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Original Article

Microbiology Section

In-*vitro* Inhibition of Biofilm Formation in *Candida albicans* and *Candida tropicalis* by Heat Stable Compounds in Culture Filtrate of *Aspergillus flavus*

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ABSTRACT

Background: Invasive candidiasis, caused mostly by *Candida albicans* and *C. tropicalis* is one of the most common causes of bloodstream infection with a substantial attributable mortality. This disease is associated with formation of structured, multilayered microbial communities known as biofilms over indwelling devices. Treatment is rendered difficult owing to factors like poor drug penetration through biofilms and high cost of the available antifungal drugs. Hence there is imminent need of developing low-cost natural compounds inhibiting Candidal biofilm formation in vitro. Organohalgen compounds derived from crude culture filtrate of *Aspergillus flavus* have been documented to impair in vitro Candidal survival.

Aim: We aimed to detect the effect of preheated and unheated crude culture filtrate of *Aspergillus flavus* on biofilm formation of *Candida albicans* and *C. tropicalis* in vitro.

Setting and Designs: Ours was a laboratory-based observational study with clinical isolates of the microorganisms selected randomly.

Material and Methods: In this study, we showed for the first time by microtitre plate method that heat stable compounds which were present in preheated and unheated culture filtrates of *Aspergillus flavus* inhibited biofilm formation of *Candida albicans* and *C. tropicalis* and also lipase activities of these pathogens, and filtrate was non-toxic on human cell line as checked microscopically.

Statistical Analysis used: Z-test of significance was used to calculate significant difference between Candidal biofilm formation in normal liquid medium and culture filtrate, respectively.

Results and Conclusion: Heat stable compounds present in culture filtrate of *Aspergillus flavus* inhibit biofilm formation of *Candida albicans* and *C. tropicalis* and also in-vitro lipase activity of these pathogens and could pave the way for development of low-cost alternatives to treat invasive candidiasis.

Key words: Invasive candidiasis, Biofilm, *Candida spp*, *Aspergillus flavus*

INTRODUCTION

Invasive candidiasis is an emerging disease in developing countries and is now regarded as the fourth most common cause of bloodstream infection in developed countries and the third commonest cause of systemic infection in the Intensive Care Units (ICUs) [1]. According to literature available from developing as well as developed countries, *Candida albicans* and *C. tropicalis* are the two commonest species involved in invasive infection by this genus [2]. This disease entity is associated with the formation of structured, complex microbial communities known as biofilms [3]. Treating biofilm-associated invasive candidiasis is very difficult in developing countries since drugs available are prohibitively costly and have their own adverse effects [4]. Also, drug penetration through fungal biofilms is diminished due to formation of a reaction-diffusion barrier [4,5]. In addition, increase in cases of candidemia due to species other than *Candida albicans* and growing resistance to azole antifungal compounds have aggravated the problem [6]. Due to these factors, there is an urgent need of low-cost alternatives and naturally occurring compounds that are intended to inhibit or impair biofilm formation in *Candida albicans* and Candida tropicalis. Organochlorine compounds like Aspirochlorine, derived from *Aspergillus flavus* have shown significant activity against *Candida albicans* [7]. Lipase enzyme production plays an important role in pathogenesis of invasive infection caused by *Candida albicans* and Candida tropicalis in addition to biofilm formation [8]. Keeping these points in mind, we aimed to study the effect of crude culture filtrate of *Aspergillus flavus*, both plain and unheated, on biofilm formation and lipase inhibition of *Candida albicans* and *C. tropicalis*. We also aimed to detect toxic effect of this filtrate on host cell line (Hep-2 or Human laryngeal epithelioma cell line).

Previous study by the authors on inhibition of candidal biofilms by Pyocyanin obtained from *Pseudomonas aeruginosa* [9].

MATERIAL AND METHODS

Type, time and place of study:- This was a laboratory-based observational study, carried out in the Department of Microbiology, King George's Medical University, Lucknow, Uttar Pradesh, India from July 2011 to January 2013.

