J. Gen. Appl. Microbiol., **57**, 129–136 (2011)

Full Paper

Exposure of *Candida* to *p*-anisaldehyde inhibits its growth and ergosterol biosynthesis

Sheikh Shreaz,[1] Rimple Bhatia,[1] Neelofar Khan,[1] Sumathi Muralidhar,[2] Seemi F. Basir,[1] Nikhat Manzoor,[1] and Luqman Ahmad Khan[1,*]

[1] *Department of Biosciences, Jamia Millia Islamia, New Delhi–110025, India*
[2] *Regional Sexually Transmitted Disease Centre, Safdarjung Hospital, New Delhi–110029, India*

(Received October 5, 2010; Accepted February 6, 2011)

***p*-Anisaldehyde (4-methoxybenzaldehyde), an extract from *Pimpinella anisum* seeds, is a very common digestive herb of north India. Antifungal activity of *p*-anisaldehyde was investigated on 10 fluconazole-resistant and 5 fluconazole-sensitive *Candida* strains. Minimum inhibitory concentrations (MIC_{90}) ranged from 250 μg/ml to 600 μg/ml for both sensitive and resistant strains. Ergosterol content was drastically reduced by *p*-anisaldehyde—62% in sensitive and 66% in resistant strains—but did not corelate well with MIC_{90} values. It appears that *p*-anisaldehyde exerts its antifungal effect by decreasing NADPH routed through up-regulation of putative aryl-alcohol dehydrogenases. Cellular toxicity of *p*-anisaldehyde against H9c2 rat cardiac myoblasts was less than 20% at the highest MIC value. These findings encourage further development of *p*-anisaldehyde.**

Key Words——antifungal activity; *Candida*; ergosterol; *p*-anisaldehyde

Introduction

Opportunistic fungal infections resistant to standard antifungals—azoles and polyenes—have been increasingly documented in recent years (Cannon et al., 2009). Drug resistance has appeared largely due to extensive use of these antifungals and typically occurs in immunocompromised, AIDS and cancer patients. Resistance among species constitutes by for the most significant problem (Eggimann et al., 2003; Odds et al., 2003). Azole antifungals are fungistatic and are associated with drug–drug interaction. Polyene agents have adverse side effects such as hepatotoxicity and nephrotoxicity (Hoehamer et al., 2010; Wingard et al., 1999). The high toxicity and multidrug resistance associated with various standard antifungals have necessitated the search for safer alternatives in plant-derived materials.

Anise (*Pimpinella anisum* L., *Apiaceae*) is an annual herb widely cultivated in the Mediterranean rim and South Asia including India. *p*-Anisaldehyde (4-methoxy benzaldehyde) extracted from anisal seed oil and structurally related to vanillin is regarded as one of the world's principal flavoring compounds. The chemical structure of *p*-anisaldehyde differs from existing antifungals, including azoles (Fig. 1). *p*-Anisaldehyde exhibits antifungal activity against a number of yeast and mold strains in laboratory media, fruit purees and fruit juices (Fitzgerald et al., 2010; Lopez-Maro et al., 1995; Lu et al., 2010).

There are no reports on the effect of *p*-anisaldehyde on clinical candidal isolates and its possible site of action. Azole drugs inhibit ergosterol biosynthesis in fun-

* Address reprint requests to: Dr. Luqman Ahmad Khan, Department of Biosciences, Jamia Millia Islamia, New Delhi–110025, India.
Tel: +91–11–2698–1717– Ext. 3410
Fax: +91–11–2698–0229
E-mail: profkhanluqman@gmail.com

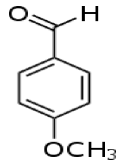


Fig. 1. Chemical structure of *p*-anisaldehyde (4-methoxybenzaldehyde).

gal cells by binding to their cytochrome P-450 sterol 14 α-demethylase (Erg 11p) (Vanden et al., 1994). Azole resistance is either because of overexpression or change in affinity in Erg 11p: In the present study we have investigated the effect of *p*-anisaldehyde on 10 fluconazole-resistant clinical isolates and 5 fluconazole-sensitive standard laboratory strains by performing growth, sensitivity assays and ergosterol quantitation.

Materials and Methods

p-Anisaldehyde was obtained from Sigma-Aldrich (US). All media constituents were obtained from HiMedia (India). All inorganic chemicals and solvents were of analytical grade and supplied by Merck (India). Fluconazole was obtained from Himedia (India).

