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ANTIFUNGAL ACTIVITY OF STILBENES AGAINST *CANDIDA ALBICANS* BY TIME KILL ASSAY

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ABSTRACT

Candida albicans is one of the most important fungi associated with oral candidiasis and the treatment of this fungi is a serious problem today because of the resistance of these fungi against conventionally used drugs. So, there is an urgent need of alternative antifungal substances especially from the natural sources. The study was conducted to examine the MFC and time kill activity of two stilbenes [3, 4', 5-trihydroxystilbene (1) and 3,5-dihydroxy-4-isopropylstilbene (2)] purified from a bacterium associated with entomopathgenic nematode against *C. albicans.* The activity was also compared to amphotericin B. The cytotoxicity of stilbenes was also tested against normal human cell lines (L231 lung epithelial and FS normal fibroblast). Results showed that stilbenes was effective against *C. albicans* with MIC and MFC of 64 and 128 µg/ml for compound 1 and 32 and 64 µg/ml for compound 2. The time kill assay of stilbenes against *C. albicans* was more effective than amphotericin B. No cytotoxicity was recorded for stilbenes upto 200 µg/ml. The strong antifungal activity and low cytotoxicity of stilbenes make it a promising agent for the treatment of *Candida*.

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 and oral candidiasis is an oral lesion caused by this organism. It has been estimated that more than 90% of human immunodeficiency virus (HIV) infected patients develop oral candidiasis often debilitating infection at some time during the progression of their disease [3, 4].

Infections caused by *C. albicans* in immune competent individuals may include oral thrush, vulvar rash, vaginitis, conjunctivitis, endophthalmitis, diaper rash, and infections of the nail, rectum, and other skin folds [5]. *Candida*s were severely affected in the treatment of patients with acute [6].

Candida spp. are the fourth leading cause of nosocomial infections in the USA and elsewhere in the world, and in those patients with candidemia, the attributable mortality rate is up to 35% [7]. Treatment of this fungal infection presents several problems. Besides the toxicity presented by amphotericin B, the widespread use of antifungal agents induced resistance to amphotericin B [8, 9].

Therefore, the clear understanding of the pharmaco-dynamic properties of antifungal agents is important for the improvement of the treatment of oropharyngeal candidiasis. However, the choices are still limited, especially due to the resistance because of the increase in the use of drugs. For this reason identification of new drugs from natural sources are needed to fight against the candidal infection in future.

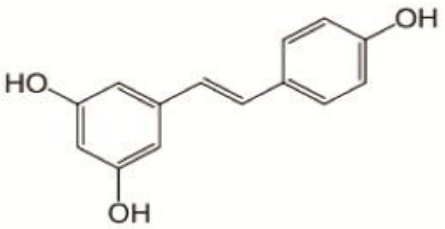
Bacteria of the genera *Xenorhabdus* and *Photorhabdus* are known to be symbiotically associated with the soil dwelling entomopathogenic nematodes (EPN) of the family steinernematidae and heterorhabditidae respectively [10]. *Xenorhabdus* and *Photorhabdus* are known to produce wide range of bioactive metabolites [11].

In the course of studies on EPN, a new entomo-pathogenic 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 [12].

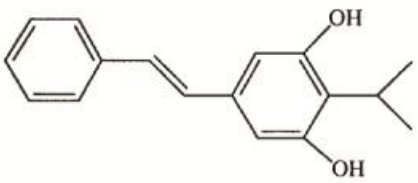
In this paper, we reported the MFC and time kill activity of two stilbene compounds against *C. albicans*. The activity of stilbenes was also compared with that of amphotericin B, an antifungal agent commonly used.

MATERIALS AND METHODS:

Test compounds: The test stilbene compounds [3,4',5-trihydroxystilbene (1) and 3,5-dihydroxy-4-isopropylstilbene (2) (**Fig. 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.



(A)



(B)

FIG. 1: STRUCTURE OF STILBENES (A) 3, 4', 5- TRIHYDROXY STILBENE, (B) 3, 5-DIHYDROXY-4-ISOPROPYLSTILBENE

Candidal culture and Antifungal Agent: 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 37°C. Amphotericin B (Sigma) was used as a standard anticandidal agent.

Antifungal assay: The macro broth dilution method was adopted to determine the susceptibility of stilbenes against *C. albicans*. The stock solutions of the stilbenes were dissolved in DMSO. The tubes containing potato dextrose broth of varying concentrations of stilbenes were inoculated with actively growing *C. albicans*. The initial density of the *Candida* was 3×10^6 colony forming unit (CFU)/ml. After 24 h incubation, the growth was monitored both visually and calorimetrically (600 nm). The MIC was defined as the lowest concentration required to arrests the growth of the fungi at the end of 24 h. For determining MFC, a 0.01 ml aliquot of the culture medium was drawn from the tubes showing no visible growth at the end of the 24 h incubation and plated on potato dextrose agar. The plates were incubated further for the appearance of fungal colonies. The MFC was defined as the lowest concentration of the antifungal agent that reduced the number of visible organisms by 99.9% [13].

