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ORIGNAL ARTICLE

Evaluation of the *in-vitro* Antifungal Activity of Selected Fungal Species Tested Against Opportunistic Human Pathogen *Candida albicans*

Aakriti Shukla*, Apoorva Pathak

ABSTRACT

Candida albicans is currently the fourth-leading cause of hospital-acquired bloodstream infections, reaching a mortality rate of up to 35–40% for systemic or disseminated infections. Systemic mycoses can occur in patients with severely impaired immune systems (AIDS), with organ or bone marrow transplants, cancer patients undergoing chemotherapy, and patients in ICU (neonates and elderly). It is, therefore, obvious that there is a substantial need for fast, effective antifungal antibiotics to combat fungal infections. The present investigation has been proposed to screen effective fungal metabolites for the control of *Candida albicans* by evaluating the potential of fungal bioactive compounds, its purification and characterization.

Keywords: Anticandidal activity, Antifungalmetabolites, Systemic mycoses.

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INTRODUCTION

The incidence of fungal infections has increased tremendously during the last decades. Mycoses are beginning to create serious problems for patients having cancer or immune-compromised or physiologically compromised.[1] Candiasis is emerging as significant to the general population. In addition, the emergence of opportunistic fungal infection associated with the acquired immune syndrome (AIDS) and AIDS-related complex (ARC) is quite alarming.[2] The incidence of fungal infection has also increased dramatically due to widespread use of broad-spectrum antibiotics, antitumor and immuno-suppressive agents, x-irradiation, steroids, and oral contraceptives, which provides the ideal conditions for growth, multiplication, and spread of the opportunistic fungal pathogen.

Many fungal species have the potential to produce a primary and secondary bioactive metabolite compound that inhibit and affects the growth, metabolism, reproduction, and survival of other types of organisms. Fungi is a very important resource for searching new biologically active compounds, and hence it uses for the production of pharmaceuticals and biochemical reagents and their lead compounds.[3] Many fungal species have a potential to exhibit anticancer, antimicrobial, antifungal or anti-inflammatory activities.[4] *Aspergillus niger, Penicilium notatum, Rhizopus nigricans, Pestiopsisetc* produces many secondary metabolite compounds that are beneficial to a human being,[5-7] A range of potential antibiotics are available for clinical use. Still many more are screened *in vitro* and *in vivo.*

Therefore, the present investigation has been proposed to screen some effective fungal metabolites for the control of *Candida albicans.*

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MATERIALS AND METHOD

Isolation and identification

All the fungal species were collected and isolated (Agrawal and Hasija 1986) from Rani Durgawati University Campus and Mycology Research Lab of Biological Science Department R.D. University Jabalpur, (M.P) and MTCC Chandigarh, The authenticity of collected fungal species were confirmed by viewing under light microscope 10x,

40x, and 100x oil immersion magnification power by field expert Dr. Jamaluddin sir (Writer 'Fungi of India').

Medium

Potato Dextrose Medium and potato dextrose broth for fungal strains, Sabouraud'sagar medium and broth medium for test fungi *candida albicans*, used as standard medium.

Screening of Fungi for and Candidal Activity

Primary Screening

Many fungal species were isolated and tested against *Candidaalbicans*; most of them give little or no inhibition against test fungi, while some have shown maximum zone of inhibition and were selected for secondary screening.[8]

Secondary screening

Best fungal species from primary screening were selected for secondary screening against test fungi. Finally, fungi which showed maximum zone of inhibition and great potential were selected for further experiments.[9]

Preparation of Cell-free Culture Filtrate (CFCF)

Cell-free culture filtrate was prepared aseptically with the help of seven days old plate culture. A disc containing fungal mycelium was cut with sterile cork borer and impregnated in Potato dextrose broth medium, then the fungal broth was kept in orbital shaking incubator (Remi, India) at 27° for 12 days (Pre calculated optimum condition for best results) at 110 rpm. CFCF was obtained by filtering the fungal broth through the Whatman filter paper.[10]

Organic Solvent Extraction

Fungal secondary metabolites were extracted from the 12 days old broth culture of selected fungal strain; solvents used in extraction were n-Butanol, Xylene, Ethyl acetate, Acetone, Chloroform. The extraction process was initiated in a volumetric flask, fungal broth and organic solvents were mixed in proportional quantity after rigorous shaking; two immiscible layers were formed. Organic solvent layer was separated carefully and subjected to vacuum rotary evaporator (temp 40° at 70–80 vacuum pressure), Fungal bioactive compound obtained after evaporation of the solvent was purified with column chromatography followed by thin-layer chromatography.