Growth conditions and media. The strains used in this study are listed in Table 1. The clinical isolates were collected from Regional STD Centre and Institute of Pathology, Safdarjung Hospital, New Delhi, India. The standard strains used in this study were obtained from Indian Institute of Integrative Medicines, Jammu, India. All of the strains were grown on yeast extract peptone dextrose (YPD) medium containing 2% (w/v) glucose, 2% peptone, and 1% yeast extract (HiMedia, India). YPD agar plates containing 2.5% agar (HiMedia) in addition were used to maintain the culture.

Determination of MIC_{90}. Microtiter assay: Cells were grown for 48 h at 30˚C to obtain single colonies, which were resuspended in a 0.9% normal saline solution to give an optical density at 600 nm (OD_{600}) of 0.1. The cells were then diluted 100-fold in YNB medium containing 2% glucose. The diluted cell suspensions were added to the wells of round-bottomed 96-well microtiter plates (100 µl/well) containing equal volumes of medium and different concentrations of test compound (Kohli et al., 2002). A drug-free control was also included. The plates were incubated at 30˚C for 24 h. The MIC test end point was evaluated both visually and by observing the OD_{620} in a microplate reader (BIO-RAD, iMark, US) and is defined as the lowest compound concentration that gave ≥90% inhibition of growth compared to the controls.

Disc diffusion halo assays. Strains were inoculated into liquid YPD medium and grown overnight at 37˚C. The cells were then pelleted and washed three times with distilled water. Approximately 10^5 cells/ml were inoculated in molten agar media at 40˚C and poured into 100-mm diameter Petri plates. Filter discs were kept on solid agar and the test compound was spotted on the disc. The test compound (10-fold more than MIC) dissolved in 10% DMSO, or solvent control (10% DMSO) was pipetted onto a 4-mm-diameter filter disc as described earlier (Ahmad et al., 2010). One hundred µg per ml of fluconazole was also on the discs to

Table 1. Minimum inhibitory concentration (MIC_{90}) of *p*-anisaldehyde against fluconazole-resistant and -sensitive *Candida* species.

	Mean MIC_{90} (µg/ml)		
Fungi	*p*-Anisaldehyde	Fungi	*p*-Anisaldehyde
C. krusei[1,a]	400	*C. guilliermondii*[9,c]	400
C. tropicalis[2,b]	500	*C. krusei*[10,c]	250
C. tropicalis[3,c]	400	*C. tropicalis* ATCC 750	600
C. albicans[4,a]	600	*C. krusei* ATCC 6258	500
C. albicans[5,a]	300	*C. glabrata* ATCC 90030	400
C. glabrata[6,b]	500	*C. albicans* ATCC 10261	450
C. glabrata[7,c]	400	*C. albicans* ATCC 44829	500
C. glabrata[8,b]	500		

[1]STD No. 1413, [2]STD No. 126/09, [3]STD No. 1118, [4]STD No. 1128, [5]STD No. 1141, [6]STD No. 608/09, [7]STD No. 2728, [8]STD No. 1121, [9]STD No. 1685, [10]STD No. 1342. [a]Invasive, [b]respiratory, [c]cutaneous.

serve as a positive control. The diameter of the zone of inhibition was recorded in mm, after 48 h and was compared with that of the control. The results are presented as mean±standard deviation of at least three replicate experiments performed on separate days.

Growth curve studies. For growth studies, 10^6 cells/ml (optical density A_{595} = 0.1) cultures of *Candida* cells were inoculated and grown aerobically in YEPD broth for control along with varied concentrations of the test compound in individual flasks. Growth was recorded turbidometrically at 595 nm using a LaboMed Inc. Spectrophotometer (USA) (Sheikh et al., 2010). The growth rate study of different *Candida* species in the absence as well as in the presence of an inhibitor was performed for each concentration in triplicate, the average of which was taken into consideration.

