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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.

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Candida; biofilm; citral; azole resistance; *Caenorhabditis elegans*; gene expression



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 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 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 monoterpene 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 notable antimicrobial activity in previous studies [15–17]. However, the mechanism of action of citral in

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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*, *CDR1* and *MDR1* 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 cell-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 phytochemical against biofilms (adhesion phase of biofilm-pressile 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, MN).

Phytochemical 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 $\geq 50\%$ 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 1×10^6 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 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-sulphophenyl)-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 μ L 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 μ L of sterile distilled water, followed by the addition of 150 μ L 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 μ L 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 μ L 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 phytochemical, 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 microtiter plates by adding 100 μ L of the adjusted inoculum into each well. After 24 h at 37°C, the microtiter plates were washed twice with sterile PBS and 100 μ L 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 \leq 0.5 synergistic; FICI $>$ 0.5 but \leq 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 microtiter plates from 100 μ L 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°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 microtiter plates. After the addition of a suspension of $1-5 \times 10^5$ colony forming units (CFU)/mL in RPMI (final volume of 200 μ L) the microtiter 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 $\geq 2 \log_{10}$ compared to the most active drug, indifference as a decrease in CFU/mL $< 2 \log_{10}$ and antagonism as an increase in CFU/mL $\geq 2 \log_{10}$ [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 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, Thorold, ON, Canada). The purity of RNA was determined by spectrophotometric analysis (NanoDrop™ 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 PrimeScript™ 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 ($2^{-\Delta\Delta Ct}$) with respect to the *ACT1* gene. The relative change in

expression was calculated with respect to the control incubated without drugs normalized to 1.

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°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 phytochemical for each well. Worm survival was visually scored on the stereomicroscope Nikon SMZ-745 (Tokyo, 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 the nematodes for 2 h at 25°C with each *Candida* isolate, which had been grown on brain heart infusion (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 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 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.

Gene	Forward sequence (5'- 3')	Reverse sequence (5'- 3')
<i>ACT1</i>	AAGAATTGATTTGGCTGGTAGAGA	TGGCAGAAGATTGAGAAGAAGTTT
<i>ERG11</i>	GGTGGTCAACATACTTCTGCTTC	GTCAAATCATTCAAATCACCACTT
<i>CDR1</i>	TGCCAAACAATCCAACAA	CGACGGATCACCTTTCATACGA
<i>MDR1</i>	GTGTTGGCCCAATTGTTTTAGTC	CCAAAGCAGTGGGGATTGTAG

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 against azole-resistant isolates such as *C. albicans* UPV 15–157 (Figure 1). Regarding the adhesion phase of biofilm, this phytochemical 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 enhances the 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* isolate. 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 the 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). The synergism between citral and fluconazole was also demonstrated against the sessile cells of the *C. albicans* UPV 15–157 isolate and was established by combining the concentrations of 0.25 mg/L of fluconazole and 256 mg/L of citral (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 biofilm metabolic activity was significantly reduced, the FICI value was > 0.5 indicating no synergistic interaction.

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. The 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 the citral and fluconazole combinations with the best results obtained, omitting other concentrations tested.

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

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

<i>Candida</i> 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
<i>C. albicans</i> UPV 05–007	256	> 1024	256	256	> 2048	256
<i>C. albicans</i> UPV 05–013	512	> 1024	128	64	> 2048	256
<i>C. albicans</i> UPV 11–342	256	> 1024	> 1024	512	> 2048	512
<i>C. albicans</i> UPV 11–345	256	> 1024	32	8	> 2048	128
<i>C. albicans</i> UPV 12–298	256	256	16	16	> 2048	256
<i>C. albicans</i> UPV 15–101	256	> 1024	> 1024	256	> 2048	64
<i>C. albicans</i> UPV 15–106	256	> 1024	128	8	> 2048	256
<i>C. albicans</i> UPV 15–157	512	> 1024	32	8	> 2048	512
<i>C. albicans</i> SC5314	512	> 1024	512	64	32	256
<i>C. albicans</i> 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
<i>C. tropicalis</i> UPV 06–115	256	> 1024	128	64	256	256
<i>C. dubliniensis</i> 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.

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

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 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-octadienal), the main phytochemical present in *C. citratus* or lemongrass, has shown different properties, including antimicrobial, anti-inflammatory and anticancer activities [14,32,33].

