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Antifungal Activity of *Zuccagnia punctata* Cav.: Evidence for the Mechanism of Action

Original Paper

Abstract

Petroleum ether and dichloromethane extracts of fruits, aerial parts and exudate of *Zuccagnia punctata* Cav. (Fabaceae) showed moderate antifungal activities against the yeasts *C. albicans*, *S. cerevisiae* and *C. neoformans* (MICs: 62.5 – 250 μg/mL) and very strong antifungal activities against the dermatophytes *M. gypseum*, *T. rubrum* and *T. mentagrophytes* (MICs: 8 – 16 μg/mL) thus supporting the ethnopharmacological use of this plant. Antifungal activity-directed fractionation of active extracts by using bioautography led to the isolation of 2′,4′-dihydroxy-3′-methoxychalcone (**1**) and 2′,4′-dihydroxychalcone (**2**) as the compounds responsible for the antifungal activity. Second-order studies included MIC_{80}, MIC_{50} and MFC of both chalcones in an extended panel of clinical isolates of the most sensitive fungi, and also comprised a series of targeted assays. They showed that the most active chalcone **2** is fungicidal rather than fungistatic, does not disrupt the fungal membranes up to 4 × MFC and does not act by inhibiting the fungal cell wall. So, 2′,4′-dihydroxychalcone would act by a different mechanism of action than the antifungal drugs in current clinical use, such as amphotericin B, azoles or echinocandins, and thus appears to be very promising as a novel antifungal agent.

Key words
Zuccagnia punctata · Fabaceae · chalcones · antifungal · bioautography · densitometry · mechanism of action.

Abbreviations

ADCM:	aerial part dichloromethane extract
AMeOH:	aerial part methanol extract
APE:	aerial part petrol extract
CC:	column chromatography
DMSO:	dimethyl sulfoxide
EDCM:	exudate dichloromethane extract
FDCM:	fruit dichloromethane extract
FMeOH:	fruit methanol extract
FPE:	fruit petrol extract
MFC:	minimum fungicidal concentration
MIC:	minimum inhibitory concentration
MOPS:	3-(*N*-morpholino)propanesulfonic acid
RDCM:	root dichloromethane extract
RMeOH:	root methanol extract
RPE:	root petrol extract
RPMI-1640:	Roswell Park Memorial Institute culture medium
SDA:	Sabouraud-dextrose agar

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Introduction

Over the past two decades, fungal infections have emerged as a major cause of morbidity and often of mortality in patients severely immunocompromised mainly by AIDS, organ transplantation or subjected to long treatments with antibacterials or invasive medical practices [1].

The limited efficacy of the available antifungal drugs [2] against the fungi causative of serious mycoses, mainly in AIDS hosts, added to the rapid emergence of resistant organisms which further diminishes therapeutic capabilities, have highlighted the need of new antifungal compounds which could constitute alternatives to the existing drugs. These structures should meet some minimal criteria, among which to be fungicidal rather than fungistatic [3] and to kill not only standardized strains but clinical isolates of the most clinical relevant fungal spp. [4] which are of major concern.

Zuccagnia punctata Cav. (Fabaceae), a monotypic sp. found at 900 – 2200 m altitude [5] in arid and semiarid areas of Argentina [6] is usually applied as antiseptic and wound healing poultices in the traditional medicine of San Juan province, Argentina [7]. Previous studies related to this ethnopharmacological use have shown that *Z. punctata* possesses antibacterial [8], [9] and also antifungal properties against phytopathogenic fungi [10], [11]. Regarding its chemical composition, flavanones, flavones, chalcones and caffeic acid esters have been previously isolated from its aerial parts [11], [12].

We report here the antifungal properties of extracts of *Z. punctata* and the bioassay-guided isolation of its most active compounds against a panel of the most prevalent fungal species in AIDS and other immunocompromised patients: *Candida albicans* and *Cryptococcus neoformans, Aspergillus flavus, A. fumigatus* and *A. niger* and those dermatophytes commonly isolated from skin infections: *Microsporum gypseum, Trichophyton mentagrophytes* and *T. rubrum* [3]. Active compounds were tested against an extended panel of clinical opportunistic pathogenic fungi at three endpoints: MIC_{100}, MIC_{80} and MIC_{50} and for all of them, the minimal fungicidal concentrations (MFC) were determined. In addition, some studies on the mode of action of the most active structures are reported too.

Materials and Methods

Plant material

Fruits (F), aerial parts (A) and roots (R) of *Z. punctata* were collected in December 2004 in Rodeo, Iglesia district, San Juan province (Argentina). The plant was identified by Dr. Gloria Barboza, National University of Córdoba, Argentina and a voucher specimen has been deposited at the herbarium of the Botanic Museum of Córdoba (CORD 1125).