Antimicrobial Assay

The *in vitro* anticandidal and antifungal activities of the crude extracts of fungi and the isolated, purified compound was evaluated by Kirby Bauer's Disc diffusion technique (Kirbyand Rollins 1996). The inoculums of test fungi was prepared 24 hours before performing the experiments onSabouraud's dextrose medium and stored at optimum temperature 27°. Inoculums of fungal strain were prepared in potato dextrose broth (Himedia), whileSabouraud's Agar medium was used in the preparation of test fungi inoculums.[11]

Fungal strains were collected from the Mycology research laboratory, Rani Durgawati University Jabalpur, while test Fungi *Candida albicans* was isolated from urine sample of infected patient affected from yeast infection and Standard Test fungi was collected from MTCC Chandigarh. Thereafter sterilized medium was poured in pre-sterilized petri plates and allowed to cool and solidify. Then the broth of test fungi was taken on a cotton swab and was spread evenly on a plate (Figures 2-7).

Dried and sterilized pretreated fungal disc (soaked overnight in fungal strain inoculums) of 5 mm diameter (Whatman filter paper), were then loaded carefully by using sterile forceps and micropipette. Discs containing the test strain were placed onSabouraud's dextrose agar medium plate uniformly.

Figure 1: *Penicillium notatum*

Figure 2: *Aspergillus versicolor*

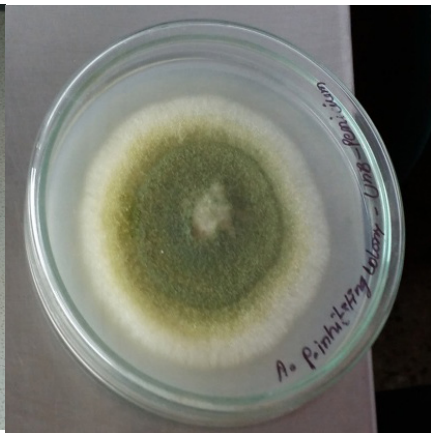


Figure 3: *Aspergillus flavus*

Antibiotic Flucanozole25(Himedia) discs were used as a standard control, and blank discs (soaked with solvents) were used as positive control, and DMSO used as a negative control. The plates were incubated for 24 hours at 37°C to allow the maximum growth of the organisms. The anticandidal activities of fungal metabolites were measured from the zone of inhibition, and all experiments were carried out in triplicate.

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is used to separate the compound present in the crude extract; in this method, separation of the compound also depends on the usage of the solvent. The drug with a concentration of 1 mg/mL was plotted on the TLC plate and dried. It was then run with a different solvent ratio. The spots were identified both in the UV light and in the iodine chamber. The R-value was