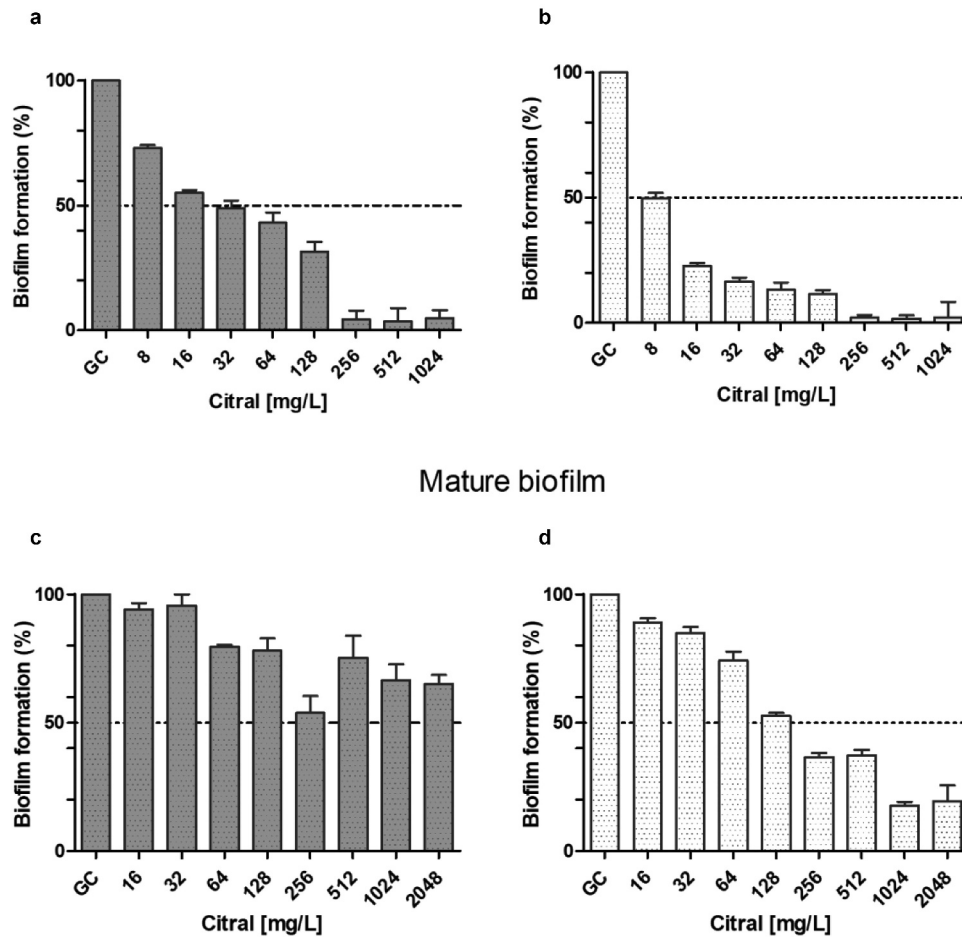


Figure 1. *In vitro* activity of citral against biofilm by the *C. albicans* UPV 15–157 azole-resistant isolate. Growth inhibition of the adhesion phase of biofilm-pre-sessile cells by biomass quantification with crystal violet assay (A) and metabolic activity determination with the XTT reduction assay (B). Growth inhibition of mature biofilm-sessile cells by biomass quantification with the crystal violet assay (C) and metabolic activity determination with the XTT reduction assay (D). GC: growth control. The title of figures a and b is missing: Adhesion phase of biofilm. Please add this title or delete the title of figures c and d: Mature biofilm.

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

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

CT: citral; FLC: fluconazole; Effect I: interpretation; IND: indifferent; SYN: synergistic. IC/MIC: inhibitory concentration/ minimum inhibitory concentration; FICI: fractional inhibitory concentration index.

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 Karuppaiyl [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*

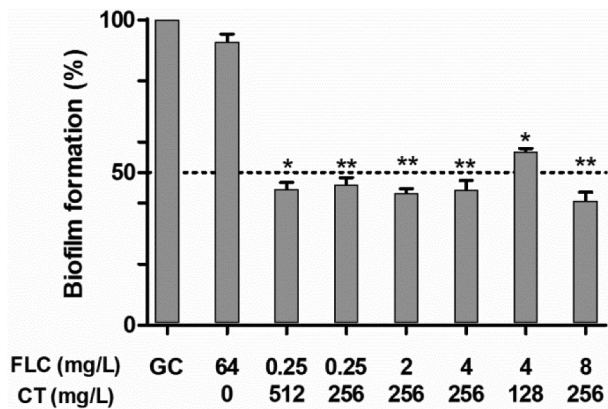


Figure 2. Activity of citral (CT) in combination with fluconazole (FLC) against mature biofilm-sessile cells of the *C. albicans* UPV 15–157 azole-resistant isolate by XTT reduction assay- metabolic activity determination. *: $p < 0.05$, **: $p < 0.01$. GC: growth control.

