

Bioautography Guided Identification of Anticandidal Compounds from *A. terreus* st. 1

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Abstract: Problem statement: Saprophytic and soil inhabiting microorganisms are prolific producers of secondary metabolites with wide range of structural and functional diversity. The objective of the current study was to screen and evaluate the anti-candidal properties of soil inhabiting fungi.

Approach: Preliminary dual culture assay and bioautography was used to screen the fungi with potential anti-fungal activity and direct detection of antifungal compound/fraction in microbial extract on thin layer chromatograms, respectively. Disk diffusion, broth dilution and germ tube inhibitory assay were used to evaluate and confirm the anticandidal activity. Partial characterization of purified extract was carried out using High Performance Liquid Chromatography (HPLC) and Fast Performance Liquid Chromatography (FPLC) techniques. **Result and Discussion:** *A. terreus* was found to produce compound with anti-candidal and germ tube inhibitory activity against human pathogen *Candida albicans* at 200 mg ml⁻¹. Further proteinase inhibitory activity of the extract, tested using plate assay and SDS-PAGE ensures the anti-candidal activity of the extract. Present study identified and confirmed the potential anticandidal activity, in terms of germ tube and proteinase inhibitory activity, of metabolites produced by *A. terreus* st.1. **Conclusion:** The bio-assay guided fractionation and purification resulted in identification of unique fraction with active anti-candidal activity. Thus this fraction may serve as one of the lead to develop novel anti-candidal therapeutics.

Key words: Anticandidal compound, germ tube assay, inhibitory activity, *Candida albicans*, soil fungi, secondary metabolites

INTRODUCTION

Fungi have been found to be the most prolific producers of secondary metabolites. It has been suggested that fungi are fundamental to the health and prosperity of every terrestrial ecosystem and are essential for their sustainability and biodiversity (Clay and Holah, 1999). Soil-derived microorganisms represent a valuable resource in the search for secondary metabolites with useful therapeutic applications. The best-known fungal secondary metabolites that are subjected to commercial production are the β -lactam antibiotics (Ramachandran *et al.*, 2007). Fungi offer the advantage that most species produce bioactive compounds during growth and metabolism allowing tracing their mycelia and metabolites in their natural substrates and habitats

(Kettering *et al.*, 2004). Isolates of *A. terreus* obtained from terrestrial habitats have been known to produce variety of secondary metabolites with various biological activity including terretonins (Li *et al.*, 2005), terrecyclic acids (Almassi *et al.*, 1996) asterredione (Wijeratne *et al.*, 2003). Bioassay guided isolation and identification of compounds are helpful to point out the specific bioactive compounds in a mixture of metabolites (Sule *et al.*, 2011; Nalina and Rahim, 2007). Hence, screening of bioactive compounds like antimicrobials from soil fungi is an effective strategy to obtain novel and potent drugs. The bioautography is one of the techniques useful in direct tracing out bioactive compounds from extracts on thin layer chromatograms (Wedge and Nagle, 2000).

Candidiasis is a most common opportunistic fungal infection of immunocompromised patients such as

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AIDS, cancer and transplant patients. Changing epidemiology, emergence of new drug resistant *Candida* spp. and differential drug resistance complicates the treatment of candidiasis. *C. glabrata*, *C. krusei*, *C. inconspicua* and *C. norvegensis* are reported to have cross drug and multidrug resistance to both the class of antifungals, azoles and echinocandins, currently used for the treatment. Hence, the objective of the current study is to screen anticandidal compounds from soil fungi and subsequently to identify the active principles, through bioautography guided fractionation, in view to develop an effective anticandidal drugs.

MATERIALS AND METHODS

Fungal strains: Soil fungi were isolated by dilution and direct plate methods as per standards described by Dhingra and Sinclair (1995). Fungi isolated from soil were identified using fungal identification manuals. Fungi were isolated and maintained in potato dextrose agar (200 g of potato per liter, 2% dextrose and 2% agar, pH 5.3). Standard test strains of *C. albicans* NCIM 3074 obtained from the National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India was used for this study. *Curvularia* strain isolated from the maize was used for the initial antifungal screening assays.

Antifungal activity: Antifungal activity of the three soil fungi (*A. terreus* st. 1, *A. terreus* st. 2 and *Penicillium* sp.) was initially screened against *Curvularia* sp. by dual culture method as described by Dhingra and Sinclair (1995).

