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Activity of carvacrol, cinnamaldehyde, citral and thymol, and their monoolein-based liposomes against oral biofilm-producing *Candida* isolates

Department of Immunology, Microbiology and Parasitology

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Abbreviations and acronyms

aa:	Amino-acid
ABC:	ATP-binding cassette super-family
AFST:	Antifungal susceptibility testing sub-committee
AgNPs:	Silver nanoparticles
Als:	Agglutinin like sequence protein
ANI:	Anidulafungin
ANOVA:	Analysis of variance
ATCC:	American Type Culture Collection
BHI:	Brain heart infusion
C_5:	Hemiterpenes
C_{10}:	Monoterpenes
C_{15}:	Sesquiterpenes
C_{20}:	Diterpenes
C_{25}:	Sesterterpenes
C_{30}:	Triterpenes
C_{40}:	Tetraterpenes
CBPs:	Clinical breakpoints
CBS:	Centraalbureau voor schimmelcultures
***CdCDR1*:**	*Candida dubliniensis Candida* drug resistance 1 gene
***CdMDR1*:**	*Candida dubliniensis* Multidrug resistance (*MDR*)1 gene
cDNA:	Complementary DNA
***CDR1*:**	*Candida* drug resistance 1 gene
***CDR2*:**	*Candida* drug resistance 2 gene
CFU:	Colony-forming units
***CgCDR1*:**	*Candida glabrata Candida* drug resistance 1 gene
***CgCDR2*:**	*Candida glabrata Candida* drug resistance 2 gene
CLSI:	Clinical and Laboratory Standards Institute
CLSM:	Confocal microscopy
Ct:	Cycle threshold
CT:	Citral
CV:	Biomass quantification by crystal violet assay
DAD:	Diode-array detector
DAPI:	4',6-diamidino-2-phenylindole
DLS:	Dynamic light scattering
DMEM:	Dulbecco's modified Eagle's medium
DMSO:	Dimethyl sulfoxide
DODAB:	Dioctadecyldimethylammonium bromide
DTS:	Dispersion technology software
ECM:	Extracellular matrix

ECOFF:	Epidemiological cut-off values of EUCAST
ECV:	Epidemiological cut-off values of CLSI
eDNA:	Extracellular DNA
ELS:	Electrophoretic light scattering
Eno1:	Enolase 1
Awp2:	Adhesin-like wall protein 2
EPA:	Environmental protection agency
Epa8:	Epithelial adhesión 8 protein
Epa19:	Epithelial adhesión 19 protein
Awp7:	Adhesin-like wall protein 7
Erg11:	14-α-lanosterol demethylase
ERG11:	14-α-lanosterol demethylase gene
***ERG1*:**	Squalene monooxygenase gene
***ERG7*:**	Lanosterol synthase gene
***ERG9*:**	Squalene synthase gene
***ERG10*:**	Acetyl-coa acetyltransferase gene
***ERG13*:**	3-hydroxy-3-methylglutaryl coenzyme a synthase
***ERG19*:**	Diphosphomevalonate decarboxylase gene
***ERG24*:**	δ-(14)-sterol reductase gene
***ERG26*:**	Sterol-4-α-carboxylate 3-dehydrogenase gene
Erg3:	C-5 sterol desaturase
***ERG6*:**	δ-(24)-sterol c-methyltransferase gene
EUCAST:	European Committee on Antimicrobial Susceptibility Testing
F:	Forward
FBS:	Foetal bovine serum
FDA:	Food and Drug Administration
FIC:	Fractional inhibitory concentration
FICI:	Fractional inhibitory concentration index
FICT:	Fluorescein-5-isothiocyanate
Fks:	β-(1,3)-D-glucan synthase
***FKS*:**	1,3-β-D-glucan-UDP glucosyltransferase gene
FLC:	Fluconazole
G:	Glucose
GAM:	Glucose-methylene blue
***glp-4*:**	Abnormal germ line proliferation-4 gene
GRAS:	Generally recognized as safe
GUT:	Gastrointestinal-induced transition yeast cells
HBB:	High biomass biofilm producer
Hc:	Henry's law constant

HEPES	1-piperazineethanesulfonic acid
HIV:	Human immunodeficiency virus
HMA:	High metabolic activity
HPLC-DAD:	High-performance liquid chromatography diode array detection
HWP1:	Hyphal wall protein 1 gene
Hwp1:	Hyphal wall protein 1
IC:	Inhibitory concentration
Int1:	Integrin-like protein 1
ISA:	Isavuconazole
***ITS1*:**	Internal transcribed spacer 1 gene
LB:	Luria bertani
LBB:	Low biomass biofilm producers
LDH:	Lactate dehydrogenase
LMA:	Low metabolic activity
LPS	Lipopolysaccharide
LUVs:	Large unilamellar vesicles
***MAT*:**	Mating type
MBB:	Moderate biomass biofilm producer
***MDR1*:**	Multidrug resistance 1 gene
MFC:	Minimum fungicidal concentration
MFS:	Major facilitator super-family
MHA:	Muller hinton agar
MIC:	Minimum inhibitory concentration
MIC2:	Minimum inhibitory concentration 2, the lowest concentration which showed ≥ 50% inhibition growth
MKA:	macrophage killing assay
MLVs:	Multilamellar vesicles diameter > 200 nm
MMA:	Moderate between 0.30 - 0.50
MO:	Monooleoyl-rac-glycerol
MOPS:	Morpholinopropanesulfonic acid
MTL:	Mating type-like
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVVs:	Multivesicular vesicles
NAC:	Non-*Candida albicans Candida*
NCPF:	National collection of pathogenic fungi
NCYC:	National collection of yeast cultures
NGM:	Nematode growth medium
NP:	Nanoparticles
NPCAR:	Nanoparticles of carvacrol

NPCIN:	Nanoparticles of cinnamaldehyde
NPCT:	Nanoparticles of citral
NPTHY:	Nanoparticles of thymol
NWT:	Non-wild type
OD:	Optical density
pb:	Pair bases
PBS:	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCR-RFLP:	PCR-restriction fragment length polymorphism
PDI:	Polydispersity index
pDNA	Plasmid DNA
Pgk:	Phosphoglycerate kinase
PK:	Pharmacokinetics
PLA:	Phospholipase A
PLB:	Phospholipase B
PLC:	Phospholipase C
PLD:	Phospholipase D
PSIC:	Pre-sessile inhibition concentration
PSMICs:	Pre-sessile mics
Rr:	Reverse
R:	Resistant
R2:	Coefficient R
RT-PCR:	Reverse transcription polymerase chain reaction
RVVC:	Recurrent vulvovaginal candidiasis
S:	Susceptible
***SADH*:**	Secondary alcohol dehydrogenase gene
SAPCD:	Secreted aspartyl proteinases *C. dubliniensis* gene
Sapcd	Secreted aspartyl proteinases *C. dubliniensis*
SAPP:	Secreted aspartyl proteinases *C.* p*arapsilosis* gene
Saps:	Secreted aspartic proteases
***SAP*:**	Secreted aspartyl proteinases gene
***SAPT*:**	Secreted aspartyl proteinases *C. tropicalis* gene
SDA:	Sabouraud dextrose agar medium
S-DD:	Susceptible dose dependent
Sek-1	SAPK/ERK kinase-1
SEM:	Scanning electron microscopy
SIC:	Sessile inhibition concentration
SMICs:	Sessile mics
SUVs:	Small unilamellar vesicles

T-ECOFF:	Tentative epidemiological cut-off values of EUCAST
Tris-HCL:	Hydroxymethyl aminomethane hydrochloride
WT:	Wild-type
XTT:	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.
YPD:	Yeast peptone dextrose
Yps:	Yapsin protease
%EE:	Encapsulation efficiency percentage
χMO:	MO molar fraction

Summary

Oral candidiasis are common fungal infections, mainly caused by *Candida albicans*. However, the epidemiology of these diseases has changed in recent decades, and the prevalence of other species of *Candida* that are less susceptible to antifungal drugs has increased. In addition, biofilm formation and resistance to antifungal drugs modify the degree of infection resolution. The search for therapeutic alternatives based on phytocompounds obtained from nature remains an ongoing challenge.

In the first part of this study, the prevalence of species closely related to *Candida glabrata* and *Candida parapsilosis* was evaluated in oral isolates from the collection of the Laboratory of Medical Mycology at University of the Basque Country UPV/EHU from 2003 to 2013. We identified one isolate of *Candida metapsilosis* and *Candida orthopsilosis* but no isolates of *Candida bracarensis* or *Candida nivariensis*. *C. glabrata* was isolated in a higher proportion from mixed cultures, while the *C. parapsilosis* complex was isolated mainly from pure culture. *C. glabrata* showed reduced susceptibility and cross-resistance to fluconazole and itraconazole. The *C. parapsilosis* complex was susceptible to fluconazole and showed cross-resistance to miconazole and itraconazole.

The second and third parts of the Thesis included the evaluation of the anticandidal activity of monoterpenes and other phytocompounds. Carvacrol, cinnamaldehyde, citral, eugenol and thymol were the most active phytocompounds against *Candida* planktonic cells. Moreover, carvacrol, cinnamaldehyde, citral and thymol acted against early biofilms. Citral was also active against mature biofilms by reducing their metabolic activity, while carvacrol and thymol not only reduced metabolic activity but also their biomass. In addition, the combined effect of citral and fluconazole against *Candida* and its effect on the expression of fluconazole resistance-associated genes *ERG11*, *MDR1* and *CDR1* were evaluated. *In vitro* and *in vivo* synergistic effects were obtained with the combination treatment of citral and

fluconazole against *Candida* planktonic and sessile cells, and in a *Caenorhabditis elegans* model of candidiasis. When combined with fluconazole, citral downregulated *MDR1* expression.

The last section included the development and characterization of DODAB:MO-based liposomes derived from carvacrol, cinnamaldehyde, citral and thymol. These nanoparticles were stable at refrigeration, with positive ζ-potential and particle sizes ranging from 497.6 to 580.3 nm. Carvacrol and thymol nanoparticles at 128 mg/L with >69% encapsulation maintained their antifungal activity. Cinnamaldehyde nanoparticles were encapsulated at the lowest rate and maintained their antifungal activity. Citral was extensively encapsulated (83.3%), but these nanoparticles did not show antifungal activity. Encapsulation significantly reduced the cytotoxicity of the phytocompounds. Thymol and carvacrol nanoparticles were the best tolerated by macrophages and improved the ability of macrophage to kill *Candida* cells with negligible stimulation of cytokine expression.

The outcomes of this Doctoral Thesis highlight the anticandidal activity of phytocompounds such as carvacrol, cinnamaldehyde, citral and thymol, free or in nanoparticles. This activity evidences their potential usefulness for the treatment of biofilm-associated candidiasis. Furthermore, these results underscore the promising application of phytocompounds in combination therapy with the most commonly used antifungal drugs.

Resumen

Las candidiasis orales son infecciones fúngicas comunes, causadas habitualmente por *Candida albicans*. Sin embargo, la epidemiología de las candidiasis orales ha cambiado en las últimas décadas y ha aumentado la prevalencia de otras especies de *Candida* menos sensibles a los fármacos antifúngicos. Además, la formación de biopelículas y la resistencia a los fármacos antifúngicos afectan a la resolución de estas enfermedades. La búsqueda de alternativas terapéuticas basadas en fitocompuestos obtenidos de la naturaleza sigue siendo un reto médico importante.

En la primera parte de este estudio se evaluó la prevalencia de especies estrechamente relacionadas con *Candida glabrata* y *Candida parapsilosis* en aislamientos orales pertenecientes a la colección del Laboratorio de Micología Médica de la Universidad del País Vasco/ Euskal Herriko Unibertsitatea UPV/EHU entre 2003 a 2013. Se identificó un aislamiento de *Candida metapsilosis* y *Candida orthopsilosis*, pero no se encontraron *Candida bracarensis* ni *Candida nivarensis*. *C. glabrata* se aisló en mayor proporción en cultivos mixtos, mientras que *C. parapsilosis* complex se aisló principalmente en cultivos puros. *C. glabrata* mostró una sensibilidad reducida y resistencia cruzada al fluconazol y al itraconazol. *C. parapsilosis* complex fue sensible al fluconazol y mostró resistencia cruzada al miconazol y al itraconazol.

La segunda y tercera parte de esta Tesis incluyeron la evaluación de la actividad anticandidiásica de los monoterpenos y otros fitocompuestos. Carvacrol, cinamaldehído, citral, eugenol y timol fueron los fitocompuestos más activos contra las células planctónicas de *Candida*. Además, carvacrol, cinamaldehído, citral y timol actuaron contra las biopelículas en su fase temprana. El citral también fue activo contra las biopelículas maduras al reducir su actividad metabólica, mientras que carvacrol y timol no solo redujeron la actividad metabólica sino también su biomasa. Además, se evaluó el efecto combinado de citral y fluconazol contra *Candida* y su efecto sobre la expresión de los genes

asociados a la resistencia al fluconazol, *ERG11*, *MDR1* y *CDR1*. Se obtuvo un efecto sinérgico *in vitro* e *in vivo* con la combinación de citral y fluconazol contra las células planctónicas y sésiles de *Candida* y la infección por *Candida* en un modelo de *Caenorhabditis elegans*. El citral combinado con fluconazol disminuyó la expresión del gen *MDR1*.

La última sección de este trabajo incluyó el desarrollo y la caracterización de liposomas basados en DODAB:MO de carvacrol, cinamaldehído, citral y timol. Estas nanopartículas fueron estables en refrigeración, con ζ-potencial positivo y un tamaño de partícula que oscilaba entre 497,6 y 580,3 nm. Las nanopartículas de carvacrol y timol de 128 mg/L con > 69% de encapsulación mantuvieron su actividad antifúngica. Las nanopartículas de cinamaldehído presentaron una menor encapsulación pero mantuvieron su actividad antifúngica. El citral fue bien encapsulado (83,3%), pero sus nanopartículas no tenían actividad antifúngica. La encapsulación redujo significativamente la citotoxicidad de los fitocompuestos. Las nanopartículas de timol y carvacrol fueron las mejor toleradas por los macrófagos y mejoraron la capacidad de éstos para eliminar las células de *Candida*, con una escasa estimulación en la producción de citoquinas.

Los hallazgos de esta Tesis Doctoral ponen de manifiesto la actividad anticandidiásica de fitocompuestos tales como el carvacrol, el cinnamaldehido, el citral y el timol, libres o en nanopartículas. Esta actividad evidencia su potencial utilidad para el tratamiento de las candidiasis asociadas a biopelículas. Además, estos resultados destacan la prometedora aplicación de los fitocompuestos en terapia combinada con los fármacos antifúngicos más communmente empleados.

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INTRODUCTION

1.1 Candidiasis

Candidiasis is a fungal disease or mycosis, caused by infections with an opportunistic fungal pathogen belonging to the genus *Candida* that range from superficial to invasive and that may be systematic and/or life-threatening (McCarty et al., 2021; Quindós et al., 2018). Although *Candida* species are frequently found as commensal yeasts in the human body, including on the skin, in vaginal and oral mucosae and in the gastrointestinal and genitourinary tracts, different factors drive a shift from their status as commensal fungi to opportunistic pathogens (Kumamoto, 2011; Vila et al., 2020). These mycoses have become increasingly common, due in part to the increase in the number of susceptible people. These at-risk individuals include immunocompromised patients undergoing immunosuppressive therapies, patients with immune system disorders and with solid organ or bone marrow transplant receptors; patients who have undergone major surgery, such as abdominal surgery; and anyone treated with broad spectrum antibiotic agents or using biomedical devices, such as prostheses (Canela et al., 2018; Reinhardt et al., 2018; Zilberberg et al., 2014). Superficial candidiasis involving oral and vaginal mucosae is often associated with discomfort and pain and typically manifests as recurrent and recalcitrant biofilm-related infections (Rosati et al., 2020; Saito et al., 2020). In addition, oral and gastrointestinal colonization by *Candida* has been associated with a higher risk and occurrence of candidaemia and invasive candidiasis (Katagiri et al., 2018; Kumamoto, 2011; Miranda et al., 2009).

Invasive candidiasis has a great impact on public health due to high morbidity and mortality rates, as it is the fourth most common bloodstream-associated infection in Europe and the USA (Jeffery-Smith et al., 2018; Lockhart, 2014; Magill et al., 2014; McCarty et al., 2021). Although *Candida albicans* is the most prevalent species causing candidiasis, the current emergence of resistant *C. albicans* isolates and resistant *Candida*

species has raised concerns over the worsening management of candidiasis (Ghazi et al., 2019; Jeffery-Smith et al., 2018; Quindós et al., 2018; Ricotta et al., 2021; Ruiz-Gaitán et al., 2018; Sanguinetti et al., 2015).

1.1.1 Oral candidiasis

Oral candidiasis is the most common mycosis that affects the oral cavity (Hu et al., 2019; Saito et al., 2020). *C. albicans* is the most prevalent species. *Candida* colonization together with local and systemic predisposing factors in patients favours the development of this infection (Vila et al. 2020). Local factors include smoking, xerostomia, mucosal disorders such as atrophy and oral lichenoid syndromes, topical and inhaled corticosteroid therapy, antineoplastic chemotherapy and radiotherapy, dental prostheses, poor oral hygiene and alterations of oral microbiota caused by an unbalanced diet (Fukushima et al., 2003; Gunsalus et al., 2015; Meira et al., 2017). In addition, extremes of age, nutritional status, endocrine or immune disorder, carcinoma and haematological malignancies are systemic factors that increase the occurrence of oral candidiasis (Meira et al., 2017; Patil et al., 2015).

Clinical manifestations of oral candidiasis are classified as primary or secondary and include acute and chronic forms (Axéll et al., 1997; Samaranayake et al., 2009) (Table 1.1). Primary oral candidiasis involves acute and chronic infections and *Candida*-associated lesions, with pseudomembranous candidiasis the most common acute form. Moreover, if two or more of these forms are present at the same time, the condition is called multifocal candidiasis (Aguirre-Urizar, 2002). Secondary oral candidiasis is a chronic mucocutaneous candidiasis with persistent or recurrent manifestations associated with HIV infection and diverse immunodeficiency disorders, such as Di George syndrome, hyperimmunoglobulin E syndrome, Nezelof's syndrome, myeloperoxidase deficiency, severe combined

immunodeficiency syndrome and endocrine disorders, such as Addison's disease and hypoparathyroidism (Patil et al., 2015).

Table 1.1. Classification of oral candidiasis. Adapted from Samaranayake et al. (2009) and Axéll et al. (1997).

Primary oral candidiasis	Secondary oral candidiasis
Acute forms	Oral manifestations of systemic mucocutaneous candidiasis
Pseudomembranous	Thymic aplasia
Erythematous	Candidiasis endocrinopathy síndrome
Chronic forms	
Hyperplastic (nodular or plaque-like)	
Erythematous	
Pseudomembranous	
Candida-associated lesions	
• Denture stomatitis	
• Angular cheilitis	
• Median rhomboid glossitis	
Keratinized primary lesions with *Candida* super infection	
Leucoplakia	
Lichenoid syndrome	
Lupus erythematosus	

1.1.2 Pseudomembranous candidiasis

Pseudomembranous oral candidiasis commonly presents as an acute infection characterized by lesions with white plaques known as "thrush" on the tongue, buccal mucosa, palate

periodontal tissues, and oropharynx that can be removed, revealing erythematous mucosa after scraping (Millsop & Fazel, 2016). Patients experience alterations in the sense of taste and burning and tingling sensations. The lesions may readily bleed, particularly in patients at the extremes of the age spectrum (i.e., new-born and elderly), after the use of inhaled steroids and in diabetes patients. Moreover, pseudomembranous candidiasis is considered an initial sign of HIV infection (presenting in more than 90% of HIV patients), and it is a common infection in patients with head and neck cancer undergoing chemotherapy and other treatments or with a condition that causes immunosuppression (Hu et al., 2019; Saito et al., 2020; Vila et al., 2020). Chronic and recurrent cases are frequent in patients with HIV infections, which lead to oesophageal candidiasis or *Candida* oesophagitis with oesophageal mucosa involvement, and these cases are characterized by dysphagia and altered nutritional status (Patil et al., 2018; Vila et al., 2020).

1.1.3 Erythematous candidiasis

Erythematous candidiasis causes pain and hyperaemic lesions located on the dorsum of the tongue, typically presenting atrophy of lingual papillae in de-papillated areas and less frequently in the buccal mucosa (Millsop & Fazel, 2016; Patil et al., 2015). This candidiasis can manifest as acute or chronic lesions that are associated with the prolonged use of broad-spectrum antibiotics, and it is therefore called "antibiotic sore tongue". Conditions such as uncontrolled diabetes, iron and vitamin B12 deficiency, HIV infection and use of corticosteroids are risk factors for manifestation of the acute form (do Egito Vasconcelos et al., 2008; Lu, 2016; Paillaud et al., 2004). Meanwhile, the chronic form is often reported in patients with HIV infection and involves lesions on the palate (Millsop & Fazel, 2016).

1.1.4 Chronic hyperplastic candidiasis

Chronic hyperplastic candidiasis is characterized by homogeneous white patches (plaque-like) or elevated fissured white patches (nodular or speckled form) that do not dislodge when scraped on oral mucosa, mainly at the retrocommissural area, followed by the sides of the tongue and palate (Aguirre-Urizar, 2002; Vila et al., 2020). These lesions are difficult to distinguish from leucoplakia. The population most at risk consists of middle-aged male smokers, whose risk of malignant transformation from epithelial dysplasia to oral squamous cell carcinoma increases to 10% (Coronado-Castellote & Jiménez-Soriano, 2013; Vila et al., 2020).

1.1.5 *Candida*-associated lesions

Denture stomatitis, the most common *Candida*-associated lesion, is characterized by chronic inflammation related to the use of ill-fitting removable dentures, which generally affects females and involves the presence of *Candida* biofilms (Ramage et al., 2004; Reinhardt et al., 2018). Denture stomatitis is typically located in the denture-bearing palatal mucosa. Other determining factors are hyposalivation and xerostomia, poor dental hygiene, and smoking. Lesions can range from pinpoint hyperaemic lesions (type I) and diffuse erythematous lesions (type II) to papillary hyperplasic or granular lesions (type III) and present in conjunction with angular cheilitis and median rhomboid glossitis (Patil et al., 2015).

Angular cheilitis is a lesion on one or both commissures of the mouth and perilabial skin, characterized by grey-white thickening and erythematous injury with pain, soreness and fissuring. *C. albicans* is often isolated with *Staphylococcus aureus* (Millsop & Fazel, 2016).

Median rhomboid glossitis, an infrequent (less than 1% incidence) and asymptomatic presentation, consists of erythematous, rhomboid-shaped lesions located in the centre rear of the dorsal side of the tongue, before the circumvallate papillae, with atrophy of filiform

papillae. The use of inhaled corticosteroids, smoking and dentures increase the predisposition to manifesting this lesion (Glick, 2015; Vila et al., 2020).

1.2 Genus *Candida*

Candida is a fungal genus that belongs to the Ascomycota division in the Fungi kingdom (Table 1.2). Although most of the species described take unicellular yeast-like form and grow in pseudohyphal form, *C. albicans* and *Candida dubliniensis* can also form germ tubes, hyphae and chlamydospores, and this ability is closely associated with virulence (Citiulo et al., 2009; Csank & Haynes, 2000; Torosantucci et al., 2004) (Figure 1.1). Moreover, *Candida tropicalis* is able to form true hyphae (Lackey et al., 2013).

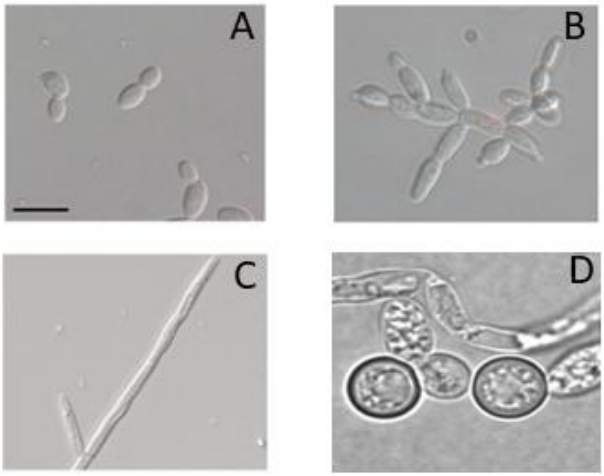

Figure 1.1. Morphology of *Candida* cells. A: yeasts, B: pseudohyphae; C: hyphae and D: chlamydospores. Adapted from Thompson et al. (2011) and Martin et al. (2005).

More than 300 *Candida* species have been described, some of which are common human commensals, but some species are associated with important human pathologies (Silva et al., 2012). The group of opportunist pathogens includes *C. albicans*, *C. dubliniensis, Candida famata, Candida glabrata, Candida guilliermondii, Candida inconspicua, Candida kefyr, Candida krusei,*

Candida lipolytica, Candida lusitaniae, Candida norvegensis, Candida parapsilosis, Candida pelliculosa, Candida rugosa, C. tropicalis and the emergent *Candida auris*. Of these species, *C. albicans* is the most frequently identified, whereas *C. glabrata, C. tropicalis, C. parapsilosis,* and *C. krusei* are critical for most non-*C. albicans Candida* (NAC) infections (Jeffery-Smith et al., 2018; Pfaller & Diekema, 2007; Quindós et al., 2018; Silva et al., 2012).

1.2.1 *Candida albicans*

C. albicans is part of the human microbiota. However, this species is also recognized as the most common pathogenic yeast and is globally involved in approximately 50% or more of candidiasis cases (Dadar et al., 2018). In the oral mucosa, it is the most frequent commensal yeast and the main cause of oral candidiasis among people in all age groups and among all clinical manifestations.

This species is diploid, its genome is composed of eight pairs of chromosomes, and its genetic plasticity is associated with chromosome length polymorphisms, reciprocal translocations, chromosomal deletions and trisomy (Selmecki et al., 2010).

Although *C. albicans* was long been considered an asexually reproducing microorganism owing to low-frequency clonal multiplication, its sexual reproduction cycle was confirmed with the finding of the mating type-like loci (*MTL*) homologous to the *Saccharomyces cerevisiae* mating-type loci (*MAT*) (Tzung et al., 2001). Four yeast morphologies of *C. albicans* have been described, including white, opaque, grey and gastrointestinal-induced transition (GUT) yeast cells. White-to-opaque changes are related to its sexual reproduction cycle, which is controlled by the *MTL locus* and is associated with fluconazole resistance due to sexual recombination between different variants (Morschhäuser, 2016).

1.2.2 *Candida glabrata* and *Candida parapsilosis* complexes

C. glabrata and *C. parapsilosis* rank second and third as the species most frequently involved in oral candidiasis, and they have been recovered from coinfections with *C. albicans* and other *Candida* species (Miranda-Cadena et al., 2018; Muadcheingka & Tantivitayakul, 2015). Both species are complexes made up of closely related *Candida* species that differ in their virulence and susceptibility patterns (Romeo et al., 2009; Tavanti et al., 2005).

The *C. glabrata* complex comprises *C. glabrata, C. bracarensis* and *C. nivariensis*. However, these two related species are not normally isolated from human oral specimens or blood (Asadzadeh et al., 2019; Miranda-Cadena et al., 2018). As explained below, *C. glabrata* has been identified as the second or third most frequently isolated *Candida* species from patients with oral candidiasis, candidaemia or vulvovaginitis, depending on the geographic location and characteristics of the population (Miranda-Cadena et al., 2018; Quindós et al., 2018; Rosati et al., 2020). Importantly, the high mortality rates associated with invasive *C. glabrata* infection is due in part to intrinsic and/or acquired resistance to triazoles, especially fluconazole, a widely used antifungal agent (Asadzadeh et al., 2019).

The *C. parapsilosis* complex comprises three closely related species: *C. parapsilosis*, *Candida metapsilosis* and *Candida orthopsilosis*. Of these yeast, greater resistance has been noted in *C. parapsilosis* than in *C. metapsilosis* or *C. orthopsilosis* (Miranda-Zapico et al., 2011), while less virulence has been ascribed to *C. metapsilosis* than to *C. parapsilosis or C. orthopsilosis*. *C. parapsilosis* and *C. metapsilosis* have been found to invade reconstituted human epidermal and oral epithelial tissues (Orsi et al., 2010). In oral candidiasis, *C. parapsilosis* is typically the most prevalent, while *C. metapsilosis* and *C. orthopsilosis* represent less than 5% of isolates obtained from the *C. parapsilosis* complex (Miranda-Cadena et al., 2018; Moris et al., 2012).

Although *Candida* species are integrated into the mucosal microbiota in general, *C. parapsilosis* can also colonize human skin, which is clinically important in critical and hospitalized patients. In these hospitalized patients, mainly neonates receiving

Table 1.2. Taxonomic classification of some *Candida* species (Cendejas-Bueno et al., 2012; Diezmann et al., 2004).

Division	Class	Order	Family	Species
Ascomycete				
	Hemiascomycetes			
		Saccharomycetales		
			Saccharomycetaceae	*Candida glabrata*
				Candida kefyr/Kluyveromyces marxianus
			Debaryomycetaceae	*Candida albicans*
				Candida dubliniensis
				Candida famata/Debaryomyces hansenii
				Candida guilliermondii/Meyerozyma guilliermondii
				Candida parapsilosis
				Candida tropicalis
			Pichiaceae	*Candida krusei/Pichia kudriavzevii*
			Dipodascaceae	*Candida lipolytica/Yarrowia lipolytica*
			Metschnikowiaceae	*Candida auris*
				Candida lusitaniae/Clavispora lusitaniae

intravenous hyperalimentation, incorrect manipulation can lead to *C. parapsilosis* involvement in catheter-associated candidaemia (Krcmery & Barnes, 2002; Quindós et al., 2018).

1.2.3 Other *Candida* species

C. dubliniensis and *Candida africana* are phenotypically closely related to *C. albicans* (Sullivan et al., 1995; Tietz et al., 2001). *C. dubliniensis* has been isolated most frequently as a cause of oral candidiasis in HIV-infected patients but also in patients suffering from denture-induced stomatitis (Gutiérrez et al., 2002; Mahelová & Růžička, 2017; Marcos-Arias et al., 2009). In addition, *C. africana* has been recovered from women with vaginitis (Naeimi et al., 2018). Current molecular techniques using a fragment of the *HWP1* gene has allowed the differentiation of clinical isolates recovered from these species (Romeo & Criseo, 2008).

C. tropicalis is becoming increasingly prevalent in oral infections. In HIV-infected, cancer and transplantation patients, *C. tropicalis* is the second or third most prevalent species (14.1%, 42.8% and 4.5%, respectively) (da Silva-Rocha et al., 2014; Jain et al., 2016; Mulu et al., 2013). The ability to form biofilm and acquire antifungal resistance has made this species the second most virulent species after *C. albicans*.

C. krusei is considered to be a potential multiresistant pathogen owing to its intrinsic resistance to fluconazole and reduced susceptibility to other azoles and polyenes. The prophylactic use of fluconazole has played a facilitating role in azole-resistant yeast selection. *C. krusei* is frequently reported to be found in patients with haematologic malignancies, elevating their risk of mortality (Pfaller et al., 2008; Zilberberg et al., 2014). Morphologically, *C. krusei* grows into elongated cells similar to those of *C. kefyr,* while most *Candida* species have ovoid cells.

C. auris is related to three other rare *Candida* species: *Candida haemulonii, Candida duobushaemulonii* and *Candida pseudohaemulonii* (in the *C. haemulonii* complex) (Cendejas-Bueno et al., 2012). Although it was isolated for the first time from the external ear canal of a Japanese patient, in recent years, *C. auris* was found to be critical for several invasive candidiasis-causing infections showing multiresistance to antifungal therapy (Jeffery-Smith et al., 2018).

1.2.4 Virulence factors

In the development of *Candida* infections, in addition to local and systemic host factors that increase the predisposition of a shift from the commensal to infectious form, it has been demonstrated that *Candida* cells play active roles adhering, infecting, and causing diseases through different structural and biological characteristics that are associated with the term "virulence factors" (Cauchie et al., 2017). Virulence factors include adhesion, production and secretion of hydrolytic enzymes, phenotypic switching, hyphal formation and the ability to form biofilms (Segal & Frenkel, 2018) (Table 1.3).

Adhesion

The cell surface hydrophobicity of *C. albicans* has been correlated *in vitro* with adhesion to HeLa cell surfaces and the inert acrylic surfaces of dentures. *C. krusei* has not been shown to adhere to inert polymers; in contrast, this species is highly correlated with adhesion to epithelial cells (Samaranayake et al., 1995). In *C. parapsilosis* isolates, cell surface hydrophobicity has been strongly correlated with adherence to polystyrene surfaces and buccal epithelial cells (Tóth et al., 2019). This adherence is attributed to electrostatic interactions with salivary flow and swallowing play roles as host defences against *Candida* colonization (Kamagata-Kiyoura et al., 2004).

On the other hand, the interaction of cell wall receptors, such as the agglutinin-like sequence (Als) protein, has been described in different *Candida* species. The agglutinin-like sequence 3 (Als3) cell wall protein, which is related to biofilm formation, is critical for *Candida* species adhesion to epithelial cells, as indicated by adhesion inhibition in the absence of this protein (Zhao et al., 2006). Moreover, this protein is involved in the cell invasion process and in iron acquisition from the host (Almeida et al., 2008). This adhesion through Als1 and Asl2 has been identified in *C. albicans* and *C. tropicalis*, showing that these proteins facilitate *Candida* species attachment to several inert materials and proteins, such as fibrinogen, fibronectin and laminin (Punithavathy & Menon, 2012; Silverman et al., 2010).

Table 1.3. *Candida* pathogenic attributes relevant to oral infection. Adapted from Vila et al. (2020).

Adhesion to oral epithelial surface
➢ Cell surface hydrophobicity (reversible adherence)
➢ Expression of cell surface adhesins (Als3, Hwp1, Epa1, etc.)
Biofilm formation
➢ Development of denture stomatitis
➢ Failure of antifungal therapy
Evasion of host defences
➢ Phenotypic switching
➢ Binding to complement
➢ Resistance to phagocytic stresses (oxidative and nitrosative stress response)
➢ Proteolytic degradation of host immune factors (antibodies, antimicrobial peptides, etc.)
Invasion and destruction of host tissue
➢ Hyphal development and thigmotropism (tissue penetration)
➢ Secretion of hydrolytic enzymes: secreted aspartyl proteinases, phospholipases and lipases (tissue degradation)
➢ Secretion of the hypha-specific toxin candidalysin
➢ Degradation of E-cadherin
➢ Induction of endocytosis

C. glabrata expresses epithelial adhesion proteins (Epa), of which Epa1 mediates 95% of yeast cell adhesion to epithelial cells *in vitro* (Cormack et al., 1999). *C. parapsilosis* can attach to silicone-made materials via glycolysis and the gluconeogenesis-regulating proteins phosphoglycerate kinase (Pgk) and enolase 1 (Eno1), and this species adheres mainly to human buccal epithelial cells through Als7, which is orthologous to *C. albicans* adhesin (Tóth et al., 2019).

Another major adhesin is hyphal wall protein 1 (Hwp1), which is involved in yeast adhesion to human buccal epithelial cells, produced by *C. albicans* and *C. tropicalis*, and related to biofilm formation (Aznita et al., 2013; Ene & Bennett, 2009). Integrin-like 1 (Int1) protein and α-1,2-mannosyltransferase encoded by the *INT1* and *MNT1* genes, respectively, are other adhesins that contribute to *Candida* infections (Mayer et al., 2013).

Morphogenesis and phenotypic switching

The change from the yeast form to the hypha form is considered an important step in the establishment of fungal infection. The change is associated with the fungal cell invasion process, endothelial rupture, evasion of host defence mechanisms, biofilm production and thigmotropism (Lackey et al., 2013). *C. albicans* is the most studied species in this field. However, it has been reported that *C. tropicalis* conserves at least 55 of the 105 genes involved in *C. albicans* filamentation (Zuza-Alves et al., 2017).

Environmental factors such as temperature (37 °C), pH (> 7) and CO_2 concentrations, as well as carbon sources, lead to and stimulate the morphogenesis process through multiple pathways (Mayer et al., 2013). This process is highly associated with virulence and has been demonstrated with mutant strains, for which diminished or no virulence was reported (Omeara et al., 2015). The yeast form is associated with adhesion, multiplication and dispersion, while the hyphal form allows *C. albicans* to avoid phagocytosis and invade

through thigmotropism specifically to intercellular junctions of host tissue, leading to infection (Bain et al., 2014; Vila et al., 2020).

Phenotypic switching consists of a change in colony morphology, with white-to-opaque switching being the most extensively studied change, which has been reported to confer plasticity through the modulation of virulence factors, such as secreted aspartic protease (Sap), during the adhesion process (Morrow et al., 1992).

Production and secretion of hydrolytic enzymes

Several hydrolytic enzymes secreted by *Candida* species involved in colonization, host tissue invasion and destruction of host tissue have been described (Ciurea et al., 2020; Tóth et al., 2017). These include lipases, phospholipases and peptidases. The aspartic protease class is a group of enzymes that degrade many host proteins and is considered an essential virulence factor.

Candida aspartic proteinases are grouped into two families, with Saps or candidapepsins being the most frequently excreted enzymes. *C. albicans* has 10 *SAP* genes in its genome, and orthologous *SAP* genes are expressed in *C. dubliniensis* (*SAPCD1* to *SAPCD4* and *SAPCD7* to *SAPCD10*), *C. parapsilosis* (*SAPP1*, *SAPP2* and *SAPP3*), and *C. tropicalis* (*SAPT1* to *SAPT4*). However, *C. glabrata* expresses a proteinase belonging to the yapsin protease family (Yps) on its cell wall but not candidapepsins (Rapala-Kozik et al., 2018). Furthermore, in studies on mucosal candidiasis, the role of *SAP1-SAP3* expression in the development of *C. albicans* infection and the role of *SAP9* and *SAP4-SAP8* genes in tissue damage have been described (Naglik et al., 2008; Schaller et al., 2003). In addition, only *SAPP1* expression in *C. parapsilosis* has been detected in an *in vitro* model, which was based on reconstituted human oral epithelium (Rapala-Kozik et al., 2017).

C. glabrata in a murine model of oropharyngeal candidiasis was not able to start the invasive process (Tati et al., 2016). However, upon coinfection with *C. albicans*, *C. glabrata* can cause oral disease. This infection process is explained in part by the combination of the expression of Als1 and Als3 adhesins/invasins in *C. albicans* hyphae with adhesins in *C. glabrata* (Epa8, Epa19, Awp2, Awp7, and CAGL0F00181) to facilitate the adherence of *C. glabrata* cells to *C. albicans* hyphae (Swidergall & Filler, 2017).

Four phospholipases (PLA, PLB, PLC, and PLD) in *C. albicans* have been demonstrated to be virulence factors in murine models (Leidich et al. 1998). *C. glabrata, C. parapsilosis, C. tropicalis, C. lusitaniae,* and *C. krusei* produce extracellular phospholipase, while *C. dubliniensis, C. glabrata, C. parapsilosis*, and *C. tropicalis* produce intracellular phospholipase orthologues of *C. albicans* PLC1 (Ghannoum, 2000). Lipases secreted by *C. albicans* are encoded by the *LIP1* to *LIP10* genes, and their expression has been studied in a murine model of oral infection, for which the results showed that the expression of these genes depends on the stage of infection (Stehr et al., 2004).

Biofilm formation

The ability to form biofilms is a determining factor in the persistence of *Candida* colonization and candidiasis. Isolates can form biofilms that are associated with significantly higher virulence, since biofilms exhibit increased antifungal resistance and confer protection from host defences. Moreover, the dispersal of more-virulent cells from biofilms contributes to the pathogenesis of candidiasis (Ramage et al., 2012a; Wall et al., 2019).

Biofilm formation has been observed on several types of medical devices, such as intravascular catheters, dentures, prosthetic heart valves, and replacement joints (Cavalheiro & Teixeira, 2018; Estivill et al., 2011). In oral candidiasis, and especially in

denture stomatitis, the relationship of biofilm to recalcitrance has more evidence (Ramage et al., 2004; Reinhardt et al., 2018). Indeed, the formation of *Candida* biofilms on oral acrylic dentures and inert materials frequently used in dental abutments and prostheses has been widely reported (Eguia et al., 2020; Hahnel et al., 2012; Nikawa et al., 2003).

A biofilm is formed by a microbial community irreversibly adhered to biotic or abiotic surfaces. *Candida* biofilms are complex structures composed of yeasts, hyphae and/or pseudohyphae surrounded by an exopolymeric matrix (Mayer et al., 2013; Nobile & Johnson, 2015). Extracellular polymeric substances, including carbohydrate and extracellular DNA (eDNA), are the main compounds of the extracellular matrix (ECM), and in conjunction with biofilm sessile cells with low growth rates and altered phenotypes have been linked to multidrug resistance (Hirota et al., 2017; Mayer et al., 2013; Ramage et al., 2012a). Biofilm formation is a complex multifaceted process that involves the expression of several genes in different steps (Table 1.3). In general, the development of a biofilm structure can be summarized as follows: Fungal cells adhere to a surface (biotic or abiotic); a basal layer is formed by fungal growth and proliferation; hyphae and/or pseudohyphae and then the ECM are produced or enter the maturation phase; and finally, sessile cells (with increased virulence) are dispersed onto other surfaces (Ramage, et al., 2012a) (Figure 1.2).

C. albicans and other *Candida* species have been described as biofilm producers; however, each species produces a biofilm with a different morphology and ECM, and their formation depends on factors such as host, surface and environmental conditions. In the case of *C. albicans*, most of the studied isolates can form biofilms on different materials, and tissues of *in vivo* models support strong biofilms that have a heterogeneous structure (blastospores and hyphae) and water channels surrounding the microcolonies. In other studies, *C. tropicalis* produced biofilms more effectively than *C. albicans*, and the *C. tropicalis*

biofilm is formed by yeast, pseudohyphae, and hyphae, with intense hyphal budding and a thick ECM (Cavalheiro & Teixeira, 2018; Marcos-Zambrano et al., 2014; Zuza-Alves et al., 2017).

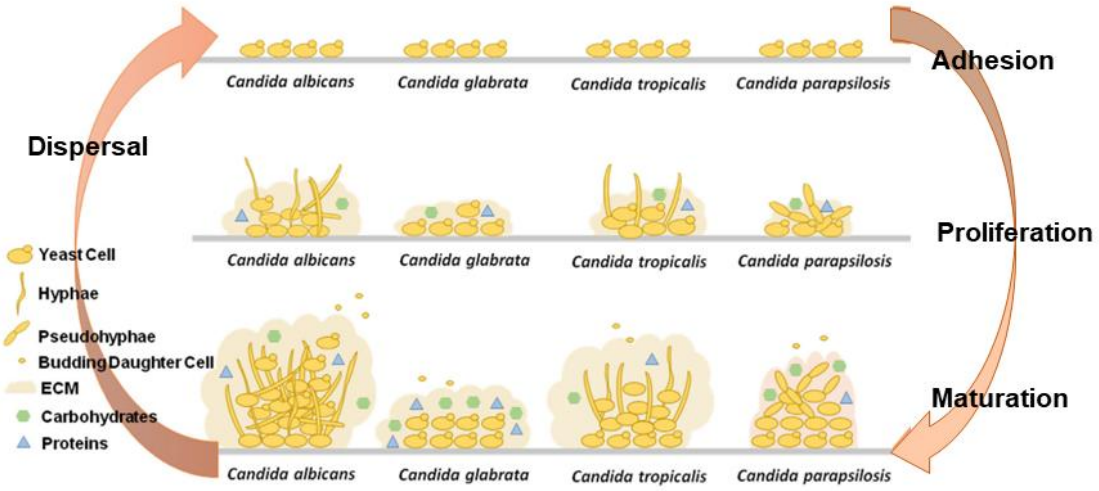

Figure 1.2. Biofilm formation of *C. albicans, C. glabrata, C. tropicalis* and *C. parapsilosis*. Adapted from Cavalheiro & Teixeira (2018).

Regarding *C. parapsilosis* biofilms, blastospores and pseudohyphae with minimal ECM are frequently observed (Cavalheiro & Teixeira, 2018; Marcos-Zambrano et al., 2014). *C. glabrata* species show highly variable biofilm production among isolates; its biofilm is formed by yeast clumped in a multilayer structure without filamentation and low total biomass (Cavalheiro & Teixeira, 2018; Marcos-Zambrano et al., 2014; Rodrigues et al., 2014). *C. dubliniensis* biofilms are formed by chains of cells with a thin extracellular matrix, and variability between clinical isolates has been observed with respect to biofilm production (Silva et al., 2017). It is noteworthy that multispecies biofilms often occur; hence a study in the oropharyngeal mucosa revealed that multispecies biofilms were a reservoir for respiratory pathogens (Hirota et al., 2017).

1.3 Antifungal agents

In oral candidiasis, topical formulations are commonly used to treat limited lesions. However, in cases of resistance to topical medications, widespread lesions related to immunosuppression and/or a risk of disseminated candidiasis, systemic antifungal therapy is required (Pappas et al., 2016). Nystatin and miconazole are the most commonly used and highly effective topical formulations. When nystatin and miconazole are prescribed, long-term treatment is required. It should be noted that miconazole can interact with other drugs, producing undesirable effects. Moreover, some topical formulations, such as amphotericin B and clotrimazole, are not available in all countries (Millsop & Fazel, 2016; Quindós et al., 2019).

Regarding systemic treatments, oral fluconazole is still the most commonly used. However, other drugs are available, such as itraconazole, voriconazole, posaconazole, isavuconazole, micafungin, anidulafungin, caspofungin and lipidic presentations of amphotericin B, some of which require intravenous administration (Pappas et al., 2016). Currently, in the development of new antifungal agents, rezafungin (a long-lasting echinocandin) and ibrexafungerp (a novel glucan synthase inhibitor) are being tested and showing interesting activity (Arendrup et al., 2020a; Berkow et al., 2017; Quindós et al., 2019). Rezafungin is being studied in a multicenter phase III trial in patients with invasive candidiasis. Ibrexafungerp is being conducted in several phase III trial, which are studying its efficacy in the treatment of recurrent vulvovaginal candidiasis, invasive candidiasis and infections refractory to other therapies as esophageal or oropharyngeal candidiasis (Seiler & Ostrosky-Zeichner, 2021).

Antifungal drugs are mainly classified on the basis of their mode of action as azoles, echinocandins, polyenes or polyenic macrolides, allylamines and pyrimidines. The latter are less commonly used drugs (Figure 1. 3).

Azoles act by inhibiting ergosterol synthesis, a constituent of the cytoplasmic membrane, and they include imidazoles (ketoconazole and miconazole) and triazoles (fluconazole, isavuconazole, itraconazole, posaconazole and voriconazole). Polyenes containing amphotericin B and nystatin lead to rupture of the fungal cell membrane by binding to ergosterol. The echinocandin group includes anidulafungin, caspofungin and micafungin, which inhibit 1,3-β-D-glucan synthesis in the cell wall. Other drugs with antifungal activity are griseofulvin, terbinafine and 5-fluorocytosine, the latter of which alters the synthesis of nucleic acids (Roemer & Krysan, 2014) (Figure 1.4).

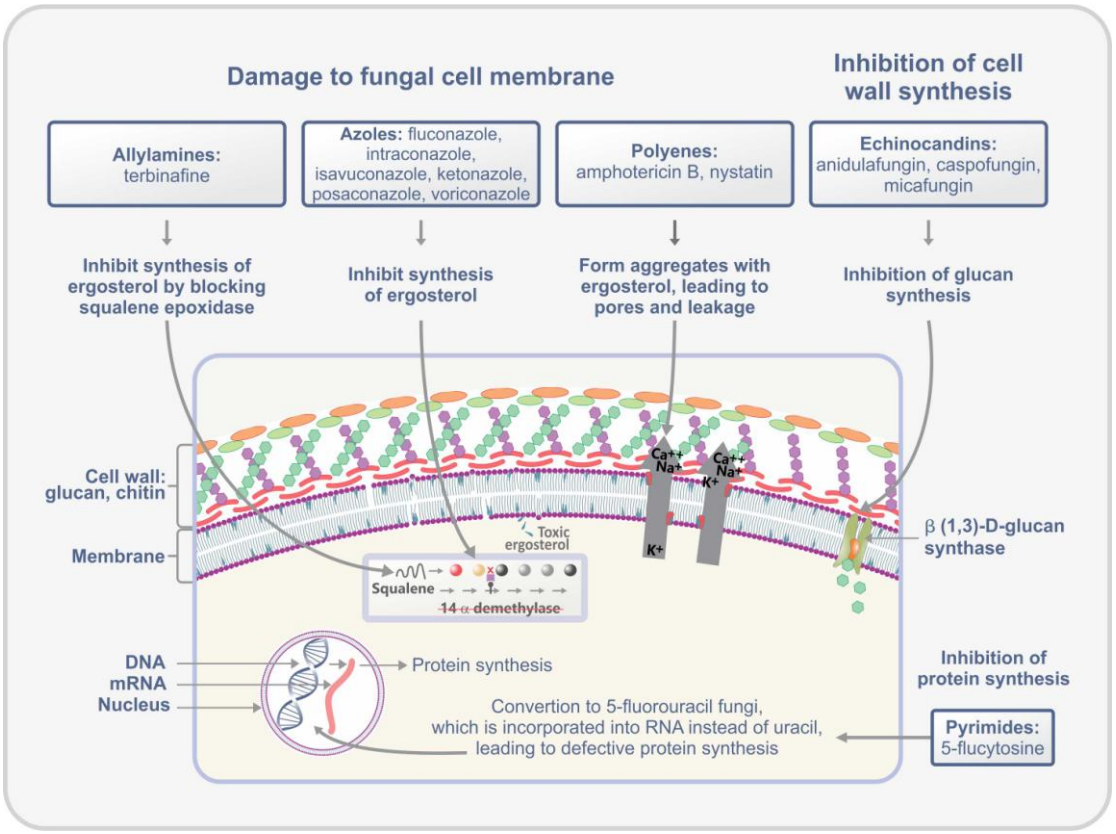

Figure 1.3. Mechanism of action of main antifungal drugs. Adapted from Antifungal therapy/Musculoskeletal Key, https://musculoskeletalkey.com/antifungal-therapy/.

1.3.1 Azoles

The mechanism of action of azoles is the targeted inhibition of 14-α-lanosterol demethylase (Erg11), a cytochrome P450-dependent system encoded by the *ERG11* gene, hindering the biosynthesis of ergosterol, a fungal-specific membrane sterol. The inhibition of ergosterol synthesis alters membrane fluidity, affecting permeability, followed by the accumulation of toxic precursors affecting cell functions and yeast viability. In general, azoles are well-tolerated fungistatic agents, although they may interfere with other drugs that also act on the cytochrome P450 system (Roemer & Krysan, 2014; Whaley et al., 2017). However, the low activity of azoles against *Candida* biofilms has been extensively described (Chandra et al., 2001; Kuhn et al., 2002).

Fluconazole

Fluconazole is a second-line triazole absorbed by the gastrointestinal membrane. It has a half-life in the body of 27-37 h and is metabolized and excreted mainly by the liver and kidneys. It is often used as a prophylactic therapy in patients at risk of candidaemia. However, its administration can lead to the selection of less susceptible or resistant *Candida* species such as *C. glabrata* and *C. krusei,* the latter exhibiting intrinsic resistance to fluconazole (Charlier et al., 2006; Orasch et al., 2018).

Isavuconazole

Isavuconazole is a triazole agent with activity against *Candida*, *Aspergillus* and other fungi. The effectiveness of isavuconazole as an oesophageal candidiasis treatment has been observed, and its success as a primary treatment for candidaemia and invasive candidiasis is being assessed (Astvad et al., 2017; Kullberg et al., 2019). Oral and intravenous formulations are available. In contrast with that of voriconazole and posaconazole, the

intravenous formulation of isavuconazole does not include sulfobutylether-β-cyclodextrin, a component associated with nephrotoxicity in other azoles (Falci & Pasqualotto, 2013).

Itraconazole

This lipophilic antifungal compound is orally administered to treat infections of several fungi, including fluconazole-resistant *Candida* species, but cross-resistance has been observed (Müller et al., 2000). Itraconazole is generally effective in the treatment of superficial mycoses and oral candidiasis in immunocompromised patients as well as in patients suffering denture stomatitis. The use of itraconazole to treat systemic infections is limited due to erratic oral absorption and pharmacological interactions. Similar to other triazoles, itraconazole is designated a category C drug (risk to foetus cannot be ruled out) because of its embryotoxicity and teratogenicity; therefore, it should not be administered during pregnancy, especially during the first trimester (Mølgaard-Nielsen et al., 2016).

Miconazole

Miconazole is a topical imidazole antifungal agent often applied as a gel, cream or lacquer formulation. In denture wearers, the lacquer form is most often recommended (Könsberg & Axéll, 1994). Its properties include activity against *Candida* species, other fungal agents and gram-positive bacteria and are important in the treatment of coinfections common in angular cheilitis. In addition, miconazole has proven to be more effective than nystatin in the treatment of pseudomembranous oral candidiasis (Quindós et al., 2019).

Posaconazole

Posaconazole is a lipophilic substance with potency and spectrum similar to those of itraconazole. Its use commonly leads to the treatment of fluconazole-resistant *Candida* infections, such as oropharyngeal and oesophageal refractory candidiasis (Skiest et al.,

2007). The liver metabolizes this drug, and its absorption is dose limited and largely subject to food intake (Li et al., 2010).

Voriconazole

Voriconazole is a triazole derived from fluconazole that exhibits broad-spectrum activity against *Candida, Cryptococcus*, dimorphic fungi, and filamentous fungi. Voriconazole has a fungistatic action against *Candida* and other fungi, and it shows fungicidal activity against *Aspergillus fumigatus* (Meletiadis et al., 2007). The combination of voriconazole with echinocandin reportedly results in a synergistic action against filamentous fungi (Philip et al., 2005). It has been prescribed orally and intravenously to treat mucosal and systematic candidiasis, invasive aspergillosis and *Scedosporium* and *Fusarium* infections. In invasive candidiasis, voriconazole treatment failure has been mainly reported in infections caused by *C. glabrata* that had been previously treated with fluconazole (Pfaller et al., 2011).

1.3.2 Echinocandins

Echinocandins comprise a group of antifungal agents derived from secondary metabolites in fungal species, namely, lipopeptides consisting of a cyclic hexapeptide core with a lipid side chain critical for the inhibition of β-(1,3)-D-glucan synthase (Fks), disrupting and inhibiting cell wall synthesis. The three main members of this group are administered intravenously as a first-line therapy in patients with invasive fungal infections and in combination with amphotericin B, voriconazole or posaconazole (Philip et al., 2005). In addition, synergist activity of anidulafungin and micafungin in combination with fluconazole and itraconazole has been described on the basis of *in vitro* assays performed to evaluate azole-resistant *Candida* isolates (Ahmadi et al., 2020). Although echinocandins have very similar antifungal activity to each other, their differences lie in their pharmacokinetics (PK), which are affected by several conditions of the patients (Muilwijk et al., 2015). In oral

candidiasis, echinocandins are used to treat oesophageal candidiasis and recalcitrant oropharyngeal candidiasis for patients who failed to respond to previous treatments with other antifungal drugs (Quindós et al., 2019).

Anidulafungin

Anidulafungin is a semisynthetic derivative obtained from *Aspergillus nidulans* (Hof & Dietz, 2009). It is intravenously administered to treat oesophageal candidiasis, invasive candidiasis associated with peritonitis and intra-abdominal abscesses in non-neutropenic patients (de la Torre & Reboli, 2007; Pfaller, 2004). Moreover, it is not necessary to adjust the dose of treatment in patients with hepatic or renal insufficiency (de la Torre & Reboli, 2007). Anidulafungin has fungicidal activity against *Candida* species and fungistatic activity against azole-resistant *Candida* species (*C. glabrata* and *C. krusei*) and polyene-resistant *Aspergillus* species such as *Aspergillus flavus, Aspergillus lentulus, Aspergillus terreus* and *Aspergillus udagawa* (Badali et al., 2009; Hof & Dietz, 2009; Pfaller, 2004). In addition, this echinocandin is active against *Alternaria*, *Curvularia* and *Bipolaris* (Espinel-Ingroff, 2003). In several studies, the antibiofilm activity of echinocandins against *Candida* species has been reported, of which anidulafungin has been demonstrated to be more effective than caspofungin and micafungin in eradicating *Candida* biofilms; however, paradoxical effects have been reported (Walraven et al., 2013).

Caspofungin

Caspofungin is a synthetically modified fermentation product, pneumocandin B0, obtained from *Glarea lozoyensis*. This echinocandin has been widely used in the clinical treatment of invasive candidiasis and aspergillosis. However, in patients with hepatic insufficiency, a reduction in the loading dose is recommended (Spriet et al., 2011). Its range of activity

covers *Candida* species, except *C. guilliermondii* and *C. parapsilosis* (Barchiesi et al., 2006). Moreover, an increase in the resistance by *C. glabrata* has been reported (Pham et al., 2014).

Micafungin

Micafungin is derived from FR901370 (WF11899A), a sulfonated hexapeptide obtained from the fungus *Coleophoma empetri*. It can be administered once daily due to its pharmacokinetic profile, no dose reduction is required for patients in renal failure or with mild or moderate liver failure, and it has low interaction rates with other drugs. After haematopoietic stem cell transplantation in either adults or infants, micafungin is noticeably effective as a prophylactic agent against *Candida* infections (Cross & Scott, 2008). Micafungin is also the only echinocandin indicated for neonates (Hope et al., 2010).

1.3.3 Polyenes or polyenic macrolides

Nystatin and amphotericin B are polyenes obtained from *Streptomyces* species that act on fungal cell membranes by combining with ergosterol and forming pores that interfere with membrane permeability and cellular transport functions. Although nystatin has a broad spectrum of action, *C. guilliermondii* and *C. lusitaniae* have been described as the *Candida* species least susceptible to these polyenic macrolides (Krcmery & Barnes, 2002). Nystatin can be administered to treat mucosal and skin mycoses through several formulations, including a cream, ointment, lozenge, oral suspension or vaginal tablet. However, due to the high concentration of glucose in the oral formulation, the risk of causing dental cavities and increased risk to patients with diabetes mellitus, this formulation is not recommended for certain patients (Millsop & Fazel, 2016).

In some countries, amphotericin B can be used topically to treat superficial mycoses, such as oral candidiasis, but the drug is not absorbed by the digestive tract. Intravenous

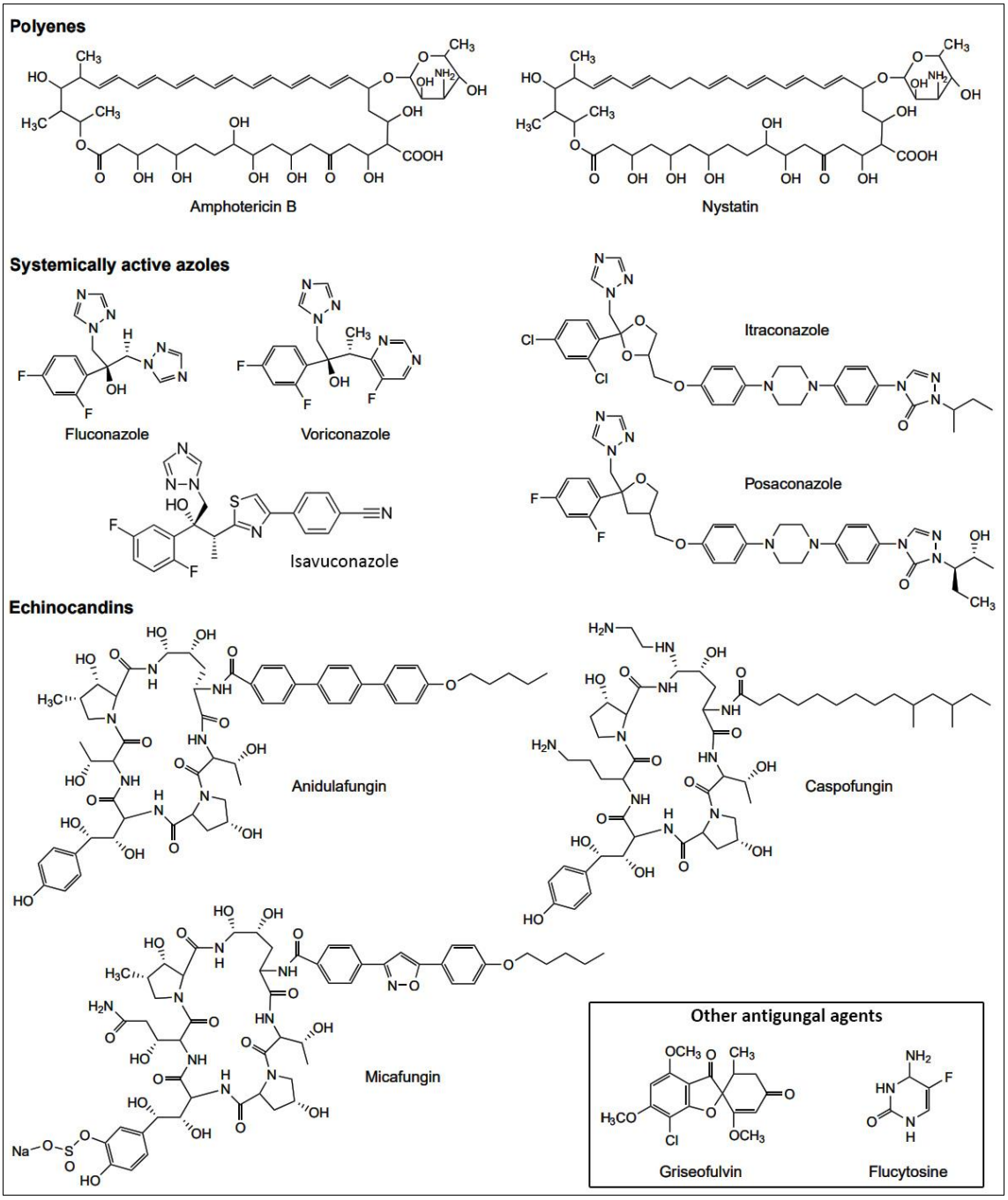

Figure 1.4. Chemical structure of the main antifungal agents. Adapted from Odds et al. (2003).

amphotericin B is used to treat disseminated or complicated *Candida* or *Aspergillus* infections, exhibiting fungicidal activity and high antibiofilm activity against *Candida* species (Kuhn et al., 2002). However, high nephrotoxicity has been associated with this treatment.

Recently, the production of three lipid formulations, liposomal amphotericin B, amphotericin B lipid complex and amphotericin B colloidal dispersion, of which liposomal amphotericin B is the most frequently administered, has contributed to the reduction in its undesirable toxic effects (Adler-Moore et al., 2019; Roemer & Krysan, 2014). Moreover, encochleated amphotericin B, an oral formulation in a phase II study, has showed remarkable reduction of toxicity and improvement in symptoms in refractory mucocutaneous candidiasis (Seiler & Ostrosky-Zeichner, 2021).

1.3.4 Other antifungal agents

5-Fluorocytosine or flucytosine

This compound is a fluoridated pyrimidine that inhibits fungal DNA and RNA synthesis and shows fungistatic activity against *Candida* species, except for *C. krusei*. Its use in combined therapy with other antifungal agents, such as amphotericin B, is recommended because both, primary and secondary resistance induced by monotherapy with flucytosine, have been reported (Mayers et al., 2017).

Griseofulvin

Griseofulvin is an aromatic polyketide synthesized from *Penicillium griseofulvum* with fungistatic activity that is orally administered to treat dermatophyte infections. Its action disrupts mitosis by inhibiting microtubule assembly and blocking tubulin, which interferes with the activity of polymerized microtubules (Odds et al., 2003).