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

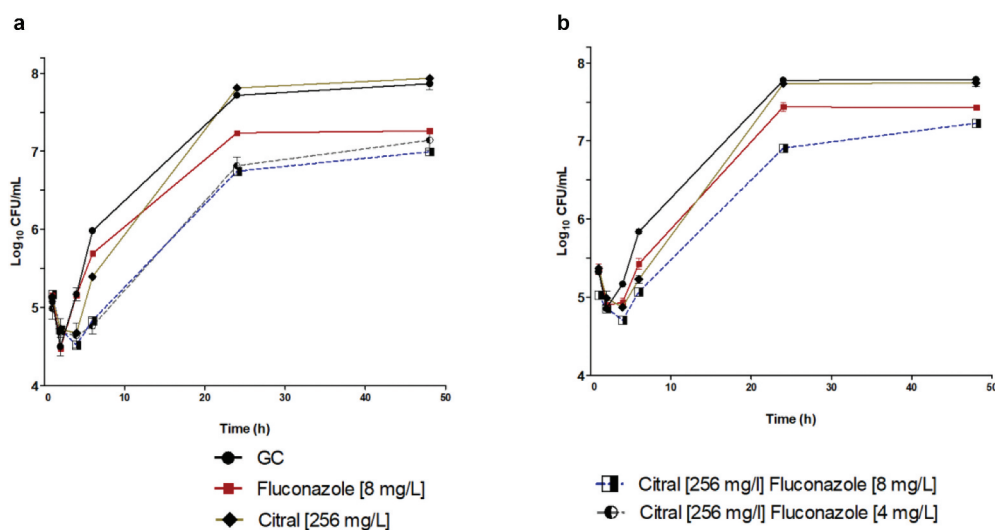


Figure 3. Time-kill curves of *C. albicans* UPV 15–157 (A) and *C. dubliniensis* UPV 11–366 (B) incubated with citral and fluconazole. Each data point represents the mean result \pm standard deviation. GC: growth control.

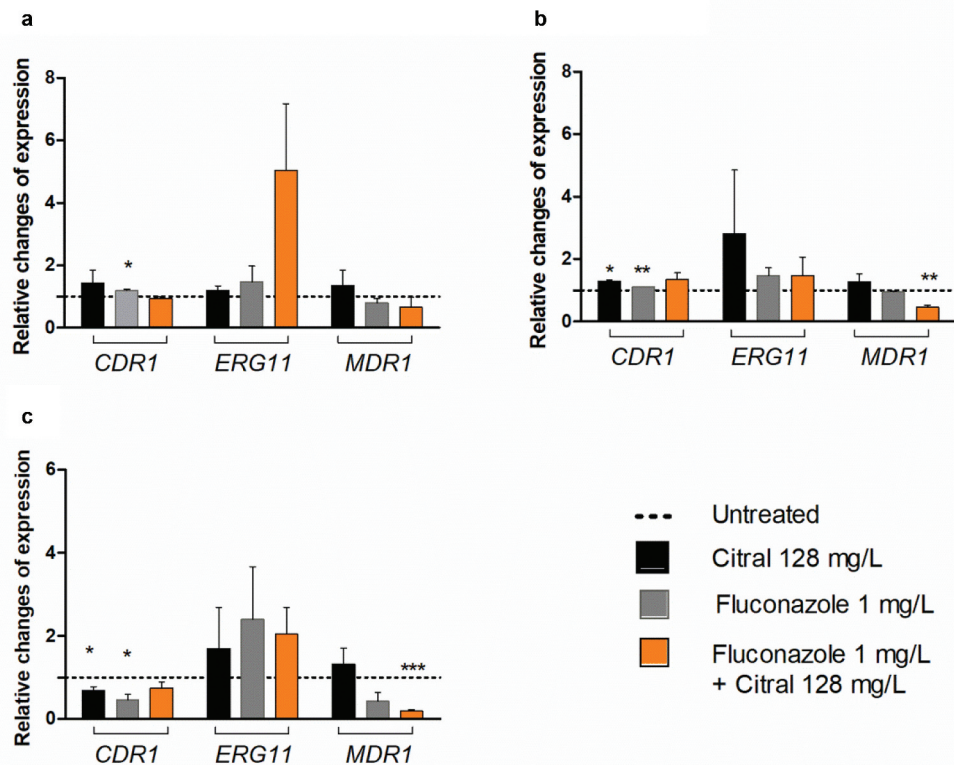


Figure 4. Relative changes in the expression of the genes *ERG11*, *CDR1* and *MDR1* of two azole-resistant isolates, *C. albicans* UPV 15-157 (A) and *C. albicans* ATCC 64124 (B), and a fluconazole-susceptible strain, *C. albicans* SC5314 (C); after incubation with citral, fluconazole and a combination 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 analysed.