Ergosterol extraction and estimation assay. A single *Candida* colony from an overnight Sabouraud dextrose agar (Difco) plate culture was used to inoculate 50 ml of Sabouraud dextrose broth (Difco) for control and for various concentrations of *p*-anisaldehyde along with positive control fluconazole (64 μg/ml). The cultures were incubated for 16 h and harvested by centrifugation at 2,700 rpm for 5 min. The net weight of the cell pellet was determined. Three milliliters of 25% alcoholic potassium hydroxide solution was added to each pellet and vortex mixed for 1 min. Cell suspensions were transferred to sterile borosilicate glass screw-cap tubes and were incubated in an 85°C water bath for 1 h. Following incubation, the tubes were allowed to cool. Sterols were then extracted by addition of a mixture of 1 ml of sterile distilled water and 3 ml of *n*-heptane followed by vigorous vortex mixing for 3 min. The heptane layer was transferred to a clean borosilicate glass screw-cap tube and stored at 20°C. Prior to analysis, a 20-μl aliquot of sterol extract was diluted fivefold in 100% ethanol and scanned spectrophotometrically between 240 and 300 nm with a spectrophotometer (Systronics UV-Visible Spectrophotometer 117). Ergosterol content is calculated as a percentage of the wet weight of the cell as described by Arthington-Skaggs et al. (1999). Values were shown in terms of mean±standard error of mean (SEM) of all three respective categories.

MTT cell viability assay. H9c2 rat cardiac myoblasts were cultured and maintained as a monolayer in Dulbecco's modified Eagle's medium (DMEM), high glucose, supplemented with 10% fetal bovine serum (heat inactivated), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B, at 37°C in humidified incubator with 5% CO_2 (Gupta et al., 2006). Cells were incubated with different concentrations of the test compound for 48 h at 37°C in a 5% CO_2 humidified incubator together with an untreated control sample. At appropriate time points, cells were washed in PBS, treated with 60 μl MTT solution (5 mg/ml, tetrazolium salt) and incubated for 45 min at 37°C. After 45 min of incubation at 37°C, the cell supernatants were discarded, MTT crystals were dissolved with acid isopropanol and the absorbance measured at 570 nm. All assays were performed in triplicate. Percent viability was defined as the relative absorbance of treated versus untreated control cells. Plates were analyzed in an ELISA plate reader (Labsystems Multiskan RC, Helsinki, Finland) at 570 nm with a reference wavelength of 655 nm.

Statistical analysis. All the experiments were performed in triplicate and the results were determined as mean±standard deviation.

Results

Minimal inhibitory concentration (MIC_{90})

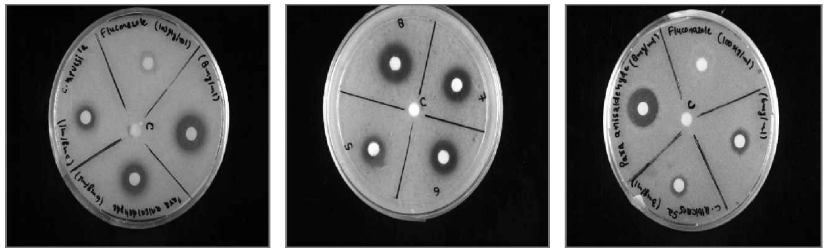
Table 1 summarizes the in vitro susceptibilities of 10 *Candida* isolates of fluconazole-resistant and 5 standard *Candida* species of the fluconazole-sensitive category against *p*-anisaldehyde. The data are reported as MIC's required to inhibit 90% growth of the *Candida* cell population in broth dilution assay. All the *Candida* species were found to be sensitive to the test compound. Species exhibiting fluconazole MIC≥64 μg/ml were considered as resistant. MIC_{90} values ranged from 250 μg/ml (*C. krusei* STD No. 1342) to 600 μg/ml (*C. albicans* STD No. 1128, *C. tropicalis* ATCC 90030).

Disc diffusion

Figure 2 gives typical results for disc diffusion assays. Table 2 summarizes in vitro susceptibilities of 10 fluconazole-sensitive and 5 fluconazole-resistant strains of *Candida* measured as the diameter of the zone of inhibition (mm). Concentration dependency was found at all tested concentrations of *p*-anisaldehyde.

Growth studies (turbidometric measurement)

Figure 3a, 3b, 3c shows the effect of different concentration of *p*-anisaldehyde on the growth pattern of resistant isolates of *C. albicans* STD No. 1141*, C. kru-*



(a) (b) (c)

Fig. 2. Disc diffusion assay of *p*-anisaldehyde.
(a) *C. krusei* STD 1413. (b) C*. albicans* ATCC 10261 (5, 6, 7)-*p*-anisaldehyde and 8-fluconazole (100 μg/ml). (c) *C. albicans* STD No. 1141.

Table 2. Disc diffusion assay of *p*-anisaldehyde and fluconazole (100 μg/ml) against different *Candida* species.