1.4 *In vitro* antifungal susceptibility testing

In vitro study of the susceptibility of antifungal drugs allows the detection of resistant clinical isolates, and it can be a critical step in the selection of an appropriate treatment for

mycoses caused by unusual fungi or resistant isolates. Different *in vitro* methodologies are used to test the susceptibility of *Candida* isolates, including disk diffusion methods and broth macro- and microdilution methods. Both diffusion and dilution methods allow the determination of the minimum inhibitory concentration (MIC) of certain antifungals and the classification of clinical isolates of *Candida* and other fungi into susceptible, intermediate or susceptible-dose dependent (S-DD), or resistant isolates.

1.4.1 The disk diffusion method

The disk diffusion method has been standardized for fluconazole, voriconazole and echinocandins by the Clinical and Laboratory Standards Institute (CLSI) (document M44-A3) (Clinical and Laboratory Standards Institute (CLSI), 2018). There are also commercially available methods for analysing antifungal susceptibility, such as Neo-Sensitabs (Rosco, Denmark) and Etest (AB Biodisk, Sweden), which are based on the disk diffusion protocol (Koga-Ito et al., 2008; Rementeria et al., 2007).

1.4.2 Microdilution susceptibility testing

Regarding microdilution methods, standardized protocols have been defined by the CLSI (document M60 and M27-A4) and the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (AFST-EUCAST E.DEF 7.3.2 Document) with modifications (Arendrup et al., 2020b; Clinical and Laboratory Standards Institute (CLSI), 2020) (Table 1.4). Furthermore, the results obtained by both methods show clinical correlation and allow suitable discrimination between wild-type strains (WT) (without phenotypically detectable acquired resistance mechanisms) and non-wild type strains (NWT) (with intrinsic resistance or acquired resistance mechanisms) (Arendrup, 2017; Rambach et al., 2011).

Table 1.4. Technical differences between CLSI and EUCAST methodologies.

Method characteristics	CLSI (M60)	EUCAST
Glucose	0.2%	2%
Inoculum size	0.5-2.5 x 10^3 CFU/mL	0.5-2.5 x 10^5 CFU/mL
Plate /reading	Round bottom/visual	Flat/spectrophotometry
Reading time	24 h	24 h
Endpoint	50% inhibition/ 90% inhibition*	50% inhibition/ 90% inhibition*

*for fungicidal agents

The CLSI documents describe the parameters within which to test antifungal agents against yeasts and moulds and provide species-specific clinical breakpoints for all three echinocandins, as well as for azoles, amphotericin B and flucytosine. Thus, the clinical breakpoints, epidemiological cut-off values (ECVs), quality control strains and reading criteria parameters are established in the M60 and M27A4 documents (Clinical and Laboratory Standards Institute (CLSI), 2020, 2017). The epidemiological cut-off values enable the detection of emerging antifungal resistance and contribute to local and global resistance surveillance (Arendrup, 2017).

Using the EUCAST method, it is possible to test the susceptibility of moulds and yeast, such as *Candida* and *Cryptococcus,* to the most commonly used antifungal drugs. Species-specific clinical breakpoints and epidemiological cut-off values (ECOFFs) are available on the EUCAST website (www.eucast.org); however, caspofungin and isavuconazole breakpoints have not been established by this method (https://mic.eucast.org/search/).

Sensititre YeastOne (Trek Diagnostics Systems Ltd, England) is a standardized commercial method with some methodological differences and modifications in terms of medium

composition and the times and forms of readouts, but exhibits good concordance with the CLSI and EUCAST methods. Moreover, automated methods such as Vitek2 (BioMérieux, France) and the ATB Fungus (BioMérieux) are currently commercially available and show a good correlation with the standardized CLSI and EUCAST method and other commercial methods such as Sensititre YeastOne (Cuenca-Estrella et al., 2010; Eraso et al., 2008).

1.4.3 Combination therapy

Combination antifungal therapy has emerged because of the search for alternatives that avoid or reduce therapeutic failures associated with long-term or high-dose conventional antifungal treatments (Lewis & Kontoyiannis, 2001). The effect of antifungal combinations has been studied with different *in vitro* assays, with the checkerboard assay being one of the most commonly applied. This method consists of calculating a fractional inhibitory concentration index (FICI) based on a two-dimensional array of serial concentrations of the test compounds. The FICI values obtained allow an assessment of whether the combination of the agents causes a greater effect than the sum of their effects alone (Johnson et al., 2004). Synergism is indicated by a FICI $\leq$ 0.5 (indicating that the combination has significantly increased the antifungal activity, in contrast with the action of each agent separately); antagonism is indicated by a FICI > 4.0 and indifferent or additive interaction is indicated by a FICI > 0.5–4.0 (Van Dijck et al., 2018).

1.5 Mechanisms of *Candida* resistance to antifungal agents

Therapeutic failure with antifungal drugs is associated with host factors such as immunodeficiency, the presence of underlying diseases that affect drug metabolism and bioavailability, and the characteristics of microorganisms, such as antimicrobial resistance (Sanguinetti et al., 2015). The identification of *Candida* species resistant to antifungal agents

and of resistant isolates of species usually susceptible to antifungal drugs has become an increasingly frequent finding.

Antifungal resistance is based on primary or secondary mechanisms, which are related to intrinsic or acquired characteristics of fungi (Rodrigues et al., 2014). These mechanisms consist of interference with the antifungal mechanism of the respective drug through modification of the metabolic pathway of the target; modification of the target drug through overexpression, structural alteration or suppression; or a decrease in the intracellular concentration of the antifungal drugs through blockades to their influx or efflux (Revie et al., 2018; Rodrigues et al., 2014).

1.5.1 Resistance mechanisms to azoles

Resistance to azoles, such as fluconazole, has been found to be intrinsic in some species, such as *C. krusei* and *C. auris,* and in some clinical and environmental isolates from other *Candida* species. In several *Candida* species, azole resistance is secondary to the use of azole prophylaxis as a long-term treatment. A global resistance rate of *C. krusei* to fluconazole of 78.3% has been reported, and an alarming 80% of *C. auris* isolates display resistance to fluconazole (Osei Sekyere, 2019; Sanguinetti et al., 2015).

C. glabrata shows reduced dose-dependent susceptibility with a rate of global resistance to fluconazole of 15.7%. In addition, *C. albicans*, *C. parapsilosis* and *C. tropicalis* display variable rates of primary resistance to fluconazole (1.4%, 2-5% and 0% to 83%, respectively) (Sanguinetti et al., 2015; Whaley et al., 2017). Furthermore, cross-resistance to azoles by *C. albicans, C. glabrata, C. parapsilosis* and *C. tropicalis* has limited the use of this group of antifungal agents to treat infections caused by these species (Pfaller & Diekema, 2007).

The main mechanisms of azole resistance in most common *Candida* species are shown in Figure 1.5 (Sanguinetti et al., 2015; Whaley et al., 2017). One mechanism of azole resistance

involves the development of bypass pathways incorporating alternative sterols in the yeast membrane upon Erg3 inactivation (Figure 1.5 A). Thus, mutations in the *ERG3* gene have been shown to prevent the formation of 14-α-methyl-3,6-diol (a toxic metabolitel) from 14-α-methylfecosterol, promoting the synthesis of fecosterol, which can replace ergosterol**.** In fluconazole-resistant isolates of *C. glabrata*, uptake of exogenous sterols helps to prevent the inhibition of endogenous sterol production by fluconazole (Figure 1.5 B). Azole resistance owing to mutations in *ERG3* has been described in a few clinical isolates of *C. albicans*.

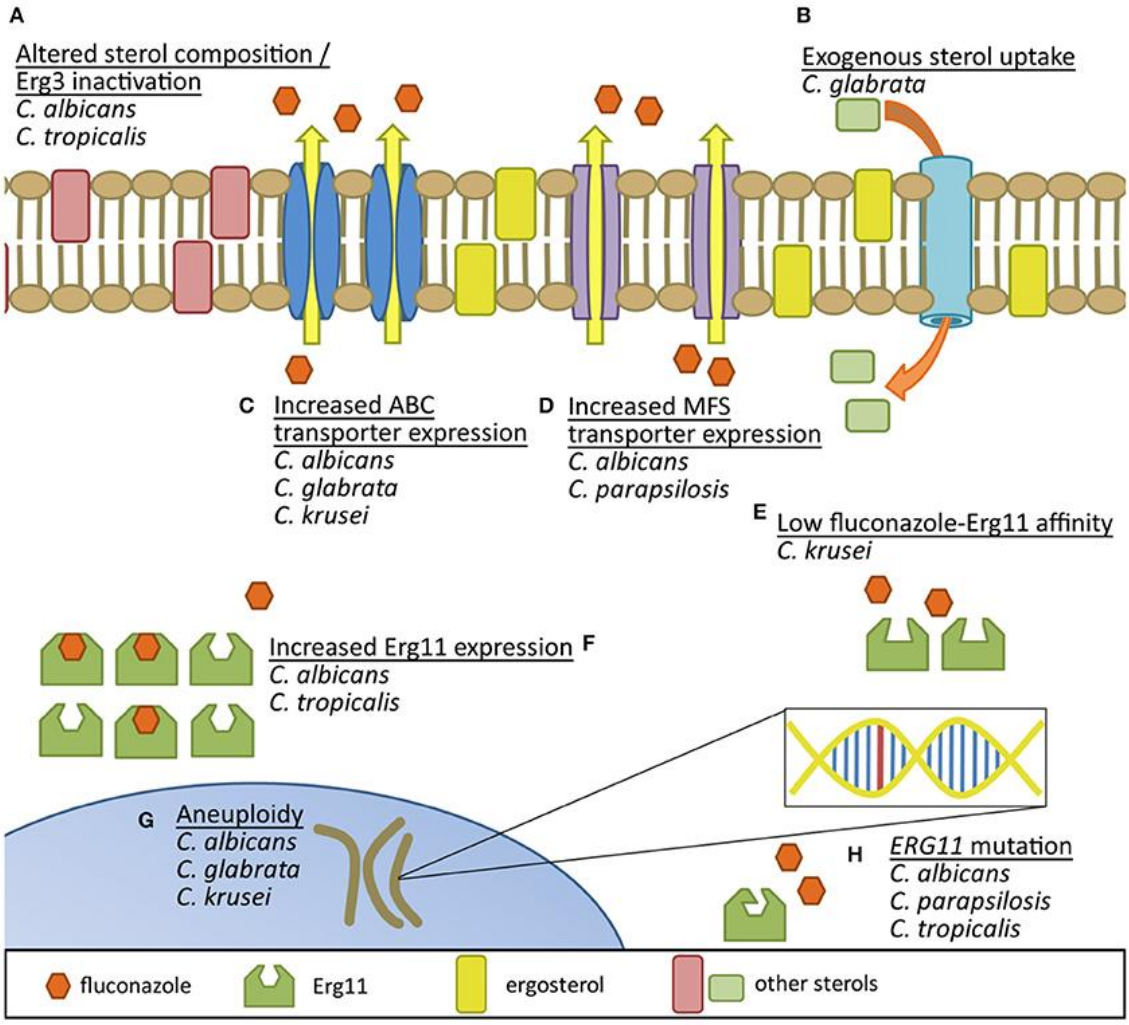

Figure 1.5. Comparison of documented fluconazole resistance mechanisms in some *Candida* species. Obtained from Whaley et al. (2017).

Enhanced induction of drug efflux pumps that transport drugs outside of the cell with a subsequent reduction in the intracellular azole concentration is the most common resistance mechanism in *Candida* species. Efflux pumps are transmembrane proteins that transport a wide variety of substrates across the cell membrane and are classified into two families, ATP-binding cassette superfamily (ABC) and major facilitator superfamily (MFS) transporters (Chaabane et al., 2019). *CDR* genes in ABC transporters encode efflux pumps and have been shown to confer *Candida* resistance to almost all azoles (Figure 1.5 C), and *MDR* genes in MFS transporters encode efflux pumps that seem to be selective for fluconazole (Figure 1.5 D). The overexpression of *CDR* and *MDR* genes confers azole resistance in *C. albicans* (*MDR1*, *CDR1*, and *CDR2*), *C. glabrata* (*CgCDR1* and *CgCDR2*) and *C. dubliniensis* (*CdMDR1* and *CdCDR1*) (Whaley et al., 2017).

Another mechanism is related to the increased expression of the Erg11 protein, an enzyme that targets lanosterol 14-α-sterol demethylase, which is encoded by the *ERG11* gene (Figure 1.5 F). In the case of *C. albicans*, mutations in the gene that encodes the zinc-cluster transcriptional regulator Upc2 mediate upregulation of ergosterol biosynthesis through the constitutive increase in the expression of the *ERG11* gene. In addition, the alteration of the Erg11 protein by mutations in the *ERG11* gene had led to lower affinity of *Candida* to fluconazole (Figure 1.5 H). Point mutations in three hot spot regions located between amino acids 105 and 165, 266 and 287, and 405 and 488 are the most frequent in *Candida* species (Chaabane et al., 2019). The intrinsic resistance of *C. krusei* to fluconazole has been attributed to the inherently low affinity of fluconazole for Erg11 binding, which reduces the inhibitory action of fluconazole (Figure 1.5 E). Furthermore, other genes involved in the ergosterol biosynthesis pathway, such as *ERG1-ERG7, ERG9, ERG10, ERG13, ERG19* and *ERG24* to *ERG26,* are less associated with azole resistance (Henry et al., 2000).

Aneuploidy in some species of *Candida* leads to genetic adaptation by increasing cell exposure to azole (Figure 1.5 G). *C. albicans* and *C. glabrata* can exhibit azole resistance by increasing copies of genes related to azole resistance by inducing aneuploidy, through chromosomal duplication or loss of heterozygosity. Moreover, respiratory and mitochondrial deficiencies have also been described as contributing to azole resistance in these species (Brun et al., 2003; Qu et al., 2012). In *C. auris,* the presence of MFS efflux pump genes, high ABC efflux pump activity in relation to *CDR1*, and point mutations Y132F and K143R in the *ERG11* gene have been described as the main mechanisms of azole resistance (Chaabane et al., 2019).

1.5.2 Resistance mechanisms to echinocandins

Echinocandins exhibit antifungal activity via inhibition of the enzyme glucan synthase, which is encoded by three related genes (*FKS1*, *FKS2* and *FKS3*). This inhibition disrupts the biosynthesis of 1,3-β-D-glucan and thus causes the loss of fungal cell wall integrity and high cellular stress. Although most *Candida* species are susceptible to echinocandins, in certain *C. parapsilosis* isolates, reduced *in vitro* susceptibility has been reported. A P660A substitution in Fks1 is critical for the reduced susceptibility phenotype (García-Effrón et al. 2008). In addition, *C. guilliermondii* also shows intrinsically reduced susceptibility associated with polymorphisms (L633M and T634A) in Fks1 in a region of polynucleotides with a high frequency of mutation known as hot spot 1 (Dudiuk et al., 2017).

Acquired echinocandin resistance has been reported in *C. auris, C. albicans, C. dubliniensis, C. kefyr, C. krusei, C. glabrata, C. lusitaniae* and *C. tropicalis* isolates (Arendrup & Perlin, 2014; Chaabane et al., 2019). The main concern is the resistance of *C. glabrata*, which due to an increase in the number of isolates with resistance to echinocandins combined with its resistance to azoles, has led to multiresistant infections (Perlin, 2015; Rivero-Menendez et

al., 2019). Mutations in specific hot spot regions of the *FKS1* gene in each *Candida* species, as well as those of *FKS2* in *C. glabrata*, causing several single amino acid substitutions, determine the degree of MIC increase, and this substitution occurs near the extracellular membrane surface within transmembrane segments 6 and 7 (Arendrup & Perlin, 2014; Rodrigues et al., 2014) (Figure 1.6). Moreover, in *C. auris*, mutations in the *FKS1* gene that cause the amino acid substitutions S639Y, S639P and S639F have been related to echinocandin resistance (Chaabane et al., 2019).

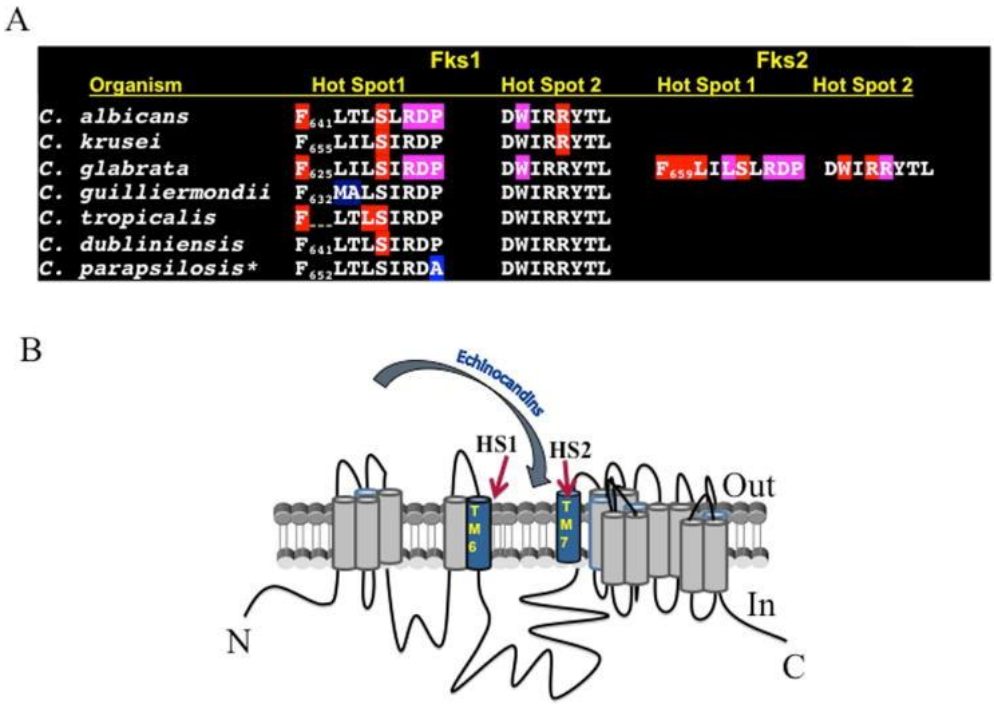

Figure 1.6. A: Amino-acid changes in hot spot regions of Fks1 and Fks2 in *Candida* species that confer clinical resistance to echinocandins. Positions associated with prominent resistance (red), weaker resistance (purple) and naturally occurring reduced susceptibility (blue). B: Schematic model of glucan synthase and predicted positions of amino acid substitutions conferring echinocandin resistance**.** Obtained from Perlin (2015).

1.5.3 Resistance mechanisms to polyenes

Resistance to polyenes by *Candida* species and other fungi is not common. However, primary resistance has been observed in some isolates of *C. lusitaniae, C. lipolytica* and

C. guilliermondii, and several *C. auris* strains are resistant to amphotericin B (Cantón et al., 2004; Chaabane et al., 2019). There are different mechanisms of resistance to polyenes, including a reduction in the amount of ergosterol and the modification of the fungal cell membrane. Additionally, mutations in the *ERG3* gene encoding C-5 sterol desaturase (Erg3), an enzyme in the ergosterol biosynthetic pathway of *C. albicans*, and a rare mutation in the *ERG6* gene in *C. glabrata* have been reported (Kelly et al., 1997; Vandeputte et al., 2007).

1.6 Terpenes and other natural extracts

Historically, plants have been used for medicinal purposes, such as prevention and alternative treatments for several infectious diseases. This medicinal practice led to the discovery of a tremendous source of phytocompounds that exhibit antimicrobial activity as well as other biological properties, such as anticancer, analgesic, insecticidal and antioxidant activity (Dhifi et al., 2016; Kumar et al., 2019; Saddiq & Khayyat, 2010; Sun et al., 2020; Suntres et al., 2015). These phytochemicals represent potential alternatives in the fight against biofilm-related infections.

Essential oils (EOs) from aromatic plants, most of which have medicinal uses, are complex mixtures of several volatile organic phytocompounds in different proportions and are produced by plants as secondary metabolites (Dhifi et al., 2016). EOs can be obtained by extraction techniques such as maceration, cold pressing, steam distillation, turbo distillation, enfleurage, hydrodistillation, solvent extraction, supercritical CO_2 and fluid extraction from different parts of plants (buds, flowers, leaves, roots, wood, and bark) (Hanif et al., 2019; Valdivieso-Ugarte et al., 2019).

In contrast to pure phytocompounds, the study of EO activity and potential applications is affected by factors that alter the EO composition in the plant, such as the plant part, species, geographical origin, season of harvesting, extraction method and protocols. All these aspects of the plant affect EO biological properties and the reproducibility between the results of different studies (Jordán et al., 2006; Mejri et al., 2010; Viljoen et al., 2005).

Phytocompounds are low-weight molecules and are classified into several groups according to their molecular structure. Most phytocompounds with antifungal activity belong to classes of phenylpropanoids, quinones, flavonoids, terpenes (terpenoids or isoprenoids), alkaloids, stilbenes and bisbibenzyls, among others (Lu et al., 2017). Of these compounds, terpenes and phenylpropanoids are the most common constituents of many EOs.

Terpenes consist of 5-carbon-base units or isoprene, which, depending on the number of isoprene units, are classified into hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30), tetraterpenes (C40) and polyterpenes (> 8 isoprene units) (Lu et al., 2017; Nagegowda & Gupta, 2020). Monoterpenes are major constituents of EOs extracted from aromatic plants and include several bioactive phytocompounds, including acyclic alcohols, such as citral, geraniol, citronellol, citronellal and linalool; monocyclic alcohols, such as menthol and terpinen-4-ol; and cyclic phenols, such as carvacrol and thymol (Nagegowda & Gupta, 2020; Ramage et al., 2012b). Phenylpropanoid acid phytocompounds, cinnamaldehyde, eugenol and methyleugenol, composed of a benzene ring with three carbon units, are also being studied because of their multiple biological characteristics (Dhara & Tripathi, 2020; Sun et al., 2020).

Phytocompounds such as carvacrol, thymol, cinnamaldehyde, citral, p-cymene, eugenol, limonene and menthol, among others, have been classified as generally recognized as safe (GRAS) for use in food (Hyldgaard, Mygind, and Meyer 2012). Hence, they are widely used

in the cosmetics and food industries, although their use as an antifungal therapy has not yet been established.

1.6.1 Carvacrol

Carvacrol (2-methyl-5-(1-methylethyl)-phenol) is mainly obtained from plants of the *Labiatae* family (oregano, thyme and savoury), such as *Origanum acutidens, Origanum dictamnus*, *Origanum vulgare*, *Origanum majorana*, *Thymbra capitata*, *Thymus vulgaris*, *Thymus zygis*, *Thymus serpyllum*, *Satureja hortensis*, and *Satureja montana* (Kordali et al., 2008; Suntres et al., 2015) (Figure 1.7).

Studies have shown that carvacrol exhibits antifungal properties against *Candida* species, *S. cerevisiae* and fungal plant pathogens (Ahmad et al., 2011; Kordali et al., 2008; Pei et al., 2020; Shaban et al., 2020). Similar to other phytocompounds, its chemical composition is associated with antimicrobial activity and often related to cell membrane disruption. In the case of carvacrol, the hydroxyl group is partially responsible for its activity (Dhifi et al., 2016; Kordali et al., 2008). Moreover, other possible mechanisms of carvacrol against *Candida* species have been described, such as the inhibition of ergosterol biosynthesis through the downregulation of the *ERG3* and *ERG11* genes; production of endoplasmic reticulum stress, resembling Ca^2+ stress; and inhibition of the target of rapamycin (TOR) pathway (Ahmad et al., 2011; Alizadeh et al., 2018; Chaillot et al., 2015; Rao et al., 2010).

1.6.2 Thymol

Thymol (2-isopropyl-5-methylphenol) is the major constituent of essential oils obtained from thyme and oregano, especially *T. vulgaris* and *O. vulgare* (Hyldgaard et al., 2012) (Figure 1.7). It exhibits several activities, including pharmacological, antibacterial, antiparasitic and antifungal activities against *Candida, Alternaria, Aspergillus, Cladosporium* and *Fusarium* (de Vasconcelos et al., 2014; Jafri & Ahmad, 2020; Marchese et al., 2016; Nagoor Meeran et al.,

2017). Similar to carvacrol (a thymol isomer), it has a similar antifungal action, inhibiting ergosterol biosynthesis and causing Ca2+ stress in yeast (Ahmad et al., 2011; Rao et al., 2010). Furthermore, thymol was able to protect against *C. albicans* infection of *in vivo* invertebrate models via the p38 MAPK signalling pathway associated with the host innate immune response (Shu et al., 2016).

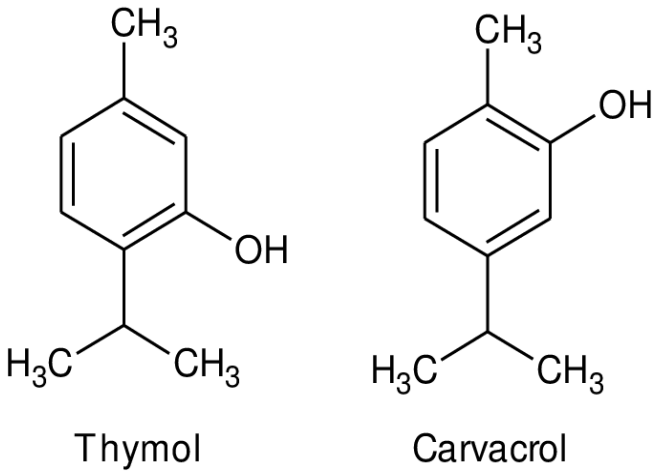

Figure 1.7. Chemical structure of thymol and carvacrol. Obtained from Shiyab et al. (2012).

1.6.3 Citral

Citral (3,7-dimethyl-2,6-octadienal) is obtained as geranial (citral A, a *trans*-isomer) and neral (citral B, a *cis*-isomer) predominantly found in lemongrass (*Cymbopogon citratus* and *Cymbopogon flexuosus*) and in plants such as *Melissa officinalis, Verbena officinalis, Lippia citriodora*, *Litsea cubeba*, *Backhousia citriodora*, and *Citrus sinensis* (Bailly, 2020; Shi et al,. 2016) (Figure 1.8). Its antifungal activity has been reported against *Aspergillus, Candida*, *Geotrichum*, *Penicillium* and *Trichophyton* (Hua et al., 2014; Hyldgaard et al., 2012; Leite et al., 2014; Tao et al., 2014). The inhibition of ergosterol biosynthesis in *Candida* cells (Rajput & Karuppayil, 2013; De Sousa et al., 2016), the disruption of cell membrane integrity (Zore et al., 2011),

but no action on fungal cell wall biosynthesis or binding to ergosterol (Leite et al. 2014; Lima et al., 2012) are some possible mechanisms of action of citral studied in *Candida*.

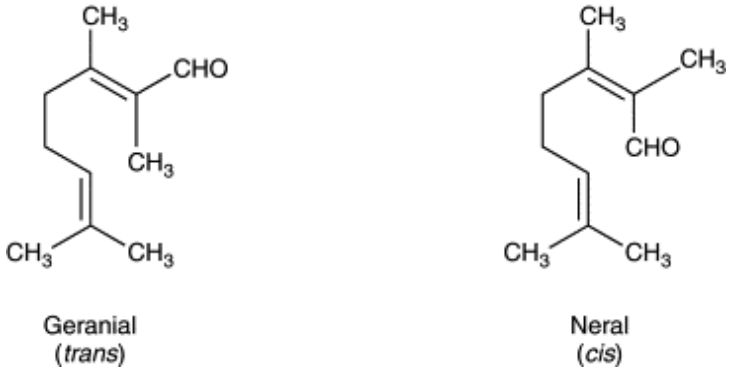

Figure 1.8. Chemical structure of citral, geranial (citral A, *trans*-isomer) and neral (citral B, *cis*-isomer). Obtained from Clarke (2008).

1.6.4 Cinnamaldehyde

Cinnamaldehyde (*trans*-3-phenyl-2-propenal) is a major constituent of trees in the genus *Cinnamomum* (*Lauraceae*) and is critical for the smell and flavour of cinnamon (Figure 1.9). Its fungal action extends to *Aspergillus, Candida*, *Cryptococcus, Fusarium, Penicillium* and *S. cerevisiae* (Shreaz et al., 2016; Wang et al., 2018). In contrast to the monoterpenes mentioned below, this phytocompound acts on the cell walls of yeasts by inhibiting the synthesis of zinc enzymes and chitin synthase isozymes as a non-competitive inhibitor of β-(1,3)-D-glucan synthase and a mixed inhibitor, respectively (Bang et al., 2000).

Moreover, the inhibition of the yeast-to-hyphae transition and subsequent biofilm formation of *Candida* cells has been demonstrated after treatment with cinnamaldehyde (Raut et al., 2014). Additionally, Shreaz et al. (2016) stated that the mode of action of cinnamaldehyde is dependent on its concentration. Thus, at lethal concentrations, cell membranes are the target, with their subsequent disruption; at high but sublethal concentrations, cinnamaldehyde acts as an ATPase inhibitor and affects ergosterol

biosynthesis in *Candida* cells, and at very low concentrations, it may inhibit ATPases and enzymes involved in cytokine interactions (Shreaz et al., 2010).

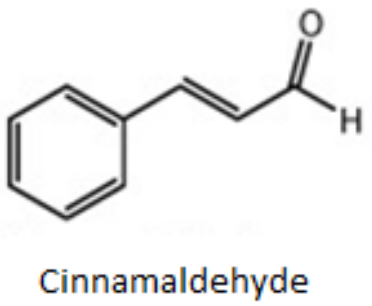

Figure 1.9. Chemical structure of cinnamaldehyde. Modified from Shreaz et al. (2016).

1.7 Nanoparticles

Nanotechnology is a field that is being increasingly studied, as it may become an excellent tool for providing solutions to problems associated with drug delivery, allowing a controlled and specific targeted solution to gene delivery applications in cancer therapy and for antimicrobial therapy (Buabeid et al., 2020; Ranghar et al., 2014; Sun et al., 2019).

Implementation of encapsulation techniques has allowed hurdles regarding the physical properties of compounds that determine bioavailability, toxicity, stability, water solubility and irritant side effects to be overcome (Adler-Moore et al., 2019; Gursul et al., 2019; Khan et al., 2017). The volatility of phytocompounds is a characteristic that makes their application difficult, and this volatility is prevalent in the most biologically active phytochemicals. High volatility is related to high instability, and these compounds can be easily affected by variations in environmental conditions (Dhifi et al., 2016). Therefore, in the case of phytocompounds, encapsulation is a significantly useful tool for producing more-stable molecules with respect to volatility because encapsulation creates a physical

barrier to oxygen, protecting bioactive components from oxidation and even enhancing their solubility and bioavailability (Gursul et al., 2019).

Several nanoparticles have been designed for drug delivery, including metal-based nanoparticles such as silver (AgNPs), gold, zinc, magnesium and titanium oxide particles; natural or synthetic polymeric-based nanoparticles such as polyethylene glycol, poly(lactide-o-glycolic) acid, alginate and chitosan; and lipid-based nanoparticles such as liposomes, solid lipids and lipid vesicles (Buabeid et al., 2020; Ivask et al., 2014; Ranghar et al., 2014). An example of a successful liposomal formulation for delivery of an antifungal agent is liposomal amphotericin B, which was developed with the aim of reducing amphotericin B toxicity and side effects by entrapping it inside a spherical unilamellar liposome (Adler-Moore et al., 2019; Adler-Moore & Proffitt, 2008).

1.7.1 Liposomal nanoparticles (DODAB:MO)

Liposomes are spherical phospholipid bilayers composed of amphiphilic molecules that can be self-assembled with an aqueous volume entrapped inside them; they can also encapsulate hydrophilic molecules into the aqueous core and hydrophobic compounds into lamellae (Beltrán-Gracia et al., 2019; Ranghar et al., 2014) (Figure 1.10).

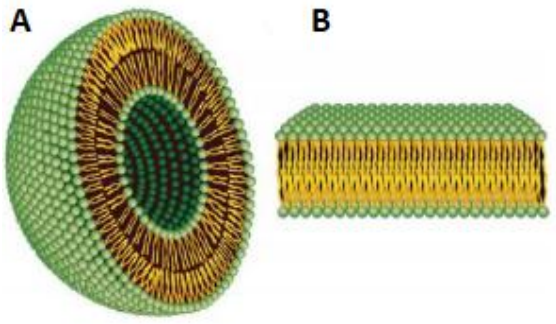

Figure 1.10. A: Schematic image of the structure of a liposome. B: Schematic image of the phospholipid bilayer structure; green represents a hydrophilic region comprising a polar headgroup, and yellow represents hydrophobic nonpolar hydrocarbon chains (tails). Adapted from Bozzuto & Molinari (2015).

Liposomes are classified according to their size and number of bilayers: multivesicular vesicles (MVVs, diameter > 1000 nm), multilamellar vesicles (MLVs, diameter > 200 nm), large unilamellar vesicles (LUVs, diameter 100–400 nm), and small unilamellar vesicles (SUVs, diameter 20-100 nm) (Daraee et al., 2016; González-Rodríguez & Rabasco, 2011; Ranghar et al., 2014).

DODAB:monoolein liposomes are cationic liposomes that consist of lipid-bilayer vesicles with a positive charge. Cationic liposomes include different types of cationic lipids in their structure, and these liposomes have been studied for their potential roles in drug and vaccine delivery, and gene therapy (Carneiro et al., 2015; Das et al., 2016; Davidsen et al., 2005; Oliveira et al., 2015). Dioctadecyldimethylammonium bromide (DODAB) is a cationic synthetic surfactant comprising a hydrophilic positively charged dimethylammonium headgroup connected to two long hydrocarbon saturated chains that constitute a tail (hydrophobic 18-carbon alkyl chains (C18:0) (Carneiro et al., 2015). Monoolein, 1-monooleoyl-rac-glycerol (MO), is a nontoxic and biodegradable lipid characterized by 9-*cis*-octadecenoic acid at the sn-1 position of glycerol (Ganem-Quintanar et al., 2000). Incorporation of monoolein has allowed the system to be stabilized, improving lateral fluidity in the DODAB bilayer, which is essential for fusion with cell membranes and subsequent introduction of the drug through the cell membrane or inside the microorganism (Oliveira et al., 2014; Ranghar et al., 2014; Silva et al., 2011) (Figure 1.11). Moreover, the presence of monoolein minimizes the cytotoxic effects related to DODAB (Carneiro et al., 2015).

A 1:2 DODAB:MO molar ratio provides the system with the special ability to retain encapsulated compounds because of the structural rigidity of the DODAB lamellar phase, and the inverted MO nonlamellar phases are critical for fluidity and positive charge, which

is required for interaction with cell surface membranes and to deliver compounds inside the target cell (Figure 1.11) (Carneiro et al., 2015).

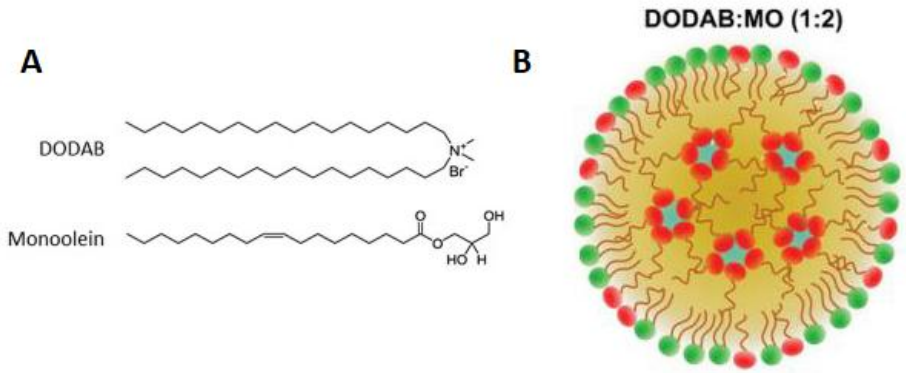

Figure 1.11. A: The chemical structures of DODAB and monoolein. B: Schematic representation of DODAB:MO (1:2) liposomes (green, DODAB, red, monoolein). Adapted from Oliveira et al. (2015, 2016).

1.8 Methods of studying phytocompounds antifungal activities

1.8.1 Susceptibility testing of *Candida* to natural compounds

Evaluation of antifungal activity can be carried out, in addition to the previously mentioned diffusion plate and broth dilution methods, by bioautography, agar absorption and vapour phase assays (Amat et al., 2017; Horváth et al., 2010). However, several studies have used variable incubation conditions, culture media and emulsifiers or solvents that have produced a variety of results, which has hampered the ability to correlate results between the studies (Van de Vel et al., 2019). Phytocompound properties such as water solubility, thermal resistance and volatility should be considered. Therefore, the study of novel compounds such as phytocompounds should be achieved using the most regulated and standardized methodology available to determine the minimum inhibitory concentration

(IC) and minimum fungicidal concentration (MFC) of phytocompounds needed to inhibit the growth of the fungus, such as those included in recent recommendations from Van Dijck et al. (2018).

Microdilution susceptibility testing is the most common methodology used for the evaluation of planktonic cells, whereas time-kill curves and the checkerboard assay are frequently used to evaluate combination therapy and define fungicidal or fungistatic activity. Moreover, methods such as quantifying microbial biomass (the crystal violet protocol) or metabolic activity (2,3-bis (2-methoxy- 4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide reduction (XTT)) are also performed to test the susceptibility of fungal biofilms to antifungal agents or compounds (Ramage et al., 2001).

1.8.2 *In vivo* method for monitoring antifungal compounds

Toxicity assessment and monitoring of the activity of new compounds in the laboratory has been habitually carried out by using *in vivo* models, including *ex vivo* systems and animal models; however, evaluation of cytotoxicity using *in vitro* cellular systems is necessary as a first screening step (Scorzoni et al., 2016). Although the most useful models are vertebrate systems, such as murine models, because they are phylogenetically closely related to humans, nonvertebrate models are less labour-intensive and advantageous options in terms of ethical requirements; these systems include *Caenorhabditis elegans*, *Drosophila melanogaster*, *Galleria mellonella, Bombyx mori* and zebrafish larvae (*Danio rerio*), (Chamilos et al., 2006; Desalermos et al., 2012; Desbois & Coote, 2012) (Figure 1.12). Of these, *C. elegans* has been widely used in the screening of large quantities of new active compounds as well as in determining the virulence of microorganisms and immune host response associated with innate host defence; hence, the use of *C. elegans* has led to greater understanding and

enhanced knowledge of the infection process and the discovery of new active compounds (Breger et al., 2007). Some phytocompounds, such as magnolol, honokiol and thymol, have been efficiently evaluated using *C. elegans* as model of *Candida* infections (Shu et al., 2016; Sun et al., 2015). Some advantages of this model include the possibility of performing high-throughput screening, toxicity analysis and the identification of key processes of virulence and innate host immunity because this worm can be infected with *Candida* and other microorganisms of interest. Nevertheless, adaptive immunity cannot be tested, nor can bioavailability or organ toxicity be assessed, which would require further study in mammalian models (Peterson & Pukkila-Worley, 2018).

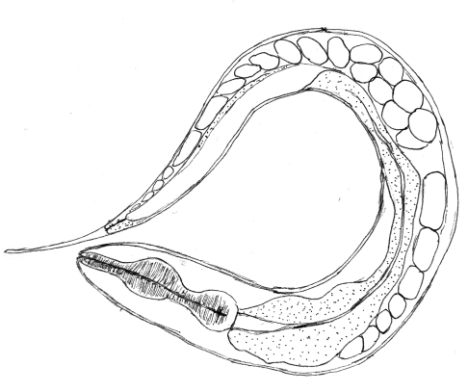

Figure 1.12. Schematic representation of *in vivo* model *C. elegans.*

2

JUSTIFICATION AND OBJECTIVES

Oral candidiasis is one of the most common mycoses, characterized to a greater extent by acute manifestations that is sometimes secondary to previous pathologies, which may develop a chronic, recurrent and recalcitrant response to treatment. For instance, chronic pseudomembranous candidiasis, typical in patients after receiving chemotherapy, and oesophageal candidiasis, a very frequent secondary complication of immunodeficiency in HIV-infected patients. These manifestations can even evolve into more serious forms, such as invasive candidiasis. Several factors influence the development of *Candida* infection, one of which is previous colonization by a *Candida* species since most cases of candidiasis are caused by strains in the microbiome of patients. In addition, other host factors determine the course of infection, such as age, dental hygiene habits, long-term antibiotic treatment, use of dental prostheses, corticoids steroid use, immunomodulatory treatments, or diseases that produce immunodeficiency.

On the other hand, *Candida* species engage mechanisms that actively act in the development of infection. The capacity to produce biofilms on different surfaces, such as catheters and dental prostheses, is among the main factors of virulence of *Candida* species and is associated with a higher resistance to antifungal drugs and evasion of the immune response of the host. Therefore, its eradication from tissues as well as from medical devices is determinant in the therapeutic success of the infection.

C. albicans is the species implicated most frequently as an aetiological agent. However, other *Candida* species have acquired prominence and complicated the treatment candidiasis, such as *C. glabrata* and *C. parapsilosis*. The colonization and infection of species such as *C. glabrata* or *C. krusei* with a significant reduction in susceptibility to azoles, including miconazole and fluconazole, and the presence of *C. albicans* isolates that exhibit resistance to azoles and other antifungal drugs used in the treatment of oral candidiasis are important therapeutic challenges. Although new drugs with antifungal activity have been studied and developed in

recent years, including azoles such as isavuconazole and echinocandins such as anidulafungin, micafungin and caspofungin, the number of available antifungal drugs is limited. Fluconazole is a triazole that induces low toxicity, is delivered by oral and parenteral administration, and shows cost-effectiveness and minimal interactions with other drugs. However, its extensive prophylactic use and long-term treatments have led, in many cases, to the development of resistance.

In the current scenario, the search for new compounds with antifungal activity is important, both for treatment with monotherapy and combination therapy with drugs currently available. In the past decade, the study of the biological and antimicrobial activity of terpenes and other phytocompounds has been relevant and currently represents a starting point for the development of new drugs and the discovery of new therapeutic targets upon the exploration of their mechanism of action. The most promising compounds should include characteristics such as low toxicity, adequate solubility for topical, oral or parenteral use and no or few side effects. Encapsulation of drugs into liposomal nanoparticles is a strategy that has improved the bioavailability and reduced the toxicity of drugs such as amphotericin B. Drug delivery by nanoparticles is a very useful tool for increasing the therapeutic efficacy of phytocompounds in the treatment of *Candida* infections.

The main objective of this PhD Thesis was to evaluate the *in vitro* and *in vivo* antifungal activity of terpenes and other phytocompounds and their nanoparticles against the main *Candida* species involved in oral candidiasis, to determine the synergistic action of these compounds with fluconazole and to study their possible mechanism of action.

To achieve the main objective, the following specific objectives were pursued:

1. To establish the prevalence of *Candida* species in addition to *C. albicans*, mainly *C. glabrata* and *C. parapsilosis* complexes in oral candidiasis and determine their *in vitro* susceptibility to the main antifungal drugs commonly used in treatment.

   To achieve specific objective 1, **study 1**, entitled **"Prevalence and antifungal susceptibility profiles of *Candida glabrata* and *Candida parapsilosis* complexes",** was carried out.

2. To determine the antifungal activity of terpenes and other phytocompounds against planktonic and sessile cells of the main *Candida* species isolated from patients with oral candidiasis.

   To achieve specific objective 2 associated with the phytocompounds carvacrol, cinnamaldehyde and thymol, **study 2,** titled **"*In vitro* activity of terpenes and other phytocompounds against *Candida*",** was performed. In the case of citral, **study 3,** titled **"*In vitro* and *in vivo* antifungal activity of citral"**, allowed the development of this specific objective.

3. To determine the *in vitro* synergistic activity of citral in combination with fluconazole against planktonic and sessile cells of *Candida* species.

4. To determine the *in vivo* synergistic activity of citral in combination with fluconazole as a treatment against *Candida* infection in a *C. elegans* animal model.

5. To evaluate the effect of citral in monotherapy and in combination with fluconazole on the expression of the *ERG11*, *CDR1* and *MDR1* genes associated with azole resistance mechanisms.

   Study 3, titled **"*In vitro* and *in vivo* antifungal activity of citral",** allowed the development of specific objectives 3, 4 and 5, in addition of objective 2.

6. To develop and characterize liposomal nanoparticles encapsulating carvacrol, cinnamaldehyde, citral and thymol and to evaluate their antifungal activity against planktonic *Candida* cells.
7. To evaluate the cytotoxicity of phytocompounds and corresponding nanoparticles in the murine macrophage cell line and the effect of these compounds and nanoparticles on the ability of macrophages to eliminate *Candida* cells.

   To achieve specific objectives 6 and 7, **study 4,** titled **"Antifungal activity of carvacrol, cinnamaldehyde, citral and thymol nanoparticles",** was performed.

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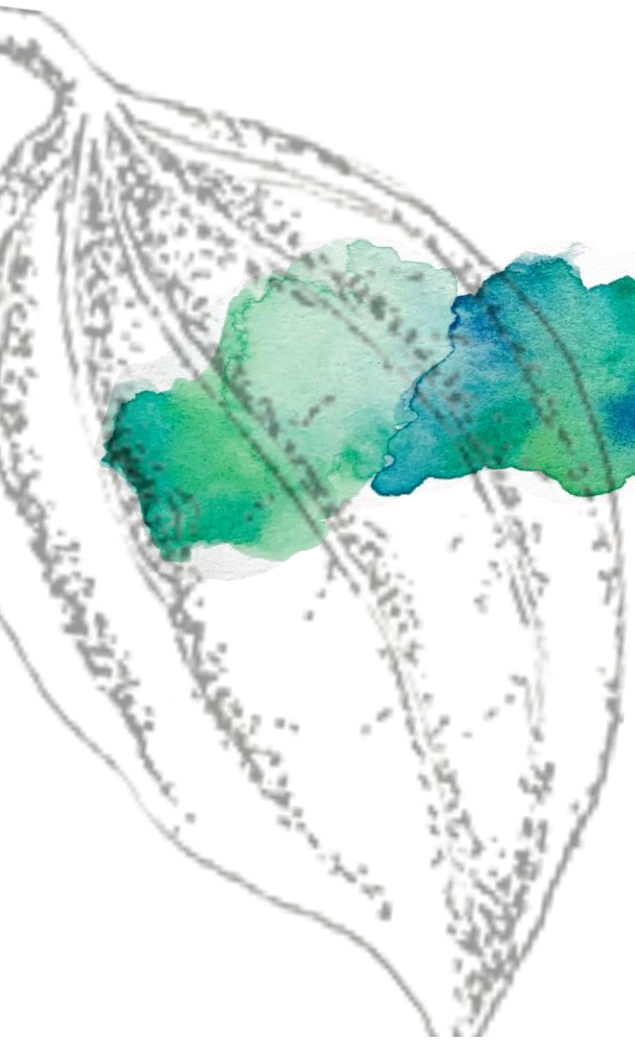


MATERIALS AND METHODS

3.1 Reference strains and clinical isolates

Candida reference strains, cell lines and model animals used in all studies included in this work are described in Table 3.1. *Candida* reference strains were obtained from the American Type Culture Collection (ATCC, USA), Centraalbureau voor Schimmelcultures (CBS, The Netherlands), National Collection of Yeast Cultures (NCYC, United Kingdom) or National Collection of Pathogenic Fungi (NCPF, United Kingdom). *C. elegans* AU37 and *E. coli* OP50 supplied by the *Caenorhabditis* Genetics Center (University of Minnesota, USA). Murine macrophage-like cell line RAW 264.7 from ATCC. The hypha-defective mutant *C. albicans* Ca2 was kindly donated by Professor Antonio Cassone, Istituto Superiore di Sanità, Rome, Italy).

All clinical isolates of *C. albicans*, *C. glabrata*, *C. dubliniensis*, *C. krusei*, *C. guilliermondii*, *C. orthopsilosis*, *C. parapsilosis*, *C. tropicalis* and *C. metapsilosis* were obtained from patients with clinically diagnosed oral candidiasis who were seeking treatment at the Dental Clinic Service of the Universidad del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU), Bilbao (Spain). The *C. auris* isolate used in the fourth study was isolated from a blood sample taken from a patient suffering candidaemia in a German hospital. In the first study, which was focused on the prevalence of oral candidiasis, a total of 211 unique isolates of *C. glabrata* and *C. parapsilosis* obtained between 2003 and 2013 were examined (patients' mean age was 59.3 years with a range of 18–87 years; 70.1% were women). In addition, in the other studies (investigations into the antifungal activity of carvacrol, cinnamaldehyde and thymol phytocompounds on *Candida* biofilms; anti-*Candida* activity after treatment with citral in monotherapy or in combination with fluconazole; and the antifungal activity of liposomes with all the phytocompounds studied), a specific selection of clinical isolates of the previously mentioned *Candida* species obtained between 2003 and 2018 were subjected to different assays. The clinical isolates are described in detail in Table 3.2.

All *Candida* isolates and reference strains were stored in vials with sterile distilled water at room temperature, and before assays and methodologies, they were cultured on Sabouraud dextrose agar medium (SDA) (Difco, USA) at 37 °C for 24 h.

Table 3.1. Microorganism/cell line/animal model reference strains.

Microorganism/cellular line /animal model and source	**Assay methodologies**	**Study**
Candida africana MYA 2669	PCR *C. albicans* complex	†
Candida albicans SC5314/ MYA-2876 from ATCC	EUCAST/ BP‡/ FICI/ TK/ MKA/ IV/ RTPCR	2,3,4
Candida albicans Ca2■	BP/ EUCAST	2,3
Candida albicans ATCC 64124	EUCAST/ FICI/ RTPCR	3
Candida albicans ATCC 90028	EUCAST/ BP/ PCR *C. albicans* complex	†,2,3
Candida bracarensis NCYC 3133	Multiplex PCR	1
Candida dubliniensis NCPP 3949	EUCAST/ BP/ PCR *C. albicans* complex	†,1,2,3
Candida glabrata ATCC 90030	Multiplex PCR/ EUCAST/ CLSI/ BP/ FICI	1,2,3
Candida krusei ATCC 6258	EUCAST/ CLSI/ BP/ FICI	1,2,3
Candida metapsilosis ATCC 96144	PCR-RFLP	1
Candida nivariensis CBS 9984	Multiplex PCR	1
Candida orthopsilosis ATCC 96141	PCR-RFLP	1
Candida parapsilosis ATCC 22019	PCR-RFLP/ BP/ EUCAST/ CLSI/ FICI	1,2,3
Caenorhabditis elegans AU37 (double mutant strain (*glp-4;sek-1*))	*In vivo* model, toxicity and management of infection	3
Escherichia coli OP50	Maintenance of *in vivo* model	3
Murine macrophage-like cell line RAW 264.7 from ATCC	Cytotoxicity/ MKA/ AAL	4

† identification of the *C. albicans* complex in all isolates belonging to the UPV/EHU collection; BP, biofilm production; BP‡, biofilm production and *in vitro* phytocompound susceptibility of sessile cells; EUCAST, *in vitro* susceptibility testing of phytocompounds and/or antifungal agents; TK, time-kill curves; MKA, macrophage killing assay; IV, *in vivo* infection model with survival assessment after treatment; and AAL, *in vitro* antifungal activity of liposomes based on EUCAST methodology; ■ hypha-defective mutant.

Table 3.2. Clinical isolates of *Candida* studied in this work.

***Candida* species isolates**	**Assay methodologies**	**Study**
Candida albicans UPV 05-007	BP‡/ EUCAST/ AAL	2,3,4
Candida albicans UPV 05-008	BP/ EUCAST	2,3
Candida albicans UPV 05-013	BP‡/ EUCAST/ AAL	2,3,4
Candida albicans UPV 06-116	BP/ EUCAST	2,3
Candida albicans UPV 11-342	BP‡/ EUCAST/ AAL	2,3,4
Candida albicans UPV 11-345	BP‡/ EUCAST/ AAL	2,3,4
Candida albicans UPV 12-298	BP‡/ EUCAST/ AAL	2,3,4
Candida albicans UPV 15-101	BP‡/ EUCAST/ AAL	2,3,4
Candida albicans UPV 15-106	BP‡/ EUCAST/ AAL	2,3,4
Candida albicans UPV 15-157	BP‡/ EUCAST/ FICI‡/ IV/ RTPCR/ TK/ AAL	2,3,4
Candida auris UPV 18-029	BP/ EUCAST/ AAL	4
Candida dubliniensis UPV 11-366	BP‡/ EUCAST/ FICI‡/ IV/ RTPCR/ TK/ AAL	2,3,4
Candida dubliniensis UPV 12-064	BP/ EUCAST	2,3
Candida dubliniensis UPV 12-090	BP/ EUCAST	2,3
Candida glabrata UPV 05-048	BP/ EUCAST	2,3
Candida glabrata UPV 05-068	BP/ EUCAST	2,3
Candida glabrata UPV 05-022	BP/ EUCAST	2,3
Candida glabrata UPV 06-024	BP/ EUCAST	2,3
Candida glabrata UPV 08-058	BP/ EUCAST/ FICI	2,3
Candida glabrata UPV 13-164	BP/ EUCAST	2,3
Candida glabrata UPV 13-184	BP/ EUCAST	2,3
Candida glabrata UPV 13-185	BP/ EUCAST	2,3
Candida glabrata UPV 13-200	BP/ EUCAST	2,3
Candida glabrata UPV 14-004	BP/ EUCAST/ FICI	2,3
Candida krusei UPV 03-242	BP/ EUCAST/ FICI	2,3
Candida krusei UPV 05-054	BP/ EUCAST	2,3
Candida krusei UPV 13-120	BP/ EUCAST/ FICI	2,3

Table 3.2. Clinical isolates of *Candida* studied in this work, continuation.

***Candida* species isolates**	**Assayed methodologies**	**Study**
Candida guilliermondii UPV 05-059	BP/ EUCAST	2,3
Candida guilliermondii UPV 05-078	BP/ EUCAST	2,3
Candida orthopsilosis UPV 12-056	BP/ EUCAST	2,3
Candida orthopsilosis UPV 12-057	BP/ EUCAST	2,3
Candida parapsilosis UPV 07-008	BP/ EUCAST	2,3
Candida parapsilosis UPV 12-296	BP/ EUCAST	2,3
Candida metapsilosis UPV 11-449	BP/ EUCAST	2,3
Candida tropicalis UPV 05-016	BP/ EUCAST/ FICI	2,3
Candida tropicalis UPV 06-115	BP/ EUCAST/ FICI/ BP‡/ AAL	2,3,4

AAL, *in vitro* antifungal activity of liposomes based on EUCAST methodology; BP, biofilm production; BP‡, biofilm production and *in vitro* phytocompounds susceptibility of sessile cells; EUCAST, *in vitro* susceptibility testing of phytocompounds and/or antifungal agents; TK, time-kill curves; IV, *in vivo* Infection model with survival analysis after treatment; FICI, checkerboard assay of planktonic cells; FICI‡, checkerboard assay against sessile cells.

3.2 Methods of *Candida* identification

Candida isolates were isolated using molecular and conventional methods. In the conventional methods of *Candida* identification, all *Candida* strains were isolated and seeded onto Candida Chromogenic Agar (Laboratorios Conda, Spain) and CHROMID Candida medium (BioMérieux, France) containing chromogenic substrates such as β-glucosaminidase, which allows species to be identified according to the colour developed by the colonies (Table 3.3). Other techniques, such as serum germ tube production, were also applied to the assays (Mackenzie, 1962). The presence of chlamydospores was determined on the basis of their morphology in an experiment in which samples were seeded on corn meal agar supplemented with Tween 80 according to Dalmau's technique and observed by microscopy (McGinnis et al., 1984).

Table 3.3. *Candida* species and the colour of the colonies produced on chromogenic media

***Candida* species**	**CHROMID Candida**	**Candida Chromogenic Agar**
Candida albicans	Cobalt blue	Green
Candida dubliniensis	Turquoise blue	Green
Candida glabrata	White	Violet
Candida guilliermondii	Pink	Pink
Candida krusei	Matt white	Matt pink
Candida parapsilosis	White	White
Candida tropicalis	Pink-violet	Bluish purple

The API ID 32C kit (BioMérieux) contains the dehydrated substrates sorbitol, D-xylose, ribose, glycerol, rhamnose, palatine, erythritol, melibiose, glucuronate, melezitose, gluconate levulinate, glucose, sorbose, glucosamine, esculin, galactose, actidione, sucrose, N-acetylglucosamine, DL-lactate, L-arabinose, cellobiose, raffinose, maltose, trehalose, 2-ketogluconate α-methyl-D-glucoside, mannitol, lactose and inositol. Through this test kit, yeasts are identified according to the patterns of carbon source assimilation (Eraso et al., 2006; San-Millán et al., 1996) (Figure 3.1).

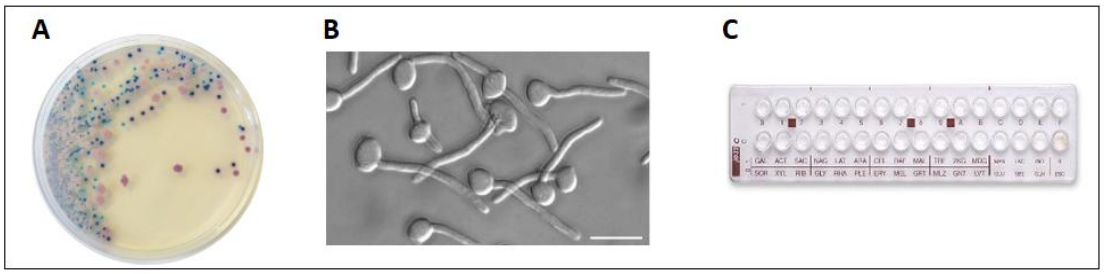


Figure 3.1. Conventional methods of *Candida* species identification. A: Chromogenic medium, B: Germ tube test at the 10 µm scale (Sudbery et al., 2004) and C: API ID32C commercial kit for the assimilation of carbon sources.

3.2.1 Molecular methods used for *Candida* species identification

The use of molecular methods allows the differentiation of closely related species to *C. albicans, C. glabrata* and *C. parapsilosis*. Isolates and reference strains were grown on SDA to obtain DNA.

3.2.1.1 Identification of *C. albicans* by PCR

C. albicans, *C. dubliniensis* and *C. africana* were identified by PCR reaction using primers CR-f and Cr-r, targeting *HWP1* gene (Romeo & Criseo, 2008) (Table 3.4). Conditions of PCR reaction were, a first step of denaturation at 95 °C for 5 min, 30 cycles of denaturation at 94 °C for 45 s, primer annealing at 58 °C for 40 s, and extension at 72 °C for 55 s, followed by a final extension at 72 °C for 10 min using a BioRad C1000™ Thermal Cycler (Bio-Rad, USA). DNA amplified products and weight marker, HyperLadder™ 100bp (Bioline, UK), were separated on a 1.3% agarose gel (Laboratorios Conda) stained with GelRed (Biotium, USA) for 160 min at 70 V, using horizontal electrophoresis cell Sub-cell GT (Bio-Rad). ChemiDoc™ (Bio-Rad) was used for visualizing the expected DNA fragments of 941, 569 and 700 bp corresponding to *C. albicans*, *C. dubliniensis* and *C. africana*, respectively (Table 3.4).

3.2.1.2 Identification of the *C. glabrata* complex by multiplex PCR

C. glabrata, C. bracarensis and *C. nivariensis*, which are phenotypically related species belonging to the *C. glabrata* complex, were identified by multiplex polymerase chain reaction (multiplex PCR) using primers GLA-f, NIV-f, BRA-f and UNI-5.8S targeting the internal transcribed spacer (ITS1) region and 5.8S rRNA gene (Romeo et al., 2009) (Table 3.4). The master mix was prepared with BioMix™ Red (Bioline) and 0.42 μM UNI-5.8S-reverse primer and each of the other three primers (GLA-f, NIV-f, and BRA-f) at a concentration of 0.21 μM. A Bio-Rad C1000™ thermal cycler was used to perform PCR, which was

programmed with a denaturation step at 95 °C for 5 min, followed by 34 cycles of 30 sec at 94 °C, annealing for 40 sec at 60 °C, elongation for 50 sec at 72 °C, and a final 10 min extension step at 72 °C. The PCR products and a weight marker, HyperLadder™ 100 bp, were separated by electrophoresis on a 2% agarose gel stained with GelRed for 180 min at 50 V in a horizontal electrophoresis Sub-Cell GT system. Visualization of the gel was performed with ChemiDoc™ image analysis equipment. DNA fragments of 397, 293 and 223 bp corresponded to *C. glabrata*, *C. nivariensis* and *C. bracarensis*, respectively, were expected (Table 3.4) (Figure 3.2 A).

3.2.1.3 Identification of *C. parapsilosis* complex by PCR-RFPL

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was the molecular technique used for the identification of *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis* species. Primers S1-F forward and S1-F reverse corresponding to a region of the secondary alcohol dehydrogenase (*SADH*) gene were used (Miranda-Zapico et al., 2011; Tavanti et al., 2005) (Table 3.4). Master mixes containing BioMix™ Red and 0.4 μM primer (forward and reverse) were subjected to PCR amplification carried out with a Bio-Rad C1000™ Thermal Cycler. The amplification was initiated with a denaturation step at 95 °C for 5 min, followed by 40 cycles of 1 min at 92 °C, 1 min at 45 °C and 1 min at 68 °C and a final extension step of 7 min at 68 °C. The amplified fragments were digested with 0.8 μl (16 units) of restriction enzyme BanI (New England Biolabs, USA) for each 25 μl of reaction mixture for 2 h at 37 °C. The DNA fragments obtained and the weight marker HyperLadder™ 100 bp were separated by electrophoresis on a GelRed-stained 1.5% agarose gel for 70 min at 90 V, and visualization was performed using ChemiDoc™ image analysis equipment. DNA fragments of 521 and 196 bp; 370, 188, 93 and 60 bp; and 716 bp corresponding to *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis* species, respectively, were expected (Table 3.4) (Figure 3.2 B).

Table 3.4. Molecular methodologies used for identifying species of *Candida* complexes.

***Candida* complex**	**Method**	**Gene**	**Primers**	**Species/size products: bp**	**Ref**
C. albicans	PCR	*HWP1* gene	**CR-Forward** 5′-GCTACCACTTCAGAATCATCATC-3′ **CR-Reverse** 5’-GCACCTTCAGTCGTAGAGACG-3′	*C. albicans*: 941 *C. africana*: 700 *C. dubliniensis:* 569	(Romeo & Criseo, 2008)
C. glabrata	Multiplex PCR	5.8S rRNA gene and ITS1 region	**GLA-Forward** 5'-CGGTTGGTGGGTGTTCTGC-3' **BRA- Forward** 5'-GGGACGGTAAGTCTCCCG-3' **NIV- Forward** 5'-AGGGAGGAGTTTGTATCTTTCAAC-3' **UNI-5.8S- Reverse** 5'-ACCAGAGGGCGCAATGTG-3'	*C. glabrata*: 397 *C. bracarensis*: 293 *C. nivariensis*: 223	(Romeo et al., 2009)
C. parapsilosis	PCR-RFLP	*SADH* gene	**S1- Forward** 5'-GTTGATGCTGTTGGATTGT-3' **S1- Reverse** 5'-CAATGCCAAATCTCCCAA-3'	*C. parapsilosis*: 521 and 196 *C. metapsilosis*: 370, 188, 93 and 60 *C. orthopsilosis*: 716	(Miranda-Zapico et al., 2011; Tavanti et al., 2005)

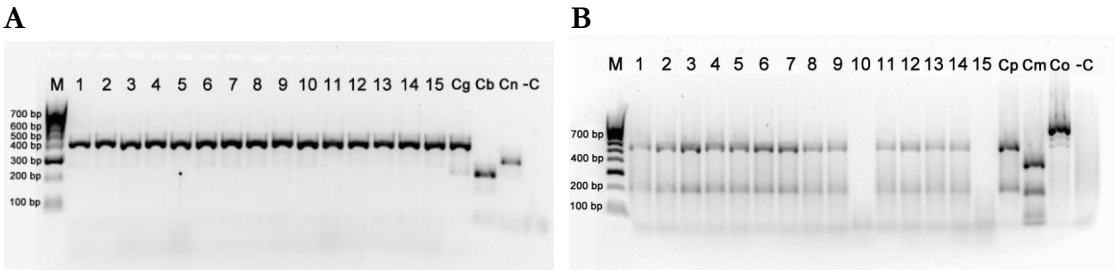


Figure 3.2 A: Agarose gel separation of isolates from the *C. glabrata* complex identified by multiplex PCR. B: Agarose gel separation of the *C. parapsilosis* complex isolates identified by PCR-RFLP. C.g, *C. glabrata*; C.b, *C. bracarensis*; C.n, *C. nivariensis*; C.p, *C. parapsilosis*; C.m, *C. metapsilosis*; C.o, *C. orthopsilosis*; M, marker HyperLadder™ 100 bp; and NC, negative control. Each number refers to a clinical isolate processed by each corresponding technique.

