Antifungal Activity and Studies on Mode of Action of Novel Xanthoxyline-Derived Chalcones

Paula Boeck[a], Paulo C. Leal[a], Rosendo A. Yunes[a], Valdir Cechinel Filho[b], Silvia López[c], Maximiliano Sortino[c], Andrea Escalante[c], Ricardo L. E. Furlán[c], and Susana Zacchino[c]

[a] Departamento de Química, Universidade Federal de Santa Catarina, Florianópolis, Brasil
[b] Núcleo de Investigações Químico-Farmacêuticas, Universidade do Vale do Itajaí, Itajaí, Santa Catarina, Brasil
[c] Farmacognosia, Facultad Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

Chalcones and chalcone-like compounds, most of them new ones, prepared by base-catalyzed condensation of appropriate aldehydes and xanthoxyline, were tested for antifungal properties against a panel of yeasts, hialohyphomycetes as well as dermatophytes with the agar dilution assay. Results indicate that neither the sole presence of a "xanthoxyline-like" substitution pattern nor a 2′-OH substituent on ring A are sufficient for these compounds to have antifungal properties. The chalcone 3-(2-chlorophenyl)-1-(2′-hydroxy-4′,6′-dimethoxyphenyl)prop-2-en-1-one, with a Cl atom in the ortho position of benzene ring B showed the best antifungal activity against standardized strains of *Trichophyton rubrum* (MIC = 12.5 μg/mL) and inhibited all of the ten clinical isolates of *T. rubrum* tested (MIC at which 50% [MIC_{50}] and 90% [MIC_{90}] of the isolates were inhibited = 12.5 and 25 μg/mL). Regarding its mode of action, the *Neurospora crassa* assay showed a blotchy appearance in the inhibition halo produced by this chalcone, strongly suggesting that it could act by inhibiting the fungal cell wall. This chalcone seems to be an hyphal malformation inducer, since a clear curling of the hypha was observed in this hazy zone at a magnification of × 400. This work strongly contributes to the knowledge of the antifungal properties of hydroxy-chalcones.

Keywords: Antifungal drugs; Dermatophytes; Chalcones; Fungal cell-wall inhibitors

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Introduction

Chalcones represent an important group of natural compounds with a variety of biological actions including antifungal activity [1−5].

Various reports about antifungal chalcones suggest that the presence of -OH groups on the aromatic rings plays a significant role, though it is not clear yet how the exact location of those groups correlates with the observed activity [1]. So, the evaluation of a series of chalcones against different *Candida* species suggested that a OH in the 2-position is required for antifungal activity [6]. However, a further report of same authors [7] showed that a 2-hydroxy group does not always confer antifungal activity. Another study concluded that among a series of chalcones tested, a 4′-hydroxychalcone was the most active [1]. However, two 2′,4′-diOH chalcones isolated from *Myrica serrata* showed marginal activities when tested against *Cladosporium cucumerinum* [8]. The majority of the studies about the antifungal properties of hydroxy-chalcones reported to date have focused on their activity against *Cladosporium cucumerinum* or yeasts like *Cryptococcus neoformans* and *Candida* spp. [8−13].

Additional studies of the antifungal properties of hydroxy-chalcone derivatives seem to be necessary, and will provide information on the structure-activity relationships of this kind of compounds.

As part of our continuing work on the isolation of bioactive compounds from South-American plants, we previously isolated 2′-OH- 4′,6′-diOMe acetophenone (xanthoxyline) in high yield from leaves and stems of *Sebastiania schottiana* [14], and then, we easily synthesized it by methylation of phloroacetophenone [15], rendering enough available amounts of this compound for its further use.

We describe here the synthesis of a series of chalcones and analogues possessing a unique OH in position 2′, by the base-catalysed condensation of appropriate aldehydes with xanthoxyline **1** as the starter ketone.

Correspondence: Susana A. Zacchino, Farmacognosia, Facultad Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531-(2000) Rosario, Argentina. Phone: +54 341 4375315, Fax: +54 341 4375315, e-mail: szaabgil@citynet.net.ar

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These xanthoxyline derived-chalcones were tested against a panel of 12 human pathogenic opportunistic fungi, including 5 dermatophyte species in order to have a view about their spectra of antifungal action.

In addition, considering that some chalcones have showed to be inhibitors of the fungal cell-wall [16], the most active compound was evaluated with the whole-cell *Neurospora crassa* assay [17, 18], an agar diffusion method which allows a macroscopical detection of fungal cell wall inhibitors. Since fungal but not mammalian cells possess a wall, its inhibition represents an ideal mode of action for antifungal drugs. The malformations of *N. crassa* hypha produced by this chalcone are reported too.

Results and discussion

Chemistry

Chalcones and chalcone-like compounds were prepared by base-catalysed condensation of the appropriate aldehyde and either xanthoxyline **1** or 3-bromo xanthoxyline **2** (Scheme 1). After purification, chalcones were obtained in 16–89% yields. Compounds **3**, **6**, **7**, **8**, **11**, and **17** have been reported in the literature [15, 19–22]. Structures **4, 5, 9, 10, 12-16, 18** and **19** are new compounds.

