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Short communication

Study on the substituents' effects of a series of synthetic chalcones against the yeast *Candida albicans*

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Abstract

A large series of chalcones were synthesized and studied for activity against *Candida albicans*. The SAR analysis showed that the antifungal activity was highly dependent on the substitution pattern of the aryl rings and correlated to a large extent with the ability of compounds to interact with sulfhydryl groups. The most active were the hydroxylated chalcones as their activity related to the location of the phenolic group in the aryl ring B as follows: *o*-OH > *p*-OH ~ 3,4-di-OH > *m*-OH. These and other correlations obtained strongly contribute to the knowledge for design of anticandidal chalcones.

Keywords: Chalcones; Antifungal activity; *Candida albicans*; SAR

1. Introduction

Candida albicans is the major human pathogen giving rise to opportunistic oral and vaginal infections. It is a leading cause of invasive fungal disease in premature infants, diabetics, surgical, AIDS and other immunocompromised patients. In these cases the intensive prophylactic use of antifungal drugs leads to emergence of resistant strains of *C. albicans*. This causes a great concern for finding suitable new therapeutics [1].

Chalcones (1,3-diaryl-2-propen-1-ones) seem to be promising antifungal drug candidates. They have been shown to inhibit the growth of various fungi and yeasts, including representatives of the *Candida* species [2,3]. It is known that the mode of the antifungal action of chalcones relates to inhibition of the fungal cell wall [4]. Recently, we have widened this knowledge showing that at least one of the steps involved in chalcone detoxification in yeasts is based on thiol alkylation [5]. From a therapeutic standpoint it is also interesting that chalcones can inhibit glutathione-*S*-transferases (GSTs), enzymes that appear to be involved in drug resistance [3]. To be of help for developing anticandidal chalcones these mechanistic data require extensive study of the structure–activity relationships (SARs).

Up-to-now the SAR analysis has shown that the anticandidal activity of chalcones was lost by their cyclization to corresponding flavanones or by reduction to dihydrochalcones [6]. These results indicate that the chalcone skeleton is fundamental for the growth inhibitory property against *Candida* spp. In addition, some contradictory data exist about the anticandidal activity of hydroxyl-chalcones. According to Sato et al. the chalcones lacking phenolic groups were either inactive or having low potency [7]. At the same time some hydroxyl-chalcones derived from plants did not show marked activity towards *C. albicans* [8,9]. Most of the naturally occurring chalcones are hydroxylated in their aryl ring A and therefore such compounds have mainly been object of antifungal studies [4]. Little is known about the position effect of a phenolic group located in the aryl ring B.

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Besides this uncertain point, the question if and how the electronic effects of the aryl substituents correlate with the anticandidal activity of chalcones still remains unclear. Obviously, additional studies are necessary to verify the substituents' effects in the aromatic rings.

In this study, we synthesized a large series of chalcones with diverse substituents in the aromatic rings, mainly in ring B or with a modified enone moiety and probed the structure–activity correlations towards the yeast *C. albicans*. Our objective was to find important SARs for developing new anticandidal agents based on the chalcone skeleton.

2. Results and discussion

2.1. Chemistry

A series of 44 chalcones and three diphenyl alkenones were easily prepared by the Claisen–Schmidt condensation between acetophenone derivatives or acetone and appropriate aryl aldehydes (Table 1, Fig. 1). Five new compounds were synthesized. The desired products were obtained on an average yield of 80% after purification. Their structures were established with UV, IR, NMR, mass spectrometry and elemental analysis. ^{1}H NMR spectra showed that typically for this reaction the *E*-isomers were specifically generated. The spectral data of the novel compounds are presented in Section 4.

2.2. Microbiology

Antifungal activity was checked by the agar cup method of Spooner and Sykes with *C. albicans* grown on Sabouraud agar [10]. Chalcones giving an inhibitory zone with a diameter (d) of at least 15 mm were initially considered as active. The minimal inhibitory concentration (MIC) of all compounds was determined by the method of serial dilutions as described by Hindler [11]. The hydroxylated chalcones gave relatively small inhibitory zones with diameters between 14 and 21 mm, which might be due to their low diffusion potential into the agar media. All these compounds were highly active when tested in liquid media (meat-pepton broth) yielding MICs around 62.5 μg/ml.