3.3 Phytocompounds and antifungal agents

Tablets of itraconazole, fluconazole, nystatin and miconazole were used in a disk diffusion test. The phytocompounds carvacrol, cinnamaldehyde, citral and thymol and the antifungal agents, anidulafungin, fluconazole and isavuconazole, all with purity greater than 90%, were tested. The techniques used included *in vitro* microdilution susceptibility testing, checkerboard assay, time-kill curves, a liposome production assay, *in vivo* toxicity measures and analysis of the treatment effects on *in vivo* infection. Eugenol, geraniol, linalool and terpinen-4-ol were assessed using *in vitro* microdilution susceptibility testing (Tables 3.5 and 3.6).

Stock solutions were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA); phytocompounds were prepared on the basis of their density in the case of liquid phytocompounds on the same day that the susceptibility testing was performed, and stock solutions of antifungal agents were stored at –70 °C for no more than one month before their use.

Table 3.5. Antifungal agents and phytocompounds.

Antifungal agent	Appearance	Purity/density	Manufacturer
Itraconazole	Tablets 10 µg	-	Rosco Diagnostica-NeoSensitabs, Denmark
Fluconazole	Tablets 25 µg	-	
Nystatin	Tablets 50 µg	-	
Miconazole	Tablets 10 µg	-	
Anidulafungin	Powder	82.4%	Pfizer, Spain
Fluconazole	Powder	98%	Sigma-Aldrich, USA
Isavuconazole	Powder	99.1%	Basilea Pharmaceutica,Switzerland
Carvacrol	Liquid	98%/0.976 g/mL	Sigma-Aldrich, USA
Cinnamaldehyde	Liquid	95%/1.05 g/mL	Sigma-Aldrich, USA
Citral	Liquid	95%/0.888 g/mL	Sigma-Aldrich, USA
Eugenol	Liquid	98%/1.067 g/mL	Sigma-Aldrich, USA
Geraniol	Liquid	98%/0.879 g/mL	Sigma-Aldrich, USA
Linalool	Liquid	97%/0.87 g/mL	Sigma-Aldrich, USA
Terpinen-4-ol	Liquid	95%/0.934 g/mL	Sigma-Aldrich, USA

Table 3.6. Description of assay methodologies, antifungal agents and phytocompounds.

Antifungal agent/ phytocompound/ nanoparticle	***Candida* planktonic cells**			***Candida* sessile cells**			**Murine macrophage line**		***In vivo* model**	***C. elegans***
	EUCAST	FICI	Time -kill	Early biofilm	Mature biofilm	Biofilm FICI	Cytotoxicity	M Killing assay	Toxicity	Treatment infection
Anidulafungin	✓			✓	✓					
Fluconazole	✓			✓	✓				✓	✓
Isavuconazole	✓			✓	✓					
Carvacrol	✓			✓	✓		✓	✓		
Cinnamaldehyde	✓			✓	✓		✓			
Citral	✓	✓	✓	✓	✓	✓	✓		✓	✓
Eugenol	✓									
Geraniol	✓									
Linalool	✓									
Terpinen-4-ol	✓									
Thymol	✓						✓	✓		
NPCarvacrol	✓						✓	✓		
NPCinnamaldehyde	✓						✓			
NPCitral	✓						✓			
NPThymol	✓						✓	✓		

NP, nanoparticle consisting of monoolein-based liposome; M Killing assay, macrophage killing assay; FICI, checkerboard assay; Treatment infection, worms with *Candida* infection treated with one agent in monotherapy or in combination (citral and fluconazole); ✓, done.

3.4 *In vitro* antifungal susceptibility testing against planktonic cells

3.4.1 The disk diffusion method

A total of 114 isolates of *C. glabrata*, 93 isolates of *C. parapsilosis*, three isolates of *C. orthopsilosis*, and one isolate of *C. metapsilosis* were evaluated by disk diffusion using tablets containing 25 µg of fluconazole, 10 µg of itraconazole, 10 µg of miconazole or 50 µg of nystatin nystatin (Rosco Diagnostica—Neo-Sensitabs). The protocol was a modified version of the CLSI M44-A2 guidelines (Clinical and Laboratory Standards Institute (CLSI), 2009; Rementeria et al., 2007) (Figure 3.3)

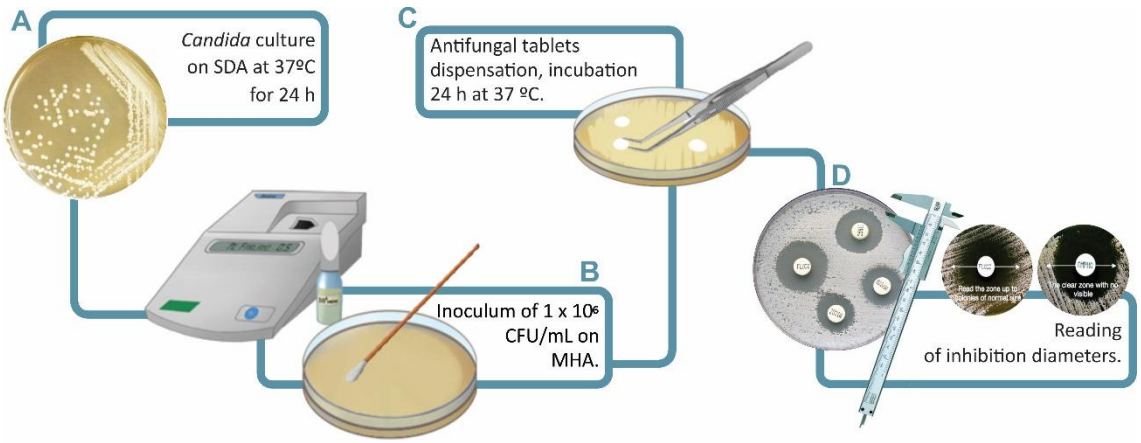


Figure 3.3. Scheme of disk diffusion procedure. A: Inoculum preparation from yeast cultured on SDA. B: Suspensions adjusted to be equivalent to the 0.5 McFarland standard were spread on MHA. C: Dispensation of antifungal agent tablets on MHA and incubated for 24 h at 37 °C. D: Inhibition diameters.

First, a yeast cell suspension in sterile saline equivalent to a 0.5 McFarland standard (approximately 1–5 $\times 10^6$ colony-forming units (CFU)/mL) was prepared from isolated colonies of each isolate previously cultured on SDA at 37 °C for 24 h. On sterile swabs,

each inoculum was spread in all directions onto Mueller-Hinton agar medium (Difco) supplemented with 2% (w/v) glucose and 0.5 µg/L methylene blue (Table 3.7). After 3 to 5 min of drying, antifungal tablets were carefully dispensed onto the plate surface and incubated for 24 h at 37 °C. Then, inhibition zone diameter guide points were measured in millimetres using a calliper, and the technical criteria such as the presence of microcolonies on the edge of the inhibition halo or large colonies on the inside the halo were ignored. In cases of no visible yeast growth, the incubation time was extended by an additional 24 h. After the results were interpreted, classification of the clinical isolates in terms of their respective susceptibilities to the antifungal agents was established according to the criteria published by the corresponding agent manufacturer (Table 3.8).

Table 3.7. Preparation of Mueller-Hinton agar (MHA) supplemented with 2% (w/v) glucose and 0.5 µg/L methylene blue.

Glucose stock solution (40%)	
➢ Glucose	40 g
➢ Distilled water	100 mL
Methylene blue solution (5 mg/mL)	
➢ Methylene blue	0.1 g
➢ Distilled water	20 mL
Glucose-methylene blue stock solution (GAM) with 4 mg/mL glucose and 10 µg/mL methylene blue	
➢ Methylene blue solution stock	200 µL
➢ Glucose stock solution	100 mL
GAM was sterilized in an autoclave GAM was added to MHA plates The solution was dispensed and spread with 1.5 mL of GAM solution over the surface of 9-10 cm MHA plates and maintained at room temperature until the liquid was completely absorbed	

In addition, quality control using *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 reference strains was performed, and the results were verified on the basis of the expected susceptibilities (Table 3.8).

Table 3.8. Criteria for the susceptibility interpretation of inhibition diameters-disk diffusion tests, based on neosensitabs protocol.

Antifungal agent		Zone diameter (mm)				Quality controls	
		S	I	S-DD	R	*C. krusei* ATCC 6258	*C. parapsilosis* ATCC 22019
Itraconazole	Tablets 10 µg	≥23		14 - 22	≤13	16 – 22	19 - 26
Fluconazole	Tablets 25 µg	≥19		15 - 18	≤14	-	22 - 33
Nystatin	Tablets 50 µg	≥15	10 - 14		No zone	18 - 24	22 - 28
Miconazole	Tablets 10 µg	≥20	12 - 19		≤11	11 - 18	13 - 20

S, susceptible; I, intermediate; S-DD, susceptible-dose dependent; R, resistant.

3.4.2 *In vitro* microdilution susceptibility testing using the CLSI method

In addition to the disk diffusion test, a total of 114 isolates of *C. glabrata*, 93 isolates of *C. parapsilosis*, three isolates of *C. orthopsilosis*, and one isolate of *C. metapsilosis* were evaluated by microdilution antifungal susceptibility testing to confirm susceptibility to fluconazole with the final concentration ranging from 0.125 to 64 µg/mL. The microdilution antifungal susceptibility was determined following the methodology described in document M27-A3 of the CLSI (CLSI 2008).

Microplates were prepared from a stock solution of fluconazole (3200 μg/mL) (Sigma-Aldrich) prepared in pure water after serial twofold dilutions of the antifungal agent. Subsequently, the content of each tube was diluted in RPMI-1640 medium buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (MOPS) at an intermediate concentration ranging from 0.25 to 128 μg/mL (Table 3.9).

Table 3.9. Preparation of RPMI-1640 medium buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (MOPS) (RPMI).

RPMI-1640 medium buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (MOPS) (RPMI)	
➢ RPMI-1640 with L-glutamine and no bicarbonate (Sigma- Aldrich)	10.4 g
➢ MOPS (Sigma- Aldrich)	34.54 g
Distilled water was added for establishing a total volume of 1 L and adjusted to pH 7.0 Sterilized by filtration and stored at 4 °C	

A 100-μL aliquot of each of these solutions was added to each well in a row of 96-well microplates with concave bottoms (U-bottom), with the 10th column having the lowest concentration, and solution of continuously higher concentration was added to the subsequent columns. In the final two columns, 100 μL of RPMI without antifungal agent was added as a fungal growth control and as an environmental sterility control (columns 11 and 12) respectively. The prepared microplates were labelled and stored at -70 °C for up to six months.

An inoculum adjusted to a final concentration of $1–5\times10^3$ CFU/mL in RPMI medium by a 1:1000 dilution was made from a yeast suspension equivalent to the 0.5 McFarland standard in tubes with 0.85% saline. Inoculation of the microplates was performed with

100 µL of adjusted inoculum in each well of columns containing the drugs and the growth control column to obtain the final concentration of fluconazole to be studied and the final inoculum density (0.125 to 64 µg/mL and 0.5-2.5 $\times 10^3$ CFU/mL, respectively). The plates were then incubated for 24 and 48 h at 37 °C. In addition, quality controls, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 reference strains, were included and verified according to the expected results of their respective susceptibility to fluconazole. Visual assessments were performed at 24 and 48 h, and the growth in the different wells was compared with the growth in the control columns.

The fluconazole MIC was determined to be the lowest concentration that causes ≥ 50% inhibition of growth (MIC2) after 24 h of growth compared to the growth without exposure to an antifungal drug. The clinical isolates were characterized in terms of their susceptibilities on the basis of clinical breakpoints (CBPs), which are often used to identify the clinical isolates that can respond to treatment with a given antimicrobial agent administered using the approved dosing regimen for the specific drug (Pfaller & Diekema, 2012). The CBP recommended in the document M27-S4 supplement of the CLSI (Clinical and Laboratory Standards Institute (CLSI), 2012) was used.

Moreover, epidemiological cut-off values (ECVs), which can be the most sensitive measure of emerging strains with decreased susceptibility to a given agent, were also assessed to categorize wild-type (WT, strains that show no mutations or acquired resistance mechanisms) and non-wild-type isolates (NWT, strains that have mutations or have acquired resistance mechanisms) because the resistance of oral *Candida* isolates to fluconazole has not been characterized (Pfaller & Diekema, 2012) (Table 3.10).

3.4.3 *In vitro* microdilution susceptibility testing using EUCAST method

Thirty five *Candida* isolates and references strains were used for assessing their *in vitro* antifungal susceptibilities to carvacrol, cinnamaldehyde, citral and thymol and the antifungal agents anidulafungin, fluconazole and isavuconazole, according to the methodology proposed by the EUCAST final documents EDef 7.2 and EDef 7.3.2 (Arendrup et al., 2020b, 2012) (Tables 3.1 and 3.2). Final concentrations of anidulafungin ranging from 0.016 to 8 mg/L, of fluconazole from 0.12 to 64 mg/L and of isavuconazole from 0.016 to 8 mg/L were used. Susceptibility to carvacrol, cinnamaldehyde, citral, eugenol, geraniol, linalool, terpinen-4-ol and thymol was assayed at concentrations ranging from 2 to 1024 mg/L.

Table 3.10. Clinical breakpoints (CBPs) and epidemiological cut-off values (ECVs) of fluconazole of *C. glabrata*, *C. parapsilosis* and quality control based on the CLSI method.

Species	CBP (µg/mL)			ECV (µg/mL)		Quality controls (MIC µg/mL)	
	S	S-DD	R	WT	NWT	*C. krusei* ATCC 6258	*C. parapsilosis* ATCC 22019
						8 – 64	0.5 – 4
C. glabrata		≤ 32	≥ 64	≤32	>32		
C. parapsilosis	≤ 2	4	≥ 8	≤2	>2		

CBP, clinical breakpoints; ECV, epidemiological cut-off values; WT, wild type; NMT, non-wild type; S, susceptible; S-DD, susceptible-dose dependent; R, resistant.

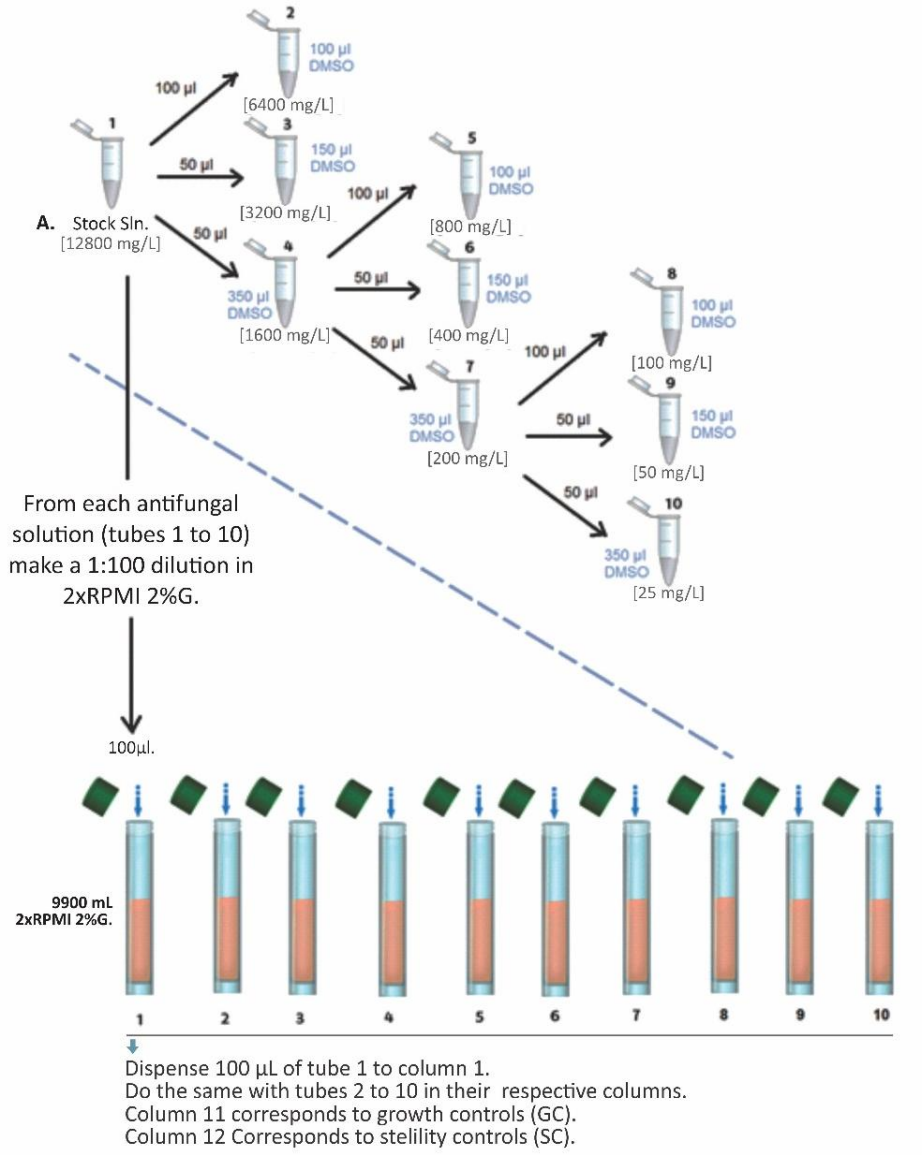
Stock solutions of anidulafungin, fluconazole, isavuconazole were 1600 mg/L, 12800 mg/L, 1600 mg/L, respectively; meanwhile, stock solutions of carvacrol, cinnamaldehyde, citral, eugenol, geraniol, linalool, terpinen-4-ol and thymol were 204800 mg/L. Both drugs and phytocompounds were dissolved in DMSO. Serial two-fold dilutions of the antifungals and the phytochemicals were prepared in double strength RPMI 1640 (with L-glutamine

and without bicarbonate) supplemented with 2% glucose (2×RPMI 2% G) and buffered to pH 7.0 with MOPS (Table 3.11). These solutions were dispensed into flat bottomed 96-well plates, 100 μL of each concentration, in each well of a column starting with column 10 in order from the lowest to the highest concentration (at a double concentration to be evaluated). In the final two columns, 100 μl of 2×RPMI 2% G with 2% DMSO and without antifungal agent were added as fungal growth control and as environmental sterility control (columns 11 and 12, respectively) (Figure 3.4). The antifungal microplates were sealed in aluminium foil and stored frozen at -70 ºC for six months.

Table 3.11. Preparation of 2×RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (MOPS) supplemented with 2% (w/v) glucose (2×RPMI 2% G).

2×RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (MOPS) supplemented with 2% (w/v) glucose (2×RPMI 2% G).	
➢ RPMI-1640 with L-glutamine and no bicarbonate (Sigma- Aldrich)	20.8 g
➢ MOPS (Sigma- Aldrich)	69.06 g
➢ Glucose	36 g
Distilled water was added for establishing a total volume of 1 L and adjusted to pH 7.0 The medium was sterilized by filtration and stored at 4 °C	

Clinical isolates were previously grown on SDA plates at 37 °C for 24 h, and cell suspensions equivalent to the 0.5 McFarland (1-5 × 10^6 CFU/mL) were prepared with sterile water. Later, an isolate solution diluted at 1:10 with sterile water was prepared from the cell suspensions, and a 100-μl aliquot was dispensed onto previously prepared microplates to obtain a final inoculum concentration between 0.5 × 10^5 and 2.5 × 10^5 CFU/mL. Sterility control and growth control wells were included in each microplate.



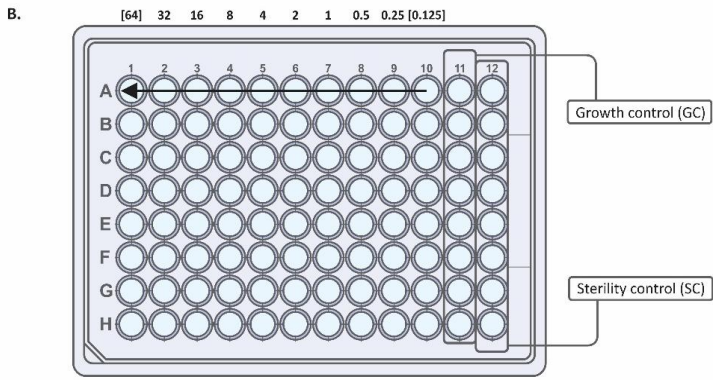


Figure 3.4. Scheme of the preparation of ten microplates with fluconazole using the EUCAST method. A: Preparation of serial twofold dilutions of antifungal agents in 2×RPMI with 2% G medium. B: Addition of antifungal solution onto the microplate. In the case of phytocompounds, the procedure was the same with an initial stock solution of 204800 mg/L.

The absorbance of each microplate was measured at a 450-nm wavelength with an iMark reader (Bio-Rad) after 24 and 48 h of incubation at 37 °C. The value of the blank was subtracted from the values for the other wells in the microplate. Absorbance values equal to or less than 0.2 after 48 h were considered indicators of a failed test. Phytocompounds were analysed in triplicate in at least three separate experiments.

The minimum inhibitory concentrations of anidulafungin, fluconazole and isavuconazole, as well as the inhibitory concentrations of carvacrol, cinnamaldehyde, citral and thymol, were calculated at 24 h after incubation on the basis of the lowest drug concentration inhibiting ≥ 50% of growth compared to the growth of the control without antifungal drugs or phytocompounds (Van Dijck et al., 2018). Additionally, the *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 reference strains were included as quality controls and verified according to their expected susceptibility to antifungal agents (Table 3.12).

Table 3.12. Expected results of the quality controls in term of the MIC for anidulafungin (ANI), fluconazole (FLC) and isavuconazole (ISA) according to the EUCAST method.

Quality controls	MIC (mg/L)		
	ANI	FLC	ISA
Candida krusei ATCC 6258	0.016-0.06	16-64	0.016-0.06
Candida parapsilosis ATCC 22019	0.25-1	0.5-2	0.008-0.03

Clinical isolates were classified as susceptible, susceptible-dose dependent and resistant using the species-specific MIC breakpoints defined by EUCAST for anidulafungin and azoles in the Clinical breakpoint version 10.0 document (European Committee for Antimicrobial Susceptibility Testing (EUCAST), 2020) (Table 3.13).

Table 3.13. EUCAST Clinical breakpoints (CBPs) and tentative epidemiological cut-offs (T-ECOFFs) of anidulafungin and fluconazole.

Species	Antifungal drug	CBPs (mg/L)		T-ECOFF (mg/L)	
		Susceptible	Resistant	Wild-type	Non wild-type
C. albicans	Anidulafungin	≤0.03	>0.03	≤0.03	>0.03
	Fluconazole	≤2	>4	≤0.5	>0.5
C. dubliniensis	Anidulafungin	-	-	-	-
	Fluconazole	≤2	>4	≤0.5	>0.5
C. glabrata	Anidulafungin	≤0.06	>0.06	≤0.06	>0.06
	Fluconazole	≤0.001	16	≤16	>16
C. guilliermondii	Anidulafungin	IE	IE	-	-
	Fluconazole	IE	IE	≤16	>16
C. krusei	Anidulafungin	≤0.06	>0.06	0.06	0.06
	Fluconazole	-	-	≤128	>128
C. parapsilosis	Anidulafungin	≤4	>4	≤4	>4
	Fluconazole	≤2	>4	≤2	>2
C. tropicalis	Anidulafungin	≤0.06	>0.06	0.06	0.06
	Fluconazole	≤2	>4	≤1	>1

3.4.4 Checkerboard assay

The antifungal activity of citral in combination with fluconazole against planktonic cells of one azole-susceptible *C. tropicalis* UPV 05-016 and six oral fluconazole-resistant isolates of *Candida*, including *C. albicans* UPV 15-157, *C. dubliniensis* UPV 11-366, *C. glabrata* UPV 08-058 and UPV 14-004, and *C. krusei* UPV 13-120 and UPV 03-242 isolates, was tested. The reference strains *C. albicans* ATCC 64124, *C. albicans* SC5314, *C. krusei* ATCC 6258 and

C. parapsilosis ATCC 22019 were also subjected to the test. The selection of clinical *Candida* isolates was carried out considering their susceptibility to fluconazole previously tested through the EUCAST method. The checkerboard assay was performed according to the final EUCAST final document EDef 7.3.2 for yeasts with some modifications (Arendrup et al., 2020b; Meletiadis et al., 2010).

The checkerboard was prepared in microtitre plates (flat bottomed 96-well plates) for multiple combinations of twofold serial dilutions of citral and fluconazole. The final concentrations ranged from 2 to 1024 mg/L and from 0.125 to 64 mg/L for citral and fluconazole, respectively.

Stock solutions of citral (409600 mg/L) and fluconazole (25600 mg/L) were prepared with DMSO, and serial twofold dilutions were obtained in double strength RPMI-1640 supplemented with 2% glucose (2×RPMI 2% G) and buffered to pH 7.0 with MOPS as described in Figure 3.4 A. From these fluconazole dilutions, 50-µL aliquots was dispensed from row G to row A (from the lowest to highest concentration) from column 1 to column 11. In the case of citral dilutions, 50-µL aliquots were dispensed from column 11 to column 2 (from lower to higher concentration) from well A to well H. In the case of the growth control (column 12) and sterility control (the H1 and H12 wells), 100 µL of 2×RPMI 2% G with 1% DMSO without antifungal agent was added (Figure 3.5).

Cell suspensions of *Candida* isolates were prepared with turbidity of the 0.5 McFarland standard (1-5 × 10^6 CFU/mL) in sterile water, from which a 1:10 dilution was prepared with sterile water. The inoculum was dispensed onto a previously prepared microplate, with one isolate per plate; thus, 100-µL aliquots were dispensed onto all wells of the microplate, except in wells H1 and H12, to reach a final inoculum of between 0.5 × 10^5 and 2.5 × 10^5 CFU/mL.

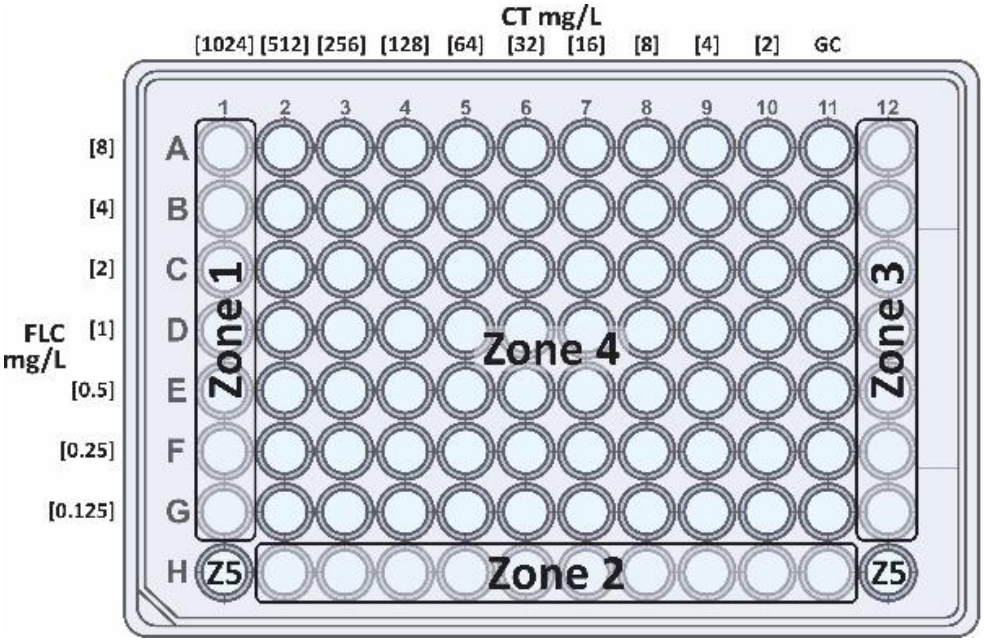
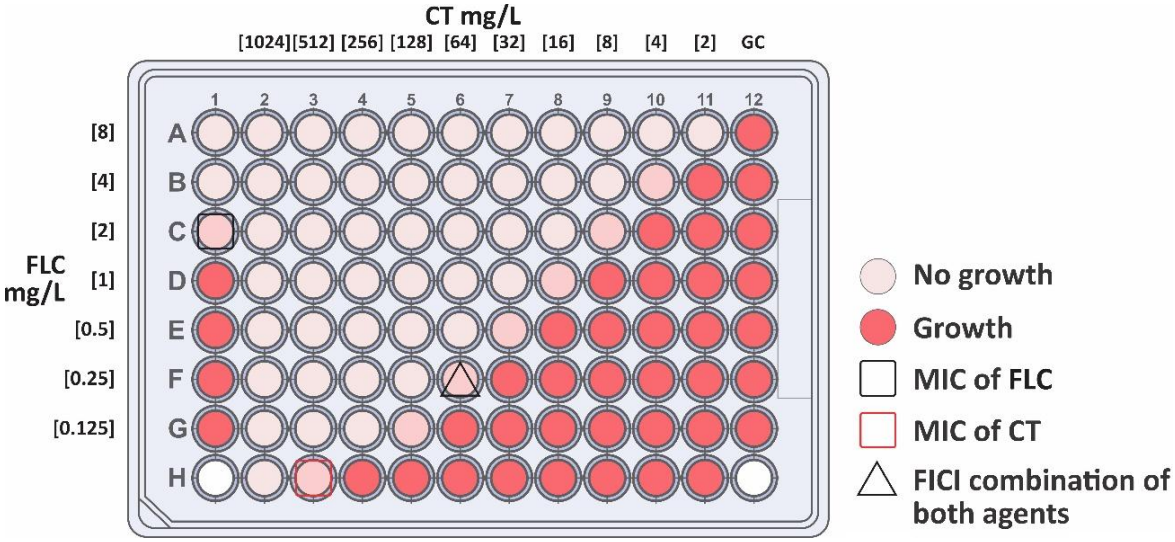


Figure 3.5. Scheme of citral (CT) and fluconazole (FLC) distribution onto microplates used for the checkerboard assay. Zone 1, where only fluconazole was dispensed in rows. Zone 2, where only citral was dispensed in columns. Zone 3, growth control. Zone 4, combinations of fluconazole and citral. Zone 5, the sterility control.

The IC and MIC were determined by optical density reading at 450-nm wavelength. The fractional inhibitory concentration index (FICI) was obtained because it represents the sum of the FICs of each drug tested, and the FIC is determined for each drug by dividing the MIC (or IC in case of a phytocompound) of each drug used in combination by the MIC (or IC in case of a phytocompound) of each drug used alone (Meletiadis et al., 2010) (Figure 3.6).

The *in vitro* interaction of the antifungal combination was interpreted as follows: FICI ≤ 0.5, synergistic; FICI > 0.5 but ≤ 4, indifferent/additive; and FICI > 4, antagonistic effect (Van Dijck et al., 2018).



$$\text{FICI} = \frac{\text{MIC FLC in combination}}{\text{MIC FLC alone}} + \frac{\text{IC CT in combination}}{\text{IC CT alone}}$$

Figure 3.6. Scheme of interpretation of checkerboard and formula for obtaining the FICI.

3.5 Biofilm production assay

Biofilm production by 35 oral isolates and reference strains previously described was assessed using the method described by Ramage et al. (2001) (Tables 3.1 and 3.2).

Each isolate and reference strain obtained from a colony grown on SDA at 30 °C for 72 h were cultured overnight at 30 °C in an orbital shaker on 5 ml of yeast peptone dextrose medium. Cells were centrifuged for 10 min at 2500 rpm and washed three times in 3 mL of sterile phosphate-buffered saline (PBS). An adjusted cellular density of 1.0×10^6 cells/mL in RPMI-1640 supplemented with L-glutamine and buffered to pH 7 with 0.165 M MOPS was established by cell recounting with a Bürker chamber (Table 3.14). *Candida* biofilms were developed on sterilized, flat-bottomed honeycomb 100-well polystyrene microtitre plates (Labsystems, Finland) by adding 100 µL of the adjusted standard cell suspension into

each well; five repetitions per strain were prepared. Two identical microtitre plates were prepared, and one plate was used to determine metabolic activity and the other was used for biomass determination. A sterility control consisting of RPMI medium without inoculum was used. In addition, *C. albicans* SC5314 and the hypha-defective mutant *C. albicans* Ca2 were placed in each microtitre plate as positive and negative controls of biofilm production, respectively.

The microtitre plates were incubated at 37 °C in a computer-controlled incubator (BioScreen C MBR, Growth Curves Ltd, Finland). After 24 and 48 h, non-adherent and loosely adherent cells were removed by washing three times with 100 μL of sterile PBS.

Table 3.14. Preparation of Yeast peptone dextrose (YPD) medium.

Yeast peptone dextrose (YPD) medium	
➢ Yeast extract	10 g ≈ 1% w/v
➢ Peptone	20 g ≈ 2% w/v
➢ Dextrose	20 g ≈ 2% w/v
Distilled water was added for establishing a total volume of 1 L Autoclave at 121 °C during 15 min	

3.5.1 Metabolic activity determination assay

The metabolic activity of the biofilm was determined using the colorimetric method described by Ramage et al. (2001) using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT, Sigma-Aldrich) (Table 3.15).

Table 3.15. Preparation of the reagents required for use in metabolic activity determination assay.

Saturated solution of Ringer's lactate	
➢ NaCl	6 g
➢ KCl	0.3 g
➢ CaCl $2H_2O$	0.2 g
➢ $C_3H_5O_3$.Na	31 g
The total volume was completed to 1 litre with the addition of distilled water The solution was autoclaved at 121 °C for 15 min	
Saturated solution of 0.5 g/l of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) in Ringer's lactate	
A total of 500 mg of XTT was added to 1 L of ringer lactate and passed through a 0.22-µm filter. The tubes were stored at -70 °C and protected from light	
Menadione 10 mM in acetone	
➢ Menadione	0.34 g
➢ Acetone	200 mL
The menadione solution was protected from light at 4 °C	

The XTT assay was performed to monitor the conversion of tetrazolium salt into water soluble formazan by living cells through their mitochondrial succinoxidase and cytochrome P450 systems. Determination of the colorimetric signal of formazan is directly related to the number of viable cells.

A saturated solution of XTT (Sigma-Aldrich) in 0.5 g/L in Ringer's lactate reagent was prepared and stored at -70 °C. Before each assay was performed, a 12-mL volume of stock XTT solution was thawed for each plate, and 1.2 µL of 10 mM menadione-acetone was added to each 12-mL stock solution to a final concentration of 1 µM. Then, 100 µL of the

XTT-menadione solution was added to each prewashed well and incubated in the dark for 2 h at 37 °C. The colorimetric changes indicating the metabolic activity of the biofilm were measured with the BioScreen C MBR microtitre plate reader at a 490-nm wavelength (Figure 3.7).

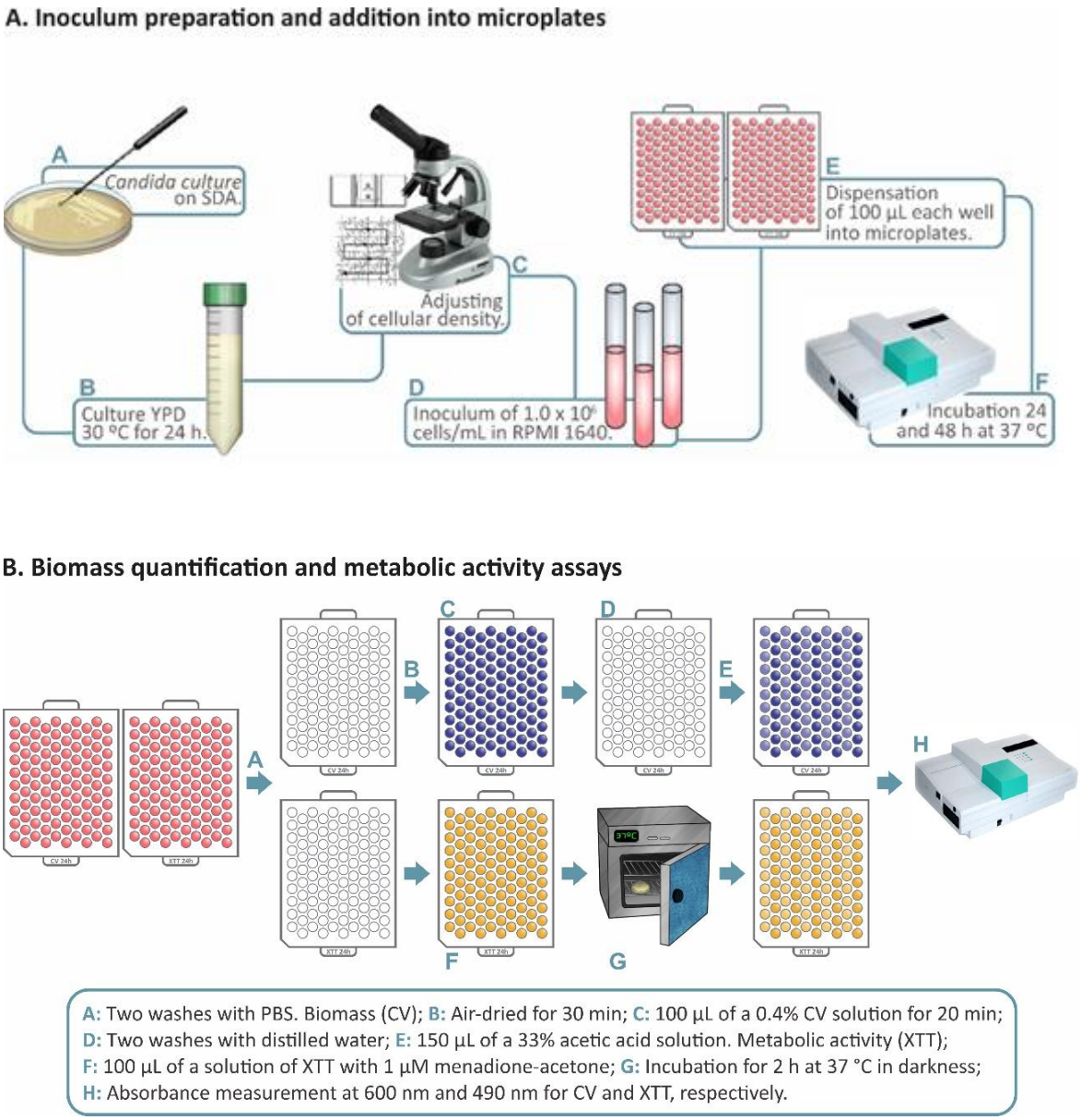


Figure 3.7. Scheme of biofilm production. A: Preparation of inoculum and dispensation into BioScreen microtitre plates. B: Incubation of isolates and XTT reduction assay-metabolic activity determination and CV assay-biomass quantification.

3.5.2 Biomass quantification assay

Biomass quantification of *Candida* biofilms was determined by following the method described by Peeters et al. (2008) using crystal violet (Table 3.16). Once non-adherent cells were removed from microplates and air-dried for 30 min, 100 μL of 0.4% crystal violet solution was added to each well and incubated for an additional 20 min at room temperature. The microplates were washed twice with 250 μL of sterile distilled water, and 150 μL of 33% acetic acid was added to each well. Absorbance of the biomass was measured with a BioScreen C MBR microtitre plate reader at a 600-nm wavelength (Figure 3.7).

Table 3.16. Preparation of the reagents required for use in biomass quantification assay.

0.4% (vol/vol) Crystal violet (CV) solution	
➢ Crystal violet (Merck Millipore, USA)	4 mL
Distilled water was added for establishing a total volume of 1 L	
The volume was completed to 1 litre with the addition of distilled water The solution was autoclaved at 121 °C for 15 min	
33% (vol/vol) acetic acid	
➢ Acetic acid (Sigma-Aldrich, USA)	33 mL
Distilled water	67 mL

3.5.3 Cut-off determination of biofilm production assay

The biofilm production of all *Candida* isolates assessed by XTT and CV assays was compared, and the production levels were divided into terciles, providing the cut-off values to classify isolates with absorbance measured at an OD of 600 nm as high biomass biofilm

producers (HBB) with absorbance > 0.50, moderate biomass biofilm producers (MBB) with absorbance between 0.30 - 0.50 and low biomass biofilm producers (LBB) with absorbance < 0.3. In addition, isolates with absorbance measured at an OD of 492 nm were classified as having high metabolic activity (HMA) with absorbance > 0.50, moderate metabolic activity (MMA) with absorbance between 0.30 and 0.50 and low metabolic activity (LMA) with absorbance < 0.3 were observed.

3.5.4 Morphology and architecture of the biofilm

The effect of phytocompounds on the morphology and architecture of the *C. albicans* SC5314 strain and *C. albicans* UPV 15-1757 isolate biofilms matured for 24 h was studied by scanning electron microscopy (SEM) and confocal microscopy (CLSM). Inoculum of 1.0×10^6 cells/mL in RPMI-1640 supplemented with L-glutamine and buffered to pH 7 with 0.165 M MOPS was used for microscopic analyses.

The mature biofilm of *C. albicans* SC5314 was treated with 2048 mg/L carvacrol, cinnamaldehyde and thymol, and the effects of these phytocompounds on *Candida* biofilms were assessed by SEM and CLSM. The effect of treating the *C. albicans* SC5314 biofilm with 2048 mg/L citral was analysed by SEM. In both experiments, untreated mature biofilms were studied as controls.

3.5.4.1 Scanning electron microscopy (SEM)

Mature biofilms were developed on nitrocellulose filters of 13 mm (Merck Millipore). After two washes with 1 mL of PBS, samples were fixed in 2% glutaraldehyde phosphate buffer solution for at least 1 h at room temperature. After three washes with 6% sucrose in Sorenson's buffer, the biofilms were dehydrated with graded ethanol (Merck Millipore, USA) solutions (50% and 70% ethanol in distilled water and 100% ethanol) for 5 min at each step and washed three times in hexamethyldisilane (Electron Microscopy Sciences,

Hatfield, USA) before air-drying (Table 3.17). Next, samples were mounted on SEM stubs and gold coated using an Emitech k550x ion sputter. Finally, images were acquired using a Hitachi S-4800 scanning electron microscope.

Table 3.17. Preparation of reagents required for fixing samples for SEM and CLSM.

2% Glutaraldehyde phosphate buffer solution (fixing solution)	
➢ Glutaraldehyde solution 25% (Merck Millipore, USA)	1 mL
➢ Sorenson's phosphate buffer	5 mL
Distilled water was added for establishing a total volume of 10 mL	
Sorenson's phosphate buffer	
➢ Na_2HPO_4	11.5 g
➢ NaH_2PO_4	2.96 g
Distilled water was added for establishing a total volume of 0.5 L	
6% (w/v) Sucrose solution in Sorenson's buffer	
➢ Sucrose (Sigma-Aldrich, USA)	6 g
➢ Sorenson's phosphate buffer	50 mL
➢ Distilled water	50 mL
10 mM Na-HEPES pH: 7.2, with 2% glucose	
➢ Glucose	20 g
➢ Na-HEPES (Millipore Sigma)	2.603 g
Distilled water was added for establishing a total volume of 1 L The buffer was adjusted to pH 7.2	

3.5.4.2 Confocal microscopy (CLSM)

Biofilms formed on 8-well tissue culture chambers (Sarstedt, Germany) were assessed by CLSM utilizing the LIVE/DEAD yeast viability kit (Thermo Fisher Scientific S.L., USA) to stain samples with FUN-1 and calcofluor white M2R according to the kit manufacturer's instructions. Biofilms were washed twice with HEPES buffer (10 mM Na-HEPES, pH: 7.2, with 2% glucose), and subsequently, 500 μL of FUN-1 and M2R white calcofluor solution (1:5) in HEPES buffer was added to each well to obtain final concentrations of 10 μM and 25 μM, respectively (Table 3.17). The samples were incubated in the dark for 30 min and then observed through standard FICT and DAPI filters with an Olympus FluoView FV500 confocal microscope. Live cells are green, and dead cells are yellow/orange.

3.6 Effect of phytocompounds and antifungal agents against *Candida* biofilms

A selection of ten *Candida* isolates, including eight *C. albican*s and one isolate of *C. dubliniensis* and *C. tropicalis* described as HMA or MMA according to their biofilm production, were analysed. *C. albicans* SC5314 and hypha-deficient *C. albicans* Ca2 were included as controls (Tables 3.1 and 3.2). A final cell density of 1×10^6 cells/mL in RPMI-1640 supplemented with L-glutamine and buffered to pH 7 with 0.165 M MOPS was used prepared for inoculation. The phytocompounds carvacrol, cinnamaldehyde, citral and thymol, as well as the antifungal agents anidulafungin and isavuconazole, were assessed during the adhesion phase (early biofilm formation) and with mature biofilm according to the method described by Van Dijck et al. (2018) and Ramage et al. (2001).

3.6.1 Early biofilm-adhesion phase of biofilm

A volume of 100 µL of each *Candida* inoculum plus 100 µL of the phytocompound/antifungal agent was added to 100-well polystyrene microtitre plates. The final concentrations of the phytocompounds ranged from 8 to 1024 mg/L and those of the antifungal agent ranged from 0.125 to 16 mg/L in RPMI-1640 supplemented with L-glutamine and buffered to pH 7 with 0.165 M MOPS. One phytocompound per microplate was evaluated, three isolates were tested in triplicate per microplate, and each microplate included a growth control and sterility control (Figure 3.8 A). After 24 h of incubation at 37 °C, non-adherent cells were removed, and the microplate was washed three times using 100 µL of PBS. Biomass quantification and metabolic activity determination assays described in sections 3.5.1 and 3.5.2 were performed to establish the effect of phytocompounds inhibiting biofilm formation.

Pre-sessile ICs (PSICs) of the phytocompounds and pre-sessile MICs (PSMICs) of the drugs were defined as the concentration of agent that causes 50% metabolic inhibition and 50% biomass reduction with respect to the controls (without compound treatment) (Figure 3.8 C).

3.6.2 Mature biofilm

Phytocompound concentrations ranging from 16 to 2048 mg/L and from 0.25 to 32 mg/L of anidulafungin and isavuconazole against the sessile cells of the mature *Candida* biofilms were tested. Biofilms development was initiated on 100-well polystyrene microtitre plates by adding 100 µL of *Candida* inoculum into each well, one isolate per microplate. After incubation for 24 h at 37 °C, non-adherent and loosely adherent cells were removed from the mature biofilm by washing each well twice with 250 µL of sterile PBS. Then, 100 µL of the phytocompound/antifungal agent at the predetermined concentrations in RPMI

medium was added to the corresponding microtitre plate to obtain plates on which biofilm growth was produced by a single strain. Three compounds were tested on each microplate in triplicate, with each microplate containing a growth control and sterility control (Figure 3.8 B). Microtitre plates were incubated for an additional 24 h at 37 °C, and the ability of the agents to inhibit mature biofilm development was determined by XTT and biomass determination assays as described in sections 3.5.1 and 3.5.2.

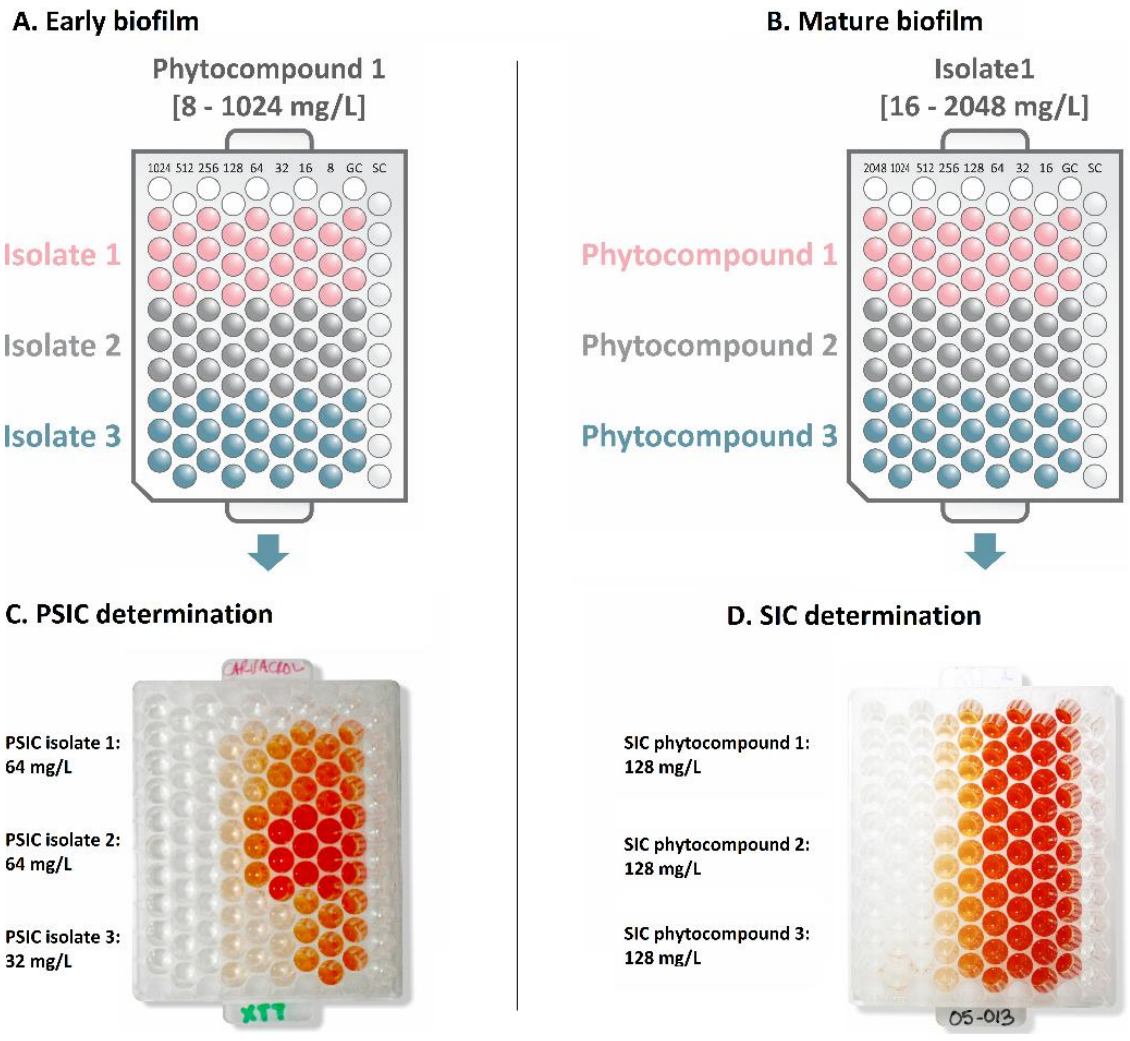


Figure 3.8 Scheme of preparation of microtiter plates (BioScreen) used for evaluation of activity of phytocompounds and drugs on A: early biofilm-adhesion phase and B: mature biofilm. C: Pre-sessile inhibition concentration, PSIC. D: Sessile inhibition concentration, SIC. Metabolic activity was determined by XTT assay.

Sessile ICs (SICs) of phytocompounds and sessile MICs (SMICs) of drugs were determined to be the concentration causing 50% metabolic inhibition and 50% biomass reduction, respectively, compared with untreated the controls (Figure 3.8 D).

3.6.3 Checkerboard assay

Citral in combination with fluconazole, with concentrations of citral ranging from 8 to 512 mg/L and those of fluconazole ranging from 0.25 to 64 mg/L, was tested against mature biofilms formed by the azole-resistant isolates *C. albicans* UPV 15-157 and *C. dubliniensis* UPV 11-366, as well as *C. albicans* SC5314. Mature biofilms (24 h old) were obtained by inoculating 100 μL of *Candida* inoculum in RPMI at 1×10^5 CFU/mL into each well, with one isolate added to each 100-well polystyrene microtitre plate. After 24 h of incubation at 37 °C, the plates were washed twice with 250 μL of sterile PBS, and subsequently, solutions of citral and fluconazole prepared in RPMI were added and incubated for 24 h at 37 °C. The effectiveness of the treatment against mature biofilm was determined by XTT and biomass determination assays; sessile ICs were estimated; and the FICI was calculated as previously described.

3.7 Time-kill curves

The killing activity of the antifungal combination of citral and fluconazole was assessed based on the procedure described by Caballero et al. (2021) and Cantón et al. (2004). Two azole-resistant isolates (*C. albicans* UPV 15-157 and *C. dubliniensis* UPV 11-366) and *C. albicans* SC5314 were studied.

In flat-bottomed 96-well microtitre plates, 100 μL of citral or fluconazole dilutions in monotherapy and in combination were dispensed to final concentrations of 8, 4 and 16

mg/L of fluconazole and 128 and 256 mg/L of citral and their combinations. Twofold dilutions of citral and fluconazole solutions were performed in RPMI with the stock solutions prepared with DMSO, as described in protocol M60 (Clinical and Laboratory Standards Institute (CLSI), 2020).

A cell solution in sterile water equivalent to the 0.5 McFarland was obtained from cells cultured overnight on SDA at 37 °C. After a 1:10 dilution in RPMI, 100 µL of this cell solution, that is, the *Candida* inoculum, was dispensed onto microplates to a final cellular density of 1 - 5 × 10^5 CFU/mL. At least three replicates were performed under each condition. Sterility control and growth control wells were included in all microplates. The microplates were incubated for 48 h at 37 °C without agitation.

The CFU/mL was determined at 0, 2, 4, 6, 24 and 48 h in aliquots (6, 10 or 15 µL, depending on the dilution) obtained from each well. Subsequent dilutions of both the control and drug aliquots were performed in PBS at different ratios: 1:100, 1:200, 1:300 and 1:500. Then, 5, 10, 25, 50 and 100 µL (depending on the dilution) of each dilution was seeded onto SDA plates in triplicate and incubated at 37 °C for 24 to 48 h.

Synergism was defined as a decrease in the CFU/mL of ≥ 2 $\log_{10}$ compared to the most active drug, inddiference was defined as a decrease in CFU/mL < 2 $\log_{10}$ and antagonism was defined as an increase in CFU/mL ≥ 2 $\log_{10}$ (Sahuquillo et al. 2006). The lower limit of accurate and detectable colony counts was 30 CFU/mL. These studies were conducted at least twice on different days.

3.8 Quantification analysis by real-time reverse transcription-polymerase chain reaction (RT-PCR)

The effect of 128 mg/L citral in monotherapy and in combination with 1 mg/L fluconazole on the expression of the *ERG11*, *MDR1* and *CDR1* genes in the fluconazole-resistant and fluconazole-susceptible *C. albicans* isolates was evaluated. In the selection of *C. albicans* strains, the results of checkerboard tests and the FICI values that indicated synergism between citral and fluconazole were considered; thus, the fluconazole susceptible *C. albicans* SC5314 and the fluconazole resistant *C. albicans* ATCC 64124 reference strains and the resistant *C. albicans* UPV 15-157 clinical isolate were analysed. The assessments were performed in duplicate.

Dilutions of citral and fluconazole alone and in combination obtained from stock solutions dissolved in DMSO were generated with 2×RPMI 2% G. Then, 500 µL of each sample under each condition was dispensed into a well in a 24-well plate to a final concentration of 128 mg/L citral alone, 1 mg/L fluconazole alone, and 128 mg/L citral plus 1 mg/L fluconazole. Each assay was performed with triplicate sets of each sample.

Adjusted inocula of 1×10^5 CFU/mL in suspension were added in triplicate in sterile distilled water at a cell density of the 0.5 McFarland standard. Then, a volume of 500 µL of each inoculum was added under each condition, and the plate was incubated for 24 h at 37 °C. In addition, growth and sterility controls were included in each assay.

After incubation, all the contents under each condition were removed from the plate and transferred to 2-mL microtubes for RNA extraction and further processing using RNAse- and DNAse-free material. RNA extraction from the samples treated with the compounds and from the control without treatment was performed using Total RNA Purification kit (Norgen, Biotek Corp., Canada) according to the manufacturer's recommendations. The purity and integrity of the RNA were determined by spectrophotometric analysis (NanoDrop™ 2000, Thermo Fisher Scientific) and measured by automated capillary electrophoresis separation using a LabChip GX Touch Analyser (Perkin Elmer, USA).

Complementary DNA (cDNA) was synthesized using a PrimeScript™ RT reagent kit (Takara Bio Inc., Japan) under the conditions described in Table 3.18.

Table 3.18. Conditions of cDNA production.

Reagents and stock concentrations	Volume (µl)
PrimeScript Buffer (5x)	2
PrimeScript RT Enzyme Mix I (50 µM)	0.5
Oligo dT Primer (50 µM)	0.5
Random 6-mers (100 µM)	0.5
RNA	6
RNAse Free H_2O	0.5
Total	10

The reaction mixture was incubated in the BioRad C1000TM thermal cycler under the following conditions: 15 min at 37 °C (reverse transcriptase) and 5 sec at 85 °C (reverse transcriptase inactivation). The cDNA samples were stored at -20 °C.

Primer sequences and the conditions employed to amplify the *ACT1*, *CDR1, ERG11*, and *MDR1* genes are described in Table 3.19 (Rocha et al., 2017). The expression levels of these genes were quantified by real-time PCR with a 7300 Fast Real-Time PCR thermal cycler (Applied Biosystems, USA). The cycling program included an initial step at 95 °C for 30 sec; 40 cycles of 95 °C for 5 sec and 55 °C for 31 sec for the *MDR1* gene (50 °C for 31 sec for the *ACT1, CDR1* and *ERG11* genes), and a dissociation stage of 95 °C for 15 sec, 60 °C for 1 min, and 95 °C for 15 sec for all these genes.

The experiments were performed in duplicate. Cycle threshold (Ct) values of the *ERG11*, *CDR1* and *MDR1* transcripts were normalized to the Ct corresponding to housekeeping gene *ACT1*. The quantification of the gene expression was analysed with the comparative

method Ct ($2^{-\Delta\Delta Ct}$) with respect to the *ACT1* gene. The relative change in expression was calculated with respect to the control incubated without drugs and normalized to 1.

Table 3.19. Conditions and primers used for amplification of the *ACT1*, *ERG11*, *MDR1* and *CDR1* genes by RT PCR.

Reagents and stock concentrations	Volume (µL)
SYBR Green Premix Ex Taq (Takara) (2x)	10
PCR Forward Primer (10 µM)	0.4
PCR Reverse Primer (10 µM)	0.4
ROX Reference Dye (50x)	0.4
cDNA	2
Sterile Milli-Q H_2O	6.8
Total	20

Gene	Primer Sequence Forward (F); Reverse (Rr)
ACT1	F: AAGAATTGATTTGGCTGGTAGAGA Rr:TGGCAGAAGATTGAGAAGAAGTTT
CDR1	F: TGCCAAACAATCCAACAA Rr: CGACGGATCACCTTTCATACGA
ERG11	F:GGTGGTCAACATACTTCTGCTTC Rr:GTCAAATCATTCAAATCACCACCT
MDR1	F:GTGTTGGCCCATTGGTTTTCAGTC Rr: CCAAAGCAGTGGGGATTTGTAG

3.9 *In vivo Caenorhabditis elegans* model

The *in vivo C. elegans* model was employed for the citral toxicity study and to determine the effect of citral in combination with fluconazole on *Candida* infections. *C. elegans* AU37, a double-mutant strain (*glp-4*; *sek-1*), was used. The *glp-4* mutation rendered these worms sterile at 25 °C. The *sek-1* mutation makes the worms more susceptible to fungal infection (Beanan & Strome, 1992; Scorzoni et al., 2013). The *C. elegans* worms were maintained on nematode growth medium (NGM) at 15 °C (Table 3.20).

Table 3.20. Preparation of the required reagents and culture media.

Nematode growth medium (NGM)	
➢ Sodium chloride-NaCl	2.4 g
➢ Peptone	2 g
➢ Bacteriological agar	3.6 g
➢ Distilled water	780 mL
The medium was autoclaved at 121 °C for 15 min A total of 800 µL of 1 M $CaCl_2$, 1 M $MgSO_4$ and 5 mg/L cholesterol solution, and 20 mL of KPO_4 buffer (each) was added when the medium was below 65 °C	
KPO_4 buffer	
➢ K_2HPO_4	36. 6 g
➢ KH_2PO_4	108.3 g
Distilled water was added for establishing a total volume of 1 L	
M9 buffer	
➢ Na_2HPO_4	6 g
➢ KH_2PO_4	3 g
➢ NaCl	5 g
➢ $MgSO_4$ 1M	1 mL
Distilled water was added for establishing a total volume of 1 L The medium was autoclaved at 121 °C for 15 min	
M9K buffer	
➢ M9 buffer	1 L
➢ Kanamycin solution (45 mg/mL)	1 mL

The NGM had been previously seeded with *Escherichia coli* OP50, as a food source for the worms. The bacterial strain had been previously seeded in Luria Bertani (LB) broth and incubated for 18 h at 30 °C. When food was scarce or the number of worms needed to be

increased for use in assays, a chunk of agar containing worms was transferred onto fresh NGM with bacterial growth following the protocol described by Stiernagle et al. (2006). After approximately 10 min, the piece of agar was removed, and the worms that had moved onto the fresh NGM were incubated for 48 h at 15 °C.

The L4 larval population was required to carry out toxicity, infection, and treatment assays at 25 ºC. Worms were recovered in M9 broth and age synchronized to the L4 larval population using an alkaline hatch hypochloride solution for lysing the gravid hermaphrodites (1 mL of 10% NaClO solution and 0.5 mL of 5 N NaOH solution). After ten minutes, the eggs were extracted by centrifugation at 3500 rpm for 30 sec, seeded on NGM without bacteria and incubated for 18 h at 15 °C. L1 worms were transferred as previously described onto NGM with *E. coli* and incubated for 72 h at 25 °C. Subsequently, worms were collected using supplemented M9 buffer, twice centrifuged at 1200 rpm for 2 min and the supernatant was discarded to eliminate *E. coli* cells. Finally, the resulting worms were transferred onto microplates for toxicity and infection assays.

3.9.1 Analysis of citral-induced toxicity in *C. elegans*

C. elegans was used to assess the toxicity of 32, 64, 128 and 256 mg/L citral and 0.5, 1, 2, 4, 8, 32, 64 and 128 mg/L fluconazole. The 24-well microplates (Tissue Culture Plate, Sarstedt Inc., USA) were prepared using 750 µL of M9K buffer (supplemented with 10 µg/mL cholesterol in ethanol and 90 µg/mL kanamycin) in each well. Worms previously obtained were transferred onto microtitre plates at 20 worms per well. The antifungal or phytocompound was dispensed at the proper concentration into each well to the desired final concentration at a final volume of 1300 µL. The microplates were incubated at 25 °C and included 0.5% DMSO as an untreated control. Worm survival was visually scored by Nikon SMZ-745 stereomicroscope every 24 h during the 96-h incubation. The nematodes

were determined to be dead when they were rod-shaped and/or did not respond to stimulation with a platinum wire pick. Survival curves of citral-exposed nematodes were compared with those of the untreated control using the Kaplan-Meier method and included the use of log-rank to test for equivalence between them..

