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

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ABSTRACT

Background: The ability 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.

Aim: 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 analysed 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.

Results: 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 the efflux pump encoded by MDR1, leading to a greater effect of fluconazole.

Conclusion: Citral in monotherapy and in combination with fluconazole could represent an interesting therapy to treat recalcitrant Candida infections associated to biofilms.

Introduction

Superficial and invasive candidiasis are very common infectious diseases [1–3], with Candida albicans as 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 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 14α-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 the 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 nerol 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 notable 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 the ERG11, CDRI and MDRI genes associated with azole resistance was also assessed.

Materials and methods

Microorganisms

Thirty-five Candida isolates from patients suffering from oral candidiasis attending the Dental Clinical Service at the University of the Basque Country (UPV/EHU) in Bilbao (Spain) were analysed. 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 of Candida metapsilosis. After testing the susceptibility of planktonic cells to citral and fluconazole, six isolates with reduced susceptibility to fluconazole were selected for assessing the activity of citral combined with fluconazole, including one isolate each of C. albicans, C. dubliniensis and two isolates each of C. glabrata and C. krusei, and one of susceptible C. tropicalis. The 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 phyto compound against biofilms (adhesion phase of biofilm-prec sseille 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 hypaphdefective 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, MN).

Phytocompound and antifungal agent

Citral (95% of purity) and fluconazole (98% of purity) were purchased from Sigma-Aldrich (MO). 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°C.

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 1,024 mg/L. C. krusei ATCC 6258 and C. parapsilosis ATCC 22019 were used as quality controls. Absorbance to the microplates was measured at 450 nm by the iMark plate reader (BioRad, CA) after 24 and 48 h of incubation at 37°C. Assays were conducted by triplicate on three independent experiments. The citral inhibitory concentration (IC) and fluconazole minimal inhibitory concentration (MIC) were determined at 24 h as the lowest drug concentration inhibiting ≥ 50% of the growth compared to controls without the compound [20]. The Minimum fungicidal concentration (MFC) was defined as the lowest concentration of antifungal agent resulting in the death of 99.9% of the inoculum [20].

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, Vantaa, Finland). A cell suspension was adjusted using a haemocytometer to a final concentration of 1 × 10⁶ 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 the metabolic activity and the other to quantify the biomass. After 24 and 48 h of incubation at 37°C, unattached or poorly attached cells were removed by washing three times with sterile phosphate buffered saline solution (PBS).

The 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-sul fophenyl)-2 H-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°C. After that, the absorbance to the microplates was measured by the microtiter plate reader BioScreen C MBR (Growth Curves Ltd, Turku, Finland) at 490 nm wavelength. The 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. The 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 the microplates was measured at 600 nm wavelength. Metabolic activity and biomass quantification were estimated according to the absorbance value after subtracting the blank absorbance. Isolates were ranked in terms of their ability to form biofilms compared to the ability of the positive control C. albicans SC5314 [18].

**Effect of citral against the adhesion phase of Candida and on mature Candida biofilms**

The activity of citral against the adhesion phase of ten Candida biofilm producer 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 × 10⁶ cells/mL) were inoculated into the 100-well polystyrene microtitre plates plus 100 µL of citral at final concentrations ranging from 8 to 1,024 mg/L. After 24 h of incubation at 37°C, pre-sessile inhibitory concentrations (PSICs), which were the concentrations producing 50% of metabolic inhibition and 50% of biomass reduction compared to controls without cytokompetitive, 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 in 100-well polystyrene microtitre plates by adding 100 µL of the adjusted inoculum into each well. After 24 h at 37°C, the microtitre plates were washed twice with sterile PBS and 100 µL of the final concentrations of citral were added, ranging from 16 to 2,048 mg/L in RPMI medium. A further incubation of 24 h at 37°C was performed. Sessile inhibitory concentrations (SICs) were determined by XTT reduction and crystal violet assays, which correspond to those concentrations that caused 50% metabolic inhibition and 50% biomass reduction compared to controls, respectively.

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

Microdilution 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. In addition, the 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 included. The checkerboard was prepared in microtitre plates for multiple combinations of two-fold serial dilutions of citral and fluconazole. The final concentrations ranged from 2 to 1,024 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.

The 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 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 produced on microtitre plates from 100 µL of a cell suspension adjusted to a concentration of 1 × 10⁶ cells/mL in RPMI of each isolate, and subsequently incubated for 24 h at 37°C. 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 further incubation of 24 h at 37°C was performed. Sessile ICs (SICs) were estimated through metabolic activity determination and biomass quantification assays. FICI was calculated as previously described.

