasmática fúngica, el cual se vio aumentado por la disfunción mitocondrial. Además, su efecto en el ensamblaje de

eiosomas pudo ser la causa del engrosamiento de la pared fúngica y los cambios en la morfología celular observados. Por otro lado, la regulación negativa de los genes codificantes para varias histonas y positiva de genes involucrados con el ensamblaje del uso mitótico puede explicar los efectos observados en el núcleo y en el ciclo celular de *C. tropicalis* luego de los tratamientos.

8. Perspectivas

La alta volatilidad, inestabilidad y baja solubilidad en agua de los AE y terpenos suponen un desafío para su uso a nivel farmacológico, la implementación de vehículos específicos o formulaciones de liberación controlada usando la nanotecnología pueden mejorar las características físicas del compuesto activo. Varios estudios respaldan la actividad antifúngica del AE de *L. origanoides* y de sus componentes mayoritarios timol, carvacrol y *p*-cimeno, no obstante, las vías metabólicas o de señalización fúngica afectadas no se han caracterizado, por lo tanto, es importante explorar dichas vías mediante herramientas transcriptómicas, proteómicas o metabolómicas para conocer los blancos específicos y dirigir las investigaciones. Así mismo, dados los efectos en la homeostasis de los lípidos y su relación con el metabolismo energético, biogénesis de membrana, señalización y división celular un análisis lipidómico de alta resolución podría arrojar información valiosa y así avanzar hacia el desarrollo de un producto farmacéutico comercializable.