3.9.2 *In vivo* activity of citral in combination with fluconazole

Biofilm producer and azole-resistant isolates, *C. albicans* UPV 15-157 and *C. dubliniensis* UPV 11-366, were selected to evaluate the activity of citral in combination with fluconazole against *Candida* infections in *C. elegans* by survival analysis (Ortega-Riveros et al. 2017; Hernando-Ortiz et al., 2020). *C. elegans* infected with *C. albicans* SC5314 were treated with citral and fluconazole in monotherapy.

An inoculum of all *Candida* strains at a cell density of 2 McFarland standards was prepared, spread on brain heart infusion (BHI) agar and incubated for 24 h at 37 °C. Worms obtained as previously described (L4 larvae) were exposed to previously incubated *Candida* strains to allow feeding for 2 h at 25 °C (Breger et al., 2007). Infected worms were washed twice with M9K buffer and transferred onto NMG without *E.coli*, where the worms were allowed time to eliminate the *Candida* cells in the cuticle.

M9K buffer (supplemented with 10 μg/mL cholesterol in ethanol) and final concentrations of 2, 64 and 128 mg/L fluconazole; 32, 64 and 128 mg/L citral; and combinations of 1 and 2 mg/L fluconazole with 32, 64 and 128 mg/L citral were dispensed into flat-bottom 24-well microplates at a final volume of 1300 μL.

Then, infected and uninfected worms were transferred onto microtitre plates, at 20 worms per well, and incubated at 25 °C. The subsequent survival study was performed as previously described. At least two replicates were analysed in independent experiments. The study included infected and untreated controls, and uninfected controls with 0.5%

DMSO. Survival curves of nematodes exposed to citral and fluconazole in monotherapy, in combination therapy and untreated were compared with each other. The Kaplan-Meier and log-rank methods were used to test the equivalence between them.

3.10 Preparation and characterization of the liposomal nanoparticles

Three concentrations of each phytocompound (carvacrol, cinnamaldehyde, citral and thymol) were selected according to the IC results obtained against planktonic *Candida* cells using the EUCAST method. The phytocompound stock solution in DMSO was diluted with sterile ultrapure water to reach the final concentration of the phytocompounds in the nanoparticles described in Table 3.21.

Table 3.21. Stock solution and final concentrations of phytocompound used for nanoparticle preparation.

Phytocompound and lipid components		**Final concentrations in nanoparticles***
Phytocompound	**Stock solution (mg/L)**	**(mg/L)**
Carvacrol	51200	32, 64 and 128
Cinnamaldehyde	25600	16, 32 and 64
Citral	102400	64, 128 and 256
Thymol	51200	32, 64 and 128
Lipid mixture	**Liposomal stock dispersion (mM)**	**(mM/mg/L)**
DODAB:MO (1:2)†	4	2 / 888

*:Nanoparticles contained also 0.5% DMSO. †: χMO = 0.330

Dioctadecyldimethylammonium bromide (DODAB) (Tokyo Kasei, Japan) and 1-monooleoyl-rac-glycerol (MO) (Sigma-Aldrich) were employed for liposome production. A stock solution of 20 mM DODAB and 20 mM MO was prepared in 10 mL of ethanol of high spectral purity (Uvasol, United Kingdom) and weighed 126.2 mg and 71.3 mg, respectively.

3.10.1 Preparation of liposomes by the lipid-film method

DODAB:MO-based liposomes were prepared using the lipid film hydration method (Bangham et al., 1965). DOBAB:MO at a molar ratio of 1:2 was selected on the basis of previous reports of accurate stability and homogeneous populations (Carneiro et al., 2015). The liposomal stock dispersion was prepared with a 4 mM total lipid concentration by transferring the required volume from DODAB and MO stock solutions into a glass tube considering an MO molar fraction (χMO) of 0.330 (DOBAB:MO molar ratio 1:2). Subsequently, the solvent (ethanol) was removed by rotary evaporation using nitrogen gas. Liposomes were obtained after hydration of the lipid film with ultrapure water at 60 °C and mixing by vortexing for 2 min (Figure 3.9).

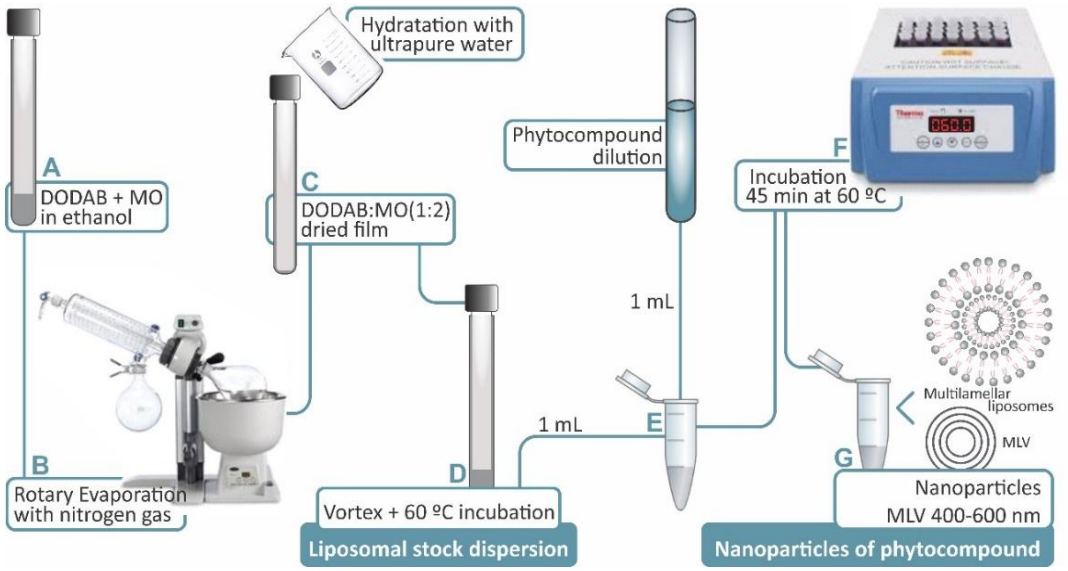


Figure 3.9. Scheme for the preparation of DODAB:MO (1:2) liposomes by the thin lipid-film hydration method.

Liposomal nanoparticle were assembled with equal volumes of liposomal stock dispersion and the phytocompound solution (diluted stock solution 100 times in ultrapure water) to obtain final formulations with 0.5% DMSO at a final lipid concentration of 2 mM (888 mg/L) and final concentrations of phytocompounds in the liposome as described in Table 3.21. These formulations were poured into 2-mL tubes and incubated for 45 min at 60 °C to allow the compounds to be absorbed. Thus, the final concentrations of nanoparticles for carvacrol and thymol were 32, 64 and 128 mg/L; the final concentrations of nanoparticles for cinnamaldehyde were 16, 32 and 64 mg/L; and the final concentrations of nanoparticles for citral, were 64, 128 and 256 mg/L. In addition, empty liposomes (2 mM) were generated with 0.5% DMSO.

3.10.2 Size, polydispersity index and ζ–potential

Each sample of nanoparticles and empty liposomes was placed into a capillary zeta cell (700 µL) and cuvettes for spectrophotometry to measure the physical parameters of the liposome surface charge, size and the polydispersity index. All characterizations were assessed five times.

The ζ-potential and the mean size of all formulations were determined by dynamic light scattering (DLS) at 25 °C with a Malvern ZetaSizer Nano ZS particle analyser. Malvern Dispersion Technology Software (DTS) was used with multiple narrow mode (high-resolution) data processing, and the mean size (nm), polydispersity index (PDI) and error values were determined. The liposome surface charge was measured indirectly by ζ-potential analysis using electrophoretic light scattering (ELS) at 25 °C. DTS with monomodal mode data processing was used to determine the average ζ-potential (mV) and error values.

3.10.3 Encapsulation efficiency (%EE)

The phytocompound encapsulation efficiency was calculated using the following equation:

$$EE(\%) = \frac{(Total\ amount\ of\ phytocompound - Free\ phytocompound)}{Total\ amount\ of\ phytocompound} \times 100$$

To measure the free phytocompounds in all the formulations, the liposomal formulations were pelleted by ultracentrifugation (1.5 mL at 100,000 g for 1 h at 4 °C) using a Sorvall MX120 micro-ultracentrifuge (Thermo Scientific). The supernatant was carefully removed and then filtered through 0.22-μm acetate cellulose filters (Millipore Merck, Germany). The concentration of the nonencapsulated phytocompounds in the supernatant was determined with high-performance liquid chromatography-diode array detection (HPLC-DAD) (Hitachi EZChrom elite, Agilent Technologies, USA).

The HPLC-DAD methodology was standardized for each phytocompound by preparing a standard curve with nonencapsulated phytocompounds on the basis of concentrations ranging from 1 to 50 mg/L without loss of linearity. Analysis was carried out using a Vydac 218TP54 column (C18, 5 μm, 4.6 mm i.d. x 250 mm), with the acetonitrile:water (50:50) mobile phase in isocratic mode at a 1 mL/min flow rate. The chromatographic runs were carried out at 30 °C for 20 min after each of three independent sample or control injections of 90 μL. A diode-array detector (DAD) was set at 210 nm for the detection of carvacrol and thymol, at 290 nm for the detection of cinnamaldehyde and at 240 nm for the detection of citral (Gaonkar et al., 2016; Gursale et al., 2010; Hajimehdipoor et al., 2010) (Table 3.22).

3.10.4 Antifungal activity of the nanoparticles against planktonic cells

Eleven *Candida* isolates were tested, including eight *C. albicans*, one *C. dubliniensis*, one *C. tropicalis* and one *C. auris* strain (Table 3.2). The *C. albicans* SC5314 reference strain was also tested. *Candida* isolates were selected according to the characteristics associated with *Candida* virulence, such as biofilm production capacity and azole resistance.

The antifungal activity of the nanoparticles against *Candida* planktonic cells was evaluated according to the EUCAST method described in a previous section (EUCAST definitive document EDef 7.3.2) (Arendrup et al., 2020b). Nanoparticles with carvacrol, cinnamaldehyde, citral and thymol, as well as empty liposomes, were tested in *Candida* strains in triplicate. Microtitre plates were prepared on the same day that an assay was performed using freshly prepared formulations and 2×RPMI 2% G medium in flat-bottomed 96-well microplates.

Table 3.22. HPLC-DAD parameters for the detection of nonencapsulated phytocompounds among the nanoparticle formulations.

Phytocompound	Wavelength detection (nm)	Coefficient (R2)	Linear range (mg/L)	Time retention (min)
Carvacrol	210	0.99	1-50	6.6
Cinnamaldehyde	290	0.98	1-50	4.4
Citral	240	0.99	1-50	5.5, 5.8
Thymol	210	0.99	1-50	6.4

Clinical isolates and reference strains cultured on SDA for 24 h at 37 °C were harvested and suspended in sterile distilled water to obtain a suspension with an optical density of 0.5 McFarland standard (1-5 × 10^6 CFU/mL). After dilution, a final inoculum with a

concentration between 0.5 and 2.5 × 10^5 CFU/mL was dispensed onto 96-well microplates previously prepared with nanoparticles and empty liposomes. Sterility control and growth control wells were included in each microplate. The absorbance values of the microplates at 450 nm were measured by an iMark microplate absorbance reader (Bio-Rad) after 24 h and 48 h of incubation at 37 °C. The value of the empty liposomes was considered a blank and was subtracted from readings of the other wells in the microplate. The IC was calculated as the lowest drug concentration causing growth inhibition of ≥ 50% after 24 h compared with the growth of the control without nanoparticles.

3.11 Cultures of the RAW 264.7 murine macrophage-like cell line

The RAW 264.7 murine macrophage-like cell line from ATCC (number TIB-71) (Raschke et al., 1978) was cultured in cell culture flasks with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated foetal bovine serum (FBS), 2% glutamine, 1% sodium pyruvate, and 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer in a 5% CO_2 atmosphere at 37 °C (Table 3.23).

Maintenance of the cell line consisted of transferring cells into a new cell culture flask, which was supplemented DMEM every 2 d. After reaching confluency, macrophages were washed three times with no supplemented DMEM. Then, supplemented DMEM was added, and the cells were harvested by scraping. Viable cells were identified by trypan blue exclusion counting with a haemocytometer and resuspended in supplemented DMEM to achieve the desired final concentration for use in each assay. The concentrations of phytocompounds and nanoparticles used in each assay are described in Table 3.24.

Table 3.23. Preparation of the required reagents and culture medium.

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1 mM sodium pyruvate and 2 mM L-glutamine	
➢ DMEM (Thermo Fisher Scientific #42430-025)	450 mL
➢ Heat-inactivated foetal bovine serum (FBS) (Valbiotech, France)	50 mL
➢ Sodium pyruvate (Sigma–Aldrich)	55 mg
Sterilized by filtration and followed by aliquoting 50 mL	
0.5% w/v MTT stock solution	
➢ MTT	50 mg
Up to 10 mL of distilled water was added and sterilization was performed by filtration	
DMEM:MTT (4:1) solution	
➢ 0.5% w/v MTT stock solution	1 mL
For larger volumes, a ratio of 1 MTT to 10 DMEM should be maintained	
Certified pyrogen-free material was used in all assays of cultured cells	

Table 3.24. Concentrations of empty liposome, phytocompounds and their nanoparticles tested in macrophage for cytotoxicity, cytokine production and macrophage killing assay.

Phytocompound or DODAB:MO liposomes	**Final tested concentrations (mg/L)**		
	Cytotoxicity	**Cytokine production**	**M killing assay**
Carvacrol	32, 64, 128	32	32, 64
Cinnamaldehyde	16, 32, 128		
Citral	64, 128, 256		
Thymol	32, 64, 128	32	32, 64, 128
NPCAR 128, 64, 32	18, 36, 72, 144	18, 36	18, 36
NPCIN 64, 32, 16	18, 36, 72, 144		
NPCT 256, 128, 64	18, 36, 72, 144		
NPTHY 128, 64, 32	18, 36, 72, 144	18, 36	18, 36, 72
Empty liposome 2mM	18, 36, 72, 144		

NPCAR, nanoparticles with carvacrol; NPCIN, nanoparticles with cinnamaldehyde; NPCT, nanoparticle with citral; NPTHY, nanoparticles with thymol.

3.11.1 Cytotoxicity assay

The cytotoxicity assay was performed to evaluate the toxic effect of empty liposomes, nanoparticles and nonencapsulated phytocompounds on macrophages.

Macrophages at a final concentration of 5×10^5 cells/mL were added in 200 μL aliquots to 96-well tissue culture plates (Thermo Fisher Scientific) and incubated overnight at 37 °C in a 5% CO_2 atmosphere. Then, the supernatant was removed, and empty liposomes, nanoparticles and non-encapsulated phytocompounds were added with supplemented DMEM in triplicate. Nanoparticles and empty liposomes (2 mM) were tested at final concentrations of 18, 36, 72 and 144 mg/L per well. The tested nanoparticles were DODAB:MO (1:2) in 0.5% DMSO with 32, 64 and 128 mg/L carvacrol; 32, 64 and 128 mg/L thymol; 16, 32 and 64 mg/L cinnamaldehyde or 64, 128 and 256 mg/L citral. Nonencapsulated phytocompounds were also evaluated at the same concentrations (Table 3.24). Untreated cells grown with supplemented DMEM were included as viability controls (considered to be 100% viable), and the control for cytotoxicity prepared with Tris-HCl was included for each replicate. Two identical culture plates were prepared and incubated, one was incubated for 24 h and the other was incubated for 48 h at 37 °C in a 5% CO_2 atmosphere. Then, culture viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (which measures cellular metabolic activity), and the impact on membrane integrity was determined by lactate dehydrogenase (LDH) assay (Figure 3.10).

3.11.1.1 Viability determination by MTT assay

RAW 264.7 cell viability was determined by MTT assay, which is performed to evaluate cellular metabolic activity. The MTT assay consists of the conversion of MTT into insoluble purple formazan crystals by mitochondrial succinate dehydrogenase activity in

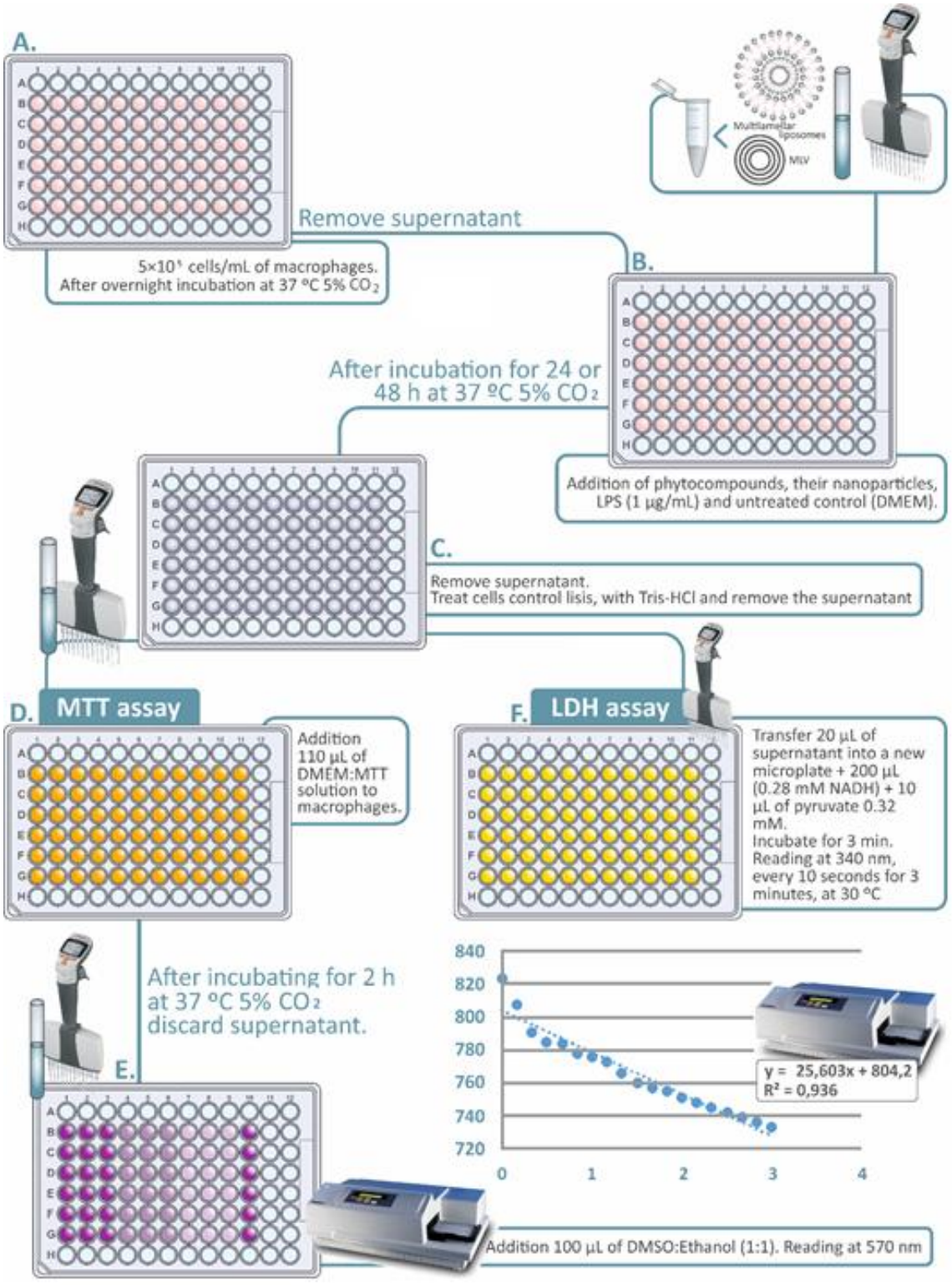


Figure 3.10. Scheme of the cytotoxicity assay of phytocompounds and nanoparticles in the macrophage-like cells model.

living cells.The measurement of the purple formazan colorimetric signal is directly related to the number of viable cells. Supernatants from cell culture plates were removed and stored for further analysis. For preparing cytotoxicity controls, designated empty wells were treated with 100 μL of Tris-HCl (0.5% w/v).

A total of 110 μL of DMEM:MTT (4:1) solution was added to each well and incubated for 2 h at 37 °C, and the supernatant was removed when incubation was complete. Enzymatic activity as determined by MTT assay was quantified after the purple formazan crystals were dissolved by adding 100 μL of a DMSO–ethanol (1:1) solution, and absorbance was measured at 570 nm (Perrie et al., 2008).

The percent of viable cells is expressed according to the following equation, as previously described (Carneiro et al., 2014):

$$Viability\ (\%) = \frac{Experimental\ value(average)\ -\ Control\ of\ cytotoxicity\ (average)}{Control\ of\ viability\ (average)\ -\ Control\ of\ cytotoxicity\ (average)} \times 100$$

3.11.1.2 Viability determination by LDH assay

Lactate dehydrogenase is a stable enzyme in the cytoplasm of all cells. When cells lose membrane integrity through external damage or apoptosis, LDH is rapidly released into the supernatant of the cell culture, and the determination of its activity is proportional to the number of damaged cells. LDH catalyses the oxidation of NADH to NAD+ in the conversion of pyruvate to lactate.

The LDH leakage assay was made according by Silva et al. (2011). The lactate dehydrogenase activity was measured with a microplate reader (Spectra Max 340PC, USA). Specifically, 200 μL of 0.28 mM NADH and 10 μL of 0.32 mM pyruvate (in phosphate buffer, pH 7.4) used as substrate, were added to 20 μL of the extracellular medium and

incubated for 3 min at 30 ºC. The reaction was measured as the decrease in absorbance rate observed at 30 °C every 10 sec for 3 min at 340 nm.

The following equation was used to determine LDH leakage into the supernatant, where V_max is the initial rate slope in m OD/min, and V_sample is the volume of the sample used in the assay, expressed in millilitres (Berry et al., 1991):

$$U/mL = (V\_\max * 0.332) / (1000 * 6.22 * V\_sample)$$

The viability percentage was calculated following the equation described by Holder et al., (2012). The activity of the LDH released into the supernatant in samples taken from wells containing the phytocompounds and liposomal formulations (msample), the activity in the samples taken from wells containing only cells (Ømin) and the maximum expected LDH value obtained from the activity in the samples taken from wells in which the cells were treated with Tris-HCl (Ømax) were documented.

$$\%viability = \left(1 - \frac{msample - Ømin}{Ømax - Ømin}\right) \times 100$$

The results are expressed as the percent of viable cells with respect to the untreated control.

3.11.2 Cytokine production assay

The cytokine production assay was performed using the supernant of culture media after the macrophages were exposed to phytocompounds and liposomes. Briefly, 200 µL of a macrophages suspension in were added to 96-well tissue culture plates (Thermo Fisher Scientific) at a final concentration of 5×10^5 cells/mL and incubated overnight at 37 °C in a 5% CO_2 atmosphere. Then, the supernatant was removed, and empty liposomes,

phytocompounds and nanoparticles with these compounds were added to the culture and incubated for 24 or 48 h at 37 °C in a 5% CO_2 atmosphere. The concentrations of the phytocompounds and nanoparticles analysed in this assay were selected according to the results of the macrophage cytotoxicity assay, and on this basis, 32 mg/L carvacrol and thymol and the liposomal formulations of 32, 64 and 128 mg/L carvacrol and thymol were evaluated.

The pro-inflammatory cytokine TNF-α, and the IL10 were quantified according to the ELISA kit manufacturer's instructions (Mouse TNFα #88-7324 and IL-10 ELISA Kit #88-7105, Thermo Scientific) on the basis of three independent assays. In this assay, macrophages were incubated with 1 mg/L LPS, which induces an inflammatory response, and untreated macrophages were used as the negative control.

3.11.3 Macrophage killing assay

The ability of macrophages to kill *Candida* after the addition of phytocompounds with or without their nanoparticles was determined according to the protocol described by McKenzie et al. (2010) with some modifications. Carvacrol, thymol and their nanoparticles were analysed at the final concentrations indicated in Table 3.24.

Briefly, an overnight macrophage culture was placed onto 96-well tissue culture plates at a concentration of 1×10^4 cells/well and incubated at 37 °C in a 5% CO_2 atmosphere. After one hour of incubation to allow macrophage adherence onto the tissue culture plate, nonencapsulated phytocompounds and nanoparticles were added to the wells. At the same time, *Candida* cells were added at a ratio of 5 *Candida* cells to 1 macrophage, using 100 μL of a final inoculum of *C. albicans* SC5314 of 5×10^5 CFU/mL in each well. After one hour of incubation, the macrophages were lysed with 10% saponin solution, and serial dilutions of the suspension were plated on YPD agar. The yeast CFUs were determined after 24 h of

incubation at 37 °C. Moreover, CFUs of *Candida* alone and from macrophages incubated with *Candida* without treatment were also performed as controls.

3.12 Statistical software programs

The statistical software programs GraphPad Prism (5.0 version, GraphPad Software, USA) and SPSS v21.0 (IBM, USA) were used for statistical analysis of the data. According to data distribution parametric or non-parametric tes were required. t Student test was used when the data showed a normal distribution. Non-parametric Kruskal Wallis test with Dunn's multiple comparison test and Mann Whitney non-parametrical test were performed when data did not show a normal distribution.The Mann-Whitney test was used to compare the relative gene expression of each gen and treatment. Survival curves were prepared by the Kaplan-Meier method and included the use of the log-rank for testing equivalency between them. Analysis of variance was made to compative analysis of different groups followed by the Bonferroni test in the MTT, LDH and macrophage killing assays. In all cases, $p < 0.05$ values were considered statistically significant.

4

RESULTS

4.1 Study 1: Prevalence and antifungal susceptibility profiles of *Candida glabrata* and *Candida parapsilosis* complexes

A total of 211 oral isolates previously identified by conventional methods as *C. glabrata* (114) and *C. parapsilosis* (97) were analysed by molecular techniques. Their patterns of antifungal susceptibility to itraconazole, fluconazole, miconazole, and nystatin were evaluated by disk diffusion testing, and their susceptibility to fluconazole was determined by microdilution testing. All *C. glabrata* isolates were identified as *C. glabrata*. Of the 97 *C. parapsilosis* isolates analysed, 93 were identified as *C. parapsilosis*, one isolate was identified as *C. metapsilosis* and three isolates were identified as *C. orthopsilosis*. *C. glabrata* isolates were recovered mainly from mixed cultures (83 of 114 isolates, 72.8%) and associated with *C. albicans* (74 of 83 isolates, 89.2%) or other *Candida* species (Table 2 from the manuscript 1, pag. 132). These isolates did not show resistance to miconazole or nystatin but showed reduced susceptibility to fluconazole and itraconazole, and some isolates presented cross-resistance (Table 3 from the manuscript 1, pag. 135).

The number of isolates of the *C. parapsilosis* complex in pure culture (51 of 97 isolates, 52.6%) was higher than that in mixed culture. The *C. parapsilosis* isolates were more frequently associated with *C. albicans* (36 of 46 isolates, 78.2%) (Table 2 from the manuscript 1, pag. 132). *C. parapsilosis* isolates were susceptible to fluconazole but showed cross-resistance to the azoles miconazole and itraconazole. *C. metapsilosis* and *C. orthopsilosis* were isolated from pure cultures and were susceptible to all the antifungal agents tested (Table 3 from the manuscript 1, pag. 135).

The results of study 1 have been published in "Archives of Oral Biology", which are presented in the following pages (manuscript 1).

Manuscript 1

Prevalence and antifungal susceptibility profiles of *Candida glabrata*, *Candida parapsilosis* and their close-related species in oral candidiasis

Katherine Miranda-Cadena, Cristina Marcos-Arias, Estibaliz Mateo, José Manuel Aguirre, Guillermo Quindós, Elena Eraso.

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Prevalence and antifungal susceptibility profiles of *Candida glabrata*, *Candida parapsilosis* and their close-related species in oral candidiasis

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ABSTRACT

Objective: To evaluate the importance of *Candida glabrata*, *Candida parapsilosis* and their close-related species, *Candida bracarensis*, *Candida nivariensis*, *Candida metapsilosis* and *Candida orthopsilosis* in patients with oral candidiasis and, to determine the *in vitro* activities of antifungal drugs currently used for the treatment.

Methods: One hundred fourteen isolates of *C. glabrata* and 97 of *C. parapsilosis*, previously identified by conventional mycological methods, were analysed by molecular techniques. *In vitro* antifungal susceptibility to fluconazole, itraconazole, miconazole, and nystatin was evaluated by CLSI M44-A2 disk diffusion test, and by CLSI M27-A3 microdilution for fluconazole.

Results: All *C. glabrata* isolates were identified as *C. glabrata sensu stricto*, 93 out of 97 C. parapsilosis isolates as *C. parapsilosis sensu stricto*, three as *C. orthopsilosis* and one as *C. metapsilosis*. *Candida glabrata* was mainly isolated in mixed cultures but *C. parapsilosis* complex was more frequent in pure culture. *Candida metapsilosis* and *C. orthopsilosis* were isolated as pure culture and both species were susceptible to all antifungal agents tested. Most *C. glabrata* isolates were susceptible to miconazole and nystatin, but resistant to fluconazole and itraconazole. Azole cross resistance was also observed. *Candida parapsilosis* isolates were susceptible to fluconazole although azole cross resistance to miconazole and itraconazole was observed.

Conclusion: This study highlights the importance of accurate identification and antifungal susceptibility testing of oral *Candida* isolates in order to have an in-depth understanding of the role of *C. glabrata* and *C. parapsilosis* in oral candidiasis.

Prevalence and antifungal susceptibility profiles of *Candida glabrata*, *Candida parapsilosis* and their close-related species in oral candidiasis

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Highlights

- *C. glabrata* and *C. parapsilosis* isolates from oral candidiasis were analysed.
- PCR-RFLP of *SADH* gene identified isolates of *C. metapsilosis* and *C. orthopsilosis*.
- *C. glabrata* was mainly isolated in mixed cultures and *C. parapsilosis* in pure cultures.
- Nystatin and fluconazole were the most active antifungal agents.
- Azole cross resistance was detected both in *C. parapsilosis* and *C. glabrata* isolates.

Abstract

Objective

To evaluate the importance of *Candida glabrata*, *Candida parapsilosis* and their close-related species, *Candida bracarensis*, *Candida nivariensis*, *Candida metapsilosis* and *Candida orthopsilosis* in patients with oral candidiasis and, to determine the *in vitro* activities of antifungal drugs currently used for the treatment.

Methods

One hundred fourteen isolates of *C. glabrata* and 97 of *C. parapsilosis*, previously identified by conventional mycological methods, were analysed by molecular techniques. *In vitro* antifungal susceptibility to fluconazole, itraconazole, miconazole, and nystatin was evaluated by CLSI M44-A2 disk diffusion test, and by CLSI M27-A3 microdilution for fluconazole.

Results

All *C. glabrata* isolates were identified as *C. glabrata sensu stricto*, 93 out of 97 *C. parapsilosis* isolates as *C. parapsilosis sensu stricto*, three as *C. orthopsilosis* and one as *C. metapsilosis*. *Candida glabrata* was mainly isolated in mixed cultures but *C. parapsilosis* complex was more frequent in pure culture. *Candida metapsilosis* and *C. orthopsilosis* were isolated as pure culture and both species were susceptible to all antifungal agents tested. Most *C. glabrata* isolates were susceptible to miconazole and nystatin, but resistant to fluconazole and itraconazole. Azole cross resistance was also observed. *Candida parapsilosis* isolates were susceptible to fluconazole although azole cross resistance to miconazole and itraconazole was observed.

Conclusion

This study highlights the importance of accurate identification and antifungal susceptibility testing of oral *Candida* isolates to have an in-depth understanding of the role of *C. glabrata* and *C. parapsilosis* in oral candidiasis.

Keywords: *Candida glabrata*; *Candida parapsilosis* complex; Oral candidiasis; Antifungal susceptibility.

1.- Introduction

Oral candidiasis is an infection caused by *Candida* which is often related to the characteristics of the patient, such as, age, immunological status, and denture wearing among other predisposing factors (Samaranayake, Keung Leung, & Jin, 2009). Oral candidiasis frequently produces discomfort, pain and dysgeusia and, manifests itself in a wide variety of chronic and acute clinical manifestations, such as pseudomembranous, erythematous or hyperplastic candidiasis. *Candida albicans* is the major aetiological agent, although other species of *Candida*, such as *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei* or *Candida glabrata* can be isolated from oral lesions (Muadcheingka & Tantivitayakul, 2015; Razzaghi-Abyaneh et al., 2014; Sadeghi et al., 2018; Samaranayake, Keung Leung, & Jin, 2009). Since 1990, changes in the distribution of *Candida* species causing invasive candidemia are being increasingly reported: *C. albicans* frequency is decreasing while that of *C. glabrata* remains stable and *C. parapsilosis* incidence has risen (Guinea, 2014; Quindós, 2014; Vaezi et al., 2017). An improvement in diagnostic procedures that enables a more rapid and accurate identification has been arising during the last 20 years by molecular and proteomic technics (Alonso-Vargas et al., 2008; Aslani et al., 2018; Chowdhary et al., 2017; Yazdanparast et al., 2015).

Development of molecular based identification methods has allowed the finding of new species phylogenetically close to *C. glabrata* and *C. parapsilosis*. The new species, *C. nivariensis* and *C. bracarensis*, are phylogenetically similar to *C. glabrata* (Alcoba-Flórez et al., 2005; Correia, Sampaio, James, & Páis, 2006); while *C. metapsilosis* and *C. orthopsilosis* are closely related to *C. parapsilosis* (Kurtzman & Robnett, 1997; Tavanti, Davidson, Gow, Maiden, & Odds, 2005). These new species are considered significant pathogens that can be isolated from oral lesions (Borman et al., 2008; Jahanshiri et al., 2018; Wahyuningsih et al., 2008).

Patient characteristics and prior antifungal therapy play an important role in the increasing isolation of these cryptic species in candidiasis (Guinea, 2014). In our setting, patients with oral candidiasis are often treated with nystatin or miconazole which are suitable topic agents for the treatment of superficial infections, while other antifungal agents such as fluconazole, itraconazole, or voriconazole are mainly indicated for the treatment of deep-seated infections or for the treatment of recalcitrant oral candidiasis when a topic treatment has failed (García-Cuesta, Sarrion-Pérez, & Bagan, 2014).

Miconazole, fluconazole and voriconazole have shown excellent *in vitro* activities against oral *Candida* isolates (Kobayashi et al., 2002; Marcos-Arias, Eraso, Madariaga, Carrillo-Muñoz, & Quindós, 2012; Tscherner, Schwarzmüller, & Kuchler, 2011). However, the reduced susceptibility of *C. glabrata* to azoles could be a problem for the treatment of infections caused by this species (Arendrup et al., 2013; Pemán et al., 2012; Pfaller et al., 2012b; Quindós, 2014; Tscherner et al., 2011).

Candida bracarensis, *C. nivariensis*, *C. metapsilosis* and *C. orthopsilosis* share many phenotypic characteristics or are undistinguishable from *C. glabrata* or *C. parapsilosis*. Hence, some oral clinical isolates routinely identified as *C. glabrata* or *C. parapsilosis* could be actually misidentified isolates of their cryptic species. Knowledge about the prevalence and distribution of these emerging species of *Candida* is still needed to elect the best antifungal treatment against them. Therefore, the present study aims to evaluate the importance of *C. glabrata*, *C. parapsilosis* and their phylogenetically close-related species in oral candidiasis, and to assess their *in vitro* susceptibility to itraconazole, fluconazole, miconazole, and nystatin.

2.- Materials and methods

2.1.- Clinical isolates

A total of 211 *C. glabrata* and *C. parapsilosis* were isolated from oral swabs of 1126 episodes of patients suffering from clinical oral candidiasis attending at the Dental Clinic Service of the Universidad del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU), Bilbao (Spain) from 2003 to 2013 (mean age: 59.3 years, range: 18–87 years, 70.1% women). Oral isolates were identified by conventional mycological methods, such as colony morphology on *Candida* Chromogenic agar (Laboratorios Conda, Spain) and ChromID Candida (BioMérieux, France), the germ tube test, microscopic morphology on corn meal agar and carbon source assimilation kit API ID 32C system (BioMérieux) (Eraso et al., 2006). These isolates, stored in the UPV/EHU yeast stock collection at room temperature in vials containing sterile distilled water, were cultured on Sabouraud dextrose agar medium (Difco, USA) at 37 °C for 24 h for molecular identification and for *in vitro* antifungal susceptibility testing.

2.2.- Candida glabrata *complex identification by 5.8S rRNA gene and the internal transcribed spacer (ITS1) analysis*

Identification of *C. glabrata* and its phenotypically related species, *C. bracarensis* and *C. nivariensis*, was performed by multiplex-polymerase chain reaction (multiplex-PCR) using four primers targeting the ITS1 region and the 5.8S ribosomal RNA gene (Table 1) previously described (Romeo, Scordino, Pernice, Lo Passo, & Criseo, 2009). Briefly, the master mixture was prepared from BioMix™ Red (Bioline Reagents Ltd, United Kingdom) with 0.42 μM of the primer UNI-5.8S-Reverse primer and 0.21 μM of the other three primers. The PCR reaction carried out with a BioRad C1000^{TM} Thermal Cycler (Bio-Rad, USA) consisted of a denaturation step at 95 °C for 5 min, followed by 34 cycles of 30 s at 94 °C, annealing for 40 s at 60 °C, elongation for 50 s at 72 °C, and a final 10 min extension step at 72 °C. The DNA amplified products were separated by electrophoresis on 2% agarose gel stained with GelRed (Biotium, USA) for 180 min at 50 V.

2.3.- Candida parapsilosis *complex identification by secondary alcohol dehydrogenase gene (*SADH*) analysis*

Clinical isolates of *C. parapsilosis* were analysed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for the identification of *C. parapsilosis sensu stricto*, *C. metapsilosis* and *C. orthopsilosis* species using specific primers for the region of the *SADH* (Table 1) (Miranda-Zapico et al., 2011; Tavanti et al., 2005). Briefly, a mixture containing BioMix™ Red (Bioline) and 0.4 μM of primers was subjected to PCR amplification carried out with a BioRad C1000^{TM} Thermal Cycler (Bio-Rad). The amplification started with a denaturation step at 95 °C for 5 min, followed by 40 cycles of 1 min at 92 °C, 1 min at 45 °C and 1 min at 68 °C; and a final extension step of 7 min at 68 °C. The amplified fragments were digested with the restriction enzyme *Ban*I (New England Biolabs, USA) for 2 h at 37 °C. The DNA fragments obtained were separated by electrophoresis on GelRed stained agarose gel at 1.5 %, for 70 min at 90 V.

2.4.- In vitro *activity of fluconazole, itraconazole, miconazole and nystatin*

All oral isolates were evaluated by disk diffusion using tablets of 25 μg of fluconazole, 10 μg of itraconazole, 10 μg of miconazole and 50 μg of nystatin, (Rosco Diagnostica-NeoSensitabs, Denmark) following a modification of the CLSI M44-A2 guidelines (Clinical and Laboratory Standards Institute (CLSI), 2009) (Rementeria et al., 2007). Mueller-Hinton agar medium (Difco) supplemented with 2% (w/v) glucose and 0.5 μg/l of methylene blue was used for disk diffusion testing. Yeast cell suspensions of 0.5 McFarland (1-5 × 10^6 CFU/ml, approximately) for each clinical isolate were prepared in sterile saline water. Inocula were spread using sterile swabs onto Mueller-Hinton plates and tablets were dispensed on the surface. In order to classify the clinical isolates in terms of their susceptibilities to these antifungal agents, after 24 and 48 h incubation at 37 °C, inhibition

zone diameters endpoints were measured in millimetres using a calliper and interpreted following the criteria published by the manufacturer. The susceptibility of isolates was categorized according to inhibition zone diameter as follows: a) fluconazole ≥ 19 mm, susceptible 15-18 mm, susceptible-dose dependent; and ≤ 14 mm, resistant; b) itraconazole ≥ 23 mm, susceptible; zone diameter 14-22 mm, susceptible-dose dependent; and ≤ 13 mm, resistant; c) miconazole ≥ 20 mm, susceptible; 12-19 mm, intermediate; and ≤ 11 mm, resistant; d) nystatin ≥ 15 mm, susceptible; zone 10-14 mm, intermediate; and < 10 mm, resistant.

In addition, *in vitro* susceptibility to fluconazole was confirmed by microdilution antifungal susceptibility testing as described in the document M27-A3 from the CLSI (Clinical and Laboratory Standards Institute (CLSI), 2008). Stock solution of fluconazole (3200 μg/ml) (Sigma-Aldrich, USA) was prepared in pure water and serial two-fold dilutions of the antifungal were made on RPMI 1640 medium (Sigma-Aldrich) buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (MOPS, Sigma-Aldrich) and added into each well of 96-well microplates. Antifungal concentrations ranged from 0.125 to 64 μg/ml and inocula were adjusted to a final concentration of 1-5 × 10^3 CFU/ml in RPMI medium. Plates were then incubated at 37 °C for 24 and 48 h. Fluconazole MIC was considered as the lowest concentration which caused ≥ 50% inhibition of growth (MIC_2) after 24 h of growth compared to the growth without antifungal drug. Clinical breakpoints (CBP) are often used to indicate those clinical isolates that are able to respond to treatment with a given antimicrobial agent administered using the approved dosing regimen for that specific drug (Pfaller & Diekema, 2012a; Turnidge & Paterson, 2007). In this study, the CBP used were the recommended in the M27-S4 supplement of CLSI (Clinical and Laboratory Standards Institute (CLSI), 2012) and are as follow: for fluconazole against *C. glabrata*,

Table 1 Primers used for identification of *C. glabrata*- and *C. parapsilosis*-complex isolates.

Name	Sequence (5’ → 3’)	Species identified	Reference
GLA-Forward	5'-CGGTTGGTGGGTGTTCTGC-3'	*Candida glabrata sensu stricto*	Romeo et al. (2009)
BRA- Forward	5'-GGGACGGTAAGTCTCCCG-3'	*Candida bracarensis*	Romeo et al. (2009)
NIV- Forward	5'-AGGGAGGAGTTTGTATCTTTCAAC-3'	*Candida nivariensis*	Romeo et al. (2009)
UNI-5.8S- Reverse	5'-ACCAGAGGGCGCAATGTG-3'	*Candida glabrata-complex*	Romeo et al. (2009)
S1- Forward	5'-GTTGATGCTGTTGGATTGT-3'	*Candida parapsilosis*-complex	Tavanti et al. (2005)
S1- Reverse	5'-CAATGCCAAATCTCCCAA-3'	*Candida parapsilosis*-complex	Tavanti et al. (2005)

susceptible-dose dependent ≤ 32 μg/ml and resistant ≥ 64 μg/ml; and against *C. parapsilosis*, susceptible ≤ 2 μg/ml, susceptible-dose dependent 4 μg/ml, and resistant ≥ 8 μg/ml. Moreover, epidemiological cut-off values (ECV) which can be the most sensitive measure of the emergence of strains with decreased susceptibility to a given agent, were also used to categorize wild-type (WT- those without mutational or acquired resistance mechanisms) and non-wild-type isolates (NWT- those having mutational or acquired resistance mechanisms) since resistance of oral *Candida* isolates to fluconazole has not been defined. The MIC for fluconazole to separate NWT isolates of *C. parapsilosis* was 2 μg/ml, and 32 μg/ml for *C. glabrata* (Pfaller & Diekema, 2012a).

2.6.- Quality control

Type strains obtained from the American Type Culture Collection (ATCC), the National Collection of Yeast Cultures (NCYC) and the Central Bureau voor Schimmel cultures (CBS) were used as quality control for the molecular identification and *in vitro* antifungal susceptibility testing: *C. albicans* ATCC 64548, *C. albicans* ATCC 64550, *C. bracarensis* NCYC 3133, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *C. metapsilosis* ATCC 96144, *C. nivariensis* CBS 9984, *C. orthopsilosis* ATCC 96141, *C. parapsilosis sensu stricto* ATCC 22019.

3.- Results

3.1.- Species identification

During 2003 to 2013, a total of 1328 clinical isolates were recovered from 1126 episodes of clinical oral candidiasis in patients attending at the Dental Clinic Service at UPV/EHU. *Candida albicans* was the most prevalent species (928 out of 1328 isolates, 70.4%) followed by *C. glabrata* (114 out of 1328 isolates, 8.6%), *C. parapsilosis* (97 out of 1328 isolates, 7.4%) and *C. tropicalis* (43 out of 1328 isolates, 3.3%). Mixed cultures of more than one isolate,

were obtained from a total of 173 out of 1126 episodes (15.3%) and *C. glabrata* or *C. parapsilosis* complexes were present in 126 of these episodes (72.8%).

All isolates previously identified as *C. glabrata* by conventional mycological methods were identified as *C. glabrata sensu stricto* by multiplex-PCR (Figure 1). Of interest was than *C. bracarensis* and *C. nivariensis* were not detected in the oral specimens from these patients. Regarding the 97 *C. parapsilosis* isolates, 93 out of 97 isolates were identified as *C. parapsilosis sensu stricto* (95.9%), three as *C. orthopsilosis* (3.1%) and one as *C. metapsilosis* (1%) by PCR-RFLP (Figure 2).

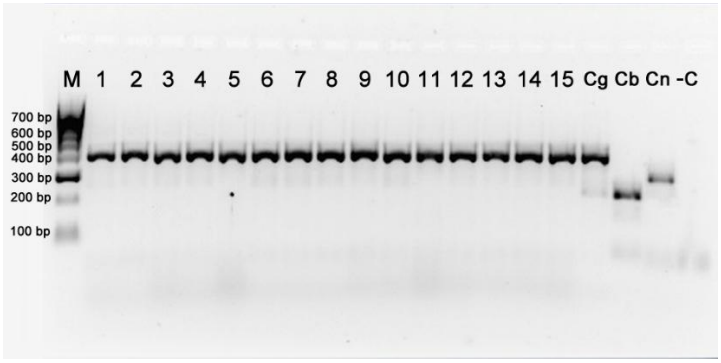

Figure 1. 5.8S rRNA gene and the internal transcribed spacer (ITS1) amplification by multiplex-PCR of oral isolates of *C. glabrata* complex. Lanes: *M* 100-bp DNA ladder, *1 to 15* oral isolates, *Cg C. glabrata* ATCC 90030, *Cb C. bracarensis* NCYC 3133, *Cn C. nivariensis* CBS 9984, *-C* negative control.

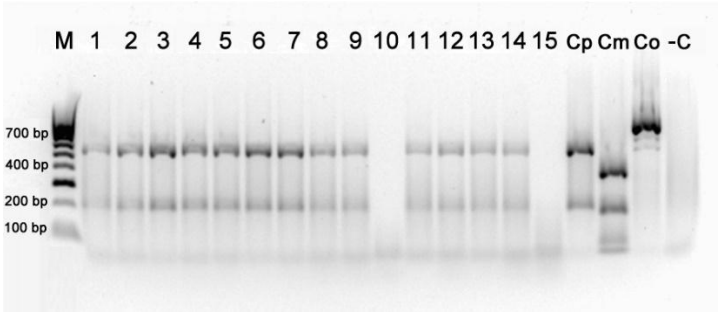

Figure 2. Secondary alcohol dehydrogenase gene (SADH) restriction profiles by PCR-RFLP of oral isolates of *C. parapsilosis* complex. Lanes: *M* 100-bp DNA ladder, *1 to 15* oral isolates, *Cp C. parapsilosis sensu stricto* ATCC 22019, *Cm C. metapsilosis* ATCC 96144, *Co C. orthopsilosis* ATCC 96141, *-C* negative control.

Most *C. glabrata* isolates were yielded as mixed cultures (83 out of 114 isolates, 72.8%) (Table 2). There were associations of up to four species of *Candida* and the most frequent was *C. albicans* (74 out of 114 total isolates, 64.9%) plus *C. glabrata* and *C. tropicalis* (11 out of 114 isolates, 9.6%). Regarding to the isolates of *C. parapsilosis* complex, the presence as pure culture (51 out of 97 isolates, 52.6%) was slightly higher than with other yeast species (46 out of 97 isolates, 47.4 %). *Candida parapsilosis* was found together with *C. albicans* in most cases (36 out of 97 total isolates, 37.1%). In eight of these 36 isolates (22.2%) both species were yielded together with other *Candida* species. Conversely, *C. metapsilosis* and *C. orthopsilosis* were always isolated as pure cultures.

3.3.- Antifungal susceptibility testing

Table 3 shows the *in vitro* antifungal susceptibility of the 114 isolates of *C. glabrata*, 93 isolates of *C. parapsilosis*, three isolates of *C. orthopsilosis* and one of *C. metapsilosis*. Figure 3 shows the isolates distribution regarding to the zone diameters obtained by disk diffusion. The reference strains used as quality controls presented the expected values (data not shown).

Nystatin showed an excellent activity against all isolates. Most isolates of *C. glabrata* were susceptible to miconazole (113 out of 114 isolates, 99.2%), and only one was intermediate (0.8%). Eight (7%) and seven (6.1%) out of 114 *C. glabrata* isolates were susceptible-dose dependent to itraconazole and fluconazole, respectively. Moreover, 14 (12.3%) and three (2.6%) *C. glabrata* isolates were resistant to fluconazole and itraconazole, respectively. On the other hand, all *C. parapsilosis* isolates were susceptible to fluconazole. However, half of *C. parapsilosis* isolates were intermediate to miconazole (46 out of 93 isolates, 49.5%) and one was resistant to this drug (1.1%). Susceptibility-dose dependent to itraconazole was

Table 2. Identification of *C. glabrata* and *C. parapsilosis*-complex isolates recovered alone or in combination with other *Candida* species between 2003 and 2013.

Species	Number of isolates
***C. glabrata*-complex**	
Pure culture	**31**
C. glabrata sensu stricto	31
Mixed culture	**80**
C. glabrata + C. albicans	57
C. glabrata + C. tropicalis	4
C. glabrata + C. krusei	1
C. glabrata + C. guilliermondii	1
C. glabrata + C. albicans + C. tropicalis	11
C. glabrata + C. albicans + C. famata	1
C. glabrata + C. albicans + C. krusei	1
C. glabrata + C. albicans + C. dubliniensis	2
C. glabrata + C. dubliniensis + C. krusei	1
C. glabrata + C. pararugosa + C. lusitaniae + C. rugosa	1
***C. parapsilosis*-complex**	
Pure culture	**51**
C. parapsilosis sensu stricto	47
C. metapsilosis	1
C. orthopsilosis	3
Mixed culture	**43**
C. parapsilosis + C. albicans	28
C. parapsilosis + C. rugosa	1
C. parapsilosis + C. guilliermondii	4
C. parapsilosis + C. litytica	1
C. parapsilosis + Saccharomyces cerevisiae	1
C. parapsilosis + Zygosaccharomyces	1
C. parapsilosis + C. albicans + C. tropicalis	1
C. parapsilosis + C. albicans + C. intermedia	1
C. parapsilosis + C. albicans + C. famata	1
C. parapsilosis + C. albicans + C. guilliermondii	1
C. parapsilosis + C. albicans + Saccharomyces cerevisiae	1
C. parapsilosis + C. albicans + Kodamaea ohmeri	1
C. parapsilosis + C. famata + C. guilliermondii	1
Mixed culture of *C. glabrata* and *C. parapsilosis*	**3**
C. glabrata + C. parapsilosis	1
C. parapsilosis + C. glabrata + C. albicans	2

detected in four *C. parapsilosis* isolates (4.3%). *Candida metapsilosis* and *C. orthopsilosis* isolates were susceptible to all antifungal agents tested.

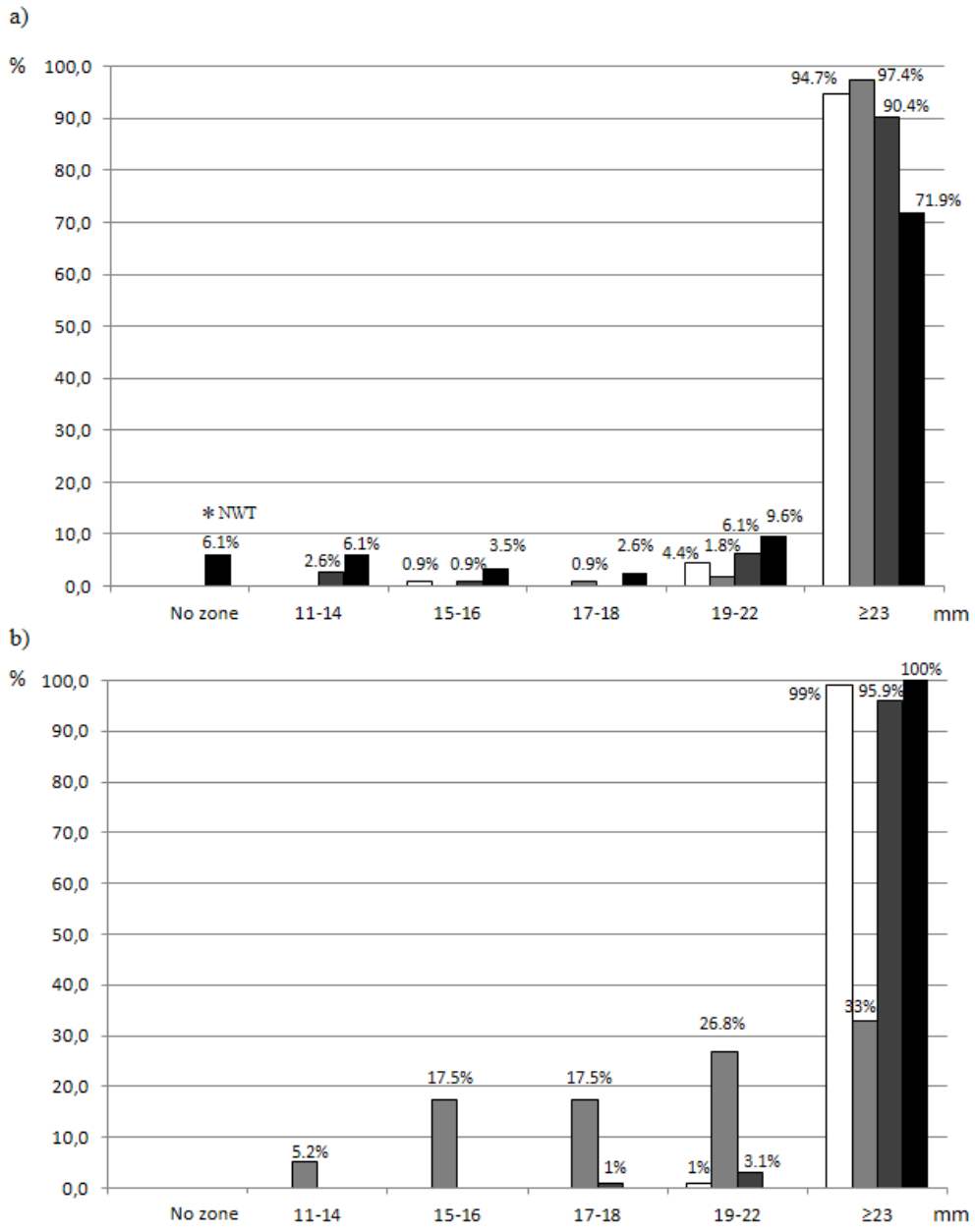

Figure 3. Isolates distribution according to zone inhibition diameter (millimetres) for fluconazole, itraconazole, miconazole, and nystatin by disk diffusion test: (a) isolates of *Candida glabrata*; (b) isolates of *Candida parapsilosis* complex. Zone diameters endpoints are indicated as susceptible (S), susceptible-dose dependent (S-DD), intermediate (I) and resistant (R) for each antifungal drug. The distribution of the two non-wild-type isolates (NWT) for fluconazole is represented by asterisk.

Fluconazole activity was also tested by microdilution method for 35 *C. glabrata* with different *in vitro* susceptibilities to this antifungal agent by disk diffusion method. These isolates were classified by disk diffusion method as 13 resistant, seven susceptible-dose dependent and 15 susceptible isolates. All susceptible and susceptible-dose dependent isolates by disk diffusion method were susceptible-dose dependent by microdilution method. Conversely, only one out of 13 resistant isolates by disk diffusion method was found to be fluconazole resistant by microdilution assay (7.7%). This resistant isolate was found as pure culture, and fluconazole MIC for this isolate was 64 μg/ml. The remaining 12 resistant isolates by disk diffusion method were found to be susceptible-dose dependent by microdilution assay. According to the CLSI interpretation criteria for microdilution assay and fluconazole, *C. glabrata* cannot be classified as susceptible, only resistant or susceptible-dose dependent. Therefore, in an attempt to analyse these 12 resistant isolates only by disk diffusion, ECVs were considered and, it was found that two isolates, classified as resistant by disk diffusion method, were categorized as NWT with MICs of 32 and 64 μg/ml and the remaining isolates were categorized as WT.

Azole cross-resistance was observed in isolates of *C. glabrata* and *C. parapsilosis*. Three out of 14 *C. glabrata* isolates resistant to fluconazole by diffusion method were also resistant to itraconazole. One of these three isolates were intermediate and the other two were susceptible to miconazole. Three out of 14 fluconazole resistant isolates were also susceptible-dose dependent to itraconazole. On the other hand, one miconazole resistant isolate of *C. parapsilosis* was susceptible-dose dependent to itraconazole and was separated from a mixed culture along with *C. albicans*. Moreover, two itraconazole susceptible-dose dependent *C. parapsilosis* isolates also were intermediate to miconazole.

Table 3. *In vitro* activity of current antifungal agents against isolates of *C. glabrata* and *C. parapsilosis*-complex.

Antifungal agents tested	Species			
	C. glabrata sensu stricto	***C. parapsilosis sensu stricto***	***C. metapsilosis***	***C. orthopsilosis***
Disk diffusion method:				
Nystatin				
Susceptible	114	93	1	3
Intermediate susceptibility	-	-	-	-
Resistant	-	-	-	-
Miconazole				
Susceptible	113	46	1	3
Intermediate susceptibility	1	46	-	-
Resistant	-	1	-	-
Itraconazole				
Susceptible	103	89	1	3
Susceptible-dose dependent	8	4	-	-
Resistant	3	-	-	-
Fluconazole				
Susceptible	93	93	1	3
Susceptible-dose dependent	7	-	-	-
Resistant	14	-	-	-
Microdilution test:				
Number of isolates	35	3		
Fluconazole				
Susceptible		3		
Susceptible-dose dependent	34	-		
Resistant	1	-		
MIC_{50}	4	-		
MIC_{90}	2	-		
MIC range	0.25-64	0.12-0.25		
MIC geometric mean	4	-		
MIC mean	4	-		

4.- Discussion

Candida albicans is the major aetiological agent of oral candidiasis but *C. glabrata* and *C. parapsilosis* are considered emerging causes of this disease presenting decreased susceptibilities to current antifungal drugs (Pfaller et al., 2012b; Sadeghi et al., 2018; Samaranayake et al., 2009). There are limited studies on the presence of species from *C. glabrata* and *C. parapsilosis* complexes in oral cavity (Borman et al., 2008; Jahanshiri et al., 2018; Wahyuningsih et al., 2008). In the present study, more than 15% of oral isolates belonged to *C. glabrata* (8.6%) and *C. parapsilosis* (7.4%) species complexes and were present in 72.8% of mixed cultures. This fact remarks the importance of these species in oral pathology and should be considered for therapeutical approach. Moreover, in the current study, inside the *C. parapsilosis* complex, *C. orthopsilosis* and *C. metapsilosis* were yielded as pure cultures from a low number of oral cavities of patients as it has been described by other authors (Ge et al., 2012; Moris et al., 2012). This event highlights the necessity of achieving a correct identification of the isolates involved in oral candidiasis because of the differences in virulence and susceptibility patterns of these species in comparison to *C. parapsilosis*. However, *C. nivariensis* and *C. bracarensis* were not present in oral specimens of patients suffering from clinical oral candidiasis in the current study. Previous studies have reported a low prevalence of *C. nivariensis* and *C. bracarensis* in the oral cavity (Borman et al., 2008; Lockhart et al., 2009; Wahyuningsih et al., 2008), the female genitourinary system (Li, Shan, Fan, & Liu, 2014; Sharma et al., 2013), or blood cultures (Li et al., 2014; López-Soria et al., 2013; Miranda-Zapico et al., 2011). The species variability found in oral cavity can be wide due to population, dietary or geographical reasons (Lockhart et al., 1999; Sharifzadeh et al., 2013).

Candida albicans can be co-isolated with other *Candida* species as it has previously been reported in other studies (Kleinegger, Lockhart, Vargas, & Soll, 1996; Qi, Hu, & Zhou,

2005; Zaremba et al., 2006). In the current study, mixed cultures were present in 173 out of 1126 episodes (15.3%). The most common association found was *C. albicans* and *C. glabrata* (64.9%), as it has been reported previously in other studies (Coco et al., 2008; Martins et al., 2010; Muadcheingka & Tantivitayakul, 2015; Zomorodian et al., 2011), followed by the association between *C. albicans* and *C. parapsilosis* with a frequency of 37.1%. Other authors reported a lower mixed colonization with other species different of *C. albicans*; however, the presence of multiple *Candida* species may contribute to their permanence in oral cavity and in case of causing oral candidiasis, a more complicate or recalcitrant episode (Lockhart et al., 1999; Martins et al., 2010).

In this study, the most frequent association with more than two species was composed by *C. albicans*, *C. glabrata* and *C. tropicalis*, as it is also reported by other authors (Muadcheingka & Tantivitayakul, 2015; Pereira et al., 2013; Sanita et al., 2011; Rabelo, Noborikawa, Silva-Siqueira, Silveira, & Lotufo, 2011). The presence of two or more species of *Candida* in oral specimens from a patient suffering from candidiasis is difficult to interpret. Probably, the apparently less pathogenic species could be an adjuvant pathogen or merely a colonizer.

Nystatin was the most active antifungal agent *in vitro* against *Candida*. This polyene is one of the first choices of treatment for mucosal and superficial candidiasis (Carrillo-Muñoz et al., 2010; das Neves et al., 2008; García-Cuesta et al., 2014; Niimi, Firth, & Cannon, 2010). Resistance to nystatin is infrequent and it has been attributed to alterations in cell membrane (Kathiravan et al., 2012; Marcos-Arias et al., 2012; Mohamadi et al., 2014). Miconazole showed good activity against *C. glabrata* and *C. parapsilosis*. Different formulations of miconazole have been used such as gel or mucoadhesive buccal tablets (Bensadoun et al., 2008; Khozeimeh, Shahtalebi, Noori, & Savabi, 2010; Miki, Ohtani, & Sawada, 2011; Vázquez & Sobel, 2012) and, although some resistant isolates of *Candida* have been reported for miconazole (Kuriyama et al., 2005; Manfredi et al., 2006; Marcos-

Arias et al., 2012), this antifungal agent exerts great inhibitory activity against most *Candida* species (Bensadoun et al., 2008; Isham & Ghannoum, 2010; Khozeimeh et al., 2010; Niimi et al., 2010; Thevissen et al., 2007; Van Roey, Haxaire, Kamya, Lwanga, & Katabira, 2004). Fluconazole is a common antifungal agent used for most oral candidiasis and has been also used for systemic *Candida* infections due to its reduced toxicity, efficacy and good tolerance (Maertens & Boogaerts, 2005). However, the widespread use of this antifungal agent has likely promoted the higher resistance rates observed (Fakhim et al., 2017; Jahanshiri et al., 2018; Silva et al., 2012). In the present study, fluconazole was very effective against *C. parapsilosis,* but its activity was not as good against *C. glabrata*. Some authors have reported that the latter species develops resistance to fluconazole during therapy and, in general, presents intrinsically low susceptibility to triazoles (Arendrup et al., 2013; Pemán et al., 2012; M. A. Pfaller & Diekema, 2007; Quindós, 2014; Tscherner et al., 2011).

