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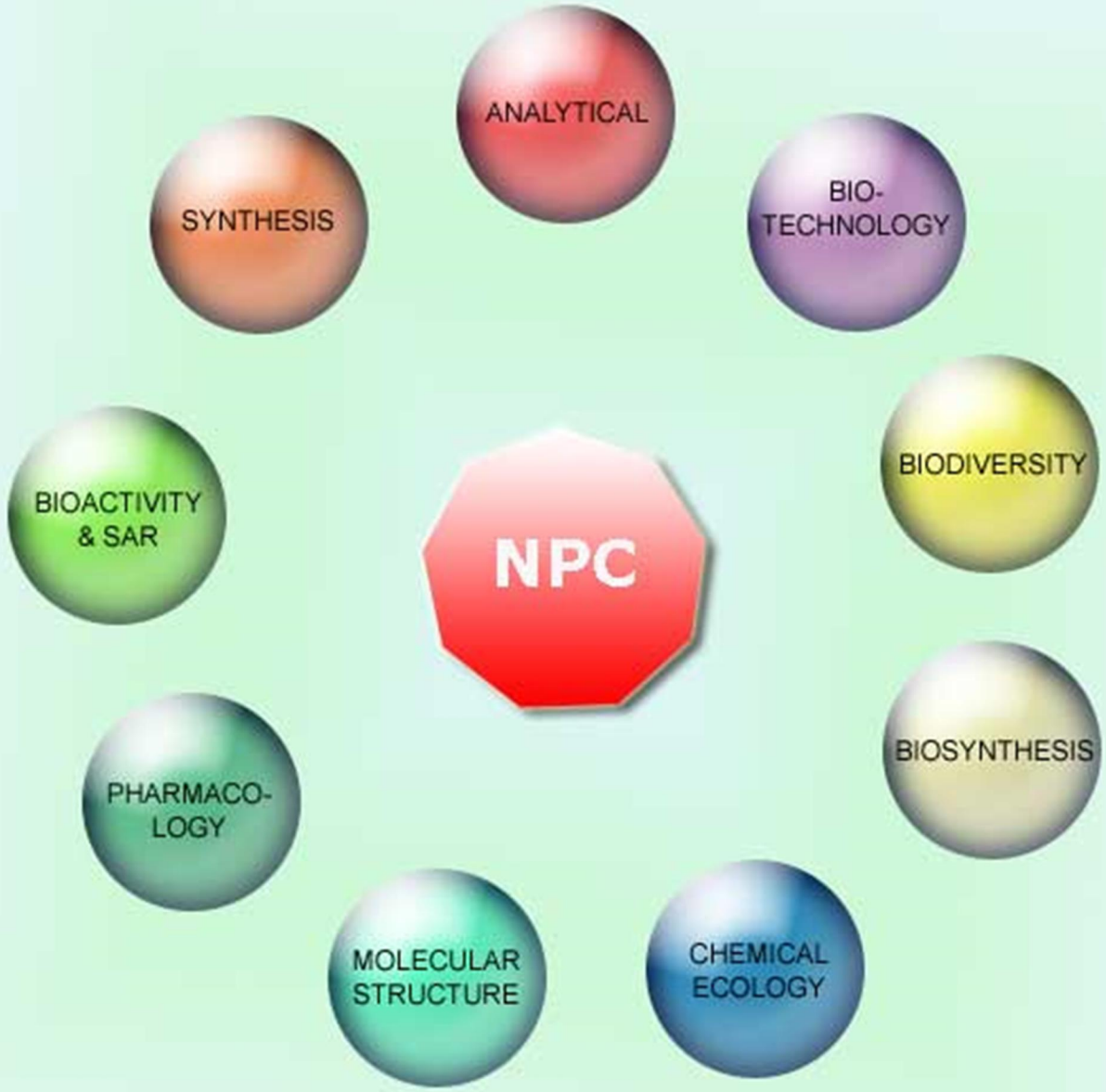