	Zone of inhibition (mm)							
p-Anisaldehyde	*C.k*[1,a]	*C.t*[2,b]	*C.t*[3,c]	*C.a*[4,a]	*C.a*[5,a]	*C.gla*[6,b]	*C.gla*[7,c]	*C.gla*[8,b]
8 mg/ml	13 (± 0.5)	11 (± 0.3)	13 (± 0.7)	8 (± 0.5)	15 (± 0.9)	10 (± 0.8)	12 (± 1.0)	9 (± 1.0)
6 mg/ml	9 (± 0.5)	6 (± 0.2)	8 (± 0.0)	3 (± 0.9)	11 (± 0.4)	4 (± 0.1)	7 (± 1.0)	4 (± 0.2)
3 mg/ml	3 (± 0.4)	1 (± 0.1)	2 (± 0.1)	0.5 (± 0.0)	5 (± 0.6)	0.5 (± 0.0)	2 (± 0.1)	1 (± 0.0)
Fluconazole	—	—	—	—	—	—	—	—
	C.gui[9,c]	*C.k*[10,c]	*C. a* 10261	*C. t* 750	*C. gla* 90030	*C. k* 6258	*C. a* 44829	
8 mg/ml	11 (± 1.5)	16 (± 0.6)	12 (± 0.5)	7 (± 0.2)	13 (± 1.0)	10 (± 0.6)	11 (± 0.5)	
6 mg/ml	6 (± 1.2)	12 (± 0.2)	7 (± 0.2)	2 (± 0.1)	8 (± 0.3)	5 (± 0.2)	6 (± 0.2)	
3 mg/ml	2 (± 0.2)	7 (± 0.4)	2 (± 0.0)	0.5 (± 0.0)	3 (± 0.1)	1 (± 0.0)	1 (± 0.0)	
Fluconazole	—	—	14 (± 0.3)	11 (± 0.5)	18 (± 0.1)	14 (± 0.2)	16 (± 0.5)	

The data represent the mean±SD of three experiments.
C.k, *C. krusei*; *C.t*, *C. tropicalis*; *C.a*, *C. albicans*; *C.gla*, *C. glabrata*; *C.gui*, *C. guillerimondii.* [1]STD No. 1413, [2]STD No. 126/09, [3]STD No. 1118, [4]STD No. 1128, [5]STD No. 1141, [6]STD No. 608/09, [7]STD No. 2728, [8]STD No. 1121, [9]STD No. 1685, [10]STD No. 1342. [a]Invasive, [b]respiratory, [c]cutaneous.

sei STD No. 1413*, C. tropicalis* STD No. 126/09 and Fig. 3d, 3e, 3f shows the effect on the growth pattern of fluconazole-sensitive *C. glabrata* ATCC 90030, *C. albicans* ATCC 10261 and *C. krusei* ATCC 6258. Significant and pronounced effect is observed for other species (data not shown). Results obtained demonstrated that the ability to kill *Candida* species is dependent on the concentration of the test compound. Control cells showed a normal pattern of growth with a lag phase of 4 h, and an active exponential phase of 8−10 h before attaining the stationary phase. An increase in the concentration of *p*-anisaldehyde leads to a significant decrease in growth with suppressed and delayed exponential phases. At MIC_{90} values complete cessation of growth was observed for all the yeast species.

Ergosterol assay

Table 3 summarizes the effect of *p*-anisaldehyde on ergosterol biosynthesis in fluconazole-susceptible, and fluconazole-resistant, *Candida* isolates. The total ergosterol content was determined for each isolate grown in varying concentrations of *p*-anisaldehyde. A dose-dependent decrease in ergosterol production was observed. The mean decrease in total cellular ergosterol content for fluconazole-susceptible standard strains at MIC_{90} value was 62% (range 53% to 73%). The mean decrease in total ergosterol content for fluconazole-resistant isolates was 66% (range 54% to 75%). Thus significant difference was seen in ergosterol biosynthesis inhibition in fluconazole-sensitive and -resistant strains. The mean decrease in total cellular ergosterol content of susceptible cells treated with fluconazole (64 μg/ml) was measured to be 99%. In contrast, for resistant isolates, the mean decrease was only 17.6%.

Toxicity profile

To evaluate the toxicity of synthesized compounds, they were tested against H9c2 cardiac myoblasts.