Fungal fermentation and extraction of metabolites: Fungus was cultured in Czepak-dox medium consist of 3% sucrose, 0.3% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄.7H₂O, 0.05% KCl and 0.001% FeSO₄. 7H₂O, with the final pH of 7.3. Cultures were allowed to grow in Roux bottles for 12 days at 25±2°C. The broth filtrate (2 L) of *A. terreus* st. 1 was separated from the mycelia by filtering through a Whatman No. 1 filter paper and an equal volume of ethyl acetate was added to the filtrates for extraction. The mixture was shaken in a separatory funnel for 30 min. The organic layer was collected and evaporated fully to get a dark brown solid, whose weight was measured to determine the yields of soluble metabolites.

Screening of anticandidal activity:

Disc diffusion method: Susceptibility disks (5 mm in diameter) were impregnated with 0.5 mg of extracted compounds dissolved in DMSO and placed on Sabouraud dextrose agar plates inoculated with the *C. albicans*. Sabouraud dextrose agar consists of 4% dextrose, 1% peptone and 2% of agar and the pH was

adjusted to 5.6. After 48 h. of incubation at 37°C plates were observed for clear zone of inhibition. Disc containing only DMSO was used as a negative control where as disk containing fluconazole (10 mg/disc) was used as positive control.

Germ tube inhibition assay: Test samples (dried extract dissolved in DMSO) of 0.1 mg mL⁻¹ concentration were used for this study. To this 990 µL of bovine serum and 10 µL of *C. albicans* mid log phase culture suspension was added and incubated for 3 h. at 37°C. The formation of germ tube was observed under the microscope after in the test samples incubation and compared with the control. The proportion of germinated and ungerminated cells was evaluated and % germination inhibition was calculated comparing with the control (without the test compound).

Proteinase inhibition assay by agar plate method Proteinase inhibition assay was performed as described by Dostal *et al.*, 2003. Briefly, the proteinase inhibition activity of ethyl acetate extract of *A. terreus* st. 1, proteinase induction agar was prepared adding 0.2% filter sterilized BSA to the medium containing 1% glucose, 3% yeast carbon base and 2% agar. The *Candida albicans* NCIM 3074 and a clinical isolates were streaked parallelly on the proteinase induction agar. Between two lanes of culture, the disc (0.5 mg) containing the ethyl acetate extract of *A. terreus* st. 1 was kept. The plates were incubated for 72 h at 37°C. The disc containing DMSO was used as negative control. After 72 h. of incubation, plates were processed by treating with 20% trichloroacetic acid and then stained with amidoblack (1.25% amidoblock dissolved in methanol containing 10% acetic acid). Further, plates were destained with 15% acetic acid and observed for the inhibition of clear zone formation by extract around the colony.

Detection of proteinase activity in SDS-PAGE

Proteinase activity of fungal compound is also detected in SDS-PAGE as described by (Korting *et al.*, 1999). *C. albicans* NCIM 3074 culture was grown in Sabouraud broth at 37°C for 48 h. The culture was centrifuged at 7000 rpm for 5 min to pellet the cells. The cell pellet was washed twice with normal saline and centrifuged to collect the pellets. Pellet was resuspended again in normal saline and cell concentration was adjusted approximately to 0.6 Optical Density at 620 nm using spectrophotometer. 100 µL of *Candida* cell suspension was added to proteinase induction medium containing 1% glucose, 3% yeast carbon base and 1% BSA. 500 µg mL⁻¹ ethyl acetate extract of *A. terreus* st. 1 in dimethyl sulfoxide was added to test tubes immediately after the

inoculation of culture. The mixture was incubated for 48 h at 37°C in a shaker at 150 rpm. Culture broth was centrifuged at 7000 rpm for 15 min to remove cells. Supernatant was retained for the future studies. Negative control containing only DMSO and positive control with pepstatin A (50 µg mL⁻¹) was run parallelly. One-dimensional SDS-PAGE was performed to detect the proteinase inhibition activity. The proteinase inhibition activity was measured in turn of no cleavage of BSA by proteinase of *Candida* comparing with control (without compound).

Purification:

Thin layer chromatography: TLC plates of 0.5 mm thickness prepared by using Silica Gel G 1 mg of crude extract was loaded in a single spot at one edge and plates were developed in a presaturated TLC chamber with dichloromethane, ethyl acetate and methanol (8:1:1 v/v/v) for separation of compounds. TLC plates were treated with vapors of iodine for compound visualization. R_f values of all the compounds were recorded. Fractions were eluted with ethyl acetate and used for further study.