Regarding to itraconazole activity, resistance and dose dependent susceptibility was observed in less than the 10% of the isolates of *C. glabrata* and *C. parapsilosis sensu stricto*. This azole has been indicated as good alternative for fluconazole resistant *Candida* isolates (Oude Lashof et al., 2004) and it has also been successfully used to treat patients with oropharyngeal candidiasis (Koks, Meenhorst, Bult, & Beijnen, 2002) and denture stomatitis (Maertens & Boogaerts, 2005).

Despite the high effectiveness of the antifungal agents tested, the azole cross-resistance observed by disk diffusion requires consideration. Six of 14 isolates of the fluconazole resistant *C. glabrata* isolates by disk diffusion method presented azole cross-resistance. Cross-resistance was mainly observed against fluconazole and itraconazole but in one isolate was extended to miconazole. Interestingly, three azole cross-resistant *C. glabrata* were isolated in association with *C. albicans* suggesting that the treatment may be effective against *C. albicans* but not against *C. glabrata* and could result in an increase of the oral

burden with this resistant species, maintaining an oral candidiasis recalcitrant to the antifungal therapy. It has been reported that the co-infection or prior infection with *C. albicans* might advantage *C. glabrata* infection (Tati et al 2016). Moreover, two *C. glabrata* isolates were cross-resistant to fluconazole and itraconazole, one of them in association with *C. krusei*; which has known intrinsic resistance to azoles (Arendrup et al., 2013; Pemán et al., 2012). Azole cross-resistance was also observed to itraconazole and miconazole in three *C. parapsilosis sensu stricto* isolates from which one was in association with *C. albicans*. Multi-species colonization can contribute to increase both the interaction with surfaces in oral cavity and the risk of being resistant to the treatment (Martins et al., 2010).

Increased isolation of non-*C. albicans* species could be related to the use of more sensitive techniques that has allowed an accurate identification of species that always have been present in oral cavity (Dahiya et al., 2003; Fakhim et al., 2018; Muadcheingka & Tantivitayakul, 2015). Alternatively, a real increase of these species can be associated to changes in the oral environment by the use of antifungal drugs or other antimicrobial compounds, such as chlorhexidine or triclosan. In this regard, this study highlights that the development and implementation of accurate identification techniques would contribute to enhancing the knowledge of the oral candidiasis aetiology and, therefore, to the best choice for the most appropriate treatment. The present study reports the increase in the frequency of *C. glabrata*, *C. parapsilosis* and their close-related species in oral candidiasis. Furthermore, the important rate of antifungal resistance observed is a clinical challenge that makes it necessary to study the in vitro susceptibility of oral *Candida* isolates to guide the selection of the most appropriate treatment.

Disclosure

All authors have read and approved the final article. Also, authors declare that they have no conflict of interests related to the present study.

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4.2 Study 2: *In vitro* activity of terpenes and other phytocompounds against *Candida*

The anti-*Candida* activities of eight phytocompounds, including carvacrol, cinnamaldehyde, citral, eugenol, geraniol, linalool, terpinen-4-ol and thymol, were analysed. All the phytocompounds tested showed antifungal activity against *Candida* planktonic cells. The most-active compounds in all *Candida* species, including azole-resistant isolates, were cinnamaldehyde, thymol and carvacrol, as determined by geometric mean values (GMs) of their IC (IC GMs: 61.5, 93.2 and 105 mg/L, respectively) (Table 4.1 and Table 1 from the manuscript 2, pag.165). Geraniol, eugenol, terpinen-4-ol and linalool presented antifungal activity with less effectiveness than the other phytocompounds tests (IC GMs: 438.9; 440.7, 894.8 and 894.8 mg/L, respectively). Fungicide activity was observed with the use of cinnamaldehyde (MFC GM: 99 mg/L), carvacrol (MFC GM: 251.0 mg/L), thymol (MFC GM: 241.2 mg/L) and eugenol (MFC GM: 991.5 mg/L) against planktonic cells (Table 4.2 and Table 2 from the manuscript 2, pag. 166). No fungicidal activity was observed with geraniol, linalool, or terpinel-4-ol at the concentrations tested (MFC range: > 1024 mg/L for all three compounds) (Table 4.2).

Regarding the classification of *Candida* isolates on the basis of their biofilm production cut -off values, different categories were identified (Figure 4.1). There were no significant differences between the results obtained after 24 and 48 h. *C. albicans* was the most prolific biofilm-producing *Candida* species (Figure 1 from the manuscript 2, pag. 163).

The anti-biofilm activity of carvacrol, cinnamaldehyde and thymol were analysed against early and mature *Candida* biofilms of ten oral biofilm-producing isolates. Carvacrol, cinnamaldehyde and thymol actively inhibited the metabolic activity of the early biofilm (GM PSIC XTT assay: 104; 97 and 157.6 mg/L); cinnamaldehyde was the most effective

reducing the biomass (GM PSIC 111 mg/L), while anidulafungin actively reduced both biomass and metabolic activity (GM PSMIC XTT assay 0.125 mg/L and CV assay 0.134 mg/L).

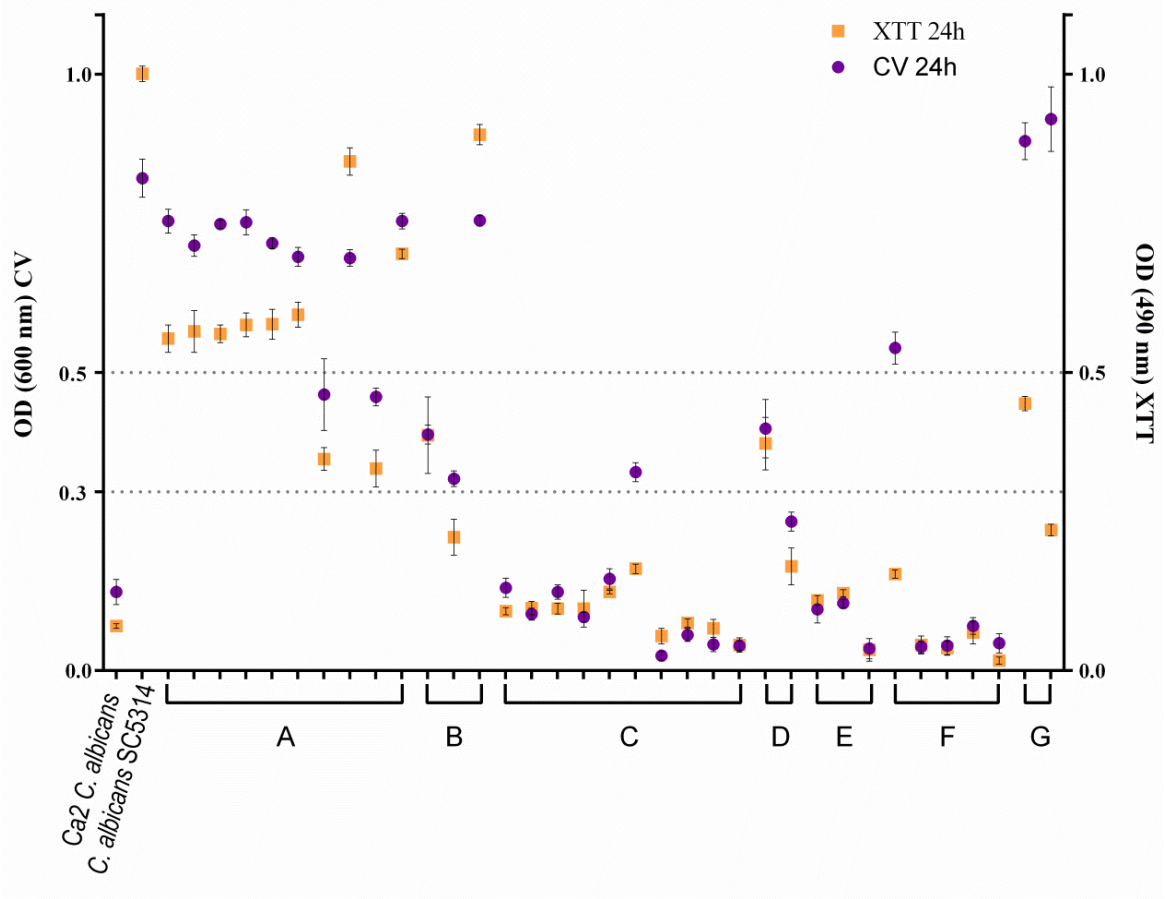

Figure 4.1. Biofilm production of *Candida* isolates and cut-off distribution with respect to XTT and CV assays. A: *C. albicans* isolates, B: *C. dubliniensis* isolates, C: *C. glabrata* isolates, D: *C. guilliermondii* isolates, E: *C. krusei* isolates, F: *C. parapsilosis* complexe isolates and G: *C. tropicalis* isolates.

Carvacrol and thymol were active against most of the mature biofilms, reducing both metabolic activity and biomass (GM SIC XTT assay 188.1 mg/L and CV assay 812.7 mg/L of carvacrol; and GM SIC XTT assay 128 mg/L and CV assay 1106 mg/L of thymol). Anidulafungin and cinnamaldehyde reduced the metabolic activity of sessile cells

to a greater extent than they reduced biomass production (GM SMIC XTT assay 0.9 mg/L and CV assay 29.6 mg/L of anidulafungin; GM SIC XTT assay 118.5 mg/L and CV assay 1896.2 mg/L of cinnamaldehyde). Isavuconazole showed the lowest activity against preformed *Candida* biofilms (Table 3 from the manuscript 2, pag. 167).

Table 4.1. IC of eugenol, geraniol, linalool and terpinen-4-ol against *Candida* species.

***Candida* isolate**		**Eugenol [2-1024 mg/L]**	**Geraniol [2-1024 mg/L]**	**Terpinen-4-ol [2-1024 mg/L]**	**Linalool [2-1024 mg/L]**
***C. albicans* (10)**	**IC GM**	580.8	795.9	1024	1024
	Range IC	512-1024	256->1024	>1024	>1024
***C. glabrata* (10)**	**IC GM**	512	724.1	776.1	724.1
	Range IC	256-1024	64->1024	64->1024	32->1024
***C. krusei* (3)**	**Range IC**	64-512	32->1024	256->1024	256->1024
***C. guilliermondii* (2)**	**Range IC**	256	128	>1024	>1024
***C. tropicalis* (2)**	**Range IC**	1024	256	1024->1024	>1024
***C. orthopsilosis* (2)**	**Range IC**	256	128	512-1024	>1024
***C. parapsilosis* (2)**	**Range IC**	256	128-512	1024->1024	>1024
***C. dubliniensis* (3)**	**Range IC**	256-512	128-256	>1024	>1024
***C. metapsilosis* (1)**	**IC**	256	128	>1024	>1024
Total	**IC GM**	440.7	438.9	894.8	894.8
	Range IC	64-1024	32-1024	62->1024	32->1024

IC: inhibitory concentration, GM: geometric mean.

Table 4.2. MFC of eugenol, geraniol, linalool and terpinen-4-ol against *Candida* species

***Candida* isolate**		**Eugenol [2-1024 mg/L]**	**Geraniol [2-1024 mg/L]**	**Terpinen-4-ol [2-1024 mg/L]**	**Linalool [2-1024 mg/L]**
***C. albicans* (10)**	**MFC GM**	1024	-	-	-
	Range MFC	1024->1024	>1024	>1024	>1024
***C. glabrata* (10)**	**MFC GM**	961.5	-	-	-
	Range MFC	512->1024	>1024	>1024	>1024
***C. krusei* (3)**	**MFC GM**	812.8	-	1024	-
	Range MFC	512->1024	>1024	1024->1024	>1024
***C. guilliermondii* (2)**	**MFC GM**	1024	-	-	-
	Range MFC	1024	>1024	>1024	>1024
***C. tropicalis* (2)**	**MFC GM**	-	-	-	-
	Range MFC	>1024	>1024	>1024	>1024
***C. orthopsilosis* (2)**	**MFC GM**	-	-	-	-
	Range MFC	>1024	>1024	>1024	>1024
***C. parapsilosis* (2)**	**MFC GM**	1024	-	-	-
	Range MFC	1024->1024	>1024	>1024	>1024
***C. dubliniensis* (3)**	**MFC GM**	1024	-	-	-
	Range MFC	1024->1024	>1024	>1024	>1024
***C. metapsilosis* (1)**	**MFC**	>1024	>1024	>1024	>1024
Total	**MFC GM**	991.5	-	1024	-
	Range MFC	512->1024	>1024	1024->1024	>1024

MFC: minimum fungicidal concentration, GM: geometric mean.

Part of the results obtained in study 2 has been submitted for publication in "Biomedicine and Pharmacotherapy", which is presented in the following pages (manuscript 2).

Manuscript 2

***In vitro* activities of carvacrol, cinnamaldehyde and thymol against *Candida* biofilms**

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In vitro activities of carvacrol, cinnamaldehyde and thymol against *Candida* biofilms

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Abbreviations: IC, Inhibitory concentration; SICs, Sessile inhibitory concentrations; PSICs, Pre-sessile inhibitory concentrations; SMIC, Sessile minimum inhibitory concentration; PSMIC, Pre-sessile minimum inhibitory concentration; CV, crystal violet assay; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium -5-carboxanilide; HBB, High biomass biofilm producer; MBB, Moderate biomass biofilm producer; LBB, Low biomass biofilm producer; HMA, High metabolic activity biofilm producer; MMA, Moderate metabolic activity biofilm producer; LMA, Low metabolic activity biofilm producer.

Highlights

- Phytocompounds are promising alternatives against *Candida* biofilms
- Carvacrol, cinnamaldehyde & thymol are fungicidal against *Candida* planktonic cells
- High activity of cinnamaldehyde in inhibiting biofilm adhesion
- Carvacrol and thymol reduce both biomass and metabolic activity of mature biofilms

Abstract: Oral candidiasis is frequently associated with *Candida* biofilms. Biofilms are microbial communities related to persistent, recalcitrant and difficult to-treat infections. Conventional treatments are not sufficient to overcome biofilm-associated candidiasis; thus, the search of new antifungal compounds is necessary. In the current study, we have evaluated the effect of three phytocompounds, carvacrol, cinnamaldehyde and thymol, against *Candida* planktonic and sessile cells. Reduction in biofilm biomass and metabolic activity was assessed during adhesion and mature biofilm phases. *Candida albicans* was the most biofilm-producing *Candida* species. All phytocompounds tested were fungicidal against *Candida* planktonic cells. Cinnamaldehyde was the most active in inhibiting biofilm adhesion, but carvacrol and thymol significantly reduced both mature biofilm biomass and metabolic activity. These results highlight the role of cinnamaldehyde, carvacrol and thymol as promising alternatives for the treatment of candidiasis due to their antibiofilm capacities, and stress the necessity to continue studies on their safety, toxicity and pharmacodynamics and pharmacokinetics.

Keywords: *Candida* biofilms; carvacrol; cinnamaldehyde; thymol; antifungal susceptibility

1. Introduction

Oral candidiasis is one of the most prevalent opportunistic infections that causes oral discomfort, pain and dysgeusia and it is often associated with a poor immune status that can lead to complications, such as esophageal or systemic candidiasis [1-3]. Risk for systemic infection increases in immunocompromised patients colonized by *Candida*. Although most oral infections are easily treatable, often follow a protracted course in those patients carrying dentures, HIV-infected or under chemotherapy [2]. Treatment of oral candidiasis is based in the correction of underlying diseases, the maintenance of good oral hygiene and the use of antifungal drugs. *Candida albicans* is the most frequent etiology, followed by *Candida glabrata* [1,4]. *C. albicans* outstands in the oral cavity due in part to its biofilm forming ability that challenges the efficacy of treatment. Extracellular polymeric substances, such as carbohydrate and extracellular DNA, often hamper antifungal penetration through biofilms extracellular matrix (ECM). This clinical concern is magnified by the emergence of azole resistant isolates and by the selection of species of *Candida* with reduced antifungal susceptibility [3]. In recent years, essential oils and their components have gathered significant attention as potential antimicrobial agents due to its relative safety, low long-term genotoxicity and scarcity of side effects [5]. Main strategies focus on studying the effectiveness of key phytocompounds against *Candida* biofilms as monotherapy or in combination with current antifungal drugs, thus limiting development of resistance or decreasing antifungal selective pressure. Some phytocompounds have been included in rinses or mouthwashes for preventing oral infections [6]. However, most studies evaluate the activity of essential oils as a whole, rather than studying key components, which could be more advantageous in safety and reproducibility [7]. Therefore, the aim of the present study was to assess the in vitro activity of carvacrol,

cinnamaldehyde and thymol, compared to anidulafungin, fluconazole and isavuconazole, against against planktonic and sessile *Candida* cells.

2. Materials and Methods

2.1. Microorganisms

Thirty-five oral isolates from patients suffering oral candidiasis attending the Dental Clinic Service at the Universidad del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU), Bilbao (Spain) were analyzed. Isolates were identified by conventional techniques, such as colony morphology on *Candida* Chromogenic agar (Laboratorios Conda, Spain) and ChromID *Candida* (BioMérieux, France), carbon source assimilation kit API ID 32C system (BioMérieux), and molecular methods (Multiplex PCR and PCR-RFLP to *C. glabrata* complex and *C. parapsilosis* complex, respectively) [4]. These isolates included 10 *C. albicans*, 10 *C. glabrata*, three isolates each of *Candida dubliniensis* and *Candida krusei*, two isolates each of *Candida guilliermondii*, *Candida orthopsilosis*, *Candida parapsilosis* and *Candida tropicalis*, and one *Candida metapsilosis*. Moreover, seven reference strains from the American Type Culture Collection (ATCC) and the National Collection of Pathogenic Fungi (NCPF), and the hypha-defective mutant *C. albicans* Ca2 (kindly donated by Professor Antonio Cassone, Istituto Superiore di Sanità, Rome, Italy) were studied. Isolates and reference strains were cultured on Sabouraud dextrose agar (Difco, Becton Dickinson, USA) at 37 °C for 24 h before testing.

2.2. Biofilm production

Biofilm production by oral isolates and reference strains was assessed. Prior to each experiment, they were cultured overnight at 30 °C in an orbital shaker on yeast peptone dextrose (YPD) medium containing 1% weight/volume (w/v) yeast extract, 2% w/v

peptone and 2% w/v dextrose. Cells were washed thrice in sterile phosphate buffered saline solution (PBS), and adjusted to a cellular density of 1.0 x 10^6 cells/ml in RPMI-1640 supplemented with L-glutamine and buffered at pH 7 with 0.165 M 3-(N-morpholino)propanesulfonic acid, MOPS (Sigma-Aldrich). *Candida* biofilms were developed in sterilized, flat-bottomed honeycomb 100-well polystyrene microtiter plates (Labsystems, Finland) by adding 100 µl of the adjusted standard cell suspension into each well. Two identical microtiter plates were prepared, one to determine metabolic activity and the other to quantify biomass. Microtiter plates were incubated at 37 °C in a computer-controlled incubator (BioScreen C MBR, Growth Curves Ltd, Finland). After 24 and 48 h non-adherent and loosely adherent cells were removed by washing three times with sterile PBS.

2.2.1. Metabolic activity determination assay

Metabolic activity of the biofilm was measured following the colorimetric method described by Ramage et al. [8]. The 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT, Sigma-Aldrich) reagent was prepared as a saturated solution at 0.5 g/l in Ringer's lactate and the solution was sterilized by filtration, aliquoted and stored at −70 °C until required. Before each assay, an aliquot of stock XTT was thawed and menadione was added to a final concentration of 1 µM. Then, 100 µl of XTT-menadione was added to each prewashed well and incubated in dark for 2 h at 37 °C. The colorimetric changes showing metabolic activity of the biofilm were measured on the microtiter plate reader BioScreen C MBR at 490 nm wavelengths.

2.2.2. Biomass quantification assay

Biomass quantification was performed following the method described by Peeters et al. [9] using crystal violet. After removing non-adherent cells, microplates were air-dried for 30

min and then, 100 µl of 0.4% crystal violet solution was added to each well and incubated for a further 20 min at room temperature. Microplates were washed twice using 250 µl of sterile distilled water and 150 µl of 33% acetic acid were afterwards added to each well. Absorbance of the biomass was measured at 600 nm wavelengths.

2.3. Phytocompounds and antifungal agents

Carvacrol, cinnamaldehyde and thymol at 98, 95 and 99% purity, respectively, were purchased from Sigma-Aldrich. Anidulafungin (Pfizer, Spain), fluconazole (Sigma-Aldrich) and isavuconazole (Basilea Pharmaceutica, Switzerland) were also used.

Stock solutions were prepared in dimethylsulphoxide (DMSO, Sigma-Aldrich). Antifungal drugs were stored at –70 ºC and phytocompounds were prepared on the same day of the susceptibility test. The tested compounds contained 0.5% DMSO, therefore all drug-free controls also included this concentration of DMSO.

2.4. In vitro *antifungal activity against planktonic cells*

In vitro antifungal susceptibility was assessed according to the methodology proposed by the European Committee for Antimicrobial Susceptibility Testing [10,11]. Final concentrations of anidulafungin ranging from 0.016 to 8 mg/l, of fluconazole from 0.12 to 64 mg/l and of isavuconazole from 0.016 to 8 mg/l were used. Susceptibility to carvacrol, cinnamaldehyde and thymol was assayed at concentrations ranging from 2 to 1024 mg/l. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality controls. Absorbance of each microplate was measured at 450 nm wavelengths by the iMark reader (BioRad, USA) after 24 and 48 h of incubation at 37 °C. Absorbance values equal to or less than 0.2 after 48 h were considered a failed test. Antifungal activities were studied in triplicate and in at least three separate experiments. Anidulafungin, fluconazole and isavuconazole minimum

inhibitory concentrations (MIC) were calculated at 24 h as the lowest drug concentration inhibiting ≥ 50% of growth in comparison to controls without antifungal drugs [10,11]. Carvacrol, cinnamaldehyde and thymol inhibitory concentrations (IC) were calculated at 24 h as the lowest drug concentration inhibiting ≥ 50% of growth in comparison to controls without phytocompounds [12]. Clinical isolates were classified as susceptible, susceptible-dose dependent and resistant using the species-specific MIC breakpoints defined by EUCAST for anidulafungin and azoles [13]. However, EUCAST breakpoints for isavuconazole have not been established.

Minimum fungicidal concentration (MFC) was calculated by seeding 100 μl of each well without growth onto Sabouraud dextrose agar plates [14]. Fungicidal activity was defined as the lowest concentration of antifungal agent resulting in the death of 99.9% of the inoculum.

2.5. Effect of phytocompounds against adhesion and mature Candida *biofilms*

Ten clinical isolates were selected according to their biofilm production in the previous assay: eight *C. albicans* and one isolate each of *C. dubliniensis* and *C. tropicalis*. *C. albicans* SC5314 and the hypha-deficient *C. albicans* Ca2 were included as controls. Activities of carvacrol, cinnamaldehyde and thymol to prevent *Candida* biofilm formation were assessed according to the method described by Van Dijck et al. [12]. Briefly, 100 μl of the adjusted standard cell suspension of each isolate were inoculated into the 100-well polystyrene microtiter plates plus 100 μl of the phytocompound at final concentrations ranging from 8 to 1024 mg/l. Pre-sessile ICs (PSICs) were those concentrations causing 50% metabolic inhibition and 50% biomass reduction with respect to controls without compound.

For the susceptibility assay against the sessile cells of the mature biofilm, concentrations of phytocompound ranging from 16 to 2048 mg/l were used. Briefly, biofilms were developed

into 100-well polystyrene microtiter plates by adding 100 µl of the adjusted *Candida* inoculum into each well. After an incubation of 24 h at 37 °C, non-adherent and loosely adherent cells were removed from the mature biofilm by washing twice with sterile PBS. Then, 100 µl of the final concentrations of phytocompounds in RPMI medium were added into microtiter plates for a further incubation of 24 h at 37 °C. Compound-free wells and biofilm-free wells were also included as positive and negative controls, respectively. Sessile ICs (SICs) were those concentrations causing 50% metabolic inhibition and 50% biomass reduction with respect to controls.

2.6. Morphology and architecture of the biofilm

Morphology and architecture of the mature 24 h biofilms of *C. albicans* SC5314 strain developed on nitrocellulose filters of 13 mm (Merck Millipore, Germany) or 8-well tissue culture chambers (Sarstetd, Germany) were studied by scanning electron microscopy (SEM) and confocal microscopy (CLSM), respectively. Mature biofilms were also treated with 2048 mg/l of carvacrol, cinnamaldehyde and thymol to observe the effect on *Candida* biofilms by SEM and CLSM.

Samples analyzed by SEM were fixed in 2% glutaraldehyde phosphate buffer solution. After three washes with 6% sucrose in Sorenson's buffer, biofilms were dehydrated using graded ethanol solutions (50% and 70% in distilled water, and 100%) for 5 min each, and washed three times in hexamethyldisilazane (Electron Microscopy Sciences, USA) before air drying. Afterwards, samples were mounted on SEM stubs and gold coated using an Emitech k550x ion sputter. Finally, images were acquired using a Hitachi S-4800 scanning electron microscope.

Biofilms analyzed by CLSM were stained with the LIVE/DEAD Yeast Viability Kit (Thermofisher Scientific S.L., USA) using FUN-1 and calcofluor white M2R following the

instructions of manufacturers. Briefly, biofilms formed on the 8-well tissue culture chambers slides were washed with buffer HEPES (10 mM Na-HEPES; pH: 7.2, with 2% glucose). Subsequently 500 µl of FUN-1 and M2R white calcofluor solution (1:5) in HEPES buffer were dispensed into each well. Samples were incubated in dark for 30 min and afterwards, they were observed with standard filter set of FICT and DAPI by an Olympus Fluoview FV500 confocal microscope. Live cells show green color and dead cells yellow or orange.

2.7. Statistical analysis

Statistics were analyzed using Graphpad Prism 5.0 version (GraphPad Software, USA). Comparisons between quantitative results were analyzed by t Student's test when data showed a normal distribution and non-parametric Kruskal Wallis test with Dunn's multiple comparison test and Mann Whitney non-parametrical test when data did not show a normal distribution. In all the cases, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Biofilm production of oral Candida *isolates*

Biofilm production of 35 isolates and reference strains was divided into terciles for CV and XTT assays. It allowed the classification of isolates as high biomass (HBB) or high metabolically active (HMA) biofilm producers when the mean absorbance (OD) was greater than 0.500; moderate biomass (MBB) or with moderate metabolic activity (MMA) biofilm producers when absorbance ranged between 0.300 and 0.499, and low biomass (LBB) or low metabolically active (LMA) biofilm producers when the mean values of the absorbance were less than 0.300 for each method (Fig. 1). *C. albicans* isolates developed the most metabolically active biofilm and showed the highest biomass production, including

the isolate resistant to fluconazole and with reduced susceptibility to isavuconazole *C. albicans* UPV 15-157 isolate, classified as HBB and HMA biofilm producer. Highest biofilm producers among *C. glabrata* isolates exhibited moderate biofilm production (MBB and LMA). *C. dubliniensis* UPV 11-366, a fluconazole resistant isolate and with reduced susceptibility to isavuconazole was HMA and HBB, while two *C. tropicalis* isolates (UPV 06-115 and UPV 05-016) and *C. parapsilosis* UPV 12-296 were HBB but their biofilms showed moderate or low metabolic activities. All isolates of *C. guilliermondii*, *C. krusei* and the rest of species inside the *C. parapsilosis* complex produced biofilms with low or moderate metabolic activity and biomass.

C. albicans produced more biofilm than *C. krusei*, *C. glabrata* and *C. parapsilosis* complex ($p < 0.0005$). Nevertheless, no significant differences were found in biofilm production among *C. albicans*, *C. tropicalis*, *C. dubliniensis* and *C. guilliermondii*.

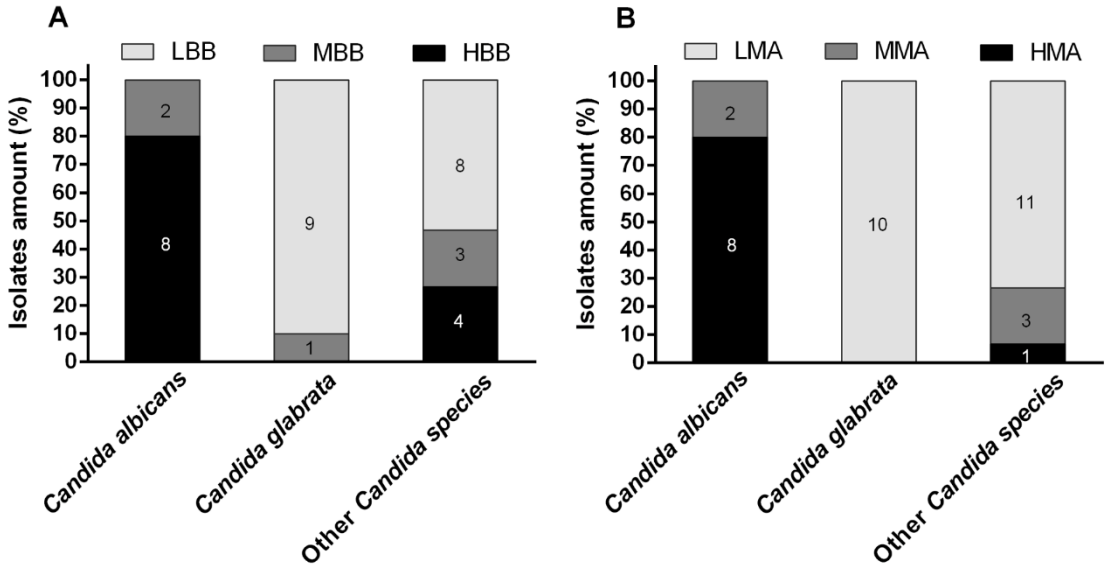

Fig. 1. Biomass (A) and metabolic activity (B) of *Candida* biofilms. Percentages and number de isolates of each *Candida* species. Isolates were classified as high (HBB), moderate (MBB) and low (LBB) biomass biofilm producers; and with high (HMA), moderate (MMA) and low (LMA) metabolic activity.

3.2. In vitro *antifungal susceptibility testing against* Candida *planktonic cells*

Table 1 shows the MIC and IC at 24 h of all oral isolates and reference strains. Carvacrol, cinnamaldehyde and thymol showed antifungal activity against planktonic cells of all isolates (geometric mean -GM- of IC were 105 mg/l; 61.5 mg/l and 93.2 mg/l, respectively), including those resistant to fluconazole isolates (*C. albicans* UPV 15-157 and *C. dubliniensis* UPV 11-366), and susceptible-dose dependent and fluconazole resistant isolates of *C. glabrata* and *C. krusei*. Cinnamaldehyde was active against the 90% of isolates studied at 64 mg/l (IC90), followed by thymol and carvacrol (IC90 128 mg/l for both compounds); therefore, cinnamaldehyde was the most active against all *Candida* species except *C. metapsilosis* (IC 256 mg/l). The activity of all phytocompounds was fungicidal against *Candida* planktonic cells (Table 2). Cinnamaldehyde was the most effective agent, followed by thymol and carvacrol (GM MFC: 99.0 mg/l, 241.2 mg/l and 251.0 mg/l, respectively).

3.3. In vitro *antifungal susceptibility testing against* Candida *sessile cells*

Ten clinical isolates, including eight *C. albicans* (one fluconazole resistant), one fluconazole resistant *C. dubliniensis* and one *C. tropicalis*, were selected for their high biofilm production to study the effect of phytocompounds and antifungal agents against adhesion and preformed biofilms (Table 3). Anidulafungin was active reducing both the biomass and the metabolic activity in the adhesion phase of *Candida* biofilm formation of all isolates (GM PSMIC 0.134 and 0.125 mg/l, respectively). Carvacrol, cinnamaldehyde and thymol were active inhibiting the metabolic activity of the adhesion phase (GM PSIC 104 mg/l, 97 mg/l and 157.6 mg/l); as well as reducing the biomass, in which the cinnamaldehyde was the most effective (GM PSIC 111.4 mg/l), although thymol and carvacrol also showed good activity (GM PSIC 147 mg/l and 157.6 mg/l, respectively).

Table 1. *In vitro* activity of carvacrol, cinnamaldehyde, thymol and current antifungal drugs against 35 isolates of *Candida.*

***Candida* species (n)**		**Carvacrol [2-1024 mg/l]**	**Cinnamaldehyde [2-1024 mg/l]**	**Thymol [2-1024 mg/l]**		**Anidulafungin [0.016-8 mg/l]**	**Isavuconazole [0.016-8 mg/l]**	**Fluconazole [0.12-64 mg/l]**
***C. albicans* (10)**	**IC GM**	128	59.7	104.0	**MIC GM**	0.02	0.03	0.35
	Mode	128	64	128	**Mode**	0.016	0.016	0.25
	Range IC	128	32 - 64	32 - 256	**Range MIC**	0.016 - 0.03	0.016 - 8	0.12 - >64
***C. glabrata* (10)**	**IC GM**	119.4	68.6	111.4	**MIC GM**	0.04	0.09	12.1
	Mode	128	64	128	**Mode**	0.06	0.125	16
	Range IC	16 - 512	64 -128	32 - 128	**Range MIC**	0.03 - 0.06	0.03 - 0.5	4 - >64
***C. krusei* (3)**	**IC GM**	80.6	50.8	64	**MIC GM**	0.04	0.1	50.8
	Mode	128	64	-	**Mode**	0.03	0.125	64
	Range IC	32 -128	32 -128	32 -128	**Range MIC**	0.03 - 0.06	0.06 - 0.12	32 - >64
***C. dubliniensis* (3)**	**IC GM**	128	64	64	**MIC GM**	0.02	0.13	1
	Mode	128	64	64	**Mode**	0.03	0.016	0.125
	Range IC	128	64	64	**Range MIC**	0.016 - 0.03	0.016 - 8	0.12 - >64
***C. parapsilosis* (2)**	**IC GM**	64	90.5	90.5	**MIC GM**	2	0.02	0.5
	Range IC	64	64 - 128	64 - 256	**Range MIC**	0.03 - 2	0.016 - 0.5	0.5 - 2
***C. guilliermondii* (2)**	**IC GM**	64	64	90.5	**MIC GM**	0.25	0.24	4
	Range IC	64	64	64 -128	**Range MIC**	0.25	0.12 - 0.5	2 - 8
***C. orthopsilosis* (2)**	**IC GM**	64	32	64	**MIC GM**	0.5	0.016	0.4
	Range IC	64	32	64	**Range MIC**	0.5	0.016	0.25 - 0.5
***C. tropicalis* (2)**	**IC GM**	128	64	128	**MIC GM**	0.02	0.016	5.7
	Range IC	128	64	128	**Range MIC**	0.016 - 0.03	0.016	0.5 - >64
***C. metapsilosis* (1)**	**IC**	64	256	64	**MIC**	0.25	0.016	2
Total	**IC GM**	105	61.5	93.2	**MIC GM**	0.05	0.05	2.4
	Mode	128	64	128	**Mode**	0.016	0.016	0.125
	Range IC	16 - 512	32 - 128	32 - 256	**Range MIC**	0.016 - 2	0.016 - 8	0.12 - >64

IC: inhibitory concentration, GM: geometric mean, MIC: minimum inhibitory concentration.

Table 2. Minimum fungicidal concentration (MFC) of carvacrol, cinnamaldehyde and thymol against 35 isolates of *Candida.*

***Candida* species (n)**		**Carvacrol**	**Cinnamaldehyde**	**Thymol**
		[2-1024 mg/l]	**[2-1024 mg/l]**	**[2-1024 mg/l]**
***C. albicans* (10)**	**MFC GM**	256	119.4	256
	Mode	256	128	256
	Range MFC	256	64 – 128	256
***C. glabrata* (10)**	**MFC GM**	238.9	119.4	238.9
	Mode	256	128	256
	Range MFC	128 - 512	64 - 128	128 - 256
***C. krusei* (3)**	**MFC GM**	256	64	256
	Mode	256	64	256
	Range MFC	256	64	256
***C. dubliniensis* (3)**	**MFC GM**	161.3	64	161.3
	Mode	128	64	128
	Range MFC	128 - 256	64	128 - 256
***C. parapsilosis* (2)**	**MFC GM**	256	128	256
	Range MFC	256	128	256
***C. guilliermondii* (2)**	**MFC GM**	362	64	256
	Range MFC	256 - 512	64	256
***C. orthopsilosis* (2)**	**MFC GM**	256	64	256
	Range MFC	256	64	256
***C. tropicalis* (2)**	**MFC GM**	362	91	256
	Range MFC	256 - 512	64 - 128	256
***C. metapsilosis* (1)**	**MFC**	256	128	256
Total	**MFC GM**	251.0	99.0	241.2
	Mode	256	128	256
	Range MFC	128 - 512	64 -128	128 - 256

GM: geometric mean, MFC: minimum fungicidal concentration.

Fig. 2 shows mature biofilm growth inhibition of ten *Candida* isolates analyzed by biomass and metabolic activity determination assays. Anidulafungin and cinnamaldehyde showed activity inhibiting the metabolic activity but were less effective reducing biomass (GM SMIC 0.9 mg/l with XTT and 29.6 mg/l ml with CV; GM SIC 118.5 mg/l with XTT and 1896.2 mg/l with CV assays, respectively). Conversely, carvacrol and thymol reduced the biomass of most of the mature biofilms, and also showed activity inhibiting metabolic

Table 3. In vitro activity of carvacrol, cinnamaldehyde, thymol and current antifungal drugs against pre-sessile and sessile *Candida* cells.

Candida isolates	Carvacrol				Cinnamaldehyde				Thymol				Anidulafungin				Isavuconazole			
	Range analysed [mg/l]				Range analysed [mg/l]				Range analysed [mg/l]				Range analysed [mg/l]				Range analysed [mg/l]			
	[8-1024]		[16-2048]		[8-1024]		[16-2048]		[8-1024]		[16-2048]		[0.125-16]		[0.25-32]		[0.125-16]		[0.25-32]	
	PSIC		SIC		PSIC		SIC		PSIC		SIC		PSMIC		SMIC		PSMIC		SMIC	
	CV	XTT	CV	XTT	CV	XTT	CV	XTT	CV	XTT	CV	XTT	CV	XTT	CV	XTT	CV	XTT	CV	XTT
***C. albicans* SC5314**	128	64	≥2048	128	64	64	≥2048	128	256	64	≥2048	128	0.125	0.125	32	2	8	0.125	≥32	≥32
C. albicans Ca2	128	256	-	-	64	64	-	-	128	128	-	-	0.125	0.125	-	-	0.25	≥16	-	-
***C. albicans* UPV 05-013**	128	64	≥2048	128	128	64	≥2048	128	128	32	≥2048	128	0.125	0.125	≥32	0.25	2	0.125	≥32	32
***C. albicans* UPV 05-007**	256	128	512	128	128	128	≥2048	64	256	256	≥2048	128	0.125	0.125	≥32	0.25	8	≥16	≥32	≥32
***C. albicans* UPV 11-342**	256	64	512	512	128	128	≥2048	256	256	128	1024	256	0.125	0.125	≥32	≥32	4	≥16	≥32	≥32
***C. albicans* UPV 11-345**	256	64	1024	512	128	128	≥2048	64	256	128	≥2048	512	0.25	0.125	≥32	0.25	8	0.125	≥32	≥32
***C. albicans* UPV 12-298**	128	128	512	128	64	64	≥2048	128	32	64	512	256	0.125	0.125	≥32	0.25	≥16	≥16	≥32	≥32
***C. albicans* UPV 15-101**	128	128	512	128	256	128	≥2048	64	128	256	512	16	0.125	0.125	16	≥32	2	0.125	1	4
***C. albicans* UPV 15-106**	128	256	512	128	128	128	1024	128	128	256	256	32	0.125	0.125	≥32	0.25	≥16	≥16	32	0.25
***C. albicans* UPV 15-157**	128	64	1024	256	128	128	≥2048	256	128	64	≥2048	256	0.125	0.125	≥32	0.25	≥16	1	≥32	≥32
GM	152.6	104	812.7	188.1	111	97	1896.2	118.5	147	157.6	1106	128	0.134	0.125	29.6	0.9	4.9	1.7	21.8	14.8
Mode	128	64	512	128	128	128	≥2048	128	128	64	≥2048	128	0.125	0.125	≥32	0.25	≥16	≥16	≥32	≥32
***C. dubliniensis* UPV 11-366**	128	32	512	128	128	32	≥2048	128	64	64	≥2048	128	0.125	0.125	≥32	16	≥16	≥16	≥32	≥32
***C. tropicalis* UPV 06-115**	128	64	512	128	128	128	≥2048	256	64	64	512	128	0.125	0.125	≥32	≥32	≥16	0.125	≥32	≥32

Minimum Pre-sessile ICs (PSICs) were those concentrations causing 50% metabolic inhibition and 50% biomass reduction with respect to controls without compound. Sessile ICs (SICs) were those concentrations causing 50% metabolic inhibition and 50% biomass reduction with respect to controls. CV: crystal violet assay - biomass quantification, XTT: XTT assay - metabolic activity determination.

activity (GM SIC 188.1 mg/l with XTT and 812.7 mg/l with CV; 128 mg/l with XTT and 1106 mg/l with CV assays, respectively); while isavuconazole was less active reducing biomass and metabolic activity of mature biofilms.

All phytocompounds were effective against the azole-resistant *Candida* isolates inhibiting significantly the metabolic activity of mature biofilm, with a reduction of 80% or more with the highest concentration assayed (2048 mg/l, figure 2). Moreover, a significant reduction was observed with 64 mg/l of the three phytocompounds against *C. albicans* UPV 15-157 isolate, and from the lowest concentration (16 mg/l) against *C. dubliniensis* UPV 11-366 isolate and against the susceptible isolates.

3.4. Morphology and architecture of the biofilm after treatment with phytocompounds

Fig. 3 shows the cell morphology and the changes in the ultrastructure of the mature *C. albicans* SC5314 biofilm treated with the respective SIC of carvacrol, cinnamaldehyde and thymol (2048 mg/l) observed by SEM. Untreated *Candida* biofilm presented filamentation, abundant hyphae with well-defined shapes and oval shaped yeast cells with smooth surfaces and polar bud scars. In biofilms treated with all phytocompounds, damage was evident presenting deformed hyphae, irregular surfaces in some sites with deposit of lytic material, reduction of number of cells and absence of hypha production. Untreated and treated biofilm showed absence of ECM due to dehydration during SEM procedures.

The mature biofilm of *C. albicans* SC5314 treated with 2048 mg/l of each phytocompound in the same conditions described above but monitored by CLSM is shown in Fig. 4. Untreated *Candida* biofilm exhibited high cell viability, while on the treated biofilms, the viability decreased significantly, and the amount of dead *Candida* cells increased. CLSM allowed to visualize cell viability of treated and untreated biofilms and confirmed the results of metabolic activity assay.

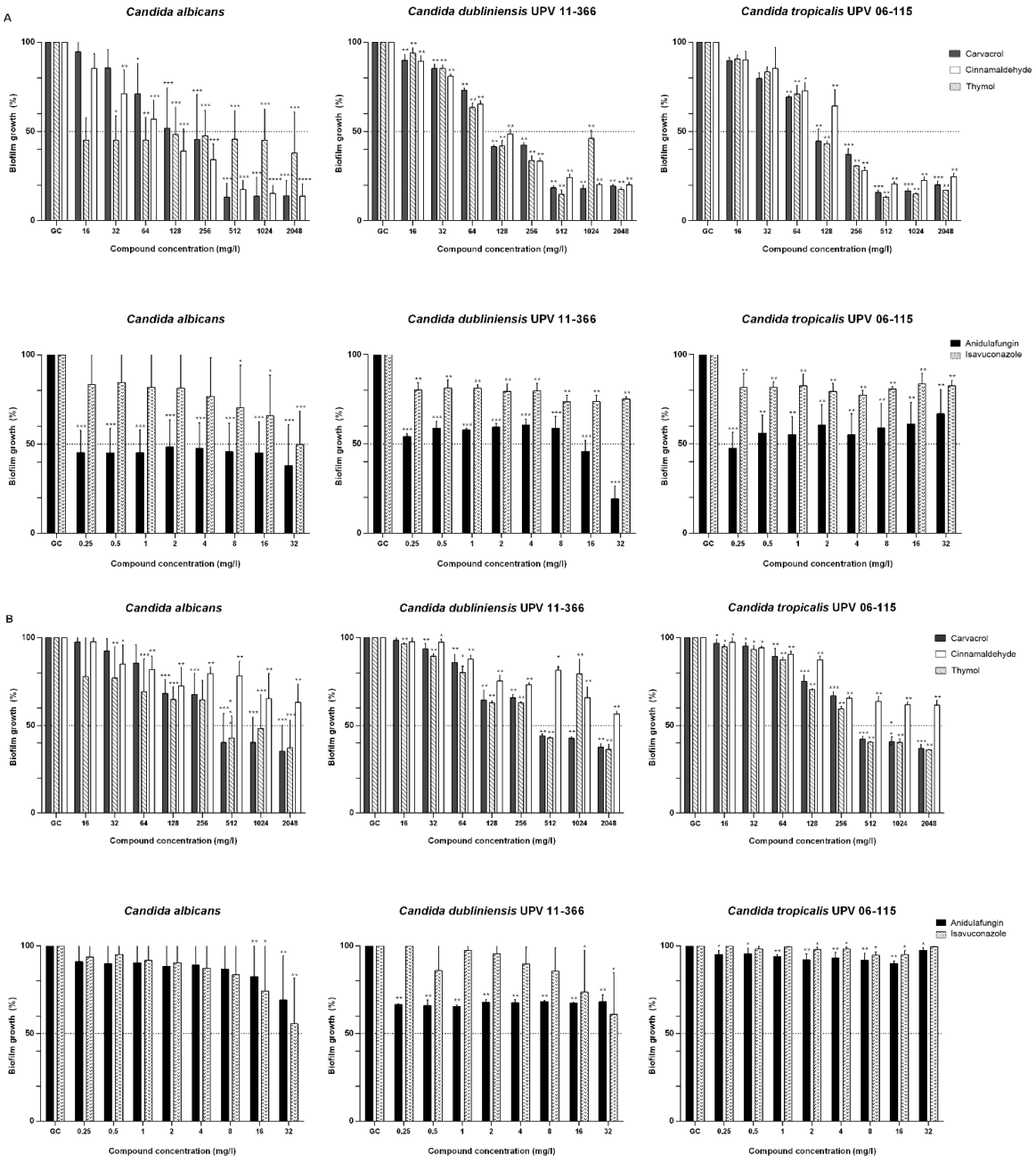

Fig. 2. Biofilm growth inhibition of mature biofilms treated with carvacrol, cinnamaldehyde, thymol, anidulafungin and isavuconazole. Metabolic activity (A) and biomass (B). *Candida albicans*: mean of eight isolates including a fluconazole resistant isolate. GC: growth control. *: $p < 0.05$, **: $p < 0.01$ respect to the growth control without antifungal agents.

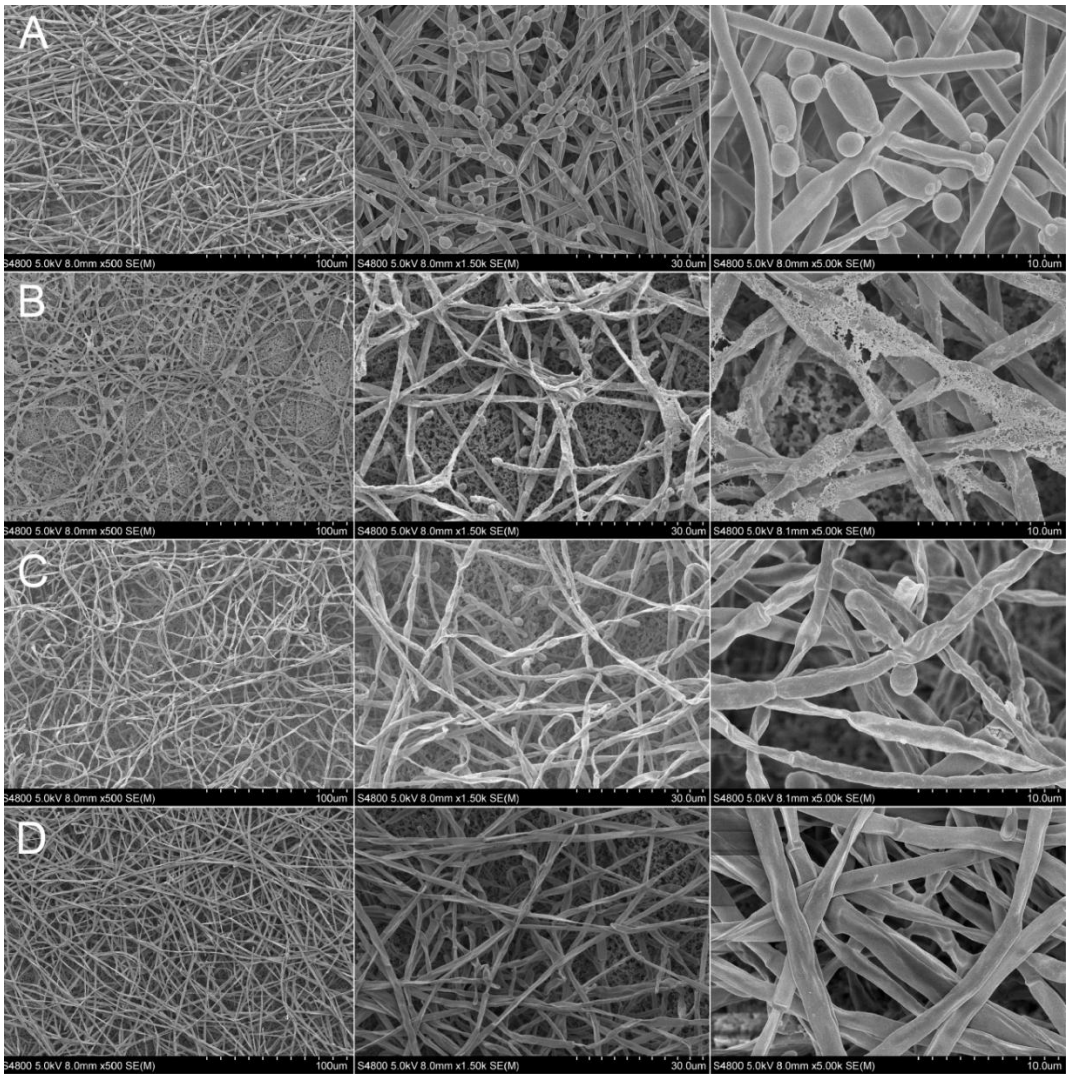

Fig. 3. Images of *C. albicans* SC5314 biofilm by scanning electron microscopic at 500x, 1000x and 5000x. Untreated biofilm (A), biofilm treated with 2048 mg/l of carvacrol (B), 2048 mg/l of cinnamaldehyde (C) and 2048 mg/l of thymol (D).

4. Discussion

Oral candidiasis is a common superficial infection in immunocompromised patients, denture wearers, and in the elderly and new-borns [1]. Pseudomembranous candidiasis, erythematous candidiasis, hyperplastic candidiasis, denture stomatitis and angular cheilitis are clinical manifestations of this *Candida* infection. Classical treatment of oral candidiasis has some important issues mainly related to the development of antifungal resistances, the

limited availability of antifungal agents and their potential toxicity, so new therapeutic alternatives should be implemented [15]. Essential oils stand out in the management of oral infections because of their antimicrobial activity and have been included in oral rinses [6,16]. However, reliable information on their potential use in antifungal therapy is scarce. These oils are complex mixtures of chemically heterogeneous components obtained from aromatic plants that have numerous biological properties including antioxidant, antimicrobial, antitumor and analgesic activities [17-21].

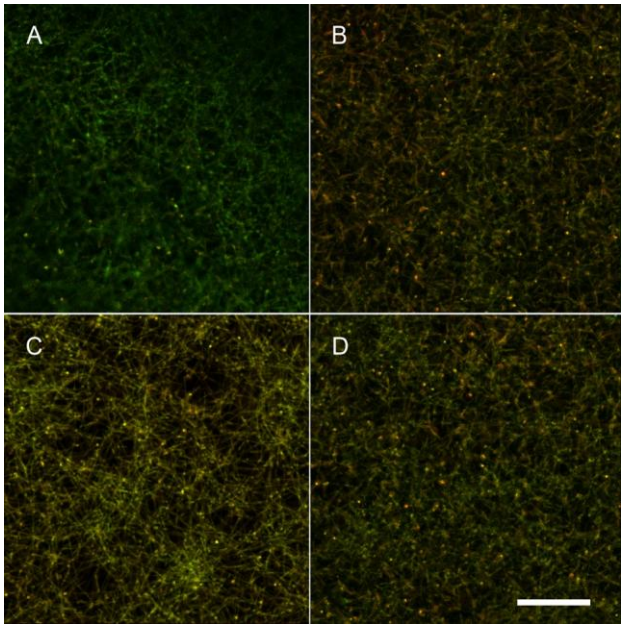

Fig. 4. CLSM analysis of C. albicans SC5314 biofilm stained by LIVE/DEAD Yeast kit. Untreated biofilm (A), biofilm treated with 2048 mg/l of carvacrol (B), 2048 mg/l of cinnamaldehyde (C) and 2048 mg/l of thymol (D). Green staining indicates live cells and yellow/orange staining indicates dead cells. Bar scale of 100 µm.

While the study of antimicrobial properties of essential oils as a whole has gained considerable interest, comparisons of published results is often difficult and has limitations that hinder its clinical application. These limitations include the variability among studies

due to different composition of chemical constituents, the influence of the time of harvesting, the part and lot of the plant used or the methodology of extraction, and the hypersensitivity reactions associated to their use [22]. On the other hand, the study of pure phytochemical compounds can present important advantages, as it facilitates the comparison of results among research and toxic side effects related to other components present in the essential oils are avoided. Moreover, previous studies have indicated a significantly superior and sustained inhibitory effect of components compared to essential oils [7].

Carvacrol and its isomer thymol are monoterpenoid phenols present in major proportion in the essential oils extracted from plants of *Lamiaceae*, *Verbenaceae*, *Scrophulariaceae*, *Ranunculaceae*, and *Apiaceae* families [20]. Cinnamaldehyde is a phenylpropanoid present in essential oils from several trees from genus *Cinnamomum* of *Lauraceae* family [21]. In the current study, carvacrol, cinnamaldehyde and thymol showed activity against planktonic and sessile *Candida* cells. In the case of planktonic cells, cinnamaldehyde presented the strongest antifungal fungicidal activity from low concentrations as reported by Rajput and Karuppayil [23], followed by thymol and carvacrol. The latter phytocompounds were also very active against all *Candida* species studied, even against isolates with reduced susceptibility or resistance to fluconazole, as previously described [24,25]. Although the mode of action seems to be directed towards ergosterol synthesis, differences and similarities between these phytocompounds could be explained by their chemical structure. Carvacrol and thymol both have hydroxyl groups and belong to the same chemical class, while cinnamaldehyde would exert its greater activity due to its aldehyde group, as hypothesized in a study of the inhibitory activity of these compounds via vapour phase mediated susceptibility assay [26].

Biofilms are communities of adherent sessile cells with different properties from those of planktonic cells [27]. Biofilm formation is an important virulence factor because the presence of these microbial communities is related to increased resistance to antimicrobial agents and recalcitrant infections such as denture stomatitis, chronic mucocutaneous candidiasis and chronic multifocal candidiasis [1]. In the current study, *C. albicans* was the most biofilm producing species, but also *C. tropicalis* isolates were high biofilm producers. *C. tropicalis* and *C. glabrata* are frequently co-isolated with *C. albicans* from patients with oral infections or colonization [4,28]. Moreover, co-infection or prior infection with *C. albicans* may facilitate *C. glabrata* infection [29]. These fungal associations could change antifungal susceptibility patterns and reduce the effectiveness of conventional treatments for candidiasis [15]. In the current study, the three phytochemicals tested and anidulafungin have shown anti-*Candida* biofilm activity, in contrast with the low antibiofilm activity of isavuconazole. The last one, a second-generation triazole, although active against planktonic fungal cells, was not active against *Candida* biofilms as reported in studies with antifungal agents of the same family [30]. Anidulafungin showed activity against almost all *C. albicans* biofilms, although it was not active against *C. tropicalis* biofilms, as described in previous reports showing that echinocandins do not eradicate or kill *C. tropicalis* biofilms [31,32]. Comparisons on biofilm production capacity among different species are subject to a number of limitations inherent to the methodologies employed due to both the methodologies employed and the specific biofilm production patterns of each species. Isolate classification according to biofilm production capacity contemplated both metabolic activity and biomass results, as recommended by other authors [33]. These procedures are broadly used in the literature, despite the lack of standardized methodologies. However, other media did not used in the present work could be tested to assess the biofilm production in order to resemble the normal conditions in the oral cavity.

Carvacrol and thymol may be efficient alternatives for eradicating biofilm-associated recalcitrant infections, considering that in our study they were more active than cinnamaldehyde in reducing metabolic activity and biomass of mature biofilm. Biomass reduction could play a crucial role in the management of recalcitrant infections, as biofilms are a source for dispersal of cells with advantageous characteristics, such as the ability to form new biofilms more efficiently, enhanced adhesion and virulence, as reported in mice models of candidiasis [27,34]. Carvacrol and thymol reduced at 24 h more than 85% of *Candida* biofilms metabolic activity of azole-susceptible isolates and until 80% of mature biofilms of azole-resistant isolates. These results are supported by the CLSM observation of *Candida* biofilms treated with these phytocompounds in which loss of cell viability was evident. Moreover, these results are in line with those described by Braga et al. [35], who observed a reduction of 45.1% in metabolic activity of *C. albicans* biofilm at 6 h of incubation with thymol, 68% inhibition at 12 h and 88.3% at 24 h. Similarly, Doke et al. [17] stated that carvacrol eugenol and thymol were very effective in reducing > 80% of metabolic activity of *C. albicans* biofilm. Dalleau et al. [24] showed that mature biofilms of *C. albicans*, *C. glabrata* and *C. parapsilosis* isolates were susceptible to carvacrol, geraniol and thymol, even the fluconazole-resistant isolates. Carvacrol and thymol are moderately water-soluble and their effectiveness against *Candida* is associated to cell membrane rupture and solubilization, together with ergosterol biosynthesis inhibition [20,36].

Cinnamaldehyde, in our study, reduced biofilm metabolic activity but its effect on biomass reduction was lower. Moreover, the highest activity of this phytocompound was obtained in the early phase, preventing the formation of biofilm. Almeida et al. [16] reported anti-*Candida* biofilm activity of cinnamon and its components, among which is cinnamaldehyde. The mode of action of cinnamaldehyde against biofilm could be related to its capacity to reduce adhesion on biotic and abiotic surfaces by downregulating *HWP1* gene [18]. This

gene is involved in hyphal formation on early stages of biofilm development. In addition, cinnamaldehyde could cause a loss in cell wall integrity by ergosterol depletion [21,23] and an apoptotic effect [18].

Oral and denture cleansers that include phytocompounds in their formula have been extensively used. However, there are interesting reports that indicate increased *C. albicans* cell counts from mixed oral biofilms exposed to denture cleanser [37]. Therefore, given the importance of *C. albicans* in the pathobiology of oral infections such as denture stomatitis, dental caries and other biofilm-related clinical presentations [38,39], a careful design of the formula of these cleansers should be advisable.

Unlike to the findings of previous studies, our study evaluated biofilm biomass as an important parameter related to the persistence of the biofilm after treatment, and not only with its metabolic activity. Hence, the present work provides consistent data on the effect of pure compounds against biofilm formation of different *Candida* species and relevant knowledge about the capacity to remove biomass of mature biofilm, thus limiting recurrence. These properties could be promising for its application in biomedical fields, such as the design of antifungal coatings for biomedical devices from pure phytocompounds avoiding the disadvantages of the essential oils described above.

Nonetheless, limitations of the use of phytocompounds from essential oils lie in its poor solubility in aqueous solution, volatility and instability, and possible hypersensitivity reactions. Furthermore, the typical dosage of oral rinses enables only short exposure to phytocompounds and may not be suitable to treat active infections. Indeed, the clinical applications of pure phytochemical compound may be attainable using mainly prolonged release formulations instead of cleanser solutions. Nanotechnology can help developing nanoparticles with less adverse effects, better bioavailability and site-specific delivery.

Cinnamaldehyde could be also included in antibiofilm material coatings, while carvacrol and thymol can be used to design curative antibiofilm therapy. Use of drug combinations, in which the phytocompounds would degrade the matrix and other drugs exert antifungal actions, could be beneficial to improve the clinical response to therapy in candidiasis associated to biofilms, where azoles have proven little or no effect [40].

5. Conclusion

The use of phytocompounds from essential oils has potential in the improvement of established infections without developing further resistance associated to treatment. Carvacrol, cinnamaldehyde and thymol are promising alternatives for candidiasis treatment: cinnamaldehyde by preventing biofilm formation and carvacrol and thymol against established biofilms. However, toxicity and viability studies must be done in animal models and cellular cultures to establish the optimal use of these phytocompounds as antifungal agents in clinical assays.

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CRediT authorship contribution statement

KM-C, CM-A, GQ and EE: Conceptualization. KM-C: Methodology. KM-C, CM-A and EM: Formal analysis and investigation. JMA-U: Resources. KM-C and CM-A: Writing—original draft preparation. EM, JMA-U, GQ and EE: Writing—review and editing. GQ and EE: Supervision and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest Statement

The authors declare that there are no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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4.3 Study 3: *In vitro* and *in vivo* antifungal activity of citral

The activity of citral combating *Candida* was evaluated *in vitro* against planktonic and sessile cells in monotherapy and in combination with fluconazole. The anti-*Candida* effect of citral was observed against planktonic and sessile cells. Citral was active against planktonic cells of all the studied isolates, even against azole-resistant isolates (GM IC: 197.89 mg/L, and GM MFC: 649.35 mg/L) (Table S2 from the manuscript 3, pag. 223).

This phytocompound was effective in reducing the biomass as well as the metabolic activity of early biofilms (PSIC: CV assay 149.3 mg/L and XTT assay 47 mg/L). Additionally, citral reduced the metabolic activity of mature biofilms to a greater extent than biofilm biomass (SIC: XTT assay 237 mg/L and CV assay 1290.2 mg/L) (Table 2 from the manuscript 3, pag. 201). *Candida* biofilms treated with 256 mg/L citral presented significant alterations in cellular architecture, such as a decreased number of blastospores and changes in mycelial surface and quantity, in contrast with the untreated biofilm observed by SEM (Figure 4.2).

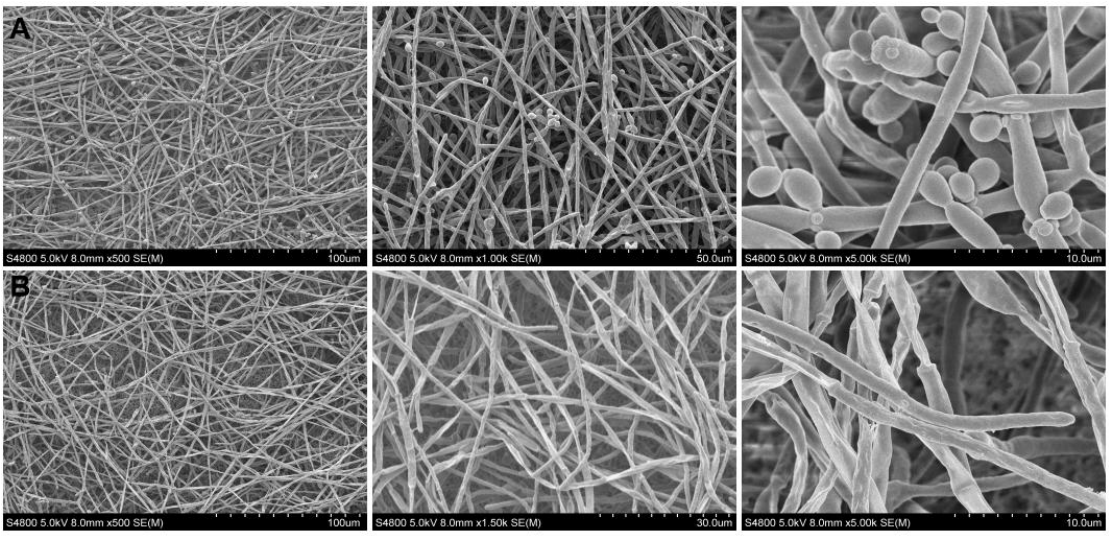

Figure 4.2. Images of *C. albicans* SC5314 biofilm taken by scanning electron microscopy at 500, 1000-1.500 and 5000x. A: Untreated biofilm, B: Biofilm treated with 256 mg/L of citral.