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 synergetic 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 log₁₀ 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 log₁₀ 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 log₁₀ 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*

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-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	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 [128]	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 [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.

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 \leq 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 phytochemical could be a good candidate to use as an antifungal treatment, since citral is generally also considered as a safe (GRAS) phytochemical, 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 (*MRP*) 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

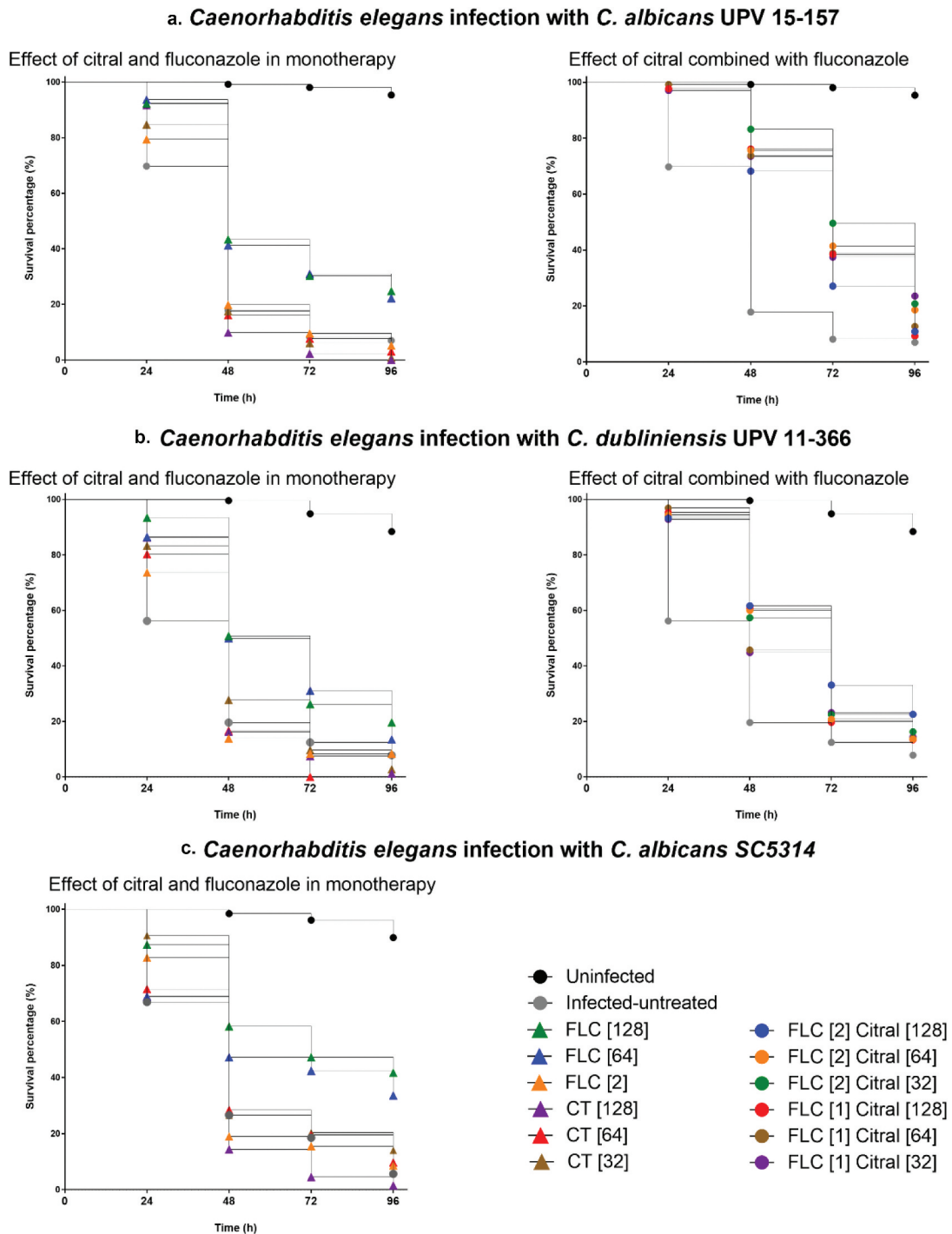


Figure 5. Efficacy of citral (CT) and fluconazole (FLC) treatment in monotherapy and/or in combination on survival curves of *C. elegans* infected with *C. albicans* UPV 15–157 (A), *C. dubliniensis* UPV 11–366 (B) and *C. albicans* SC5314 (C). Concentrations are expressed in mg/L.

