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Efficacy of *Trichoderma harzianum*, a biocontrol agent for controlling opportunistic fungal pathogens.

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Abstract

Trichoderma harzianum is widely used as a biocontrol agent for phytopathogens. In the present study, we surmise, it can be used to control human opportunistic pathogens. Extraction with fungus mycelium was performed with the Soxhlet method. The inhibitory concentration was tested on *Candida sps*, including multidrug-resistant *C. auris* strain and gram-positive bacteria. The results suggest that the maximum zone of inhibition is 12 mm and 8 mm for fungus and bacteria, respectively. A high-performance thin-layer chromatography (HPTLC) experiment suggests the presence of anthraquinone and stigmasterol in the extract. We anticipate the zone of inhibition is due to these active ingredients. Our result unveiled the previous connotation and suggested perhaps *Trichoderma sps.* It can be used to treat human fungal pathogens.

Keywords: Trichoderma, human fungal pathogens, Non-albicans Candida, Secondary metabolites.

INTRODUCTION

Opportunistic pathogens, as the name depicts, affect humans due to an immunocompromised state. Most of them are normal flora of the body, but due to several intrinsic and extrinsic factors become pathogenic and cause serious diseases. Microorganism, bacteria, fungi, viruses, and protozoa are normal flora of human body (1). Most of the studies are focused on bacteria or viruses, and fungal diseases are often neglected. As most of the fungal diseases cause superficial infections, which are easily curable. However, recent trends suggest invasive fungal diseases are responsible for high mortality in immunocompromised patients (2).

To explore other means to control the opportunistic pathogens, we explored a well-known biocontrol agent *Trichoderma harzianum*. It is a mycoparasite, is a potent biocide, and successfully used against phytopathogens. The various mechanism involved in mycoparasitism is antibiosis, mycotoxin, secondary metabolites and cell wall degrading enzymes (3). The various secondary metabolites derived from *T. harzianum* are shown to be antimicrobial are namely palmitic acid, anthraquinone, pyrone, furanone, stigmasterol, harzianopyridone or its derivates (4). Most of the studies about antibiosis or mycoparasitism are either focused on the interaction of *Trichoderma* with plant or phytopathogens (5). Since *Trichoderma* can restrict the growth or kill the phytopathogens, we hypothesize it could also be able to restrict the growth of human fungal pathogens. Similar reports were earlier reported on the same theme (6–8), however studies focused on non-albicans candida as claimed in the present study are meager.

MATERIALS AND METHODS

Media components and Strains

The media components used in the study are listed below in table 1. The components were procured form HIMEDIA®. The media components were all prepared in double distilled water, and an appropriate pH was adjusted whenever required. The media was autoclaved at 121°C at 15 psi pressure for 15-20 minutes.

Growth Conditions

The strains obtained were mentioned in Table 2. The yeast and bacterial strains were grown and maintained in yeast extract peptone dextrose and nutrient broth medium, respectively. The *T. harzianum* strain was grown in 500 mL of potato dextrose broth medium at 28°C for 10-12 days to obtain the mycelium. The mycelium was collected by passing the medium through the Whatman filter paper. The collected mycelium was dried in a hot air oven at 55°C for 4-5 h.

Antimicrobial susceptibility assay

The antimicrobial property was performed using the disc-diffusion antibiotic susceptibility method using Muller Hinton (MH) and Sabouraud Dextrose (SD) media for bacteria and fungi, respectively. The mycelium was placed in the Soxhlet apparatus, ~5 g of mycelium was extracted with 100 mL of ethyl acetate and methanol for 30 cycles. The extract was concentrated in the rotary evaporator 40 rpm at 45°C and further dried in hot air oven to obtain a dry powder. A stock solution of 10.0 mg/mL was prepared in dimethyl sulfoxide (DMSO) and used for disc diffusion assay. The antimicrobial assay was performed by overnight grown culture equivalent to OD_{600nM} 0.025 and 0.05 was spread plated on MH and SD agar plates for bacteria and fungi respectively. A saturated 6 mM sterile disc impregnated with crude lysate was placed on the plate. The zone of inhibition was calculated after 24 h incubation at 37 °C and 28 °C for bacteria and fungi respectively. Based on the zone of inhibition, we have calculated the minimum inhibitory concentration. The formula for calculation of zone of inhibition=Zone of inhibition of the extract-Zone of inhibition of control. The control here is the negative control of DMSO used for the disc diffusion assay. The amount of extract used is deduced with the stock solution used for the assay.