A synergistic effect was obtained from by the checkerboard assay results for the combination of citral and fluconazole against planktonic cells of different fluconazole-resistant *Candida* species, except *C. krusei* isolates (Table 3 from manuscript 3, pag. 203). The time-kill curves results for the combination of citral and fluconazole did not show a significant decrease in CFU/mL compared to fluconazole alone. However, the highest decrease was observed in the combination of 256 mg/L of citral and 8 mg/L of fluconazole against azole-resistant isolates for 6 h and 24 h (Figure 3 from manuscript 3, pag. 205).

In addition, the combination of citral at 0.25 mg/L and fluconazole at 256 mg/L reduced the metabolic activity on all mature *Candida* biofilms, as determined by the XTT reduction assay (synergism at FICI 0.5 in *C. albicans* UPV 15-157), but the biofilm biomass was not affected (Figure 2 from manuscript 3, pag. 204, and Figure 4.3).

The synergism of citral and fluconazole was assayed in the treatment of *Candida* infection in *C. elegans* model. These combinations, at 32 or 128 mg/L citral and 2 mg/L fluconazole, also led to a synergistic effect on azole-resistant *Candida* infection in the *in vivo* model (Figure 5 and Table 4 from manuscript 3, pag. 209 and 208, respectively).

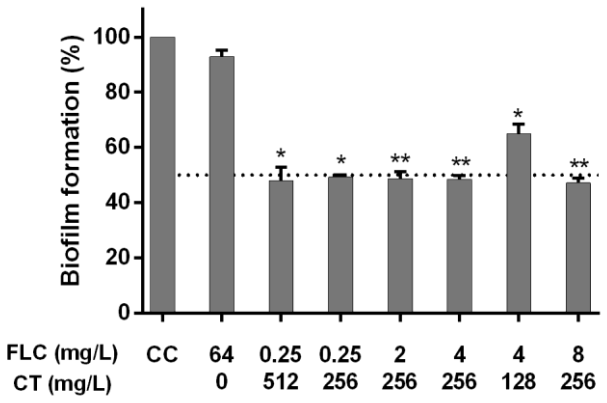

Figure 4.3. Activity of citral (CT) in combination with fluconazole (FLC) against mature biofilm of the *C. dubliniensis* UPV 11-366 azole-resistant isolate by XTT reduction assay. *: $p < 0.05$, **: $p < 0.01$. GC: growth control.

Moreover, the mechanism of action of citral on the expression of *CDR1, ERG11* and *MDR1* genes, related to fluconazole resistance, was analysed. Downregulation of *MDR1* was noticed in *Candida* cells after treatment with the combination of 128 mg/L of citral plus 1 mg/L of fluconazole (Figure 4 and 6 from manuscript 3, pag. 206 and 215, respectively).

The results obtained in study 3 have been submitted for publication, which are presented in the following pages (manuscript 3).

Manuscript 3

In vitro and in vivo anti-*Candida* activity of citral in combination with fluconazole

Katherine Miranda-Cadena, Cristina Marcos-Arias, Aitzol Perez-Rodriguez, Iván Cabello-Beitia, Estibaliz Mateo, Elena Sevillano, Lucila Madariaga, Guillermo Quindós, Elena Eraso.

Submitted for publication, 2021.

In vitro and in vivo anti-*Candida* activity of citral in combination with fluconazole

Running title: Antifungal activity of citral

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***Abbreviations*:** IC, Inhibitory concentration; SICs, Sessile inhibitory concentrations; PSICs, Pre-sessile inhibitory concentrations; CV, crystal violet assay; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium -5-carboxanilide; CT, citral.

Highlights:

- The combination of citral and fluconazole is synergistic against planktonic and sessile *Candida* cells.
- Citral downregulates *MDR1* expression in fluconazole-resistant *Candida albicans*.
- Citral in combination with fluconazole was an effective treatment against *Candida* infection.

Abstract

The aim of this study was to evaluate the antifungal activity of citral, a phytocompound present in lemongrass essential oil, in monotherapy and combined with fluconazole against azole-resistant *Candida* planktonic cells and biofilms. The effect of citral combined with fluconazole was also analyzed with regard to the expression of fluconazole resistance-associated genes in *Candida albicans* and the effectiveness of the combination therapy in a *Caenorhabditis elegans* model of candidiasis. Citral reduced biofilm formation at initial stages and the metabolic activity of the mature biofilm. The combination of citral with fluconazole was synergistic, with a significant increase in the survival of *C. elegans* infected with *Candida*. RNA analysis revealed a reduction of the expression of efflux pump encoded by *MDR1*, leading to a greater effect of fluconazole. In conclusion, citral in monotherapy and in combination with fluconazole could represent a promising therapy to treat recalcitrant *Candida* infections associated to biofilms.

Keywords: *Candida*, biofilm, citral, azole resistance, *Caenorhabditis elegans*, gene expression.

1. Introduction

Superficial and invasive candidiasis are very common infectious diseases [1–3], being *Candida albicans* a frequent etiological agent of these clinical entities [1,3,4]. The shift from colonization to *Candida* infection is related to local and systemic factors of the patient, including the treatment with immunosuppressive therapies, broad spectrum antibiotics or the use of dentures [5]. The ability of *C. albicans* and other species of *Candida* to develop biofilms on inert surfaces or living tissues favors recalcitrant and chronic candidiasis associated, in many instances, with resistance to current antifungal therapy [5–7].

Fluconazole is one of the first-line antifungal agents for treating candidiasis. This triazole interferes with the biosynthesis of ergosterol inhibiting the lanosterol 14-α-demethylase (Erg11), an essential cytochrome P450 enzyme encoded by *ERG11* [8]. Fluconazole resistance of *Candida* biofilms is due to several complex mechanisms, including increased metabolic activity and genetic alterations, such as overexpression of genes implicated in efflux pumps in the early stages of biofilm formation [6,9]. In mature biofilms, resistance is associated with a variation in sterol composition of the extracellular polymeric matrix that hinders access and internalization of fluconazole in the sessile cell [10]. In the case of planktonic cells, several mechanisms have been described in *C. albicans*, including *ERG11* point mutations, overexpression of Erg11 mediated by the zinc-cluster transcriptional regulator Upc2, overexpression of Mdr1 and Cdr1p/Cdr2 efflux pumps, inactivation of the *ERG3* gene, aneuploidy (related to Chr5) and/or loss of heterozygosity [8].

With regard to oral candidiasis, fluconazole resistance has been reported in *C. albicans* isolates recovered from patients receiving previous fluconazole treatments [11]. In addition, oral candidiasis could be a potential source for candidemia in immunocompromised patients and, therefore, an adequate and effective treatment is of high relevance [12].

Phytocompounds from different plants have been reported as an alternative treatment for candidiasis, alone or in combination with fluconazole, due to their antifungal properties [13]. One of these phytocompounds is citral, a monoterpenoid aldehyde, which occurs as geraniol or citral A (*trans*-isomer) and neral or citral B (*cis*-isomer) in the essential oils of lemongrass (*Cymbopogon citratus* and *Cymbopogon flexuosus*) and other plants [14]. This phytocompound has demonstrated a promising antimicrobial activity in previous studies [15–17]. However, the mechanism of action of citral in combination with fluconazole against *Candida* species has not been clarified.

The aim of this study was to evaluate the in vitro and in vivo antifungal activity of citral, in monotherapy and in combination with fluconazole, against biofilm forming *Candida* isolates. The effect of citral on the expression of *ERG11*, *CDR1* and *MDR1* genes associated with azole resistance was also assessed.

2. Materials and methods

2.1 Microorganisms

Thirty-five *Candida* isolates from patients suffering oral candidiasis attending the Dental Clinical Service at the University of the Basque Country (UPV/EHU) in Bilbao (Spain) were analyzed. These isolates included 10 *C. albicans*, 10 *Candida glabrata*, three isolates each of *Candida dubliniensis* and *Candida krusei*, two isolates each of *Candida guilliermondii*, *Candida orthopsilosis*, *Candida parapsilosis* and *Candida tropicalis*, and one *Candida metapsilosis*. After testing the susceptibility of planktonic cells to citral and fluconazole, six isolates with reduced susceptibility to fluconazole were selected for testing the activity of citral combined with fluconazole, including one isolate each of *C. albicans*, *C. dubliniensis* and two isolates each *C. glabrata* and *C. krusei,* and one susceptible *C. tropicalis*. This combined

activity of citral with fluconazole was also tested against sessile cells-biofilms of two *C. albicans* isolates (UPV 15-157 and UPV 11-336). On the other hand, ten isolates previously classified as high or moderate metabolic biofilm producers and high biomass biofilm producers were included in the present work to test the effect of this phytocompound against biofilms (adhesion phase of biofilm-pre-sessile cells and mature biofilm-sessile cells) [18]. Reference strains obtained from the American Type Culture Collection (ATCC) were also included in the study: *C. albicans* ATCC 64124, *C. albicans* SC5314 (also identified as ATCC MYA-2876), *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, and the hypha-defective mutant *C. albicans* Ca2 (kindly donated by Professor Antonio Cassone, Istituto Superiore di Sanità, Rome, Italy) (Table S1).

The in vivo studies were performed in a candidiasis model on *Caenorhabditis elegans*. The double mutant *C. elegans* AU37 strain (*glp-4;sek-1*) and the *Escherichia coli* strain OP50 used as a food source for the nematodes were supplied by the *Caenorhabditis* Genetics Center (University of Minnesota, USA).

2.2 Phytocompound and antifungal agent

Citral (95% of purity) and fluconazole (98% of purity) were purchased from Sigma-Aldrich (USA). Stock solutions were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich). The citral was prepared on the day of use, while fluconazole was stored for up to one month at –70 ºC.

2.3 *In vitro antifungal susceptibility testing*

In vitro antifungal activity against planktonic cells was tested according to the European Committee for Antimicrobial Susceptibility Testing (EUCAST) broth microdilution method described in the document EDef 7.3.2 [19]. The final concentrations of fluconazole ranged from 0.12 to 64 mg/L, while those of citral ranged from 2 to 1024

mg/L. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality controls. Absorbance of the microplates was measured at 450 nm by the iMark plate reader (BioRad, USA) after 24 and 48 h of incubation at 37 °C. Assays were conducted by triplicate on three independent experiments. Citral inhibitory concentration (IC) and fluconazole minimal inhibitory concentration (MIC**)** were calculated at 24 h as the lowest drug concentration inhibiting ≥ 50% of growth compared to controls without the compound [20]. Minimum fungicidal concentration (MFC) was defined as the lowest concentration of antifungal agent resulting in the death of 99.9% of the inoculum [20].

2.4 *Development of* Candida *biofilms*

The ability to produce biofilm was evaluated in 35 oral isolates as previously described [18]. *Candida* biofilm-producing tests were developed in sterilized, flat-bottomed honeycomb 100-well polystyrene microtiter plates (Labsystems, Finland). A cell suspension was adjusted using an hemocytometer to a final concentration of 1×10^6 cells/mL using RPMI-1640 supplemented with L-glutamine and buffered at pH 7 with 0.165 M of 3-(N-morpholino) propanesulfonic acid (MOPS, Sigma-Aldrich). One hundred μL of this cell suspension were added into each well of the plates. Two identical microtiter plates were prepared, one to determine metabolic activity and the other to quantify biomass. After 24 and 48 h of incubation at 37 ºC, unattached or poorly attached cells were removed by washing three times with sterile phosphate buffered saline solution (PBS).

Metabolic activity of the biofilm was tested following the protocol previously described [21]. Briefly, 100 μL of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT, Sigma-Aldrich) with 1 μM of menadione were added to each prewashed well and incubated in the dark for 2 h at 37 ºC. After that, the absorbance of microplates was measured on the microtiter plate reader BioScreen C MBR (Growth

Curves Ltd, Finland) at 490 nm wavelengths. Biomass quantification was measured according the method previously described using crystal violet [22]. The prewashed microplates were air-dried for 30 min, after that, 100 μL of 0.4% crystal violet solution were added to each well and incubated for 20 min at room temperature. Microplates were washed twice using 250 μL of sterile distilled water, followed by the addition of 150 μL of 33% acetic acid to each well. The absorbance of microplates was measured at 600 nm wavelengths.

2.5 Effect of citral against adhesion phase and mature Candida *biofilms*

The activity of citral against the adhesion phase of ten biofilm producer *Candida* isolates was evaluated following a method previously described [20,21]. Briefly, 100 μL of the adjusted cell suspension of each isolate (final concentration of 1×10^6 cells/mL) were inoculated into the 100-well polystyrene microtiter plates plus 100 μL of citral at final concentrations ranging from 8 to 1024 mg/L. After 24 h of incubation at 37 ºC, pre-sessile inhibitory concentrations (PSICs), which were the concentrations producing 50% of metabolic inhibition and 50% of biomass reduction with respect to controls without phytocompound, were determined by XTT reduction and crystal violet assays, respectively. *C. albicans* SC5314 and the hypha-defective mutant *C. albicans* Ca2 were included as controls.

For studying the citral activity against mature biofilms, *Candida* biofilms were developed on 100-well polystyrene microtiter plates by adding 100 μL of the adjusted inoculum into each well. After 24 h at 37 ºC, the microplates were washed twice with sterile PBS and 100 μL of the final concentrations of citral were added, ranging from 16 to 2048 mg/L in RPMI medium. A further incubation of 24 h at 37 ºC was performed. Sessile inhibitory concentrations (SICs) were determined by XTT reduction and crystal violet assays, which

correspond to those concentrations that caused 50% of metabolic inhibition and 50% of biomass reduction compared to controls, respectively.

2.6 Antifungal activity of citral in combination with fluconazole against planktonic and sessile cells

Checkerboard assay, based on the document EDef 7.3.2 from EUCAST for yeasts, was used to evaluate the antifungal activity of citral in combination with fluconazole against planktonic cells [19,23]. One azole-susceptible *C. tropicalis* isolate and six oral fluconazole-resistant isolates of *Candida*, including *C. albicans* UPV 15-157, *C. dubliniensis* UPV 11-366, *C. glabrata* UPV 08-058, *C. glabrata* UPV 14-004, *C. krusei* UPV 03-242, and *C. krusei* UPV 13-120 isolates were tested. Moreover, reference strains *C. albicans* ATCC 64124, *C. albicans* SC5314, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019, and the hypha-deficient *C. albicans* Ca2 strain were also included. The checkerboard was prepared in microtitre plates for multiple combinations of two-fold serial dilutions of citral and fluconazole. Final concentrations ranged from 2 to 1024 mg/L and from 0.125 to 64 mg/L of citral and fluconazole, respectively. ICs and MICs were determined by reading the optical density at 450 nm wavelength with a spectrophotometer.

Fractional inhibitory concentration index (FICI) represents the sum of the FICs of each drug tested. For calculation of the FICI, the FIC was obtained by dividing the MIC or IC of each drug when used in combination by the MIC or IC of each drug when used alone [23]. The in vitro interaction of the antifungal combination was interpreted as follows: FICI ≤ 0.5 synergistic; FICI > 0.5 but ≤ 4 indifferent/additive, and FICI > 4 antagonistic [20].

The evaluation of the antifungal activity of citral in combination with fluconazole was tested against sessile cells of mature biofilms of the azole-resistant isolates *C. albicans* UPV 15-157 and *C. dubliniensis* UPV 11-366 and the reference strain *C. albicans* SC5314. *Candida*

biofilms were formed on microtiter plates from 100 μL of a cell suspension adjusted to a concentration of 1×10^6 cells/mL in RPMI of each isolate, and subsequently incubated for 24 h at 37 ºC. After two washing steps with sterile PBS, final concentrations of citral (from 8 to 512 mg/L) and fluconazole (from 0.25 to 64 mg/L) were added, and a further incubation of 24 h at 37 ºC was performed. Sessile ICs (SICs) were estimated through metabolic activity determination and biomass quantification assays. FICI was calculated as previously described.

2.7 Time-kill assay

The killing activity of the combination of citral and fluconazole was tested against strains *C. albicans* UPV 15-157, *C. dubliniensis* UPV 11-366 and *C. albicans* SC5314. Final concentrations of 4, 8 and 16 mg/L of fluconazole and 128 and 256 mg/L of citral and their combinations were assayed in flat bottomed 96-well microtiter plates. After the addition of a suspension of $1\text{-}5\times10^5$ colony forming unit (CFU)/mL in RPMI (final volume of 200 μL) the microplates were incubated during 48 h at 37 °C without agitation. Aliquots from each well and condition were obtained at 0, 2, 4, 6, 24 and 48 h. The number of CFU/mL was determined after subsequent dilution on PBS and inoculation on Sabouraud dextrose agar plates, which were incubated at 37 °C for 24 to 48 h [24].

Synergism was defined as a decrease in CFU/mL ≥ 2 $\log_{10}$ compared to the most active drug, indifference as a decrease in CFU/mL < 2 $\log_{10}$ and antagonism as an increase in CFU/mL ≥ 2 $\log_{10}$ [24]. The lower limit of accurate and detectable colony count was 30 CFU/mL. These studies were conducted in at least two independent assays.

2.8 Quantification analysis by real-time reverse transcription-polymerase chain reaction (RT-PCR)

Cell suspensions of *C. albicans* SC5314, *C. albicans* ATCC 64124 and *C. albicans* UPV 15-157 were adjusted to a final concentration of 1×10^5 CFU/mL, prepared in RPMI 1640 medium in triplicate. The inocula were incubated for 24 h at 37 °C with 1 mg/L of fluconazole, 128 mg/L of citral and the combination of both concentrations. Untreated cell suspensions were used as control.

RNA was extracted from the samples incubated with the compounds and from the control, using the Total RNA Purification kit (Norgen, Biotek-Corp, Canada). The purity of RNA was determined by spectrophotometric analysis (NanodropTM 2000, Thermo Fisher Scientific, USA) and the RNA integrity was measured by automated capillary electrophoresis separation using LabChip GX Touch Analyzer (Perkin Elmer, USA). The complementary DNA (cDNA) was synthesized using the PrimeScriptTM RT Reagent kit (Takara Bio Inc., Japan). Primer sequences used to amplify the *ACT1*, *ERG11*, *CDR1* and *MDR1* genes are listed in Table 1 [25]. The expression levels of these genes were quantified by real-time PCR in the 7300 Fast Real-Time PCR thermocycler (Applied Biosystems, USA). Cycling profile included an initial step at 95 °C 30 sec; 40 cycles of 95 °C 5 sec, 50 ºC 31 sec (55 °C 31 sec for *MDR1*); and dissociation stage of 95 °C 15 sec, 60 °C for 1 min, and 95 °C 15 sec.

The experiments were performed in duplicate. Cycle threshold (Ct) values of *ERG11*, *CDR1* and *MDR1* transcripts were normalized to the Ct corresponding to the housekeeping-*ACT1*. The quantification of the gene expression was analyzed with the comparative method Ct ($2^{-\Delta\Delta Ct}$) with respect to the *ACT1* gene. The relative change in expression was calculated with respect to the control incubated without drugs normalized to 1.

Table 1. Sequence of primers to amplify the *ACT1*, *ERG11*, *CDR1* and *MDR1* genes by RT-PCR method.

Gene	Forward sequence (5'- 3')	Reverse sequence (5'- 3')
ACT1	AAGAATTGATTTGGCTGGTAGAGA	TGGCAGAAGATTGAGAAGAAGTTT
ERG11	GGTGGTCAACATACTTCTGCTTC	GTCAAATCATTCAAATCACCACCT
CDR1	TGCCAAACAATCCAACAA	CGACGGATCACCTTTCATACGA
MDR1	GTGTTGGCCCATTGGTTTTCAGTC	CCAAAGCAGTGGGGATTTGTAG

2.9 In vivo activity of citral in combination with fluconazole

The effect of citral in combination with fluconazole against *Candida* infection was assayed in the *C. elegans* model. Survival analysis was determined as previously described [26]. Age synchronous populations of L4-larvae, prepared using an alkaline hatch hypochloride solution for lysing the gravid hermaphrodites, were used to conduct the assays.

C. elegans was used to assess the toxicity of 32, 64, 128 and 256 mg/L of citral and 0.5, 1, 2, 4, 8, 32, 64 and 128 mg/L of fluconazole. The worms cultivated on nematode growth medium (NGM) agar plates were washed with M9 buffer to remove any residual *E. coli* cells and transferred to microtiter plates, in a quantity of 20 worms for each well, and incubated at 25 ºC. The microtiter plates contained worms, M9 buffer, 10 μg/mL cholesterol in ethanol, 90 μg/mL kanamycin and the corresponding concentration of antifungal or phytocompound for each well. Worm survival was visually scored on the stereomicroscope Nikon SMZ-745 (Japan) every 24 h of incubation until 96 h. The nematodes were scored as dead when they were rod-shaped and/or did not respond to stimulation with a platinum wire pick.

C. elegans was also used to evaluate the effect of citral in combination with fluconazole against *C. albicans* UPV 15-157 and *C. dubliniensis* UPV 11-366 infections. The effect of citral and fluconazole in monotherapy was tested against infection of fluconazole

susceptible *C. albicans* SC5314 as a control of methodology. The infection was performed by feeding of nematodes for 2 h at 25 ºC with each *Candida* isolate, which was previously grown on brain heart infusion (BHI) plates for 24 h at 37 ºC. After that, the worms were transferred into microtiter plates, 20 worms per well containing M9 buffer and a final concentration of 2, 64 and 128 mg/L of fluconazole; 32, 64 and 128 mg/L of citral and the combinations of 1 and 2 mg/L of fluconazole with 32, 64 and 128 mg/L of citral. Groups of uninfected nematodes and infected but untreated nematodes were also analyzed in the presence of 0.5% DMSO as controls. These plates were incubated at 25 ºC, and the survival study was performed as previously described. At least twice independent replicates were performed for each assay.

2.10 Statistics

Statistical analyses were performed using the SPSS v21.0 software (IBM, USA) and Graphpad Prism 5.0 version (GraphPad Software, USA). Comparisons between quantitative results were determined by t Student test when the data showed a normal distribution. The Mann-Whitney test was used to compare the relative gene expression of each gen and treatment. Survival curves were prepared by the Kaplan-Meier method and included the use of the log-rank for testing equivalency between them. In all the cases, $p < 0.05$ was considered statistically significant.

3. Results

3.1 Inhibitory effect of citral against planktonic and sessile cells of Candida

Citral had inhibitory effect against planktonic cells of the 35 oral isolates, including those which were azole-resistant (geometric mean –GM– of inhibitory concentration (IC) and minimum fungicide concentration: 197.9 mg/L and 649.4 mg/L, respectively). In addition, it showed a fungicidal activity mainly against the azole-resistant isolates of *C. glabrata* and *C. krusei* (Table S2).

The effect of citral was assessed against ten *Candida* isolates previously selected for their biofilm production, including eight *C. albicans*, one *C. dubliniensis* and one *C. tropicalis*. Citral showed activity against adhesion phase of biofilms (pre-sessile cells) and mature *Candida* biofilms (sessile cells) (Table 2). Citral was able to reduce metabolic activity to a greater extent than biomass in both the adhesion and mature biofilm assays. This activity was even observed against azole-resistant isolates such as *C. albicans* UPV 15-157 (Figure 1). Regarding adhesion phase of biofilm, this phytocompound showed GMs lower than the IC observed for planktonic cells (PSIC: XTT assay 47 mg/L and CV assay 149.3 mg/L versus IC 256 mg/L). The higher concentration of citral did not reduce the biomass of the mature biofilm of most isolates, except for the *C. tropicalis* isolate and *C. albicans* SC5314 strain.

Table 2. Effect of citral against planktonic and sessile cells of different *Candida* isolates.

***Candida* isolates**	**Planktonic cells**		**Sessile cells**			
	IC (mg/L)	**MFC (mg/L)**	**Adhesion phase PSIC (mg/L)**		**Mature biofilm SIC (mg/L)**	
			CV	**XTT**	**CV**	**XTT**
***C. albicans* UPV 05-007**	256	> 1024	256	256	> 2048	256
***C. albicans* UPV 05-013**	512	> 1024	128	64	> 2048	256
***C. albicans* UPV 11-342**	256	> 1024	> 1024	512	> 2048	512
***C. albicans* UPV 11-345**	256	> 1024	32	8	> 2048	128
***C. albicans* UPV 12-298**	256	256	16	16	> 2048	256
***C. albicans* UPV 15-101**	256	> 1024	> 1024	256	> 2048	64
***C. albicans* UPV 15-106**	256	> 1024	128	8	> 2048	256
***C. albicans* UPV 15-157**	512	> 1024	32	8	> 2048	512
***C. albicans* SC5314**	512	> 1024	512	64	32	256
C. albicans Ca 2	32	128	-	-	-	-
GM	256	724.1	149.3	47	1290.2	237
Range	32– 512	128 - > 1024	16 - > 1024	8 - 512	32 - > 2048	64 - 512
Mode	256	> 1024	128	8	> 2048	256
***C. tropicalis* UPV 06-115**	256	> 1024	128	64	256	256
***C. dubliniensis* UPV 11-366**	256	512	32	8	> 2048	256

IC: inhibitory concentration, GM: geometric mean, PSIC: inhibitory concentration of pre-sessile cells, inhibitory concentration of sessile cells, CV: crystal violet method/biomass quantification assay, XTT: tetrazolium salt reduced to formazan method/metabolic activity determination assay.

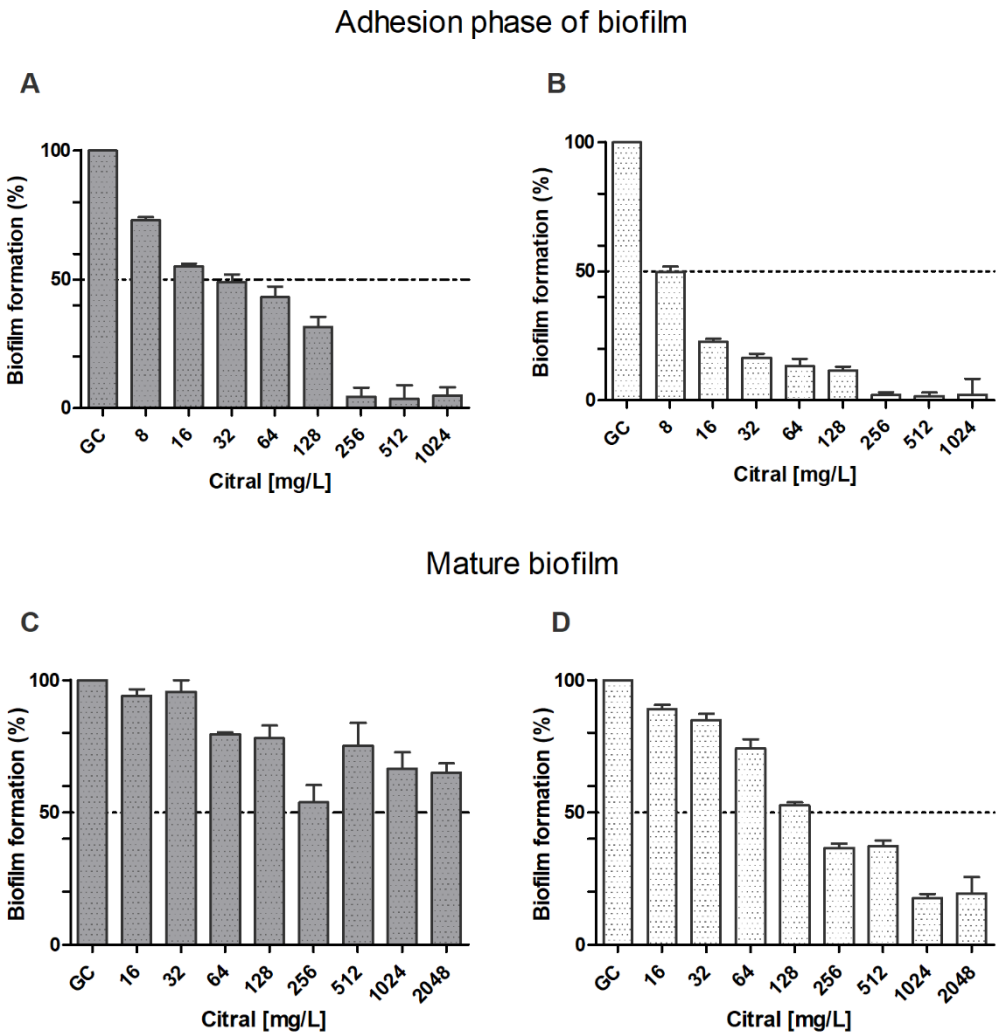

Figure 1. In vitro activity of citral against biofilm by *C. albicans* UPV 15-157 azole-resistant isolate. Growth inhibition of adhesion phase of biofilm-pre-sessile cells by biomass quantification with crystal violet assay (A) and metabolic activity determination with XTT reduction assay (B). Growth inhibition of mature biofilm-sessile cells by biomass quantification with crystal violet assay (C) and metabolic activity determination with XTT reduction assay (D). GC: growth control.

3.2 Citral enhances fluconazole effect against Candida

The activity of citral in combination with fluconazole was tested against planktonic cells of six isolates with reduced susceptibility to fluconazole, including one *C. albicans* isolate, two *C. glabrata*, two *C. krusei,* one *C. dubliniensis*, and one susceptible *C. tropicalis*. The combination of citral and fluconazole was active against planktonic cells of most

fluconazole-resistant *Candida* isolates (62.5%, 5/8), showing a synergistic effect in most of cases with 128 mg/L of citral and 1 mg/L of fluconazole. In the case of *C. krusei* isolates, no synergistic effect was shown (Table 3).

Table 3. Combination of antifungal activities of citral with fluconazole against planktonic *Candida* cells.

***Candida* isolate**	**IC/MICs-Alone**		**MIC-In combination**		**FICI**	**Interpretation**
	CT	**FLC**	**CT**	**FLC**		
***C. albicans* ATCC 64124**	512	16	128	1	0.31	SYN
***C. albicans* SC5314**	256	0.25	128	0.125	1.00	IND
***C. parapsilosis* ATCC 22019**	512	2	256	0.25	0.63	SYN
***C. krusei* ATCC 6258**	128	32	128	0.125	1.00	IND
***C. albicans* UPV 15-157**	512	> 64	128	1	0.26	SYN
***C. dubliniensis* UPV 11-366**	512	> 64	128	2	0.27	SYN
***C. glabrata* UPV 08-058**	256	> 64	2	8	0.07	SYN
***C. glabrata* UPV 14-004**	512	16	128	1	0.31	SYN
***C. krusei* UPV 13-120**	64	64	64	0.125	1.00	IND
***C. krusei* UPV 03-242**	32	> 64	32	0.125	1.00	IND
***C. tropicalis* UPV 05-016**	512	1	128	0.25	0.50	SYN

CT: citral; FLC: fluconazole; IND: indifferent; SYN: synergic. IC/MICs: inhibitory concentration/ minimum inhibitory concentration; FICI: fractional inhibitory concentration index.

The synergism between citral and fluconazole was also demonstrated against the sessile cells of the isolate *C. albicans* UPV 15-157 and was established by combining the concentrations of 0.25 mg/L of citral and 256 mg/L of fluconazole (FICI 0.5) (Figure 2). In addition, this combination reduced the metabolic activity of mature biofilms of azole-resistant *Candida* isolates but not their biomass. In the case of the isolate *C. albicans* UPV

11-366, although the reduction in biofilm metabolic activity was significantly reduced, the FICI value was > 0.5 indicating a no synergistic interaction.

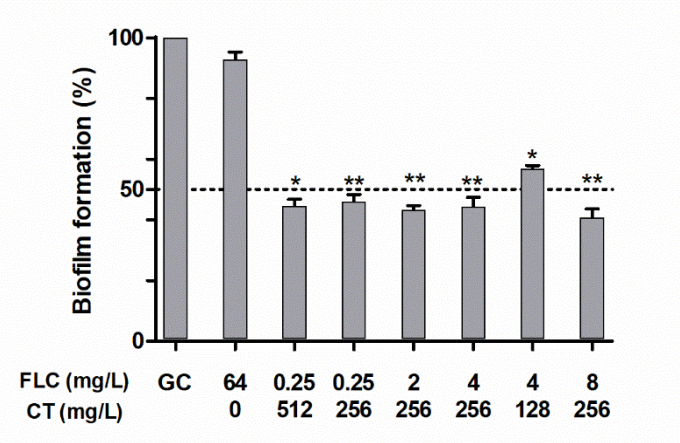

Figure 2. Activity of citral (CT) in combination with fluconazole (FLC) against mature biofilm-sessile cells of the *C. albicans* UPV 15-157 azole-resistant isolate by XTT reduction assay-metabolic activity determination. *: $p < 0.05$, **: $p < 0.01$. GC: growth control.

The killing activity of the antifungal combination was assessed by the analysis of the time-kill curves of two azole-resistant *Candida* isolates (*C. albicans* UPV 15-157 and *C. dubliniensis* UPV 11-366) and *C. albicans* SC5314. Cells were incubated with 4, 8 and 16 mg/L of fluconazole and 128 and 256 mg/L of citral. The highest decrease in CFU/mL (< 1 $\log_{10}$ CFU/mL) was observed for the combination of 256 mg/L of citral with 8 mg/L of fluconazole against both *Candida* isolates at 6 h and 24 h. However, no synergism was found compared to the activity of fluconazole alone. Figure 3 describes time-kill curves of citral and fluconazole combinations with best results obtained, omitting other concentrations tested.

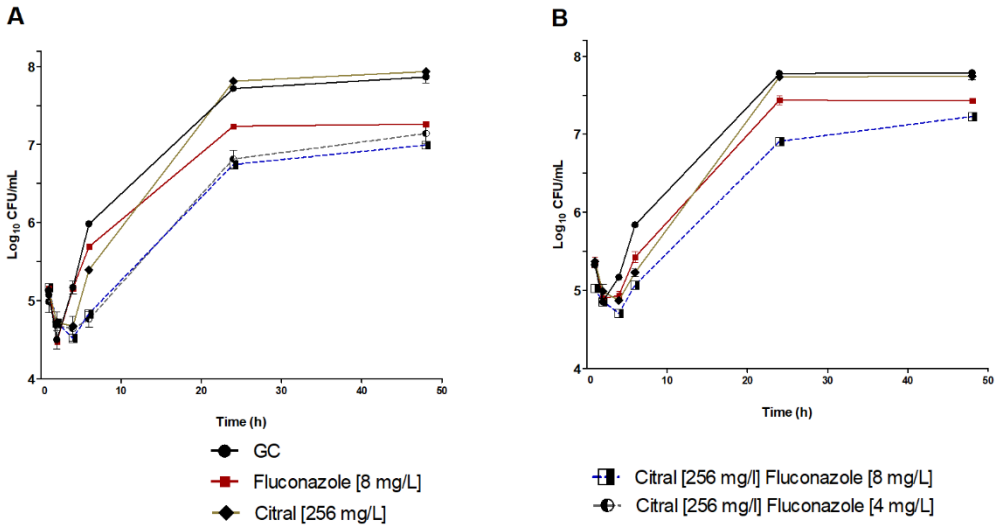

Figure 3. Time-kill curves of *C. albicans* UPV 15-157 (A) and *C. dubliniensis* UPV 11-366 (B) incubated with citral and fluconazole. Each data point represents the mean result ± standard deviation. GC: growth control.

3.3 Citral downregulates MDR1 *expression in* Candida *cells treated with the combination of citral and fluconazole*

The effect of citral in combination with fluconazole on the expression of *ERG11, MDR1* and *CDR1* genes was analyzed by quantification analysis by real-time reverse transcription-polymerase chain reaction**.** After the treatment of *Candida* cells with 128 mg/L of citral in combination with 1 mg/L of fluconazole, the *MDR1* expression level was decreased in the three *C. albicans* studied (fluconazole susceptible *C. albicans* SC5314, fluconazole resistant *C. albicans* ATCC 64124 and fluconazole resistant *C. albicans* UPV 15-157) and except to *C. albicans* UPV 15-157, the reduction was significant. Overexpression of *ERG11* was observed after the treatment with fluconazole and citral, in monotherapy or in combination. However, this upregulation was not significant. The levels of expression of *CDR1* of the *C. albicans* susceptible strain decreased in the presence of fluconazole alone and in combination with citral. In *Candida* resistant isolates, the expression of *CDR1* after

incubation with the combination of fluconazole and citral remained relatively constant or increased slightly compared to untreated cells (Figure 4).

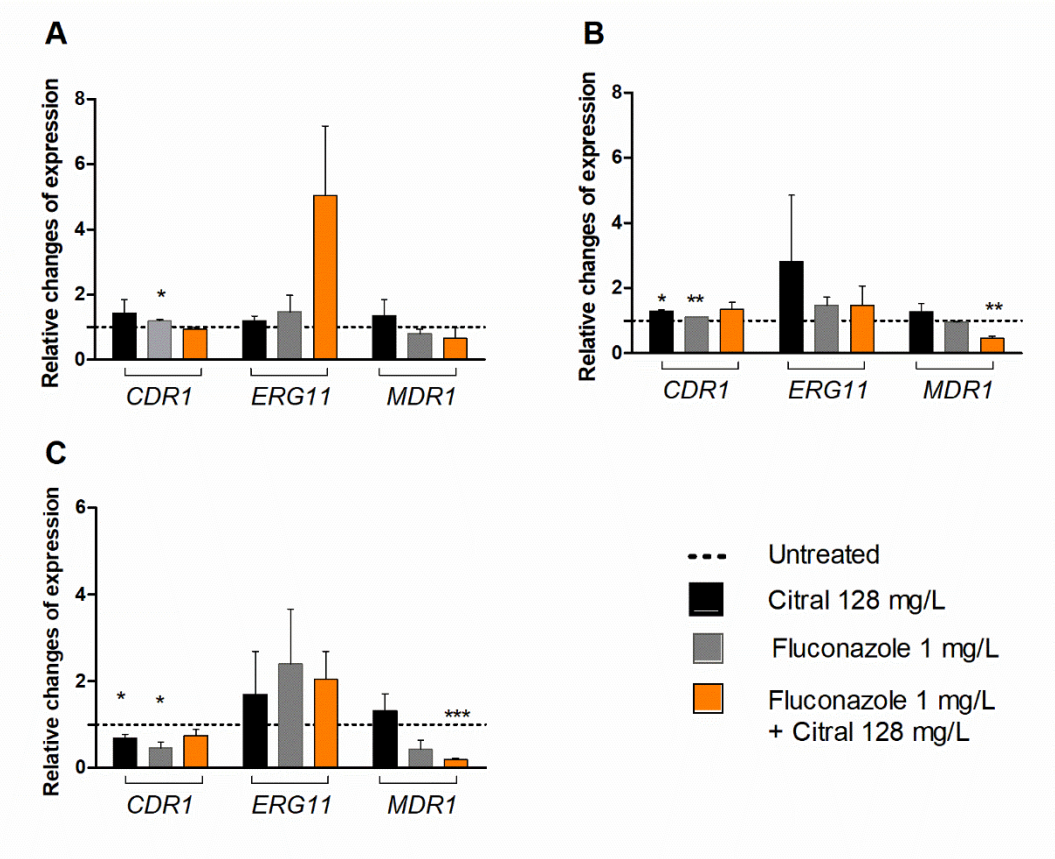

Figure 4. Relative changes in the expression of genes *ERG11*, *CDR1* and *MDR1* of two azole-resistant isolates, *C. albicans* UPV 15-157 (A) and *C. albicans* ATCC 64124 (B), and a fluconazole-susceptible strain, *C. albicans* SC5314 (C); after incubation with citral, fluconazole and a combination of both. *p < 0.05, **p < 0.01, ***p < 0.001, the relations were made between the control without treatment and the expression of the different genes analyzed.

3.4 Citral acts synergistically with fluconazole in the treatment of azole-resistant Candida *infection in a* Caenorhabditis elegans *model*

In vivo *C. elegans* model was used to evaluate the effect of the combination of citral and fluconazole against *C. albicans* UPV 15-157 and *C. dubliniensis* UPV 11-366 infections. Furthermore, the effect of citral and fluconazole in monotherapy against *C. albicans* SC5314

infection and the citral and fluconazole toxicity were studied. Citral concentrations equal to or less than 128 mg/L were nontoxic to *C. elegans* (Table S3). Thus, *C. elegans* worms exposed to these concentrations showed survival rates of about 94% up to 96 h of exposition (94.3, 96.8, and 95.8 % with 128, 64 and 32 mg/L of citral, respectively). The fluconazole concentration of 128 mg/L significantly reduced the survival of *C. elegans* at 96 h ($p = 0.002$), while the lower concentrations of fluconazole did not present toxicity during the 96 h of the assay. *Candida* azole-resistant isolates and the susceptible strain *C. albicans* SC5314 were able to infect *C. elegans* with significant mortality (Table 4).

All combinations of citral and fluconazole used to treat these infected worms significantly increased the survival of nematodes (Figure 5). Furthermore, the treatment with 2 mg/L of fluconazole plus 32 mg/L of citral resulted in a survival rate of 23.5% (at 96 h) with 79.9 h of mean lifespan of nematodes, compared to 7% (at 96 h) with 47 h of mean lifespan of untreated nematodes infected with *C. albicans* UPV 15-157. In the case of nematodes infected with *C. dubliniensis* UPV 11-366, after the treatment with the combination of fluconazole 2 mg/L with citral 128 mg/L, the survival rate was 22.6% (at 96 h) with 69.1 h of media lifespan of nematodes, compared to 7.8% (at 96 h) with 47 h of mean lifespan of untreated nematodes infected. However, there were not significant differences of survival among untreated worms versus worms treated with citral in monotherapy or with the lowest concentrations of fluconazole ($\leq$ 2 mg/L).

Table 4. Survival of nematodes treated with different concentrations of citral and fluconazole.

Treatments [mg/L]	Survival (%) Time (h)											
	24			48			72			96		
	A	B	C	A	B	C	A	B	C	A	B	C
Uninfected	100	100	100	99.2	99.6	98.4	98.1	94.8	96.1	95.3	88.4	89.9
Infected-	69.8	56.2	66.9	17.8	19.6	26.6	8.1	12.4	18.5	7	7.8	5.6
FLC [128]	92.2	93.4	87.4	43.4	50.8	58.3	30.2	26.2	47.2	24.8	19.7	41.7
FLC [64]	93.7	86.5	68.8	41.3	50	47.2	31	31.1	42.4	22.2	13.5	33.6
FLC [2]	79.4	73.6	82.8	19.9	13.9	19	9.6	8.3	15.5	5.1	8.3	8.6
CT [128]	91.7	86.3	68.9	9.8	16.3	14.4	2.3	7.5	4.5	0	1.3	1.5
CT [64]	92.3	80.3	71.5	16.2	16.7	28.5	7.7	0	19.5	3.1	0	9.8
CT [32]	84.7	83.3	90.6	17.6	27.8	26.6	6.1	9.7	20.3	0.8	2.8	14.1
FLC [2] Citral	100	93.2		68.2	61.7		27.1	33.1		10.9	22.6	
FLC [2] Citral [64]	100	94.4		75.7	60		41.4	20.8		18.6	13.6	
FLC[2] Citral [32]	100	93.8		83.2	57.4		49.6	22.5		20.8	16.3	
FLC [1] Citral	97.7	95.3		76.2	60.6		38.5	19.7		9.2	13.4	
FLC [1] Citral [64]	99.2	96.9		73.8	45.7		38.9	20.2		12.7	14	
FLC [2] Citral [32]	97.1	92.8		73.5	44.8		37.5	23.2		23.5	14.4	

CT: citral; FLC: fluconazole; A: *C. albicans* UPV 15-157; B: *C. dubliniensis* UPV 11-366; C: *C. albicans* SC5314. Untreated: 0.5% DMSO. Data represent the mean of at least twice independent assays.

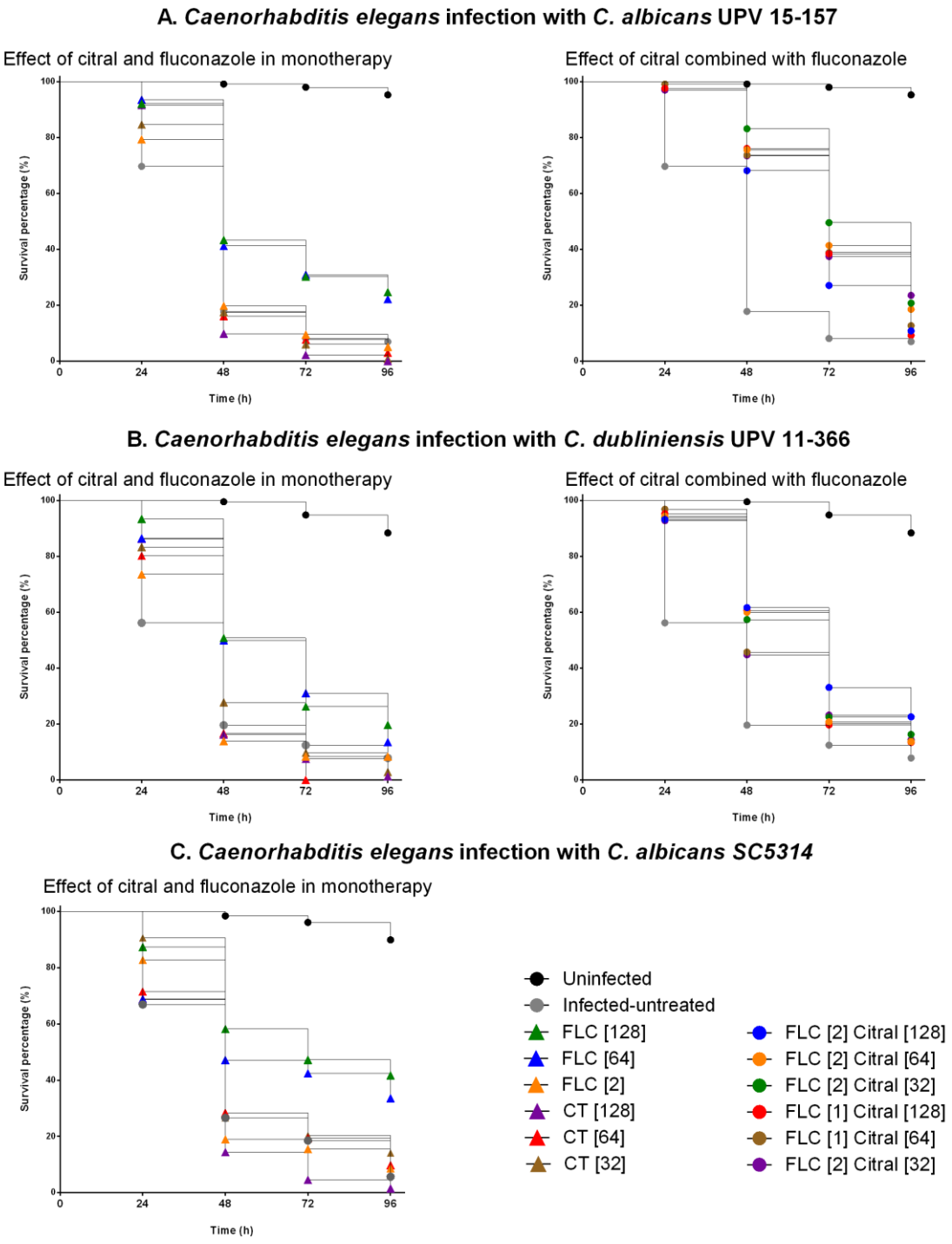

Figure 5. Efficacy of citral (CT) and fluconazole (FLC) treatment in monotherapy and/or in combination on survival curves of *C. elegans* infected with *C. albicans* UPV 15-157 (A) *C. dubliniensis* UPV 11-366 (B) and *C. albicans* SC5314 (C). Concentrations are expressed in mg/L.

4. Discussion

Oral candidiasis includes frequent acute and chronic manifestations such as pseudomembranous and erythematous candidiasis [5]. In contrast, secondary chronic mucocutaneous candidiasis, with persistent or recurrent relapses, are infrequent and are associated with Th17 CD4+ cells functional immunodeficiency [27,28]. Clinical cure rates of more than 80% have been described using fluconazole in patients suffering from AIDS; however, in patients suffering malignant tumors, the rates can be considerably lower [2]. Fluconazole has advantages such as cost-effectiveness, limited toxicity and high bioavailability that support its extensive use to treat several fungal diseases, although its frequent use as prophylactic therapy has favored azole-related resistance, mainly in *C. albicans* and *C. dubliniensis* [29–31].

Several natural compounds have been studied as therapeutic alternatives against resistant candidiasis with promising results [13]. Citral (3,7-dimethyl-2,6-octadienal), the main phytocompound present in *Cymbopogon citratus* or lemongrass, has shown different properties, including antimicrobial, anti-inflammatory and anticancer activities [14,32,33].

In the current study, citral inhibited the growth of planktonic cells from all *Candida* species. The values of IC GM 256 mg/L and MFC GM 776.05 mg/L obtained against *C. albicans* isolates, are in concordance with the studies carried out by Lima et al. (2012) and Rajput and Karuppayil (2013) [34,35]. However, other authors have reported lower IC and MFC values of citral than the obtained in our study (IC: 64 mg/L and MFC: 256 mg/L; IC and MFC of 32 mg/L, respectively) that could be related to isolate origin and methodological differences [16,33]. Furthermore, in our study, citral was active even against fluconazole resistant isolates, including *C. dubliniensis, C. krusei* and *C. glabrata*, which is very important due to their increasing prevalence in candidiasis. In fact, previous colonization with

C. albicans may facilitate *C. glabrata* persistence within the oral cavity [7,36]. Hence, the effect detected in this study could be very helpful in these cases.

Biofilm formation is considered an important factor in the virulence of *Candida* because its complex matrix protects the cells from external stresses and the host immune response [21]. Besides, *Candida* is able to produce biofilms on abiotic surfaces of material used in the manufacture of abutments and prostheses [37]. Biofilms represent persistent sources of infection due to the possibility of dispersion of cells from them; therefore, treatment strategies to prevent biofilm formation and eradicate the mature biofilm are essential. In our study, citral was effective in preventing biofilm formation and eradicating biomass of mature biofilm, although it was less active in the last case. These findings differ with the results reported on a previous study, in which it was observed less than 30% reduction in biofilm formation but higher activity against mature biofilms [15]. These authors evaluated the effectiveness of citral without considering its effect on biomass reduction and only used two isolates of *C. albicans*, while in our study, twelve *Candida* isolates were tested and both, biomass and metabolic activity, were determined in order to assess the reduction of mature biofilm more accurately. Citral was able to reduce the metabolic activity and biomass of the most of the *C. albicans* and *C. tropicalis* biofilms, as previously reported [17]. Despite the fact that citral was not very active removing the biomass of mature biofilms, it was effective in preventing the establishment of early-stage biofilms, considerably reducing biomass and metabolic activity. Therefore, citral could be considered for use as a treatment for biofilm-related infections such as oral candidiasis rather than for prophylactic use in biomedical devices, since citral acts not only against planktonic cells, but also by inhibiting biofilm formation, against preformed biofilms, and by preventing the possible establishment of biofilms from dispersed sessile cells.

The therapeutic problem arising from azole-resistant isolates requires new targets and the development of new therapeutic approaches to achieve reduction of the use or dosage of antifungal drugs against candidiasis. With this purpose, the combination of citral and fluconazole was evaluated in order to search synergistic effects. The results showed a synergistic interaction against planktonic cells of resistant isolates of *C. albicans*, *C. dubliniensis* and *C. glabrata*, also fluconazole susceptible *C. parapsilosis* and *C. tropicalis*. Other authors have described synergism against resistant *C. tropicalis* and *C. albicans* isolates using concentrations of citral at 128 mg/L and 90 mg/L, with reduction of fluconazole MIC from 4 to 32-fold, respectively [38,39]. However, in our study there was a greater reduction (for up to 128 folds) in the required concentration of fluconazole in synergism (from MIC > 64 and 16 mg/L in monotherapy to 1 to 2 mg/L in combination with 128 mg/L of citral), even in *C. krusei* isolate with no synergetic effect, the fluconazole MIC was sharply reduced 1024-fold.

The synergistic effect of citral in combination with fluconazole against *Candida* biofilms has been less studied than against planktonic cells. In our study, the combination of 0.25 mg/L of fluconazole and 256 mg/L of citral was found to reduce the mature biofilm in a synergistic way, in contrast with the findings of a previous study that only observed synergistic effect of eugenol and cinnamaldehyde with fluconazole against *C. albicans* biofilm, but not with citral [15].

Overall, in the evaluation of the in vitro effect of the combination of citral and fluconazole, a synergistic effect was observed against planktonic and sessile cells as stated previously. The time-kill curves of the azole resistant isolates demonstrated that this effect should be considered fungistatic, as the reduction of 0.8 $\log_{10}$ CFU/mL by the combination of 256 mg/L and 8 mg/L of fluconazole was not significant. Nonetheless, in other studies, the reported results of citral alone on time-kill curves are diverse in terms of growth reduction.

For example, it was reported a significant reduction of > 1 $\log_{10}$ of growth from 6 h of treatment with citral in monotherapy [39], a fungicidal activity of citral from 4 h at the IC (64 mg/L) [33], and also a reduction in the growth of a clinical isolate equal to 3 $\log_{10}$ CFU/mL after 2 h of exposure to the IC and IC×2 of citral [16]. Finally, in another study it was also described fungicidal activity against half of the tested isolates using IC, while against the remaining isolates, IC×2 and 120 min exposure were required to reach fungicidal effect [40]. Since in our study the objective was to evaluate synergism at concentrations similar to those obtained by checkerboard assay, concentrations lower than the IC of citral in monotherapy were tested in the time-kill curves of the *C. albicans* isolates. Hence our results on fungicidal effect of citral were lower than the reported by other authors that used concentrations equal to or higher than the IC.

Our findings suggest that the fungicidal effect might be isolate-dependent as in some cases is necessary IC×2 or more to obtain fungicidal activity as previously reported [33]; it could also depend on the methodology, as time-kill curves allow a continuous following over time in contrast to MFC, that gives an end point reading.

The in vitro synergistic effect of citral and fluconazole was also evident in our in vivo model. The effect of the treatment of the infection in *C. elegans* caused by azole-resistant, biofilm-producing *C. albicans* and *C. dubliniensis* isolates, with citral concentrations from 32 to 128 mg/L in combination with 1 or 2 mg/L of fluconazole resulted in a significant increase in lifespan. Nevertheless, as expected, the use of fluconazole concentrations of 1 or 2 mg/L was not effective in increasing the survival of worms infected with fluconazole-resistant *Candida* isolates.

Additionally, a low toxicity was observed in *C. elegans* at citral concentrations ≤ 128 mg/L, in concordance with the results previously reported in Swiss albino mice, where citral (oral administration of 5, 50 and 500 mg/kg body weight) was well-tolerated [41]. Therefore,

this phytocompound could be a good candidate to use as an antifungal treatment, since citral is also considered a "*generally recognized as safe"* (GRAS) phytocompound, extensively used as additive in food, pharmacy and cosmetic industry [42].

Despite the antifungal activity of citral has been reported against *Candida* species, other yeasts and filamentous fungi, its mechanism of action is not well explored. Citral mechanism of action against *C. albicans* was not related to modifications of the fungal cell wall nor to binding to ergosterol [33,34]. Some authors indicated that citral activity was mediated by the inhibition of ergosterol biosynthesis in *Candida* [35,38], *Penicillium italicum* [43] and *Aspergillus ochraceus* [44]. Disruption of cell membrane integrity, and loss of cellular components and induction of apoptosis have also been suggested [40].

Considering the aforementioned, citral might act mainly by altering the fungal cell membrane, which, in a combined treatment with fluconazole, would favor the increase of the intracellular concentration of fluconazole. However, expression profiles of genes associated with *C. albicans* resistance when using combined fluconazole and citral treatment had not yet been described. In the current study, we analyzed the effect of citral in combination with fluconazole on the regulation of some main genes involved in fluconazole resistance in *C. albicans* (Figure 6).

Overexpression of efflux pumps is frequently described as a mechanism of resistance to several antimicrobial drugs, due to the expulsion of the drug leading to a low drug accumulation. Two types of multidrug transporters are described in *Candida*, the ATP-binding cassette transporters (-ABC- encoded by *CDR1* and *CDR2*) and the major facilitators (encoded by *MDR1*). The Cdr1 and Cdr2 transporters have several azoles as substrate, while the Mdr1 transporters are specific to fluconazole [8].

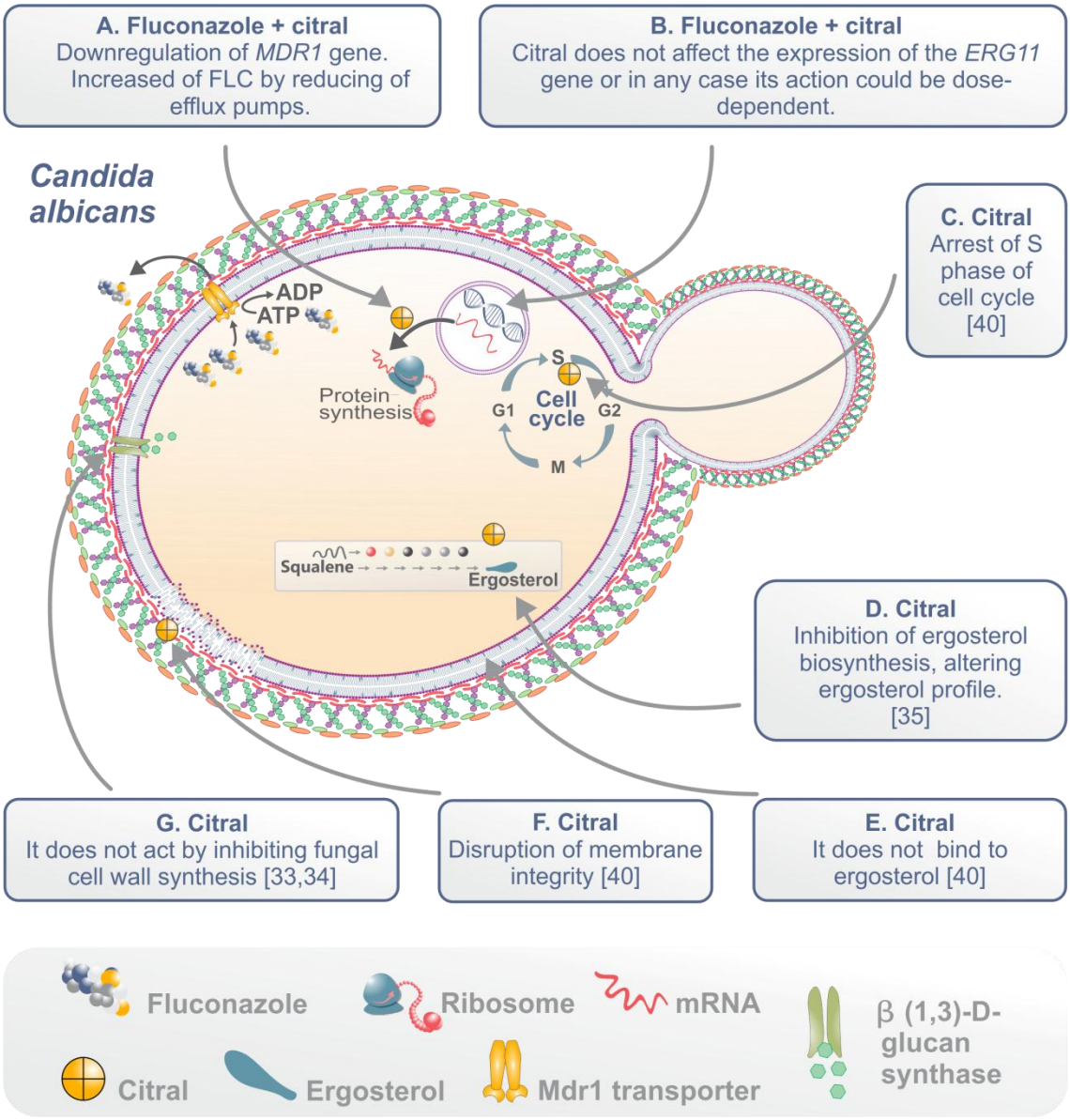

Figure 6. Schematic representation of the mechanisms of action of citral in monotherapy and in combination with fluconazole against *C. albicans*. Mechanisms described in the present work (A and B) and literature review (C-G). FLC: fluconazole.

In the current study, when a fluconazole susceptible strain was treated with fluconazole, there was a reduction of *CDR1* and *MDR1* expressions, while the resistant isolates treated with fluconazole showed an overexpression of *CDR1*, while *MDR1* expression was kept relatively constant. The role of *MDR1* in the synergism of citral and fluconazole was evidenced, since the treatment with this combination produced a significant reduction of its

expression in both susceptible and resistant strains, meanwhile the expression of *CDR1* was not significantly affected in any case. In a similar way to our findings in *C. albicans*, citral interferes the multidrug resistance in *Penicillium expansum*, by down-regulated expression of transporters (multidrug resistance protein (*MRP*) genes) [45].

With regard to the involvement of citral in the expression of *ERG11* gene, our findings were not conclusive. The relative changes in expression were not significant in any case, despite the fact that a slight *ERG11* upregulation was observed. This could be due to the low concentrations of fluconazole used in this study [46]. However, in a previous study using carvacrol, down-regulated expression of *ERG3* and *ERG11* was described at different concentrations (IC, 25 mg/L, and 0.5×IC) [47]. Although *ERG11* encodes an essential enzyme in the *C. albicans* pathway and the *Hot-spot* mutations and its overexpression are associated with fluconazole resistance, there are also about 20 genes involved in ergosterol biosynthesis, which have not been included in this study. Hence, if citral interferes with the ergosterol pathway, it should be independent to *ERG11* or likely dose dependent, and other *ERG* genes should be considered. In addition, it is relevant to note that antifungal resistance is often the result of the sum of several mechanisms, and further study would be necessary for a better understanding.

5. Conclusions

The low toxicity of citral along with the in vitro and in vivo synergistic effect with fluconazole makes citral a potential candidate to treat *Candida* infections in combination with fluconazole. In addition, the knowledge of its effect downregulating *MDR1* gene, which encodes Mdr1 efflux pump, contributes to identify its antifungal mechanism of action.

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pathway in *Candida albicans* and *Candida dubliniensis* biofilms exposed to fluconazole. Mycoses 2009;52:118–28. https://doi.org/10.1111/j.1439-0507.2008.01550.x.

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Table S1. *Candida* isolates, reference strains and methodologies used in this study.