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

Citral inhibited *Candida* biofilm formation in the early stages as well as reduced the metabolic activity of the

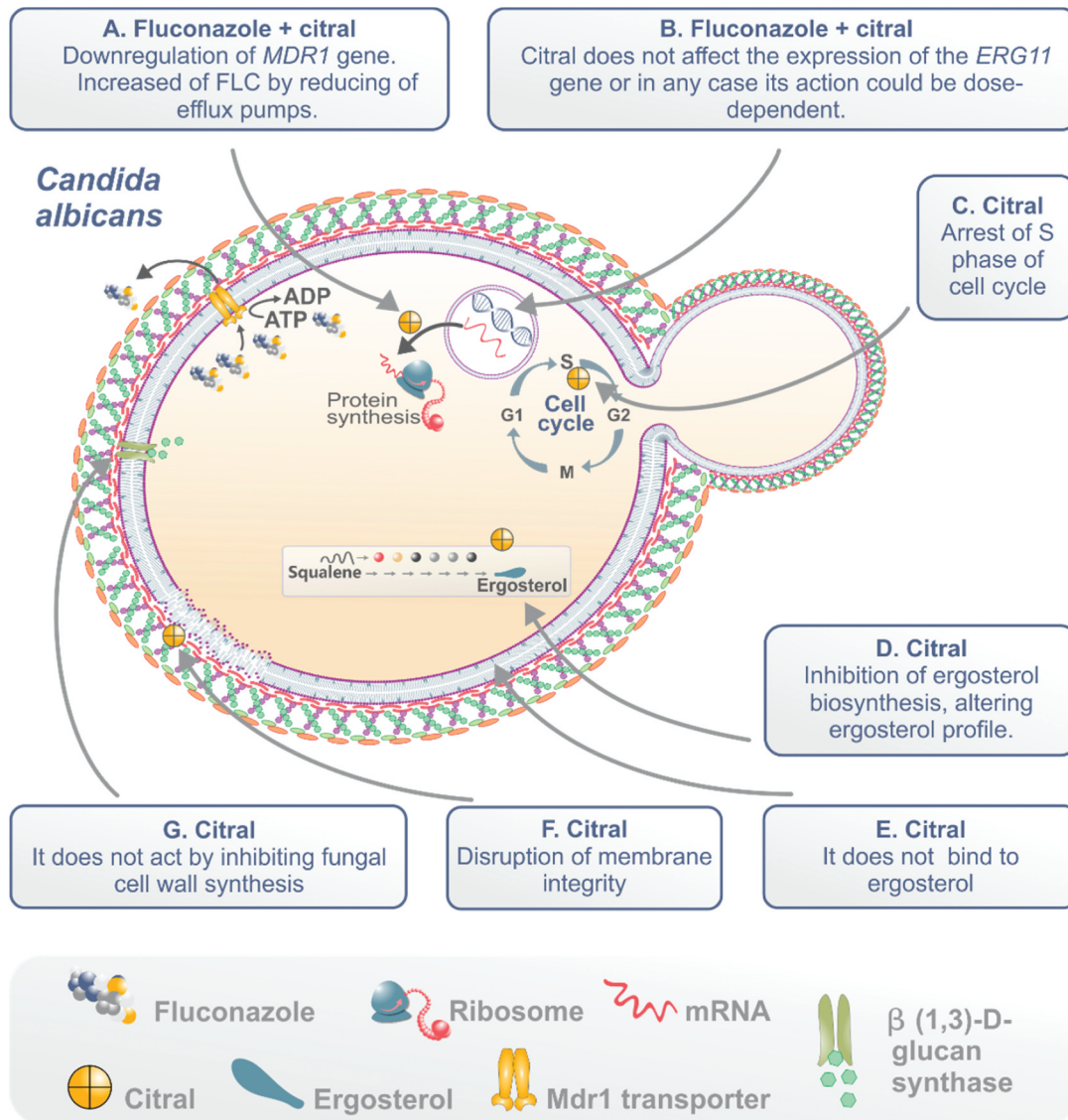


Figure 6. Schematic representation of the mechanisms of action of citral in monotherapy and in combination with fluconazole (FLC) against *C. albicans*. Mechanisms described in the present work (**A** and **B**) and literature review (**C** [40]; **D** [35]; **E** [33,34]; **F** [40]; **G** [33,34]).

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 downregulating 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


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