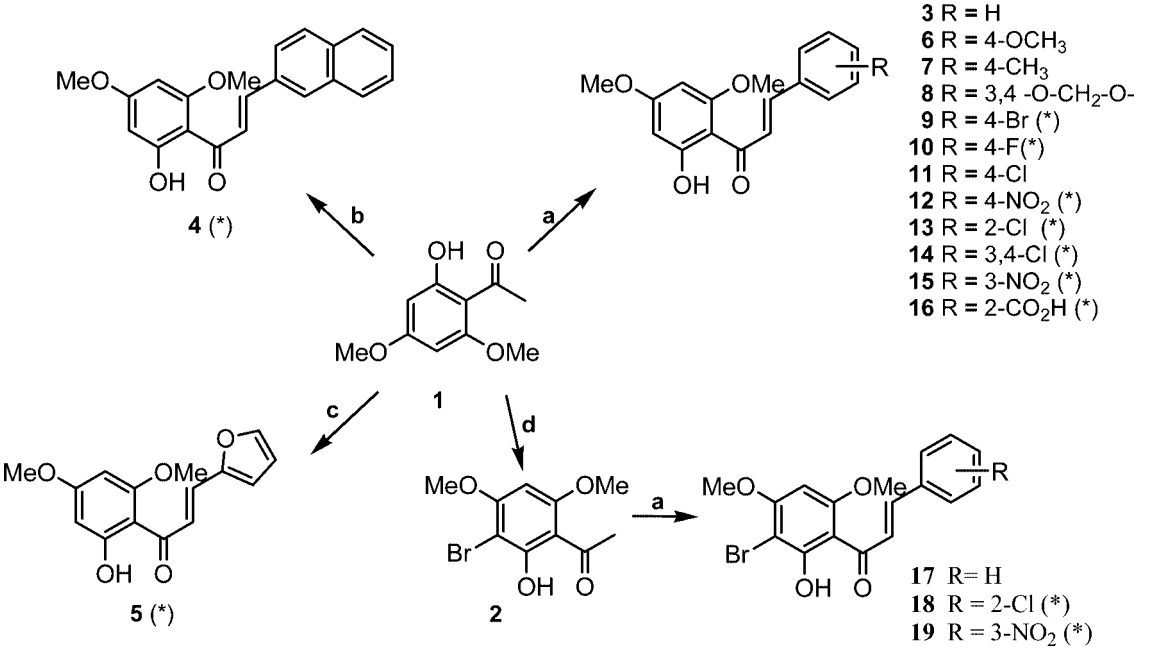
Structures of the products were established using IR, UV and NMR spectroscopies, and elemental analysis. Inspection of the ^{1}H NMR spectra suggested that chalcones were geometrically pure (E isomer, $J_{H\alpha\text{-}H\beta}$ = 15–16 Hz), except for compounds **6, 8** and **12** (Tables 1 and 2 show the spectroscopic data of novel compounds).

Antifungal evaluation

The antifungal properties of compounds **3**–**19** were evaluated using the agar dilution method against a panel of twelve fungal species: three human opportunistic pathogenic yeasts, three hialohyphomycetes and five dermatophytes. To carry out the antifungal evaluation, concentrations of compounds up to 250 μg/mL were incorporated to growth media according to reported procedures [23]. Amphotericin B, terbinafine and ketoconazole were used as positive controls.

In order to evaluate the antifungal activity of these chalcones and analogues, the effect of different structural modifications were considered: (a) replacement of the benzene ring B by alternative aromatic systems such as naphthalene or furane; (b) variations of the substitution pattern of the benzene ring B and (c) variations on the ring A by substitution with bromine in the 3′-position.

Results showed that none of the compounds tested was effective against the yeasts *C. albicans*, *S. cerevisiae* or *C. neoformans* nor against the filamentous fungi *A. niger*, *A. fumigatus* or *A. flavus*. In contrast, different antifungal activities were observed for the compounds of the series against dermatophytes (Table 3).



Scheme 1. Synthetic route to xanthoxyline-derived chalcones and analogues. Reagents: a) CHO-Ph-R, NaOH/EtOH; b) 2–naphthaldehyde, NaOH/EtOH; c) 2-furaldehyde, NaOH/EtOH; d) Br_2/ AcOH. (*) New compounds.

Table 1. ^{1}H NMR Data (δ in ppm, $CDCl_3$) of compounds**: 4**, **5**, **9**, **10**, **12**–**16**, **18**, **19**†.

	4	5	9	10	12	13	14	15	16	18	19
H3·	5.99 d; J = 2.2 Hz	5.99 d; J = 2.2 Hz	5.96 d; J= 2.2 Hz	6.91 d; J = 2.2 Hz	6.17 d; J= 2.2 Hz	6.0 d; J = 2.2 Hz	5.97 d; J = 2.2Hz	5.98 d; J= 2.2Hz	6.09 d; J = 2.2Hz	–	–
H5·	6.13 d; J = 2.2 Hz	6.03– d; J = 2.2 Hz	6.11 d; J = 2.2 Hz	6.08 d; J = 2.2 Hz	6.42 d; J = 2.2 Hz	6.15 d; J = 2.2 Hz	6.14 d; J = 2.2 Hz	6.12 d; J = 2.2Hz	6.12 d; J = 2.2Hz	6.05 s	6.10 s
Hα	7.47–7.99 m	7.57 d; J = 15.37 Hz	7.68 d; J = 15.51 Hz	7.01–7.42 m	7.98 d; J = 15.51 Hz	7.91 d; J = 15.67 Hz	7.62 d; J = 15.60 Hz	7.74 d; J = 15.68 Hz	7.81 d; J = 15.49 Hz	7.81 d; J = 15.58 Hz	8.48–8.60 m
Hβ	7.47–7.99 m	7.79 d; J = 15.37 Hz	7.87 d; J = 15.51 Hz	7.01–7.42 m	8.26 d; J = 15.51 Hz	8.19 d; J = 15.67 Hz	7.85 d; J = 15.60 Hz	7.98 d; J = 15.68 Hz	8.44 d; J = 15.49 Hz	8.17 d; J = 15.58 Hz	8.48–8.60 m
H1	7.47–7.99 m	–	–	–	–	–	–	–	–	–	–
H2	–	–	7.47–7.51 m	7.01–7.42 m	6.75 d J = 8.85 Hz	–	7.43–7.51 m	–	–	–	8.48–8.60 m
H3	7.47–7.99 m	6.50 d; J = 8.4 Hz	7.47–7.51 m	7.01–7.42 m	7.25 d J = 8.85 Hz	7.30–7.75 m	–	8.46 s	7.96 d; J = 7.87 Hz	7.28–7.70 m	
H4	7.47–7.99 m	6.67 t; J = 8.4 Hz	–	–	–	7.30–7.75 m	–	8.22 d; J = 7.75 Hz	7.47–7.81 m	7.28–7.70 m	8.48–8.60 m
H5	7.47–7.99 m	7.51 d; J = 8.4 Hz	7.47–7.51 m	7.01–7.42 m	7.25 d J = 8.85 Hz	7.30–7.75 m	7.43–7.51 m	7.84–7.88 m	7.47–7.81 m	7.28–7.70 m	7.43–7.51 m
H6	7.47–7.99 m	–	7.47–7.51 m	7.01–7.42 m	6.75 d J = 8.85 Hz	7.30–7.75 m	7.43–7.51 m	7.84–7.88 m	7.47–7.81 m	7.28–7.70 m	8.48–8.60 m
H7	7.47–7.99 m	–	–	–	–	–	–	–	–	–	–
H8	7.47–7.99 m	–	–	–	–	–	–	–	–	–	–
OCH_3	3.95 s	3.91 s	3.91 s	3.83 s	3.97 s	3.94 s	3.92 s	3.94 s	3.93 s	3.98 s	4.03 s
OCH_3	3.85 s	3.83 s	3.84 s	3.79 s	3.93 s	3.88 s	3.84 s	3.85 s	3.84 s	3.98 s	4.01 s