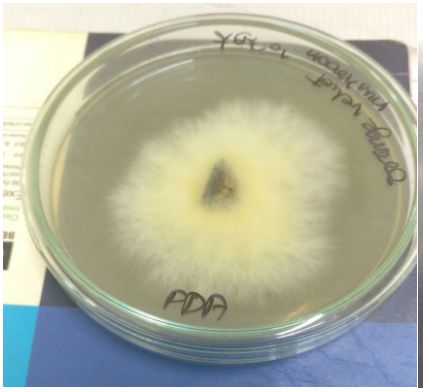


Figure 4: *Orange wild mushroom*

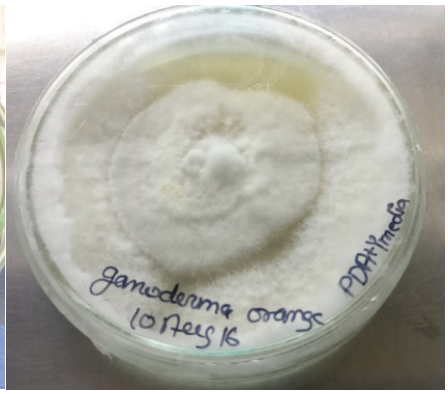


Figure 5: *Ganoderma lucidum*

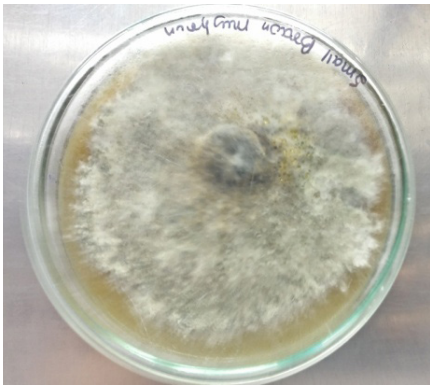


Figure 6: *Brown wood Mushroom*

Table 1: Fungal species isolated and screened against *Candida albicans*

S.no	*Fungal Species screened against Candida albicans*	*Activity or control (primary and secondary screening)*
1.	*Alternaria alternata*	Present
2.	*Aspergillus niger*	Present
3.	*Aspergillus fumigatus*	Present
4.	*Aspergillus versicolor*	Present
5.	*Aspergillus niger*	Present
6.	*Aspergillus flavus*	Absent
7.	*Penicillum notatum*	Present
8.	*Penicillium roqueforti*	Present
9.	*Peniciliumdigitatum*	Absent
10.	*Cladosporium cladosporioides*	Absent
11.	Fusarium oxysporum	Absent
12.	*Fusarium roseaum*	Absent
13.	*Geotricumcandidum*	Present
14.	*Alternaria alternata*	Absent
15.	*Choletotricumcurvulie*	Absent
16.	*Myrothecium sp.*	Present
17.	*Mucor sp.*	Absent
18.	*Rhizopus nigricans*	Present
19.	*Rhizopus stolonifer*	Present
20.	*Pestiopsissp*	Present
21.	*Ganoderma lucidum*	Present
22.	*Muscudoralbus*	Absent
23.	*Polyporus sp.*	Absent
24.	*Phoma sp.*	Absent
25.	*Trichoderma viridae*	Present

calculated using the formula:
R value = Distance traveled by the solute / Distance traveled by the solvent

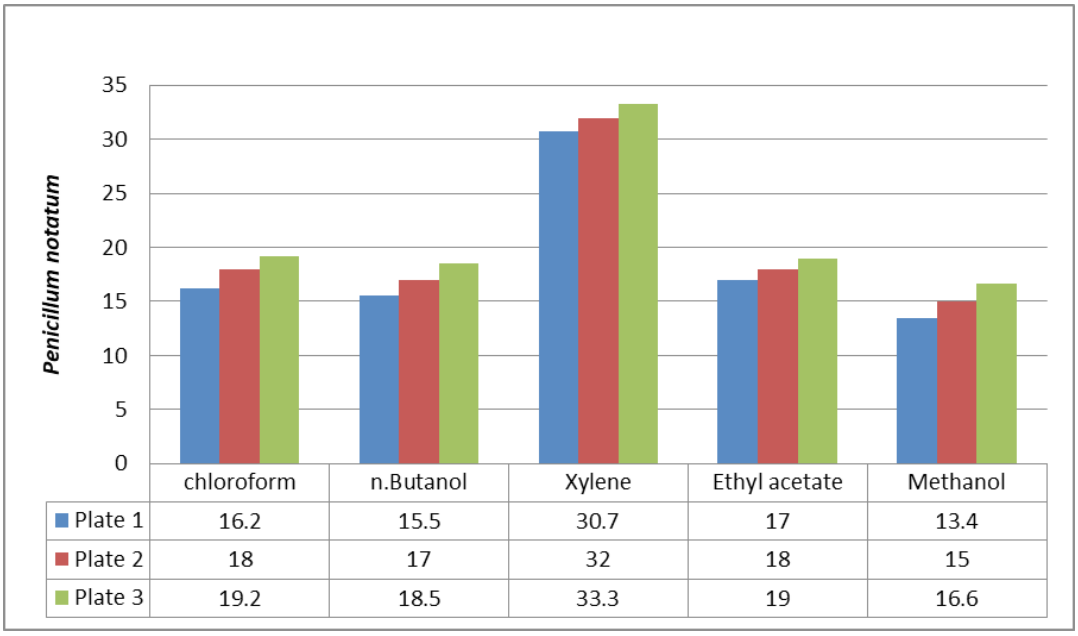
High-performance Liquid Chromatography Analysis