2.3. Structure–activity relationships

2.3.1. Electronic effects of the p-substituents in B ring

Our study of the electronic effects of the aryl substituents of chalcones was based on the knowledge that the antifungal mechanism of these compounds is partially by thiol alkylation [5]. Such reactions may occur between chalcones and the sulfhydryl groups of intracellular thiols like cysteine, homocysteine and glutathione, representing a prime line of cell defence. Thiol alkylation of chalcones is a Michael addition at their electrophilic centre C-β resulting in adducts' formation. It may be facilitated by EW groups located in *p*-position in the aryl ring B and hampered by *p*-electron donating (ED) groups in the same ring. Meanwhile, the electronic effects of the substituents in ring A if there are any should be negligible.

To verify the hypothesis that the anticandidal activity of chalcones might be based on the better acceptance of Michael reaction we studied variously substituted chalcones. Twenty-two of the compounds had *p*-EW or *p*-ED group in ring B and were differently substituted in ring A (Table 2). As expected, the chalcones with *p*-EW group were much more active than those having *p*-ED substituent. However, either type of substituted chalcones was less effective than the unsubstituted chalcone **1**. Its higher activity may be due to inhibition on the enzymes involved in chalcone detoxification, such as the GSTs. GSTs contribute to phase 2 cell biotransformation of endo- and xenobiotics by conjugating these compounds with the reduced glutathione. Inhibition of GSTs by xenobiotics makes the yeast cell more susceptible for alkylating agents and can thus have toxic consequences [12]. It is known that within a series of chalcones with various *p*- or *p*′-groups the unsubstituted chalcone has shown the highest inhibitory activity against GSTs [13,14].

As it can be seen from Table 2 the chalcones with *p*-chloro group were as active as the chalcones with *p*-ED group probably because of the poor electron withdrawing ability of the halogen substituent. The most active ones were the chalcones with *p*-hydroxyl group. Obviously, their activity cannot be ascribed to increased electrophilicity of the C-β atom and must depend on some other properties of their molecules.

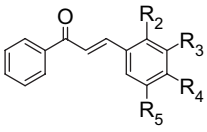
As expected, the electronic effects of substituents in the aryl ring A were not significant. The *p*′-chloro group did not affect the antifungal activity of the chalcones with *p*-EW or *p*-OH group in the ring B and depleted the activity of the other compounds. The chalcones with methoxylated ring A were inactive, which might be due to sterical reasons.

2.3.2. Effect of the position of hydroxyl groups

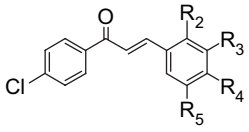
Several studies including this one reveal the importance of phenolic groups for the antifungal activity of chalcones against various *Candida* spp. [3]. According to some authors anticandidal chalcones must possess 2-hydroxyl function [6,7] while Bilgin et al. have demonstrated that the precise location of the hydroxyl group was not important [15].

To clarify this matter a number of chalcones with hydroxyl groups in different positions in ring B were examined for activity against *C. albicans*. Regarding to the position of the phenolic group the antifungal activity decreased in the following order: *o*-OH (**8**) > *p*-OH (**10**) ~ 3,4-di-OH (**11**) > *m*-OH (**9**) (Table 3). Interestingly, this result correlated with the suggestion of Dinkova-Kostova et al. that the presence of *o*-, but not *m*- or *p*-hydroxyl group had a powerful accelerating effect on the reactivity of chalcones with sulfhydryl groups [16]. These authors have proposed a mechanism by which the *o*-hydroxyl groups are involved in extensive intermolecular hydrogen bond formation facilitating the addition of thiols. However, it is very likely that the antifungal action of *o*-hydroxyl-chalcones works in a multiple mechanism including not only thiol alkylation. This view is supported by the fact that these compounds are also known as strong inhibitors of GSTs and are supposed to chelate the metals in metalloproteases [3].

