

Investigation of Antifungal Activity of Stilbenes Alone and in Combination with Clotrimazole against *Candida albicans*

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ABSTRACT

Candidiasis is a term describing infections by yeasts from the genus *Candida*, and the type of infection encompassed by candidiasis ranges from superficial to systemic. Treatment of such infections often requires antifungal agents such as clotrimazole, but increased use of these drugs has led to a selection of *Candida* sp. with increased resistance to these drugs. Antibiotics have been effective in treating infectious diseases, but resistance to these drugs has led to the emergence of new and the reemergence of old infectious diseases. In this study, we used two stilbenes [3,4',5-trihydroxystilbene (1) and 3,5-dihydroxy-4-isopropylstilbene (2)] purified from a bacterium associated with entomopathogenic nematode (EPN), to demonstrate both its intrinsic antifungal activity and its synergy with the clotrimazole, in the treatment of *Candida albicans* *in vitro*. Our results demonstrated that significant synergistic effect exists between stilbenes and Clotrimazole against *C. albicans*. The time kill assay also supports the synergistic activity. The cytotoxicity of stilbenes was also tested against normal human cell lines (L231 lung epithelial and folliculo-stellate (FS) normal fibroblast) and no cytotoxicity was recorded for stilbenes up to 200 µg/mL.

Keywords: anticandidal activity, normal human cells, oral *candida* synergistic activity

INTRODUCTION

Candidiasis, the main opportunistic fungal infection has steadily increased over the past 30 years (Ghannoum 2001; Melo *et al.* 2004). Among the many species, *Candida albicans* is the most important pathogen and oral candidiasis is an oral lesion caused by this organism. Oral candidiasis is most commonly associated with individuals infected with the human immunodeficiency virus (HIV), and it is also seen in infants, patients with diabetes mellitus, and those receiving broad-spectrum antibiotics. However, oral candidiasis is relatively uncommon in the general population (Pelletier *et al.* 2000), despite the fact that *C. albicans* can be recovered in the alimentary canal of healthy individuals in over 50% of cases (Pelletier *et al.* 2000; Barchiesi *et al.* 2002). *Candida* spp. are the fourth most common agent of hospital-acquired bloodstream infections (Banerjee *et al.* 1991; Edmond *et al.* 1999). Specifically, *C. albicans* is the most frequently isolated yeast from clinical specimens, but non-*C. albicans* species are more commonly isolated from blood, urine, skin and upper respiratory tract (Ng *et al.* 1999).

Treatment of this fungal infection presents several problems. Despite the extensive usage of clotrimazole in HIV-infected patients, little is known about the emergence of microbial resistance to this topically administered imidazole. While considerable attention has been directed to the emergence of resistance to fluconazole and itraconazole in HIV-infected patients, there have been virtually no studies investigating clotrimazole. Azoles disrupt ergosterol biosynthesis in fungi resulting in the formation of cell membrane with altered structure and function (Odds *et al.* 2003). However, the choices are still limited, especially due to the resistance because of the increase in the use of drugs (Pelletier *et al.* 2000). Increasing usage of anticandidal agents, the number and variety of azole-resistant fungal strains have increased. Moreover, these antifungal drugs often have side effects, and the search for newer treatment regimes for safer and

more effective treatment is warranted.

Bacteria of the genera *Xenorhabdus* and *Photorhabdus* are known to be symbiotically associated with the soil dwelling entomopathogenic nematodes (EPN) of the family steinernematidae and heterorhabditidae respectively (Marokhazi *et al.* 2004). *Xenorhabdus* and *Photorhabdus* are known to produce a wide range of bioactive metabolites (Forst and Neilson 1996). In the course of studies on EPN, a new entomopathogenic nematode belonging to the genus *Rhabditis* and subgenus *Osccheius* was isolated from sweet potato weevil grubs collected from Central Tuber Crops Research Institute (CTCRI) farm, Thiruvananthapuram. The bacterium associated with the EPN was identified as *Bacillus* sp. The cell free culture filtrate and the two stilbene compounds isolated from this bacterium were found to have antifungal activity (Kumar *et al.* 2011).

In the present study, *in vitro* anticandidal activity of stilbenes alone and in combination with clotrimazole against *C. albicans* was investigated.

MATERIALS AND METHODS

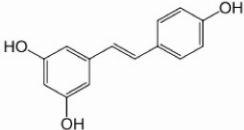
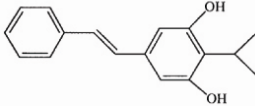
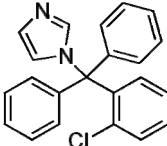
Test compounds

The test stilbene compounds [3,4',5-trihydroxystilbene (1) and 3,5-dihydroxy-4-isopropylstilbene (2) (Table 1)] were isolated and purified from the cell free culture filtrate (Tryptic soya broth) of a bacterium associated with a novel EPN, *Rhabditis* (*Osccheius*) sp. and chemical structures of the compounds were established on the basis of spectral analyses. Clotrimazole (Sigma Aldrich, St. Louis, MO, USA) (Table 1) was used as a standard anticandidal agent.

