Antagonistic Activity of Soil Fungal Metabolite Against *Candida albicans*

Prayan Jain
Asstt. Professor in Biotechnology
University Institute of Engineering and Technology
Kurukshetra University
Kurukshetra

R.K. Pundir
Lecturer in Biotechnology
University Institute of Technology and Management
Bhor Saidan
Kurukshetra

Abstract: *In the present study, soil molds were evaluated for their antifungal activity against three Candida albicans strains. Of the 200 fungal isolates, 10 isolates showed inhibitory activity. Of these 10 isolates, Aspergillus sp. was found to be most effective against all the three Candida albicans strains with zone of inhibition varying between 28mm and 30mm. On comparison, fungal metabolite of Aspergillus was found to be most effective than the commercially available drugs. It may be suggested that antimicrobial metabolite obtained from the soil fungus, might be used in the prevention and treatment of C. albicans infections.*

Keywords: *Candida albicans, soil fungi, antimicrobial metabolite*

1. INTRODUCTION

The dimorphic fungus *Candida albicans* is a commensal of the human oral, gastrointestinal, vaginal, cutaneous, and mucosal surfaces. The increased prevalence of local and systemic disease caused by *C. albicans* has resulted in numerous new clinical syndromes; the expression of which depends primarily on the immune status of the host. In immunocompetent as well as immunocompromised individuals, *C. albicans* causes cutaneous or subcutaneous infections such as vaginitis or oral thrush or infections of the nails and skin[1]. *Candida albicans*, is also prevalent in the saliva and dental biofilm of caries active individuals[2]. In patients receiving broad-spectrum antibiotics or undergoing cancer chemotherapy, *C. albicans* can enter the bloodstream to cause serious systemic invasive disease. Due to the difficulty in identifying antifungal targets, unique to fungi that are not shared with the human host, only a restricted number of antifungal agents have been widely used for treating *C. albicans* systemic infections. The management of serious and life-threatening invasive candidiasis remains severely hampered by delays in diagnosis and the lack of reliable diagnostic methods that allow detection of both *fungemia* and tissue invasion by *C. albicans*[3]. Difficulty in the treatment of *C. albicans* infections is compounded by the fact that they are resisant to many major classes of antifungal drugs, including azoles. Antifungal drug resistance in *C. albicans* continues to increase in response to the widespread application of azole therapeutics among immunosuppressed patients[4,5]. Systemic infections due to *Candida albicans* and resistance to antifungals is on the rise in India. *Candida albicans* accounts for 40-60% of yeasts isolated in India and increasing resistance to azoles and amphotericin B has been reported.

Microorganisms continue to be important natural sources of therapeutically active molecules. Soil is a diverse medium composed of many minerals and substrates essential for metabolic pathways of prokaryotic and eukaryotic inhabitants[7]. The abiotic and biotic diversity present in this medium makes it difficult for the isolation of all the microbial communities present. Research has demonstrated that not even 1% of the entire soil community has been identified[7]. There is a great opportunity for discovering new groups of microorganisms with industrial and clinical importance in soil. A number of drugs have been discovered from soil inhabiting microorganisms-- bacteria especially actinomycetes and molds[7]. In lieu of the above justifications for the continuous search for new isolates from soil having antimicrobial activity, the present paper highlights the bioactivity of soil molds against *Candida albicans* through the production of antimicrobial metabolite and comparison with commercially available antibiotics which are being used for treatment of Candida infections.

2. MATERIALS AND METHODS

Collection of Soil samples

Soil samples were collected from Delhi, Rajasthan, Haryana, Punjab, Uttar Pradesh, and Himachal Pradesh. During the field trips, 200 soil samples were collected in sterile polyethylene bags from various sites rich in organic matter e.g. areas receiving industrial wastes, mushroom farm, crop fields, rotten wood soil, leaf litter, farmhouse backyards, household wastes and vegetables

refuses. After collection, the sample bags were labelled with date and site of collection.

Isolation of Microorganisms from Soil

The serial dilution agar plate method was used for the isolation of fungi from the soil samples[8]. Potato dextrose agar (PDA) (HiMedia), supplemented with streptopenicillin, was employed for the isolation of soil fungi. Ten grams of soil (finely pulverized and air dried) was suspended in 90 ml sterilized distilled water blank no. 1 and shook vigorously on a magnetic stirrer for 20-30 minutes to obtain uniform suspension of microorganisms. 10ml of suspension was transferred, while in motion, from the stock suspension (No. 1), into sterile water blank number 2 with sterile pipette under aseptic conditions to make 1:100 (10^{-2}) dilution and shook it well for five minutes. Further dilutions from 10^{-3} to 10^{-7} were made by pipetting 10 ml suspension into sterile water blanks numbered 3, 4, 5, 6 and 7 from water blanks labeled 2, 3, 4, 5 and 6 respectively. Finally 1 ml aliquots of the suspension of final five dilutions i.e. 10^{-3} to 10^{-7} were added to labelled and sterilized Petri plates. Approximately, 20-25 ml cooled (45°C) molten PDA (with streotopenicillin) were added to each Petri plate and mixed gently by rotation. After solidification of the agar media, the inoculated PDA plates were incubated in an inverted position at 25°C for 6 days.