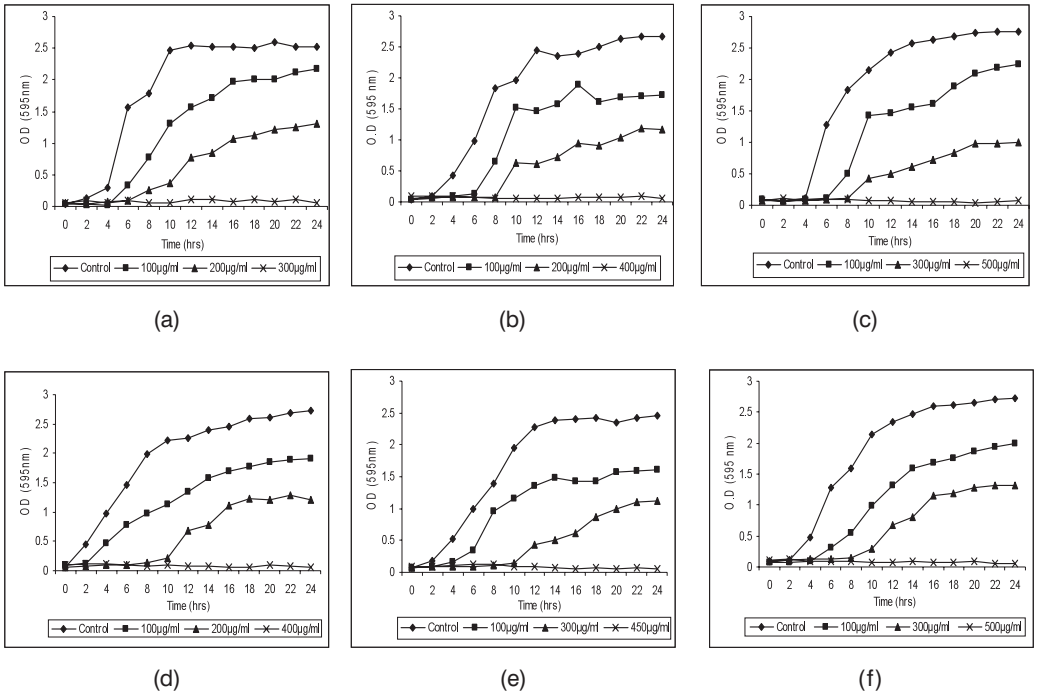


Fig. 3. Effect of *p*-anisaldehyde on growth of different *Candida* species.

Growth curve plotted against absorbance at 595 nm and time (h) shows complete inhibition of growth at MIC_{90} values. (a) Against *C. albicans* STD No. 1141. (b) Against *C. krusei* STD No. 1413. (c) Against *C. tropicalis* STD No. 126/09. (d) Against *C. glabrata* ATCC 90030. (e) Against *C. albicans* ATCC 10261. (f) Against *C. krusei* ATCC 6258.

Subconfluent populations of H9c2 cells were treated with increasing concentrations of the compound and the number of viable cells was measured after 48 h by MTT cell viability assay. The concentration range of the compound was 10−320 µg/ml. Figure 4 depicts *p*-anisaldehyde showing a viability of 100% at the concentration range of 10 µg/ml and at the same concentration the reference compound fluconazole showed a viability of 98%. At concentration of 20, 40, 80 and 160 µg/ml fluconazole showed 94%, 87%, 71% and 66% viability while as *p*-anisaldehyde offered a remarkable viability of 100%, 97%, 93% and 89% respectively. *p*-Anisaldehyde shows negligible toxicity even at values approaching its MIC. At 320 µg/ml toxicity of *p*-anisaldehyde was very low (15%) compared to fluconazole (52%).

Discussion

Minimum inhibitory concentrations of *p*-anisaldehyde (4-methoxybenzaldehyde) (Fig. 1) for 10 fluconazole-resistant strains of *Candida* were found to be in the range of 250 µg/ml to 600 µg/ml; MIC value for fluconazole-sensitive laboratory strains ranged from 400 to 600 µg/ml. Thus no major difference in *p*-anisaldehyde sensitivity was seen in fluconazole-resistant and -sensitive strains. *p*-Anisaldehyde MICs agree well with those determined for *S. cerevisiae* in the range of 256 µg/ml (Lu et al., 2010). Antifungal activity in solid media examined by disc diffusion assay yields similar results. On a concentration basis *p*-anisaldehyde is, however, found to be less effective than fluconazole.

We demonstrate that *p*-anisaldehyde exhibits fungicidal and not fungistatic activity, by halo assay and growth curve studies against recently obtained clinical isolates. Our results show that the growth is inhibited by *p*-anisaldehyde in 5 laboratory wild-type *Candida* strains and 10 (resistant), recently obtained clinical isolates, and the halo produced was completely clear, an indication of potential fungicidal activity, whereas in contrast fluconazole showed no clearing or a very turbid halo in the case of resistant species (Fig. 2a and 2c), an indication of the fungistatic nature of fluconazole (Onyewu et al., 2000).