Test organism

The *Candida* strain used in the study was *C. albicans* MTCC 277 and was subcultured in potato dextrose agar and broth (Hi-media Laboratories Limited, Mumbai, India) at 37°C for 24–48 h to ensure viability and purity prior to testing.

Table 1 Stilbenes and Clotrimazole used in this study.

Antibiotics and stilbenes	Antimicrobial class	Target	Structure
3,4',5-trihydroxystilbene	Poly phenols	Unknown	
3,5-dihydroxy-4-isopropylstilbene	Poly phenols	Unknown	
Clotrimazole	Azoles	Cell wall Figure from Wikimedia	

Inoculum preparation

Stock inoculum suspensions of the *C. albicans* was prepared by picking five colonies from 24-h cultures grown on potato dextrose agar at 37°C and suspending in 5 mL of sterile saline (0.85%). Cell density was adjusted with spectrophotometric method at 600 nm wavelength to achieve the turbidity equivalent 0.5 McFarland standard. The dilution of *C. albicans* stock suspension was adjusted from 1×10^6 to 5×10^6 cells/mL (Arumugam *et al.* 2012).

Synergy study (checkerboard method)

Combinations of stilbenes and clotrimazole were tested by the checkerboard method against the *C. albicans* in potato dextrose broth (Pillai and Moellering 2005). The stilbenes and clotrimazole were mixed in 1:1 ratio. The combined study for *C. albicans* was tested in triplicates (Lee *et al.* 2012). The concentration of the individual compound in the combination of stilbenes and clotrimazole in which the growth of organisms is completely inhibited is taken as the MIC of the individual compound in the combination. Drug interaction was regulated as synergistic, additive, indifferent or antagonistic on the basis of the fractional inhibitory concentration (FIC) index.

The fractional inhibitory concentration was calculated as follows (Lee *et al.* 2012):

FIC of compound a (FIC_a) = MIC of compound a in combination/
MIC of compound a alone

FIC of compound b (FIC_b) = MIC of compound b in combination/
MIC of compound b alone

The sum of fractional inhibitory concentration (FICs) indices of two compounds in the combination was calculated as follows:

FIC_a + FIC_b = FICs.

Two drugs or bioactive compounds are defined as having synergistic effect if the FIC index was less than or equal to 0.5, additive if the FIC index was greater than 0.5 and less than or equal to 1.0, indifferent if the FIC index was greater than 1.0 and less than or equal to 2.0, and antagonistic if the FIC index was greater than 2.0 (George *et al.* 1996).

Time kill assay

The potential of compound carryover during the plating process were determined by following (Klepser *et al.* 1998). Briefly, three to five colonies of *C. albicans* grown for 24 to 48 h on potato dextrose agar (PDA) were suspended in 5 ml of saline, and the fungal suspension was counted using a hemocytometer (Glass Agencies,

Haryana, India). Dilutions yielded a starting inoculum of approximately 1×10^6 CFU/mL. *C. albicans* was exposed to two stilbenes and clotrimazole alone as well as in combinations. Test solutions were placed on a shaker and incubated at 37°C. At predetermined time points (0, 6, 12 and 24 h) after the incubation, 100 µl volumes were removed from each test suspension, serially diluted in sterile saline and plated on potato dextrose agar plates for colony count determination. Plates were incubated at 37°C for 24 h. The broth without any agent was used as the control for *C. albicans* growth at each time point. The data were plotted as log CFU/mL versus time (h) for each time point. Tests were performed three times.