**Time-kill assay**

The killing activity of the combination of citral and fluconazole was tested against the 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 microtitre plates. After the addition of a suspension of 1–5 × 10³ colony forming units (CFU)/mL in RPMI (final volume of 200 µL) the microtitre plates were incubated for 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 in PBS and incubation on Sabouraud dextrose agar plates 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, indiffere-
ence as a decrease in CFU/mL < 2 log_{10} and antagon-
ism 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.

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
susensions 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, Thorold, ON, Canada). The purity of RNA
was determined by spectrophotometric analysis
(NanoDropTM 2000, Thermo Fisher Scientific,
Waltham, MA) and the RNA integrity was measured
by automated capillary electrophoresis separation
using the LabChip GX Touch Analyser (Perkin
Elmer, Waltham, MA).

The complementary DNA (cDNA) was synthesized using
the PrimeScriptTM RT Reagent kit (TaKaRa Bio Inc., Shiga, Japan).
Primers 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, Foster City, CA).
The cycling profile included an initial step at 95°C 30s; 40
cycles of 95°C 5s, 50°C 31s (55°C 31s for MDR1); and
dissociation stage of 95°C 15s, 60°C for 1 min, and
95°C 15s. The experiments were performed in
duplicate. The cycle threshold (Ct) values of the ERG11,
CDR1 and MDR1 transcripts were normalized to the Ct
corresponding to the housekeeping-ACT1. The quantification of the gene expression was analysed with the comparative method Ct (2^{-ΔΔCt})
with respect to the ACT1 gene. The relative change in
expression was calculated with respect to the control
incubated without drugs normalized to 1.

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 hermaph-
rodites, 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°C. 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 (Tokyo, Japan) every 24 h of incuba-
tion 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
ciral 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 fluco-
nazole susceptible C. albicans SC5314 as a control
of methodology. The infection was performed by feeding
the nematodes for 2 h at 25°C with each Candida
isolate, which had been grown on brain heart infu-
sion (BHI) plates for 24 h at 37°C. After that, the
worms were transferred to microtiter plates, 20
worms per well containing M9 buffer and a final
centration 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 analysed
in the presence of 0.5% DMSO as controls. These
plates were incubated at 25°C, and the survival

| Table 1. Sequence of primers to amplify the ACT1, ERG11, CDR1 and MDR1 genes by the RT-PCR method. | Forward sequence (5'-3') | Reverse sequence (5'-3') |
|---|---|---|
| ACT1 | AAGAATTGATTGCGTGGTAGAGA | TGGCGAGAAGTGGAGAGAAAGTTT |
| ERG11 | GTGTTGACATACCTCCTGCTC | GTCATACATGCTATCAACCT |
| CDR1 | TGCCAAAACATCCCAACA | CGACGATGGTTTCATCAAGA |
| MDR1 | GTGTTCGCCCATTGTTTTCAGTC | CCAAAGCGATGGGATTTTAG |
study was performed as previously described. At least twice independent replicates were performed for each assay.

**Statistics**

Statistical analyses were performed using the SPSS v21.0 software (IBM, NY) and the GraphPad Prism 5.0 version (GraphPad Software, San Diego, CA). 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 expression of each gene 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.

**Results**

**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 the 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 10 Candida isolates previously selected for their biofilm production, including eight *C. albicans*, one *C. dubliniensis* and one *C. tropicalis*. Citral showed activity against the adhesion phase of biofilms (pre-sessile cells) and mature Candida biofilms (sessile cells) (Table 2). Citral was able to reduce the metabolic activity to a greater extent than the biomass in both the adhesion and mature biofilm assays. This activity was even observed againstazole-resistant isolates such as *C. albicans* UPV 15–157 (Figure 1). Regarding the 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 the *C. albicans* SC5314 strain.

**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 the ERG11, MDR1 and CDR1 genes was analysed by quantification analysis by the 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 for *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 the 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).

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

The 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 11–366, in the case of nematodes infected with C. dubliniensis UPV 11–366, after the treatment with the combination of fluconazole 2 mg/L and 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 (≤ 2 mg/L).

**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 from 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 in resistant candidiasis with promising results [13]. Citral (3,7-dimethyl-2,6-octadienial), the main phyto compound present in C. citratus or lemon grass, has shown different properties, including antimicrobial, anti-inflammatory and anticancer activities [14,32,33].