Isolation and identification of the microorganisms:- *Candida albicans*, Candida tropicalis and *Aspergillus flavus* isolates were recovered from different clinical samples received in the laboratory, like blood, urine and sputum by culturing them on SDA (Saboraud's Dextrose Agar with Emmon's modification). *Candida albicans* and *C. tropicalis* were identified by Dalmau slit inoculation technique, Germ tube test and sugar fermentation and assimilation tests. *Candida albicans* was identified by positive germ tube test, single terminal chlamydospore formation by Dalmau technique after 48 hours at 25^{0}C and positive fermentation and assimilation of glucose and maltose with production of acid and gas, while *C. tropicalis* was identified by negative germ tube test, no chlamydospore formation with positive pine-forest like pseudohyphal pattern by Dalmau technique and positive fermentation of glucose, maltose and sucrose with formation of acid, gas and thick surface pellicle [9]. Ten(10) isolates each of *Candida albicans*, *Aspergillus flavus* and *C. tropicalis* were randomly selected for this study out of these isolates.

Test for Biofilm formation in *Candida spp*.:- In the given study, the microtitre plate model was employed for in vitro formation and demonstration of biofilms Ramage et al., [10]. At first, yeast isolates were suspended in YPD liquid medium (1% yeast extract, 2% peptone, 2% dextrose, all weight/volume) overnight and incubated at 37^{0}C (1 loopful of yeast colony in 5 ml of YPD broth). *Aspergillus flavus* isolate (1 loopful in 5 ml) was inoculated onto PBST(Phosphate Buffered Saline with 0.02% Tweeen-20, weight/volume) containing 10% serum and incubated at 37^{0}C for 3 days. Then culture filtrate was prepared by centrifuging the suspension at 3000 rpm for 5 minutes and passing the supernatant through membrane (syringe) filter of pore size 0.22 µm (Micro-Por Minigen Syringe Filter, Genetix Biotech Asia, New Delhi). One part of this filtrate was left unheated and another part was heated at 100^{0}C for 20 minutes and subsequently cooled. Yeast cell turbidity was adjusted to 106 cells/ml in a) YPD broth, b) unheated culture filtrate, c) heated culture filtrate. Then 100 µl of each of these suspensions was dispensed in wells of a 96-welled flat-bottomed polystyrene microtitre plate (Nunclon A/S, Kampstrupvej, Denmark), keeping one well with 100µl of physiological saline as blank control. Following an incubation of 90 minutes at 37^{0}C, wells were washed 3 times by PBS (Phosphate Buffered Saline, pH 7.2) to remove non-adherent cells and reloaded with respective liquid media (YPD broth, unheated and heated culture filtrate) and incubated again. Washing and reloading were repeated at intervals of 24 and 48 hours. After that, the wells were washed and stained with 100 µl of 1% safranine (weight/volume) in 95% ethanol. After staining for 1 minute, excess stain was washed thrice with PBS and the wells were observed under inverted microscope at 20X magnification. Reading of wells was also noted in a spectrophotometer at 450 nm wavelength (iMark MicroPlate reader, Bio-Rad,USA).

Assay for lipase activity:- For detection of lipase, *Candida albicans* and *C. tropicalis* isolates were suspended in YPD broth(1 loopful in 2 ml YPD), incubated overnight at 37^{0}C and 1 loopful was streaked to Lipase agar, prepared as described by Muhsin et al., [11]. Thereafter the inoculated media were incubated at 25^{0}C for 2 days. A positive lipase activity was defined as production of visible opaque zone of haziness around the colonies due to formation of a complex by released fatty acids and Calcium chloride (CaCl2). The yeast isolates were also suspended similarly in unheated and heated culture filtrate (1 loopful in 2 ml filtrate), incubated and streaked onto lipase agar. All the experiments were first carried out with control strains (*Candida albicans* ATCC 90028, Candida tropicalis ATCC 200026 and *A. flavus* ATCC 40050) and then with laboratory isolates. All the procedures were performed in triplicate.

Assay for toxicity of the filtrate on Hep-2 (Human Laryngeal epithelioma) cell line:

To check toxicity of the filtrate on Hep-2 cell line, the monolayer in small tubes were incubated initially for 1 hour at 37^{0}C with100 µl filtrate, washed with PBS and reloaded with 2 ml MEM (Eagle's Minimum essential medium), followed by incubation for 2 days at 37^{0}C and observation every 6 hours, under inverted microscope at 20X magnification.