Three general mechanisms of azole resistance have been described for *Candida* sp. The first is alteration in the target enzyme, 14 α-demethylase, leading to its over-expression and reduced susceptibility to azole

Table 3. % decrease in ergosterol content of control cells and treated samples.

p-Anisaldehyde					
C. krusei STD No. 1413		*C. tropicalis* STD No. 126/09		*C. tropicalis* STD No. 1118	
Sample	% decrease	Sample	% decrease	Sample	% decrease
Control	0	Control	0	Control	0
100 µg/ml	11.04 ± 0.382	100 µg/ml	7.11 ± 1.070	100 µg/ml	10.64 ± 0.221
200 µg/ml	32.40 ± 0.810	300 µg/ml	26.02 ± 0.277	200 µg/ml	31.00 ± 0.170
400 µg/ml	71.14 ± 0.135	500 µg/ml	63.21 ± 1.601	400 µg/ml	70.14 ± 0.115
C. albicans STD No. 1128		*C. albicans* STD No. 1141		*C. glabrata* STD No. 608/09	
Control	0	Control	0	Control	0
100 µg/ml	7.11 ± 0.305	100 µg/ml	12.23 ± 0.476	100 µg/ml	7.11 ± 0.678
400 µg/ml	21.09 ± 1.143	200 µg/ml	33.49 ± 0.070	300 µg/ml	27.02 ± 1.897
600 µg/ml	54.43 ± 1.459	300 µg/ml	73.14 ± 0.455	500 µg/ml	62.45 ± 0.349
C. glabrata STD No. 2728		*C. glabrata* STD No. 1121		*C. guilliermondii* STD No. 1685	
Control	0	Control	0	Control	0
100 µg/ml	9.54 ± 0.382	100 µg/ml	8.11 ± 0.734	100 µg/ml	8.64 ± 0.879
200 µg/ml	30.56 ± 0.675	300 µg/ml	25.67 ± 0.617	200 µg/ml	29.78 ± 1.170
400 µg/ml	69.23 ± 0.335	500 µg/ml	60.87 ± 1.541	400 µg/ml	67.64 ± 0.835
C. krusei STD No. 1342		*C. albicans* ATCC 10261		*C. glabrata* ATCC 90030	
Control	0	Control	0	Control	0
100 µg/ml	13.91 ± 0.342	100 µg/ml	10.43 ± 0.223	100 µg/ml	12.19 ± 0.102
200 µg/ml	35.72 ± 0.784	300 µg/ml	31.11 ± 0.511	200 µg/ml	36.60 ± 0.117
250 µg/ml	75.89 ± 1.211	450 µg/ml	68.23 ± 0.987	400 µg/ml	72.07 ± 1.569
C. albicans ATCC 44829		*C. tropicalis* ATCC 750		*C. krusei* ATCC 6258	
Control	0	Control	0	Control	0
100 µg/ml	9.12 ± 0.233	100 µg/ml	8.11 ± 0.212	100 µg/ml	8.45 ± 0.255
300 µg/ml	25.70 ± 0.987	300 µg/ml	21.45 ± 0.349	300 µg/ml	23.00 ± 0.542
500 µg/ml	59.00 ± 0.509	600 µg/ml	53.29 ± 1.145	500 µg/ml	58.15 ± 0.942

The data represent the mean±SD of three experiments.

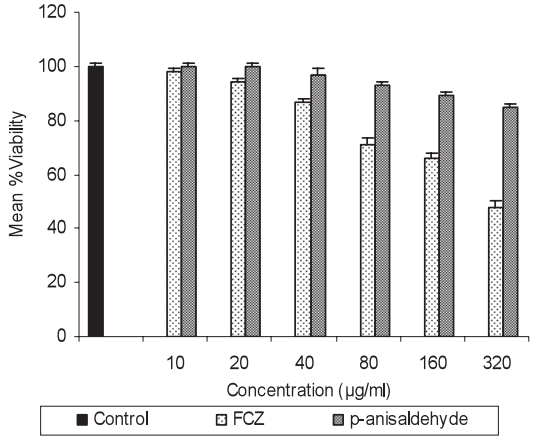


Fig. 4. Percentage of viable cells after 48 h pre-treatment of H9c2 myoblasts with fluconazole and *p*-anisaldehyde evaluated by MTT assay.

