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Original Article

The killer yeast *Wickerhamomyces anomalus* Cf20 exerts a broad anti-*Candida* activity through the production of killer toxins and volatile compounds

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Abstract

Candidiasis is a group of opportunistic infections caused by yeast of the genus *Candida*. The appearance of drug resistance and the adverse effects of current antifungal therapies require the search for new, more efficient therapeutic alternatives. Killer yeasts have aroused as suitable candidates for mining new antifungal compounds. Killer strains secrete antimicrobial proteins named killer toxins, with promissory antifungal activity. Here we found that the killer yeast *Wickerhamomyces anomalus* Cf20 and its cell-free supernatant (CFS) inhibited six pathogenic strains and one collection strain of *Candida* spp. The inhibition is mainly mediated by secreted killer toxins and, to a lesser extent, by volatile compounds such as acetic acid and ethyl acetate. A new large killer toxin (>180 kDa) was purified, which exerted 70–74% of the total CFS anti-*Candida* activity, and the previously described glucanase KTCf20 was inhibitory in a lesser extent as well. In addition, we demonstrated that Cf20 possesses the genes encoding for the β-1,3-glucanases WaExg1 and WaExg2, proteins with extensively studied antifungal activity, particularly WaExg2. Finally, the 10-fold concentrated CFS exerted a high candidacidal effect at 37°C, completely inhibiting the fungal growth, although the nonconcentrated CFS (RCF 1) had very limited fungistatic activity at this temperature. In conclusion, *W. anomalus* Cf20 produces different low and high molecular weight compounds with anti-*Candida* activity that could be used to design new therapies for candidiasis and as a source for novel antimicrobial compounds as well.

Key words: killer yeasts, killer toxins, volatile compounds, *Wickerhamomyces anomalus*, antifungals, candidiasis.

Introduction

Candidiasis refers to cutaneous, mucosal, and internal organ infections caused by fungi of the *Candida* genus, which can occur at any age and usually occur in the setting of easily identifiable risk factors for infection. *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis* are the most relevant pathogenic species and recently, the emerging pathogen *C. auris* has been added to the list, as a major pathogen associated to

mortal nosocomial outbreaks on five continents. *Candida* spp. are common commensal organisms in the skin and gut microbiota, and disruptions in the cutaneous and gastrointestinal barriers (e.g, owing to gastrointestinal perforation) promote the establishment of disease. In the case of invasive candidiasis, the spectrum of disease ranges from minimally symptomatic candidemia to fulminant sepsis with an associated mortality exceeding 70%.[1–6]

The increasing antifungal resistance further complicates an appropriate antifungal therapy. High azole and/or echinocandin usage in hospitals has led to a rise in the number of strains that are resistant to these first-line antifungals.[2,7] Furthermore, current antifungal drugs are associated to adverse drug reactions such as hypokalemia, infusion reaction, nephrotoxicity, hepatotoxicity, and gastrointestinal affections among others.[2,8,9] Due to these limitations and the morbidity/mortality associated to candidiasis, there is an urgent need for novel antifungal drugs and more efficient therapies.

In this regard, there is an increasing interest in killer toxins (KTs) as a promising source of novel clinical therapeutic agents. KTs are antimicrobial proteins secreted by killer yeasts, which kill or inhibit the growth of other microorganisms, and their mechanisms are mediated by interaction with specific receptors on the cell wall of sensitive cells.[10] KTs have been isolated and studied in more than 20 genera since their discovery in 1963 in *Saccharomyces cerevisiae*, including *Candida*, *Hanseniaspora*, *Kluyveromyces*, *Pichia*, *Wickerhamomyces*, *Williopsis*, *Zygosaccharomyces*, among others.[11,12] Their genetic determinants may include chromosomal genes (KHR, KHS, PaKT, WaExg1, WaExg2), dsRNA plasmids (K1, K2, K28, Klus), or dsDNA linear plasmids (PaT, PiT, zymocin). The lethal mechanisms of KTs are highly varied and include the formation of pores in cytoplasmic membrane and depletion of essential ions; cell cycle arrestment in G1 or S phase; hydrolysis of tRNA and rRNA, inhibition of β-1,3-glucan-synthase and hydrolysis of β-1,3- and/or β-1,6-glucans of the cell wall.[10,12–14] Despite the lethal mechanisms, all known KTs first interact with a primary receptor located on the cell wall of the sensitive cells which include β-1,3- and/or β-1,6-glucans, mannan, mannoproteins, and chitin.[10] For example, β-1,3-glucan has emerged as the primary target of some KTs produced by *Wickerhamomyces* and *Williopsis* species.[15] Most killer toxins are protease-susceptible and display their activity only within narrow pH and temperature ranges, usually below physiological limits. To the best of our knowledge, the only report of the therapeutic effectiveness of a killer toxin in mammals is the curative topical application of concentrated *Pa*KT against cutaneous infections by *Malassezia* spp. in animal models is.[16,17] However, due to their diverse mechanisms of action, killer toxins may offer a source for the development of novel clinical antimycotic agents, as demonstrated with *Pa*KT-derived killer antibodies and killer peptides against *C. albicans* in different studies.[17–19] Therefore, it is worth the study of killer yeasts and their toxins with promising antifungal activity.

Recently, our previous studies demonstrated that *W. anomalus* Cf20 exerts an extensive inhibitory activity against spoilage wine yeasts by a β-1,3-glucanase killer toxin.[20] In this approach, the aim was to evaluate the anti-*Candida* activity of *W. anomalus* Cf20 cell-free supernatant and preliminarily characterize the main inhibitory compounds in it.

Methods

Yeast strains and growth media

The yeast strains used in this study are described in Table 1. *W. anomalus* Cf20 and the remaining killer strains were isolated from winery surfaces from Northwest and Cuyo regions of Argentina. Reference killer strains *S. cerevisiae* YAT 679 (K1 type), *S. cerevisiae* NCYC 738 (K2 type), *C. glabrata* NCYC 388 (K4 type), and *Kluyveromyces marxianus* NCYC 587 (K6 type) were obtained from yeasts collections. *Candida* spp. strains were cordially given by CERELA and Cátedra de Micología of Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán (Argentina). All strains were cultured in YPD broth (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose). When required, the pH was buffered with 0.1 M citric acid/dibasic sodium phosphate. For the study of killer activity, YPD-MB agar (YPD supplemented with 30 mg/l methylene blue and 20 g/l agar) buffered at pH 4.5 and YPD-NaCl agar (YPD-MB agar supplemented with 1, 2 or 3% NaCl) were used. Yeasts were stored in YPD supplemented with 20% glycerol at −80°C.