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

| Candida isolates | Planktonic | Sessile | Sessile cell | Mature biofilm SIC (mg/L) |
|------------------|------------|---------|-------------|-------------------------|
|                   | IC (mg/L)  | MFC (mg/L) | Adhesion phase PSIC (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 | 256 |
| 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 | 256 |
| 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 Ca2 | 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.
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. and Rajput and Karuppayil [34,35]. However, other authors have reported lower IC and MFC values of citral than those 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 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 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 in 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 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 the fluconazole MIC from 4 to 32-fold, respectively [38,39]. However, in our study there was

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

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.
a greater reduction (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 the C. krusei isolate with no synergistic effect, the fluconazole MIC was sharply reduced 1,024-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 microdilution 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 the fungicidal effect of citral were lower than those 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 IC×2 or more was necessary 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 citral-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 generally also considered as a safe (GRAS) phytocompound, extensively used as additive in food, pharmacy and the cosmetic industry [42]. However, local reaction or side effects in humans can represent some limitations of its implementation. In that context the nanotechnology might improve its availability and reduce possible side effects [43].

Despite the antifungal activity of citral 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 neither 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* [44] and *Aspergillus ochraceus* [45]. Disruption of the 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 analysed the effect of citral in combination with fluconazole on the regulation of some major 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].

In the current study, when a fluconazole susceptible strain was treated with fluconazole, there was a reduction of the *CDR1* and *MDR1* expressions. The resistant isolates treated with fluconazole showed an overexpression of *CDR1*, while the *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, while the expression of *CDR1* was not significantly affected in any case. In a similar way to our findings in *C. albicans*, citral interferes with the multidrug resistance in *Penicillium expansum*, by down-regulated expression of transporters (multidrug resistance protein (MDR) genes) [46].

With regard to the involvement of citral in the expression of the *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

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

| Treatments [mg/L] | 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-untreated| 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   | 91.4   | 87.4   | 43.4   | 50.8   | 58.3   | 30.2   | 26.2   | 47.2   | 24.8   | 19.7   | 41.7   |
| FLC (46)          | 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    | 9.5    | 3.1    | 0      | 9.8    |        |
| CT [32]           | 84.7   | 83.3   | 90.6   | 16.7   | 27.8   | 26.6   | 6.1    | 9.7    | 20.3   | 0.8    | 2.8    | 14.1   |
| FLC [2] Citral [128]| 92.0  | 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 [128]| 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 [1] 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 two independent assays.
was observed. This could be due to the low concentrations of fluconazole used in this study [47]. 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) [48]. 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 about 20 genes involved in the 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.

Conclusions
Citrin inhibited Candida biofilm formation in the early stages as well as reduced the metabolic activity of the
mature biofilm. In the model of invasive candidiasis in *C. elegans*, the treatment with the combination of citral and fluconazole was synergistic and significantly increased the survival of the worms. 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 down-regulating of the *MDR1* gene, which encodes the Mdr1 efflux pump, contributes to identify its antifungal mechanism of action.

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**Author contributions**

Conceptualization, K.M.-C., C.M.-A., G.Q., and, E.E.; methodology, K.M.-C., A.P.-R. and I.C.-B.; formal analysis, K.M.-C., C.M.-A. and E.M.; investigation, K.M.-C., C.M.-A. and E.M.; data curation, K.M.-C.; writing—original draft preparation, K.M.-C. and C.M.-A.; writing—review and editing, E.M., E.S., L.M., G.Q. and E.E.; supervision, G.Q. and E.E.; project administration, G.Q.; funding acquisition, G.Q. and E.E. All authors have read and agreed to the published version of the manuscript.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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References

[1] McCarty TP, White CM, Pappas PG. Candidemia and invasive candidiasis. Infect Dis Clin North Am. 2021;35:389–413.

[2] Saito H, Shodo R, Yamazaki K, et al. The association between oral candidiasis and severity of chemoradiotherapy-induced dysphagia in head and neck cancer patients: a retrospective cohort study. Clin Transl Radiat Oncol. 2020;1:13–18.

[3] Hu L, He C, and Zhao C, et al. Characterization of oral candidiasis and the Candida species profile in patients with oral mucosal diseases. Microb Pathog. 2019;134:103575.

[4] Miranda-Cadena K, Marcos-Arias C, and Mateo E, et al. Prevalence and antifungal susceptibility profiles of Candida glabrata, Candida parapsilosis and their close-related species in oral candidiasis. Arch Oral Biol. 2018;95:100–107.

[5] Vila T, Sultan AS, Montelongo-Jauregui D, et al. Oral candidiasis: a disease of opportunity. J Fungi (Basel, Switzerland). 2020;6:1–28.

[6] Cavalheiro M, Teixeira MC. Candida biofilms: threats, challenges, and promising strategies. Front Med. 2018;5:1–15.