Table 1: Growth Media used in the study

Name	Media composition
YEPD/ YPD (broth)	10 g yeast extract, 20 g peptone, 20 g dextrose
YEPD / YPD (agar)	10 g yeast extract, 20 g peptone, 20 g dextrose, and 20 g agar
CHROMagar	15 g peptone, 4 g Yeast extract,1 g Dipotassium hydrogen phosphate, 7.22 g chromogenic mixture, 0.5 g chloramphenicol, 15 g Agar
LB Broth	10 g Casein enzymic hydrolysate, 5 g yeast extract, 10 g Sodium chloride.
LB Agar	10 g Casein enzymic hydrolysate, 5 g yeast extract, 10 g Sodium chloride, 15 g Agar.
Muller Hinton Agar	2 g Beef extract, 17.5 g Casein hydrolysate, 1.5 g Starch, 17 g Agar
Sabouraud dextrose agar	40 g Dextrose, 10 g Peptone, 15 g Agar

Table 2: List of strains used in the study

Strain name	Received from
Fungus	
T. harzianum	School of Agriculture, LPU, Punjab, India
S. cerevisiae	MTCC*-172
Candida albicans	NCCPF-400063
C. tropicalis	NCCPF B-28
C. auris	NCCPF-470097
C. krusei	NCPPF-440040
Bacteria	
B. subtilis	MTCC-121
E. faecalis	MTCC-2729
B. bifidum	NCL-5671

*MTCC-Microbial Type Culture Collection, Chandigarh, India
NCCPF-National Culture Collection of Pathogenic Fungi
NCL-National Chemical Laboratory, Pune, India

High-performance thin-layer chromatography (HPTLC)

HPTLC analysis was performed with CAMAG-Linomat and TLC Scanner in pre-coated silica gel 60 F254 of uniform thickness 0.2 mm and a size of 5 X 10 cm. The method was standardized by running different solvents at 254 and 366 nm. Approximately 10 ml of samples and the standard solution was used for the spotting. After saturation time the spots were analyzed in UV spectra for the similarly peaks and retardation factor (Rf) value.

RESULTS AND DISCUSSION

To study the efficacy of *T. harzianum,* we selected fungal strains *Saccharomyces cerevisiae*, *Candida albicans*, *Candida auris*, *Candida tropicalis*, *Candida krusei* and three bacterial strains, *Bacillus subtilis*, *Enterococcus faecalis* and *Bifidobacterium bifidum* enlisted in table 2. Out of these *S. cerevisiae* and *B. subtilis* are rare to cause any infections in humans. Other strains are known to cause serious infection in humans, especially in immunocompromised patients (2,9–11).

Our results suggest that ethyl acetate is effective as compared to the methanolic extract. The extract is more fungistatic as compared to bacteriostatic, as we did not observe any zone of inhibition with *B. subtilis*. At 12.5 mg/mL effective concentration, the maximum zone of inhibition was 12 mm for *S. cerevisiae* and lowest 8 mM for *C. auris* and *C. krusei* for ethyl acetate extract (Figure 1 A). Similarly, the maximum zone of inhibition was 8 mm for *B. bifidum and E. faecalis* for ethyl acetate and methanolic extract respectively (Figure 1 C & D). The most important finding is that it is fungistatic to *C. auris* a multidrug-resistant fungus (Figure 1 A & B). Our results give an important clue that *T. harzianum* is a potent antifungal agent for the management of *C. auris, C. tropicalis and C. krusei* drug-resistant pathogens (9),(12,13).

It has been reported that *Trichoderma sps.* Produce several secondary metabolites and some of them are antifungals such as peptides, polyketides, isoprenoid and pyrones (5). To test secondary metabolites, we tested two active components, as reported earlier (14). Our result suggests that anthraquinone and stigmasterol are present in the ethyl acetate lysate (Figure 2). We only choose ethyl acetate as it is more potent compared to methanol. Anthraquinone and its derivates were reported as potent antifungal and antibacterial, especially to gram-positive bacteria (15).