Screening of killer yeasts against *Candida* spp.

Killer yeasts were grown for 16 hours in YPD buffered at pH 4.5. The 30 μl of cultures were spotted on a lawn of each *Candida* spp. strain in YPD-MB agar pH 4.5, including the strain *C. glabrata* NCYC 388. Plates were incubated 48 hours at 20°C. Results were expressed as: sensitive (+), when a clear inhibition halo was observed around the killer strain colony, and resistant (−) when no halo was observed. The length of the halo was radially measured from the edge of the colony and killer activity was classified as: low (+, 1 to 2 mm); medium (2+, 2 to 3 mm); high (3+, 3 to 4 mm); very high (4+, more than 4 mm).

Production of cell-free supernatant (CFS)

YPD broth pH 3.0 was inoculated with 1% overnight culture *W. anomalus* Cf20 (OD$_{600}$ 3) and incubated for 96 hours at 25°C. The culture was centrifuged at 8000 × *g* for 15 minutes at room temperature to separate cells and to obtain the cell-free supernatant (CFS). CFS was sterilized by filtration through 0.22 μm filters, fractioned and stored at −20°C until use.

Quantification of killer activity

For all experiments, the killer activity (KA) was first quantified against the sensitive yeast *Meyerozyma guilliermondii* Cd6 by the agar diffusion test in YPD-NaCl 3% at 20°C according to the formula: KA (aU/ml) = $10^{(D+5.64)/6.64}$ (r^2 = 99%), where D is the diameter of the inhibition halo in mm and 1 aU is the amount of killer toxin capable of producing an inhibition halo of 1 mm.[21] The KA on *Candida* spp. strains was examined in YPD-NaCl 1, 2 and 3%.

Table 1. Yeast strains.

Phenotype	Strain	Origin
Killer (K+)	*S. cerevisiae* YAT 679 (K1)	YAT
	S. cerevisiae NCYC 738 (K2)	NCYC
	C. glabrata NCYC 388 (K4)	NCYC
	K. marxianus NCYC 587 (K6)	NCYC
	C. valida M3	Winery (Mendoza, Argentina)
	R. mucilaginosa M6	Winery (Mendoza, Argentina)
	S. cerevisiae M12	Winery (Mendoza, Argentina)
	S. cerevisiae Cf8	Winery (Cafayate, Argentina)
	S. cerevisiae Cf13	Winery (Cafayate, Argentina)
	C. oregonensis Cf3	Winery (Cafayate, Argentina)
	C. intermedia Cf4	Winery (Cafayate, Argentina)
	W. anomalus Cf20	Winery (Cafayate, Argentina)
	C. intermedia Cd13	Winery (Cafayate, Argentina)
	P. mexicana Cd19	Winery (Cafayate, Argentina)
Sensitive (R−)	*M. guilliermondii* Cd6	Winery (Cafayate, Argentina)
Pathogenic (clinical isolates)	*C. albicans* CDP1	CERELA[a]
	C. albicans FBUNT1	FBQF[b]
	C. albicans 1	CERELA
	C. albicans 78	CERELA
	C. tropicalis FBUNT3	FBQF
	C. krusei FBUNT2	FBQF

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Inhibition in liquid cultures

CFS supplemented with 1% NaCl (CFS-NaCl, 2×10^4 aU/ml) was inoculated with 1% overnight cultures of *Candida* spp. strains (OD_{600} 1). Depending on the experiments, cultures were incubated at 20, 30 or 37°C for 48 hours. Heat-inactivated CFS-NaCl (100°C, 15 minutes) was used as control and the growth was monitored by OD_{600} measurements in 96-wells plates. Inhibition was quantified according to the formula: I% = 100-$(A_T/A_C) \times 100$, where A_T and A_C are the OD_{600} of treatment and control at 48 hours of incubation, respectively.[21] When required, the viability of strains during incubation was examined by plate counting in YPD agar.

Microscopy

Samples were prepared inoculating 50 ml of CFS-NaCl 1% (2×10^4 aU/ml) with 1% overnight cultures of *C. albicans* 78 and *C. tropicalis* FBUNT3 (OD_{600} 1). Cultures were incubated at 20°C for 2 hours and centrifuged at $12,000 \times g$ for 5 min. Cells were suspended in 10 ml of saline solution for fluorescence microscopy and transmission electron microscopy.

Epifluorescence microscopy. The 50-μl aliquots of cell suspension were stained with propidium iodide (PI) and SYTO9 (LIVE/DEAD Fungalight Yeast viability kit, Invitrogen) according to the manufacturer's recommendations. Samples were observed in an Olympus BX51 epifluorescence Microscope. The ex-

citation wavelength was set at 488 nm and emission was adjusted to 500 nm for SYTO 9 and 617 nm for PI. A number of fields were randomly selected for each sample and cells were observed with a 1003 objective lens and a numerical aperture of 1.4.

Transmission electron microscopy (TEM). 10 ml samples were centrifuged, and cells were chemically fixed in 4% glutaraldehyde and further processed for TEM in CIME (Centro Integral de Microscopía Electrónica, Universidad Nacional de Tucumán, Argentina). Samples were analyzed using a Zeiss EM109 transmission electron microscope (Zeiss, Oberkochen, Germany).

Measurement of % dead cells by fluorescence spectroscopy

CFS-NaCl was concentrated ultrafiltration units (MWCO 10 kDa, Amicon, Millipore, Burlington, MS, USA) until concentration factors of 1, 2, 5, and 10 were reached. Relative concentration factor (RCF) was determined based on the concentration factor obtained by ultrafiltration. An RCF of 1 relates to the inhibitory activity of nonconcentrated CFS.[22] 2 ml of CFS at each RCF and respective heat-inactivated controls were inoculated with 1% overnight cultures (OD_{600} 1) of *C. albicans* 78 and *C. tropicalis* FBUNT3. Cultures were incubated at 20°C for 2, 4 and 8 h. After incubation, 0.2 μl of the fluorescent dies PI and SYTO9 were added to cultures and incubated for 15 minutes in a dark room. Excitation/emission wavelengths were set at 535/617 nm and 480/500 nm for IP and SYTO9,

respectively, in a photon counting spectrofluorometer (ISS PC1 Spectrofluorometer, ISS Inc., Champaign, IL, USA). Emission fluorescence intensity (FI) was monitored. Corrected FI was calculated as follows: $FI = FI_T - FI_C$, where FI_T and FI_C are the fluorescence intensity of samples and control, respectively. For calculation of % dead cells, a standard curve % dead cells vs IP/SYTO9 FI ratio was prepared ($R^2 = 0.99$), and values obtained in experiments were interpolated in the standard curve.