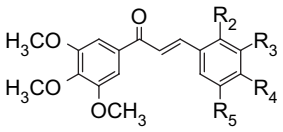
Table 1
Substitution pattern of the chalcones studied against *C. albicans*



X



Y



Z

Chalcone	Type	R_2	R_3	R_4	R_5
1	X	H	H	H	H
2	X	H	H	Cl	H
3	X	H	$-OCH_2O-$		H
4	X	H	H	$N(CH_3)_2$	H
5	X	H	H	$NHCOCH_3$	H
6	X	H	H	NO_2	H
7	X	H	H	CN	H
8	X	OH	H	H	H
9	X	H	OH	H	H
10	X	H	H	OH	H
11	X	H	OH	OH	H
12	X	OH	OCH_3	H	H
13	X	H	OH	OCH_3	H
14	X	H	H	OCH_3	H
15	X	H	H	CH_3	H
16	Y	H	H	H	H
17	Y	H	H	Cl	H
18	Y	H	$-OCH_2O-$		H
19	Y	H	H	$N(CH_3)_2$	H
20	Y	H	H	$NHCOCH_3$	H
21	Y	H	H	NO_2	H
22	Y	H	H	CN	H
23	Y	OH	H	H	H
24	Y	H	OH	H	H
25	Y	H	H	OH	H
26	Y	H	OH	OH	H
27	Y	OH	OCH_3	H	H
28	Y	H	OH	OCH_3	H
29	Y	H	OCH_3	OH	H
30	Y	H	OCH_3	OH	OCH_3
31	Y	H	OCH_3	H	H
32	Y	H	H	OCH_3	H
33	Y	H	OCH_3	OCH_3	H
34	Y	H	OCH_3	OCH_3	OCH_3
35	Y	H	H	CH_3	H
36	Z	H	H	H	H
37	Z	H	H	Cl	H
38	Z	H	$-OCH_2O-$		H
39	Z	H	H	$N(CH_3)_2$	H
40	Z	H	H	$NHCOCH_3$	H
41	Z	H	H	CN	H
42	Z	H	OCH_3	H	H
43	Z	H	H	OCH_3	H
44	Z	H	H	CH_3	H

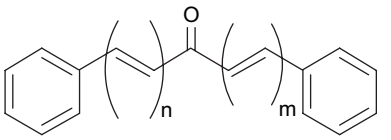
This table represents the structures of all the chalcones synthesized.

In contrast, in our previous study we have shown that *m*-OH group in ring B was the most favourable for the antifungal activity of hydroxyl-chalcones against a large number of yeast strains belonging to species other than *Candida* [5]. This difference underlines the importance of the yeast genotype along with the necessity of SAR studies.

The introduction of *p*′-Cl group in the aryl ring A almost equalized the MIC values: *o*-OH (**23**) ∼ *p*-OH (**25**) ∼ 3,4,-di-OH (**26**) > *m*-OH (**24**). Presence of the halogen was advantageous only when it was inserted into the chalcone with *m*-OH group (**9**) as this led to a two-fold increase of its activity.

2.3.3. Effect of the combination of hydroxyl and methoxy groups in ring B

Table 3 shows the potency of chalcones having one hydroxyl and one methoxy group in vicinal positions. For the hydroxyl group it was observed that the location at C-2 was the most favourable, followed by positions C-4 and C-3, of



45, n=0; m=2; **46**, n=m=1; **47**, n=m=2

Fig. 1. Chalcones with elongated conjugated linker between the aromatic rings. It presents the formulas of three diphenyl alkenones synthesized and studied against *Candida albicans*.

which the substitution at C-4 led to much more active compounds. It turned out that the best surrounding for the *p*-hydroxyl group was two vicinal methoxy groups.

Introduction of *p*′-Cl group led to an eight-fold decrease in the activity of compound **12** having *o*-OH and *m*-OCH_3 groups and did not affect the activity of the chalcone **13** with *m*-OH and *p*-OCH_3 functions.

The introduction of one, two or three methoxy groups or a 3,4-methylenedioxy group in ring B of the non-hydroxylated chalcones produced inactive compounds, which was in consistence with the electronic effects of these substituents.