Cytotoxicity test

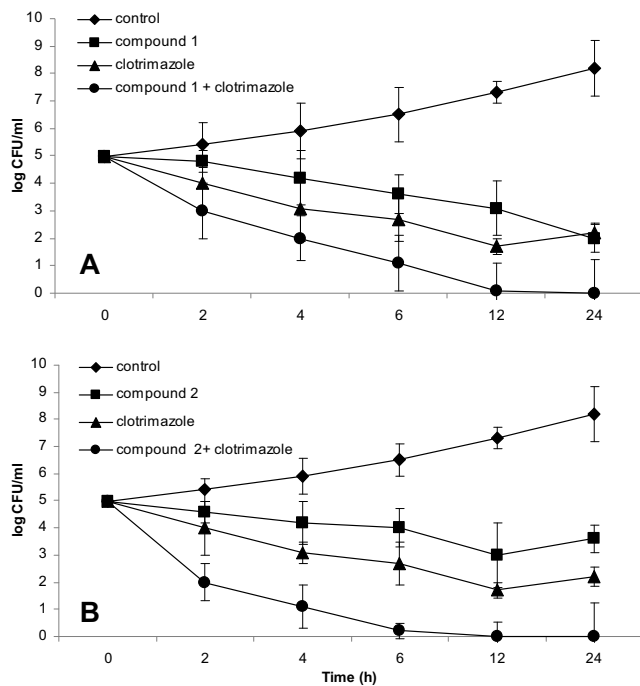
The MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay was used to determine the cytotoxicity of stilbenes. L231 lung epithelial cell and folliculo-stellate (FS) normal fibroblast cell line were used for testing. The MTT assay is based on the ability of mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and to form dark blue formazan crystals which are largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilization of the cells by the addition of a detergent results in the liberation of the crystals. The number of surviving cells is directly proportional to the level of the formazan product formed. The colour can then be quantified by a simple colorimetric assay using a multi-well scanning spectrophotometer (Bio-rad ELISA reader 680, California, USA). Briefly, cells (5×10^3 /well) were seeded in 0.2 mL of the medium (DMEM with 10% PBS) in 96-well plates, treated with drugs for 72 h. and after incubation, cytotoxicity was measured. For this after removing the drug containing media, 25 µL of MTT solution (5 mg/mL in phosphate buffered saline (PBS) and 75 µL of complete medium were added to wells (untreated and treated) and incubated for 2 h. At the end of incubation MTT lysis buffer was added to the wells (0.1 mL/well) and incubated for another 4 h at 37°C. At the end of incubation, the optical densities at 570 nm were measured using a plate reader (Bio-Rad). The relative cell viability in percentage was calculated (A_{570} of treated sample/ A_{570} of untreated sample) \times 100 (Anto *et al.* 2003).

Statistical analysis

All statistical analyses were performed with statistical packages for social sciences (SPSS) (Version 17.0; SPSS, Inc., Chicago, IL, USA). Data for time kill analysis was presented as means \pm standard deviations. Statistical significance was defined as $P < 0.05$ by Duncan's test.

Table 2 Synergistic effects of stilbenes with Clotrimazole against *Candida*.

Organism	Agent	MIC/MFC ($\mu\text{g/mL}$)		FIC ²	FICI	Outcome
		Alone	Combination ¹			
<i>C. albicans</i>	Compound 1	64/128	8/16	0.12/0.12	0.18/0.18	Synergistic/synergistic
	Clotrimazole	32/64	1/2	0.06/0.06		
<i>C. albicans</i>	Compound 2	32/64	2/4	0.06/0.06	0.18/0.18	Synergistic/synergistic
	Clotrimazole	64/128	2/4	0.12/0.12		

**Fig. 1** Time kill assay. Time kill curve of *Candida albicans* plotted as the number of remaining viable cells (CFU/mL) against time. (A) compound 1 and Clotrimazole, (B) compound 2 and Clotrimazole.

RESULTS

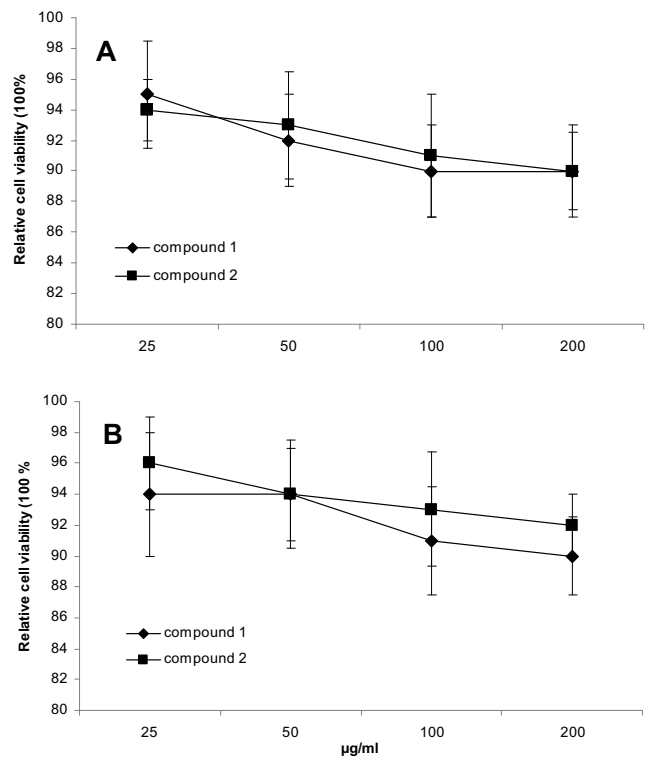
Synergistic activity of stilbenes and clotrimazole in combination and alone were presented in **Table 2**. MIC of compound 1 and clotrimazole was 64 and 32 $\mu\text{g/mL}$, respectively, whereas in combination the MIC was reduced to 8 and 1 $\mu\text{g/mL}$, respectively. Almost the same result was obtained for compound 2 and clotrimazole. The two stilbenes in combination recorded synergy and no additive or antagonistic effect was observed.