Characterization of inhibitory compounds

Sequential ultrafiltration. CFS was sequentially centrifuged using ultrafiltration units with 100, 50, 30, and 10 kDa cutoff (Amicon Merck Millipore, Burlington, MS, USA) in order to estimate a molecular size range to the active compounds in it. Each concentrated fraction was diluted 1:10 in BCF pH 3.0 and centrifuged again in order to eliminate remnants of lower size-range molecules from it. The active fractions were identified by quantification of their KA against *Candida* spp. strains.

Biochemical characterization. The effects of proteolytic enzymes trypsin, chymotrypsin, pepsin and proteinase K (Sigma-Aldrich, Saint Louis, MO, USA), temperature (20–100°C) and pH (1.0 to 7.0) on the stability of anti-*Candida* activity of CFS were examined as described by Fernández de Ullivarri et al. [20] KA against *Candida* spp. strains was quantified after treatments.

Purification of killer toxin. In sum, 1 l of 96% cold ethanol was dropwise added to 1 l of CFS at 4°C with constant stirring during 2 hours. The preparation was centrifuged at 8000 × g at 4°C for 30 minutes. The precipitate was dissolved in 100 ml of BCF-Urea buffer (0.1 M citric acid/dibasic sodium phosphate pH 3.0, 2 M urea) and centrifuged again. The resulting supernatant was washed and concentrated to 30 ml in BCF pH 3.0 by ultrafiltration (Amicon YM50, cut-off 50 kDa, Millipore, Burlington, MS, USA). The resulting concentrate was applied through a 15 × 300 mm Sephadex G-100 column equilibrated with BCF pH 3.0 using the same buffer as mobile phase at 1 ml/min. Fractions were analyzed by UV spectrophotometry at 280 nm in a Beckman DU 640 UV/Vis Scanning Spectrophotometer (Beckman, Brea, CA, USA). Protein fractions were examined for KA as described before. Active fractions were pooled and concentrated 10 × at 25°C for 7 hours in a SpeedVac Savant SPD 121P (Thermo Scientific, Thermo Electron Corporation, Columbus, OH, USA). For experiments, dilutions of the concentrated KT were prepared in BCF-NaCl 1% pH 3.0 (BCF-KT) or YPD-NaCl 1% pH 3.0 (YPD-KT). Protein concentration was quantified by the Bradford method, samples were analyzed by SDS-PAGE in continuous 6% polyacrylamide gels and stained with Coomassie Brilliant Blue (R-250) or silver staining.

Glucanases genetic determinants. DNA from *W. anomalus* Cf20 was isolated following the protocol described by

Querol et al. [23] Primers PEXG1 (WaEXG1 gene) and PEXG2 (WaEXG2 gene) were designed with Primer 3 plus software: PEXG1-F (GTGTCCCAAAGAACTTTTTCCA), PEXG1-R (GGATTTCCAGAGGCAGCACT), PEXG2-F (TGGAT-CAAGCTTTAGAATGGGCT), PEXG2-R (AACGAGAACCAC-GACCAACA). Polymerase chain reaction (PCR) conditions were: 95°C for 5 minutes; 35 cycles at 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 1 minute; a final extension at 72°C for 10 minutes. PCR products were separated in 1.5% agarose gels. Amplicons were purified and sequenced to compare with sequences of database using BLASTn tool. The Access Numbers in GenBank database are: Cf20-EXG1 MN486485 and Cf20-EXG2 MN486486.

Low molecular weight (LMW) metabolites. Acetic acid and ethyl acetate in CFS were quantified by gas chromatography (GC) following the protocol described by Mendoza et al. [24] To test the inhibitory activity of volatile compounds, a control fraction was prepared: 10 ml of CFS were ultrafiltered with 3 kDa ultrafiltration units. The < 3 kDa fraction was evaporated to dryness in a SpeedVac Savant SPD 121P, resuspended in 10 ml of sterile MilliQ H_2O, and sterilized by filtration through 0.22 μm filters. The treated and untreated <3 kDa fractions were inoculated with 1% overnight cultures of *C. albicans* 78 and *C. tropicalis* FBUNT3 (OD_{600} 1). The treated <3 kDa fraction was used as control and the inhibitory activity at 37°C was calculated as described previously for liquid cultures.

Statistical analysis. Experiments were performed by triplicate in two independent replications. One-way analysis of variance (ANOVA) was applied to the experimental data, and Tukey test was performed for multiple comparisons with the software Infostat (Infostat, Cordoba, Argentina). Differences were considered statistically significant for P-value < .01.

Results

Screening of inhibitory activity of killer yeasts

Fifteen killer strains were used to evaluate their inhibitory activity against a panel of six clinical strains of *Candida* spp. and the collection strain *C. glabrata* NCYC 388 (Table 2). *W. anomalus* Cf20 was the only killer yeast capable of inhibiting the seven strains of *Candida* spp., producing larger inhibition zones compared to the other killer yeasts as well. The killer yeasts K1, K3, and M3 inhibited *C. albicans* 1, 78 and *C. glabrata* NCYC 388; however, smaller halos were found in these cases compared to those of Cf20. Therefore, *W. anomalus* Cf20 was selected for further experiments and cell-free supernatant (CFS) was produced for its characterization. We evaluated the effect of NaCl on the inhibitory activity of CFS and found that 1% NaCl increased the inhibition. Higher NaCl concentrations were inhibitory for all the *Candida* spp. strains, since no growth was observed in the

Table 2. Inhibitory activity of different killer yeasts on pathogenic strains of *Candida* spp.

Indicator strain	Killer strain[a]														
	K1	K2	K4	K6	M3	M6	M12	Cf3	Cf4	Cf8	Cf9	Cf13	Cf20	Cd13	Cd19
C. albicans CDP1	−	−	−	−	−	−	−	−	−	−	−	−	2+	−	−
C. albicans FBUNT1	−	−	−	−	−	−	−	−	−	−	−	−	2+	−	−
C. albicans 1	+	−	−	+	+	−	+	−	−	−	−	+	2+	+	+
C. albicans 78	+	−	−	+	+	−	+	−	−	+	−	−	3+	−	−
C. krusei FBUNT2	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
C. tropicalis FBUNT3	−	−	−	−	−	−	−	−	−	−	−	−	4+	−	−
C. glabrata NCYC 388	+	+	−	+	+	+	−	−	+	2+		2+	3+	+	+

[a]Killer activity classified, based on the radial length of the clear zone between the colony and the halo, as: low (+, 1 to 2 mm); medium (2+, 2 to 3 mm); high (3+, 3 to 4 mm); very high (4+, more than 4 mm); no activity (−).