Preparation of extracts

Air-dried ground F (100 g), A (300 g) and R (285 g) were extracted successively with petroleum ether (PE) (Ciccarelli; San Lorenzo, Argentina), dichloromethane (DCM) (Anedra; San Fernando, Argentina) and methanol (MeOH) (Anedra), under reflux; filtered and evaporated in a rotary evaporator Büchi R-205 (Flawil, Switzerland) to afford FPE (0.66 g, 0.66 % w/w respective of dry starting material), FDCM (3.45 g, 3.45 %), FMeOH (18.90 g, 18.90 %), APE (11.39 g, 3.79 %), ADCM (30 g, 10 %), AMeOH (67.8 g, 22.6 %), RPE (1.71 g, 0.60 %), RDCM (1.52 g, 0.53 %) and RMeOH (62.57 g, 22 %) extracts. In addition, a resinous exudate (E) was obtained by dipping the A of fresh plants (250 g) in DCM at 30 °C during 60 s. After filtration and evaporation under reduced pressure, 36 g (14.40 %) of a semi-solid yellow orange residue (EDCM) was obtained.

Bioassay-guided fractionation of EDCM

EDCM (35 g) was purified by column chromatography (CC) [500 g of silica gel 60 H (Merck, Buenos Aires) column length 108 cm, diameter 5 cm] and eluted with 1000 mL of a PE-EtOAc (Ciccarelli) gradient (100 % PE to 100 % EtOAc). After TLC comparison [TLC pre-coated silica gel 60 F_{254} plates from Merck, elution solvent EtOAc:PE (2 : 8)], detection under UV light and spraying with diphenylboric acid-ethanolamine complex in MeOH (Sigma-Aldrich, Corp.; St. Louis, MO, USA), fractions with similar TLC patterns were combined and tested for antifungal activity. Two fractions (8 and 9) out of the 14 obtained fractions, were submitted to further purification. Repeated CC [Sephadex LH-20 (Sigma-Aldrich), column length 46 cm, diameter 2 cm], eluted with 250 mL of PE:MeOH:$CHCl_3$ (2 : 1 : 1) of fraction 8, led to the isolation of 800 mg (0.32 % w/w referred to fresh starting material) of 2′,4′-dihydroxy-3′-methoxychalcone (**1**). The active fraction 9 (2.63g) was similarly purified to afford 600 mg of 2′,4′-dihydroxy-3′-methoxychalcone (**1**) (0.24 %) and 420 mg (0.16 %) of 2′,4′-dihydroxychalcone (**2**). Pure compounds were identified by comparison of their spectroscopic data, including ^{1}H- and ^{13}C-NMR and micromelting point with reported data [13], and were compared with authentic samples [11].

Antifungal evaluation

Microorganisms and media: Strains from the American Type Culture Collection (ATCC), Rockville, MD, USA, CEREMIC (C), Center of Reference in Micology (Rosario, Argentina) and Malbrán Institute (M) (Buenos Aires, Argentina) were used. The first panel was composed of the following strains: *Candida albicans* ATCC10231, *Saccharomyces cerevisiae* ATCC9763, *Cryptococcus neoformans* ATCC32264, *Aspergillus flavus* ATCC9170, *Aspergillus fumigatus* ATTC26934, *Aspergillus niger* ATCC9029, *Trichophyton rubrum* C110, *Trichophyton mentagrophytes* ATCC9972 and *Microsporum gypseum* C115. The second panel included strains of 7 *Candida albicans*, 7 non-*albicans Candida* spp. and 11 *Cryptococcus neoformans* sp. (voucher numbers in Table **2**). Strains were grown for 48 h on Sabouraud-chloramphenicol agar (Difco Lab.; Detroit, MI, USA), slants at 30 °C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid; Nepean, Canada) and subcultured every 15 days to prevent pleomorphic transformations. Inocula of cell or spore suspensions were obtained and quantified as reported previously [14], [15]. The inocula sizes are detailed below.

Antifungal susceptibility tests – bioautography: Warm agar-Sabouraud (Britania; Buenos Aires, Argentina) with 0.02 % phenol red (Sigma-Aldrich Chem Co) (1 mL/cm^2) containing a *C. neoformans* ATCC 32264 inoculum of $1 - 5 \times 10^5$ cells/mL, was distributed over developed TLC plates [PE:EtOAc (60 : 40)]. After solidification of the media, the TLC plates were put into sterile

petri dishes with covers [16] and incubated overnight at 28 °C. Subsequently, bioautograms were sprayed with an aqueous solution (1 mg/mL) of methylthiazolyltetrazolium chloride (MTT; Sigma) and incubated 2 h at 28 °C. Yellow inhibition zones appeared against a dark brown background [17].

