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Research Article

IN VITRO INVESTIGATION OF ANTIFUNGAL ACTIVITY OF STILBENES ALONE AND IN COMBINATION WITH AMPHOTERICIN B 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 amphotericin B, but increased use of these drugs has led to a selection of yeasts 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 amphotericin B, in the treatment of *C. albicans in vitro*. Our results demonstrated that significant synergistic effect exists between stilbenes and Amphotericin B 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 FS normal fibroblast) and no cytotoxicity was recorded for stilbenes up to 200 µg/mL.

Keywords: Stilbenes, Amphotericin B, Synergistic, *C. albicans.*

INTRODUCTION

Nowadays, fungal infections such as candidiasis are becoming more prevalent in normal and immunocompromised hosts[1]. *Candida* spp. are the fourth most common agent of hospital-acquired bloodstream infections[2,3]. Treatment of this fungal infection presents several problems. For nearly 30 years, amphotericin B, which causes significant nephrotoxicity and other side effects, was the sole drug available to treat *Candida* spp. Besides the toxicity presented by amphotericin B, the widespread use of antifungal agents induced resistance to amphotericin B[4,5]. However, the choices are still limited, especially due to the resistance because of the increase in the use of drugs.

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[6]. *Xenorhabdus* and *Photorhabdus* are known to produce a wide range of bioactive metabolites[7]. 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[8].

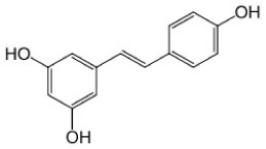
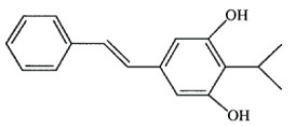
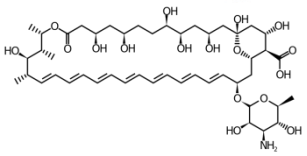
In the present study, in vitro anticandidal activity of stilbenes alone and in combination with amphotericin B against *Candida albicans* was investigated.

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. Amphotericin B (Sigma Aldrich) (Table 1) was used as a standard anticandidal agent.

Table 1: Stilbenes and Amphotericin B used in this study

S. No.	Antibiotics and stilbenes	Antimicrobial class	Target	structure
1	3,4',5-trihydroxystilbene	Poly phenols	Unknown	
2	3,5-dihydroxy-4-isopropylstilbene	Poly phenols	Unknown	
3	Amphotericin B	Polyene	Ergosterol binding	

Test organism

The *Candida* strain used in the study was *Candida albicans* MTCC 277 and was subcultured in potato dextrose agar and broth (Himedia Laboratories Limited, Mumbai, India) at 37ºC 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 37ºC 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[9].

Drug interaction study (checkerboard method)

Combinations of stilbenes and amphotericin B were tested by the checkerboard method against the *C. albicans* in potato dextrose broth[10]. The stilbenes and amphotericin B are 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 amphotericin B 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:

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[11].

Time kill assay

The potential of compound carryover during the plating process were determined by following[12]. Dilutions yielded a starting inoculum of approximately 1×10^6 CFU/mL. *C. albicans* was exposed over time to two stilbenes and amphotericin B alone as well as to their combinations. Tests were performed at 0, 2, 4, 6, 12 & 24 h. 100 µL samples 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.

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 FS normal fibroblast cell line 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 (ELISA reader). Briefly, cells (5x10^3 /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)[13].

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 $p< 0.05$.

RESULTS

The synergistic activity of stilbenes and amphotericin B in combination and alone were presented in Table 2. For compound 1 MIC was 64 µg/mL and Amphotericin B 64 µg/mL, whereas in combination the MIC was reduced to 8 µg/mL and 4 µg/mL respectively. Almost same result was obtained for compound 2 and amphotericin B. 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 *Candida* was killed by exposing to stilbenes and amphotericin B (Fig 1). For stilbene 1 and amphotericin B maximum reduction in the Candidal growth was at 12 and 24 h. At 24 h this combination completely killed (99.9 % reduction of the starting inoculum) the *Candida.* For stilbene 2 and amphotericin B maximum reduction in the Candidal growth was at 6 and 12 h and completely killed *Candida* at 12 h. The time kill assay that demonstrates the rate of killing showed the compound 2 and amphotericin B to be more effective than compound 1 and amphotericin B. Regrowth was observed for *Candida* treated with amphotericin B alone after 12 h.

The cytotoxic activity of stilbenes was tested against FS normal fibroblast and L231 by MTT assay. The data showed that there is no significant reduction in the number of cells up to 200 µg/mL (Fig 2).