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The Effect of *Zuccagnia punctata*, an Argentine Medicinal Plant, on Virulence Factors from *Candida* Species

Nuño Gabriela[a], Alberto María Rosa[a,b,c], Zampini Iris Catiana[a,b,c], Cuello Soledad[a], Ordoñez Roxana Mabel[a,b,c], Sayago Jorge Esteban[a,b,c], Baroni Veronica[d], Wunderlin Daniel[d] and Isla María Ines[a,b,c #]

[a]*INQUINOA (CONICET)*, [b]*Facultad de Ciencias Naturales*, [c]*Facultad de Bioquímica, Química y Farmacia. Universidad Nacional de Tucumán. San Miguel de Tucumán. Tucumán. Argentina*
[d]*ISIDSA, SECyT; ICYTAC-CONICET; Facultad de Ciencias Químicas - Universidad Nacional de Córdoba, Córdoba. Argentina*

misla@tucbbs.com.ar

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Zuccagnia punctata Cav. has been used as a traditional medicine in Argentina for the treatment of bacterial and fungal infections. In this study, we evaluated the ability of *Z. punctata* extract (ZpE) and compounds isolated from it to inhibit the growth and virulence factors of *Candida* species. ZpE showed inhibitory activity against planktonic cells of all assayed *Candida* species with MIC values of 400 µg/mL and with MFC values between 400 and 1,200 µg/mL. The principal identified compounds by HPLC-MS/MS and UV-VIS were chalcones (2′,4′-dihydroxy-3′-methoxychalcone, 2′,4′- dihydroxychalcone), flavones (galangin, 3,7-dihydroxyflavone and chrysin) and flavanones (naringenin, 7-hydroxyflavanone and pinocembrine). These compounds were more effective as inhibitors than the extracts upon biofilm formation as well as on preformed *Candida* biofilm and yeast germ tube formation. Furthermore, ZpE and chalcones are able to inhibit exoenzymes, which are responsible for the invasion mechanisms of the pathogens. All these effects could moderate colonization, thereby suppressing the pathogen invasive potential. Our results indicate that ZpE and chalcones could be used in antifungal therapy.

Keywords: *Candida*, Virulence factors, Chalcones, *Zuccagnia punctata*.

Mucosal and systemic fungal infections have been reported to be caused by the opportunistic pathogen *Candida* spp [1a]. Recent evidence suggests that most diseases produced by this pathogen are associated with biofilm growth [1b]. Biofilm-associated infections are frequently resistant to conventional antibiotic therapy [1c]. Decreased susceptibility of sessile cells to antimicrobial agents, including amphotericin B, fluconazole, itraconazole and ketoconazole, compared with that of planktonic cells has been extensively reported over the past decade [1d]. Since the number of therapeutic options for *Candida* related infections is scarce, new treatment should be explored.

Zuccagnia punctata Cav. (Fabaceae) is a monotypic species widely distributed in western Argentina, commonly known as jarilla pispito, puspus and jarilla macho [2a]. This species has been extensively used as a traditional medicine in Argentina for the treatment of bacterial and fungal infections, and to treat asthma, arthritis and rheumatism [2b]. Antioxidant properties [2c], activity against plant fungal pathogens [2d-f], and antiulcer [2g], antigenotoxic [2h] and antibacterial activity against antibiotic resistant Gram-negative bacteria [2i] and *Streptococcus pneumoniae* [2j] were reported for *Z. punctata* extracts. Some bioactive phenolic constituents of *Z. punctata* were also reported [2d, 2i, 2f, 2k].