Bioautography: One dimensional bioautography for ethyl acetate extract of *A. terreus* st. 1 was performed using *Curvularia* sp., as indicator fungus as described above by loading 2 mg of organic extract. The chromatograms were uniformly sprayed with potato dextrose broth (2% dextrose, potato extract, 0.1% tween 80 and 0.06% agar). by Wedge and Negle, 2000. *Curvularia* spore suspension (~10⁶ cells/ml) grown in Thin layer chromatogram was developed using method The chromatograms were kept for incubation aseptically in a moist chamber for 48-72 h at 25±2 °C. The growth of *Curvularia* over silica gel of chromatogram was noticeable with a black colored growth against white background. The antifungal activity of the separated compounds was recorded as absence of growth over the active principle (white clear zone).

HPLC and FPLC analysis: The active component from TLC is fractionated by reverse phase high-performance liquid chromatography (RP-HPLC) (Thermo spectrasystem, USA) using a C4 reversed-phase column (Waters, USA) and 10%-100% acetonitril gradient with 0.1% tetrafluoroacetic acid as mobile phase. Further, fraction was subjected to preparative reversed-phase FPLC (Pharmaciabiotech, USA) using C4 column (Waters, USA), with 10-100% acetonitril and 0.1% tetrafluoroacetic acid mobile phase at 20 mL per minute flow rate to separate each fraction. FPLC fractions were concentrated in vacuum and tested for anticandidal activity against *C. albicans*.

Broth microdilution assay: Broth microdilution assay was done according to the method of the National Committee for Clinical Laboratory Standards with slight modification. The fractionated compound obtained from HPLC (Fd) was dissolved in DMSO and concentration of compound was made from 0.1-0.5 mg mL⁻¹ in Sabouraud Dextrose (SD) broth. 0.01 mL of washed cell suspension of *Candida* (cell density approximately 10⁶ mL⁻¹) was added to each tube and total volume made up to a one ml. Tubes were incubated at 37°C in a shaker at 150 rpm for 48 h. Biomass was separated by centrifugation for 5 min at 5,000 rpm in room temperature. Pellet suspended in 0.1 ml of fresh SD broth was spread on SDA plates. Finally, SDA plates were incubated at 37°C for 48 h. Inhibition activity of extracts in broth culture was recorded.

RESULTS

Three fungal strains were isolated from paddy field soil of Bennatte farm, Thammadikoppa, Shimoga District, Karnataka State. Two isolates of the fungi identified as *A. terreus* and another isolate identified as *Penicillium* species. The fungus *A. terreus* are identified by its marked buff coloured colony on PDA, biserial-columnar conidial heads, hyaline and septated hyphae Fig. 1. Sporangiophores are smooth-walled and hyaline, terminating in mostly globose vesicles. Initial screening of all three fungi in dual culture method, showed potential antifungal activity against *Curvularia* sp. Figure 2 clearly depicts the antifungal effect of *A. terreus* st. 1 against *Curvularia* sp., screened by using dual culture method. *A. terreus* st. 1 was cultivated on Czepak-dox medium yielded reddish orange colored culture broth and recovery of ethyl acetate soluble metabolites resulted in the 90 mg of extract/L of culture *A. terreus* st. 1.



Fig. 1: Morphology of *A. terreus* st. 1

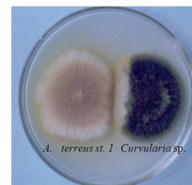


Fig. 2: Antifungal activity of *A. terreus* st. 1 against *Curvularia* sp. by dual culture method

The screening of anticandidal activity of ethyl acetate extract of three fungi by disc diffusion assay indicated the anticandidal activity only in *A. terreus* st. 1. *A. terreus* st. 1 showed 16 mm diameter zone of inhibition against *C. albicans* Table 1. Though, *A. terreus* st. 2 and *Penicillium* sp. showed antifungal activity against *Curvularia* sp in dual culture method, there is no marked anticandidal activity observed in these two fungal species upon screening using disc diffusion method. Further, broth dilution test with ethyl acetate extract of *A. terreus* st. 1 against *Candida* indicated MFC value a 0.5 mg mL^{-1} . Interesting results were observed when ethyl acetate extract of *A. terreus* st. 1 was assessed for its germ tube inhibitory activity. 78.7% germ tube inhibition was recorded when tested at 0.25 mg mL^{-1} of extract and the inhibitory activity went up to 88.9% at 1 mg mL^{-1} concentration of extract Fig. 3 and 4. Further, anticandidal activity was confirmed by testing proteinase inhibitory activity using plate assay Fig. 5 and activity was observed in terms of no clear zone formation near vicinity of extract. SDS-PAGE assay the proteinase inhibition activity of metabolites indicated as no cleavage of the Bovine Serum Albumin (BSA) in test sample as visualized in SDS polyacrylamide gel Fig. 6, compared to cleavage of BSA by *Candida* proteinases in untreated sample. The proteinase activity was recorded in 0.5 mg/disc of extract by agar plate assay and 0.5 mg mL^{-1} in liquid medium.