† Measured at 200 MHz. J = Coupling constants.

Table 2. ^{13}C NMR Data (δ in ppm, $CDCl_3$) of compounds **4**, **5**, **9**, **10**, **12**–**16**, **18** and **19**†.

	4	5	9	10	12	13	14	15	16	18	19
C=O	193.25	192.74	193.00	193.05	192.21	193.01	191.96	191.78	199.66	193.25	199.99
C1′	107.07	106.99	106.99	106.91	107.21	106.99	106.22	106.20	106.00	106.00	107.02
C2′	163.20	163.21	163.15	163.12	160.05	163.17	162.45	162.45	162.43	162.90	162.39
C3′	94.50	94.41	94.10	94.45	94.41	94.49	93.81	93.84	93.57	92.80	92.12
C4′	169.14	169.09	169.13	169.08	169.51	169.14	168.47	168.46	167.15	163.93	163.14
C5′	91.99	91.88	92.03	91.94	89.52	91.99	91.37	91.37	90.60	87.80	87.16
C6′	166.93	166.85	167.07	166.93	163.08	167.08	166.53	166.65	167.15	163.92	163.09
Cα	124.41	129.63	128.34	128.12	123.82	127.65	129.22	129.78	122.43	127.72	124.81
Cβ	143.17	145.32	141.47	141.69	139.05	138.55	139.23	138.68	149.67	139.49	140.20
C1	128.44	–	135.21	132.48	147.22	136.05	135.69	137.43	133.94	136.21	134.85
C2	134.86	152.93	130.36	130.73	131.24	134.53	130.83	122.08	128.93	134.33	121.00
C3	124.41	113.20	132.79	116.43	122.44	130.94	133.75	148.71	129.00	131.06	148.30
C4	129.29	116.11	124.88	161.94	149.55	131.35	133.12	123.99	125.50	131.62	122.55
C5	128.44	145.32	132.79	116.43	122.44	128.49	132.04	130.51	132.00	128.60	130.40
C6	127.35	–	130.36	130.73	131.24	130.73	129.67	134.01	133.94	130.22	131.00
C7	127.83	–	–	–	–	–	–	–	–	–	–
C8	129.29	–	–	–	–	–	–	–	–	–	–
C9	134.11	–	–	–	–	–	–	–	–	–	–
C10	133.77	–	–	–	–	–	–	–	–	–	–
OCH_3	56.60	56.45	56.57	56.52	56.26	56.56	55.94	55.82	55.44	57.08	56.76
OCH_3	56.30	56.24	56.30	56.26	56.18	56.30	55.64	55.56	55.30	56.85	56.18
COOH	–	–	–	–	–	–	–	–	172.00	–	–

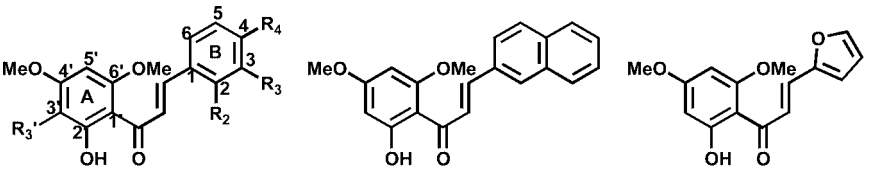
† Measured at 50 MHz (compounds **4**, **5**, **9**, **10**, **13**–**16** and **18**) and at 100 MHz (compounds **12** and **19**).

The lack of activity of chalcones **3**, **6**–**8**, **14** and **19** indicates that the presence of a 2′-OH in the chalcone is not enough for these compounds to have antifungal properties.

Regarding the influence of different B-ring systems alternative to benzene B, chalcone **4** with naphthalene as B-ring was devoid of antifungal activity. When B-ring was a furane (**5**), the structure possessed marginal activity, this result differing from previous reports for an analogous structure [1].

Concerning the influence of substituents on the benzene ring B, some interesting conclusions could be extracted: compounds holding electron withdrawing groups (EWGs)

Table 3. MIC values (μg/mL) of xanthoxyline-derived chalcones and analogues acting against dermatophytes†.



Cp	Type	$R_{3'}$	R_2	R_3	R_4	*E. f.*	*T. m.*	*T.r.*	*M.c.*	*M.g.*
3	A	H	H	H	H	>250	>250	>250	>250	>250
4	B	–	–	–	–	>250	>250	>250	>250	>250
5	C	–	–	–	–	125	250	250	250	>250
6	A	H	H	H	OCH_3	>250	>250	>250	>250	>250
7	A	H	H	H	CH_3	250	250	250	250	250
8	A	H	H	$O-CH_2-O$		>250	>250	>250	>250	>250
9	A	H	H	H	Br	50	50	50	50	250
10	A	H	H	H	F	62.5	62.5	62.5	100	>250
11	A	H	H	H	Cl	100	100	125	125	>250
12	A	H	H	H	NO_2	62.5	62.5	100	250	>250
13	A	H	Cl	H	H	100	50	**12.5**	100	250
14	A	H	H	Cl	Cl	>250	>250	250	>250	>250
15	A	H	H	NO_2	H	250	>250	125	>250	>250
16	A	H	COOH	H	H	125	250	250	125	250
17	A	Br	H	H	H	25	50	25	50	250
18	A	Br	Cl	H	H	25	50	50	50	250
19	A	Br	H	NO_2	H	>250	>250	>250	>250	>250
Amp						6.25	0.3	25	>50	6.25
Ket						12.5	25	15	15	6.25
Terb						0.004	0.04	0.01	0.01	0.04