Similarly, stigmasterol was shown to be antibacterial (16). The presence of anthraquinone and stigmasterol (Peak 6 and 9, Figures 2, C & D) in *Trichoderma* is a clear indication that the fungistatic activity is due to these compounds. However, in the present study, we have not studied the different types and subclasses of the two tested compounds. The present investigation intended to test whether *Trichoderma* is a potent antimicrobial agent against human opportunists' pathogens, and our results corroborate the hypothesis. Further studies are required to delineate the exact mechanism and mode of the action.

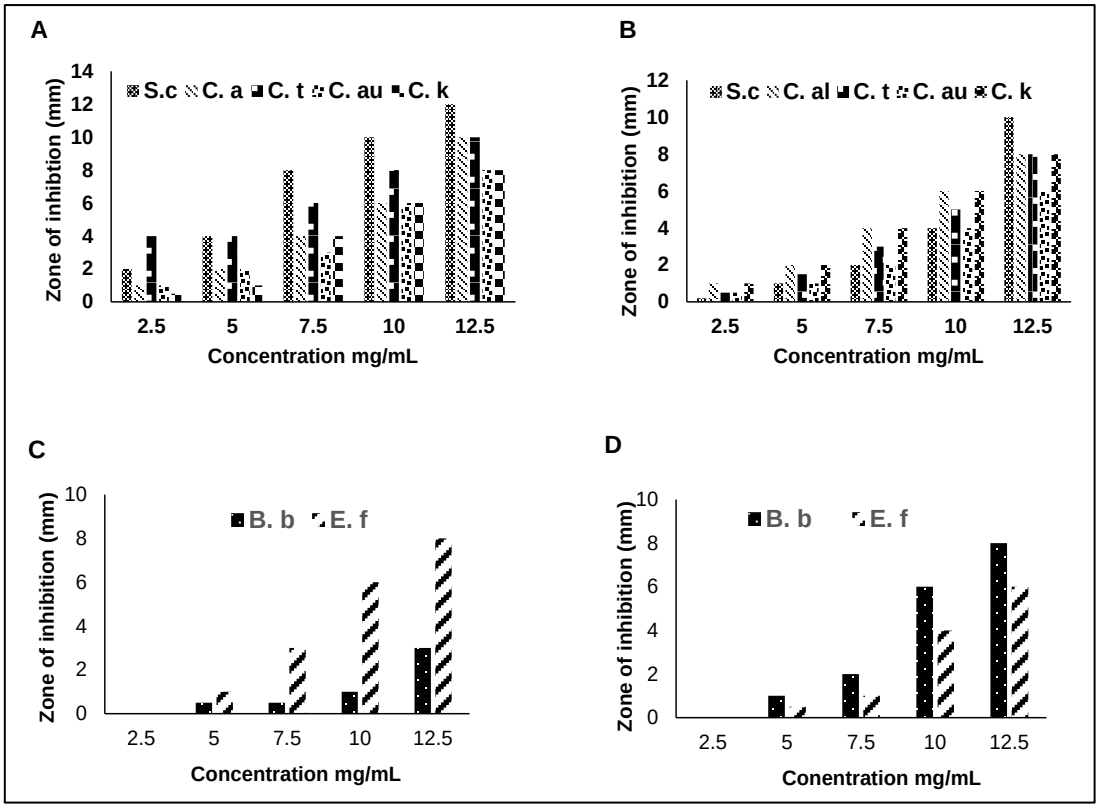
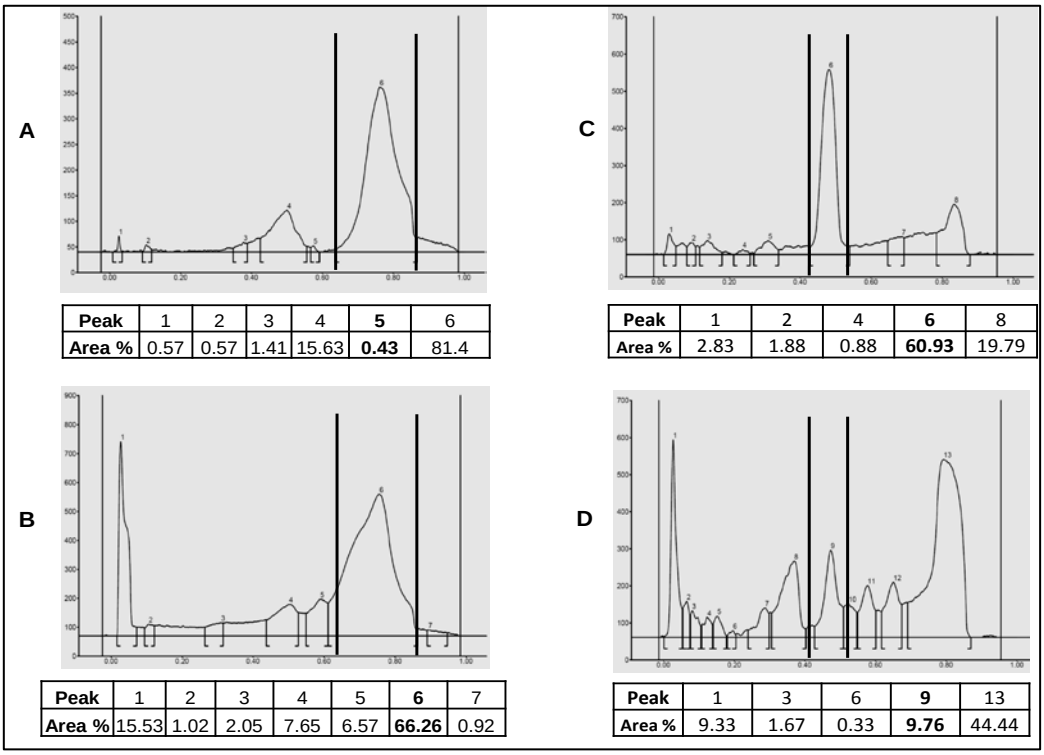