Candida	***Candida* planktonic cells**			***Candida* sessile cells**			**RT-PCR**	**In vivo model**
	EUCAST	FICI	Time-kill	Adhesion of biofilm	Mature biofilm	Biofilm FICI	*MDR1*, *CDR1* and *ERG11* genes	Treatment infection
Candida albicans UPV 05-007	✓			✓	✓			
Candida albicans UPV 05-008	✓							
Candida albicans UPV 05-013	✓			✓	✓			
Candida albicans UPV 06-116	✓							
Candida albicans UPV 11-342	✓			✓	✓			
Candida albicans UPV 11-345	✓			✓	✓			
Candida albicans UPV 12-298	✓			✓	✓			
Candida albicans UPV 15-101	✓			✓	✓			
Candida albicans UPV 15-106	✓			✓	✓			
Candida albicans UPV 15-157	✓	✓	✓	✓	✓	✓	✓	✓
Candida dubliniensis UPV 11-366	✓	✓	✓	✓	✓	✓		✓
Candida dubliniensis UPV 12-064	✓							
Candida dubliniensis UPV 12-090	✓							
Candida glabrata UPV 05-048	✓							
Candida glabrata UPV 05-068	✓							
Candida glabrata UPV 05-022	✓							
Candida glabrata UPV 06-024	✓							
Candida glabrata UPV 08-058	✓	✓						
Candida glabrata UPV 13-164	✓							
Candida glabrata UPV 13-184	✓							
Candida glabrata UPV 13-185	✓							
Candida glabrata UPV 13-200	✓							
Candida glabrata UPV 14-004	✓	✓						
Candida krusei UPV 03-242	✓	✓						
Candida krusei UPV 05-054	✓							
Candida krusei UPV 13-120	✓	✓						
Candida guilliermondii UPV 05-059	✓							
Candida guilliermondii UPV 05-078	✓							
Candida orthopsilosis UPV 12-056	✓							
Candida orthopsilosis UPV 12-057	✓							
Candida parapsilosis UPV 07-008	✓							
Candida parapsilosis UPV 12-296	✓							
Candida metapsilosis UPV 11-449	✓							
Candida tropicalis UPV 05-016	✓	✓						
Candida tropicalis UPV 06-115	✓			✓	✓			
Reference strains								
Candida albicans SC5314	✓	✓	✓	✓	✓	✓	✓	✓
Candida albicans Ca2	✓							
Candida albicans ATCC 64124	✓	✓					✓	
Candida krusei ATCC 6258	✓	✓						
Candida parapsilosis ATCC 22019	✓	✓						

.**Table S2**. *In vitro* activity of citral and fluconazole against 35 isolates of *Candida.*

***Candida* species (n)**	**Citral [2-1024 mg/L]**				**Fluconazole [0.12-64 mg/L]**	
***C. albicans* (10)**	**IC GM**	256	**MFC GM**	776.05	**MIC GM**	0.35
	Mode	256	**Mode**	1024	**Mode**	0.25
	Range IC	128 – 512	**Range MFC**	256->1024	**Range MIC**	0.12 - >64
***C. glabrata* (10)**	**IC GM**	238.86	**MFC GM**	477.71	**MIC GM**	12.1
	Mode	256	**Mode**	256	**Mode**	16
	Range IC	128 – 512	**Range MFC**	256->1024	**Range MIC**	4 - >64
***C. krusei* (3)**	**IC GM**	80.63	**MFC GM**	304.44	**MIC GM**	50.8
	Mode	-	**Mode**	256	**Mode**	64
	Range IC	32 – 256	**Range MFC**	256-512	**Range MIC**	32 - >64
***C. dubliniensis* (3)**	**IC GM**	287.35	**MFC GM**	1024	**MIC GM**	1
	Mode	256	**Mode**	>1024	**Mode**	0.125
	Range IC	128 – 256	**Range MFC**	>1024	**Range MIC**	0.12 - >64
***C. parapsilosis* (2)**	**IC GM**	256	**MFC GM**	1024	**MIC GM**	0.5
	Range IC	256	**Range MFC**	>1024	**Range MIC**	0.5 - 2
***C. guilliermondii* (2)**	**IC GM**	64	**MFC GM**	322.54	**MIC GM**	4
	Range IC	32 – 128	**Range MFC**	256-512	**Range MIC**	2 - 8
***C. orthopsilosis* (2)**	**IC GM**	181.02	**MFC GM**	1024	**MIC GM**	0.4
	Range IC	128 – 256	**Range MFC**	>1024	**Range MIC**	0.25 - 0.5
***C. tropicalis* (2)**	**IC GM**	181.02	**MFC GM**	1024	**MIC GM**	5.7
	Range IC	128 – 256	**Range MFC**	>1024	**Range MIC**	0.5 - >64
***C. metapsilosis* (1)**	**IC**	256	**MFC**	>1024	**MIC**	2
Total	**IC GM**	197.89	**MFC GM**	649.35	**MIC GM**	2.4
	Mode	256	**Mode**	1024	**Mode**	0.125
	Range IC	32 – 512	**Range MFC**	256 - >1024	**Range MIC**	0.12 - >64

IC: inhibitory concentration. GM: geometric mean, MIC: minimum inhibitory concentration. IC: inhibitory concentration. MFC: minimum fungicidal concentration.

Table S3. Survival rate of nematodes in contact with different concentrations of citral and fluconazole.

Survival rate (%)				
Tested concentrations	**Time (h)**			
	24	**48**	**72**	**96**
Untreated	100.0	100.0	99.2	98.4
CT [256 mg/l]	92.4	91.1	89.9	88.6
CT [128 mg/l]	97.0	97.0	96.4	95.8
CT [64 mg/l]	100.0	100.0	98.7	96.8
CT [32 mg/l]	100.0	100.0	96.7	94.3
FLC [128 mg/l]	97.7	95.4	95.4	88.5
FLC [64 mg/l]	98.6	97.1	95.7	95.7
FLC [32 mg/l]	100.0	100.0	96.7	96.7
FLC [8 mg/l]	100.0	100.0	96.8	96.8
FLC [4 mg/l]	100.0	100.0	96.6	96.6
FLC [2 mg/l]	100.0	100.0	98.6	95.8
FLC [1 mg/l]	100.0	100.0	100.0	100.0
FLC [0.5 mg/l]	100.0	100.0	100.0	100.0

CT: citral; FLC: fluconazole. Untreated: 0.5 % DMSO. Data represent the mean of at least twice independent assays.

4.4 Study 4: Antifungal activity of carvacrol, cinnamaldehyde, citral and thymol nanoparticles

The DODAB:MO system was used to develop carvacrol, cinnamaldehyde, citral and thymol liposomes. Parameters of nanoparticles such as stability, size, ζ-potential and encapsulation efficiency were evaluated. In addition, the anti-*Candida* activity and cytotoxicity of phytocompounds and their nanoparticles were performed.

DODAB:MO-based liposomes with carvacrol, cinnamaldehyde, citral and thymol were stable at 4 °C, with PDI values < 0.6 and ζ-potentials from + 46.9 to + 55.6 mV, of which thymol nanoparticles were the largest at a size of 580.3 ± 17.4 nm, followed by cinnamaldehyde, carvacrol and citral nanoparticles (567.9 ± 13.9, 510 ± 9.6 and 497.6 ± 13.8 nm, respectively).

High %EE was obtained for nanoparticles with 128 mg/L of carvacrol and thymol (70.8 and 69.1%, respectively) without loss of anti-*Candida* activity (GM ICs of 101.6 mg/L for carvacrol and 71.8 mg/L for thymol). Although nanoparticles with 256 mg/L of citral presented the best %EE (83.3%), antifungal activity was not observed. Cinnamaldehyde nanoparticles presented the lowest %EE (20.6 to 44.1%) and maintained their antifungal activity (Table 3 from manuscript 4, pag. 246).

Macrophages tolerated thymol and carvacrol nanoparticles the best, showing higher cell viability than those treated with other nanoparticles or nonencapsulated phytocompounds, according to the MTT assay results (83.5-112.8% and 101-137% viable macrophages with thymol and carvacrol nanoparticles, respectively). Additionally, nanoparticles composed of 18 mg/L DODAB:MO with 64 mg/L carvacrol notably improved the ability of macrophages to kill *Candida* cells, similar to nanoparticles composed of 18 mg/L DODAB:MO with 32 or 128 mg/L thymol (Figure 11 from manuscript 4, pag. 248). These

nanoparticles did not stimulate the production of IL-10. Although negligible production of TNFα was found upon treatment with carvacrol nanoparticles, this outcome was not considered a harmful effect (Figure 9 and 10 from manuscript 4, pag. 245 and 247, respectively).

The results of study 4 have been published in "Antimicrobial Agents and Chemotherapy", which are presented in the following pages (manuscript 4).

Manuscript 4

Development and characterization of monoolein-based liposomes of carvacrol, cinnamaldehyde, citral, or thymol with anti-*Candida* activities

Katherine Miranda-Cadena, Marisol Dias, Augusto Costa-Barbosa, Tony Collins, Cristina Marcos-Arias, Elena Eraso, Célia Pais, Guillermo Quindós, Paula Sampaio.

Antimicrobial Agents and Chemotherapy, 2021; 65: e01628-20.

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EXPERIMENTAL THERAPEUTICS

Development and Characterization of Monoolein-Based Liposomes of Carvacrol, Cinnamaldehyde, Citral, or Thymol with Anti-*Candida* Activities

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ABSTRACT There is an increasing need for novel drugs and new strategies for the therapy of invasive candidiasis. This study aimed to develop and characterize liposome-based nanoparticles of carvacrol, cinnamaldehyde, citral, and thymol with anti-*Candida* activities. Dioctadecyldimethylammonium bromide- and monoolein-based liposomes in a 1:2 molar ratio were prepared using a lipid-film hydration method. Liposomes were assembled with equal volumes of liposomal stock dispersion and stock solutions of carvacrol, cinnamaldehyde, citral, or thymol in dimethyl sulfoxide. Cytotoxicity was tested on RAW 264.7 macrophages. *In vitro* antifungal activity of liposomes with phytocompounds was evaluated according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology using clinical isolates of *Candida albicans*, *Candida auris*, *Candida dubliniensis*, and *Candida tropicalis*. Finally, the ability of macrophage cells to kill *Candida* isolates after addition of phytocompounds and their nanoparticles was determined. Nanoparticles with 64 μg/ml of cinnamaldehyde, 256 μg/ml of citral, and 128 μg/ml of thymol had the best characteristics among the formulations tested. The highest encapsulation efficiencies were achieved with citral (78% to 83%) and carvacrol (66% to 71%) liposomes. Carvacrol and thymol in liposome-based nanoparticles were nontoxic regardless of the concentration. Moreover, carvacrol and thymol maintained their antifungal activity after encapsulation, and there was a significant reduction (~41%) of yeast survival when macrophages were incubated with carvacrol or thymol liposomes. In conclusion, carvacrol and thymol liposomes possess high stability, low cytotoxicity, and antifungal activity that act synergistically with macrophages.

KEYWORDS *Candida*, antifungal activity, phytocompounds, liposomes, carvacrol, cinnamaldehyde, citral, thymol, macrophages

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Development and characterization of monoolein-based liposomes of carvacrol, cinnamaldehyde, citral, or thymol with anti-*Candida* activities

Running title: Liposomes with antifungal phytocompounds

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ABSTRACT

There is an increasing need for novel drugs and new strategies for the therapy of invasive candidiasis. This study aimed to develop and characterize liposome-based nanoparticles of carvacrol, cinnamaldehyde, citral or thymol with anti-*Candida* activities. Dioctadecyldimethylammonium bromide- and monoolein-based liposomes in a 1:2 molar ratio were prepared using a lipid-film hydration method. Liposomes were assembled with equal volumes of liposomal stock dispersion and stock solutions of carvacrol, cinnamaldehyde, citral or thymol in dimethyl sulfoxide. Cytotoxicity was tested on RAW 264.7 macrophages. In vitro antifungal activity of liposomes with phytocompounds was evaluated according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology using clinical isolates of *Candida albicans*, *Candida auris*, *Candida dubliniensis* and *Candida tropicalis*. Finally, the ability of macrophage cells to kill *Candida* after the addition of phytocompounds and their nanoparticles was determined. Nanoparticles with 64 μg/ml of cinnamaldehyde, 256 μg/ml of citral and 128 μg/ml of thymol had the best characteristics among the formulations tested. The highest encapsulation efficiencies were achieved with citral (78 to 83%) and carvacrol (66 to 71%) liposomes. Carvacrol and thymol in liposome-based nanoparticles were nontoxic regardless of the concentration. Moreover, carvacrol and thymol maintained their antifungal activity after encapsulation and there was a significant reduction (~41%) of yeast survival when macrophages were incubated with carvacrol or thymol liposomes. In conclusion, carvacrol and thymol liposomes possess high stability, low cytotoxicity, and antifungal activity that act synergistically with macrophages.

KEYWORDS: *Candida*, antifungal activity, phytocompounds, liposomes, carvacrol, cinnamaldehyde, citral, thymol, macrophages.

INTRODUCTION

Candida is a common commensal of human skin and mucosae that can cause superficial and invasive infections (1–4). Invasive candidiasis is an important public health problem because of its high mortality and morbidity (5–7). *Candida albicans* infection is the most frequent cause of candidiasis, but an increasing etiological relevance of other species of *Candida*, such as *Candida parapsilosis*, *Candida glabrata*, or *Candida auris*, is reported (8–10). The emergence of these species complicates the management of candidiasis due to their potential multidrug-resistance (8, 11). Moreover, there are a limited number of antifungal drugs, many of them with moderate efficacy, which are not free from adverse effects and drug interactions. These facts highlight the need to search for alternative therapies or synergistic combinations of antimicrobial agents.

Many phytocompounds have antimicrobial activities (12–14) and some of them, such as carvacrol, cinnamaldehyde, citral and thymol, have shown promising antifungal activities (13–15). Their incorporation into nanoparticles, like liposomes, can improve their therapeutic efficiency against candidiasis, decreasing allergic reactions or toxicity. Liposomes are lipid bilayer vesicles that have been employed to enhance the delivery of antifungal drugs, such as amphotericin B (16). The system dioctadecyldimethylammonium bromide (DODAB) and monoolein (MO), a cation liposome, which consists of lipid-multilayer vesicles with a positive charge, has been used for drug delivery with promising results (17–20). DODAB is a synthetic amphiphilic lipid, including a hydrophilic positively charged dimethyl ammonium group attached to two hydrophobic 18-carbon-long acryl chains. MO is used as a stabilizer as it confers fluidity to the DODAB system by favouring lipid chain mobility and improving the fusion of the liposomes with cell membranes (21). In addition, application of DODAB:MO system in the production of nanoparticles of phytocompounds as antifungal therapy, represents a biodegradable and biocompatible

novel alternative with respect to the most common used metallic or synthetic nanomaterials. The aim of this study is to develop and characterize DODAB:MO nanoparticles of carvacrol, cinnamaldehyde, citral and thymol for drug delivery and to test their cytotoxicity on murine macrophages to identify the best formulation with anti-*Candida* activity.

RESULTS

The characteristics of empty liposome with 0.5% dimethyl sulfoxide (DMSO) DODAB:MO and the nanoparticles with three concentrations of carvacrol, cinnamaldehyde, citral or thymol are presented in Fig. 1 to 4.

DODAB:MO (1:2) empty liposomes had a mean size of 545 ± 17 nm in the first day of production, a ζ-potential of 48.2 ± 2.1 mV and polydispersity index (PDI) of 0.46 ± 0.06, with time the ζ- potential remained similar, with no significant differences, but the mean size of the liposomes reduced to 508 ± 29 nm, right after the second week ($P < 0.05$) and remaining stable thereafter. The PDI value remained similar during the first 4 weeks but was reduced in week 5. Empty liposomes seem to stabilize with time. Nanoparticles with carvacrol 128 μg/ml were more stable in terms of sizes than formulations with lower concentrations, but the ζ-potential was more unstable with time. Nanoparticles with 32 μg/ml carvacrol (lower concentration) were more variable in size over time ($P < 0.0001$), but more stable in terms of ζ-potential.

This behaviour was similar for nanoparticles of cinnamaldehyde, citral and thymol; and the formulation with the greatest stability over time had the highest compound concentration. Thus, nanoparticles with 64 μg/ml of cinnamaldehyde, 256 μg/ml of citral and 128 μg/ml of thymol had the best characteristics among the formulations tested, with thymol

nanoparticles being the largest (580.3 ± 17.4 nm), followed by cinnamaldehyde (567.9 ± 13.9 nm), carvacrol (510 ± 9.6 nm) and citral (497.6 ± 13.8 nm) nanoparticles. Curiously, for all compounds at 5 weeks postproduction, the PDI was lower for the two highest concentrations, indicating size stabilization.

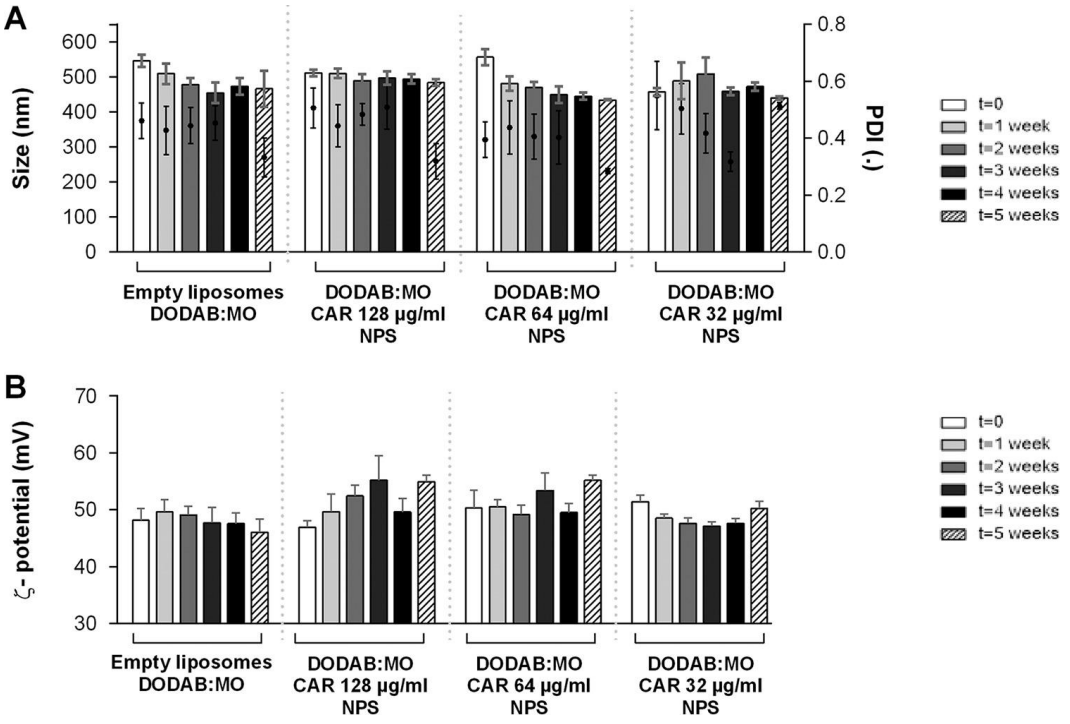

FIG 1 Size and PDI (A) and ζ-potential (B) of carvacrol (CAR) nanoparticles (NPS) prepared with three different concentrations and analyzed weekly for 5 weeks. Empty liposomes and nanoparticles were prepared by hydration of DODAB:MO (1:2) 2-mM film with 0.5% DMSO in ultrapure water. Values are the results of three independent assays.

The parameters of high-performance liquid chromatography diode array detection (HPLC-DAD) for each phytocompounds are described in Table 1. In the case of citral, two peaks at 240 nm were observed due to the presence of isomers E (geranial or citral A) and Z (neral or citral B) (Fig. 5). Encapsulation was higher with the highest concentrations for all phytocompounds tested, with nanoparticles with 256 µg/ml of citral presenting the highest

encapsulation efficiency (%EE) (83.2%), whereas all nanoparticles with cinnamaldehyde presented a lower %EE of 20.6% to 44.1% (Table 2).

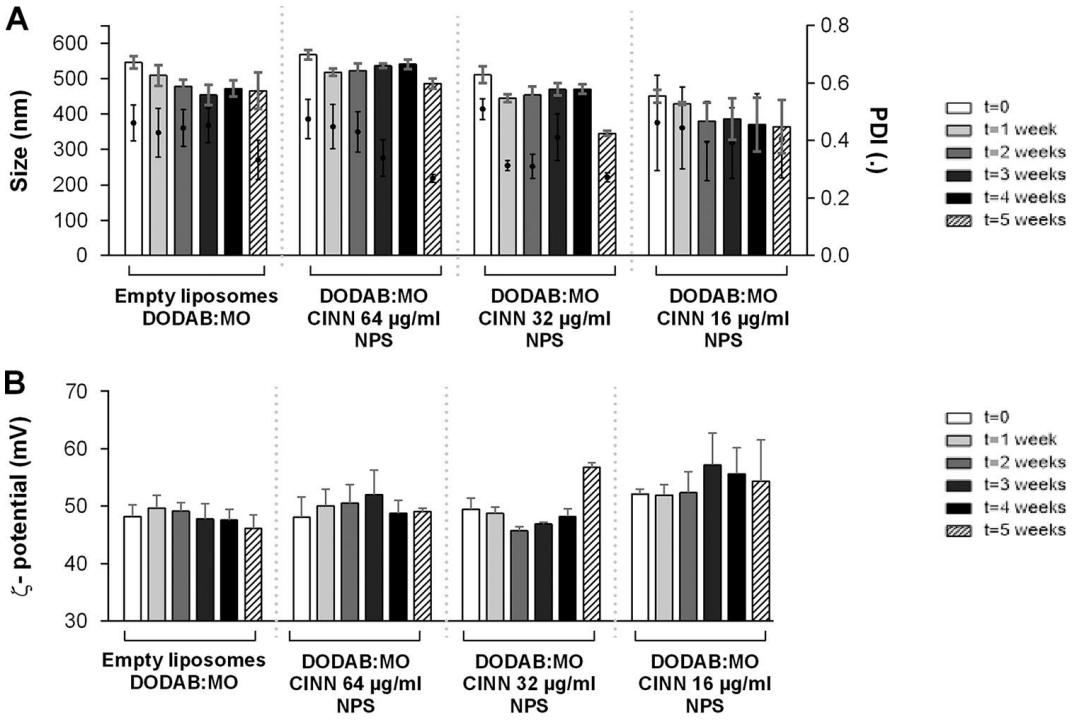

FIG 2 Size and PDI (A) and ζ-potential (B) of cinnamaldehyde (CINN) nanoparticles (NPS) prepared with three different concentrations and analyzed weekly for 5 weeks. Empty liposomes and nanoparticles were prepared by hydration of DODAB:MO (1:2) 2-mM film with 0.5% DMSO in ultrapure water. Values are the results of three independent assays.

TABLE 1. HPLC-DAD parameters for the detection of phytocompounds.

Phytocompound	Wavelength detection (nm)	Coefficient (R^2)	Linear range (µg/ml)	Time retention (min)
Carvacrol	210	0.99	1-50	6.6
Cinnamaldehyde	290	0.98	1-50	4.4
Citral	240	0.99	1-50	5.5, 5.8
Thymol	210	0.99	1-50	6.4

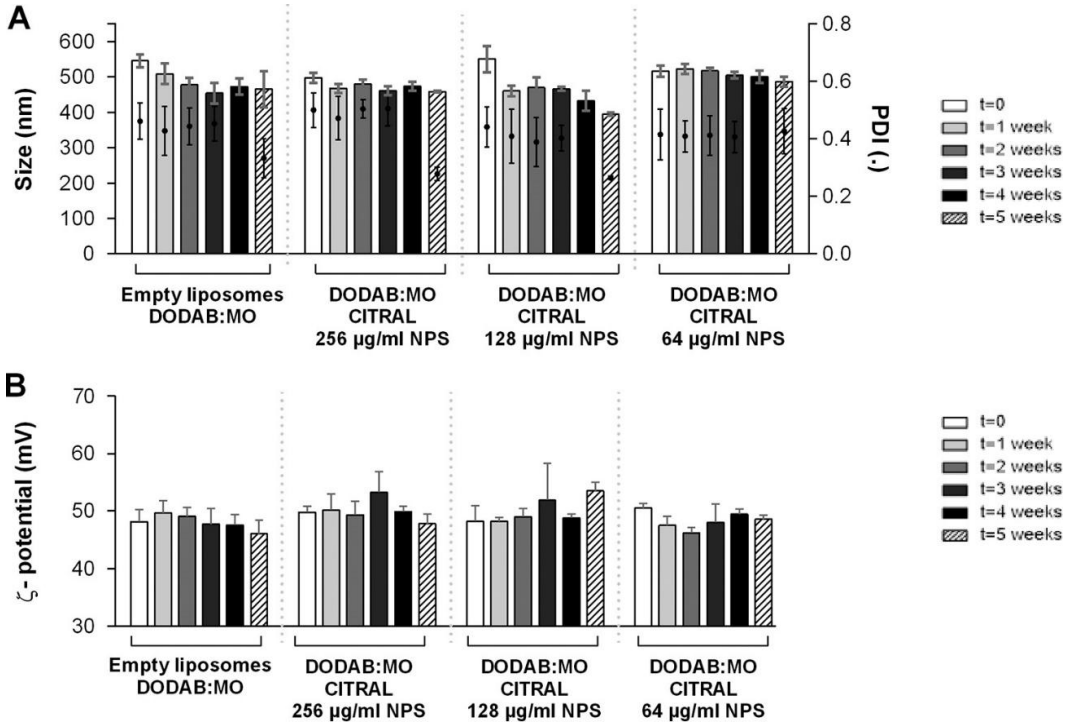

FIG 3 Size and PDI (A) and ζ-potential (B) of citral nanoparticles (NPS) prepared with three different concentrations and analyzed weekly for 5 weeks. Empty liposomes and nanoparticles were prepared by hydration of DODAB:MO (1:2) 2-mM film with 0.5% DMSO in ultrapure water. Values are the results of three independent assays.

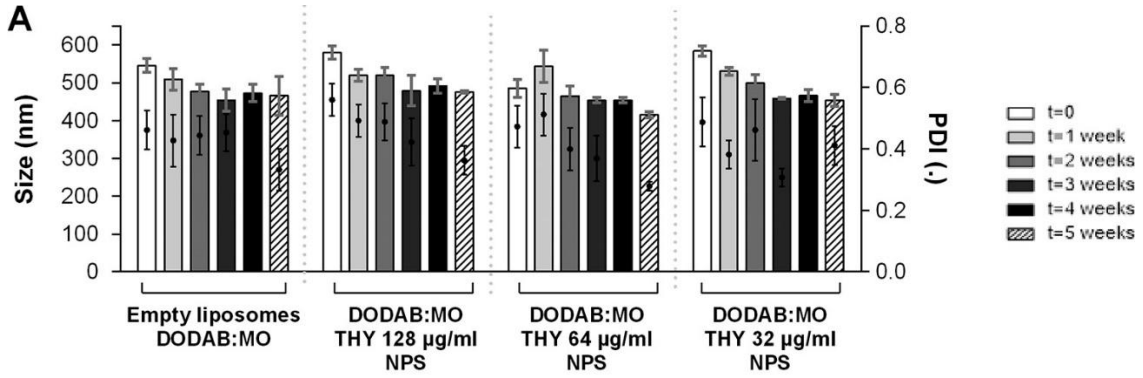

FIG 4 Size and PDI (A) and ζ-potential (B) of thymol (THY) nanoparticles (NPS) prepared with three different concentrations and analyzed weekly for 5 weeks. Empty liposomes and nanoparticles were prepared by hydration of DODAB:MO (1:2) 2-mM film with 0.5% DMSO in ultrapure water. Values are the results of three independent assays.

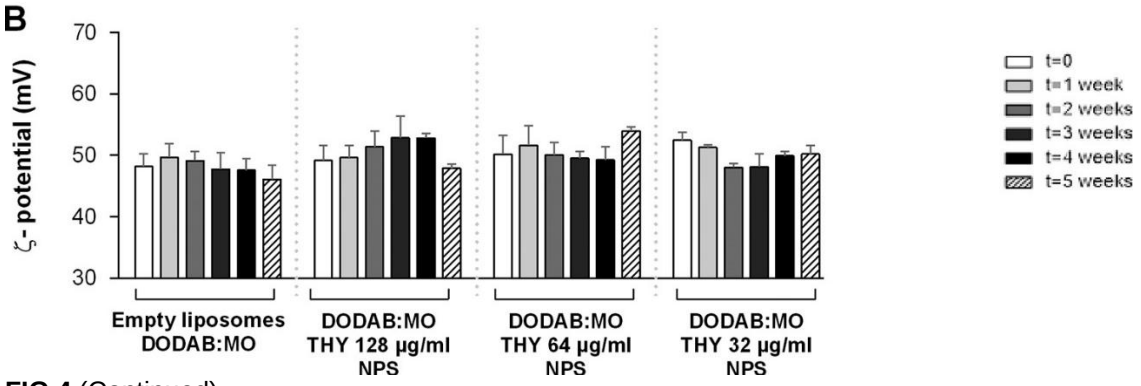

FIG 4 (Continued).

TABLE 2. Encapsulation efficiency of phytocompound nanoparticles.

Phytocompound and concn (µg/ml) in nanoparticles	%EE[α]	SD
Carvacrol		
32	65.8	0.8
64	73.5	2.2
128	70.8	5.0
Cinnamaldehyde		
16	20.6	0.1
32	30.3	1.5
64	44.1	2.7
Citral		
64	77.9	0.1
128	79.4	0.1
256	83.2	1.2
Thymol		
32	68.3	1.3
64	56.6	0.8
128	69.1	1.1

[α]%EE: Encapsulation efficiency

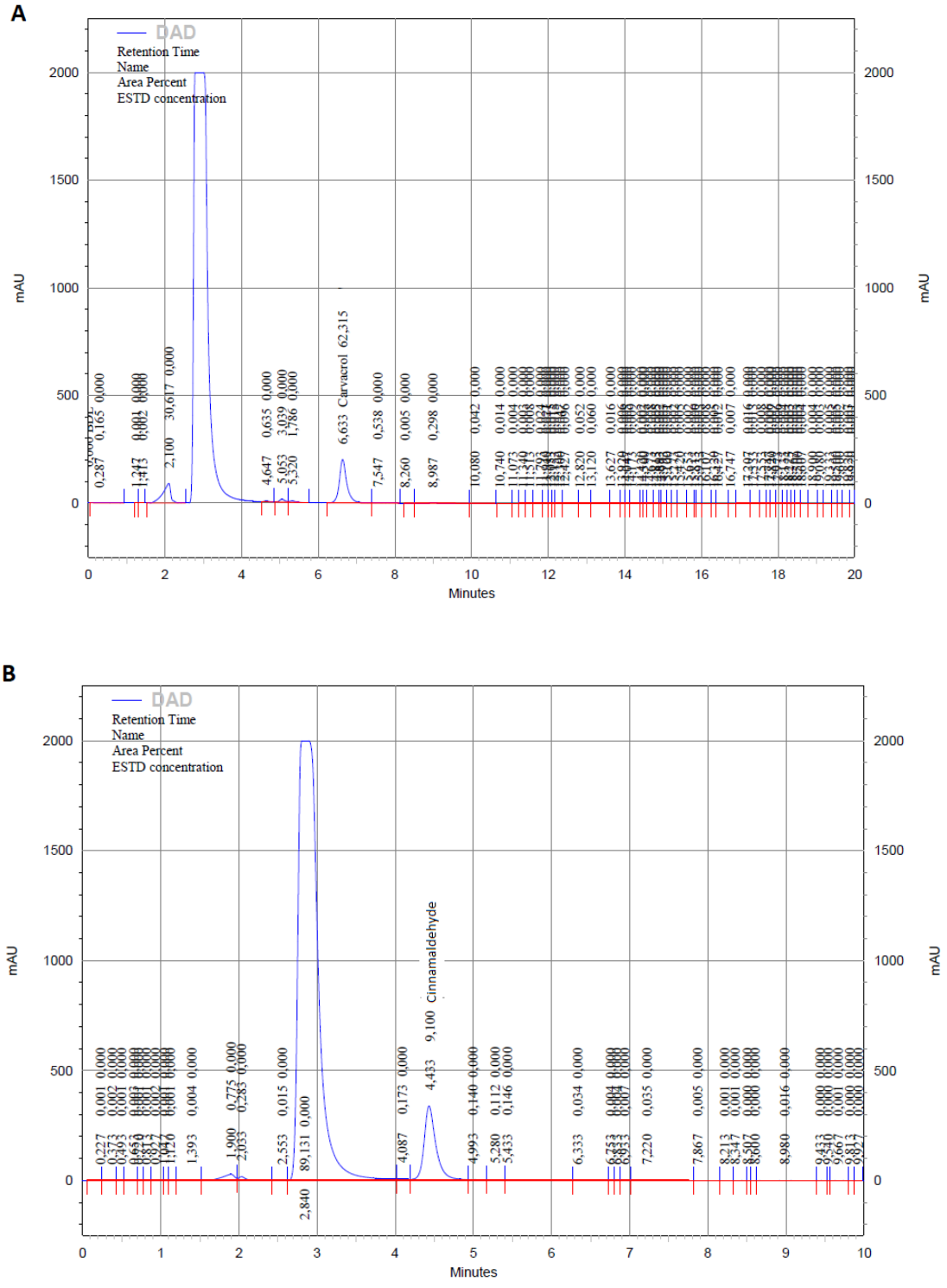

FIG 5 Chromatograms of carvacrol at 210 nm (A), cinnamaldehyde at 290 nm (B), citral at 240 nm (C) and thymol at 210 (D) by HPLC-DAD of supernatants of correspondent nanoparticles after ultracentrifugation. In all cases, the highest peak corresponds to the DMSO content.

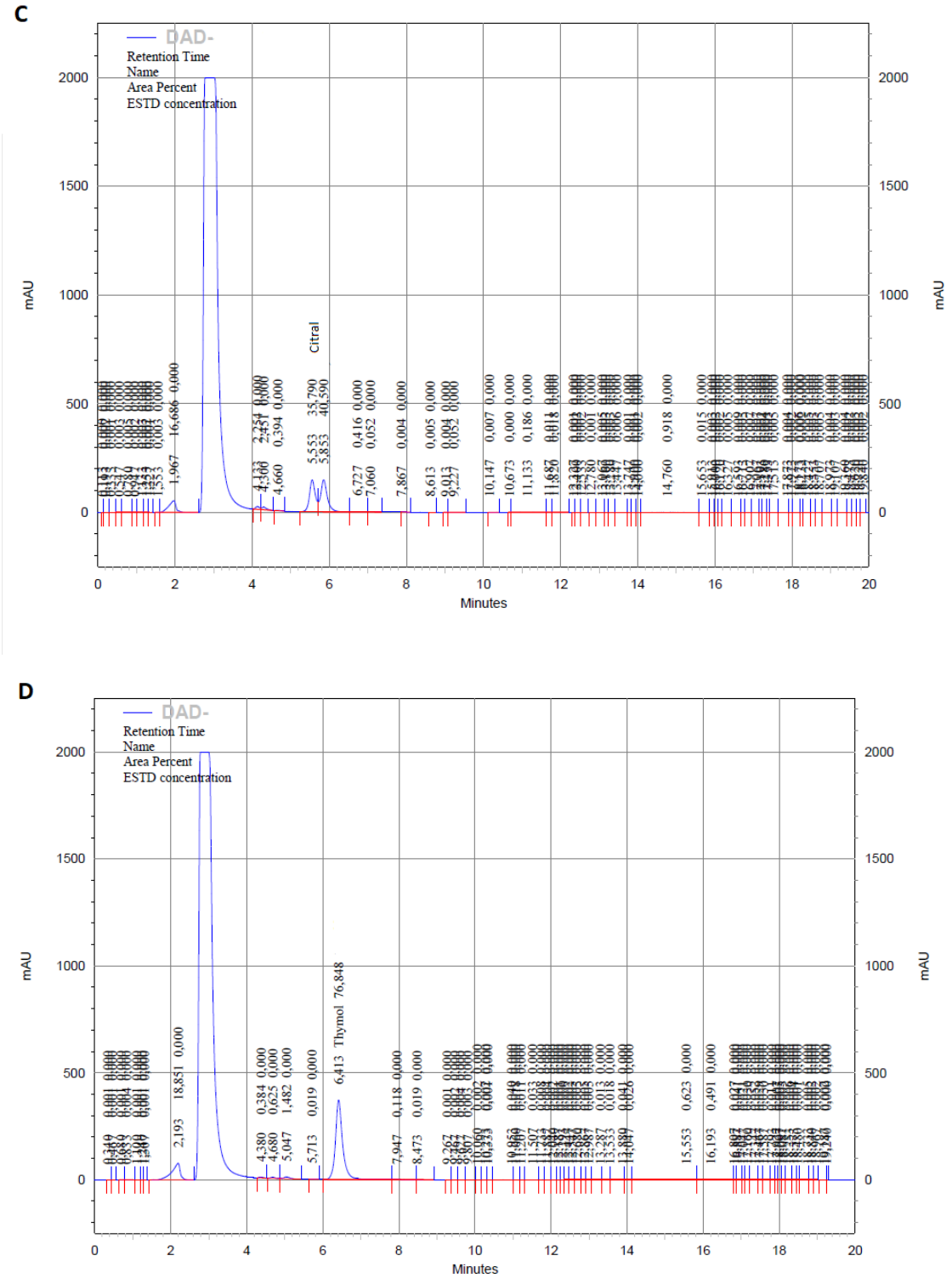

FIG 5 (Continued)

RAW 264.7 cell viability was significantly reduced ($P < 0.05$) after treatment with the highest concentrations of nonencapsulated phytocompounds (NEPs) tested, with survival at <80% the limit of accepted cytotoxicity. This cytotoxicity was dose dependent. Because no differences were found between 24 and 48 h, further studies were determined at 24 h (Fig. 6).

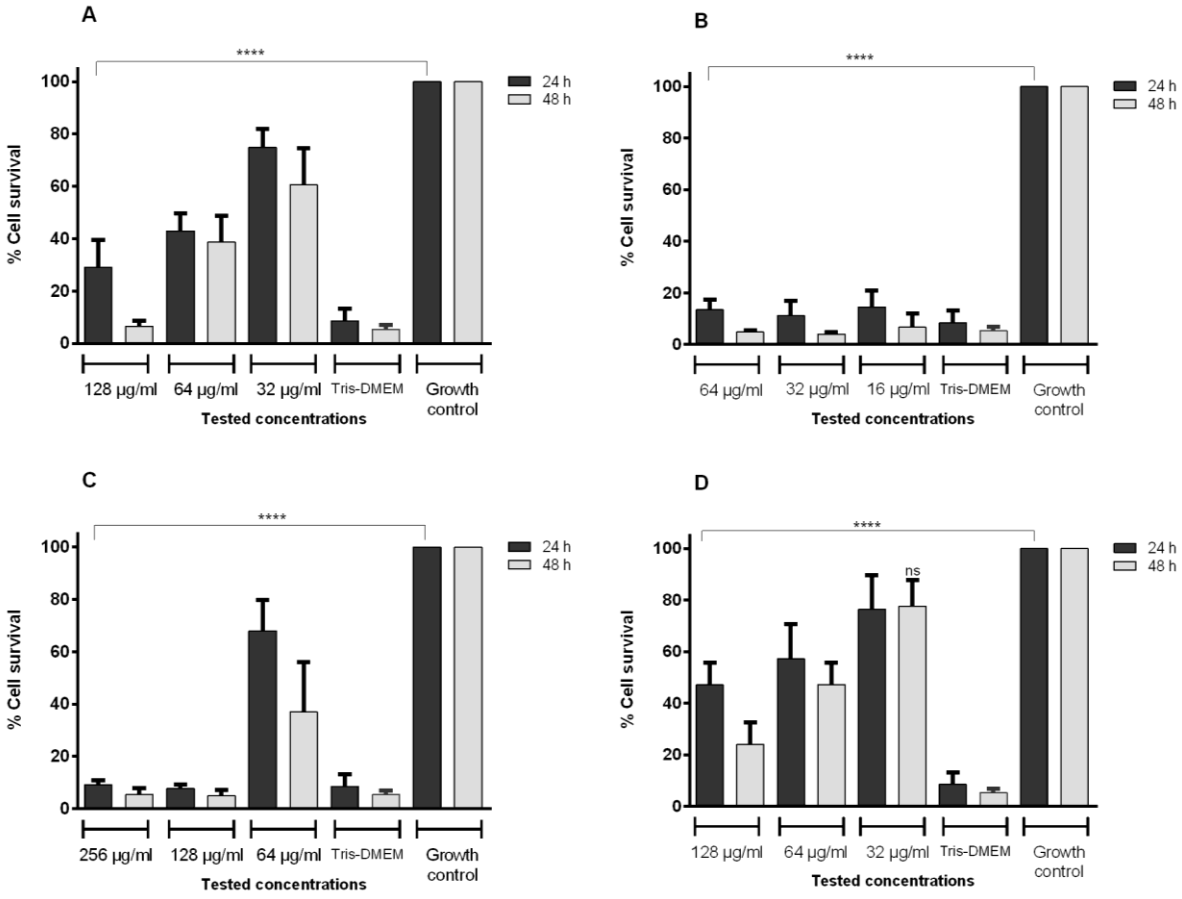

FIG 6 Viability by MTT assay of RAW 264.7 cells after 24 and 48 h of incubation with different concentrations of nonencapsulated phytocompounds: carvacrol (A), cinnamaldehyde (B), citral (C), and thymol (D). ****, $P < 0.0001$. Differences were considered in comparison with the correspondent growth control at 24 or 48 h of incubation. Values are the results of three independent assays

Viability of cells incubated with the encapsulated phytocompounds was also dose dependent and overall significantly greater (Fig. 7). However, incubation with 144 µg/ml of

lipids (DODAB:MO empty liposomes) reduced viability to < 50%. At this lipid concentration, all nanoparticles with phytocompounds were cytotoxic due to both lipids and phytocompounds. DODAB:MO nanoparticles with 18 and 36 µg/ml of lipids, including carvacrol and thymol, were nontoxic, with > 80% macrophage survival. Furthermore, these nanoparticles were better tolerated by the cells than NEPs.

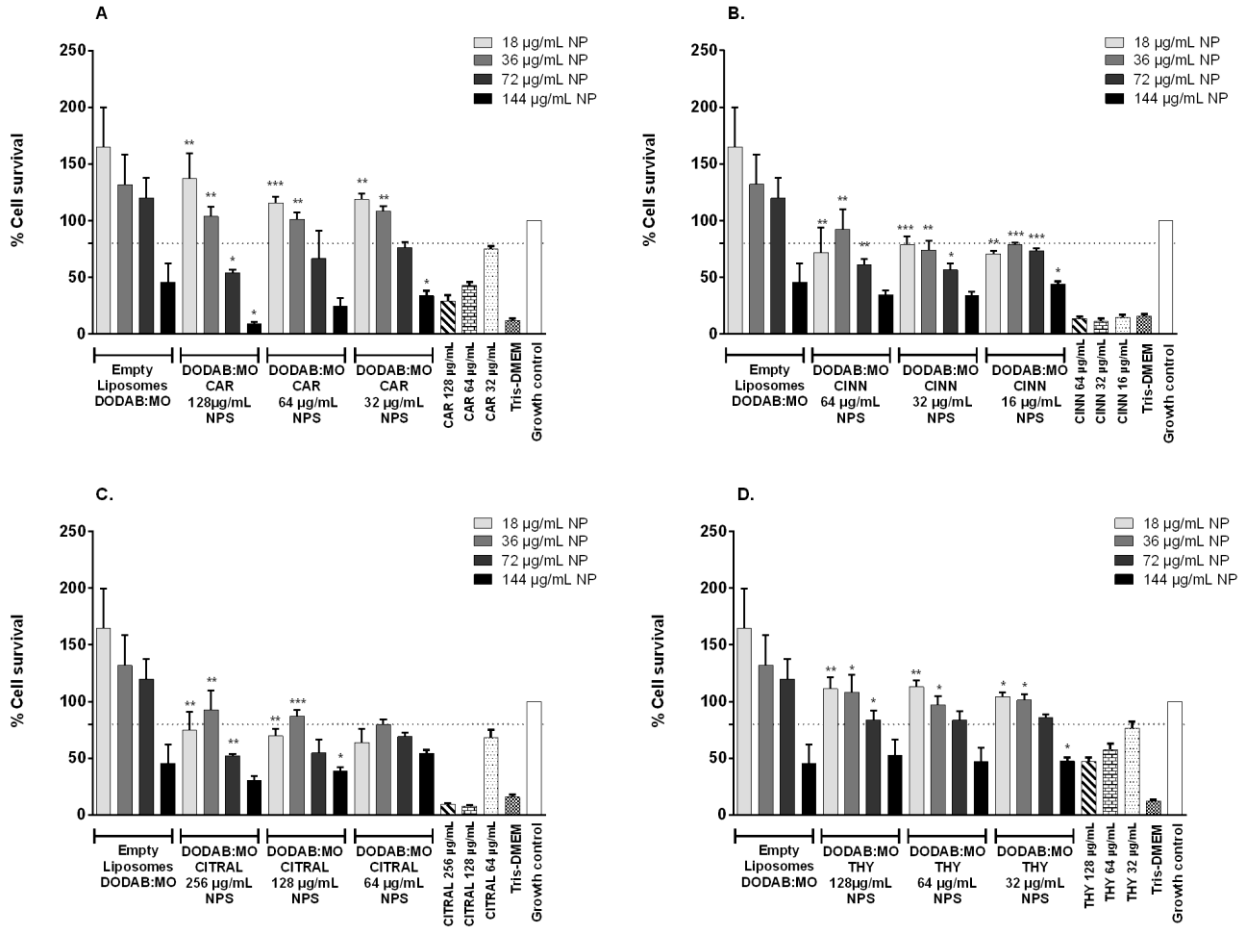

FIG 7 Viability by MTT assay of RAW 264.7 cells after 24 h incubation with nanoparticle formulations prepared with three different concentrations. A: carvacrol, B: cinnamaldehyde, C: citral and D: thymol. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. The differences were considered in comparison with the correspondent concentration of non-encapsulated compound. Values are the results of three independent assays.

Analysis of cell viability by lactate dehydrogenase (LDH) of citral and cinnamaldehyde nanoparticles confirmed the results observed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay that they are more cytotoxic (Fig. 8). Although encapsulation significantly enhanced cell viability, particularly for the highest concentrations of the phytocompounds, they are still considered toxic. For 64 μg/ml of citral, the corresponding nanoparticles were even more cytotoxic macrophages than the nonencapsulated compound. A similar result has been observed with the MTT assay for this formulation. All cinnamaldehyde nanoparticle formulations were significantly less toxic than the corresponding NEP. However, only nanoparticles with 64 μg/ml of cinnamaldehyde were considered noncytotoxic, in agreement with MTT results (92.3%) with 36 μg/ml of lipids (Fig. 7 and 8).

Cell survival rates after treatment with encapsulated phytocompounds and NEPs were taken into consideration in order to test cytokine production; IL-10, a known anti-inflammatory cytokine; and tumor necrosis factor-α (TNF-α), a known proinflammatory cytokine. Therefore, only conditions with > 80% survival in both cell toxicity assays were analyzed, which corresponded only to nanoparticles with 32 μg/ml of carvacrol or thymol. Macrophages incubated with lipopolysaccharide (LPS) were considered positive controls and macrophages alone were negative controls. No compounds or nanoparticles were able to induce IL-10 production at both 2 and 48 h (Fig. 9).

All quantifications were < 32 pg/ml, which was the detection limit. TNF-α levels were also below the detection limit (8 pg/ml) for all conditions tested at 24 h; however, after 48 h of incubation with these compounds and nanoparticles, a slight increase in proinflammatory cytokine was observed (Fig. 10A). Carvacrol nanoparticles at 48 h induced a smaller amount of TNF-α production than the nonencapsulated carvacrol, particularly with

liposomes at 36 µg/ml of lipid ($P < 0.05$) versus liposomes at 18 µg/ml of lipid. The most significant reduction in TNF-α production was observed with nanoparticles loaded with 32 and 128 µg/ml of carvacrol ($P < 0.0001$). With liposomes at 18 µg/ml, a small reduction in TNF-α production was observed only with 32-µg/ml carvacrol nanoparticles ($P < 0.05$) (Fig. 10A).

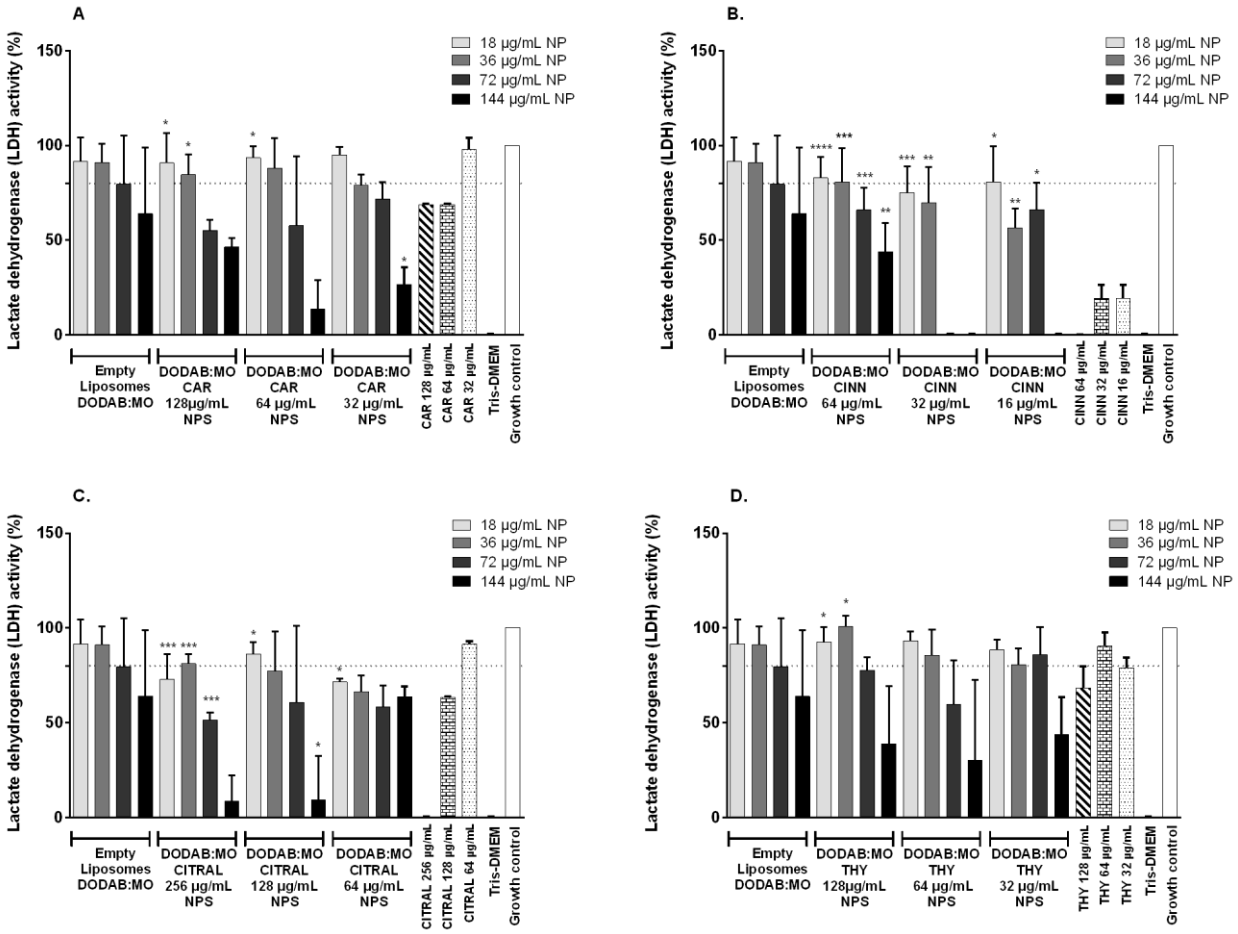

FIG 8 Viability by LDH assay of RAW 264.7 cells after 24 h of incubation with nanoparticle formulations prepared with three different concentrations: carvacrol (A), cinnamaldehyde (B), citral (C), and thymol (D). Differences were considered in comparison with the correspondent concentration of nonencapsulated compound. Values are the results of three independent assays. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

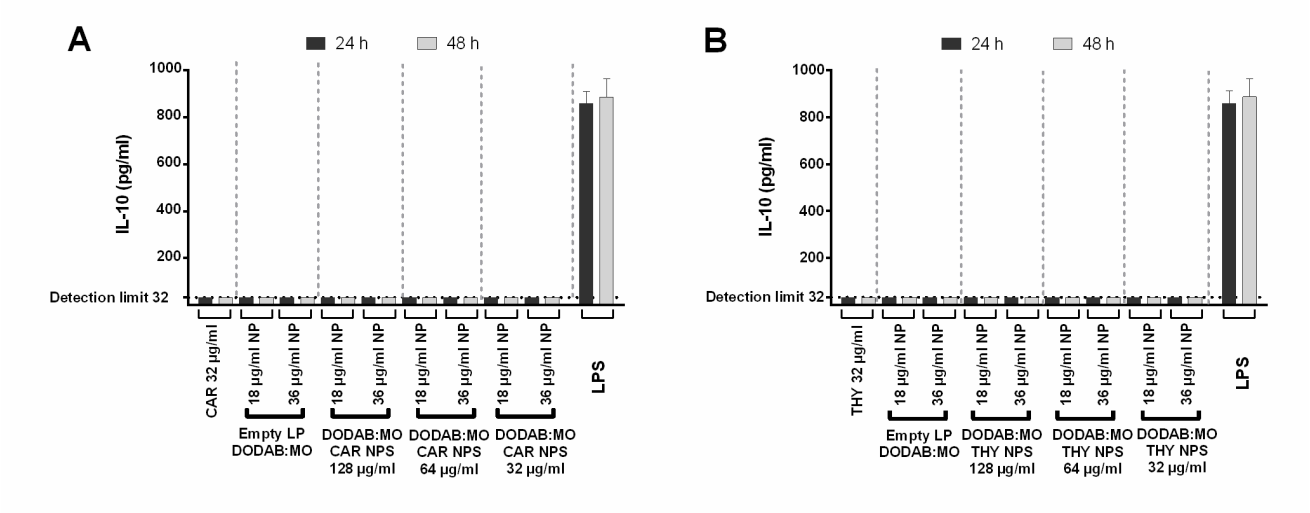

FIG 9 Production of IL-10 by RAW 264.7 cells after incubation with carvacrol (CAR) (A) and thymol (THY) (B) for 24 and 48 h. LPS, 1 µg/ml. Results indicate the mean ± SEM of three measurements from three independent experiments. Differences were considered between nanoparticles and 32 µg/ml nonencapsulated phytocompound at 24 or 48 h of incubation, respectively.

In the case of thymol nanoparticles, undetected levels of TNF-α were observed at 24 h with 128 µg/ml thymol regardless of the lipid concentration; however, a slight increase in TNF-α was observed at 64 and 32 µg/ml thymol encapsulated with 36 µg/ml of lipid ($P < 0.0001$) (Fig. 10B). At 48 h, the same pattern of TNF-α increase was observed as for carvacrol nanoparticles. Of interest, all thymol nanoparticles induced a much smaller amount of TNF-α production than NEPs or empty liposomes ($P < 0.0001$) (Fig. 10B).

Antifungal activities of encapsulated phytocompounds and NEPs are described in Table 3. NEPs were active against *Candida* isolates with mode inhibitory concentrations (IC) of 64 µg/ml for cinnamaldehyde, 128 µg/ml for carvacrol or thymol, and 256 µg/ml for citral. Nanoparticles were prepared with the same amount of phytocompounds: nanoparticles with 64 µg/ml of cinnamaldehyde were more active against planktonic cells of *Candida*, followed by 128 µg/ml thymol or carvacrol nanoparticles, as observed with NEPs..

TABLE 3. IC of encapsulated and nonencapsulated phytocompounds against *Candida* planktonic cells al 24 h.

Candida isolates	IC (μg/ml)							
	Carvacrol		Cinnamaldehyde		Citral		Thymol	
	DODAB:MO	NEP[α]	DODAB:MO	NEP	DODAB:MO	NEP	DODAB:MO	NEP
***C. albicans* UPV 05-007**	>128	128	64	64	>256	256	128	128
***C. albicans* UPV 05-013**	128	128	64	64	>256	512	32	32
***C. albicans* UPV 11-342**	>128	128	64	64	>256	256	128	128
***C. albicans* UPV 11-345**	128	128	64	64	>256	256	64	128
***C. albicans* UPV 12-298**	128	128	64	64	>256	256	128	128
***C. albicans* UPV 15-101**	128	128	64	64	>256	256	32	128
***C. albicans* UPV 15-106**	32	128	64	64	>256	256	64	128
***C. albicans* UPV 15-157**	>128	128	64	64	>256	256	128	128
***C. albicans* SC5314**	32	128	64	64	>256	512	64	128
Mode	128	128	64	64	>256	256	128	128
IC range	32 to >128	128	64	64	>256	256 - 512	32 to >128	32 - 128
IC geometric mean	94.1	128	64	64	256	298.6	74.7	109.7
***C. auris* UPV 18-029**	>128	128	64	64	256	128	32	128
***C. dubliniensis* UPV 11-366**	128	128	64	64	>256	256	>128	64
***C. tropicalis* UPV 06-115**	128	128	64	64	>256	256	64	128

[α]NEP: nonencapsulated phytocompounds.

However, for some isolates, ICs were lower with phytocompound nanoparticles, particularly with thymol. Conversely, citral nanoparticle ICs for all strains analyzed were > 256 μg/ml.

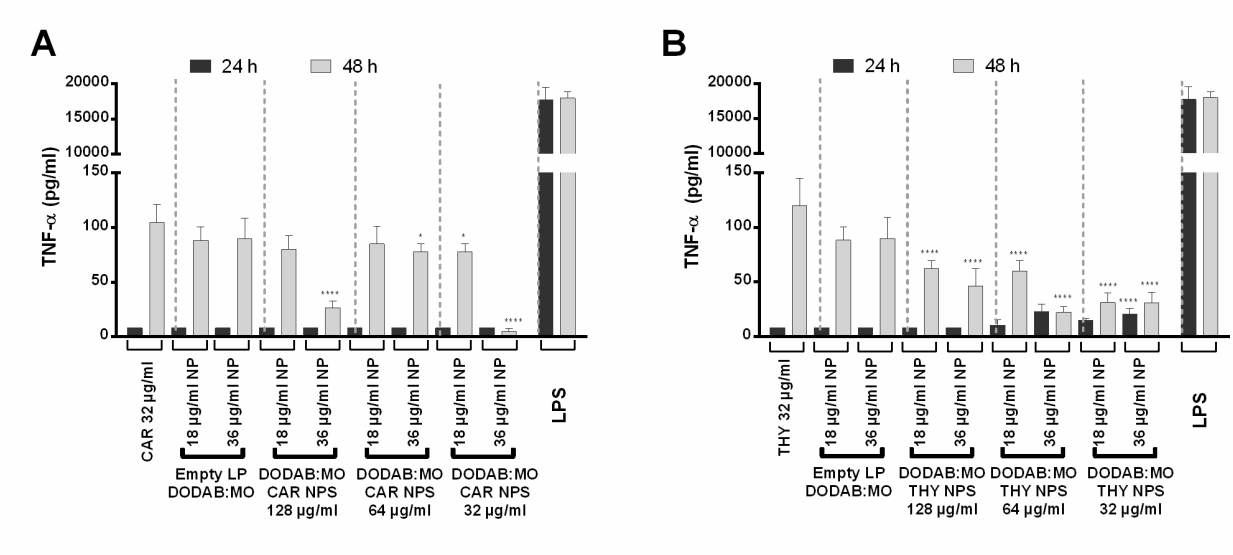

FIG 10 Production of TNF-α by RAW 264.7 cells after incubation with carvacrol (CAR) (A) and thymol (THY) (B) for 24 and 48 h. LPS, 1 μg/ml. Results indicate the mean ± SEM of three measurements from three independent experiments. *, $P < 0.05$, ****, $P < 0.0001$. Differences were considered between nanoparticles and 32 μg/ml nonencapsulated phytocompound at 24 or 48 h of incubation, respectively

The ability of macrophages to kill *Candida* cells with the help of the phytocompounds or their corresponding nanoparticles was also evaluated. Only nanoparticles with 32 and 64 μg/ml of carvacrol or thymol, noncytotoxic, were tested. Although showing some cytotoxicity, the corresponding NEPs were tested for comparison. Macrophages treated with carvacrol nanoparticles reduced *Candida* survival compared with the corresponding nonencapsulated compound. The nanoparticles of 18 μg/ml of DODAB:MO and 64 μg/ml carvacrol induced the highest reduction. This formulation was able to reduce ~38% of viable yeast cells compared with controls and ~25% compared with NEPs ($P < 0.001$) (Fig. 11A). Thymol and carvacrol nanoparticles similarly enhanced the ability of

macrophages to kill yeast cells. Nanoparticles composed of 18 μg/ml of DODAB:MO and 32 or 128 μg/ml of thymol had the greatest reduction (~41%) in *Candida* survival (Fig. 11B).

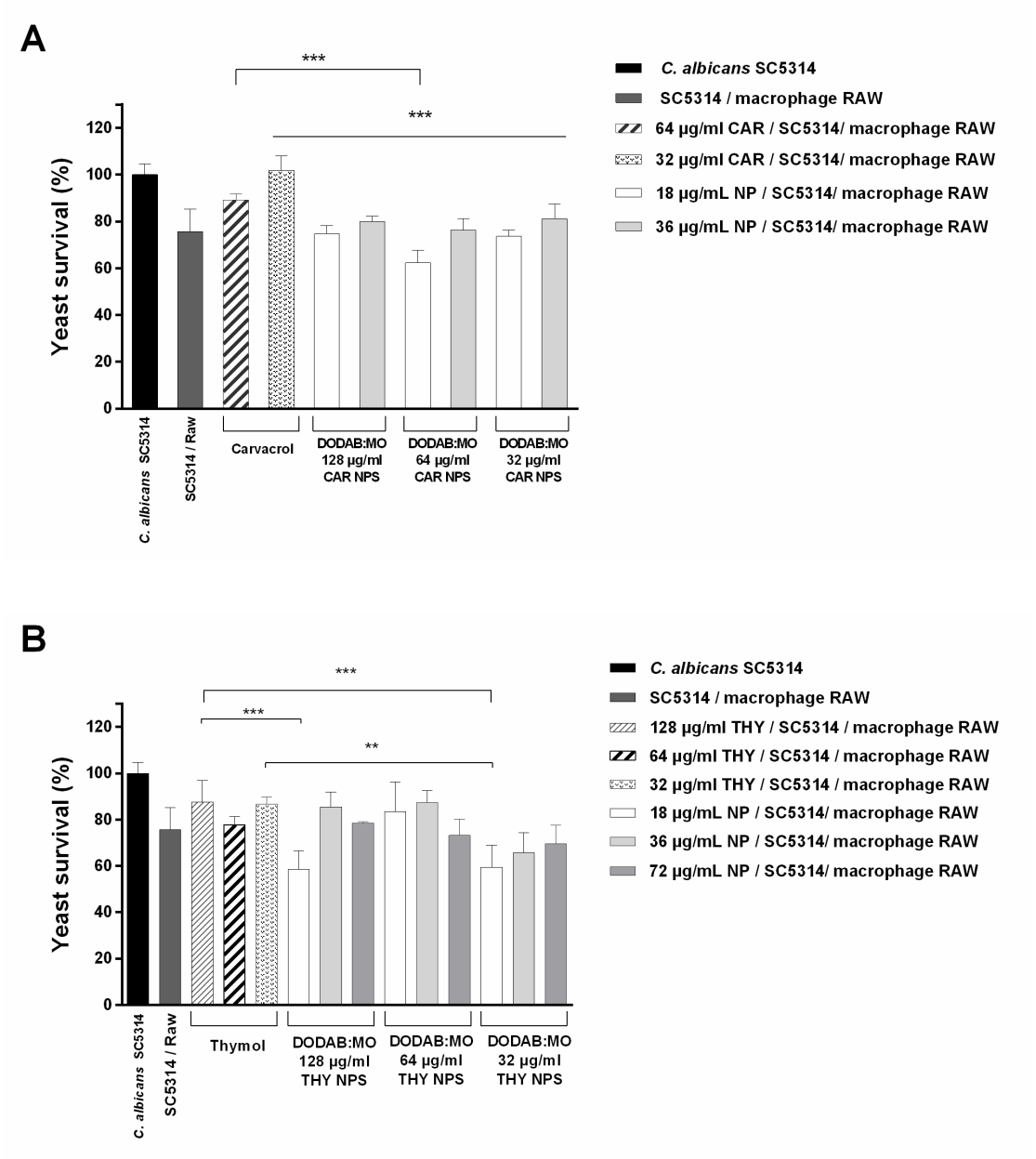

FIG 11 Results of macrophage killing assay with the carvacrol (CAR) (A) and thymol (THY) (B) nanoparticles (NPS) and corresponding nonencapsulated phytocompound. **, $P < 0.01$, ***, $P < 0.001$.