In this study, we evaluated the ability of *Z. punctata* extracts and their major constituents to eradicate established biofilms and to inhibit biofilm formation by *Candida* strains and other virulence factors. *Z. punctata* extract (ZpE) showed inhibitory activity against planktonic cells of all assayed *Candida* species with MIC values of 400 µg/mL and with MFC values between 400 and 1,200 µg/mL. The extract was separated by silica gel column chromatography into ten fractions (F-1 to F-10). In general, fractions F-6 and F-7 were more abundant (1,600 and 624.8 mg /5g ZpE, respectively) and more effective in inhibiting planktonic growth of nine strains of *Candida*. The MIC values ranged between 50 and 100 µg/mL for F-6 and between 200 and 400 µg/mL for F-7 and F-8. The other fractions were less active and even inactive on some *Candida* species. MFC values of fractions F-6 and F-7 (50-400 µg/mL) were between two and twelve folds lower than those obtained for the extract. Based on this information, fractions F-6 and F-7 were selected to continue with the activity-guided isolation of anti-*Candida* agents.

Fraction F-6 was subjected to Sephadex LH-20 column chromatography and eight sub-fractions (I to VIII) were obtained. The MIC/MFC values for the more active sub-fractions (F-6 VI and F-6 VII) were between 25 and 200 µg/mL/50 and 200 µg/mL (Table 1). Both sub-fractions were re-chromatographed on Sephadex LH 20. 2′,4′-Dihydroxy-3′-methoxychalcone (DHMC) was identified in fraction F-6-VI-D, while fraction F-6-VII-C contained 2′,4′-dihydroxychalcone (DHC) and 7-hydroxyflavanone (7-HF).

Fraction F-7 was also re-chromatographed on Sephadex LH-20 yielding 9 sub-fractions, four of which were active against all assayed *Candida* strains with MIC/MFC values between 50 and 200 µg/mL/100 and 400 µg/mL (Table 1). The compounds identified in these fractions were: DHC, DHMC, 7-HF, 3,7-dihydroxyflavone, naringenin, pinocembrine, chrysin and galangin.

DHC showed the highest antimicrobial activity with MIC values between 25 and 100 µg/mL, followed by DHMC with MIC values between 50 and 200 µg/mL (Table 1). The MFC values were between 50 and 200 µg/mL. However, 7-HF and 3,7- dihydroxy-flavone were inactive against all *Candida* strains. The anti-*Candida* activity of chalcones was lost by their cyclization to the corresponding flavanone or flavone.

For this reason, we selected 2′,4′-dihydroxychalcone and 2′,4′-dihydroxy-3′-methoxychalcone to continue with the study of their effect on the production of virulence factors by *Candida*. Among human fungal pathogens, *C. albicans* is the species most frequently associated with biofilm formation and this has a significant impact

Table 1: Antifungal activity of *Zuccagnia punctata* extracts (ZpE), fractions and major isolated compounds

Samples	1	2	3	4	5	6	7	8	9
	MIC (µg/mL)								
ZpE	400	400	400	400	400	400	400	400	400
DHC	50	50	50	50	25	25	25	25	100
DHMC	100	100	100	200	100	100	50	100	50
F-6	100	100	100	100	100	100	100	100	50
F-7	200	200	200	200	200	200	200	200	200
F-8	400	400	400	400	400	400	400	400	200
6-VI-D	100	100	100	200	100	100	50	100	50
6-VII-C	50	100	50	25	25	50	25	50	100
7-V	200	200	200	200	200	200	200	200	100
7-VI	100	200	200	200	200	200	200	200	200
7-VII	100	100	100	100	100	100	100	100	200
7-IX	100	100	50	50	100	50	100	100	100

(1 and 2) *C. albicans* (F100, F101, respectively); (3 and 4) *C. tropicalis* (F300, F301); (5) *C. krusei* (F400); (6) *C. parasilopsis* (F500); (7) *C. glabrata* (F200); (8) *C. guilliermondii* (F600), *(9) C. albicans* ATCC 10231. DHC: 2′,4′ -dihydroxychalcone; DHMC: 2′,4′-dihydroxy- 3′- methoxychalcone.