inhibition (Sanglard et al., 1998; White, 1997). Decreased drug accumulation, mediated by either diminished uptake or increased efflux of the drug, is the second mechanism (Pfaller et al., 1998; Sanglard et al., 1995). The third is a deficiency in C5 (6) sterol desaturase, which suppresses the accumulation of toxic sterol intermediates, as a result of azole-mediated 14 α-demethylase inhibition (Geber et al., 1995). The SQM is capable of detecting increased resistance due to any of the above mechanisms based on its ability to detect intracellular ergosterol following the exposure of the organisms to fluconazole.

Sub-confluent populations of H9c2 rat cardiac myoblast cells provide a handy tool for toxicity studies of the compounds. The in vitro MTT cell viability assay is a possible screening assay for gauging in vivo toxicity to host cells. The comparative study of the test com-

pound with the conventional antifungal drug indicates that the test compound was significantly less toxic. The potential killing activity of *p*-anisaldehyde and its less cytotoxic effect encouraged us to study its mode of action. This work constitutes the first attempt to assess the antifungal role of *p*-anisaldehyde by studying its effect on sterol biosynthesis. Analysis of sterols obtained from all fluconazole-susceptible, and fluconazole-resistant, *Candida* isolates showed no major differences in either the sterol contents or the sterol patterns of the untreated controls of the isolates. Growth of *Candida* in the presence of sub-inhibitory and MIC_{90} concentrations of *p*-anisaldehyde altered the sterol patterns of both fluconazole-susceptible, and fluconazole-resistant, isolates. Studying the effect of this compound at various sub-inhibitory concentrations on the sterols of the tested *Candida* strains showed that this compound acts in a dose-dependent fashion to decrease ergosterol content. Near equal reduction of ergosterol in fluconazole-sensitive and -resistant strains at MIC—62% and 66% respectively—suggests that *p*-anisaldehyde acts by a more general mechanism. Absence of corelation between ergosterol content and MIC further re-inforces the argument. Several putative aryl-alcohol dehydrogenases are induced by a factor ranging 2.6 to 4.1 following exposure to *p*-anisaldehyde (Lu et al., 2010). This may lead to a decrease in NADPH concentration, which is vital for ergosterol biosynthesis. *p*-Anisaldehyde showed negligible toxicity as compared to fluconazole even at concentration values approaching MIC.

In summary, *p*-anisaldehyde is found to be an effective antifungal against both fluconazole-resistant and -sensitive strains of *Candida*. Ergosterol biosynthesis is strikingly reduced by *p*-anisaldehyde and its cause appears to be decreased NADPH routed through up-regulation of aryl-alcohol dehydrogenases.

Acknowledgments

This work was partially supported by ICMR grant No. 59/24/2008/BMS/TRM to N. M. and L. A. K. and UGC (India) grant 33-223/2007 to Dr. L. A. Khan and Dr. Niknat Manzoor. The authors wish to thank Dr. Inshad Khan from IIIM, Jammu, India for providing the standard strains used in this study.

References

Ahmad, A., Khan, A., Manzoor, N., and Khan, L. A. (2010) Evolution of ergosterol biosynthesis inhibitors as fungicidal against *Candida*. *Microb. Pathog.,* **48**, 35–41.

Arthington-Skaggs, B. A., Jradi, H., Desai, T., and Morrison, C. J. (1999) Quantification of ergosterol content: Novel method for determination of Fluconazole susceptibility of *Candida albicans*. *J. Clin. Microbiol.*, **37**, 3332–3337.

Cannon, R. D., Lamping, E., Holmes, A. R., Niimi, K., Baret, P. V., Keniya, M. V., Tanabe, K., Niimi, M., Goffeau, A., and Monk, B. C. (2009) Efflux-mediated antifungal drug resistance. *Clin. Microbiol. Rev*., **22**, 291–321.

Eggimann, P., Garbino, J., and Pittet, D. (2003) Epidemiology of *Candida* species infections in critically ill non-immunosuppressed patients. *Lancet Infect. Dis.,* **3**, 685–702.

Fitzgerald, D. J., Stratford, M., Gasson, M. J., and Narbad, A. (2004) The potential application of vanillin in preventing yeast spoilage of soft drinks and fruit juices. *J. Food Prot.,* **67**, 391–395.

