

INVESTIGATION OF ANTIFUNGAL ACTIVITY OF STILBENES ALONE AND IN COMBINATION WITH FLUCONAZOLE AGAINST *CANDIDA ALBICANS*

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ABSTRACT

Objective: The objective of the present study was to evaluate the synergistic activity of two stilbenes in combination with fluconazole against *Candida albicans*.

Methods: 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 synergistic interaction with the fluconazole, in the treatment of *C. albicans in vitro*. The cytotoxicity of stilbenes was also tested against normal human cell line (FS normal fibroblast and L231 lung epithelial cell line).

Results: Our results demonstrated that significant synergistic antifungal activity exists between the two stilbenes and fluconazole against *C. albicans*. The time kill assay also supports the synergistic activity. No cytotoxicity was recorded for stilbenes and stilbenes plus fluconazole up to 200 µg/mL. But fluconazole alone recorded significant cytotoxicity.

Conclusion: These results suggest that stilbenes combined with antibiotics may be microbiologically beneficial. These findings have potential implications in delaying the development of resistance as the anticandidal effect is achieved with lower concentrations of both drugs.

Keywords: Anticandidal activity, Normal human cells, Stilbenes, Fluconazole

INTRODUCTION

Candidiasis, the main opportunistic fungal infection has steadily increased over the past 30 years [1-2]. Among the many species, *Candida albicans* is the most important pathogen. Oral candidiasis is an oral lesion is one of main pathological phenomena caused by this organism. Oral candidiasis is most commonly associated with people infected with the human immunodeficiency virus (HIV), infants (oral thrush), patients with diabetes mellitus and those receiving broad-spectrum antibiotic treatment. But mostly oral candidiasis is uncommon in the general population, despite the fact that *Candida albicans* can be recovered in the alimentary canal of healthy individuals in over 50% of cases [3]. *Candida* spp. are the fourth most common agent of hospital-acquired bloodstream infections [4-5]. Specifically, *Candida 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 [6]. Treatment of this fungal infection presents several problems. Despite the extensive usage of fluconazole in HIV-infected patients, little is known about the emergence of microbial resistance to this topically administered fluconazole. 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 fluconazole. Azoles disrupt ergosterol biosynthesis in fungi resulting in the formation of cell membrane with altered structure and function [7]. However, the choices are still limited, especially due to the resistance because of the increase in the use of drugs [3]. With 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 [8]. *Xenorhabdus* and *Photorhabdus* are known to

produce a wide range of bioactive metabolites [9]. In the course of studies on EPN, a new entomopathogenic nematode belonging to the genus *Rhabditis* and subgenus *Oscheius* 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 [10]. In the present study, in vitro anticandidal activity of stilbenes alone and in combination with fluconazole against *Candida 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 (Oscheius)* sp. and chemical structures of the compounds were established on the basis of spectral analyses [11]. Fluconazole (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 *Candida albicans* MTCC 277, which were procured from the Microbial Type Culture Collection and Gene Bank (MTCC) Division, CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India. 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.

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 [12].

Synergy study (checkerboard method)

Combinations of stilbenes and fluconazole were tested by the checkerboard method against the *C. albicans* in potato dextrose broth [13].

The stilbenes and fluconazole were mixed in 1:1 ratio. The combined study for *C. albicans* was tested in triplicates. The concentration of the individual compound in the combination of stilbenes and fluconazole 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 [14]:

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

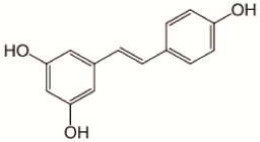
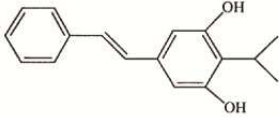
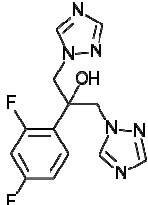
The sum of fractional inhibitory concentration (FICs) indices of two compounds in the combination was calculated as follows: FIC_a + FIC_b = FICs.

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 [15].