on morbidity and mortality [3a]. Yet, recent results have emphasized an important role of the extracellular matrix in the tolerance of *C. albicans* biofilms to antifungals, especially those of the azole and polyene classes [3b]. As presented in Table 2, the biofilm formation was inhibited betwen 50 and 40% by 100 and 50 µg/mL of ZpE. These concentrations are four- and eight- fold lower than the ZpE MIC value (400 µg/mL). The isolated compounds (chalcones) were more effective than the extracts in inhibiting biofilm formation. These results suggest that exposure of *Candida* cells to sub-MIC concentrations of these agents can reduce the adherence ability of the cells compared with the unexposed controls. Since adherence represents a major step in biofilm formation, these agents might be used to prevent *Candida* biofilm-associated infections.

Table 2: Percentage of biofilm formation inhibition, biomass reduction and metabolism decrease by *Zuccagnia punctata* extract (ZpE) and metabolites.

	DHC		DHMC		ZpE	
	Compounds concentration (µg/mL)					
	6.25	12.5	12.5	25	50	100
	Effect upon biofilm formation					
	Biofilm inhibition (%)					
C. albicans	33.9	43.9	46.7	86.7	46.9	39.8
	Effect upon preformed* Candida *biofilm					
	Biomass reduction (%)					
C. albicans	20	59.4	35	76.6	35	71.2
	Metabolism reduction (%)					
C. albicans	25	55	10	39	30	60

2′,4′-dihydroxychalcone (DHC), 2′,4′- dihydroxy-3′-methoxychalcone (DHMC).

The effect of Zp extract and fractions on preformed biofilm was also evaluated. The maximum reduction of biofilm biomass was observed at 100 µg/mL (71.2%), 25 µg/mL (76.6%) and 12.5 µg/mL (59.4%) for ZpE, DHMC and DHC, respectively. The effect of extracts and isolated metabolites was more evident for biomass reduction than for *Candida* metabolism (Table 2).

The morphological switch between the yeast and the hyphae morphology (yeast–hyphae-dimorphism) is one of the most important and well-known virulence factors in *C. albicans* [3c]. The yeast cells are more easily distributed in the bloodstream, and hyphae allow penetration of host tissues to accede to nutrient sources. Our research also showed the ability of extracts and isolated compounds to inhibit yeast germ tube formation. The ZpE concentration that was able to inhibit germ tube formation was lower than that necessary to inhibit yeast growth. The IC_{50} value for ZpE was 100 µg/mL (Figure 1 A). The chalcone concentration necessary to produce 50% inhibition of yeast germ tube formation (12.5 µg/mL) was similar to that necessary to produce 50% inhibition of *C. albicans* biofilm formation (Figure 1B).

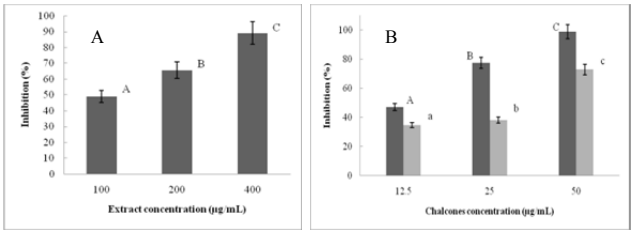

Figure 1: Effect of *Zuccagnia punctata* extract (ZpE) and isolated compounds on *Candida albicans* ATCC 10231 hyphal development. Inhibition percentage after 3 h incubation with ZpE (A),(■) 2′,4′-Dihydroxychalcone and (■), 2′,4′-Dihydroxy-3′-methoxychalcone (B).

Candida species are able to secrete many exoenzymes such as phospholipase and hemolysin, which are considered to be important virulence factors in their pathogenesis [3d-e]. ZpE and chalcones are able to inhibit these exoenzymes (Table 3). In general, ZpE and pure compounds were more effective in inhibiting hemolysin than phospholipase. However, the isolated compounds were more active than ZpE in inhibiting the secretion of both enzymes. The inhibitory effect on this virulence factor is highly significant because approximately 80% of *Candida* species isolated from chronic diseases exhibit phospholipase activity [3f]. Mane *et al* [3g] reported that 100% of *C. albicans* isolates had beta hemolytic activity.