[7] Silva S, Rodrigues CF, Araujo D, et al. Candida species biofilms’ antifungal resistance. J Fungi (Basel, Switzerland). 2017:3:8.

[8] Whaley SG, Berkow EL, Rybak JM, et al. Azole antifungal resistance in Candida albicans and emerging non-albicans Candida species. Front Microbiol. 2017;7:1–12.

[9] Shi C, Liu J, Li W, et al. Expression of fluconazole resistance-associated genes in biofilm from 23 clinical isolates of Candida albicans. Brazilian J Microbiol. 2019;50:157–163.

[10] Mukherjee PK, Chandra J, Kuhn DM, et al. Mechanism of fluconazole resistance in Candida albicans biofilms: phase-specific role of efflux pumps and membrane sterols. Infect Immun. 2003;71:4333–4340.

[11] Salari S, Khosravi AR, Mousavi SAA, et al. Mechanisms of resistance to fluconazole in Candida albicans clinical isolates from Iranian HIV-infected patients with oropharyngeal candidiasis. J Mycol Med. 2016;26:35–41.

[12] Katagiri H, Fukui K, Nakamura K, et al. Systemic hematogenous dissemination of mouse oral candidiasis is induced by oral mucositis. Odontology. 2018;106:389–397.

[13] Lu M, Li T, Wan J, et al. Antifungal effects of phyto-compounds on Candida species alone and in combination with fluconazole. Int J Antimicrob Agents. 2017;49:125–136.

[14] Bailly C. Targets and pathways involved in the antitumor activity of citral and its stereo-isomers. Eur J Pharmacol. 2020;871:172945.

[15] Khan MS, Ahmad I. Antibiofilm activity of certain phyto-compounds and their synergy with fluconazole against Candida albicans biofilms. J Antimicrob Chemother. 2012;67:618–621.

[16] Freire JCP, Junior JKDO, Silva DDF, et al. Antifungal activity of essential oils against Candida albicans strains isolated from users of dental prostheses. Evid Based Complement Altern Med. 2017;2017:7158756.

[17] Chatraith A, Gangwar R, Kumar P, et al. In vitro anti-biofilm activities of citral and thymol against Candida tropicalis. J Fungi. 2019;5:13.

[18] Miranda-Cadena K, Marcos-Arias C, Mateo E, et al. In vitro activities of carvacrol, cinnamaldehyde and thymol against Candida biofilms. Biomed Pharmacother. 2021;143:112218.

[19] Arendrup MC, Meletiadis J, Mouton JW, et al. EUCAST definitive document E.DEF 7.3.2 Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts. 2020. [cited 2021 Jul 13]. Available from: https://www.eucast.org/astofungis/methodssantifungalsusceptibilitytesting/susceptibilitytestingofyeasts/

[20] Van Dijck P, Skjøllem J, Cammue BPA, et al. Methodologies for in vitro and in vivo evaluation of efficacy of antifungal and antibiofilm agents and surface coatings against fungal biofilms. Microb Cell. 2018;5:300–326.

[21] Ramage G, Vandewalle K, Wickes BL, et al. Characteristics of biofilm formation by Candida albicans. Rev Iberoam Micol. 2001;18:163–170.

[22] Peeters E, Nelis HJ, Coenye T. Comparison of multiple methods for quantification of microbial biofilms grown in microtitre plates. J Microbiol Methods. 2008;72:157–165.

[23] Meletiadis J, Pourmaras S, Roiilides E, et al. Defining fractional inhibitory concentration index cutoffs for additive interactions based on self-drug additive combinations, Monte Carlo simulation analysis, and in vitro-vivo correlation data for antifungal drug combinations against Aspergillus fumigatus. Antimicrob Agents Chemother. 2010;54:602–609.

[24] Caballero U, Kim S, Eraso E, et al. In vitro synergistic interactions of isavuconazole and echinocandins against Candida auris. Antibiotor. 2021;10:355.

[25] Rocha MFG, Bandeira SP, de Alencar LP, et al. Azole resistance in Candida albicans from animals: highlights on efflux pump activity and gene overexpression. Mycosec. 2017;60:462–468.

[26] Hernandez-Ortiz A, Mateo E, Ortega-Riveros M, et al. Caenorhabditis elegans as a model system to assess Candida glabrata, Candida nivariensis, and Candida bracarenis virulence and antifungal efficacy. Antimicrob Agents Chemother. 2020;64:e00824–20.

[27] Sharon V, Fazel N. Oral candidiasis and angular cheilitis. Dermatol Ther. 2010;23:230–342.
[28] Patil S, Rao RS, Majumdar B, et al. Clinical appearance of oral Candida infection and therapeutic strategies. Front Microbiol. 2015;6:1–10.