DISCUSSION

Different compounds with antimicrobial activity are obtained from plants: carvacrol and thymol from *Lamiaceae*, *Verbenaceae*, *Scrophulariaceae*, *Ranunculaceae* and *Apiaceae*, cinnamaldehyde from *Lauraceae* and citral from many citrus fruits and *Cymbopogon citratus* (13, 14, 22–24). Antifungal activities of these phytocompounds have been reported against *Candida* planktonic and sessile cells (25–27). However, their high volatilization, chemical instability, sparing water solubility, cytotoxicity and irritant effects are some drawbacks for use in medical therapy (13, 24, 28, 29).

Encapsulation in liposomes has been used as pharmaceutical drug delivery system to protect the bioactive compounds and to increase the permeation rate of drugs, increasing the mean retetion time at the desired medical target (30). DODAB:MO (1:2) liposome formulation is very efficient in producing nanoparticles because of the high capacity of encapsulating compounds by forming homogeneous multilamellar vesicles (31). In the current study, the highest encapsulation efficiencies were achieved with citral (78% to 83%) and carvacrol (66% to 71%) liposomes. High encapsulation efficiency in soy phosphatidylcholine liposomes of carvacrol (~98%) and citral (86%) has been described (32, 33). The same has been observed for thymol (79%) in lipoid S100 and cholesterol liposomes (34). Moreover, a %EE of <48% in cinnamaldehyde liposomes has been shown (35).

Encapsulation protects phytocompounds against thermal- and/or photodegradation; increases their stability; extends the final-product shelf life (36); and, according to our outcomes, significantly reduces the cytotoxicity in mammalian cells of carvacrol, cinnamaldehyde, and thymol prepared with DODAB:MO liposomes. Carvacrol and

thymol nanoparticles prepared with 18 and 32 μg/ml of DODAB:MO liposomes were considered nontoxic regardless of the concentration of the phytocompound used.

In addition, carvacrol and thymol maintained and increased their antifungal activity after encapsulation, in agreement with previous studies (37–39). The improvement in antifungal activity was identified mainly when the liposome formulations of thymol were used against *C. albicans*, *C. tropicalis*, and *C. auris* isolates, with up to 4-fold reductions in ICs compared with those of nonencapsulated thymol; likewise, liposome formulations of carvacrol showed up to 4-fold reduction of the required concentration compared with NEPs against two *C. albicans* isolates. This fact can be related to positive ζ-potential of nanoparticles, since it is a relevant factor for the antifungal effect, enabling interaction with the negatively charged fungal surface (40). Cinnamaldehyde DODAB:MO liposomes also maintained their anti-*Candida* activity as well as multilamellar cinnamaldehyde liposomes (29).

Converse to the findings reported in other studies (32, 41), citral showed high encapsulation efficiency but very low antifungal activity in the current study. This suggests that citral in DODAB:MO liposomes does not easily get access to the extracellular medium to be active, in contrast to the nanoemulsion formulations and soy phosphatidylcholine liposomes.

Before testing whether the noncytotoxic formulations could help macrophages kill *Candida* cells, we tested whether they could be inflammatory. At 24 h of incubation, no significant TNF-α production was observed by macrophages treated with carvacrol or thymol. Encapsulation did not enhance TNF-α production; on the contrary, it significantly reduced this proinflammatory cytokine, particularly with nanoparticles of thymol. In all cases, TNF-α production was significantly less than that assessed in the presence of LPS.

TNF-α is a potent pleiotropic and proinflammatory cytokine, one of the most abundant early mediators in inflamed tissue, produced mainly by cells of the monocyte lineage (42). Hence, slight TNF-α production from macrophages, such as that obtained for carvacrol and thymol liposomes, can lead to cell recruitment and antifungal action of macrophages and other inflammatory cells without severe deleterious inflammation (43). Reduction of yeast survival when macrophages were incubated with carvacrol and thymol liposomes was significant compared with that of the corresponding nonencapsulated compounds. This result indicates that even if the IC obtained with the encapsulated compounds is not significantly reduced or the encapsulation efficiency is not high, liposomes seem to be beneficial in host-pathogen interaction, helping macrophage killing of *Candida* cells.

The DODAB:MO system has been described as an efficient delivery system because of its high interaction with mammalian cells and their internalized formulations by endocytosis into fungal cells (19, 44). These features could be responsible for the improved bioavailability of these phytocompounds within the macrophage and the subsequent interaction with *Candida* cells, enabling their antifungal activity. However, antifungal mechanisms of these nanoparticles have not yet been defined.

In conclusion, our data confirm that carvacrol and thymol liposomes possess high stability, low cytotoxicity, and antifungal activity that act synergistically with macrophages. These phytocompounds can be promising therapeutic alternatives for candidiasis.

MATERIALS AND METHODS

Preparation and characterization of liposomes. DODAB-based (Tokyo Kasei, Japan) and MO-based (Sigma-Aldrich) liposomes were prepared using a lipid-film hydration method (45). The liposomal stock dispersion was prepared at 4 mM total lipid

concentration. Briefly, an MO molar fraction (χMO) of 0.330 (DOBAB:MO molar ratio of 1:2) was dissolved in ethanol (high spectral purity; Uvasol, UK) and mixed in a glass tube. The solvent was then removed by rotary evaporation using nitrogen gas. Liposomes were obtained after hydration of lipid film with ultrapure water at 60 °C, vortexed for 2 min. Liposome-based nanoparticles were assembled with equal volumes of liposomal stock dispersion and the phytocompound solution to obtain formulations with 0.5% DMSO (Sigma-Aldrich, USA) and a final lipid concentration of 2 mM (888 μg/ml). Stock solutions of carvacrol (51,200 μg/ml), cinnamaldehyde (25,600 μg/ml), citral (102,400 μg/ml), and thymol (51,200 μg/ml) were prepared in DMSO. The final concentrations were 32, 64, and 128 μg/ml for carvacrol and thymol; 16, 32, and 64 μg/ml for cinnamaldehyde; and 64, 128, and 256 μg/ml for citral. These formulations were incubated for 45 min at 60 °C to allow for compound absorption. Moreover, empty liposomes were developed and characterized at 2 mM with 0.5% DMSO.

Mean size, PDI, and error values of the nanoparticles conserved at 4 °C were determined by dynamic light scattering at 25 °C with a Malvern ZetaSizer Nano ZS particle analyzer every week from the first day of production until 5 weeks later. The charge of the liposome surface was measured indirectly by ζ-potential analysis, using electrophoretic light scattering at 25 °C. Malvern dispersion technology software was used with multiple-narrow-mode (high-resolution) data processing for size and PDI, whereas monomodal data processing was used for average ζ-potential and error values. All characterization was performed in quintuplicate.

The prepared formulations were pelleted by ultracentrifugation (100,000 × *g* for 1 h at 4 °C), after which the supernatant was removed and then filtered using 0.22-μm acetate cellulose filters (Millipore Merck, Germany). The concentration of NEP in the supernatant was determined by HPLC-DAD (Hitachi EZChrom Elite, Agilent Technologies, USA).

The methodology was standardized for each phytocompound by preparing a standard curve for all. The analysis was carried out using the Vydac 218TP54 column (C18, 5 µm, 4.6 mm inside diameter × 250 mm), with acetonitrile and water (50:50) mobile phase in isocratic mode at 1 ml/min flow. A diode-array detector was set at 210 nm to detect carvacrol and thymol, 290 nm for cinnamaldehyde, and 240 nm for citral (46–48). The chromatographic runs were carried out at 30 °C for 20 min after an injection of 90 µl of samples or controls in three independent experiments.

Cytotoxicity assay. The murine macrophage-like cell line RAW 264.7 from strain ATCC TIB-71 (49) was cultured in cell-culture flasks with Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, USA) supplemented with 10% heat-inactivated fetal bovine serum (Valbiotech, USA), 2 mM L-glutamine (1%), 1 mM sodium pyruvate (1%), and 10 mM HEPES buffer (1%) (Sigma-Aldrich, USA) in 5% CO_2 at 37°C. After confluent growth, macrophage cells were recovered and washed with DMEM. Then, viable cells were determined with Trypan blue exclusion by counting in a hemocytometer, and a final concentration of 5×10^4 cells/ml in DMEM was prepared and 200 µl dispensed onto 96-well tissue culture plates (Thermo Fisher Scientific).

The culture plates with macrophages were incubated overnight at 37 °C and 5% CO_2; after that, the supernatant was removed, and empty liposomes, nanoparticles, and NEPs were added with DMEM in triplicate. Different concentrations of carvacrol, cinnamaldehyde, citral, thymol, their nanoparticles (DODAB:MO [1:2] 0.5% DMSO), and empty liposomes 2 mM (18, 36, 72, and 144 µg/ml) were evaluated.

Cell line RAW 264.7 metabolic activity was determined using MTT. The impact on membrane integrity was assessed by LDH assay after 24 and 48 h of incubation. Enzymatic activity over MTT was quantified after solubilization of MTT formazan by adding DMSO-

ethanol (1:1) solution, and absorbance was measured at 570 nm (50). The control of viability (100%) was the untreated cells, and the control of cytotoxicity was the cells treated with Tris-HCl. Results of the percentage of viability were expressed as described previously (51), according to the following equation:

$$Viability\ (\%) = \frac{Experimental\ value\ (average) - Control\ of\ cytotoxicity\ (average)}{Control\ of\ viability\ (average) - Control\ of\ cytotoxicity\ (average)} \times 100$$

The LDH leakage assay was performed by measuring LDH activity in the extracellular medium at 30°C in a microplate reader (Spectra Max 340PC) at 340 nm, employing pyruvate 0.32 mM (in phosphate buffer, pH 7.4) as the substrate. Results were expressed as the percentage of viability compared with the control without treatment.

Cytokine production. Proinflammatory cytokine TNF-α was quantified according to the manufacturer's instructions (mouse TNF-α and IL-10 enzyme-linked immunosorbent assay kit, Thermo Scientific) from three independent assays on 96-well tissue culture plates with macrophages and empty liposomes, nanoparticles, and NEPs after 24 h of incubation, as described above. Macrophages incubated with 1 μg/ml LPS were used as positive controls, and macrophages alone were used as negative controls.

Antifungal activity of nanoparticles. *In vitro* antifungal activity against *Candida* planktonic cells was evaluated according to EUCAST methodology (52, 53). Eight *C. albicans*, one *C. dubliniensis*, one *C. tropicalis*, and one *C. auris* clinical isolates and the high biofilm-producer strain *C. albicans* ATCC SC5314 were tested. *C. albicans* UPV 15–157, *C. dubliniensis* UPV 11–366, and *C. auris* UPV 18-029 were resistant to fluconazole (MIC, ≥ 64 μg/ml). To determine susceptibility, carvacrol, cinnamaldehyde, citral, and thymol were assayed at 2-fold serial concentrations ranging from 1 to 1.024 μg/ml. Liposomal formulations were tested in triplicate, including empty liposomes in RPMI 1640

(with L-glutamine and without bicarbonate; Sigma-Aldrich) supplemented with 2% glucose and buffered to pH 7.0 with 3-*N*-morpholinepropanesulfonic acid (Sigma-Aldrich). Briefly, a cell inoculum of 0.5 to 2.5 × 10^5 cells/ml from each *Candida* isolate cultured at 24 h and 37 °C in Sabouraud dextrose agar (Difco, USA) was dispensed onto the previously prepared 96-well microplates with the phytocompounds and nanoparticles. Sterility and growth control wells were included in each microplate. Absorbance at 450 nm after 24 and 48 h of incubation at 37 °C was measured with an iMark microplate absorbance reader (Bio-Rad, USA). ICs were calculated as the lowest phytochemical and nanoparticle concentrations inhibiting ≥ 50% growth after 24 h compared with controls.

Macrophage killing assay. The ability of macrophage cells to kill *Candida* isolates after the addition of phytocompounds and their nanoparticles was determined according to a previously described protocol (54). Briefly, an overnight macrophage culture was disposed at a concentration of 1 × 104 cells/well of macrophages onto 96-well tissue culture plates and incubated at 37 °C and 5% CO_2 atmosphere. After 1 h of incubation, to allow macrophage adherence onto the tissue culture plate, the NEPs and the nanoparticles were added into the wells. At the same time, *Candida* cells were added to macrophages at a ratio of 5:1, using 100 µl of 5 × 10^5 cells/ml inoculum of *C. albicans* SC5314 in each well. After 1 h of incubation, the macrophages were lysed with 10% saponin solution (Sigma-Aldrich), and serial dilutions of the suspension were plated on yeast extract-peptone-dextrose agar (Sigma-Aldrich). CFUs were determined after 24 h of incubation at 37 °C, including CFUs from macrophages incubated with *Candida* without treatment and *Candida* alone as control.

Statistical analysis. Comparative analysis of different groups was made using analysis of variance followed by the Bonferroni test (GraphPad Prism 5.0, USA). Unless otherwise stated, results shown are from at least two independent experiments with three replicates. In all cases, *P* values of <0.05 were considered statistically significant.

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We have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the current manuscript.

Note Added after Publication

In the original published version of this paper, incorrect files were used for Fig. 6 to 10. In addition, "$P < 0.5$" should be "$P < 0.05$" in the legends of Fig. 7, 8, and 10, and in the 5th paragraph of the Results section, "64" in the 2nd-to-last sentence should be "32" and "$P < 0.5$" in the last sentence should be "$P < 0.05$." All of these corrections have been made in this version of the article.

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DISCUSSION

Oral candidiasis is a common infection affecting the oral mucosa especially in patients with immunodeficiencies, at the extremes of life and/or denture wearers (Khedri et al., 2018; Lu, 2016; Vila et al., 2020; Zomorodian et al., 2011). *Candida* is common in the oral microbiota without causing disease. However, a significant imbalance between the immune system and the oral microbiota is conducive to the development of candidiasis (Millsop & Fazel, 2016; Vila et al., 2020). Prior colonization with *Candida* is indispensable for the development of infection (Eggimann & Pittet, 2014; Fanello et al., 2006; Kumamoto, 2011; Nucci & Anaissie, 2001). In the process from mucosal colonization to *Candida* infection, several determining factors are involved, such as the virulence factors of this fungus and/or the state of the immune system, which can be conditioned by diseases and other underlying physiological factors (Samaranayake et al., 2009; Sharon & Fazel, 2010). In prosthetic stomatitis, the use of dental prostheses is associated with an increased predisposition to *Candida* colonization and infection. Several factors, such as decreased salivary flow, poorly fitting dentures, inadequate oral hygiene, smoking, age, and treatment with antibiotics or immunosuppressive drugs, contribute to the development of this disease (Altarawneh et al., 2013; Ramage et al., 2001; Reinhardt et al. 2018).

In patients with underlying diseases, oral candidiasis is usually more severe. In patients undergoing radiotherapy treatment for oral, oropharyngeal, or neck and head cancer, oral candidiasis can reach an incidence rate of between 25 and 50% (Kawashita et al., 2018; Mañas et al., 2012; Nicolatou-Galitis et al., 2001; Nishii et al., 2020). Similarly, in HIV-infected patients, moderate and severe candidiasis lesions are frequent, with a prevalence that can approach 90%. Pseudomembranous candidiasis and erythematous candidiasis are the most common forms in clinical presentations, and they are associated with a high risk of developing complications with other more-severe lesions, such as candidiasis

oesophagitis (Glick, 2015; Khedri et al., 2018; Lauritano et al., 2020; Ottria et al., 2018; Quindós et al., 2019).

C. albicans is the species most frequently implicated in the aetiology of oral candidiasis (Ottria et al., 2018; Patil et al 2018). However, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* have been increasing identified as aetiologic agents (Hu et al., 2019; Patil et al., 2018). Overall, proper diagnosis and timely treatment are important to achieve effective resolution of oral candidiasis, and accurate identification of the species involved is becoming increasingly important for proper therapy.

For this PhD Thesis, we studied the importance of different *Candida* species isolated from oral samples of patients with suspected oral candidiasis between 2003 and 2013 and the *in vitro* susceptibility profile of these species to antifungal drugs. *C. albicans* was found to be the most prevalent species (70.4%), followed by *C. glabrata* (8.6%) and *C. parapsilosis* (7.4%). In addition, *C. glabrata* and *C. parapsilosis* were present in high proportions in mixed cultures (60.6%). The high level of *C. glabrata* and *C. parapsilosis* is particularly relevant because of their low susceptibility to antifungal drugs and because their presence in mixed infections may affect treatment. Other studies have found a similar distribution of *Candida* species in oral isolates. Muadcheingka & Tantivitayakul (2015) found that *C. glabrata* accounted for 15.2% and *C. parapsilosis* accounted for 3.2% of the isolates they obtained. In a study by Jahanshiri et al. (2018), *C. glabrata* was the third most frequently identified species (15%) in oral isolates obtained from head and neck cancer patients with oropharyngeal candidiasis.

Other authors have found these species in similar frequencies but had included *C. nivariensis* and *C. bracarensis*, species closely related to *C. glabrata* and *C. orthopsilosis,* and *C. metapsilosis*, related to *C. parapsilosis*, in their studies (Asadzadeh et al 2019; Borman et al., 2008; Ge et al., 2012; Gupta et al 2020; Lockhart et al., 2009; Mashaly et al 2019; Moris et al., 2012; Sadeghi et al., 2018; Wahyuningsih et al., 2008).

In our study, *C. parapsilosis* was the most frequently isolated species among the species belonging to the *C. parapsilosis* complex. The low prevalence of *C. orthopsilosis* and *C. metapsilosis* in our study was consistent with the findings described by Moris et al. (2012), who found differences with *C. parapsilosis* in terms virulence and *in vitro* susceptibility to antifungal drugs. Other studies have not isolated *C. orthopsilosis* and *C. metapsilosis* from oral isolates (Feng et al., 2012; Ge et al., 2012; Sadeghi et al., 2018).

Within the *C. glabrata* complex, *C. glabrata* remains the most prevalent species. In our study, *C. nivariensis* and *C. bracarensis* species were not isolated in oral samples from patients with oral candidiasis. The low prevalence or absence of these species in the oral cavity has also been described by Lockhart et al. (2009), Asadzadeh et al. (2019) and Mashaly et al. (2019). Some isolated cases of *C. nivariensis* colonization of the mucosa or candidiasis have been described (Borman et al., 2008; Gupta et al., 2020; Wahyuningsih et al., 2008). Differences in species distribution have been correlated with several factors, such as diet, geographic location, the population studied, and with different yeast virulence factors (Guinea et al., 2014).

In many cases, several *Candida* species have been found to be infecting the oral cavity, and coinfections with two species are the most common cases, including *C. albicans* with *C. tropicalis*, *C. albicans* with *C. krusei*, *C. albicans* with *C. glabrata*, and *C. albicans* with *C. parapsilosis* (Coco et al., 2008; Hu et al., 2019; Martins et al., 2010; Muadcheingka & Tantivitayakul, 2015; Zomorodian et al., 2011). In the present study, the association of *C. albicans* and *C. glabrata* was the most common (64.9%), followed by *C. albicans* with *C. parapsilosis* (37.1%). Previous colonization with *C. albicans* is exploited by *C. glabrata* in the process of invasion of oral tissues and development of infection because *C. glabrata* cells binds to hyphae produced by *C. albicans* (Tati et al., 2016). This effect has also been observed in a rat model of prosthetic stomatitis, where co-colonization with *C. albicans* and

C. glabrata progressed to oral candidiasis, whereas colonization with *C. glabrata* alone was not associated with appreciable inflammatory changes (Yano et al., 2019).

The presence of species less susceptible to antifungal treatments, such as *C. glabrata* or *C. krusei*, alone or together with *C. albicans*, has been associated with increased severity of infection, recurrence and therapeutic failure, especially in patients who had received prophylaxis or prior azole treatment (Aslani et al., 2018; Junqueira et al., 2012; Redding et al., 2004).

Candida virulence factors are determining mechanisms in the colonization and invasion of tissues, evasion of the immune response, and permanence in biomedical devices (Vila et al., 2020). Adhesins allow binding to tissues, while proteases and phospholipases facilitate invasion. *C. albicans* produces enzymes such as Saps and PLB1 and adhesion proteins such as ALs3 and Hwp1, which are related to the infection process, evasion of the immune response and tissue damage (Mayer et al., 2013; Rapala-Kozik et al., 2017; Vila et al., 2020). However, other species have also evolved mechanisms of colonization and infection. In *C. dubliniensis*, phospholipases homologous to those of *C. albicans* have been described: Sapcd1 to Sapcd4 and Sapcd7 to Sapcd10. In other species, phospholipases have been observed less frequently (Rapala-Kozik et al., 2017; Vidotto et al., 2004). Epa1 expression in the cell wall of *C. glabrata* is involved in its adherence to the oral epithelium, whereas in *C. tropicalis*, Als1-3 and Hwp1 proteins are responsible for binding to tissues and biomaterials (Swidergall & Filler, 2017; Zuza-Alves et al., 2017). *C. parapsilosis* binding to the oral epithelium is mediated mainly through CpAls7, a protein homologous to that of *C. albicans* (Tóth et al., 2019).

The production of biofilms favours the permanence of the fungus in tissues and biomedical devices, contributing directly to the pathogenesis of disease (Nobile et al., 2015). Since microorganisms do not usually grow as single cells, biofilm formation is very

common in the oral microbiota (O'Donnell et al., 2015). Biofilms structures are composed of a community of interacting microorganisms tightly attached to biotic or abiotic surfaces surrounded by an extracellular matrix of microbial origin composed of polysaccharides, proteins, glycoproteins and free DNA. Their complex composition limits the penetration of antifungal drugs, favouring resistance, limiting the immune response and protecting microorganisms against changes in the surrounding environment (Nobile & Johnson, 2015; Ramage et al., 2012).

In study 2 of this PhD Thesis, the biofilm production capacity of several oral *Candida* isolates was evaluated. However, there are important limitations to comparing biofilms of different *Candida* species solely on the basis of their metabolic activity; therefore, their biomass was also studied and compared (Ramage, 2016). *C. albicans* showed the greatest production of both biomass and highest metabolic activity among the isolates studied. Certainly, this characteristic of *C. albicans* makes it more successful as a colonizer and as a cause of disease. Moderate and high metabolic activity of biofilms has been observed to play a predictive role in invasive candidiasis-related mortality (Nobile & Johnson, 2015; Vitális et al., 2020). Swindell et al. (2009) have found an association between *C. albicans* biofilm production and a higher mortality rate in patients receiving parenteral nutrition since it induces germination and biofilm formation. In addition, biofilm production has been correlated with higher mortality rates in patients who have been treated with azoles or had a high Apache II score (Rajendran et al., 2016; Tascini et al., 2017; Tumbarello et al., 2012).

The ability to transform yeast to hyphae endows *C. albicans* with a considerable advantage in the formation of robust biofilms, and the presence of a higher hyphal content is related to a stronger compressive strength, which makes biofilms more resistant to removal (Paramonova et al., 2009). On the other hand, certain proteins, such as regulatory Efg1 and

Hwp1, play important roles in the transformation of yeast into hyphae, which is necessary for the formation of biofilms (Nobile et al., 2006; Ramage et al., 2002). For non-*C. albicans Candida* species, the ability to produce biofilms affects virulence to a greater extent than that of *C. albicans* (Pannanusorn et al., 2013). Other species, such as *C. tropicalis* and *C. dubliniensis*, are also hyphae and pseudohyphae producers, and many of their isolates develop biofilms (Galán-Ladero et al., 2018). In this Thesis work, *C. tropicalis* isolates produced biofilms with abundant biomass but were metabolically less active than those of *C. albicans*. Only one of the *C. dubliniensis* isolates was classified as high biomass biofilm producer and having high metabolic activity. The other isolates were categorized as moderate- or low-biofilm producers.

C. parapsilosis develops abundant biofilms on different materials used in the manufacture of catheters and other biomedical devices (Toth et al., 2019). However, the oral isolates of the *C. parapsilosis* complex evaluated produced low-abundance biofilms, and only one isolate of *C. parapsilosis* was categorized as high biomass biofilm producer and having low metabolic activity. This variability among isolates has been previously described and may be due to the clinical origin of the isolates (Lattif et al., 2010; Silva et al., 2009). For example, blood isolates form biofilms with high metabolic activity, abundant yeast content and a high concentration of carbohydrates in the extracellular matrix (Cavalheiro & Teixeira, 2018). This variability is also observed with *C. krusei*, but in this Thesis study, the isolates evaluated were not biofilm producers, whereas in other studies, they have been described (Pannausorn et al., 2012; Silva et al., 2009).

The eradication of biofilms remains a problem with difficult resolution and high epidemiological relevance (Ramage et al., 2014; Tascini et al., 2018). Biofilms decrease the effectiveness of antimicrobial treatment, which is hampered by biofilm structural and functional characteristics. The dense layer of extracellular components surrounding sessile

cells acts as both a physical and biological defence mechanism to evade the immune response and antimicrobial action of antifungal drugs. Thus, significantly increased *in vitro* resistance to azoles of sessile cells compared to planktonic cells has been described (Ramage et al., 2011). Azoles such as fluconazole, voriconazole and posaconazole are characterized by their interruption of different phases in ergosterol biosynthesis, a sterol necessary for fungal cell membrane stability. Disruption of this biosynthesis results in the accumulation of toxic intermediary metabolites with a fungistatic effect. When biofilms are formed, the cell density is high, and there is an abundant extracellular matrix with a reduced growth rate. Drug targets are not accessible, and therefore, drug action is diminished. In addition, a change in gene expression is observed, with overexpression of targets and increased sterol content in the cell membrane, as well as the presence of persister cells (Silva et al., 2017). This poor *in vitro* antifungal activity of fluconazole and isavuconazole against *Candida* biofilms, in terms of both biomass reduction and metabolic activity, was verified by this PhD Thesis work.

In the present study on the prevalence and antifungal susceptibility of *Candida* isolates from patients with oral candidiasis, cross-resistance to azoles was observed. These cross-resistance cases mostly involved fluconazole and itraconazole in *C. glabrata* and itraconazole and miconazole in *C. parapsilosis*. Cross-resistance may be observed in isolates of *C. albicans* when the patient has received long-term treatment with fluconazole (Cartledge et al., 1997).

Miconazole and nystatin are frequently used to treat mild forms of oral candidiasis. Other newer azoles, such as posaconazole and isavuconazole, are very useful in the treatment of invasive candidiasis. However, they are not effective against biofilms (Kullberg et al., 2019). Echinocandins are especially useful in the treatment of severe candidiasis, such as oesophageal candidiasis. However, the lack of oral and/or intravenous formulations and poorly accessibility in some countries limits the widespread use of echinocandins (Quindós

et al., 2019). Our results showed that anidulafungin reduced the metabolic activity of biofilms of most *C. albicans* isolates, however this echinocandin had no antifungal effect on *C. dubliniensis* and *C. tropicalis*. Other studies have described variable activity of micafungin and anidulafungin against biofilms: they reduce the metabolic activity of biofilms of some *Candida* species, but their effect on biomass reduction is lower (Prażyńska et al., 2018; Valentín et al., 2016). Some antifungal drugs are being evaluated for human use, such as Ibrexafungerp, which shows promising results in the treatment of infections caused by *Candida* and *Aspergillus* (Davis et al., 2019).

Humans have constantly resorted to the raw materials around them, such as plants, in the search for compounds with antimicrobial properties. Throughout history, compounds with antibiotic properties have been obtained. Among these compounds, essential oils with antimicrobial properties are promising (Aziz et al., 2018; Kumar et al., 2019; Nabavi et al., 2015; Valdivieso-Ugarte et al., 2019). Essential oils are obtained from different parts of the plant, concentrating a mixture of volatile organic compounds that serve as defence mechanisms against different threats. In plants, essential oils are found in the cytoplasm of secretory cells in hairs or trichomes, epidermal cells, inner secretory cells and secretory sacs (Dhifi et al., 2016).

Essential oils are mixtures that vary in composition, which may result in differences in desired antimicrobial activity under varying climatic conditions or with different extraction processes or plant varieties (Jordan et al., 2006; Viljoen et al., 2005). The study of the phytocompounds present in essential oils is crucial to evaluate their antimicrobial activity, discern the action of the most-active compounds and discard the compounds with no activity or that may be associated with adverse reactions.

The most common phytocompounds are terpenes, aldehydes, phenols, ethers, ketones, esters and amines (Dhifi et al., 2016). In this Thesis work, the antifungal activity of

carvacrol, cinnamaldehyde, citral, eugenol, geraniol, linalool, terpinen-4-ol and thymol, which are present in high concentrations in essential oils of major aromatic plants, was evaluated. Carvacrol and thymol are monoterpenes with similar antimicrobial activity, largely due to their chemical composition as cyclic phenol isomers (Nagegowda & Gupta, 2020; Suntres et al., 2015). Citral, geraniol, and linalool are monoterpenes, specifically acyclic alcohols (Aprotosoaie et al., 2014; Chen & Viljoen, 2010). Terpinen-4-ol is a monoterpene with a monocyclic alcohol structure (de Groot & Schmidt, 2016). Cinnamaldehyde and eugenol are phenylpropanoid acids (Dhara & Tripathi, 2020; Sun et al., 2020).

The antimicrobial activity of phytocompounds that inhibit the adhesion of microorganisms on biomedical devices and dental prostheses has been described (de Vasconcelos et al., 2014; Raut et al., 2013). These phytocompounds have been incorporated into different mouthwashes, tissue conditioners and aliphatic polyester membranes (surfaces coated with a film with antibiofilm properties) or mucoadhesive systems (de Fátima Souto Maior et al., 2019; Obaidat et al., 2011; Scaffaro et al., 2018). However, there are few studies on their usefulness in the treatment of oral candidiasis (Chami et al., 2005).

In our study, all tested phytocompounds showed antifungal activity against planktonic cells of different *Candida* species. The most active compounds with fungistatic and fungicidal activity were cinnamaldehyde, thymol, carvacrol and citral. This higher activity is consistent with previously described results (Chatrath et al., 2019; de Castro et al., 2015; Freire et al., 2017; Khan et al., 2012; Khan & Ahmad, 2012; Leite et al., 2014; Rajput & Karuppayil, 2013; Shreaz et al., 2010; Zore et al., 2011). Terpinen-4-ol and linalool showed lower antifungal activity and, together with geraniol, exerted no or low fungicidal effects against *Candida*, even at the highest concentration tested. Our results differ from those published by Zore et al. (2011) who studied *C. albicans* and suggested that geraniol and linalool

exerted higher fungicidal activity than citral or citronellal linalyl acetate. Similarly, Leite et al. (2015) observed fungicidal activity with 32 mg/L of geraniol.

Although other authors have described the antifungal activity of terpinen-4-ol, high concentrations were necessary. Moreover, this phytocompound has been found to be active against a few *Candid*a species, including *C. glabrata*, *C. orthopsilosis* and *C. krusei* (Francisconi et al., 2020; Hammer et al., 2004; Marcos-Arias et al., 2010).

In our study carvacrol, cinnamaldehyde, citral and thymol inhibited biofilm formation and were also shown to have an effect against mature biofilms. Cinnamaldehyde was characterized by its ability to reduce the adhesion of biofilm and reduce biofilm metabolic activity and biomass. Carvacrol and thymol inhibited the formation of biofilms and reduced metabolic activity more than biomass, consistent with reports by other authors (Doke et al., 2014). However, of all the compounds tested, citral markedly reduced metabolic activity in the biofilm formation phase at very low concentrations, which were similar or even lower than those shown to be effective against planktonic cells. In contrast, Khan & Ahmad (2012) observed better inhibitory activity of cinnamaldehyde and eugenol in *C. albicans* biofilm formation compared to citral; the latter required up to a fourfold increase in IC to be effective against *Candida* biofilms.

In the case of mature biofilm, carvacrol caused the greatest reduction in biomass, followed by thymol, and both reduced metabolic activity, an effect that has also been described previously (Braga et al., 2008; Dalleau et al., 2008; Jafri & Ahmad, 2020; Khan & Ahmad, 2012). Cinnamaldehyde significantly reduced metabolic activity without reducing biofilm biomass, similar to citral. However, citral reduced the biomass and metabolic activity of *C. albicans* SC5314 biofilms, as also described by Gao et al. (2020); they observed a reduction in mixed *C. albicans* SC5314 and *S. aureus* biofilm treated with citral of up to 87%, accounting for cell viability, and a reduction in biomass of up to 74%.

The increase in the SICs of phytocompounds with respect to the IC of planktonic cells was not as dramatic, contrary those expected for antifungal drugs such as azoles: the SMICs of isavuconazole as much as 2000-fold greater than the MICs. This increase in phytocompound effectiveness was mostly two- or fourfold the IC for metabolic activity or from 4- to 32-fold the IC with respect to biomass. In some cases, the SIC was equal to the IC or was even lower.

Phytocompounds and essential oils act mainly by destabilizing the integrity of the yeast cell membrane, increasing its permeability, and by inhibiting germ tube formation, fungal proliferation and cellular respiration. However, their effects may be closely related to their chemical structures (Gallucci et al., 2014; Nazzaro et al., 2017; Rao et al., 2010). Some authors attribute the bioactivity of phenolic compounds to the hydroxyl group attached to the benzene ring but also to other factors, such as the presence of substituents that have an ionizing effect, capable of generating free radicals and transformation into methylene quinones (Gallucci et al., 2014; Rao et al., 2010). The degree of cytotoxicity and antifungal activity has been related to the oxidation of phenols to methylene quinines. Compounds that form stable methylene quinines show better antifungal activity. Phenols with alkyl groups with isopropyl substituents that are oxidized by enzymes present in *Candida* more readily form stable methylene quinones than methyl groups with ortho-methoxyl group substitutions (Gallucci et al., 2014).

Khan & Ahmad (2012) suggested that since phytocompounds are also active against sessile cells, which are characterized by low ergosterol content, their mechanism of action does not greatly affect ergosterol biosynthesis. In the same vein, Chatrath et al. (2019) observed that the expression of *ERG11/CYT450*, cell membrane biosynthesis genes in *C. tropicalis*, was not affected by thymol treatment. The positive regulation of thymol on cell wall-

related *CNB1* tolerance genes and the results of a sorbitol protection assay suggest that this phytocompound acts on the cell wall without affecting the cell membrane.

However, the proposal of Khan & Ahmad (2012) stands in opposition with the findings of other authors. De Castro et al. (2015) and Ahmad et al. (2011) suggested that carvacrol and thymol isomers inhibit ergosterol biosynthesis in *C. albicans* without directly binding to ergosterol and that this inhibition is dose-dependent. The dose-dependent action of these phytocompounds may be explained in part by the tolerance of *C. albicans* to 14α-sterol demethylase deficiency (Shimokawa et al., 1989).

In addition, carvacrol and thymol possess more than one mechanism of action, damaging the cell membrane through pore formation, oxidative stress, and endoplasmic reticulum stress via the unfolded protein response (UPR) signalling pathway and inducing cell apoptosis (Chaillot et al., 2015; Gallucci et al., 2014; Khan et al., 2015; Marchese et al., 2016; Niu et al., 2020). The cytotoxicity of carvacrol and thymol has been related to the action of more stable methylene quinones resulting from the oxidation of alkylphenols with isopropyl substitutions (Gallucci et al., 2014).

Cinnamaldehyde shows mechanisms of action similar to those of carvacrol and thymol, which induce loss of membrane and cell wall integrity, change in the sterol profile and induction of *Candida* cell apoptosis (Chen L. et al., 2019; Deng et al., 2018; Khan & Ahmad, 2012; Khan et al., 2013; Khan et al., 2017; Rajput & Karuppayil, 2013; Shreaz et al., 2016). The inhibition of adhesion by cinnamaldehyde is related to decreased expression of the *HWP1* gene, which encodes the Hwp1 protein required for filamentation and biofilm formation. Increased secretion of farnesol, involved in cellular *quorum sensing*, which is induced by Dpp3 expression, has also been observed (Khan et al., 2017; Ying et al., 2019).

Several studies have described citral interference with ergosterol biosynthesis but not direct ergosterol binding in *C. albicans*, *C. tropicalis*, *Penicillium italicum* and *Aspergillus ochraceus* (Chatrath et al., 2019; De Sousa et al., 2016; Hua et al., 2014; Rajput & Karuppayil., 2013; Tao et al., 2014). Furthermore, other authors proposed that citral may be critical for cell membrane disruption, disrupting the cell cycle of *C. albicans* and inducing cell apoptosis of *Geotrichum citriaurantii* without affecting the cell wall (Leite et al., 2014; Lima et al., 2012; Rajput & Karuppayil, 2013; Zhou et al., 2014; Zore et al., 2014).

These mechanisms of action have been observed in *Candida* isolates susceptible or resistant to antifungal drugs. For example, in fluconazole-resistant isolates of *C. tropicalis*, citral inhibited ergosterol biosynthesis without binding directly to the cell wall or cell membrane. In the case of resistant *Candida* isolates, cinnamaldehyde inhibits glucose-stimulated H+ efflux (De Sousa et al., 2016; Gallucci et al., 2014; Shreaz et al., 2016). Indeed, in this Thesis work, the phytocompounds carvacrol, thymol, cinnamaldehyde and citral were found to be active against planktonic and sessile cells of azole-resistant *C. albicans* and *C. dubliniensis* isolates. Similarly, Dalleau et al. (2008) described a reduction in the metabolic activity of biofilms of fluconazole-resistant isolates of *C. albicans* treated with carvacrol, thymol and geraniol of greater than 80%. These findings support the use of phytocompounds in the treatment of recalcitrant candidiasis and the possibility of their use in combination therapy with antifungal drugs.

The use of combination therapies has allowed the cure of some recalcitrant candidiasis and the reduction in toxicity or adverse effects associated with high doses of antifungal drugs. Combination therapies are based on the synergistic effect that two compounds may exert through their antifungal action. With this objective in mind, in this Thesis work, the antifungal effect of the combination of fluconazole with citral was analysed. This combination included a widely used and inexpensive antifungal drug, fluconazole, with

citral, a monoterpene presents in several oral hygiene preparations. Citral shows synergistic action with fluconazole against planktonic and sessile *Candida* cells. In addition, it allowed a higher survival of *C. elegans* infected with azole-resistant *Candida* strains. The synergistic action of fluconazole and citral against *Candida* planktonic cells was observed with concentrations of 2 to 256 mg/L of citral and 0.25 to 8 mg/L of fluconazole. The studied fluconazole-resistant isolates of *C. albicans*, *C. dubliniensis*, *C. tropicalis* and *C. glabrata* were susceptible to the combination of fluconazole and citral, achieving up to a 128-fold reduction in fluconazole MIC. However, this combination had no synergistic effect against *C. krusei*, a species characterized by its primary resistance to fluconazole. This finding raises the possibility that differences in the the mechanisms of resistance to fluconazole among *Candida* species may interfere with the combined effect of these compounds, which would be interesting to evaluate in the future. Moreover, results obtained in other studies show that the synergism of citral and fluconazole can be strain-dependent. For example, De Sousa et al. (2016), found synergism of this combination against *C. tropicalis* ATCC 13803; while Khan et al. (2012) and Zore et al. (2011) observed synergism in *C. albicans* isolates, however, a low number of isolates were analysed in both studies.

The results obtained by the checkerboard technique in this work contrast in part with those observed by lethality curves for the two selected azole-resistant *Candida* isolates. For these isolates, no synergistic effect was observed in the combinations analysed by the time-kill curve technique. However, there was evidence of an improvement in the fungistatic activity of the combination compared to the activity of the antifungal drug in monotherapy and a similar behaviour between the time-kill curves of both isolates. The time-kill curve technique describes synergism as a more than 2 log reduction in the growt with respect to the growth of the most active treatment. In addition, this technique allows the identification of changes in tolerance to antimicrobial drugs over the study time, allows the

assessment of cell viability, and provides information on the most active concentration of the drug and the time in which fungicidal or fungistatic activity is achieved (Gil-Alonso et al., 2015). The checkerboard methodology allows obtaining a static point value of the antifungal action of multiple combinations of two antifungal drugs, more easily than time-kill curves. However, only a measure of cell optical density is obtained, and cell viability is not assessed, leading, in some cases, to the observation of discrepancies between methodologies. Li et al. (2008) found discordance between both methodologies after analysing the combination of cyclosporin A and fluconazole against in *Candida* isolates susceptible to fluconazole. Other authors have observed concordance between both methods evaluating the combination of phytocompounds with fluconazole (Lu et al., 2017).

Khan & Ahmad (2012) did not find synergism with the combination of citral and fluconazole against *Candida* biofilms. However, in the present Thesis work, we observed that the combination of 0.25 mg/L of fluconazole with 256 mg/L of citral was synergistic against *C. albicans* and *C. dubliniensis* biofilms. This combination decreased the metabolic activity of the biofilm with up to a 256-fold reduction in fluconazole SMIC. The combined use of citral and fluconazole had a minor effect on biofilm biomass, as had been observed with the use of citral. This fact could represent a disadvantage with respect to the complete eradication of mature biofilms or in limiting the cell spreading process.

Infection models, as in the case of the candidiasis model in *C. elegans*, have allowed the evaluation of different parameters of importance due to their simple use and great versatility compared to models in mammals. For instance, the pathogenicity and virulence of the main pathogenic *Candida* species or the therapeutic efficacy of new molecules with antifungal activity have been effectively tested (Elkabti et al., 2018; Hernando-Ortiz et al., 2020; Ortega-Riveros et al., 2017; Scorzoni et al., 2013; Sun et al., 2018). However, this

model has some disadvantages, such as the impossibility of using a defined inoculum, because the infection is carried out by ingestion of the yeasts, the pharmacological doses used in the treatment of candidiasis cannot be controlled either, and a temperature lower than human temperature is needed for the adequate life of the nematode (Madende et al., 2020; Mohammad et al., 2019; Peterson & Pukkila-Worley, 2018).

Most *Candida* species of medical interest cause infection in *C. elegans* with high mortality (Freire et al., 2017; Hernando-Ortiz et al., 2020, 2021; Ortega-Riveros et al., 2017; Souza et al., 2018). Our results of *C. albicans* candidiasis using this animal model showed the highest mortality: more than 30% of nematode survival was reduced in the first 24 h and more than 94% at 96 h. Furthermore, biofilm-producing isolate, azole-resistant *C. dubliniensis* UPV 11-366 caused high mortality from the first 24 h, reducing survival by 40% and up to 92% at 96 h compared to the results of Ortega-Riveros et al. (2017), who observed survival greater than 50% for infection with the reference strain *C. dubliniensis* NCPF 3949 after 120 h. Sun et al. (2015) and Ortega-Riveros et al. (2017) observed the highest mortality caused by *C. albicans* infection in agreement with our outcomes.

The use of concentrations of citral and fluconazole safe for the nematodes allowed the study of combinated therapy to treat *Candida* infection. Combined treatment with these compounds against *C. albicans* and *C. dubliniensis* candidiasis significantly improved nematode survival. However, when they were used in monotherapy, the same results were not obtained and, as previously described by Sun et al. (2018) with 1 and 2 mg/L fluconazole, cure of infection by fluconazole-resistant strains was not reached. These results reinforce the synergism observed *in vitro* and strengthen the possibility of the use of citral in combination treatments with fluconazole to combat candidiasis.

Molecular techniques have been successfully employed in microbiological diagnosis. Their use has allowed the identification of phylogenetically closely related species and the study

of their epidemiological role. In addition to their diagnostic use, molecular analyses are used for identifying mechanisms of resistance to antifungal drugs, typing clinical isolates and elucidating of biological processes as determined by gene expression levels.

Citral appears to exert its antifungal action on the cell membrane and ergosterol biosynthesis pathway (Rajput & Karoppagil, 2013; Zore et al., 2011). For this reason, the gene expression of some of the main genes involved in fluconazole resistance in *C. albicans*, such as the *CDR1, MDR1* and *ERG11* genes is important to study, particularly how it is affected by the use of citral at active concentrations in planktonic cells and its combination with fluconazole.

The antifungal effect of azoles has been shown to be related to the production of toxic sterols and increased levels of reactive oxygen species (ROS). Fluconazole acts on the enzyme 14α-demethylase (Erg11) and inhibits ergosterol synthesis, creating favourable conditions for the toxic sterol 14α methylergosta-8-24(28)-dienol to accumulate in fungal cells with a fungistatic effect (Alizadeh et al., 2017; Bhattacharya et al., 2020). Ergosterol is an essential component in yeast cell and mitochondrial membranes. More than 20 genes are involved in ergosterol biosynthesis, of which *ERG11* is highly relevant because it encodes the enzyme Erg11, which catalyses the oxidative removal of the 14α-methyl group from lanosterol, converting it to ergosterol (Alizadeh et al., 2017).

In general, the resistance of *C. albicans* to antifungal drugs is due to a set of different mechanisms. In the case of resistance to fluconazole, the mechanisms associated with its target include, alterations in ergosterol arising from hotspot mutations or increased ergosterol production due to overexpression of genes involved in biosynthesis. Other mechanisms include overexpression of ABC transporters- and MF transporters genes and activation of cellular changes that reduce fluconazole toxicity or allow fluconazole-induced stress tolerance (Cowen et al., 2015; Whaley et al., 2017).

Overexpression of *ERG11* as a mechanism of resistance to fluconazole may be due to two causes: amplification of the *ERG11* gene (multiple copies of the gene) or overexpression (Whaley et al., 2017).

In *C. albicans* ATCC 64124, homozygous mutations have been described, and three variations leading to amino acid substitutions in the Erg11 protein sequence have been shown to be critical for its resistance to fluconazole (Kakeya et al., 2000). In *C. albicans* UPV 15-157, fluconazole-resistant isolate used in this study, no substitutions related to azole resistance have been identified (Arrieta, 2018). In both of these strains, as well as in *C. albicans* strain SC5314, the relative changes in *ERG11* gene expression were not significant, despite its apparent upregulation. Therefore, it is not possible to conclude that citral, in combination with fluconazole, affects the regulation of *ERG11* expression, regardless of the presence or absence of mutations. In resistant isolates treated with fluconazole, an increase in *ERG11* gene expression can be expected as part of the resistance mechanism. However, in our study, no upregulation of this gene was observed, an outcome that can be explained by the low concentrations of fluconazole, 1 to 2 mg/L, used in our study (Borecká-Melkusová et al., 2009).

When we analysed the effect of citral in monotherapy on both fluconazole-resistant and fluconazole-susceptible *C. albicans* strains, slight or negligible upregulation in *ERG11* was observed. In *C. tropicalis*, Chatrath et al. (2019) observed that citral induced a significant overexpression of the *ERG11* and *CYT450* genes. The latter encodes an Erg11-dependent enzyme. However, according to these authors, this overexpression of the *ERG11* gene should not be considered to indicate activation of a tolerance mechanism to the phytocompound.

The mechanism of action of citral on the expression of genes associated with ergosterol biosynthesis appears to be dependent on *Candida* species. Furthermore, there are

differences with respect to other terpenes, such as carvacrol, which causes downregulation of *ERG3* and *ERG11* genes in *C. albicans* at concentrations ≤ 25 mg/L, or thymol, which does not influence *ERG11* or *CYT450* expression in *C. tropicalis* (Alizadeh et al., 2018; Chatrath et al., 2019).

Combined treatment with fluconazole and citral significantly reduced *MDR1* expression without affecting *CDR1* expression. Citral reduces the number of fluconazole-specific Mdr1 transporters (major facilitators) in the membrane. Our observations suggest that citral limits the efflux of fluconazole from the cell by reducing the synthesis of transporters with a subsequent increase in the intracellular concentration of fluconazole. This response destabilizes the plasma and mitochondrial membranes and increases the production of free radicals and cell toxicity. However, it cannot be ruled out that citral interferes with ergosterol biosynthesis. Its effect on *ERG11* gene expression may be dose-dependent, or citral may interfere with the inhibition of the expression of other genes involved in ergosterol biosynthesis that we have not evaluated.

Similar mechanism has also been described in citral-treated *Penicillium expansum*, in which it reduced the expression of transporter genes and, as a result, decreased resistance to several drugs (Wang et al., 2018). Other monoterpenes, such as carvacrol and thymol, share this mechanism of action. These isomers interfere with the membrane transport system, reducing the expression of the *CDR1* and *MDR1* genes in fluconazole-resistant *Candida* isolates (Ahmad et al., 2013).

With respect to the safety of using these phytocompounds in humans, carvacrol, cinnamaldehyde, citral and thymol have been assigned to the category generally recognized as safe (GRAS) by the FDA (21 CFR 182.10, 21 CFR 182.20; 21CFR182.60 and 21CFR172.515, synthetic flavouring substances and adjuvants) (Food and drug administration, FDA, 2020a, 2020b, 2020c, 2020d). This criterion supports their safe use as

additives in the human food industry; however, these phytocompounds are also being widely used in the cosmetic and pharmaceutical industries.

Citral was safe for use in *C. elegans* at concentrations at or below 128 mg/L. The lack of citral toxicity has also been described by Gupta et al. (2017), who observed that citral concentrations of 5 to 500 mg/kg body weight orally administered were well tolerated in mice. However, a different effect was obtained in murine macrophages treated with different concentrations of citral (including IC). In these cases, citral decreased cell survival at all concentrations tested, and even at the lowest concentration, cell survival did not reach 80%. A similar outcome in these cells was observed with carvacrol and thymol, with cell survival not exceeding 80% at the lowest concentration of 32 mg/L. Despite these results, the toxicity of thymol and carvacrol was found to be lower than that of citral, results also observed by Kumari et al. (2017); these authors found that thymol at concentrations greater than 283.65 mg/L reduced the survival of human keratinocyte cell lines (HaCaT) and human embryonic kidney cells (HEK-293) by more than 50%. In the case of cinnamaldehyde, we observed that the cytotoxicity was significantly higher than that of the other phytocompounds evaluated, and even at a concentration of 16 mg/L, survival did not exceed 20%.

It has been observed that the cytotoxic effect of the aforementioned phytocompounds is concentration-dependent. Usach et al. (2020) did not detect toxicity at concentrations equal to or less than 20 mg/L of citral in culture of keratinocytes *in vitro*, whereas at a concentration of citral equal to or greater than 57.41 mg/L, survival was reduced to 50% or more in HaCaT and HEK-293 cell cultures (Kumari et al., 2017). However, at or below 113.5 μM (~15 mg/L) of cinnamaldehyde, HaCaT cell survival was not affected (da Nóbrega Alves et al., 2020).

Encapsulation by liposomes is an important advance to overcome the disadvantage of dose-dependent cytotoxicity, as observed with amphotericin B (Allen & Cullis, 2013). Encapsulation of these phytocompounds would also reduce their volatility and hydrophobicity and the chemical and physical properties that increase their instability in the presence of heat, light and oxygen (Sebaaly et al., 2015; Turek & Stintzing, 2012). Encapsulation of the phytocompounds with the DODAB:MO system significantly improved macrophage survival, noting that the higher the %EE, the greater the reduction in cell toxicity, as previously described by other authors (Usach et al., 2020). The DODAB:MO system employs multilamellar nanoparticles capable of encapsulating different molecules, including proteins and hydrophobic molecules such as phytocompounds, which are protected inside the liposome by binding to the phospholipid layer, stabilized by the presence of MO (Barbosa et al., 2019; Carneiro et al., 2015). Encapsulation was efficient for citral, carvacrol and thymol and, to a lesser extent, for cinnamaldehyde. Higher concentrations of the phytocompound were associated with higher encapsulation efficiency, a fact that may correlate with the higher availability of the compound in solution. In general, in the encapsulation of these phytocompounds with the DODAB:MO system, the efficiency depends on the concentration of the phytocompound tested and on the physicochemical interactions between liposomes and phytocompounds. At low concentrations, such as those used for cinnamaldehyde, there was lower encapsulation efficiency, while at high concentrations, such as those used for citral, the phytocompound was better encapsulated. The percentage of encapsulation was similar between carvacrol and thymol. It has been described that the presence of a hydroxyl group and a low Henry's law constant (Hc) value, such as those found for carvacrol and thymol, improve their encapsulation in liposomes (Hammoud et al., 2019). Despite the fact that thymol and carvacrol are isomeric compounds, these compounds differ subtly in their

enthalpy of vaporization, PKa, dipole, charge oxygen phenolic, volume (Å2), minimal projection area (Å2), maximal projection area (Å2) and electronegativity (eV) (Gallucci et al., 2014). Variations in encapsulation efficiency using the same concentrations may be associated with these differences between isomers, so it would be interesting in the future to evaluate if the previously mentioned parameters could be more decisive in the encapsulation process.

Encapsulation significantly reduced the toxicity of all phytocompounds. The use of 36 mg/L of the 128 and 256 mg/L citral nanoparticle formulations, with more than 80% EE, increased cell survival more than fivefold compared to the free compound. Carvacrol and thymol nanoparticles, with an encapsulation efficiency close to 70%, reduced toxicity, increasing cell survival to values close to 100% or more, using 18 and 36 mg/L of the 128NP, 64NP and 32NP formulations of both phytocompounds.

The cytotoxicity of cinnamaldehyde was drastically reduced after encapsulation, even though it was 44.1% efficient. At this encapsulation efficiency, the free phytocompound was still high enough in the formulation to reduce cytotoxicity and achieve cell survival in excess of 80%. It would be interesting to evaluate in the future if encapsulating concentrations higher than 64 mg/L increases the %EE, complying with the observed trend that the higher the concentration is, the better the encapsulation. In addition, it should be analysed whether the reduction of free compound in the nanoparticle formulation could reduce the adverse effects previously described for cinnamaldehyde concentrations higher than 2%, such as oral toxicity in animals, acute dermal toxicity in humans, severe irritation and dermal sensitization (Environmental protection agency, EPA, 1995).

Depending on the liposome system used for the production of liposomal formulations of these phytocompounds, different percentages of encapsulation efficiency have been

described. A high encapsulation efficiency, higher than 98%, has been described for carvacrol and thymol in soybean phosphatidylcholine-based liposomes (Ayres Cacciatore et al., 2020; Heckler et al., 2020). In liposomes of lipoid S100 and cholesterol, thymol encapsulation was high, with a percentage of 79% EE (Hammoud et al., 2019). A moderate encapsulation, 67% EE, was described for carvacrol in liposomes of Phospholipon 90H (P90H) and 1,2-dimyristoyl-sn-glycero-3- phospho-(1'-rac-glycerol) sodium salt (DMPG), with a 1:1 ratio (Baranauskaite et al., 2018). Likewise, the encapsulation of cinnamaldehyde in lecithin and lecithin liposomes with sodium carboxymethyl cellulose (CMCNa-П) was moderate, ~ 48% EE (Chen W. et al., 2019; Wang et al., 2019). On the other hand, low encapsulation for carvacrol, 4.16% EE, has been described with the use of L-a-phosphatidylcholine and cholesterol liposomes (Liolios et al., 2009). Even the absence of encapsulation for thymol and carvacrol has been described by Coimbra et al. (2011) using liposomes consisting of dipalmitoylphosphatidylcholine (DPPC), egg-phosphatidyl-choline-35 (EPC-35) and poly(ethyleneglycol)-2000-distearoylphosphatidylethanolamine (PEG2000-DSPE) and cholesterol.

Usach et al. (2020) developed citral nanoparticles in soybean phosphatidylcholine liposomes (~86% EE), which exhibited comparable antifungal activity with the non-encapsulated compound. However, in this Thesis work, after encapsulation of citral in the DODAB:MO system, the antifungal activity of citral was reduced against most *Candida* species, except *C. auris*, compared to that of the non-encapsulated compound. The high encapsulation efficiency of citral nanoparticles reduced their antimicrobial activity by reducing the molecular interaction of the compound with the fungal cell membrane. In *C. auris,* the mechanism of action may be associated with other types of interactions that have not been described thus far.

Cinnamaldehyde retained its antifungal activity, probably due to the higher concentration of free compound in the formulation and because the mechanism of action of this phytocompound on planktonic cells is not affected by encapsulation. Khan et al. (2017) observed that a liposomal formulation of phosphatidylcholine and cholesterol exhibited an antimicrobial effect against *C. albicans* that exceeded that of free cinnamaldehyde, although these researchers did not indicate the %EE in this study.

The antifungal effect of carvacrol and thymol was preserved or increased after encapsulation with up to a 4-fold reduction in IC. This effect of thymol nanoparticles was observed against *C. albicans*, *C. tropicalis* and *C. auris*. Carvacrol nanoparticles were more active against *C. albicans* than non-encapsulated carvacrol. Liolios et al. (2009) observed that antimicrobial and antifungal activity against *C. albicans* ATCC 10231, *C. tropicalis* ATCC 13801 and *C. glabrata* ATCC 28838 was preserved with the application of carvacrol and thymol liposomes. Engel et al. (2017) described thymol/carvacrol liposomes retaining their antimicrobial activity against *S. aureus* (IC 0.66 mg/mL), but these liposomes were less effective than the free compound in reducing adhesion to stainless steel. In contrast, Ayres Cacciatore et al. (2020) found that carvacrol nanoparticles were less active against bacteria of interest in the food industry.

Interactions between nanoparticles and microorganisms play important and decisive roles in antimicrobial action. In addition to a higher surface-to-volume ratio and high reactivity compared to the non-encapsulated compound, the DODAB:MO nanoparticles presented ζ-positive potential, which favoured binding with the negatively charged yeast surface. This fact facilitates that nanoparticles enter the cytosol through an endocytosis mechanism (Barbosa et al., 2019; Ing et al., 2012).

The antimicrobial response is mediated by cells and molecules within the complex immune system. The correct fusion of DODABMO liposomes to the cell membrane of

macrophages and T lymphocytes has been proven, and they show efficiency as pDNA transfection vectors (Carneiro et al., 2015; Silva et al., 2011).

Monoolein-based liposomes do not trigger a detrimental inflammatory response in murine macrophage culture, either with empty or thymol- and carvacrol-loaded liposomes. Moreover, thymol and carvacrol nanoparticles exert a beneficial effect on cell recruitment with slightly elevated TNFα values, but these levels are significantly lower than those induced by molecules such as highly antigenic LPS. In addition, nanoparticles with 64 mg/L of carvacrol or 32 or 128 mg/L of thymol improved the fungicidal activity of macrophages in macrophage killing *assays*, showing a reduction in *Candida* survival compared to the non-encapsulated compound.

Liposomal formulations of carvacrol and thymol would exert a positive effect on the immune response, enhancing the fungicidal activity of macrophages and activating their pro-inflammatory activity. In addition, these liposomal formulations could add therapeutic advantages in superficial candidiasis, such as promoting healing action by increasing keratinocyte recruitment to the site of the lesion. This action has been described by other authors studying citral nanoparticles and *Thymus* essential oil (Manconi et al., 2018; Palmas et al., 2020).

The stability of the nanoparticles is essential for the prospect of scaling up their production and efficient preservation in different matrices for topical use or in other types of formulations. In our study, the monoolein-based liposomes of carvacrol and thymol were stable upon refrigeration, with particle sizes of nanoparticle ranked from 510 to 580 nm, with particle size stabilization throughout five weeks of storage. These properties demonstrate the great therapeutic potential of liposomal formulations in the DODAB:MO system, particularly with respect to their biodegradability, lack of toxicity and immunogenicity, and good biocompatibility. Deepening the knowledge of their antifungal

activity is still necessary for their translation into therapeutic alternatives and to evaluate several crucial parameters, such as their stability, mechanism of action, pharmacodynamics and pharmacokinetics, as well as to improve the efficiency of their production.

In general, this Thesis work studied the importance of the different species of *Candida* involved in oral candidiasis and their susceptibility to antifungal drugs. Based on this problem, some phytocompounds were evaluated in their use as a therapeutic alternative for candidiasis. This evaluation has included the study of their antifungal activity against biofilms and in combination therapy with fluconazole. The most active phytocompounds, carvacrol, cinnamaldehyde, citral and thymol, have been encapsulated in liposome-based drug delivery systems. The mechanism of action of citral, one of the most active phytocompounds, has also been studied. In the development of this study, strengths and some methodological limitations can be evidenced, being part of the latter, the reduced number of isolates used in some techniques or limitations inherent to the methodologies. For instance, the standardization between laboratories of the methodology used in the production of biofilms is still a work in progress for the scientific community. However, as we have commented above, our studies included the most robust and widely used methodologies.

In the case of the analysis of the action of citral on the expression of genes associated with fluconazole resistance, we studied a small number of resistant isolates. This fact focuses new challenges on the continuation of this work. One of them corresponds to the possibility of continuing this study in biofilms or to the evaluation of other *ERG* genes involved in the ergosterol pathway. In addition, the evaluation in invertebrate models generates results in a simpler and ethically sustainable way. Nevertheless, for a broader analysis of the activity of free and encapsulated compounds, the use in mammals would be relevant and corresponds to a further step to continue this Thesis work. Therefore, this line

could allow further study of immunological interactions and a better understanding of the antifungal activity of the most active phytocompounds.

6

CONCLUSIONS

1. *C. glabrata* was the second most prevalent species in oral candidiasis after *C. albicans*. However, *C. bracarensis* and *C. nivariensis*, species closely related to *C. glabrata*, were not found colonizing the oral mucosa.
2. *C. parapsilosis* was the third species causing oral candidiasis. The emerging species *C. metapsilosis* and *C. orthopsilosis* showed low prevalence in oral candidiasis and were susceptible to all the antifungals tested.
3. Nystatin was the most active antifungal drug. *C. glabrata* showed reduced susceptibility to azoles, primarily fluconazole and itraconazole. The *C. parapsilosis* complex was susceptible to fluconazole, but some isolates of *C. parapsilosis* showed intermediate susceptibility and even resistance to miconazole and itraconazole.
4. Phytocompounds are promising therapeutic alternatives for candidiasis. All phytocompounds studied in this work showed anti-*Candida* activity against planktonic cells. Cinnamaldehyde, carvacrol, thymol, citral and eugenol also showed fungicide activity.
5. Citral, carvacrol, cinnamaldehyde and thymol actively inhibited biofilm formation. Citral was also active against preformed biofilms by reducing their metabolic activity, while carvacrol and thymol also reduced their biomass.
6. Citral was capable of synergistically increasing the fungistatic effect of fluconazole against planktonic and sessile *Candida* cells, when used in combination. In addition, a synergistic effect was also demonstrated *in vivo* in the treatment of *Candida* infection in *Caenorhabditis elegans*.
7. Citral increases the efficacy of fluconazole through a synergistic action probably related to an increased susceptibility of *Candida* and to a decrease in fluconazole efflux caused by reduced expression of transporters.

8. The DODAB:MO system was an efficient system for encapsulating carvacrol, citral, thymol and, to a lesser extent, cinnamaldehyde. Encapsulation significantly reduced the cytotoxicity of all the phytocompounds while maintaining their antifungal activity.
9. Carvacrol and thymol liposomes are the best candidates for treating candidiasis because they are stable upon increased encapsulation and well tolerated by macrophages, improving the ability of macrophages to kill *Candida* cells without a detrimental stimulation in cytokine expression.

7

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