HPLC analysis was performed for confirmation of crude results. Samples of the extracts were dissolved in the desired volume with mobile phase (1 mL/samples), filtered through a 0.45µm disposable syringe filter into the chromatograph. Aliquots of10 µl were injected on HPLC column and analyzed using Shimadzu liquid Chromatograph, which was equipped with an LC-20Ad pump unit, a Rheodine injector, SPD detector, and with LC Solutions. A reverse-phase DC 18 column (150 × 3.9 mm, 5 µm) was used, at room temperature. The mobile phase used was acetonitrile and ethyl acetate (75:25 flow rate of 1 mL/min) for 10 min. The absorbance of samples and standard (Citrinin standar d used) was detected at 360 nm range. Retention times of each samples and peak areas were calculated by LC software. By analysis of a sample with a standard presence of citrinin in the samples identified.

RESULTS AND DISCUSSION

Anticandidal Assay

The present investigation records the antifungal activities of fungal extracts against *Candida albicans*. However, the degree of inhibition varied depending upon the concentration of the crude extracts. Different solvents such as Chloroform, ethyl acetate, *n*.Butanol, Xylene, Methanol, and Acetone were used in extraction of fungal active metabolite compounds



	chloroform	n.Butanol	Xylene	Ethyl acetate	Methanol
Plate 1	16.2	15.5	30.7	17	13.4
Plate 2	18	17	32	18	15
Plate 3	19.2	18.5	33.3	19	16.6

Figure 7: Graphical representation of *Penicillium notatum* extracts of different solvents is shown in next page, out of all fungal strains *Penicillium notatum* have given the best zone of inhibition against *Candida albicans.*

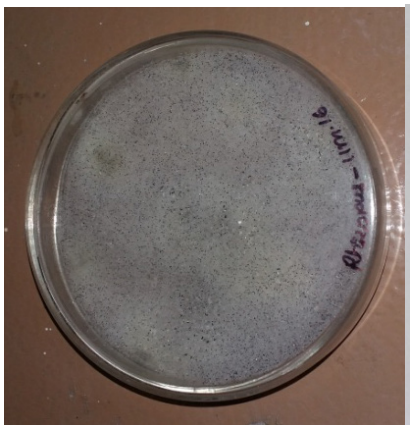


Figure 8: *Rhizopus nigricans*

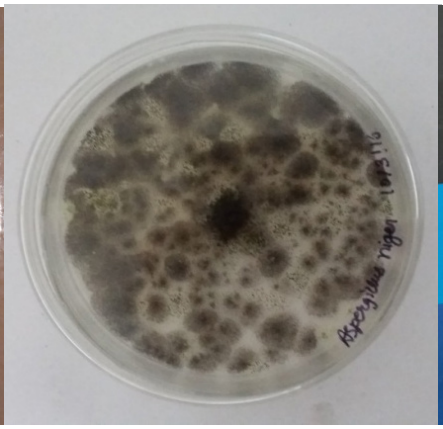


Figure 9: *Aspergillus niger*

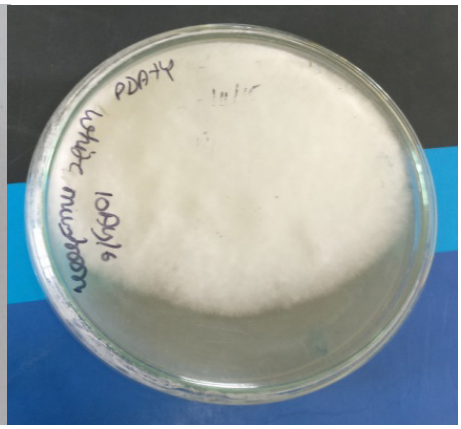


Figure 10: *Muscudoralbus*

and their activities were tested against Test fungi *Candida albicans*. Xylene extract of *Penicillium notatum* exhibited maximum zone of inhibition against *C.albicans*, while Ethyl acetate crude extracts of *Trichoderma viridae* showed minimum growth suppression (Table 2). (Figure 15) These results are in accordance with the earlier report.[11] *Aspergillus fumigates* and *A.niger* was also found equally effective in controlling the growth of *C.albicans*. The fungal isolates tried against *C.albicans* were found potentially effective in controlling its growth and development.[12-15]