Table 3: Inhibition of phospholipase activity exhibited by *Candida*.

	µg/mL	Hemolisis inhibition percentage (%)	Phospholipase inhibition percentage (%)
ZpE	50	41.7±2.1	20.0±1.0
	100	41.7±3.3	20.0±1.6
	200	50.0±2.5	33.0±3.0
2′,4′-dihydroxy chalcone	12.5	10.0±0.8	27.0±2.1
	25	50.0±3.5	27.0±1.9
	50	58.4±2.9	27.0±1.6
2′,4′-dihydroxy- 3′-methoxychalcone	6.25	33.0±2.6	0.0
	12.5	33.0±1.6	27.0±2.4
	25	42.0±2.1	27.0±1.4

In conclusion, all the demonstrated effects of ZpE and the metabolites isolated from it could moderate colonization, thereby suppressing the invasive potential of *Candida*. Our results could validate the antifungal activity of *Z. punctata* and demonstrated that the major constituents could contribute to the activity of the complex antifungal mixture.

Experimental

Plant material: The aerial parts of *Zuccagnia punctata* Cav. were collected from January to February 2011 at 1,800 m above sea level (m.a.s.l) in Tucumán, Argentina. A voucher specimen N° 605935/LIL was deposited in the Herbarium of "Fundación Miguel Lillo", Tucumán, Argentina. The plant was authenticated by Dr Soledad Cuello. Leaves and stems (aerial parts) were dried at 40°C.

Extraction and fractionation of bioactive compounds: In brief, the dried plant material (150 g) was powdered and macerated with dichloromethane (800 mL) for 40 min, shaken at room temperature and then filtered to obtain *Zuccagnia* extract (ZpE). ZpE was fractionated on a silica gel column and eluted by using a gradient of petroleum ether (PE): ethylacetate (EtAc) (80:20, 60:40, 40:60, 20:80) // EtAc // MeOH// H_2O. Ten fractions were obtained (F1 to F10) based on TLC profiles revealed with Natural Products reagent (NP – 1% methanolic solution of diphenylboric acid aminoethyl ester). Fractions **6** and **7** were separated on Sephadex LH-20 using as a mobile phase PE: chloroform ($CHCl_3$): MeOH (2:1:1).

Fraction 6: Fractions of 10 mL were collected and combined, based on TLC profile, into sub-fractions 6-**I** to 6-**VIII**. The **6-VI** sub-

fraction was re-chromatographed on Sephadex LH-20; 7 sub-fractions were obtained (6-VI-**A** to 6-VI–**G**). The **6-VII** sub-fraction was also re-chromatographed on Sephadex LH-20; 4 new sub-fractions were obtained (6-VII-**A** to 6-VII-**D**).

Fraction 7: Fractions of 10 mL were collected and combined into sub-fractions 7-I to 7-IX based on TLC profile. All sub-fractions were suspended in DMSO to obtain stock solutions of 24 mg/mL. The solutions were kept at 4°C for further experimental use.