Microbroth dilution assays: The broth microdilution assay was carried out following the standard conditions described by the CLSI (formerly NCCLS) [14] with sterile, 96-well, round-bottomed plates (Corning; New York, NY, USA) and standard RPMI-1640 (Sigma) buffered to pH 7 with MOPS as the test medium. Stock DMSO solutions of samples were two-fold diluted with RPMI-1640 in the wells to obtain a concentration range from 250 – 0.98 μg/mL and a final DMSO concentration < 2%. 100 μL of each inoculum suspension (at a final concentration $1-5\times10^4$ CFU/mL) were added to each well. A growth control (extract or compound-free, containing medium, inoculum and the same amount of DMSO used to dilute the samples), and a sterility control (sample, medium and sterile water instead of inoculum) were included for each isolate. Ketoconazole (Janssen Pharmaceutica; Beerse, Belgium), terbinafine (Novartis; Buenos Aires, Argentina), amphotericin B, itraconazole, and 5-fluorocytosine (the three drugs from Sigma), were used as positive controls.

Microtiter trays were incubated at 35 °C for yeasts and hialohyphomycetes and at 28 – 30 °C for dermatophyte strains in a moist, dark chamber. MICs were visually recorded at 48 h for yeasts, and at a time according to the control fungus growth, for the rest of fungi. MIC_{80} and MIC_{50} values (the lowest concentration of a compound that caused 80 or 50% reduction, respectively, of the growth control well) were determined with a VERSA Max microplate reader (Molecular Devices; Sunnyvale, CA, USA). MFC was determined by plating in duplicate 5 μL from each clear well of MIC determinations, onto a 150-mm RPMI-1640 agar plate buffered with MOPS (Remel; Lenexa, KS, USA). After 48 h at 30 °C, MFCs were determined as the lowest concentration of each compound showing no growth. Samples with MICs ≤ 250 μg/mL were considered active. All tests were performed in duplicate. The experiments were approved by the ethics committee of the Center of Reference in Micology (Rosario).

Sorbitol assay

Duplicate plates containing two-fold diluted compounds as to determine MIC were prepared. To one set but not to the other was added 0.8M sorbitol (Sigma). *C. albicans* ATCC10231 was used as the test fungal strain. MICs were determined for both series at 2 and 7 days [18].

Cellular leakage effect

Cells of *S. cerevisiae* after 18 h growth, washed with MOPS and resuspended in MOPS, were used to prepare the inoculum [19]. Eppendorf tubes (final volume 500 μL) containing inocula (5×10^4 cells/mL) and chalcone **2,** at 1×, 4×and 10×MFC were left from 2 to 24 h. At each period of time, Eppendorf tubes were centrifugued (5 min at 3000 rpm) and the supernatants (200μL) were drawn on the wells of a 96-well microplate and thoroughly mixed. The extractable 260 nm-absorbing materials were determined in duplicate with a Beckman Coulter DTX 880 Multimode Detector, considering as 100% release the absorbance produced by cells treated with 1.2 N $HClO_4$ at 100 °C, 30 min [20]. Results were the media of both measures.

Results and Discussion

The antifungal evaluation of *Z. punctata* extracts showed (Table **1**) that root extracts did not display any antifungal activity up to 250 μg/mL but, in contrast, PE and DCM extracts of fruits (FPE, FDCM), aerial parts (APE, ADCM), and exudate (EDCM) showed interesting antifungal activities against the yeasts *C. albicans*, *S. cerevisiae* and *C. neoformans* (MICs: 62.5 – 250 μg/mL) and a very strong antifungal activity against the dermatophytes *M. gypseum*, *T. rubrum* and *T. mentagrophytes* (MICs: 8 – 16 μg/mL). The ten extracts (including inactive and active ones) were developed on a TLC and a bioautography using *C. neoformans* was performed on it. This bioassay showed again (Fig. **1B**, available in color as Supporting Information) that only FPE, FDCM, APE, ADCM and EDCM (lanes 1, 2, 4, 5 and 7, respectively) possess antifungal activity, giving us the additional information that the activity of all active extracts would be due to two bands which can be clearly observed under 365 nm in Fig. **1A**. Both bands were quantified by densitometry using Gel-Pro 32 software (Media Cybernetics; Silverspring, MD, USA), showing that lane 7 (EDCM) possessed the highest amount of the sum of both bands. Based on these results, EDCM was submitted to bioactive-guided fractionation in order to isolate the compounds responsible for the activity.