Purification and maintenance of fungal isolates

The fungal colonies appearing on the PDA plates were transferred aseptically to fresh PDA plates (one fungal colony on each plate) and incubated at 25°C for 6 days. The fungal colonies were transferred on PDA slants and incubated at 25°C for 6 days and then maintained at 4°C in a regrigerator for further studies.

Identification of fungal isolates

All the fungal cultures were identified up to generic level following volumes/monographs/manuals[9,10].

Procurement of microbial cultures and preparation of microbial inoculum following 0.5 McFarland standard

The three strains of *Candida albicans* (MTCC Nos. 3017, 227 and 183) were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. The slants of Malt extract agar were used for preserving *C. albicans* strains. All the slants were kept at 4°C in the refrigerator.

The inoculum (1.5×10^8 cuf/ml) of *C. albicans* strain was adjusted according to 0.5 McFarland standard[11]. The McFarland tube was stored at 4-5°C and was stored at 4-5°C and was prepared afresh after every 3 to 4 months.

Evaluation of antimicrobial activity of fungal isolates by using agar overlay method

The antimicrobial activity of purified fungal isolates was evaluated by using agar overlay method[12,13]. The fungal isolates tested for antimicrobial activity were inoculated into the center of the presolidified PDA plates and incubated at 25°C for 3 to 4 days. The plates were overlaid with a thin film of molten malt extract agar medium (45°C) containing 24 hrs old cultures of test pathogens. All the overlaid plates were incubated at 35°C for 24 hrs to allow the growth of test pathogens. Antimicrobial activity of fungal isolates against test pathogens was indicated by zone of growth inhibition around the fungal colonies showing positive test for its antimicrobial activity[12].

Selection of promising fungal isolates

Of the 200 soil samples screened, 127 isolates of fungi were obtained following serial dilution agar plate method.

Antimicrobial metabolite production of promising fungus isolate

The production of antimicrobial metabolite by the selected fungal isolate was studied in Potato dextrose broth[14,15]. Two hundred ml of each broth was taken in 500 ml Erlenmeyer flasks. These flasks were autoclaved at 121°C, for 15 minutes. Each flask was inoculated with six mm disk of the fungus inoculum grown on PDA. The inoculated flasks were incubated at 25°C for 8 days under stationary condition. The broth was filtered through sterilized Whatman filter paper 1 and the culture filtrates were then tested for antimicrobial activity against test pathogens using agar well diffusion assay. The wells of 6 mm diameter were punched in the solidified malt extract agar plates containing test pathogens adjusted at 0.5 McFarland standard, with the help of sterilized cork-borer. 100µl of culture filtrate was added into the well with the help of a sterilized micropipette. The inoculated plates were kept in an upright position in an incubator until the filtrate diffused into the agar at least 3 to 4 hours. The plates were observed for the diameter of zone of growth inhibition around the well which was measured in mm by using HiMedia ZoneScale[16].

Extraction of antimicerbial metabolite with various solvents

Five different organic solvents namely acetone (100%) (Ranbaxy), chloroform (100%) (Ranbaxy), ethyl accetate (100%) (Rantaxy), dichloromethane (100%) (Ranbaxy) and dimethylsulphoxide (100%) (Ranbaxy) were tested for extraction of antimicrobial metabolite from selected fungus[17,18]. Fermentation broth and solvent (1:1) were taken in separating funnel and shaken vigorously for 5 minutes and kept without any disturbance for another 15 minutes to separate the solvent from aqueous phase. Antimicrobial activity of each extract obtained with differnt solvents was carried out by using agar well diffusion assay. The maximum extraction of antimicrobial metabolite was observed with chloroform which exhibited the best antimicrobial activity against all the test pathogens. The chloroform extract was dried by the repeated adition of Na_2SO_4 and concentrated by distillation which resulted in golden oily metabolite.

Thin Layer Chromatography (TLC)

The golden coloured oily metabolite obtained after chloroform extraction was subjected to thin layer chromatography to detect the various components present in the crude antimicrobial fungal metabolite[7,17,19].