Table 3. Effect of NaCl on inhibitory activity of CFS against pathogenic strains of *Candida* spp.

Indicator strain	NaCl concentration[a]				CFS[b]
	0%	1%	2%	3%	
C. albicans CDP1	+	2+	ng	ng	1.4×10^4
C. albicans FBUNT1	+	2+	ng	ng	1.5×10^4
C. albicans 1	+	2+	ng	ng	1.5×10^4
C. albicans 78	+	2+	ng	ng	2.3×10^4*
C. krusei FBUNT2	+	2+	ng	ng	1.1×10^4
C. tropicalis FBUNT3	+	2+	ng	ng	3.0×10^4*
C. glabrata NCYC 388	+	2+	ng	ng	2.1×10^4*

[a]Killer activity of CFS in YPD-MB pH 4.5 agar plates supplemented with different NaCl concentrations. Normal (+), increased inhibitory activity (2+) or growth of indicator strain not detected (ng).
[b]Killer activity of cell-free supernatant (CFS) expressed in aU/ml.
*Values with statistically significant difference (ANOVA, Tukey test, $P < .01$).

plates. Further quantification of the KA of CFS in YPD-NaCl 1% showed that *C. albicans* 78, *C. tropicalis* FBUNT3, and *C. glabrata* NCYC 388 are the most sensitive strains (Table 3).

Inhibitory activity of CFS

Effect on growth and viability

The growth of *C. albicans* 78 and *C. tropicalis* FBUNT3 in CFS-NaCl at 20, 30 and 37°C was studied. The inhibitory effect decreased with increasing of temperature. Inhibition values of 74–80% and 10–12% were observed at 20 and 37°C, respectively (Fig. 1A). In order to determine whether the effect at 20°C was fungistatic or fungicidal, the viability of strains was determined along the incubation (Fig. 1B). After 48 hours of treatment, strains 78 and FBUNT3 showed a loss of viability of 1.4 and 1.8 log units in relation to controls, respectively, indicating a fungistatic effect on both yeasts. Nevertheless, during the first 2 hours of incubation, FBUNT3 displayed a 1-log-unit viability decrease compared to its initial count. Thereafter, the culture started to grow until 24 hours of incubation. This short-term
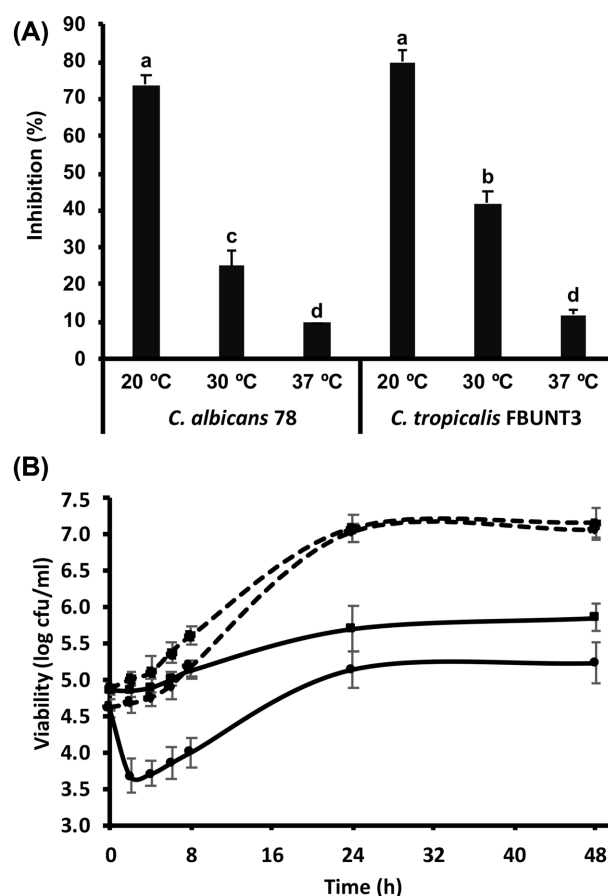

Figure 1. Effect of *Wickerhamomyces anomalus* Cf20 cell-free supernatant supplemented with 1% NaCl (CFS-NaCl, treatment) or heat-inactivated CFS-NaCl (control) on the growth of *Candida albicans* 78 and *C. tropicalis* FBUNT3 cultures. (A) % Inhibition of treated cultures compared to control cultures at 20, 30 or 37°C for 48 h, calculated by measurements of absorbance at 600 nm. Bars with the same letter represent no significant difference. (B) Viability (expressed as log cfu/ml) of *C. albicans* 78 (squares) and *C. tropicalis* FBUNT3 (circles) in treatment (solid lines) or control cultures (dashed lines) at 20°C for 48 h. Data are the mean of three biological replicates. Error bar is the standard deviation. Data were analyzed by one-way ANOVA (Tukey test, $P < .01$). Values of each column marked by different letters are significantly different.

effect on FBUNT3 explained its higher growth inhibition compared to 78 (Fig. 1A).

Cellular effects

In order to better understand the results observed in the growth curves, the effects of CFS-NaCl on the yeasts at a cellular level were evaluated after 2 hours of incubation. In the fluorescence microscopy analysis, treated samples of *C. tropicalis* displayed a greater number of cells with internalized PI, which indicated cell death and direct or indirect disruption of membrane integrity. Most *C. albicans* cells were viable in both treatment and control samples. However, treated samples displayed less cells than controls, congruent with the lower growth rate observed in the viability counts (Fig. 2A).

TEM analysis showed that both samples treated with supernatant had more cells with thinner cell walls than controls. In addition, *C. tropicalis* cells displayed cellular disorganization, chromatin condensation, nuclear fragmentation, and apoptotic bodies, which could indicate an apoptotic process induced by the supernatant (Fig. 2B). However, further experiments should be performed to elucidate the killing mechanism(s) of CFS-NaCl on *Candida* spp. strains.

Dose-dependent antifungal effect

Fluorescence spectroscopy and turbidimetry assays were performed to evaluate cell death and growth curves of samples treated with CFS with increasing RCF (Fig. 3). For both strains, the percentage of dead cells was positively correlated with RCF, indicating that CFS exerted a fungicidal effect on *C. albicans* as well. *C. tropicalis* was more sensitive than *C. albicans* at all tested concentrations except at RCF 10, which produced similar values (>92%). These results were consistent with the growth curves at different RCFs. Yeast growth decreased with increasing RCFs, and RCF 10 was the only concentration in which no growth was detected by photometry after incubation. These cultures were further inoculated into fresh YPD medium and no growth was observed after incubation either. Furthermore, in order to test the inhibitory effect of RCF 10 at human physiological temperature, growth curves were performed at 37°C, and no growth was observed for either strain.