Time kill assay

The potential of compound carryover during the plating process were determined by following [16]. Briefly, three to five colonies of *Candida* isolates grown for 24 to 48 h on SDA were suspended in 5ml of saline, and the fungal suspension was counted using a hemacytometer. Dilutions yielded a starting inoculum of approximately 1×10^6 CFU/mL. *C. albicans* was exposed over time to two stilbenes and fluconazole alone as well as to their 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, 100µl 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 *Candida* 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.

Table 1: Stilbenes and fluconazole 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 |  |
| Fluconazole | Azoles | Ergosterol |  |

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, fluconazole and stilbenes plus fluconazole. FS normal fibroblast and L231 lung epithelial cell lines were used for testing. 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 color 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 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 x100) [17].

Statistical analysis

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

RESULT AND DISCUSSION

Synergistic activity of stilbenes and fluconazole in combination and alone were presented in Table 2. MIC stilbene 1 and Fluconazole was 64 µg/mL and 32 µg/mL, respectively, whereas in combination the MIC was reduced to 8 µg/mL and 1 µg/mL respectively. Almost the same result was obtained for stilbene 2 and fluconazole. The two stilbenes in combination recorded synergy and no additive or antagonistic effect was observed.

Table 2: Synergistic effects of stilbenes with fluconazole against *Candida*

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

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

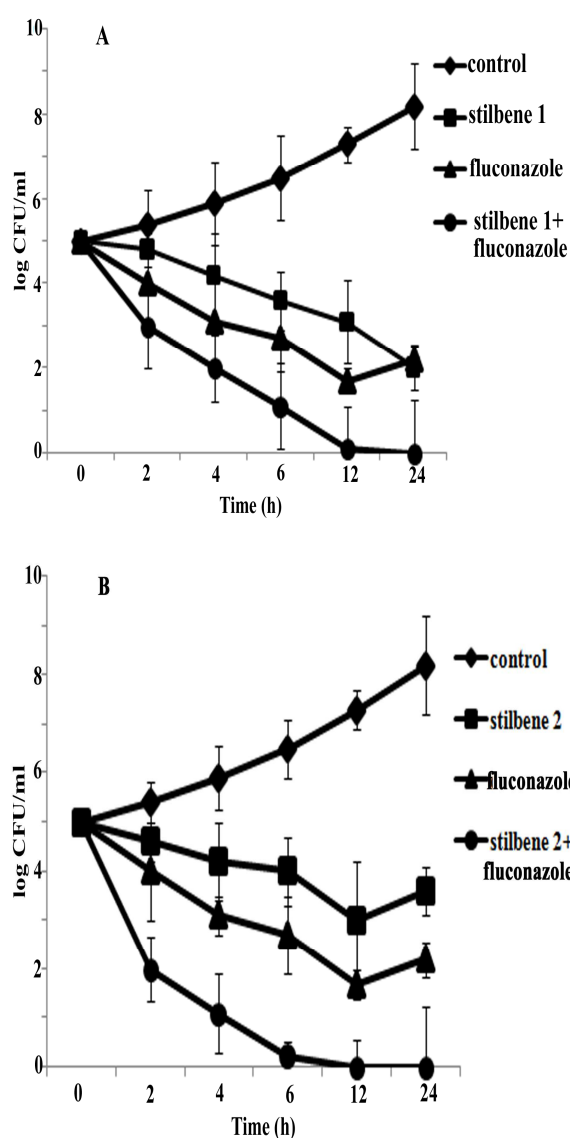


Fig. 1: Time kill assay. Time kill curve of *C. albicans* plotted as the number of remaining viable cells (CFU/mL) against time. [A]- Stilbene 1 and Fluconazole, [B]- stilbene 2 and Fluconazole.

The cytotoxic activity of stilbenes, fluconazole and stilbenes plus fluconazole was tested against FS normal fibroblast and L231 lung epithelial cell line by MTT assay. The data showed that there is no significant reduction in the number of cells up to 200 $\mu\text{g/mL}$ for stilbenes and stilbenes plus fluconazole (Fig 2a&b). But fluconazole alone recorded significant cell death from 25 $\mu\text{g/mL}$.