Compound identification: An Agilent Series 1200 LC System (Agilent, USA) coupled to a MicrOTOF Q II (Bruker Daltonics, USA) was used for HPLC-ESI-MS/MS analyses. The HPLC system consisted of a micro vacuum degasser, binary pumps, an autosampler (40 µL sample loop), a thermostated column compartment, and a diode array detector (DAD). The mass spectrometer was equipped with an electrospray ion source, and a qTOF analyzer was used in MS and MS/MS modes for the structural analysis of phenolics. HPLC analyses were performed on a thermostated (40°C) Phenomenex Luna C18 250 × 4.6 mm (5 µm) column at a 0.4 mL/min flow rate using 0.5% (v/v) formic acid (solvent A) and MeOH (solvent B) with the following composition gradient: starting with 20% and changing to 50% B for 3 min, kept for 5 min, followed by a second ramp to 80% B in 5 min, kept for 17 min, a third ramp to 20% B in 1 min, and remaining in this condition for 10 min before the next run. The injection volume was 40 µL. ESI-MS detection was performed in the negative ion mode with mass acquisition between 100 and 1500 Da. Nitrogen was used as a drying and nebulizing gas (7 L/min and 3.5 Bar, respectively), at 180°C. For MS/MS experiments, fragmentation was achieved by using an Auto MS^2 option. Detection was carried out with a DAD ranging between 200 and 700 nm. Compounds were identified by comparing their mass spectral data with those stored in database libraries and/or by interpretation of the UV data and mass spectra. The standards, quercetin, naringenin, pinocembrin, crisine, galangin, 2′,4′-dihydroxychalcone and 2′,4′-dihydroxy-3′-methoxy-chalcone, were prepared at a stock concentration of 1000 mg/L. Calibration standard samples were prepared by appropriate dilutions with methanol from the stock solutions and filtered through Millipore paper (0.45 µm) before use. MS analysis was used for compound quantification with a specific calibration curve (when reference compounds were not available, the calibration of structurally related substances was used). Compound concentrations were calculated in triplicate and the mean value calculated in each case.

Antimicrobial assays

Microorganisms: One *C. albicans* strain from the American Type Culture Collection (ATCC 10231) and 8 clinical isolates, *C. albicans* (F_{100}, F_{101}), *C. glabrata* (F_{200}), *C. tropicalis* (F_{300}, F_{301}), *C. krusei* (F_{400}), *C. parasilopsis* (F_{500}) and *C. guilliermondii* (F_{600}) were collected from patients from the Hospital del Niño Jesús, San Miguel de Tucumán, Argentina. The inocula were adjusted to the desired cellular density by counting in a hematocytometer.

Activity upon planktonic cells: The anti-*Candida* activity of ZpE and fractions was analyzed by a broth microdilution method according to the CLSI reference M27-A3 [4a]. The method was performed on microplates. Samples were dissolved in 1% DMSO to obtain final concentrations between 200 and 1,200 µg dry weight (DW) per mL (µgDW/mL) for the ZpE and between 25 and 400 µgDW/mL for fractions and pure compounds. The inoculum (100 µL of Sabouraud dextrose broth containing 5×10^5 CFU/mL) was added to each well. Plates were aerobically incubated at 28°C for 48 h. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of extract without visible growth at macroscopic level. Aliquots of 10 µL of each well suspension which showed negative-visible growth after 24 h incubation were inoculated onto the surface of Sabouraud dextrose agar. The lowest concentration of samples without fungal growth was recorded as the minimum fungicidal concentration (MFC).

Effects of the extract and selected major constituents upon Candida biofilm formation: The effect of extract and isolated compounds on biofilm formation by *C. albicans* F100 was analyzed on microtiter plates [4b]. *Candida* suspension (100 µL of 1×10^6 cells/mL in Sabouraud dextrose broth) was added to 100 µL of different ZpE dilutions (up to 200 µg/mL) and to isolated compounds (up to 50 µg/mL), and then mixed. The plates were incubated for 48 h at 37°C. After biofilm formation, the medium was aspirated, and non-adherent cells were removed by washing with sterile PBS. The effect was determined by using 100 µL of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium hydroxide (XTT, 0.5 mg/mL). The plates were incubated in the dark for 4 h at 37°C. A colorimetric change in the XTT- reduction assay was measured in a microplate reader (BioTek instruments, Inc., Vermont, USA) at 492 nm. The inhibition percentage of each sample was calculated.