Partial characterization of inhibitory factors in CFS

Killer toxins

A sequential ultrafiltration of CFS using different cut-off membranes was performed, obtaining fractions >100, 50–100, 30–50, 10–30, and <10 (kDa). Fractions >100, 50–100, and 30–50 displayed 70–74, 14–16, and 12–14% of the total anti-*Candida* activity by agar diffusion tests, respectively. The inhibitory activity of these fractions, as well as that of whole CFS, increased in presence 1% NaCl in agar plates. The proteinaceous nature of the active fractions was evaluated by protease treatment, as well as their stability at different temperatures and pHs (Table 4).

Fraction >100 kDa was inactivated only by proteinase K, while the other two fractions were inactivated by all proteases tested.

Since most of the activity was retained in the fraction >100 kDa, we proceeded to purify its killer toxin. The protein concentration of the active elution fractions in the SEC chromatography quantified by the Bradford method was very low (∼10 μg/ml) despite their high absorbance at 280 nm (Fig. 4A). In addition, no bands were detected in the SDS-gels after Coomassie Blue staining (CBB R-250) and only a tenuous diffuse band higher than the 180 kDa band of the ladder was observed after silver staining. The single peak in the chromatogram and the absence of other protein bands in the gels suggest homogeneity in the purification (Fig. 4B).

PCR was performed on total DNA in order to evaluate the presence exo-*β*-1,3-glucanase encoding genes *WaEXG1* and *WaEXG2*.[22] Figure 4C depicts that two amplicons were amplified from the DNA of Cf20. Amplicons sequencing confirmed 100% identity to the genes from *W. anomalus* BS91 (GenBank ID: JQ734563 and JQ734566 for *WaEXG1* and *WaEXG2*, respectively) and strain K (GenBank ID: AJ002195 and AJ222862 for *WaEXG1* and *WaEXG2*, respectively).

LMW compounds

Ethyl acetate and acetic acid concentrations were quantified in CFS by GC and found at 3.6 and 6.4 mM, respectively. Although no inhibitory activity was found for the <30 kDa fractions (10–30 and <10 kDa) by the agar diffusion test, a slight inhibition (11–16%) was found for the <3 kDa fraction by the liquid culture test, indicating that volatile compounds have an inhibitory effect on the growth of *Candida* strains (Table 4).

Discussion

The killer yeast *W. anomalus* Cf20 and its CFS possess a broad and potent inhibitory spectrum against pathogenic strains of *Candida* spp. The spectrum included different species such as *C. glabrata*, *C. tropicalis*, *C. krusei* and 4 strains of *C. albicans* (Table 2) The inhibition increased in presence of 1% NaCl (Table 3); however, the effect of higher NaCl concentrations could not be evaluated due to the high osmotic sensitivity of *Candida* spp. strains. The sensitivity of *Candida* spp. strains to killer toxins and the broad inhibitory spectrum of *W. anomalus* killer toxins have been described in the literature, including inhibition against fungi and bacteria.[25–29] Particularly, *W. anomalus* Cf20, isolated from winery environments, has been demonstrated to exert an extensive inhibitory activity against many wine-related yeasts species.[20] The increased inhibitory activity of killer toxins in high concentrations of osmotic agents such as glucose or NaCl have been previously reported. The higher osmotic pressure may make the cells of the sensitive yeast strains more susceptible to the toxic effects of a killer toxin, or conversely, the killer toxin may make the cells more sensitive to osmotic pressure.[30–33]
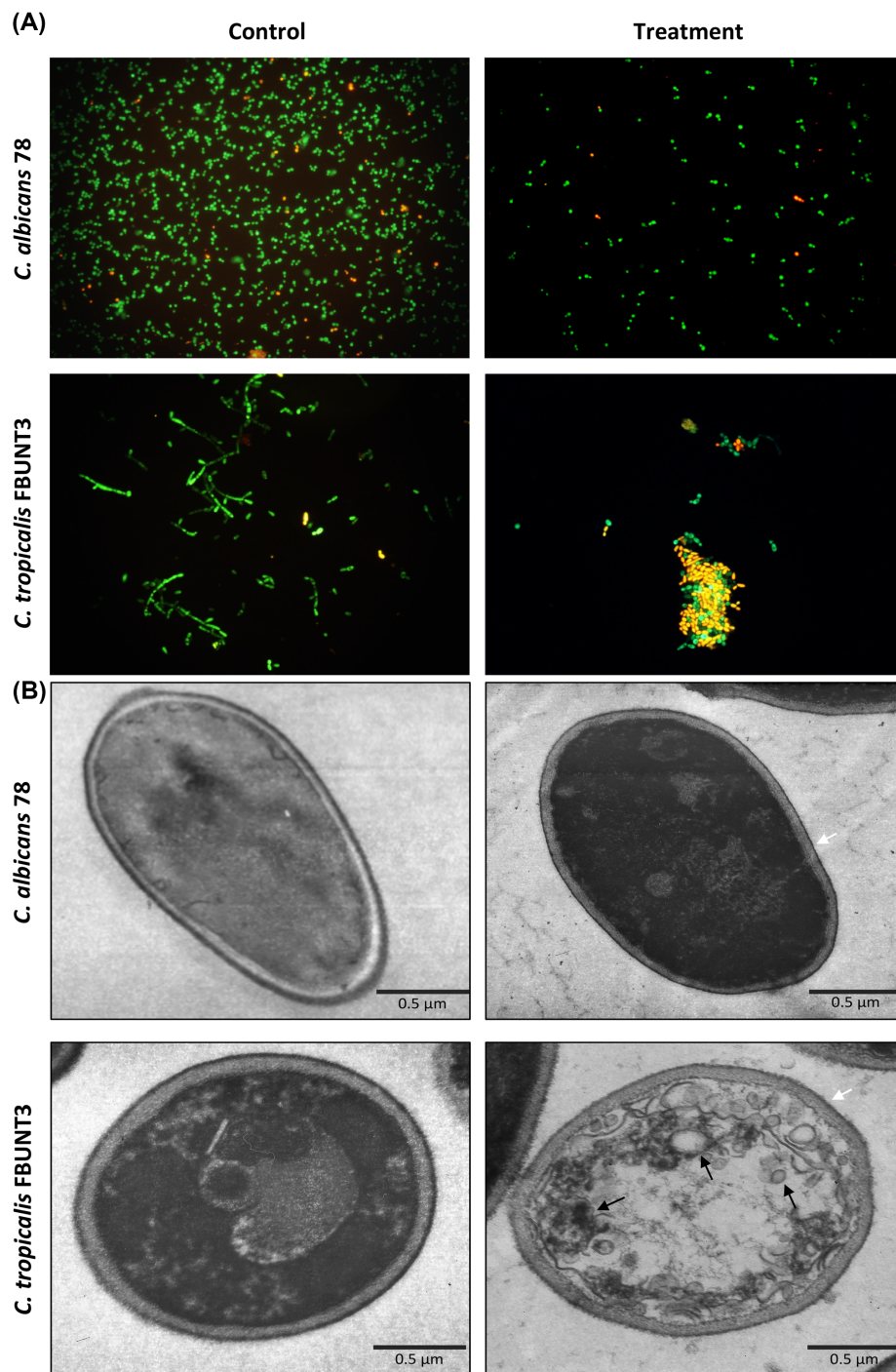
Figure 2. *Candida albicans* 78 and *C. tropicalis* FBUNT3 cells incubated in *Wickerhamomyces anomalus* Cf20 cell-free supernatant supplemented with 1% NaCl (CFS-NaCl, treatment) or heat-inactivated CFS-NaCl (control) for 2 h at 20°C observed by: (A) Epifluorescence microscopy, yeasts double-stained with propidium iodide and SYTO9. (B) Transmission electron microscopy (TEM). Black arrows indicate cellular disorganization, chromatin condensation, nuclear fragmentation and white arrow indicates cell-wall thinning. This Figure is reproduced in color in the online version of *Medical Mycology*.