Solvent Extraction Method

In the present study, the crude extracts of *Rhizopus nigricans, Aspergillus niger, A. stolonifer, A.fumigatus, Penicillium notatum, Ganoderma lucidum* produced inhibitory activity against human pathogen *Candida albicans*. By using solvent solvents extraction protocol, the crude fungal extraction produced yield enough for the experimental study and it is the most commonly used and cheap, simple method of fungal active metabolite separation. (Figure 11).

Figure 11: Solvent extraction method

Figure 12: Column Chromatograph

Figure 13: Thin layer chromatography

Table 2: Anticandidal activity of different solvent extracts against *Candida albicans* (The table explains the present study of crude extracts of *Rhizopus nigricans, Aspergillus niger, A. stolonifer, A.fumigatus, Penicillium notatum, Ganoderma lucidum* produced inhibitory activity against human pathogen *Candida albicans*, By using solvent solvents extraction protocol, the crude fungal extraction produced yield enough for the experimental study and it is the most commonly used and cheap, simple method of fungal active metabolite separation).

S. No	*Fungal strains*	*Chloroform*	*n.Butanol*	*Xylene*	*Ethyl acetate*	*Methanol*	*Blank disc (loaded with solvent)*	*Positive control (Fluca-nozole)*	*Negative control (DMSO)*
1.	*Rhizopus nigricans*	14 ± 1.8	11 ± 0.66	14 ± 1.8	10 ± 0.87	13 ± 1.5	No Zone of inhibition in mm	25 mm standard zone of inhibition	No Zone of inhibition in mm
2.	*Aspergillus niger*	14 ± 0.87	10 ± 0.91	13 ± 1.8	11 ± 0.91	9 ± 0.66			
3.	*Penicillum notatum*	18 ± 1.8	17 ± 1.5	32 ± 1.3	18 ± 1	15 ± 1.6			
4.	*Ganoderma lucidum*	14 ± 0.66	14 ± 1.8	13 ± 0.56	16 ± 0.91	10 ± 0.61			
5.	*Aspergillus fumigatus*	16 ± 1.8	11 ± 0.66	11 ± 1.5	12 ± 0.66	17 ± 0.87			
6.	*Trichoderma viridae*	10 ± 1.5	12 ± 0.87	14 ± 0.66	5 ± 1.8	12 ± 0.66			
7.	*Rhizopus stolonifer*	12 ± 0.87	11 ± 1.8	16 ± 0.87	7 ± 1.5	6 ± 1.8			