Geber, A., Hitchcock, C. A., Swartz, J. E., Pullen, F. S., Marsden, K. E., Kwon-Chung, K. J., and Bennett, J. E. (1995) Deletion of the *Candida glabrata ERG3* and *ERG11* genes: Effect on cell viability, cell growth, sterol composition, and antifungal susceptibility. *Antimicrob. Agents Chemother.*, **39**, 2708–2727.

Gupta, M. K., Neelakantan, T. V., Sanghamitra, M., Tyagi, R. K., Dinda, A., Mualik, S., Mukhopadhyay, C. K., and Goswami, S. K. (2006) An assessment of the role of reactive oxygen species and redox signaling in norepinephrine-induced apoptosis and hypertrophy of H9c2 cardiac myoblasts. *Antioxid. Redox. Signal*., **8**, 1081–1093.

Hoehamer, C. F., Edwin, D. C., George, M. H., and David Rogers, P. (2010) Changes in the proteome of *Candida albicans* in response to azole, polyene, and echinocandin antifungal agents. *Antimicrob. Agents Chemother.*, **54**, 1655–1664.

Kohli, A., Smriti, K., Mukhopadhyay, K., Rattan, A., and Prasad, R. (2002) In vitro low-level resistance to azoles in *Candida albicans* is associated with changes in membrane lipid fluidity and asymmetry. *Antimicrob. Agents Chemother.*, **46**, 1046–1052.

Lopez-Malo, A., Alzamora, S. M., and Argaiz, A. (1995) Effect of natural vanillin on germination time and radial growth of moulds in fruit based agar systems. *Food Microbiol.,* **12**, 213–219.

Lu, Y., Na, G., Yang, Y., Xiuping, W., Rizeng, M., Junwen, F., Fa, G., Wang, X., Jingbo, L., and Deng, X. (2010) Microarray analysis of *p*-anisaldehyde-induced transcriptome of *Saccharomyces cerevisiae. J. Ind. Microbiol. Biotechnol.*, **37**, 313–322.

Odds, F. C., Brown, A. J., and Gow, N. A. (2003) Antifungal agents: Mechanisms of action. *Trends Microbiol.*, **11**(6), 272–279.

Onyewu, C., Blankenship, J. R., Poeta, M. D., and Heitman, J. (2000) Ergosterol biosynthesis inhibitors become fungicidal when combined with calcineurin inhibitors against *Candida albicans*, *Candida glabrata*, and *Candida krusei. Antimicrob. Agents Chemother.*, 956–964.

Pfaller, M. A., Jones, R. N., Messer, S. A., Edmond, M. B., and Wenzel, R. P. (1998) National surveillance of nosocomial blood stream infection due to species of *Candida* other than *Candida albicans*: Frequency of occurrence and antifungal susceptibility in the SCOPE Program. *Diagn. Microbiol. Infect. Dis.*, **30**, 121–129.

Sanglard, D., Ischer, F., Koymans, L., and Bille, J. (1998) Amino acid substitutions in the cytochrome P-450 lanosterol 14 alpha-demethylase (CYP51A1) from azole-resistant *Candida albicans* isolates contribute to resistance to azole antifungal agents. *Antimicrob. Agents Chemother.*, **42**, 241–253.

Sanglard, D., Kuchler, K., Ischer, F., Pagani, J. L., Monod, M., and Bille, J. (1995) Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob. Agents Chemother.*, **39**, 2378–2386.

Sheikh, S., Sheikh, R. A., Bhatia, R., Hashmi, A. A., Manzoor, N., and Khan, L. A. (2010) Anticandidal activity of cinnamaldehyde, its ligand and Ni(II) complex: Effect of increase in ring and side chain. *Microb. Pathog.*, **49**, 75–82.

Vanden, B. H., Marichal, P., and Odds, F. C. (1994) Molecular mechanisms of drug resistance in fungi. *Trends Microbiol.,* **2**, 393–400.

White, T. C. (1997) The presence of an R467K amino acid substitution and loss of allelic variation correlates with an azole-resistant lanosterol 14 alphade methylase in *Candida albicans*. *Antimicrob. Agents Chemother.*, **41**, 1488–1494.

Wingard, J. R., Kubilis, P., Lee, L., Yee, G., White, M., Walshe, L., Bowden, R., Anaissie, E., Hiemenz, J., and Lister, J. (1999) Clinical significance of nephrotoxicity in patients treated with amphotericin B for suspected or proven aspergillosis. *Clin. Infect. Dis.,* **29**, 1402–1407.