Repeated bioassay-guided CC of EDCM led to only two active fractions (8 and 9) and, from them, 2′,4′-dihydroxy-3′-methoxychalcone (**1**) and 2′,4′-dihydroxychalcone (**2**) (Fig. **2**) were isolated as the compounds responsible for the activity. The rest of fractions, that might contain other non-polar phytochemicals, were inactive. The structure of each active compound was elucidated by ^{1}H- and ^{13}C-NMR and the spectra were compared with those reported in the literature [13]. Results of the antifungal activity of both chalcones in the same panel used for the extracts, are shown in Table **1**, rows 11 and 12. Chalcone **1** displayed a selective and strong activity against *M. gypseum, T. mentagrophytes* and *T. rubrum* with MIC = 8 μg/mL. Chalcone **2** showed a broader spectrum of activity inhibiting the yeasts *C. albicans*, *S. cerevisiae* and *C. neoformans* (MICs: 16 – 31.2 μg/mL) and dermatophytes (MICs: 4 μg/mL).

In order to gain insight into the possibility of these compounds to be clinically useful, chalcones **1** and **2** were also tested against clinical isolates of the most sensitive fungal species obtained from immunocompromised, infected patients. Compound **2** was tested against 14 *Candida* strains, seven *C. albicans* and seven non-*albicans Candida* spp. and 11 strains of *C. neoformans* by using three endpoints: MIC_{100}, MIC_{80} and MIC_{50} (the minimum concentration of compounds that inhibit 100, 80 and 50% of growth respectively). The application of less stringent endpoints such as MIC_{80} and MIC_{50} has been shown to consistently represent the *in vitro* activity of compounds [14] and many times provides a better correlation with other measurements of antifungal activity such as the MFC [21]. The selection of *Candida* and *Cryptococcus* strains for the second panel was due to the relevance of these fungi in the epidemiology of fungal infections. *C.*

Table **1** *In vitro* evaluation (MIC values in μg/mL) of the antifungal activity of different extracts of *Zuccagnia punctata* using the broth microdilution methods M-27A2 and M-38A recommended by CLSI (formerly NCCLS)

Extract	*Cr.n*	*C.a*	*S.c*	*A.fl*	*A.n*	*A.fu*	*M.g*	*T.r*	*T.m*
FPE	62.5	250	125	250	> 250	> 250	16	16	16
FDCM	62.5	62.5	62.5	250	> 250	> 250	16	8	8
FMeOH	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250
APE	125	125	62.5	> 250	> 250	> 250	16	16	16
ADCM	62.5	62.5	62.5	250	> 250	> 250	16	16	16
AMeOH	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250
EDCM	62.5	62.5	62.5	250	250	> 250	16	16	16
RPE	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250
RDCM	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250
RMeOH	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250
1	> 250	> 250	> 250	> 250	> 250	> 250	8	8	8
2	16.0	31.2	16	62.5	> 250	> 250	4	4	4
Amp	0.25	1	0.50	0.50	0.50	0.50	0.12	0.07	0.07
Ket.	0.25	0.50	0.50	0.12	0.50	0.25	0.04	0.02	0.02
Terb.							0.04	0.01	0.04

C.a = *Candida albicans* ATCC 10231; S.c = *Saccharomyces cerevisiae* ATCC 9763; Cr.n = *Cryptococcus neoformans* ATCC; 32264; A.fl = *Aspergillus flavus* ATCC 9170; A.n = *Aspergillus niger* ATCC 9029; A.fu = *Aspergillus fumigatus* ATCC 26934; M.g = *Microsporum gypseum* C 115; T.r = *Trichophyton rubrum* C113; T.m = *Trichophyton mentagrophytes* ATCC 9972; Amp = amphotericin B; Ket = ketoconazole; Terb = terbinafine.

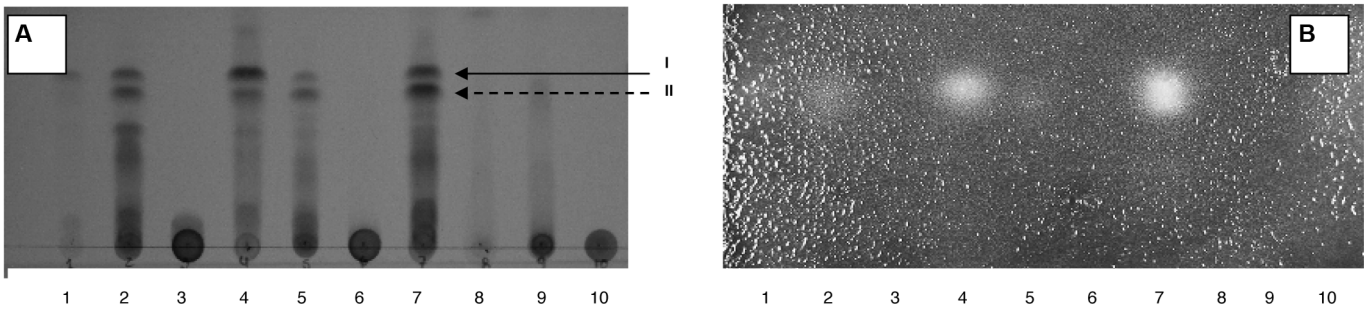
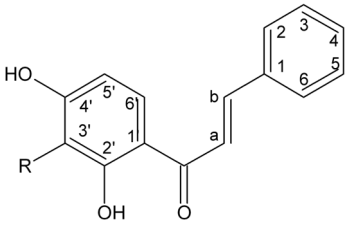