† Abbreviations: *M.c.* – *Microsporum canis* C 112, *M.g.* – *Microsporum gypseum* C 115, *T.r.* – *Trichophyton rubrum* C113, *T.m.* – *Trichophyton mentagrophytes* ATCC 9972; *E.f.* –*Epidermophyton floccosum* C 114. Amp – Amphotericin, Ket – Ketoconazole, Terb – Terbinafine.

such as bromine, fluorine, chlorine, or a nitro group in the 4-position (compounds **9**–**12**) improved the antifungal properties compared with the non ring B-substituted chalcone **3**. In contrast, compounds with electron-donating substituents such as methoxy, methyl, and methylenedioxy groups in the same position of B-ring (chalcones **6**, **7**, and **8** respectively) were all inactive.

The effect of the position of the EWG on the antifungal activity could be observed within the group of chlorinated compounds **11, 13**, and **14** where the antifungal activities follow the order 2-Cl (**13**) > 4-Cl (**11**) > 3, 4-di Cl (**14**). Similarly the 4-NO_2 chalcone **12** showed better activities than chalcone **15** with a nitro group in the 3-position. The observed effect of EWGs on the para-position of ring B in a chalcone structure is in agreement with a previous report on the antifungal activities of non-hydroxylated chalcones [16]. However, the increased activities showed by compounds holding a EWG in the o-position of ring B are somehow contradictory to the previously observed correlation between planarity of chalcones and their antifungal activity [16]. This fact deserves further studies.

Modification of ring A by introducing a bromine substituent in the 3′-position of chalcone **3** significantly improved the antifungal properties: compound **17** is 5–10 times more active against four different species of fungi. However, the effect of such substitution was not significant for other compounds (compare activities of compounds **13** vs. **18**, and **15** vs. **19**).

The selective activity of compound **13** against *T. rubrum* is particularly interesting since this fungus is responsible of approximately 80-93% of chronic and recurrent dermatophyte infections in human beings [24]. It is the ethiological agent of tinea unguium (producer of invasive nail infections), tinea manuum (palmar and interdigital areas of the hand infections) and tinea pedis (Athlete′s foot), the last one being the most prevalent fungal infection in developed countries, and the first one accounting for 50% and 90% of all fingernail and toenail infections respectively [24].

In order to gain insight into the capacity of compound **13** in acting not only against standardized strains, but against clinical isolates of *T. rubrum*, we tested it against ten clinical

Table 4. MIC values of chalcone 3-(2-Chlorophenyl)-1-(2′-hydroxy-4′,6′-dimethoxyphenyl)prop-2-en-1-one (**13**), against 10 different clinical strains of *T. rubrum* from Centro de Referencia Micológica (CEREMIC), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional Rosario.

Voucher specimen	MIC [μg/mL]
T. rubrum C 110	12.5
T. rubrum C 133	12.5
T. rubrum C 134	25.0
T. rubrum C 135	25.0
T. rubrum C 136	50.0
T. rubrum C 137	25.0
T. rubrum C 138	12.5
T. rubrum C 139	25.0
T. rubrum C 140	12.5
T. rubrum C 141	12.5

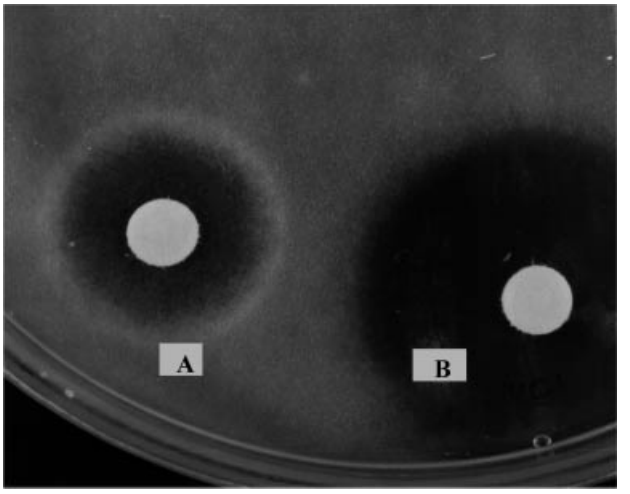


Figure 1. A. Blotchy appearance of the inhibition halo produced by chalcone [3-(2-Chlorophenyl)-1-(2−hydroxy-4,6-dimethoxyphenyl)prop-2-en-1-one] (**13**) in the *Neurospora crassa* assay. **B.** Clear halo produced by ketoconazole.

strains of *T. rubrum* isolated from skin infections of different immunocompromised patients. Results showed (Table 2) that chalcone **13** inhibited all the clinical strains of *T. rubrum* tested, with MICs between 12.5-50 μg/mL being the minimum concentration that inhibits 50% (MIC_{50}) and 90% (MIC_{90}) of the strains tested = 12.5 and 25 μg/mL respectively (Table 4).

Considering our previous finding [16] that non-hydroxylated chalcones were inhibitors of the fungal cell wall, we tested structure **13** for its capacity of inhibiting the fungal cell-wall. Since mammalian cells do not possess a wall [25], this is the intrinsic target to develop highly selective and useful drugs in medicinal investigation, and antifungal structures acting by this mechanism are actively sought.

Amongst methods that deal with this mode of action, we have chosen the *Neurospora crassa* assay [17] an agar diffusion method which allows a macroscopical detection of fungal cell wall inhibitors. *Neurospora crassa* usually grows as long hypha in a diffuse or branched way. When it grows in the presence of certain inhibitors of its wall, the hyphal growth is inhibited, and fungi grow as protoplasts. Macroscopically they can be seen as a blotchy or hazy appearance around the paper disk. The microscopic observation of this hazy zone usually shows morphological changes of the hypha [26].