[29] Paul S, Kannan I, Mohanram K. Extensive ERG11 mutations associated with fluconazole-resistant Candida albicans isolated from HIV-infected patients. Curr Med Mycol. 2019;2019:1–6.

[30] Deorukhkar S, Rouhani S. Fluconazole resistance in Candida species: ten years’ experience at a rural tertiary care teaching hospital in India. J Infectious Dis Pathog. 2017;1:102.

[31] Orasch C, Mertz D, Garbino J, et al. Fluconazole non-susceptible breakthrough candidemia after prolonged low-dose prophylaxis: a prospective FUNGINOS study. J Infect. 2018;76:489–495.

[32] Saddiq AA, Khayyat SA. Chemical and antimicrobial studies of monoterpenes: citral. Pestic Biochem Physiol. 2010;98:89–93.

[33] Leite MC, Bezerra AP, de Sousa JP, et al. Evaluation of antifungal activity and mechanism of action of citral against Candida albicans. Evid Based Complement Altern Med. 2014;2014:378280.

[34] Lima IO, De Medeiros Nobrega F, Araújo De Oliveira W, et al. Anti-Candida albicans effectiveness of citral and investigation of mode of action. Pharm Biol. 2012;50:1536–1541.

[35] Rajput SB, Karuppayil SM. Small molecules inhibit growth, viability and ergosterol biosynthesis in Candida albicans. Springerplus. 2013;2:2–6.

[36] Tati S, Davidow P, McCall A, et al. Candida glabrata binding to Candida albicans hyphae enables its development in oropharyngeal candidiasis. PLoS Pathog. 2016;12: e1005522.

[37] Eguia A, Arakistain A, De-la-pinta I, et al. Candida albicans biofilms on different materials for manufacturing implant abutments and prostheses. Med Oral Patol Oral Cir Bucal. 2020;25:e13–20.

[38] De Sousa J, Costa A, Leite M, et al. Antifungal activity of citral by disruption of ergosterol biosynthesis in fluconazole resistant Candida tropicalis. Plant Prot Innsitute HAS. 2016;11:1–11.

[39] Khan MS, Malik A, Ahmad I. Anti-candidal activity of essential oils alone and in combination with amphotericin B or fluconazole against multi-drug resistant isolates of Candida albicans. Med Mycol. 2012;50:33–42.

[40] Zore GB, Thakre AD, Jadhav S, et al. Terpenoids inhibit Candida albicans growth by affecting membrane integrity and arrest of cell cycle. Phytomedicine. 2011;18:1181–1190.

[41] Gupta P, Patel DK, Gupta VK, et al. Citral, a monoterpenoid aldehyde interacts synergistically with norfloxacin against methicillin resistant Staphylococcus aureus. Phytomedicine. 2017;34:85–96.

[42] National Toxicology Program. NTP toxicology and carcinogenesis studies of citral (microencapsulated) (CAS No. 5392-40-5) in F344/N rats and B6C3F1 mice (feed studies). Natl Toxicol Program Tech Rep Ser. 2003;505: 1–268.

[43] Miranda-Cadena K, Dias M, Costa-Barbosa A, et al. Development and characterization of monoterpene-based liposomes of carvacrol, cinnamaldehyde, citral, or thymol with anti-Candida activities. Antimicrob Agents Chemother. 2021;65:e01628–20.

[44] Tao N, OuYang Q, Jia L. Citral inhibits mycelial growth of Penicillium italicum by a membrane damage mechanism. Food Control. 2014;41:116–121.

[45] Hua H, Xing F, Selvaraj JN, et al. Inhibitory effect of essential oils on Aspergillus ochraceus growth and ochratoxin A production. PLoS One. 2014;9:108285.

[46] Wang Y, Feng K, Yang H, et al. Effect of cinnamaldehyde and citral combination on transcriptional profile, growth, oxidative damage and patulin biosynthesis of Penicillium expansum. Front Microbiol. 2018;9:1–14.

[47] Borecká-Melkusová S, Moran GP, Sullivan DJ, et al. The expression of genes involved in the ergosterol biosynthesis pathway in Candida albicans and Candida dubliniensis biofilms exposed to fluconazole. Mycoses. 2009;52:118–128.

[48] Alizadeh F, Khodavandi A, Esfandyari S, et al. Analysis of ergosterol and gene expression profiles of sterol Δ5,6-desaturase (ERG3) and lanosterol 14a-demethylase (ERG11) in Candida albicans treated with carvacrol. J Herbmed Pharmacol. 2018;7:79–87.