Time Kill Assay: The potential of compound carryover during the plating process were determined by following [14]. Dilutions yielded a starting inoculum of approximately 1×10^6 CFU/ml. The antifungal activity of compounds was studied over a range of multiples of MIC ie. 1×MIC to 4×MIC. Tests were performed at 0, 2, 4, 6, 12 & 24 h. 100 µl sample was 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 colour 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) [15].

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 $p< 0.05$.

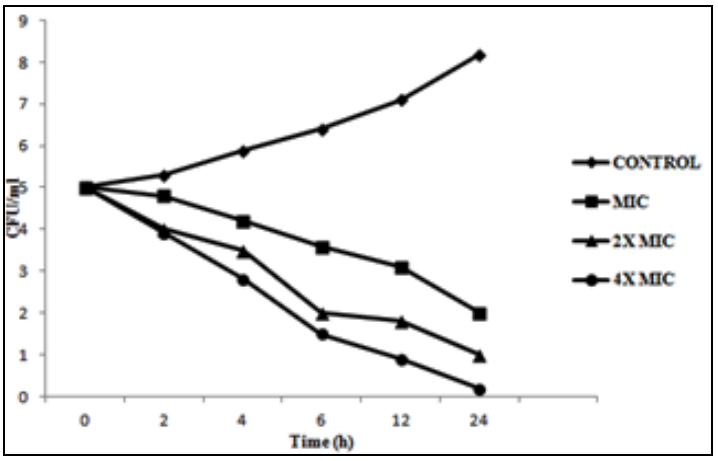
RESULTS: MIC and MFC values obtained for stilbenes and amphotericin B were presented in **Table 1**. The compound 1 was active against *C. albicans* with the MIC and MFC of 64 and 128 µg/ml, whereas the compound 2 was active against *C. albicans* with the MIC and MFC of 32 and 64 µg/ml. Amphotericin B was used as a positive antifungal agent in the study with an MIC and MFC of 64 and 128 µg/ml. The result indicated that MIC and MFC values of the compound 2 were better than amphotericin B. But for the compound 1 MIC and MFC values are equal to that of amphotericin B.

TABLE 1: THE MIC AND MFC (µg/ml) OF STILBENES AGAINST *C. ALBICANS* COMPARED WITH AMPHOTERICIN B

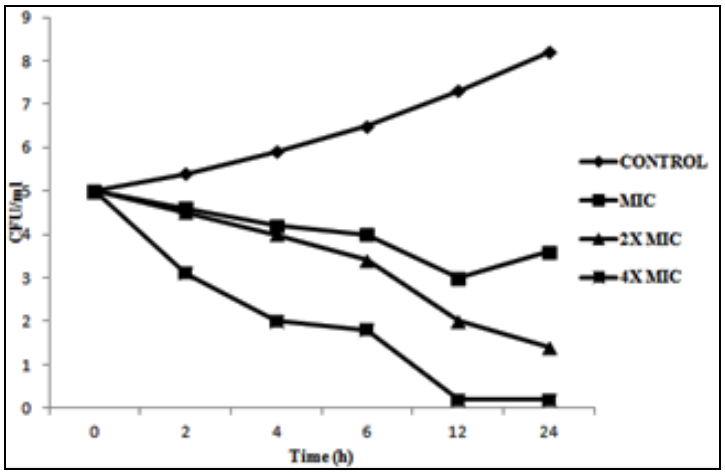
	Compound 1		Compound 2		Amphotericin B	
Pathogen	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans	64	128	32	64	64	128

The time kill assay was conducted to determine the rates at which *Candida* was killed exposed to stilbenes and amphotericin B (**Fig. 2**). At 6 h, the group treated with compound 1 at 2×MFC and 4×MFC showed a dramatic decrease in the viable cell counts, whereas the group treated with compound 2 at 2×MFC and 4×MFC showed the reduction in viable cell count only after 12 h. But amphotericin B showed the reduction of viable cell count at 12 h at 4×MFC. At 24 h the compound 1 completely killed (99.9 % reduction of the starting inoculum) the *Candida*, whereas compound 2 completely killed the *C. albicans* at 12 h.

But amphotericin B showed the maximum deduction at 12 h and regrowth was observed after 12 h. No regrowth was observed for the stilbenes which clearly showed that the activity produced by the stilbenes is long lasting. The time kill assay that demonstrates the rate of *Candida* killing showed the compound 2 to be more effective than compound 1 and amphotericin B. The time kill assay against *Candida* demonstrated that stilbenes were more effective than amphotericin B.