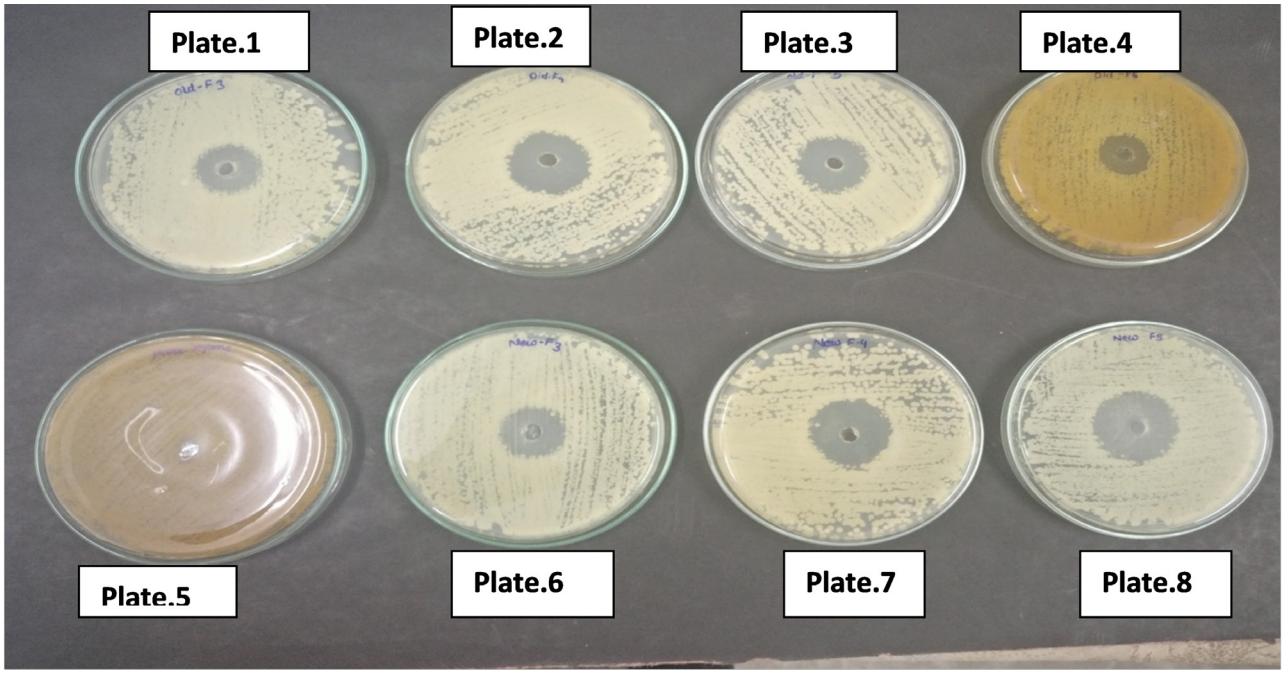


Figure 14: Anticandidal activity (Expressed by zone of inhibition in mm) of pure Fungal strains

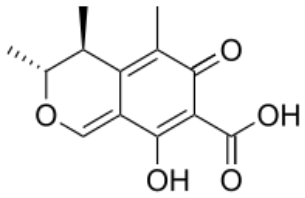


Figure 15: Citrinin structural diagram(Wikipedia image)

Column chromatography and Thin layer chromatography (TLC)

The results of column chromatography (Figure 12) of selected Fungal strain (*Penicillium notatum* which showed the maximum potential of inhibition against *Candida albicans*) shown, total six number of chromatographic bands were eluted out from the column chamber (loaded with 80–100 mesh silica gel) and activity of each eluted bands were tested by agar well diffusion assay, the active eluted bands were then purified again by running them in column again, by stepwise elution with mobile phase an yellow crude extracted at room temperature which was then filtered repeatedly to obtain pure active compound, final purification of active isolated elutes was done by TLC (Figure 14) with silica gel TLC plates, Then the RF values of isolated bands was compared with standard RF value Chart (reference). The mobile phases used in chromatographies were combination of n.Butanol: Ethanol: Acetone (120:33:57), stationary phases was silica gel, iodine vapor chamber used in formation of TlCband. Rf values were calculated by formula, and total three bands of RF values, 3.2cm, 0.685cm, 0.812cms were measured. Each band of TLC plates was scrapped out and again their activity was tested against *Candida albicans.* Final isolated active compound were identified and characterized by FTIR Spectroscopy and GCMS technique.

High-performance Liquid Chromatography Analysis

The detection of active compounds present in the purified compound obtained after thin layer chromatography was analyzed with High-performance liquid chromatography. By complete analysis of test samples presence of Citrinin is detected at 0.76nm. The quantification of Citrinin was done with using HPLC. The retention times of the culture extracts of fungal metabolite is compaired with a standard sample of citrinin(0.86nm). The HPLC elution profiles from the broth of *P. notatum* isolates from all the tested plant extracts showed the same retention time as of standard citrinin retention time = 3.8 minutes.

It has been assumed that some inhibitory metabolites are secreted by the fungal strains. Further studies are possible for the study of such metabolites in detail (Figure 16).