Results showed that chalcone **13** produced a blotchy zone around the paper disk (Figure 1A and B), indicating that the mode of action could be associated with the inhibition

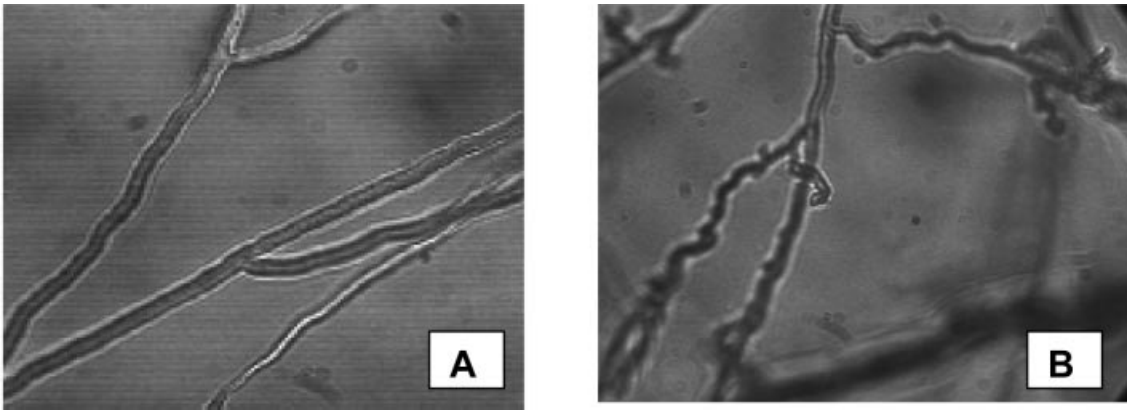


Figure 2. Microscopic appearance of *Neurospora crassa* hypha in the absence (**A**) or in the presence (**B**) of chalcone [3-(2-chlorophenyl)-1-(2-hydroxy-4,6-dimethoxyphenyl)prop-2-en-1-one] (**13**) observed under a light microscope at magnification ×400.

of cell wall polymers or assembly [18]. The microscopic observation of this hazy zone showed a marked curling of the hypha (Figure 2A and B), a defect in the directionality of hypha growth, this giving a new sign that chalcone **13** could act inhibiting the synthesis or assembly of the fungal cell wall polymers. It is important to take in account that some hyphal malformation inducers have proved to be inhibitors of (1,3)β-glucan synthase [27], the enzyme that catalyzes the synthesis of (1,3)β-glucan, main polymer of the fungal cell wall.

Conclusion

This work strongly contributes to the knowledge of the antifungal properties of hydroxy-chalcones by testing the antifungal activity of a series of 17 synthetic chalcones and analogues based in the natural product xanthoxyline (**1**). Results showed that the sole presence of an OH in the 2′-position is not enough for hydroxy-chalcones to possess antifungal activity. Nevertheless, certain members of the series showed moderate activity against dermatophytes. Chalcone **13**, with a Cl- in the *o*-position of ring B, showed the lowest MIC against *T. rubrum* and inhibited all of the ten clinical isolates of *T. rubrum* tested. Regarding its mode of action, it is interesting to note that the most active member of the series produced a blotchy appearance of the inhibition halo in the *Neurospora crassa* assay, clearly suggesting that its mode of action could be the inhibition of the synthesis or assembly of the polymers of the fungal cell wall. The microscopic appearance of this hazy zone showed curling of the hypha. Since fungal but not mammalian cells possess a wall, results obtained in targeted assays with chalcone **13** open the possibility of using this compound as a starting point for the development of safe antifungal agents, since it would attack selectively the fungi without inhibiting any biochemical system of the host.

These data together with previous results obtained by us [16] clearly indicate that hydroxy and non-hydroxy chalcone-like compounds possess antifungal properties with a mode of action related unless in part, to the inhibition to the synthesis or ensamble of polymers of the fungal cell-wall.

Experimental

General experimental procedures

Melting points were determined with a Microquimica MG APF-301 apparatus (Palhoça, Santa Catarina, Brasil) and are uncorrected. Infrared (IR) spectra were recorded with a FT Perkin Elmer 16 PC spectrometer (Connecticut, USA). Nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded on a Bruker AC-200 F (Rheinstetten, Germany) instrument with tetramethylsilane as internal standard.

Elemental analyses were obtained on a Perkin Elmer 2400 (Thermoquest Italia, Milano, Italia). Percentages of C and H were in agreement with the product formula (within ±0.4% of theoretical values).

The purity of the synthesized substances was monitored by thin-layer chromatography (TLC) using silica (Macherey-Nagel GmbH, Inc-USA) pre-coated aluminum plates (Alugram® SIL G/UV254 layer 0.2 mm) with several solvent systems of different polarity. Compounds were visualized with ultraviolet light (254 nm) and using ferric chloride (Ridel-DE Haën AG, Seelze-Hannover-Germany) solution and purified by recrystallization from ethyl ether or hexane (Dinâmica-São Paulo-Brasil).

All the aromatic aldehydes used are commercially available (Sigma-Aldrich Química, S.A., Alcobendas, Madrid). Xanthoxyline (**1**) was isolated from leaves and stems of *Sebastiania schottiana* according to a reported procedure [14]. 2-Hydroxy-3-bromo-4,6-dimethoxy-acetophenone (**2**) was prepared from **1** according to a literature procedure [15].

*Synthesis of chalcones **3**–**16***

A solution of xanthoxyline **1** (0.18 g; 0.92 mmol), EtOH (15 mL), NaOH (0.1 g; 2.5 mmol, containing a minimum amount of H_2O), and the appropriate aldehyde (0.95 mmol) was stirred at room temperature for 1–23 h. The crude product was isolated by acidification of the cool diluted solution, and recrystallized from ethyl ether or hexane.