Fig. **1** **A** TLC of extracts of *Zuccagnia punctata Cav.* developed with PE:EtOAc (60:40). From fruits, lanes 1: FPE (petroleum ether extract); 2: FDCM (dichloromethane extract); 3: FMeOH (methanol extract). From aerial parts, lanes 4: APE; 5: ADCM; 6: AMeOH, 7: exudate (EDCM) (obtained by dipping in DCM). From roots: lanes 8: RPE; 9: RDCM; 10: RMeOH. Continuous arrow indicates Band I. Dotted arrow indicates Band II. **B** Bioautography of the TLC showed in **A**, with *Cryptococcus neoformans* ATCC 32264 (For a color version see Figure **S1** in the Supporting Information).



1, R = OCH_3
2, R = H

Fig. **2** Structure of the antifungal chalcones isolated from *Zuccagnia punctata* Cav.

albicans was in the past, associated with invasive mycoses but at present, non-*albicans Candida* spp. (*C. tropicalis*, *C. glabrata*, *C. parapsilopsis*, *C. krusei* and others) comprise more than half of the isolates of candidosis in immunocompromised hosts [22]. In turn, *C. neoformans* remains an important life-threatening complication, particularly for patients who have undergone transplantation of solid organs, and therefore, new compounds acting against this fungus are highly welcome [23].

Our results showed (Table **2**) that MIC values of compound **2** were particularly low against *C. neoformans* (MICs between 1.9 and 31.2 μg/mL). In addition, it was fungicidal against all strains tested, with MFC values ranging from 31.2 to 62.5 μg/mL.

Chalcones **1** and **2** displayed very strong activities against all clinical strains of *T. mentagrophytes* and *T. rubrum* (Table **3**) with MIC values between 1.9 and 15.6 μg/mL and MFCs between 1.9 and 7.8 μg/mL. These strong activities are particularly interesting from the point of view of the ethnopharmacological use of *Z. punctata*, since these fungi are responsible for approximately 80–93% of chronic and recurrent dermatophyte infections in human beings. They are the etiological agent of *Tinea unguium* (producer of invasive nail infections), *Tinea manuum* (palmar and interdigital areas of the hand infections) and *Tinea pedis*

Table **2** Minimal inhibitory concentrations (MIC_{100}, MIC_{80} and MIC_{50}) and minimal fungicidal concentrations (MFC) of 2′,4′-dihydroxy-3′-methoxychalcone (**1**) and 2′,4′-dihydroxychalcone (**2**) from *Zuccagnia punctata* against clinical isolates of *Candida* genus and *Cryptococcus neoformans*

Strain	*Voucher specimen*	*2*				*AMP*	*Ket*	*Itz*	*5FC*
		MIC_{100}	MIC_{80}	MIC_{50}	*MFC*	MIC_{100}	MIC_{100}	MIC_{100}	MIC_{100}
C. albicans	ATCC 10231	31.2	15.6	7.8	62.5	1.0	0.5	–	–
C. albicans	C 125 – 2000	31.2	31.2	31.2	31.2	0.78	6.25	–	–
C. albicans	C 126 – 2000	31.2	31.2	31.2	31.2	1.56	1.56	–	–
C. albicans	C 127 – 2000	31.2	31.2	31.2	31.2	0.78	6.25	–	–
C. albicans	C 128 – 2000	31.2	31.2	31.2	31.2	2.56	6.25	–	–
C. albicans	C 129 – 2000	31.2	31.2	31.2	31.2	0.78	12.5	–	–
C. albicans	C 130 – 2000	31.2	31.2	31.2	62.5	0.39	6.25	–	–
C. glabrata	C 115 – 2000	31.2	31.2	31.2	31.2	0.39	1.56	–	–
C. parapsilopsis	C 124 – 2000	31.2	31.2	31.2	125	0.78	0.78	–	–
C. lusitaniae	C 131 – 2000	31.2	31.2	31.2	31.2	0.39	25	–	–
C. colliculosa	C 122 – 2000	31.2	31.2	31.2	31.2	0.36	0.78	–	–
C. krusei	C 117 – 2000	31.2	31.2	31.2	31.2	0.39	50	–	–
C. kefyr	C 123 – 2000	31.2	31.2	31.2	125	0.78	0.78	–	–
C. tropicalis	C 131 – 1997	31.2	15.6	15.6	125	0.5	1.25		
C. neoformans	ATCC 32264	15.6	15.6	15.6	31.2	0.25	0.25	0.15	
C. neoformans	IM 983040	31.2	15.6	15.6	31.2	0.13	–	< 0.015	7.8
C. neoformans	IM 972724	15.6	7.8	7.8	31.2	0.06	–	0.25	3.9
C. neoformans	IM 042074	31.2	15.6	7.8	31.2	0.25	–	< 0.015	3.9
C. neoformans	IM 983036	31.2	15.6	3.9	31.2	0.13	–	< 0.015	7.8
C. neoformans	IM 00319	31.2	15.6	3.9	31.2	0.25	–	< 0.015	n.t.
C. neoformans	IM 972751	31.2	15.6	7.8	31.2	0.25	–	< 0.015	15.6
C. neoformans	IM 031631	31.2	15.6	3.9	31.2	0.13	–	0.25	7.8
C. neoformans	IM 031706	31.2	31.2	3.9	31.2	0.25	–	0.50	7.8
C. neoformans	IM 961951	31.2	13.2	1.9	62.5	0.06	–	< 0.015	3.9
C. neoformans	IM 052470	31.2	15.6	3.9	62.5	0.50	–	< 0.015	7.8