CONCLUSION

Candida albicans is normally present 80% in the human body in cellular form, but in favorable temperature, it forms pseudohyphae and causes infection. Its overgrowth causes diseases like vaginities, oral thrush in infants and

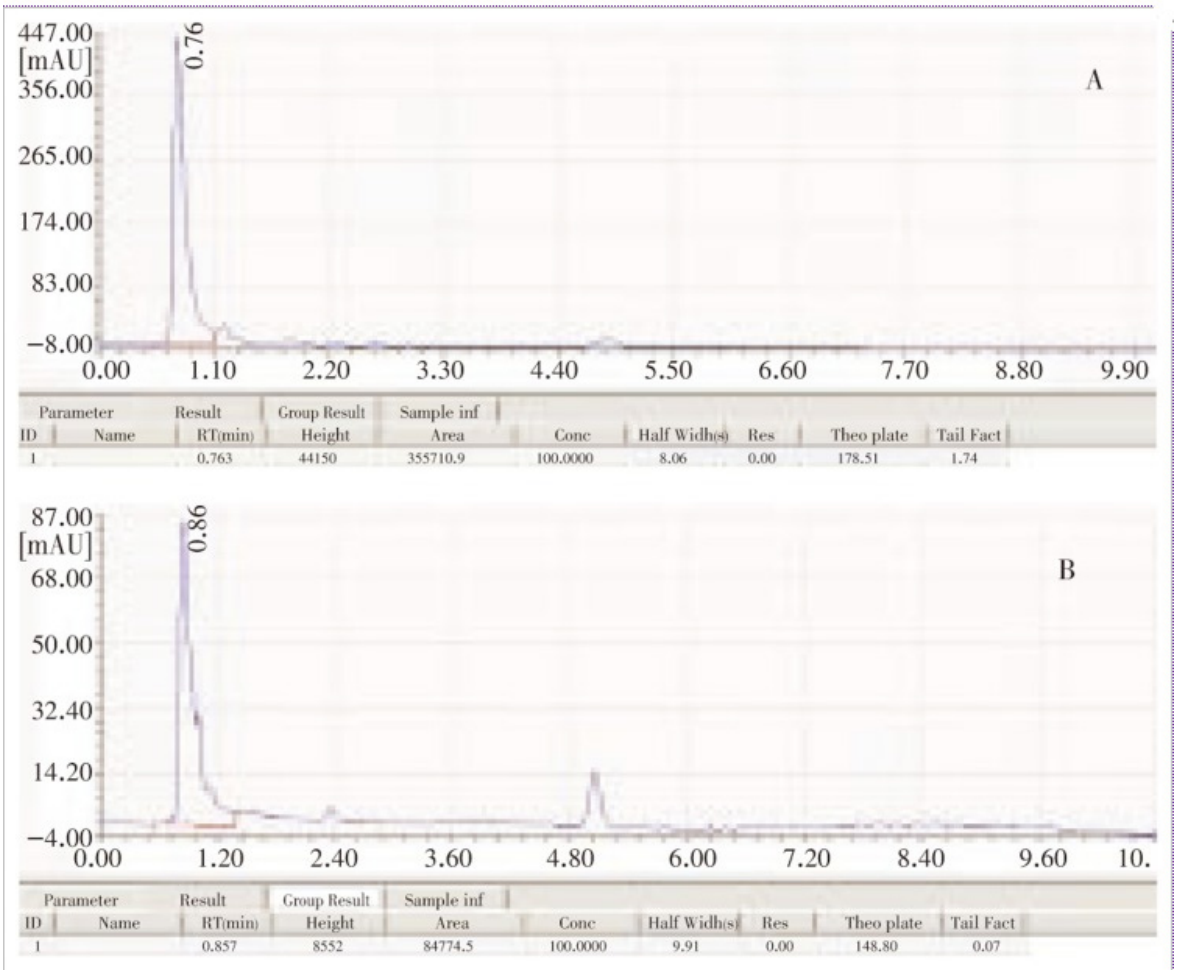


Figure 16: HPLC data of ***Penicillium notatum*** compared with ***citrinin***standard, A-Standard plot, B- Identified pure compound

dreadful diseases in human immunodeficiency virus (HIV) and cancer patients.

The results of our study clearly demonstrated that different extracts of *Rhizopus stolonifer*, *R.nigricans, Aspergillusniger, A.Fumigatus, Penicillum notatum* exhibit anticandidal activity which might be helpful in preventing the growth of these organisms causing various diseases and can be used in alternative system of medicine.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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