When the influence of temperature on the anti-*Candida* activity of CFS-NaCl was evaluated, it was found that inhibition percentage of CFS on *Candida* spp. increased with decreasing of temperature (Fig. 1A). These results agree with the behavior of most reported killer yeast and killer toxins, which are more active at lower temperatures.[10] The growth curves at 20°C showed

that *C. tropicalis* FBUNT3 and *C. albicans* 78 have different sensitivity to CFS-NaCl; the former underwent a loss of viability at 2 hours of incubation while the latter had a reduced growth rate (Fig. 1B). These results were confirmed by fluorescence spectroscopy (Fig. 3A), live/dead staining in epifluorescence microscopy, and transmission electron microscopy analysis.
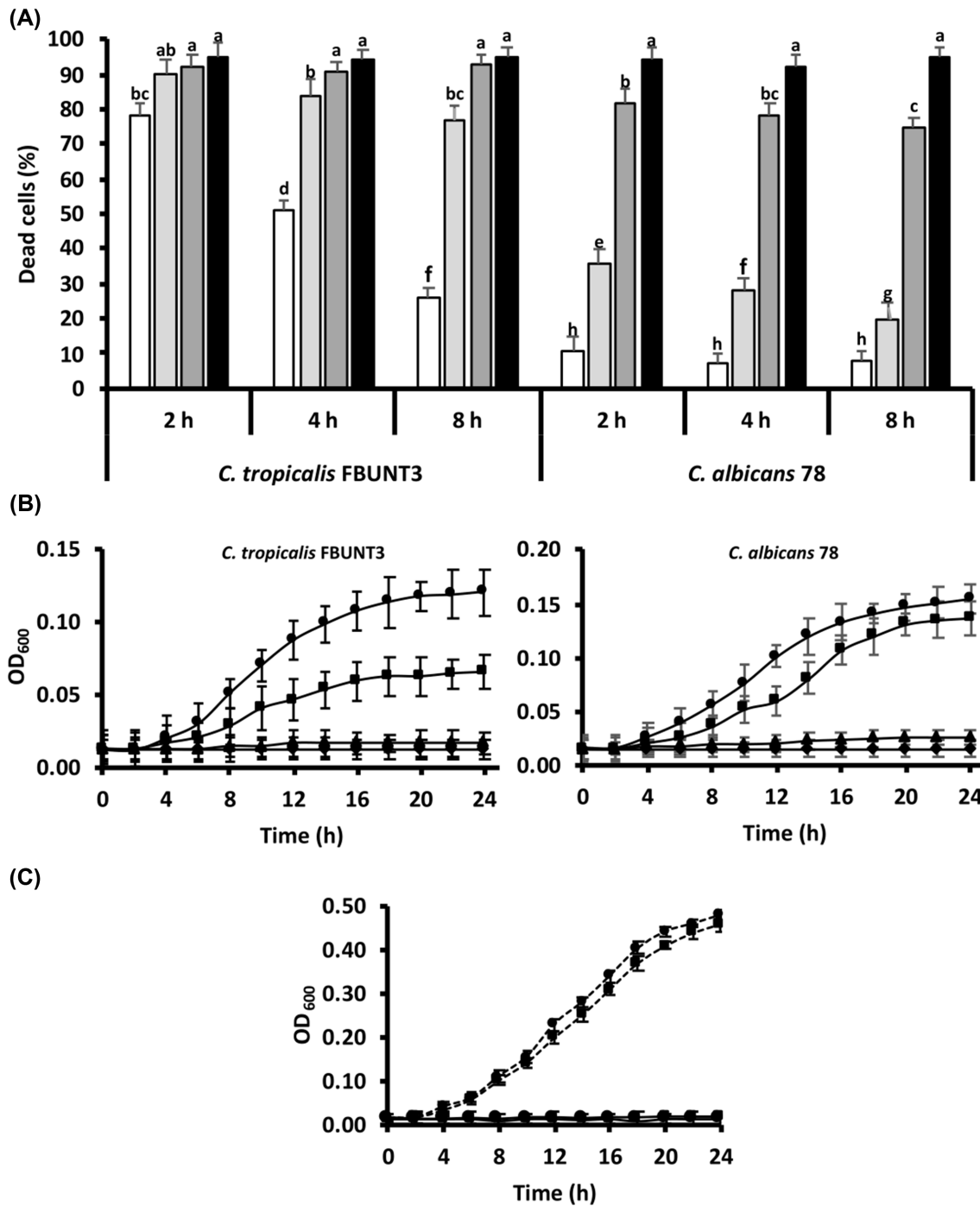
Figure 3. Dose-dependent effect of CFS. (A) % dead cells and (B) growth of *Candida albicans* 78 and *C. tropicalis* FBUNT3 cultures incubated for 2, 4, 8 h or 24 h at 20°C in 1- (white bars, circles), 2- (light gray bars, squares), 5- (dark gray bars, triangles) and 10-fold (black bars, diamonds) concentrated *Wickerhamomyces anomalus* Cf20 cell-free supernatant supplemented with 1% NaCl (CFS-NaCl), obtained by ultrafiltration with 10kDa-cutoff units. For (A), cells were double-stained with propidium iodide (PI) and SYTO9, emission fluorescence intensity (FI) was collected in ten iterations in a photon counting spectrofluorometer and PI/SYTO9 FI ratio values were interpolated in a standard curve % dead cells vs IP/SYTO9 FI ratio. (C) Growth of *C. albicans* 78 (squares) and *C. tropicalis* FBUNT3 (circles) cultures in 10-fold concentrated CFS-NaCl (solid lines) or heat-inactivated 10-fold concentrated CFS-NaCl (dashed lines) at 37°C. Data are the mean of three biological replicates measured. Error bar is the standard deviation. Data were analyzed by one-way ANOVA (Tukey test, $P < .01$). Values of each column marked by different letters are significantly different.