Effects of the extract and selected major constituents upon preformed Candida biofilms: Antifungal susceptibility assay of sessile cells was performed as previously reported [4b]. Biofilms were formed on microtiter plates. Cell suspensions (100 µL of ~1×10^6 cells/mL) of *C. albicans* F100 were incubated for 48 h at 37°C. After biofilm formation, the medium was aspirated, and non-adherent cells were removed by washing with sterile PBS. The extract (up to 200 µg/mL) and isolated compounds (up to 50 µg/mL) were then added to the biofilms in serially double-diluted concentrations and incubated for 24 h at 37°C.

Effect upon metabolic activity of biofilm: The effect of each sample against preformed *Candida* biofilm was determined by using 100 µL of XTT (0.5 mg/mL). The plates were incubated in the dark for 4 h at 37°C. A colorimetric change in the XTT-reduction assay was measured in a microplate reader (BioTek instruments, Inc., Vermont, USA) at 492 nm [4b].

Effect upon biofilm total biomass: The biofilm was treated with methanol and then with 0.02% crystal violet (CV). The bound CV was released with 33% acetic acid and absorbance was measured at 590 nm [4c]

Determination of germ tube formation in the presence of Zuccagnia punctata extract and its major constituents: *C. albicans* ATCC 10231 suspension (100 µL of 10^6 cells/mL) was added to 100 µL of each sample (400 µg/mL for ZpE and 50 µg/mL for 2′,4′-dihydroxychalcone and 2′,4′ -dihydroxy-3′- methoxychalcone). All tubes were incubated at 37°C for 15 min with agitation. Following this limited exposure, all agents were removed by 2 dilution cycles (with sterile PBS) and centrifuged for 10 min at 5,000 ×*g*. Pellets were re-suspended in PBS. Yeast suspension (50 µL) was added to 200 µL of human serum and incubated at 37°C for 3 h. Then, the cells were washed 3 times and re-suspended in PBS. Microscopic images of treated and control cells were analyzed by counting the number of hyphae-forming cells [4d]. The means and standard deviations were calculated for 6–8 images comprising a total of more than 1,000 cells.

Phospholipase activity test: *C. albicans* F100 phospholipase activity in the presence of ZpE and isolated compounds was

determined by using the plate method [4e, 3f]. Yeast suspension (100 µL of 10^7 cell/mL) was added to 100 µL of PBS, DMSO or each agent, reaching a final concentration of 50 to 200 µg/mL for ZpE; 12.5 to 50 µg/mL for 2′,4′-dihydroxychalcone and 6.25 to 25 µg/mL for 2′,4′-dihydroxy-3′-methoxychalcone. Then, the tubes were incubated at 37°C for 60 min. Following this limited exposure, all agents were removed by 2 dilution cycles (with sterile PBS) and centrifuged for 10 min at 5,000 ×*g*. Then, 10 µL of the suspension (10^7 cell/mL) was spot-inoculated on the culture media (Sabouraud containing egg yolk) and incubated at 37°C for 5 days. On day 5, a precipitate halo around the colonies was measured and phospholipase activity was scored by the ratio of colony diameter to colony diameter plus precipitation zone.

Hemolytic activity test: A blood plate assay was used for the evaluation of hemolysin production [3f]. Seven mL sheep blood was added to 100 mL of Sabouraud dextrose agar supplemented with 3% glucose. *C. albicans* F100 suspension (100 µL of 10^7 cell/mL) was added to 100 µL of each agent reaching a final concentration of 50 to 200 µg/mL for ZpE; 12.5 to 50 µg/mL for 2′, 4′-dihydroxychalcone and 6.25 to 25 µg/mL for 2′,4′-dihydroxy-3′-methoxychalcone. Then, the tubes were incubated at 37°C for 60 min. Following this limited exposure, all agents were removed by 2 dilution cycles (with sterile PBS) and centrifuged for 10 min at 5,000 ×*g*. The pellet was re-suspended in 100 µL of PBS. Then, 10 µL of the suspension was spot-inoculated onto the plate media and incubated at 37°C for 48 h. A ring of lyses around the colonies was considered for hemolytic activity.

All experiments were repeated three times, each in triplicate.

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