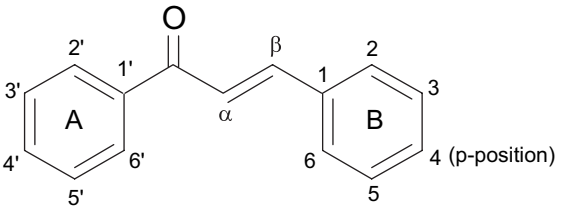
2.3.4. Influence of the position of the keto-group and length of the conjugated linker between both aromatic rings

From a structural point of view chalcones could be also considered as aryl styryl ketones. Some non-aryl, but conjugated styryl ketones are known to display activity against *C. albicans* as they inhibit one or more of the enzymes in the glutathione metabolic pathway [17,18]. Therefore we decided to check if elongation of the conjugated linker between the aromatic rings of chalcones would increase their activity. We synthesized the diphenyl alkenones **45**, **46** and **47** (Fig. 1) and examined their activity against *C. albicans*. Surprisingly, none of these compounds showed any activity when tested in high concentration (2000 μg/ml). This result indicates that insertion of one or more double bonds from either side of the carbonyl group is unfavourable and supports the fact that the diphenyl-2-propen-1-one skeleton is fundamental for the antifungal activity of chalcones.

3. Conclusion

This is the first SAR study to show that the potency of the chalcones against *C. albicans* to a large extent depends on their ability to interact with sulfhydryl groups. This conclusion was based on how the electronic effects of the aryl substituents influenced the antifungal activity. The hydroxyl-chalcones were the most active compounds. Their antifungal activity strongly correlated with the location of the hydroxyl group in ring B. The *o*-position was most favourable, which supported the presumption for a thiol alkylation of these chalcones. Insertion of one and more double bonds between the phenyl rings did not produce active compounds. Although our study shows that chalcones most probably interact with thiol cellular components of *C. albicans* we consider that the mechanism of their action is multiple and complex and deserves further investigation.

Table 2
Electronic effects of *p*-substituents in ring B on the activity of the chalcones against *C. albicans*



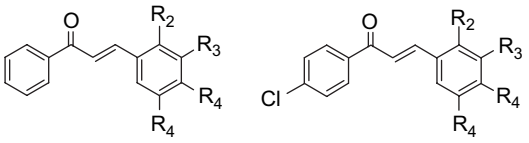
Ring A[a]	Ring B							
	Unsubstituted ring B	Electron withdrawing groups in *p*-position in ring B						
	−H		$-NO_2$		−CN		−Cl	
	d_{inh} (mm)	MIC (μg/ml)	d_{inh} (mm)	MIC (μg/ml)	d_{inh} (mm)	MIC (μg/ml)	d_{inh} (mm)	MIC (μg/ml)
4′-H	30 ± 0	125	17 ± 1	250	17 ± 1	250	0	500
4′-Cl	0	500	15 ± 1	250	23 ± 1	250	0	500
3′,4′,5′-Trimethoxy-	0	500	n.d.	n.d.	0	500	0	500

	Electron donating groups in *p*-position in ring B					
	−OH		$-N(CH_3)_2/-OCH_3$		$-CH_3/-NH(CO)CH_3$	
	d_{inh} (mm)	MIC (μg/ml)	d_{inh} (mm)	MIC (μg/ml)	d_{inh} (mm)	MIC (μg/ml)
4′-H	15 ± 1	62.5	0	500	0	500
4′-Cl	21 ± 1	62.5	0	500	0	500
3′,4′,5′-Trimethoxy-	n.d.	n.d.	0	500	0	500

The anticandidal activities of the chalcones having *p*-electron withdrawing or *p*-electron donating group in the aryl ring B are compared. The aryl ring A of the same compounds is unsubstituted or having 4′-chloro or 3′,4′,5′-trimethoxy-groups.

[a] The substitution patterns of the aryl ring A respond to the types X, Y and Z from Table 1.

Table 3
Effect of the position of the phenolic group and its surrounding in ring B on the chalcone activity against *Candida albicans*



X Y

Compound	Type	R_2	R_3	R_4	R_5	d_{inh} (mm)	MIC (μg/ml)
8	X	OH	H	H	H	21 ± 1	31.25
9	X	H	OH	H	H	17 ± 1	250
10	X	H	H	OH	H	15 ± 1	62.5
11	X	H	OH	OH	H	15 ± 1	62.5
12	X	OH	OCH_3	H	H	12 ± 0	62.5
13	X	H	OH	OCH_3	H	0	500
23	Y	OH	H	H	H	16 ± 0	62.5
24	Y	H	OH	H	H	20 ± 0	125
25	Y	H	H	OH	H	21 ± 1	62.5
26	Y	H	OH	OH	H	14 ± 0	62.5
27	Y	OH	OCH_3	H	H	0	500
28	Y	H	OH	OCH_3	H	0	500
29	Y	H	OCH_3	OH	H	19 ± 1	250
30	Y	H	OCH_3	OH	OCH_3	15 ± 1	125
31	Y	H	OCH_3	H	H	15 ± 1	500
33	Y	H	OCH_3	OCH_3	H	0	500
18	Y	H	$-OCH_2O-$		H	0	500
34	Y	H	OCH_3	OCH_3	OCH_3	0	500

The table represents data about the anticandidal activity of the chalcones having hydroxyl groups or a combination of hydroxyl and methoxyl groups in ring B.