*1-(2′-Hydroxy-4′,6′-dimethoxyphenyl)-3-(2-naphthyl)prop-2-en-1-one (**4**)*

Yellow solid, mp. = 110–112°C; UV λ_{max} 336 (4.05); IR (KBr) 1638 (C=O), 1586 (C=C) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 14.35 (s, 1H, OH), 7.47–7.99 (m, 9H, Hβ, Hα, H1, H3, H4, H5, H6, H7, H8), 6.13 (d, 1H, *J* = 2.26 Hz, H5′), 5.99 (d, 1H, *J* = 2.26 Hz, H3′), 3.95 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 193.25 (C=O), 169.14 (C4′), 166.93 (C6′), 163.20 (C2′) , 143.17 (Cβ), 134.86 (C2), 134.11 (C9), 133.77 (C10), 129.29 (C4, C8), 128.44 (C1, C5), 127.83 (C7), 127.35 (C6), 124.41 (C3, Cα), 107.07 (C1′), 94.50 (C3′), 91.99 (C5′), 56.30, 56.60 (OCH_3). $C_{21}H_{18}O_4$ Calc. C 75.43, H 5.43, Found C 75.52, H 5.47. Yield = 30%.

*3-(2-Furyl)-1-(2′-hydroxy-4′,6′-dimethoxyphenyl)prop-2-en-1-one (**5**)*

Yellow solid; mp. = 92-94°C; UV λ_{max} 363 (3.42); IR (KBr) 1626 (C=O), 1586 (C=C) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 7.79 (d, 1H, *J* = 15.37 Hz, Hβ), 7.57 (d, 1H, *J* = 15.37 Hz, Hα), 7.51 (d, 1H, *J* = 8.4 Hz, H5), 6.67 (t, 1H, *J* = 8.4 Hz, H4), 6.50 (t, 1H, *J* = 8.4 Hz, H3), 3.91 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 192.74 (C=O), 169.09 (C4′), 166.85 (C6′), 163.21 (C2′), 152.93 (C2), 145.32 (Cβ, C5), 129.63 (Cα), 116.11 (C4), 113.20 (C3), 106.99 (C1′), 94.41 (C3′), 91.88 (C5′), 56.45 (OCH_3), 56.24 (OCH_3). $C_{15}H_{14}O_5$ Calc. C 65.69, H 5.1, Found C 64.76, H 5.18. Yield = 52%.

*3-(4-Bromophenyl)-1-(2′-hydroxy-4′,6′-dimethoxyphenyl)prop-2-en-1-one (**9**)*

Orange solid; mp. = 150–151°C; UV λ_{max} 338 (4.08); IR (KBr) 1632 (C=O), 1588 (C=C) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 14.21 (s, 1H, OH), 7.87 (d, 1H, *J* = 15.51 Hz, Hβ), 7.68 (d, 1H, *J* = 15.51 Hz, Hα), 7.47–7.51 (m, 4H, H2, H3, H5, H6), 6.11 (d, 1H, *J* = 2.2 Hz, H5′), 5.96 (d, 1H, *J* = 2.2 Hz, H3′), 3.91 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 193 (C=O), 169.13 (C4′), 167.07 (C6′), 163.15 (C2′), 141.47 (Cβ), 135.21 (C1), 132.79 (C3, C5), 130.36 (C2, C6), 128.34 (Cα), 124.88 (C4), 106.99 (C1′), 94.1 (C3′),

92.03 (C5′), 56.57 (OCH_3), 56.30 (OCH_3). $C_{17}H_{15}BrO_4$ Calc. C 56.22, H 4.16, Found C 56.04, H 4.21. Yield = 38%.

*3-(4-Fluorophenyl)-1-(2′-hydroxy-4′,6′-dimethoxyphenyl)prop-2-en-1-one (**10**)*

Yellow solid; mp. = 140-141 °C; UV λ_{max} 339 (4.00); IR (KBr) 1632 (C=O), 1572 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 13.72 (s, 1H, OH), 7.01−7.42 (m, 6H, Hβ, Hα, H2, H3, H5, H6), 6.08 (d, 1H, *J* = 2.22 Hz, H5′), 6.91 (d, 1H, *J* = 2.22 Hz, H3′), 3.83 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 193.05 (C=O), 169.08 (C4′), 166.93 (C6′), 163.12 (C2′), 161.94 (C4), 141.69 (Cβ), 132.48 (C1), 130.73(C2, C6), 128.12 (Cα), 116.43 (C3, C5), 106.91 (C1′), 94.45 (C3′), 91.94 (C5′), 56.52 (OCH_3), 56.26 (OCH_3). $C_{17}H_{15}FO_4$ Calc. C 67.54, H 5.00, Found C 67.50, H 5.04. Yield = 87%.

*1-(2′-Hydroxy-4′,6′-dimethoxyphenyl)-3-(4-nitrophenyl)prop-2-en-1-one (**12**)*

Orange solid; mp. = 295−296 °C; UV λ_{max} 379 (3.80) R (KBr) 1664 (C=O), 1584 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.26 (d, 1H, *J* = 15.51 Hz, Hβ), 7.98 (d, 1H, *J* = 15.51 Hz, Hα), 7.25 (d, 2H, *J* = 8.85 Hz, H3, H5), 6.75 (d, 2H, H2, H6), 6.42 (d, 1H, *J* = 2.2 Hz, H5′), 6.17 (d, 1H, *J* = 2.2 Hz, H3′), 3.97 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 192.21 (C=O), 169.51 (C4′), 163.08 (C6′), 160.05 (C2′), 149.55 (C4), 147.22 (C1), 139.05 (Cβ), 131.24 (C2, C6), 123.82 (Cα), 122.44 (C3, C5), 107.21 (C1′), 94.41 (C3′), 89,52 (C5′), 56.26 (OCH_3), 56.18 (OCH_3).$C_{17}H_{15}NO_6$ Calc. C 62.00, H 4.59, N 4.25, Found C 61.98, H 4.56, N 4.23. Yield = 16%.