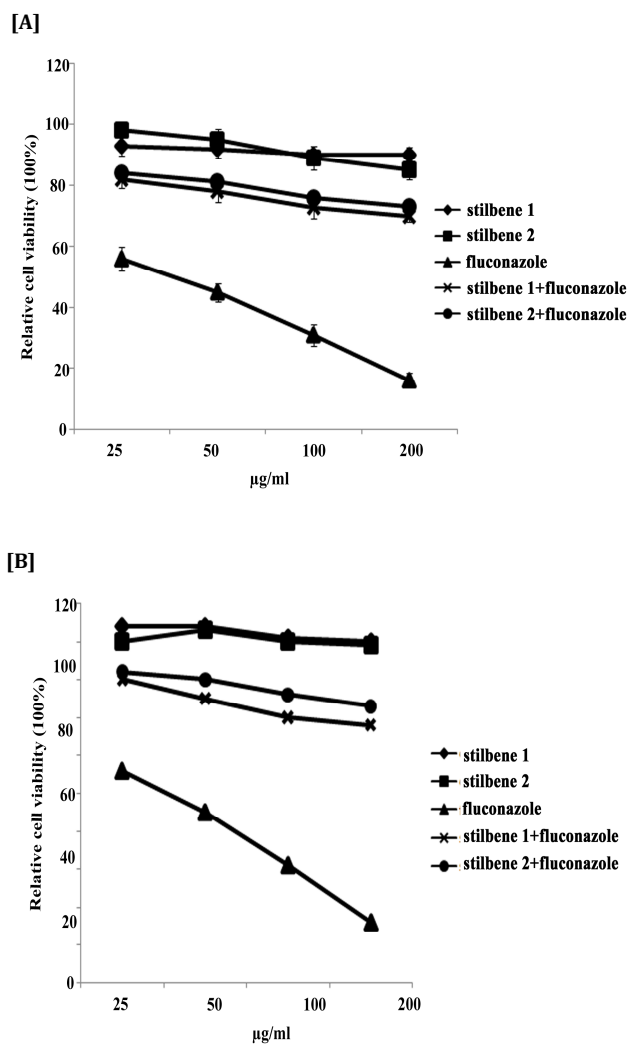


Fig.2: The cytotoxicity of stilbenes against normal human cell lines [A] FS normal fibroblast and [B] L231 lung epithelial cell line

Invasive fungal infections such as candidiasis have increased in prevalence worldwide over the last two decades, and consequently, the use of antifungal drugs increased [18-19]. Microbial and plant metabolites have led to a doubling of the human lifespan during the 20th century, reduced pain and suffering, and revolutionized medicine [20]. 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 [19]. Triazole antifungal drugs such as fluconazole, and also imidazoles like ketoconazole, have significant roles in the treatment of candidiasis and other invasive fungal infections [7,21], 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 [22-23]. Overtime, under some clinical settings, the efficacy of azoles has decreased due to increased resistance to the antifungals [7,24]. Since 1970, the rate of *Candida* infection increased significantly due to widespread use of immunosuppressive therapies, uncontrolled use of broad-spectrum antibacterial agents, the use of intravenous devices and AIDS. Because of the drug resistance and increase in fungal infections necessitated the search for new, safer, and more potent antifungal agents with novel mechanism of action to treat serious fungal infections [25]. For nearly 30 years, azoles, which causes significant nephrotoxicity, were the sole drug available to treat many serious fungal infections. But there is currently no information at all about the anticandidal activity of stilbenes in combination with fluconazole. In the present study, stilbenes were synergistic with the fluconazole against *Candida* sp. The combined effect of stilbenes and fluconazole exhibited good synergistic activity towards *C. albicans*. For nearly 50 years, fluconazole has been employed as a potent fungicidal agent to treat many serious fungal infections. However, the use of fluconazole is limited because of high toxicity to the patient such as in bringing about the hemolytic effect [26]. Combined effect study of stilbenes and fluconazole reduced the amount of both compounds and this will reduce the side effects caused by fluconazole to the patients. The cytotoxicity study of stilbenes also recorded nil effect. This clearly indicated that stilbenes are safe for the treatment of *candida*.

CONCLUSION

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 fluconazole *in vitro* should also be investigated in *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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