Table 4. Partial characterization of ultrafiltration fractions from cell-free supernatant.

Fraction (kDa)	>100	50–100	30–50	10–30	<10	<3
Activity (%)	70–74*	14–16*	12–14*	–*	–*	11–16#
NaCl Stability	+	+	+	nd	nd	nd
pH	2.0–5.0	2.0–4.5	2.0–4.5	nd	nd	nd
Temperature (°C)	35	35	35	nd	nd	nd
Proteasesˆ						
trypsin	ns	s	s	nd	nd	nd
chymotrypsin	ns	s	s	nd	nd	nd
pepsin	ns	s	s	nd	nd	nd
proteinase K	s	s	s	nd	nd	nd

*Evaluated in agar plates (agar diffusion test). Values represent the distribution (%) of inhibitory activity among the fractions.

#Evaluated in liquid cultures. Values represent the % inhibition compared to a growth control.

+, Activity is enhanced in presence of 1% NaCl.

ˆSensitive (s) or not sensitive (ns) to protease.

nd, not determined.

CFS-NaCl produced both fungistatic or fungicidal activity on *Candida* spp. in a strain-dependent manner, inducing cytotoxic effects on *C. tropicalis* FBUNT3 that resemble to those of apoptotic processes (Fig. 2). Previous studies from our group on the killer toxin KTCf20, purified from CFS, produced similar cytotoxic effects on *M. guilliermondii* Cd6,[20] suggesting that KTCf20 may have a role in the anti-*Candida* activity of CFS. These results also indicate that *C. tropicalis* FBUNT3 is intrinsically more sensitive to CFS-NaCl than *C. albicans* 78. In contrast, the concentrated CFS (RCF 10) exerted fungicidal activity on both strains and retained a remarkable inhibitory activity at 37°C (Fig. 3) despite it was lost to a large extent at RCF 1 at this temperature (Fig. 1A). Similar results were reported by Sawant et al.,[34] who described that the purified and concentrated killer toxin from *W. anomalus* WC65 (*P. anomala*) had fungicidal activity against *C. albicans* in contrast to the fungistatic activity of the crude toxin. Likewise, the differential effects observed in our study seem to be related to the CFS concentration as well as to the intrinsic resistance mechanism of each strain, that is, dose- and strain-dependent.

A partial characterization of anti-*Candida* compounds in different ultrafiltration fractions was carried out. The sensitivity of fractions >30 kDa to proteases confirmed that these inhibitory compounds in CFS are proteinaceous in nature; hence they are killer toxins.[10] It was previously underlined that we proceeded to purify the killer toxin from fraction >100 kDa (Fig. 4A,B). It is well reported that heavily glycosylated proteins are very underestimated by the Bradford assay, and Coomassie-based and traditional silver staining methods weakly or even fail to stain them as well.[35–37] The results suggest that the purified killer toxin could be a glycoprotein, a common characteristic for many killer toxins.[10,38] Killer toxins with large masses like this one are very

unusual and, to the best of our knowledge, Kh-I and Kh-II are the only two extensively glycosylated killer toxins larger than 180 kDa (both 300 kDa) from *W. anomalus* described in the literature. Kh-II and the purified toxin have similar characteristics: not sensitive to trypsin, chymotrypsin or pepsin; stability between pH 2.0 and 5.0 and up to 35°C.[39] In previous studies we demonstrated that KTCf20 is a protein larger than 50 kDa, stable between pH 2.0 and 4.5, and up to 35°C and sensitive to all the proteases tested, the same characteristics as fraction 50–100 (Table 4). In addition, the cellular effects observed in the TEM analysis for CFS resemble those observed for KTCf20 but milder. These data confirm that the >180 kDa toxin is different from KTCf20, and that KTCf20 is highly likely to be the anti-*Candida* killer toxin contained in fraction 50–100 kDa.

Since exo-β-1,3-glucanases are commonly found in *W. anomalus* strains, we evaluated the presence of the *WaEXG1* and *WaEXG2* genes, which encode this type of killer toxins (Fig. 4C). The results indicate that Cf20 possesses the genes that encode WaExg1 and WaExg2, the 58 and 49 kDa exo-β-1,3-glucanases, respectively.[22,40,41] Additional experiments should be conducted to confirm whether these genes are functional and whether their products correspond to anti-*Candida* killer toxins present in CFS.

W. anomalus is known to produce and secrete several LMW metabolites.[42] In previous studies we found that *W. anomalus* Cf20 produces high concentrations of the antimicrobial molecules ethyl acetate and acetic acid in pure cultures in grape musts.[20] These compounds were identified in CFS and it was demonstrated that volatile compounds in fraction <3 kDa have anti-*Candida* activity (Table 4). Evidence has revealed the occurrence of an apoptotic phenotype in different yeasts that is inducible with environmental stresses such as H_2O_2, weak small organic acids and their salts. Different studies reported that 40–60 mM acetic acid induced apoptosis in pathogenic *Candida* spp. strains.[43–46] Although the concentration in CFS was 3.6 mM, similar levels of this compound have exerted inhibitory effects on other yeasts such as *S. cerevisiae*, hampering vesicle exit from the endosome to the vacuole by intracellular acidification.[45,47] The antiyeast activity of ethyl acetate has not been widely described in the literature. Studies on ethyl acetate producers, such as *W. anomalus* and *Kluyveromyces marxianus* indicate that, as expected, these species resist high concentrations of this ester (17 g l^{-1}, 193 mM). Nevertheless, different studies have reported efficient anti-mold activity of ethyl acetate produced by *W. anomalus*, some of them at concentrations like those found by us.[42,48,49] To the best of our knowledge, only one study has evaluated the effect of this compound on *C. albicans*, reporting a MIC ≤ 5%; however, lower concentrations were not evaluated.[50] An additive or synergistic effect of LMW compounds with killer toxins is not discarded and will be addressed in future studies.