*3-(2-Chlorophenyl)-1-(2′-hydroxy-4′,6′-dimethoxyphenyl)prop-2-en-1-one (**13**)*

Yellow solid; mp. = 136−137 °C; UV λ_{max} 336 (3.68); IR (KBr) 1630 (C=O), 1556 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 14.25 (s, 1H, OH), 8.19 (d, 1H, *J* = 15.67 Hz, Hβ), 7.91 (d, 1H, *J* = 15.67 Hz, Hα), 7.30−7.75 (m, 4H, H3, H4, H5, H6), 6.15 (d, 1H, *J* = 2.2 Hz, H5′), 6.0 (d, 1H, *J* = 2.2 Hz, H3′), 3.88 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 193.01 (C=O), 169.14 (C4′), 167.08 (C6′), 163.17 (C2′), 138.55 (Cβ), 136.05 (C1), 134.53 (C2), 131.35 (C4), 130.94 (C3), 130.73 (C6), 128.49 (C5), 127.65(Cα), 106.99 (C 1′), 94.49 (C3′), 91.99 (C5′), 56.56 (OCH_3), 56.30 (OCH_3). $C_{17}H_{15}ClO_4$ Calc. C 64.06, H 4.74, Found C 63.87, H 4.80. Yield = 89%.

*3-(3,4-Dichlorophenyl)-1-(2′-hydroxy-4′, 6′-dimethoxyphenyl)prop-2-en-1-one (**14**)*

Yellow solid; mp. = 120−123 °C; UV λ_{max} 343 (3.80); IR (KBr) 1622 (C=O), 1586 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 14.14 (s, 1H, OH), 7.85 (d, 1H, *J* = 15.60 Hz, Hβ), 7.62 (d, 1H, *J* = 15.60 Hz, Hα), 7.43−7.51 (m, 3H, H2, H5, H6), 6.14 (d, 1H, *J* = 2.2 Hz, H5′), 5.97 (d, 1H, *J* = 2.2 Hz, H3′), 3.92 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 191.96 (C=O), 168.47 (C4′), 166.53 (C6′), 162.45 (C2′), 139.23 (Cβ), 135.69 (C1), 133.75 (C3), 133.12 (C4), 132.04 (C5), 130.83(C2), 129.22 (Cα), 106.22 (C1′), 93.81 (C3′), 91.37 (C5′), 55.94 (OCH_3), 55.64 (OCH_3). $C_{17}H_{14}Cl_2O_4$ Calc. C 57.81, H 4.00, Found C 57.32, H 4.09. Yield = 47%

*1-(2′-Hydroxy-4′, 6′-dimethoxyphenyl)-3-(3-nitrophenyl)prop-2-en-1-one (**15**)*

Orange solid; mp. = 171−172 °C; UV λ_{max} 335 (3.74); IR (KBr) 1640 (C=O), 1580 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 14.09 (s, 1H, OH), 8.46 (s, 1H, H2), 8.22 (d, 1H, *J* = 7.75 Hz, H4), 7.98 (d, 1H, *J* = 15.68 Hz, Hβ), 7.84−7.88 (m, 2H, H5, H6), 7.74 (d, 1H, *J* = 15.68 Hz, Hα), 6.12 (d, 1H, *J* = 2.2 Hz, H5′), 5.98 (d, 1H, *J* = 2.2 Hz, H3′), 3.94 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 191.78 (C=O), 168.46 (C4′), 166.65 (C6′), 162.45 (C2′), 148.71 (C3), 138.68 (Cβ), 137.43 (C1), 134.01 (C6), 130.51 (C5), 129.78 (Cα), 123.99 (C4), 122.08 (C2), 106.20 (C1′), 93.84 (C3′), 91.37 (C5′), 55.82 (OCH_3), 55.56 (OCH_3). $C_{17}H_{15}NO_6$ Calc. C 62.00, H 4.59, N 4.25, Found C 60.08, H 4.55, N 4.26. Yield = 57%.

*2-[-3-(2′-Hydroxy-4′,6′-dimethoxyphenyl)-3-oxoprop-1-enyl]-benzoic acid (**16**)*

Yellow solid; mp. = 160−161 °C; UV λ_{max} 291 (3.82) IR (KBr) 1640 (C=O), 1560 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 13.99 (s, 1H, OH), 8.44 (d, 1H, *J* = 15.49 Hz, Hβ), 7.81 (d, 1H, *J* = 15.49 Hz, Hα), 7.96 (d, 1H, *J* = 7.87 Hz, H3), 7.47−7.81 (m, 3H, H4, H5, H6), 6.12 (d, 1H, *J* = 2.2 Hz, H3′), 6.09 (d, 1H, *J* = 2.2 Hz, H5′), 3.93 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) d 199.66 (C=O), 167.15 (C6′, C4′), 162.43 (C2′), 149.67 (Cβ), 133.94 (C1, C6), 132 (C5), 129 (C3), 128.93 (C2), 125.50 (C4), 125.04 (C4, C6), 122.43 (Cα), 106 (C1′), 93.57 (C3′), 90.60 (C5′), 55.44 (OCH_3), 55.30 (OCH_3). $C_{18}H_{16}O_6$ Calc. C 65.85, H 4.91, Found C 65.00, H 4.84. Yield = 44%.