MIC_{100}, MIC_{80} and MIC_{50} = concentrations of a compound that caused 100, 80 or 50% reduction of the growth respectively.
Within Voucher specimen column: ATCC = American Type Culture Collection (Rockville, MD, USA); C = Center of Mycological Reference (Rosario, Argentina); IM = Malbrán Institute (Buenos Aires, Argentina); n.t. = not tested.
Amp = amphotericin B; Ket = ketoconazole; Itz = itraconazole; 5FC = 5-fluorocitosine.

(athlete's foot), the last one being the most prevalent fungal infection in developed countries [24].

Some targeted-assays were performed on the most active chalcone **2** in order to probe its mechanism of action. Considering our previous findings [25] that non-phenolic synthetic chalcones were inhibitors of the synthesis of polymers of the fungal cell wall, we tested the most active chalcone **2** in the whole-cell sorbitol protection assay. In this screen, sorbitol-protected cells can grow in the presence of an inhibitor of the fungal cell wall, while they are inhibited in its absence. This effect is detected by the great difference observed between the MICs obtained with and without sorbitol. Chalcone **2** appeared not to act by this mechanism, since the MICs in both experiments were identical (= 31.2 μg/mL) [18].

Considering the lipophilic character of chalcones and their possibility of interacting with fungal membranes, we studied the effect of 1×, 4× and 10× MFC of chalcone **2** on the irreversible damage to the fungal membrane by measuring the leakage of 260 nm-absorbing intracellular materials released to the medium [20] at different time intervals from 2 to 24 h. Results showed (Fig. **3**) that chalcone **2** produced a time-dependent damage to the fungal membrane only at 10× MFC (11.33 to 97.45% at 2 and 24 h, respectively). It did not produce any damage either at 1× or at 4× MFC. Perchloric acid was considered to produce 100% cellular leakage.

In conclusion, the antifungal spectra, potency and fungicidal properties of 2′,4′-dihydroxy-3′-methoxychalcone (**1**) and 2′,4′-dihydroxychalcone (**2**) isolated from *Z. punctata* evidenced that they could have potential as antifungal agents for human beings. Regarding the mode of action, the results reported here show that both chalcones have a different mode of action than the polyene and azole class existing drugs, since chalcone **2** did not disrupt membranes at concentrations such as 1× and 4× MFC as amphotericin B does [19] and both chalcones are fungicidal and not fungistatic, an important difference with azoles [3].

Since many known biochemical and molecular targets for antifungal compounds exist and many efforts are being directed toward the identification and development of new ones, it is not possible to suggest a mechanism of action for chalcone **2**. Never-

Table **3** Minimal inhibitory concentrations (MIC_{100}) and minimal fungicidal concentrations (MFC) of 2′,4′-dihydroxy-3′-methoxy-chalcone (**1**) and 2′,4′-dihydroxychalcone (**2**) from *Zuccagnia punctata* against clinical isolates of *Trichophyton rubrum* and *T. mentagrophytes*