Determination of Minimum inhibitory concentration of antimicrobial fungal metabolite

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation[11]. MIC of the powdered antimicrobial metabolite against all the test pathogens was determined by a macrodilution broth assay[11]. In this method, the test concentrations of white powdered antimicrobial metabolite were made from 64 to 0.125 µg/ml in ten sterile tubes labeled 1-10. 100 µl sterile Mueller Hinton broth (HiMedia) was poured in each sterile tube and then 200µl metabolite was added in tube 1. Two fold serial dilutions were carried out from tube 1 to the tube 10 and excess broth (100µl) was discarded from last tube no. 10. To each tube, 100µl of inoculum, adjusted to 0.5 McFarland standard was added. Positive control (containing inoculum but no metabolite) and negative control (containing metabolite but no inoculum) were made for the comparison. All the tubes were incubated for 24 hr at 37°C.

Comparative evaluation of commercial drugs with powdered antimicrobial metabolite

The comparison of antimicrobial activity of commercial antibiotics/drugs and powdered antimicrobial metabolite extracted from selected fungal strain using disk diffusion assay against the strains of *C. albicans*[20,21]. The commercial antibiotics were tested at the following concentrations which were the MIC concentrations denoted on the HiMedia disks: Ketoconazole (10 µg), itraconazole (10 µg), clotrimazole (10 µg) and amphotericin B (100 µg). 20 ml Mueller Hinton agar (MHA) (HiMedia) and 500 µl of each test culture of 24 hrs incubation adjusted at 0.5 MfFarland standard were mixed thoroughly and poured in sterilized and labelled Petri plates.

Preparation of powdered antimicrobial metabolite disk for evaluation

The powdered antimicrobial metabolite disks were prepared by punching the sterile Whatman filter paper 1, following autoclaving of the disk and then dipping the sterile disk with the help of sterile forcep into dimethylsulphoxide solution having metabolite at the concentration of 1 µg/ml and then the disk was air dried for 5 minutes to evaporate the solvent. The disks of various commercial antibiotics and Whatman filter paper no. 1 disk of powdered metabolite were put on labelled and solidified MHA plates aseptically. The loaded plates were incubated at 35°C for 24 hours and observed for the diameter of zone of growth inhibition of *C. albicans* strains[22].

3. RESULTS AND DISCUSSION

The emergence of antibiotic-resistant of *C. albicans* is seemingly inevitable, and results, within a few decades, in decreased efficacy and withdrawal of the antibiotic from widespread usage. The traditional answer to this problem has been to introduce new antibiotics that kill the resistant microorganisms. For the time being, the majority of new antibiotics that reach the market place are likely to be structural analogues of existing families of antibiotics or new compounds, both natural and non-natural which are screened in a conventional way against live multiplying microorganisms[6,23].

Soil sustains an immense diversity of microbes, which to a large extent, remains unexplored, Fungi are well known as prolific producers of biologically active natural products[22]. Soil occupied by roots has a complex ecosystem and it is not surprising that its inhibitants have evolved chemical defenses against each other[24]. The warfare between the host, pathogen and

antagonistic microorganisms around the roots is as unexhaustic source of antibiotics. Most of the naturally occurring antibiotics have been isolated from soil microorganisms[24].

In the present study, the isolation of antibiotic producing soil fungi for controlling *C. albicans* included species of *Aspergillus, Penicillium, Alternaria, Fusarium, Mucor, Rhizopus, Cladosporium, Cephalosporium, Gliocladium,* and *Colletotrichum*.

Out of 127 fungal isolates, the 10 fungal isolates: 7, 12, 19, 28, 107 (*Aspergillus* spp.), 50 (*Cephalosporium* sp.), 70, 85, 98 (*Penicillium* spp.) and 103 (*Cladosporiun* sp.) were effective against *C. albicans* strains on the basis of diameter of zone of growth inhibition (Table 1).

Isolates 7, 50, 103 and 107 were effective against all the three strains of *C. albicans*. Isolate 7 produced zones of growth inhibition ranging between 13 and 14 mm. Isolate 50 produced zones ranging between 9 and 11mm. Isolate 103 showed activity in the form of zone of growth inhibition ranging between 14 and 15mm. Isolate 107 was most effective against *C. albicans* strains with zone of inhibition varying between 30 and 35mm.

Isolates 19, 28 and 85 were effective against two strains of *C. albicans* and produced zones of inhibition varying from 10 to 15mm. Isolates 12 and 70 were active against one strain of *C. albicans* producing zones of growth inhibition varying between 12 and 14mm.

On the basis of the results obtained in the form of diameter of zone of growth inhibition of test pathogens, fungal isolate 107 was found to be most effective of all the soil fungal isolates (Table 1). The minimum inhibitory concentration of the fungal metabolite against three strains of *C. albicans* was determined as 4µg/ml (Table 2).