4. Experimental protocols

Infrared and UV spectra were recorded on Bruker IFS 113V and Helios gamma UV–vis spectrophotometers, respectively. ^1H and ^{13}C NMR spectra were obtained with Bruker AM 250 spectrometer with tetramethylsilane as internal reference. Chemical shifts are given in ppm (δ-scale); coupling constants (J) are in Hz. Splitting patterns are described as singlet (s), doublet (d), triplet (t) and multiplet (m). Mass spectral analyses were accomplished on a Hewlett–Packard 5972 using Mass Selective Detector with EI (70 eV). The melting points were obtained using Mel-Temp 1102D-230 VAC and were uncorrected. The reactions were monitored on silica gel 60 F_{254} using PE/acetone 7:3 or toluene/Et_2O 4:1. Flash chromatography was performed for purification of the chalcones on silica gel 60 (230–400 mesh ASTM) using eluent PE/acetone 7:3.

4.1. Chemistry

4.1.1. General procedure for synthesis of chalcones

Acetophenone (97 μl, 0.83 mmol), *p*-chloroacetophenone (84 μl, 0.65 mmol) or 3,4,5-trimethoxyacetophenone (150 mg, 0.71 mm) was added to equimolar quantities of appropriate aryl aldehydes or cinnamaldehyde and dissolved in MeOH (0.8 ml). To this solution 6 M NaOH (0.4 ml) was added and the reaction mixture was stirred for 40 min and then kept in refrigerator overnight. The product crystals were filtrated and washed carefully with ice water and cold MeOH to neutral reaction. The resulting chalcones were purified by recrystallization except for the compounds **9**, **19** and **20**, which were subjected to flash chromatography over silica gel using toluene, toluene/Et_2O 8:1 or PE/acetone 3:1 as the eluent [5].

4.1.2. Synthesis of diphenyl alkenones *45*, *46* and *47*

Benzaldehyde or cinnamaldehyde (1.46 mmol) was dissolved in 1.0 ml MeOH and acetone (0.73 mmol), and 1.4 ml 6 M NaOH was added. In a few minutes solids formed. The products were isolated after filtration, washing the crystals with cold methanol and recrystallization from MeOH.

4.1.3. Structural data

*4.1.3.1. p-Cyanochalcone (**7**).* Yield 98%, white crystals, m.p. 140–141 °C (MeOH). UV (MeOH): 225, 230, 305 nm. IR (KBr): 2210 (C≡N), 1650 (C=O), 1592 (C=C) cm^{-1}. MS *m/z* [$M^{+\bullet}$] 233 (100), [M – 77]$^+$ 156 (39), [M – 105]$^+$ 128 (33), [M – 128]$^+$ 105 (53), [M – 156]$^+$ 77 (59). ^1H NMR ($CDCl_3$, 250 MHz): δ 8.00–8.05 (m, 2H, H-2′, H-6′), 7.77 (d, $J = 15.8$ Hz, 1H, H-β), 7.67–7.75 (m, 3H, H-2, H-6, H-4′), 7.60 (d, $J = 15.8$ Hz, 1H, H-α), 7.44–7.65 (m, 4H, H-3′, H-5′, H-3, H-5). ^{13}C NMR ($CDCl_3$, 250 MHz): δ 189.7 (C=O), 142.0 (C-β), 139.2, 137.6 (C-1′, C-1), 133.3 (C-4′), 132.6 (C-3, C-5), 128.8, 128.7, 128.5 (C-2′, C-3′, C-5′, C-6′, C-2, C-6), 125.1 (C-α), 118.3 (C≡N), 113.5 (C-4). Anal. for $C_{16}H_{11}NO$. Calc. (%): C, 82.38; H, 4.75; N, 6.00. Found (%): C, 82.49; H, 4.96; N, 6.15.