Strain	*Voucher specimen*	*1*		*2*		*Terb MIC_{100}*
		MIC_{100}	*MFC*	*MIC_{100}*	*MFC*	
T. rubrum	C 113	7.8	15.6	3.9	7.8	0.06
T. rubrum	C 110	3.9	7.8	1.9	7.8	0.06
T. rubrum	C 135	3.9	7.8	3.9	7.8	0.06
T. rubrum	C 136	3.9	7.8	3.9	7.8	0.06
T. rubrum	C 137	3.9	3.9	1.9	3.9	0.06
T. rubrum	C 139	1.9	7.8	1.9	7.8	0.12
T. rubrum	C 140	1.9	3.9	1.9	1.9	0.03
T. rubrum	C 133	3.9	7.8	3.9	7.8	0.06
T. rubrum	C 141	3.9	7.8	3.9	7.8	0.06
T. rubrum	C 134	1.9	3.9	3.9	7.8	0.12
T. rubrum	C 138	3.9	7.8	1.9	3.9	0.03
T mentagrophytes	ATCC 9972	7.8	15.6	3.9	3.9	
T mentagrophytes	C 108	3.9	3.9	1.9	7.8	0.06
T mentagrophytes	C 364	3.9	15.6	1.9	7.8	0.06
T mentagrophytes	C 539	3.9	15.6	3.9	7.8	0.06
T mentagrophytes	C 738	1.9	1.9	1.9	3.9	0.06
T mentagrophytes	C 943	3.9	7.8	1.9	7.8	0.06
T mentagrophytes	C 726	3.9	7.8	3.9	7.8	0.12
T mentagrophytes	C 189	3.9	7.8	1.9	3.9	0.03

MIC100 = concentration of compound that inhibits 100% of the growth respectively; MFC = minimum fungicidal concentration; Terb = Terbinafine.

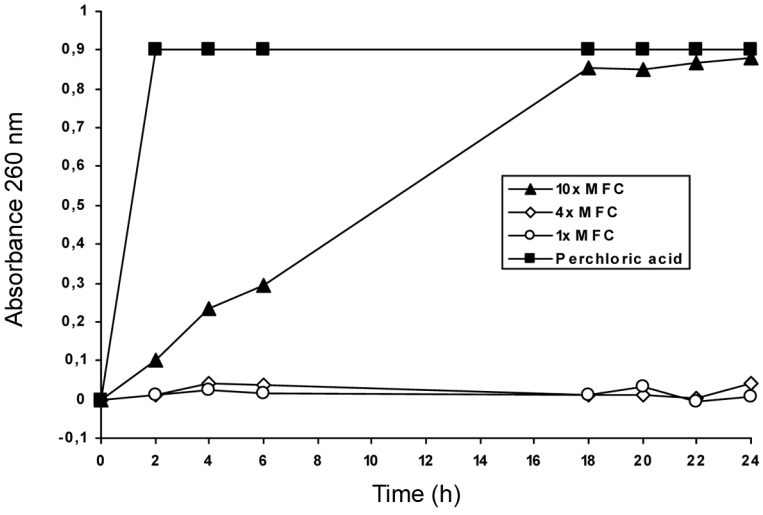


Fig. **3** Release of 260 nm UV-absorbing materials from cells of *Saccharomyces cerevisiae* incubated with 1×, 4× and 10×MFC (2 to 24 h) by 2,4-dihydroxychalcone (2) isolated from *Zuccagnia punctata* Cav. aerial parts DCM exudate.

theless, their lipophilic character added to the fact that they do not produce any damage in the membrane leads to the conclusion that they may traverse the membranes and interact with intracellular targets such as pathways of the intermediary metabolism (nucleic acid, amino acid or polyamine synthesis), microtubule formation or any other process that takes place inside the fungal cells [26], [27].

These results give support to the ethnopharmacological use of *Z. punctata* as antiseptic and wound-healing poultices in the traditional medicine of San Juan province, Argentina showing that fruits, aerial parts and exudate (which are the plant components that are most traditionally used) display antifungal activities mainly against skin-infecting fungi such as *T. rubrum* and *T. mentagrophytes*. The antifungal activity showed also against clinically important yeasts (*Candida* spp and *C. neoformans*), producers of systemic fungal infections in immunocompromised hosts, could be an important finding for future developments.

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References

[1] Kontoyiannis D, Mantadakis E, Samonis G. Systemic mycoses in the immunocompromised host: an update in antifungal therapy. J Hosp Infect 2003; 53: 243 – 58.

[2] Patterson T. Advances and challenges in management of invasive mycoses. Lancet 2005; 366: 1013 – 5.

[3] Ablordeppey S, Fan P, Ablordeppey J, Mardenborough L. Systemic antifungal agents against AIDS-related opportunistic infections: current status and emerging drugs in development. Curr Med Chem 1999; 6: 1151 – 95.

[4] Odds F, Motyl M, Andrade R, Bille J, Cantón E, Cuenca-Estrella M et al. Interlaboratory comparison of results of susceptibility testing with caspofungin against *Candida* and *Aspergillus* species. J Clin Microbiol 2004; 42: 3475 – 82.

[5] Kiesling R. Flora de San Juan, Vol. 1. Buenos Aires: Vazquez Manzini; 1994 : 286.