A



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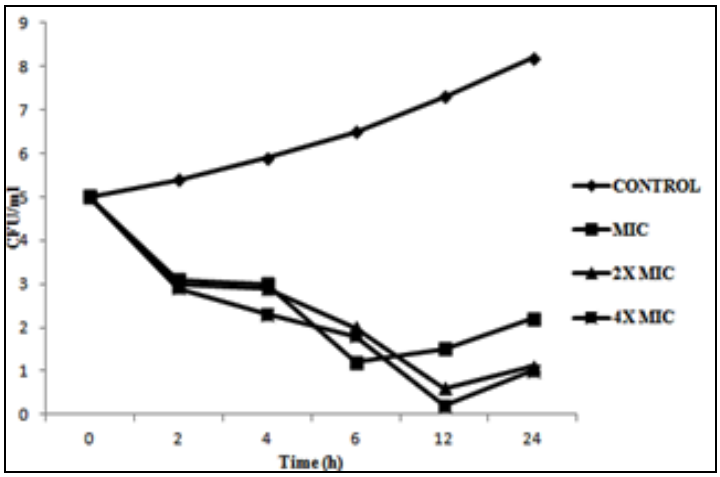

C

FIG. 2: TIME KILL ASSAY. TIME KILL CURVE OF *C. ALBICANS* PLOTTED AS THE NUMBER OF REMAINING VIABLE CELLS (CFU/ML) AGAINST TIME

A- Compound 1, B- Compound 2 and C- Amphotericin B

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. 3**).

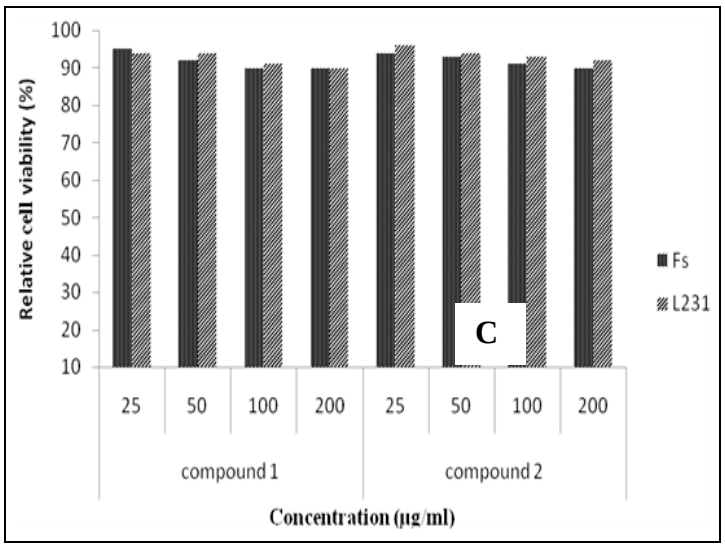

FIG 3: THE CYTOTOXICITY OF STILBENES AGAINST NORMAL HUMAN CELL LINES. HISTOGRAM REPRESENTS THE RELATIVE CELL VIABILITY IN PERCENTAGE DETERMINED BY MTT ASSAY

DISCUSSION: Candidal yeasts constitute a part of the normal oral flora and are considered as harmless commensal microbes in the oral cavity. However, this opportunistic yeast species could induce oral infections when the ecological balance is disturbed causing changes in host defense mechanisms [16]. Oral candidiasis is most commonly associated with individuals infected with the human immunodeficiency virus, and it is also seen in infants, patients with diabetes mellitus, and those receiving broad-spectrum antibiotics [17]. The emergence of resistant candidal strains towards antifungal agents has increased great concern among scientists, medical and dental practitioners, and this has lead to the search of new antifungal agents from natural resources especially from microbes. Natural products are currently in great demand for research purposes due to the huge and extensive biological properties which has medicinal and commercialization values.

The results of this study have elucidated that stilbenes exhibited good antifungal activity towards *C. albicans* species compared to amphotericin B. For the time kill assay, we compared the antifungal activity of stilbenes with that of amphotericin B in order to find out a new natural antifungal agent. The anticandidal activity of compound 1 was previously reported by [18] and in our study also observed the similar pattern of results. The Strong antifungal activity of compound 2 against *Aspergillus flavus, Candida tropicalis* etc. was reported by [19].

The activity of compound 2 was more effective than amphotericin B. 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 [20]. For the time kill assay, we compared the antifungal activity of stilbenes with that of amphotericin B in order to find out the efficiency of the stilbene compounds against oral candida.

The time killing activity of compound 2 was more effective than compound 1 and amphotericin B. However we found that regrowth was observed for amphotericin B after 12 h. This might be due to the onset of these agents was not longer than 12 h. On the other hand, stilbenes exert rapid action even after 12 h. This property makes stilbenes a promising antifungal agent against oral candida. Furthermore, additional information from further studies on the mechanism of action of stilbenes, might contribute to its use as an alternative to the conventional antifungal drugs for the management of candidiasis. Therefore, stilbenes have a great potential to be considered in the development of oral health care products.

The emergence of higher resistance level of *C. albicans* and the non-*C. albicans* (NAC) species towards many commonly prescribed antifungal agents has become a great concern to society [21]. This is said to be due to the consequence of the heavy usage of antifungal agents such as amphotericin B and fluconazole [22]. Therefore, there is an increased need in obtaining alternative antifungal substances which can act effectively against oral *Candida* sp.

CONCLUSION: In conclusion, the present study showed that stilbenes were effective against *C. albicans.* Regarding cytotoxicity stilbenes was not toxic to normal human cell lines. Thus, the strong, rapid antifungal activity may make it a good candidate drug molecule against *C. albicans.*

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