*4.1.3.2. 4-Acetamido-3′,4′,5′-trimethoxychalcone (**40**).* Yield 85%, yellow crystals, m.p. 186–188 °C (MeOH). UV (MeOH): 345 nm. IR (KBr): 3350 (NH), 1700 (NH–C=O, amide-I), 1643 (C=O), 1550 (C=C), 1496 (C–N–H, amide-II), 1300 (CH_3) cm^{-1}. MS *m/z* [$M^{+\bullet}$] 355 (100), [M – 15]$^+$ 340 (50), [M – 31]$^+$ 324 (22), [M – 43]$^+$ 312 (19), [M – 57]$^+$ 298 (24), [M – 160]$^+$ 195 (15), [M – 167]$^+$ 188 (6), [M – 188]$^+$ 167 (5). ^1H NMR ($CDCl_3$, 250 MHz): δ 7.87 (bs, 1H, NH), 7.76 (d, $J = 15.5$ Hz, 1H, H-β), 7.60 (s, 4H, H-2, H-3, H-5, H-6), 7.40 (d, $J = 15.5$ Hz, 1H, H-α), 7.26 (s, 2H, H-2′, H-6′), 3.92 (s, 9H, 3 × CH_3O), 2.19 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$, 250 MHz): δ 189.3 (C=O), 168.6 (CONH), 153.1 (C-3′, C-5′), 144.2 (C-β), 142.4 (C-4), 140.2 (C-4′), 133.6 (C-1′), 130.6 (C-1), 129.4 (C-2, C-6), 120.6 (C-α), 119.7 (C-3, C-5), 106.1 (C-2′, C-6′), 60.9 (CH_3O), 56.4 (2 × CH_3O), 24.6 (CH_3). Anal. for $C_{20}H_{21}NO_5$. Calc. (%): C, 67.59; H, 5.96; N, 3.94. Found (%): C, 67.86; H, 6.14; N, 3.65.

*4.1.3.3. 4-Cyano-3′,4′,5′-trimethoxychalcone (**41**).* Yield 91%, pale yellow crystals, m.p. 171–173 °C (MeOH). UV (MeOH): 308 nm. IR (KBr): 2214 (C≡N), 1657 (C=O), 1575 (C=C), 1336 (C–O–Ar) cm^{-1}. MS *m/z* [$M^{+\bullet}$] 323 (100), [M – 15]$^+$ 308 (32), [M – 31]$^+$ 292 (15), [M – 43]$^+$ 280 (35), [M – 128]$^+$ 195 (29), [M – 167]$^+$ 156 (20), [M – 195]$^+$ 128

(22). ^{1}H NMR (DMSO-d_6, 250 MHz): δ 7.77 (d, $J = 15.5$ Hz, 1H, H-β), 7.72 (s, 4H, H-2, H-3, H-5, H-6), 7.54 (d, $J = 15.5$ Hz, 1H, H-α), 7.27 (s, 2H, H-2′, H-6′), 3.95 (s, 6H, CH_3O), 3.94 (s, 3H, CH_3O). ^{13}C NMR (DMSO-d_6, 250 MHz): δ 188.4 (C═O), 153.2 (C-3′, C-5′), 143.0 (C-1), 141.9 (C-β), 139.2 (C-4′), 132.8 (C-1′), 132.7 (C-3, C-5), 129.6 (C-2, C-6), 124.8 (C-α), 118.3 (C≡N), 113.5 (C-4), 106.3 (C-2′, C-6′), 61.0 (CH_3O), 56.4 ($2 \times CH_3O$). Anal. for $C_{19}H_{17}NO_4$. Calc. (%): C, 70.58; H, 5.30; N, 4.33. Found (%): C, 70.56; H, 5.51; N, 4.04.