[6] Burkart A. Las leguminosas argentinas silvestres y cultivadas.; 2a Ed Buenos Aires: ACME Agency; 1952: 184 – 5

[7] Del Vitto L, Petenatti E, Petenatti M. Recursos herbolarios de San Luis (República Argentina). Primera parte: plantas nativas. Multequina 1997; 6: 49 – 66.

[8] Alcaraz LE, Blanco SE, Puig ON, Tomás F, Ferreti FH. Antibacterial activity of flavonoids against methicillin-resistant *Staphylococcus aureus* strains. J Theor Biol 2000; 205: 231 – 40.

[9] Zampini IC, Vattuone MA, Isla MI. Antibacterial activity of *Zuccagnia punctata* Cav. ethanolic extracts. J Ethnopharmacol 2005; 102: 450 – 6.

[10] Quiroga EN, Sampietro AR, Vattuone MA. Screening for antifungal activities of selected medicinal plants. J Ethnopharmacol 2001; 74: 89 – 96.

[11] Svetaz L, Tapia A, López SN, Furlán RLE, Petenatti E, Pioli R et al. Antifungal chalcones and new caffeic acid esters from *Zuccagnia punctata* acting against soybean infecting fungi. J Agric Food Chem 2004; 52: 3297 – 300.

[12] Pederiva R, Giordano O. 3,7-Dihydroxy-8-methoxyflavone from *Zuccagnia punctata*. Phytochemistry 1984; 23: 1340 – 1.

[13] Pederiva R, Kavka J, D'Arcangelo A. Chalconas y flavanonas aisladas de *Larrea nítida* Cav. An Asoc Quím Argentina 1975; 63: 85 – 90.

[14] NCCLS. National Committee for Clinical and Laboratory Standards. Method M-27-A2 for yeasts. 2002: Vol. 22(15); 1 – 29. NCCLS and method M-38A for filamentous fungi. 2002; 22(16): 1 – 27.

[15] Wright LR, Scott EM, Gorman SP. The sensitivity of mycelium, arthrospores, and microconidia of *Trichophyton mentagrophytes* to imidazoles determined by *in vitro* tests. J Antimicrob Chemother 1983; 12: 317 – 27.

[16] Rahalison L, Hamburger M, Hostettmann K, Monod M, Frenk E. A bioautographic agar overlay method for the detection of antifungal compounds from higher plants. Phytochem Anal 1991; 2: 199 – 203.

[17] Saxena G, Farmer S, Towers GHN, Hancock REW. Use of specific dyes in the detection of antimicrobial compounds from crude plant extracts using a thin layer chromatography agar overlay technique. Phytochem Anal 1995; 6: 125 – 9.

[18] Frost D, Brandt K, Cugier D, Goldman R. A whole cell *Candida albicans* assay for the detection of inhibitors towards fungal cell wall synthesis and assembly. J Antibiot 1995; 48: 306 – 10.

[19] Carson CF, Mee BJ, Riley TV. Mechanism of action of *Melaleuca alternifolia* (Tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage and salt tolerance assays and electron microscopy. Antimicrob Agents Chemother 2002; 46: 1914 – 20.

[20] Lunde C, Kubo I. Effect of polygodial on the mitochondrial ATPase of *Saccharomyces cerevisiae*. Antimicrob Agents Chemother 2000; 44: 1943 – 53.

[21] Ernst E, Roling E, Petzold R, Keele D, Klepser M. *In vitro* activity of micafungin (FK-463) against *Candida* spp.: microdilution, time-kill, and postantifungal-effect studies. Antimicrob Agents Chemother 2002; 46: 3846 – 53.

[22] Almirante B, Rodríguez D, Park B, Cuenca-Estrella M, Planes A, Amela L et al. Epidemiology and predictors of mortality in cases of *Candida* bloodstream infection: results from population-based surveillance, Barcelona, Spain, 2002 – 2003. J Clin Microbiol 2005; 43: 1829 – 35.

[23] Singh N. Treatment of opportunistic mycoses: how long is long enough? Lancet Infect Dis 2003; 3: 703 – 8.

[24] Weitzmann I, Summerbell R. The dermatophytes. Clin Microb Rev 1995; 8: 240 – 59.

[25] López S, Castelli MV, Zacchino SA, Domínguez J, Lobo G, Charris J et al. *In vitro* antifungal evaluation and structure-activity relationships of a new series of chalcone derivatives and synthetic analogues, with inhibitory properties against polymers of the fungal cell wall. Bioorg Med Chem 2001; 9: 1999 – 2013.

[26] Groll A, De Lucca A, Walsh T. Emerging targets for the development of novel antifungal therapeutics. Trends Microbiol 1998; 6: 117 – 23.

[27] Di Domenico B. Novel antifungal drugs. Curr Opin Microbiol 1999; 2: 509 – 15.