Statistical tests: Z –test of significance was applied in order to calculate the difference in biofim forming ability of the yeasts in YPD broth and the heated and unheated culture filtrates, respectively [12].

RESULTS

As observed spectrophotometrically and microscopically, biofilm formation in *Candida albicans* and *C. tropicalis* were impaired significantly by *Aspergillus flavus* filtrate, both heated and unheated. The difference was found to be statistically significant. The results are shown in [Table/Fig-1]. There was no visible zone of haziness around colonies of *Candida spp*. on Muhsin's Lipase agar after initial incubation in culture filtrate of *Aspergillus flavus*, indicating that lipase enzyme of *Candida spp*. is inhibited by the filtrate.

The filtrate, both heated and unheated was found not to induce any visible cytopathic effect on Hep-2 cell line as observed under inverted microscope.

Mean O.D.	In YPD	In unheated culture filtrate	In preheated culture filtrate	p value
Candida albicans	3.427	0.056	0.364	<0.05
Candida tropicalis	3.5	0.387	0.168	<0.05

[Table/Fig-1]: O.D. of *C. albicans* and *C. tropicalis* in YPD, unheated and preheated *A. flavus* culture filtrate

DISCUSSION

Invasive candidiasis is an emerging infectious disease in developing countries with a crude mortality of about 40% [13]. Due to factors like the ongoing HIV epidemic, haematological malignancies and an increasingly ageing population, invasive fungal infections have become common in present days [14]. Of all candidemia cases, about 35-50% cases of bloodstream infection occur in the ICU setting with high attributable mortality [15]. Treatment of this disease is very challenging due to formation of complex structured communities called biofilms in 20-70% cases that retard drug diffusion and protect the fungus from host immune defenses, and toxicity of the antifungal drugs administered [16,4,5]. For example, Amphotericin B is an effective antifungal to treat candidemia but can be nephrotoxic in up to 80% cases [17]. Other drugs like Echinocandins (Caspofungin, Micafungin and others) are effective against candidal biofilms but are very costly which limits their routine usage in developing countries and permits usage only in reserved cases [18,19]. *Candida spp*. produce several virulence factors like Secreted aspartyl proteases (SAP) and lipases among others that helps the pathogen establish infection in the susceptible host [20,8]. So there is an imminent need of developing new antifungal agents that would aim at inhibiting biofilm formation, one of the main pathogenic mechanism of *Candida spp*. and inhibit enzymes like lipase.

Aspirochlorine, a chemical belonging to gliotoxin family of mycotoxins and obtained from culture filtrate of *A. flavus*, has been shown to be toxic to *Candida spp*. by inhibition of the pathogen's protein synthesis [21]. In our study, the factors inhibiting this biofilm formation in the yeast pathogens were found to be heat-stable. Furthermore, as far as we know, there is no published literature regarding inhibition of biofilm formation and lipase activity of *Candida albicans* and *C. tropicalis* by culture filtrate of *Aspergillus flavus*. Further work needs to be carried out to study the toxic effects of this filtrate on the host, by seeing the effect of the filtrate on human erythrocytes,and white blood cells and other living systems like animal models. Since this filtrate was found to be non-toxic on host cell lines, it can be precoated on the surface of indwelling intravascular devices to inhibit biofilm formation in *Candida spp*. in vivo, too. Being heat stable, the compounds in the filtrate could inhibit candidal biofilm development in febrile states also. Similar inhibition of Candidal biofilm in vitro has been shown by using Pyocyanin obtained from *Pseudomonas aeruginosa* [22].These findings could show the path of discovering new, cost-effective strategies to prevent disease pathology in invasive candidiasis.

CONCLUSION

This filtrate was found to be non-toxic to host cell lines. It can be precoated on the surfaces of indwelling intravascular devices, to inhibit biofilm formation in *Candida spp*. in vivo, too. Being heat

stable, the compounds in the filtrate could inhibit Candidal biofilm development in febrile states also. These findings could show the path for discovering new, cost-effective strategies for preventing disease pathology in invasive Candidiasis.