Figure 1. The MIC of *T. harzianum* extract using the Soxhlet method for the solvent. (A & C) Ethyl acetate. (B &D) Methanol. The bar depicts the MIC based on the zone of the inhibition (X-axis) and concentration of extract (mg/mL), *Saccharomyces cerevisiae* (S. c), *Candida albicans* (C. a), *Candida tropicalis* (C. t), *Candida auris* (C. au), *Candida krusei* (C. k), *Bifidobacterium bacter* (B. b), *Enterococcus faecalis* (E.f).



Peak	1	2	3	4	**5**	6
Area %	0.57	0.57	1.41	15.63	**0.43**	81.4

Peak	1	2	4	**6**	8
Area %	2.83	1.88	0.88	**60.93**	19.79

Peak	1	2	3	4	5	**6**	7
Area %	15.53	1.02	2.05	7.65	6.57	**66.26**	0.92

Peak	1	3	6	**9**	13
Area %	9.33	1.67	0.33	**9.76**	44.44

Figure 2. HPTLC analysis of a crude extract of *T. harzianum*. (A & C) Anthraquinone and Stigmasterol standards. (B & D) *T. harzianum* extract. The corresponding peaks are depicted below in the table with the percentage area covered by each peak. The number (1, 2, 3, etc.) denotes the pick, and the bared area is the actual peak of the molecule, also highlighted bold in the table. For simplicity, only the relevant peaks are depicted in the table.

Recent reports suggest that *Trichoderma sps* also cause diseases in immunocompromised patients (17,18). It would be quite interesting to study whether the secondary metabolites reported in the present study or reported earlier (14) could able to restrict the growth of clinical isolates of *Trichoderma* and other fungi.

CONCLUSIONS

The present investigation gives a new direction for the isolation of therapeutic molecules for the treatment of pathogenic organisms. The study unveiled that *Trichoderma sps*, which were considered only biocontrol agents for phytopathogens, are also mycopathogens and bacteriostatic for human pathogens.

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Conflict of Interest Statement

The authors declare no competing and conflict of interest.

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