The fungal isolate 107 was identified as species of the genus *Aspergillus*, which is a deuteromycetous mold, belonging to the class *Hyphomycetes*, order *Moniliales* and family *Moniliaceae* and which shows optimum growth on Potato dextrose agar at 25°C. *Aspergillus* species isolated from various soils have been found to produce antibacterial, antifungal and antitumour metabolites[25]. Species of *Aspergillus* are known to produce mycotoxim[26], organic acids and antibiotics[25]. Production of mycotoxin and antimicrobial compounds by *A. flavus* and *A. niger* is well documented[27]. *A. niger* produce a mixture of organic acids including citric acid and gluconic acid which effectively degrade and release nutrients from mineral sources which might be inhibitory to other fungi in rhizosphere[28]. Similarly, *A. versicolor* produce mycoverilin, active against *Trichophyton rubrum*[29]. *Aspergillus candidus* produce citrin, kojic acid and two antibiotics, chloroflavonin active against fungi and candidulin, a metabolite active against Gram negative and Gram-positive bacteria[29].

Table 1. Antifungal activity of ten selected soil molds against three strains of *Canadida albicans*

Isolate	No.Zone of growth inhibition (mm)[a]		
	Ca1-*Candida albicans* (MTCC No. 3017	Ca2- *C. albicans* (MTCC No. 227)	Ca3- *C. albicans* (MTCC No. 183)
7	3±0.57	4±0.81	4+0.57
12	14±0.81	NA	NA
19	10±0.81	11±0.57	NA
28	11±0.57	10±0.57	NA
50	11±0.57	9±0.57	10±0.81
70	12±0.37	NA	NA
85	14±0.57	NA	15±0.57
98	10±0.81	15±0.57	15±0.57
103	8±0.57	15±0.37	14±0.57
107	30±0.57	35±0.57	30±0.37

Table 2. Evaluation of Minimum Inhibitory Concentration (MIC) of white powdered metabolite against *C. albicans*

Organism	Concentration of metabolite (µg/ml)										
	0.125	0.25	0.5	1.0	2.0	4.0	8	12	32	64	MIC
Ca1	+	+	+	+	+	-	-	-	-	-	4
Ca2	+	+	+	+	+	-	-	-	-	-	4
Ca3	+	+	+	+	+	-	-	-	-	-	4

- No growth; + Growth

The sterilized Whatman filter paper no. 1 disk of white powdered antimicrobial metabolite made from *Aspergillus* sp. at the concentration of 4μg/ml in DMSO (MIC of fungal metabolite), was compared with commercially available drugs/antibiotics for its antimicrobial activity by using Kirby-Baeur method. The results in the form of diameter of zones of growth inhibition (measured by HiMedia Zone Scale) are shown in table 3. at 4μg/ml concentration of fungal metabolite when tested against yeasts, showed growth inhibition zones in *C. albicans* strain 1 (MTCC No. 3017) (22 mm), *C. albicans* strain 2 (MTCC No. 227) (23 mm) and *C. albicans* strain 3 (MTCC No. 183) (22 mm). The antifungal drugs ketoconazole and itraconazole (10 μg) showed growth inhibition zones against the yeasts ranging from 10 to 12 mm. Amphotericin B (100 μg) showed no growth inhibition zones against any strain which indicated resistance of yeasts to this drug. The results inferred that white powdered metabolite purified from *Aspergillus* sp., when compared with commercial drugs, was found to be better in its antimicrobial action in the present work.

4. CONCLUSIONS

It may be suggested from the present study that antimicrobial substances obtained from the soil molds might be used in the prevention and treatment of dental caries and it may also be suggested from the present study that further research is needed to acquired the biochemical and molecular characteristics of the bioactive components and to determine the merits and demerits to be used for the treatment and control of dental caries. *In vivo* studies of bioactive components are recommended for further commercialization of the active component in pharmaceutical industries.

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Table 3. Comparative evaluation of antifungal activity of white powdered metabolite with commercial drugs/antibiotics by using disk diffusion assay against *Candida albicans* strains

Commercial	Zone of growth inhibition (mm)[a]			
	Conc. of antibiotics (μg)	*Canadida albicans* (MTCC No. 3017)	*C. Albicans* (MTCC No. 227)	*C. albicans* (MTCC No. 183)
Clotrimazole	10	10±0.57	10±0.57	12±0.57
Ketoconazole	10	10±0.37	R	R
Fluconazole	10	15±0.81	13±0.57	12±0.37
Amphotericin B	100	R	R	R
Itraconazole	10	R	R	R
Metabolite	10	22±0.57	21±0.57	22±0.57

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