*Synthesis of chalcones **18**, **19***

A solution of 2-hydroxy-3-bromo-4,6-dimethoxyacetophenone **2** (0.2 g; 0.73 mmol), EtOH (15 mL), NaOH (0.1 g; 2.5 mmol, containing a minimum amount of H_2O), and the appropriate aldehyde (0.95 mmol) was stirred at room temperature for 2−3 h. The crude product, isolated by acidification of the cool diluted solution, was recrystallized from ethyl ether and hexane

*1-(3′-Bromo-2′-hydroxy-4′,6′-dimethoxyphenyl)-3-(2-chlorophenyl)prop-2-en-1-one (**18**)*

Orange solid; mp. = 210−212 °; UV λ_{max} 283 (4.12); IR (KBr) 1624 (C=O), 1556 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 14.67 (s, 1H, OH), 8.17 (d, 1H, *J* = 15.58 Hz, Hβ), 7.81 (d, 1H, *J* = 15.58 Hz, Hα), 7.28−7.70 (m, 4H, H3, H4, H5, H6), 6.05 (s, 1H, H5′), 3.98 (s, 6H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 193.25 (C=O), 163.93 (C4′), 163.92 (C6′), 162.90 (C2′), 139.49 (Cβ), 136.21 (C1), 134.33 (C2), 131.62 (C4), 131.06 (C3), 130.22 (C6), 128.60 (C5), 127.72 (Cα), 106 (C1′), 92.80 (C3′), 87.80 (C5′), 57.08 (OCH_3), 56.85 (OCH_3). $C_{17}H_{14}BrClO_4$ Calc. C 51.35, H 3.55, Found C 51.45, H 3.58. Yield = 47%.

*1-(3′-bromo-2′-hydroxy-4′,6′-dimethoxyphenyl)-3-(3-nitrophenyl)-prop-2-en-1-one (**19**)*

Orange solid; mp. = 264 °C; UV λ_{max} 288 (3.25)IR (KBr) 1632 (C=O), 1558 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 14.55 (s, 1H, OH), 8.48−7.60 (m, 6H, Hβ, Hα, H2, H5, H4, H6), 6.10 (s, 1H, H5′), 4.03 (s, 3H, OCH_3), 4.01 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 199.99 (C=O), 163.14 (C4′), 163.09 (C6′), 162.39 (C2′), 148.30 (C3), 140.20 (Cβ), 134.85 (C1), 131.00 (C6), 130.40 (C5), 124.81 (Cα), 122.55 (C4), 121 (C2), 107.02 (C1′), 92.12 (C3′), 87.16 (C5′), 56.76 (OCH_3), 56.18 (OCH_3). $C_{17}H_{14}BrNO$ Calc. C 50.02, H 3.46, N 3.43, Found C 50.10, H 3.44, N 3.40. Yield = 71%.

Biological evaluation

Microorganisms and media

The microorganisms used for the fungistatic evaluation were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) or were clinical isolates and were kindly provided by Centro de Referencia Micológica (C, CEREMIC, Facultad de

Ciencias Bioquímicas y Farmacéuticas, Suipacha 531, (2000) Rosario, Argentina: *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763, *Cryptococcus neoformans* ATCC 32264, *Aspergillus flavus* ATCC 9170, *Aspergillus fumigatus* ATCC 26934, *Aspergillus niger* ATCC 9029 and *Trichophyton mentagrophytes* ATCC 9972. Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C. Cell suspensions in sterile distilled water were adjusted to give a final concentration of 10^6 viable yeast cells/mL [28]. *Microsporum canis* C 112, *Trichophyton rubrum* C 113, 110, 133, 134, 135, 136, 137, 138, 139, 140 and 141; *Epidermophyton floccosum* C 114, *Microsporum gypseum* C 115, and *Candida tropicalis* C 131.

The strains were maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid) and subcultured every 15 days to prevent pleomorphic transformations. Spore suspensions were obtained according to reported procedures [28] and adjusted to 10^6 spores with colony forming ability/mL.

Antifungal assays

The antifungic activities of chalcones were evaluated with the agar dilution method by using Sabouraud-chloramphenicol agar for yeasts and filamentous fungi according with reported procedures [23, 29−31]. Stock solutions of pure compounds were diluted in DMSO to give serial decreasing dilutions ranging from 0.10 to 250 μg/mL. The final concentrations of DMSO in the assay did not exceed 2%. Using a micropipette, an inoculum of 5 μL of the yeast, cell or spore suspensions was added to each Sabouraud-chloramphenicol agar tube. The antifungal agents ketoconazole (Janssen Pharmaceutical, Beerse, Belgium), amphotericin B (Sigma Chemical Co, St Louis, MO, USA) and terbinafine (Novartis, Buenos Aires, Argentina) were included in the assays as positive controls for all fungi. Drug-free solution was also used as a blank control. The tubes were incubated for 24, 48 or 72 h at 30 °C (according to the control fungus growth) up to 15 days for dermatophyte strains.

MIC (Minimal Inhibitory Concentration) was defined as the lowest compound concentration, which completely inhibited the fungal growth after incubation time. MIC_{50} and MIC_{90} are the lowest compound concentrations at which 50 and 90% of the clinical isolates were inhibited respectively [32].

Neurospora crassa assay [17]

Thirty mL of a medium containing 0.5% proteose-peptone # 3 (Britania cat. B # 02-0700), yeast extract (Britania cat # 01-006-05), 4.0% sucrose (reagent grade) and 1.5% agar (Merck cat. # 1613) was autoclaved (115 °C, 15 min), inoculated with 30 μL of spore inoculum of *Neurospora crassa* (IM70 ATCC 9279) at 40 °C and then layered on a petri dish (diameter 9 cm). After the medium has solidified, 1/4" filter disks (Baxter cat # F-2882-1) were applied to media. DMSO solutions of samples were spotted on disks (25 μg/disk). Disks containing DMSO as negative controls were included in the assay. 1.25 μL cilofungin (20 mg/mL) and miconazole (Sigma cat # M-3512) were spotted on disks for positive control and to produce a clear zone respectively. Zones of inhibition were examined macroscopically for hazy or mottled appearance following incubation of the plates at room temperature for 24 h with light. In those cases where hazy zones were observed, the microscopic appearances of the fungi were observed. For preparing spore inoculum, *N. crassa* M70, ATCC 9279 was grown in a medium containing 0.25% (w/w), 0.25% (w/w) yeast extract (w/w), 1% sucrose (w/w) and 1.5% agar (w/w). 4-5days of incubation at rom temperature and light produced an orange mat of hyphal growth with spores. These were harvested with a buffer containing 0.075 g/100 mL K_2HPO_4 (reagent grade), 0.10 g/100 mL KH_2PO_4 (reagent grade) in a solution of glycerol:H_2O 15:85. Spore suspension free of hypha was prepared as described in Microorganisms and media.

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