REFERENCES

[1] Lewis RE. Overview of the changing epidemiology of candidemia. *Curr Med Res Opin*. 2009; 25:1732-40.
[2] Farroqi JQ, Jabeen K, Saeed N, Iqbal N, Malik B, Lockhart S, et al. Invasive Candidiasis in Pakistan: Clinical characteristics, species distribution and antifungal susceptibility. *J Med Microbiol*. 2013 62:259-68.
[3] Sardi JC, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJ. Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J Med Microbiol*. 2013; 62(1):10-24.
[4] Wilke MH. Treatment and Prophylaxis of Invasive candidiasis with Anidulafungin, Caspofungin and Micafungin and its impact on Use and costs-Review of the literature. *Eur J Med Res*. 2011;16: 180-86.
[5] Al-Fattani MA, Douglas LJ. Penetration of Candida Biofilms by Antifungal Agents. Antimicrob. *Agents Chemother*. 2004; 48 :3291-97.
[6] Kanafani ZA, Perfect JR. Resistance to Antifungal Agents: Mechanisms and Clinical Impact. *Clin Inf Dis*. 2008; 46:120–8.
[7] Klausmeyer P, Mc Cloud TG, Tucker KD, Cardellina JH, Shoemaker RH. Aspirochlorine Class Compounds from *Aspergillus flavus* Inhibit Azole-Resistant *Candida albicans*. *J. Nat. Prod*. 2005; 68:1300–02.
[8] Gacser A, Stehr F, Kroger C, Kredics L, Schafer W, Nosanchuk JD. Lipase 8 Affects the Pathogenesis of *Candida albicans*. *Infect Immun*. 2007;75: 4710–18.
[9] Bhattacharyya S, Gupta P, Banerjee G, Jain A, Singh M. Inhibition of Candida biofilms by Pyocyanin: an in-vitro study. *Int J Curr Res Rev*. 2013;5:31-36.
[10] Raju SB, Rajappa S. Isolation and Identification of Candida from the Oral Cavity. *ISRN Dentistry*. 2011.
[11] Ramage G, Walle KV, Wickes BL, Lopez-Ribot JL. Standardized method for in-vitro antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrobial Agents Chemother*.; 45:2475-79.
[12] Muhsin TM, Aubaid AH, Al-Duboon AH. Extracellular enzyme activities of dermatophytes and yeast isolates on solid media. *Mycoses*. 1997; 40: 465-69.
[13] Mahajan BK. Sampling Variability and Significance. In Methods in Biostatistics For Medical Students and Research Workers. 7th ed New Delhi. J*aypee Brothers Medical Publishers.* (P) Ltd.2010 .
[14] Pfaller MA, Diekema DJ. Epidemiology of Invasive Candidiasis: a Persistent Public Health Problem. *Clin Microbiol Rev*. 2007 ; 20: 133–63.
[15] Chowta MN, Adhikary P, Rajeev A, Shenoy AK. Study of risk factors and prevalence of invasive candidiasis in a tertiary care hospital. *Indian J Crit Care Med*. 2007;11:67-73.
[16] Bouza E, Munoz P. Epidemiology of candidemia in intensive care units. *Int J Antimicrob Agents*. 2008 ;32 Suppl 2:S87-91.
[17] Lincoln NJ, Andes MKD. Beta -1,3 glucan as a test for central venous catheter biofilm infection. *J Infect Dis*. 2007;195:1705.
[18] Luber AD, Maa L, Lam M, Guglielmo BJ. Risk factors for Amphotericin B- induced nephrotoxicity. *J Antimicrob Chemother*. 1999;43: 267-71.
[19] Chatzimoschou A, Katragkou A, Simitsopoulou M, Antachopoulos C, Georgiadou E, Walsh TJ, et al. Activities of Triazole-Echinocandin Combinations against Candida Species in Biofilms and as Planktonic Cells. *Antimicrob Agents Chemother*. 2011;55:1968–74.
[20] Grover ND. Echinocandins: A ray of hope in antifungal drug therapy. *Indian J Pharmacol*. 2010; 42: 9–11.
[21] Chen SC, Joosten LAB, Kullberg BJ, Netea MG. Interplay between *Candida albicans* and the Mammalian Innate Host Defense. *Infect Immun*. 2012;80: 1304-13.
[22] Monti F, Ripamonti F, Hawser SP, Islam K. Aspirochlorine: a highly selective and potent inhibitor of fungal protein synthesis. *J Antibiotics*. 1999 ;52:311-8.

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