*4.1.3.4. 3,3′,4′,5′-Tetramethoxychalcone (**42**).* Yield 78%, yellow crystals, m.p. 65–67 °C (MeOH). UV (MeOH): 320 nm. IR (KBr): 1650 (C═O), 1571 (C═C), 1329 (C–O–Ar), 1214 (C–O) cm^{-1}. MS *m/z* [M$^+$] 328 (100), [M − 15]$^+$ 313 (41), [M − 31]$^+$ 297 (46), [M − 43]$^+$ 285 (22), [M − 133]$^+$ 195 (27), [M − 167]$^+$ 161 (16), [M − 195]$^+$ 133 (10). ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.78 (d, $J = 15.5$ Hz, 1H, H-β), 7.46 (d, $J = 15.5$ Hz, 1H, H-α), 7.35 (t, $J = 8.0$ Hz, 1H, H-5), 7.22–7.27 (m, 3H, H-2′, H-6′, H-6), 7.17 (t, $J = 2.0$ Hz, 1H, H-2), 6.97 (dd, 1 H, $J_1 = 2.8$ Hz, $J_2 = 8.0$ Hz, H-4), 3.95 (s, 6H, $2 \times CH_3O$), 3.94 (s, 3H, CH_3O), 3.83 (s, 3H, CH_3O). ^{13}C NMR ($CDCl_3$, 250 MHz): δ 189.2 (C═O), 159.9 (C-3), 153.1 (C-3′, C-5′), 144.6 (C-β), 142.4 (C-4′), 136.2 (C-1), 133.4 (C-1′), 129.9 (C-4), 122.1 (C-α), 121.0 (C-6), 116.0 (C-4), 113.7 (C-2), 106.0 (C-2′, C-6′), 60.9 (CH_3O), 56.4 ($2 \times CH_3O$), 55.3 (CH_3O). Anal. for $C_{19}H_{20}O_5$. Calc. (%): C, 69.50; H, 6.14. Found (%): C, 69.61; H, 6.40.

*4.1.3.5. 3′,4′,5′-Trimethoxy-4-methylchalcone (**44**).* Yield 96%, white crystals, m.p. 120–123 °C (MeOH). UV (MeOH): 325 nm. IR (KBr): 1650 (C═O), 1575 (C═C) cm^{-1}. MS *m/z* [M$^+$] 312 (100), [M − 15]$^+$ 297 (72), [M − 31]$^+$ 281 (25), [M − 43]$^+$ 269 (29), [M − 117]$^+$ 195 (26), [M − 167]$^+$ 145 (19), [M − 195]$^+$ 117 (13). ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.79 (d, $J = 15.5$ Hz, 1H, H-β), 7.54 (d, $J = 8.0$ Hz, 2H, H-2, H-6), 7.43 (d, $J = 15.5$ Hz, 1H, H-α), 7.27 (s, 2H, H-2′, H-6′), 7.22 (d, $J = 8.3$ Hz, 1H, H-3, H-5), 3.94 (s, 6H, $2 \times CH_3O$), 3.93 (s, 3H, CH_3O), 2.39 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$, 250 MHz): δ 189.3 (C═O), 153.1 (C-3′, C-5′), 144.8 (C-β), 142.4 (C-4′), 141.1 (C-4), 133.6 (C-1), 132.1 (C-1′), 129.7 (C-3, C-5), 128.4 (C-2, C-6), 120.8 (C-α), 106.1 (C-2′, C-6′), 60.9 (CH_3O), 56.4 ($2 \times CH_3O$), 21.5 (CH_3). Anal. for $C_{19}H_{20}O_4$. Calc. (%): C, 73.06; H, 6.45. Found (%): C, 73.10; H, 6.49.

4.2. Microbiology

The strain *C. albicans* Berhaut 62I was obtained from the collection of the Stefan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria.

4.2.1. Agar cup method

Two hundred microliter suspension of the fungus (10^7 cells/ml) was plated on agar layer in Petri dishes (10 cm in diameter). Five wells per dish were prepared, each 10 mm in diameter. One hundred microliter of each sample, dissolved in 96% EtOH (5000 μg/ml) was added to the appropriate well. For pre-diffusion the Petri dishes were placed at 4 °C for 2 h. The antifungal activity was estimated by the diameter of inhibitory zones in the agar layer after incubation at 37 °C for 48 h. Control experiments were carried out with the pure solvent.

4.2.2. Measurement of MIC

MIC was determined by serial dilution of each chalcone (0.0–2000 μg/ml) in test tubes using meat-pepton broth. Each test tube was inoculated with fungal suspension containing 10^5 cells/ml and incubated at 37 °C for 24 h. The lowest dilution that visibly showed no growth compared to drug-free broth inoculated with microbial suspension was considered the MIC. For more precise detection, tubes that showed no visible growth were streaked on fresh meat-pepton agar plates, incubated at 37 °C for 24 h and checked for growth.

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