In summary, CFS produced fungistatic or fungicidal effects on *Candida* spp. in a strain and dose-dependent manner, in which
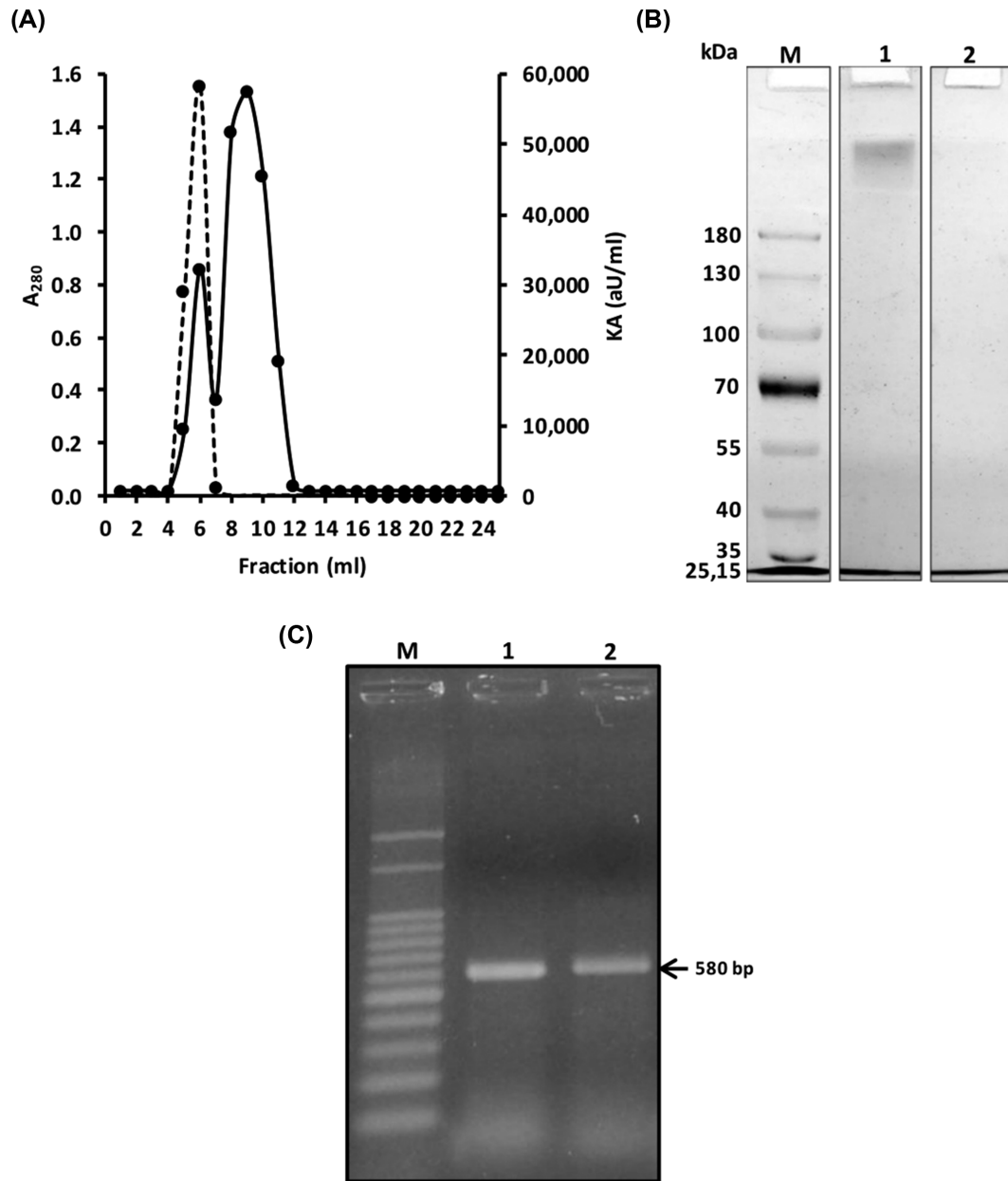
(A)

(B)

(C)

Figure 4. (A) Chromatogram from Sephadex G-100 column of a concentrated *Wickerhamomyces anomalus* Cf20 supernatant after ethanol precipitation and ultrafiltration (100 kDa cutoff) showing absorbance at 280 nm (solid line) and killer activity (dashed line) from eluates. (B) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (6%) of pooled and concentrated active fractions from Sephadex G-100 chromatography. M, molecular mass standard (Thermo Fisher); lane 1, silver staining; lane 2, Coomassie Blue R-250 staining. This figure is a composite image of three distant lanes in the same gel. (C) Agarose gel of PCR partial amplification of WaEXG genes from *W. anomalus* Cf20; M, 100 pb DNA molecular marker (Promega); Lane 1, WaEXG1; Lane 2, WaEXG2.

the cytotoxic effects induced by unconcentrated CFS resemble those of apoptotic processes. This inhibition is mainly mediated by killer toxins secreted to the extracellular medium and, in a lesser extent, by secretion of low molecular weight compounds as well, such as acetic acid and ethyl acetate. Purification and partial characterization of the main anti-*Candida* killer toxin described a protein probably highly glycosylated and unusually large with characteristics like those of Kh-II, produced by *W. anomalus* NCYC 435.[39] KTCf20, the β-glucanase killer toxin described in our previous study, exerted anti-*Candida* activity

as well.[20] In addition, we found that Cf20 possesses the genes encoding for the β-1,3-glucanases WaExg1 and WaExg2, proteins with extensively studied antifungal activity.[22,40,41,51] The inhibitory activity of CFS increased by addition of 1% NaCl, consistent with the presence of toxins that affect the cell wall integrity, making the cell more sensitive to osmotic pressure. Although it was not the focus of this approach, it is worth noting the high sensitivity of all *Candida* strains to higher NaCl concentrations, which could be useful for the design of novel topical anti-*Candida* therapies. Finally, the concentrated CFS

(RCF 10) exerted total candidacidal effect at 37°C, although the nonconcentrated CFS (RCF 1) had very limited fungistatic activity at this temperature.

In conclusion, *W. anomalus* Cf20 produces a variety of low and high molecular weight compounds with broad anti-*Candida* activity that could be used for treating *Candida* infections. *In vitro* and *in vivo* experiments will be performed in future studies to test their therapeutic activity alone or in combination with current antifungal therapeutics. Although the potential antigenicity and lability of proteins under physiological conditions would limit their use to topical applications, novel derivatives such as killer antibodies and antifungal peptides could be created for systemic applications as well. Considering the need for novel antifungal alternatives, killer yeasts and their metabolites are promising sources for mining novel compounds and developing new therapies for candidiasis and other fungal infections.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Ethical statement

This article does not contain any studies involving human participants or experimental animals.

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