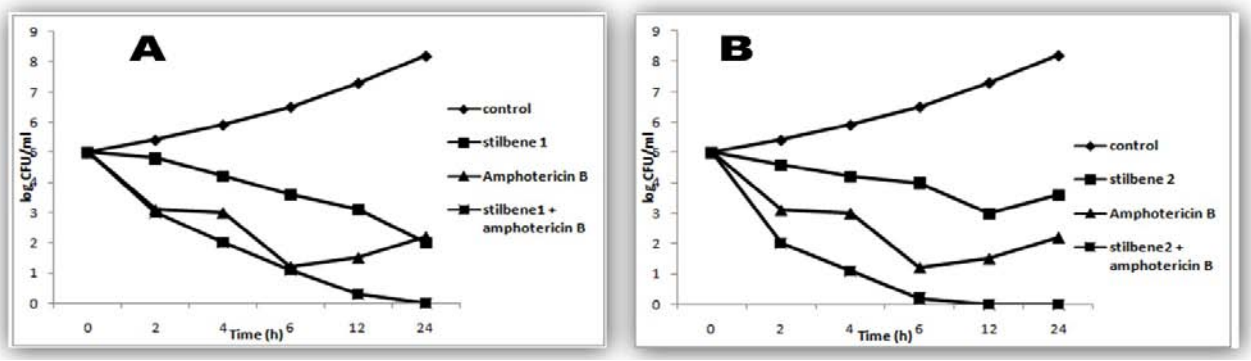


Fig. 1: Time kill assay. Time kill curve of *C. albicans* plotted as the number of remaining viable cells (CFU/mL) against time. [A]- stilbene 1 and Amphotericin B, [B]- stilbene 2 and Amphotericin B.

Table 2: Synergistic effects of stilbenes with Amphotericin B against *candida*

Organism	Agent	MIC/MFC (µg/mL)		FIC[2]	FICI	Outcome
		Alone	Combination[1]			
C. albicans	Compound 1	64/128	8/16	0.12/0.12	0.18/0.18	Synergistic/synergistic
	Amphotericin B	64/128	4/8	0.06/0.06		
C. albicans	Compound 2	32/64	2/4	0.06/0.06	0.18/0.18	Synergistic/synergistic
	Amphotericin B	64/128	8/16	0.12/0.12		

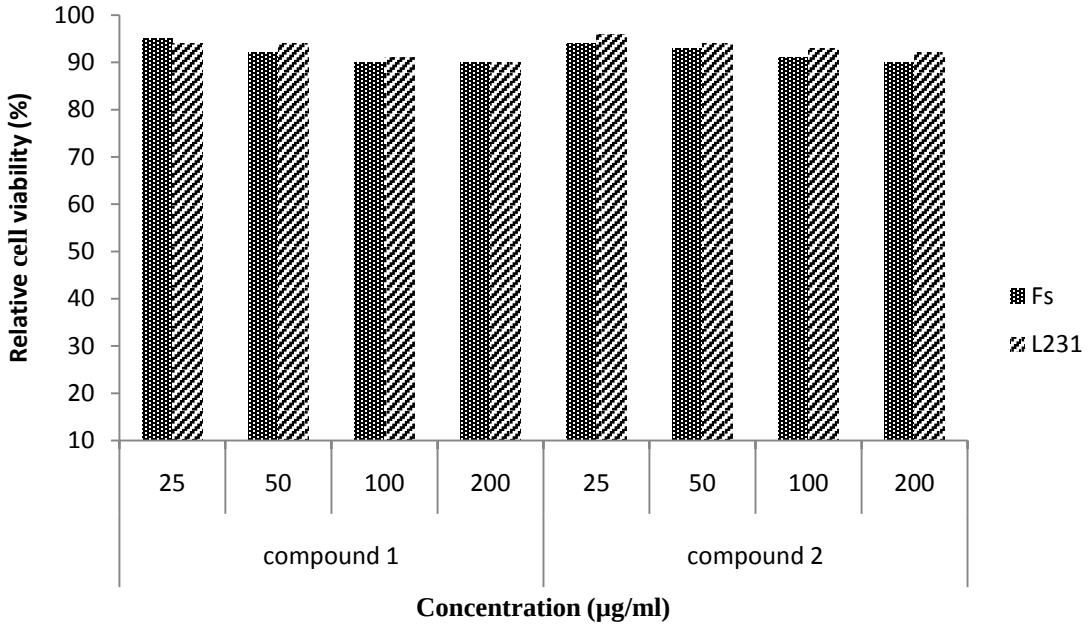


Fig. 2: The cytotoxicity of stilbenes against normal human cell lines. Histogram represents the relative cell viability in percentage determined by MTT assay.

DISCUSSION

Invasive fungal infections such as candidiasis have increased in prevalence worldwide over the last two decades, and consequently, the use of antifungal drugs increased[14,15]. Microbial and plant metabolites have led to a doubling of the human lifespan during the 20th century, reduced pain and suffering, and revolutionized medicine[16]. 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.

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[17]. For nearly 30 years, amphotericin B, 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 amphotericin B. In the present study, stilbenes were synergistic with the amphotericin B against *Candida* sp.

The combined effect of stilbenes and amphotericin B exhibited good synergistic activity towards *C. albicans*. For nearly 50 years, amphotericin B has been employed as a potent fungicidal agent to treat many serious fungal infections. However, the use of amphotericin B is limited because of high toxicity to the patient such as in bringing about hemolytic effect[18]. Combined effect study of stilbenes and amphotericin B reduced the amount of both compounds and this will reduce the side effects caused by amphotericin B 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 amphotericin B *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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