The time kill assay was conducted to determine the rates at which *C. albicans* was killed by exposing to stilbenes and clotrimazole (**Fig. 1**). For compound 1 and clotrimazole maximum reduction in the *C. albicans* growth was at 6 and 12 h. At 24 h this combination completely killed (99.9% reduction of the starting inoculum) the *Candida*. For compound 2 and clotrimazole maximum reduction in the *Candida* growth was at 6 h and completely killed *Candida* at 12 h. The time kill assay that demonstrates the rate of killing showed the compound 2 and clotrimazole to be more effective than compound 1 and clotrimazole. Regrowth was observed for *Candida* treated with clotrimazole alone after 12 h.

The cytotoxic activity of stilbenes was tested against FS normal fibroblast and L231 lung epithelial by MTT assay. The data showed that there is no significant reduction in the number of cells up to 200 $\mu\text{g/mL}$ (**Fig. 2**).

DICUSSION

Invasive fungal infections such as candidiasis have increased in prevalence worldwide over the last two decades, and consequently, the use of antifungal drugs increased (Lebouvier *et al.* 2007; Pereira-Cencil *et al.* 2008). Microbial and plant metabolites have led to a doubling of the

**Fig. 2** The cytotoxicity of stilbenes against normal human cell lines. (A) Folliculo-stellate (FS) normal fibroblast, (B) L231 lung.

human lifespan during the 20th century, reduced pain and suffering, and revolutionized medicine (Demain and Zhang 2005). Over the years, natural products have accounted for the majority of major therapeutic modalities and are currently in great demand for research purposes due to the huge and extensive biological properties which has medicinal and commercialization values. This success is largely due to their structural complexity and clinical specificity.

Invasive fungal infections such as candidiasis have increased in prevalence worldwide over the last two decades, and consequently, the use of antifungal drugs such as azoles has increased (Lebouvier *et al.* 2007; Pereira-Cencil *et al.* 2008). Triazole antifungal drugs such as fluconazole, and also imidazoles like ketoconazole, have significant roles in the treatment of candidiasis and other invasive fungal infections (Odds *et al.* 2003; He *et al.* 2007), but sometimes with the use of these agents, clinically important toxic effects such as skin rash, nausea, elevated liver enzyme (for fluconazole) gynecomastia, adrenal insufficiency and hepatotoxicity (for ketoconazole) are seen (Al-Mohsen and Hughes 1998; Cauffman and Lynch 2001). Overtime, under some clinical settings, the efficacy of azoles has decreased due to increased resistance to the antifungals (Rogers and Galgiani 1986; Odds *et al.* 2003).

Since 1970, this rate increased significantly due to more widespread use of immunosuppressive therapies, indiscriminate use of broad-spectrum antibacterial agents, the common use of indwelling intravenous devices and immunosuppressive viral infections such as AIDS. These developments and the associated increase in fungal infections necessitated the search for new, safer, and more potent agents to combat serious fungal infections (Beck-Sague *et al.* 1993). For nearly 30 years, clotrimazole, which causes

significant nephrotoxicity, was the sole drug available to treat serious fungal infections. But there is currently no information at all about the anticandidal activity of stilbenes in combination with clotrimazole. In the present study, stilbenes were synergistic with the clotrimazole against *C. albicans*.

The combined effect of stilbenes and clotrimazole exhibited good synergistic activity towards *C. albicans*. For nearly 50 years, clotrimazole has been employed as a potent fungicidal agent to treat many serious fungal infections. However, the use of clotrimazole is limited because of high toxicity to the patient such as in bringing about haemolytic effect (Adams and Kwon 2003). Combined effect study of stilbenes and clotrimazole reduced the amount of both compounds and this will reduce the side effects caused by clotrimazole to the patients. The cytotoxicity study of stilbenes also recorded nil effect. This clearly indicated that stilbenes are safe for the treatment of *candida*.

The results from the present study warrant further investigations, on the possible synergistic effects of stilbenes with another type of antifungal drugs. The observed synergism between stilbenes and clotrimazole *in vitro* should also be investigated in an *in vivo* animal model of candidiasis. Moreover, further experiments could be performed in order to elucidate the molecular mechanisms underlying this synergistic effect.

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