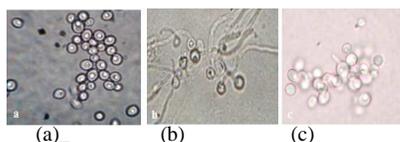


Fig. 3: Effect of ethyl acetate extract of *A. terreus* st. 1 on germ tube formation of *C. albicans* NCIM 3074 (a) Negative Control (b) Positive Control (c) Treatment

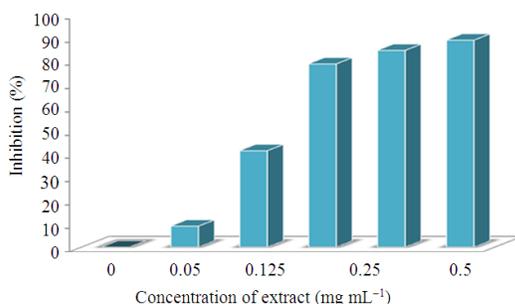


Fig. 4: Effect of ethyl acetate extract of *A. terreus* st. 1 on germ tube formation of *C. albicans* NCIM 3074

The ethyl acetate extract of *A. terreus* st. 1 was fractionated using silica gel thin layer chromatography (TLC) to identify and characterize the active metabolites with anticandidal activity. The extract was separated into six fractions (F1-F6) with Rf value 0.16, 0.26, 0.70, 0.84, 0.92 and 0.98 in TLC. Among the six fractions, F3 of the *A. terreus* st. 1 showed significant anticandidal activity (19 mm dia. inhibition zone) in disc diffusion assay and in broth dilution test it completely inhibited the *C. albicans* at 0.25 mg mL^{-1} of compound Table 2. Bioautography of the extract of *A. terreus* st. 1 tested against *Curvularia*, confirmed the antifungal activity of specific fraction on TLC. Inhibition of fungus growth was detected as white patches indicative of the presence of antifungal compound. The fraction F3 was identified to have antifungal activity against *Curvularia*, where clear white patches of inhibited area was observed in contrast to black colored growth of fungus Fig. 7.

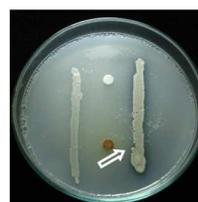


Fig. 5: Plate assay for testing proteinase inhibitory activity of ethyl acetate extract of *A. terreus* st. 1 in *C. albicans* NCIM 3074 (Arrow indicate inhibition of proteinase activity)

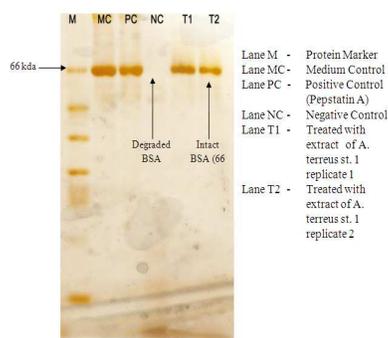


Fig. 6: SDS-PAGE for testing proteinase inhibitory activity of ethyl acetate extract of *A. terreus* st. 1 in *C. albicans* NCIM 3074

Table 1: Anticandidal activity of fungal extracts by disc diffusion assay

Extract (0.5 mg/disc)	Diameter of inhibition zone (mm)
<i>Penicillium</i> sp.	0
<i>A. terreus</i> st. 1	16
<i>A. terreus</i> st. 2	0
Control-DMSO	0
Fluconazole	0

Table 2: Bioassay of TLC purified fractions of *A. terreus* st. 1

TLC fraction	DMSO	F1	F2	F3	F4	F5	F6
Rf value		0.16	0.26	0.70	0.84	0.92	0.98
Disc diffusion assay (0.5 mg/disc)	-	-	-	+	+	-	-
				(19 mm)	(8 mm)		
Broth test (0.5mg mL ⁻¹)	-	-	-	+	-	-	-

(+) Presence of anticandidal activity, (-) Absence of anticandidal activity

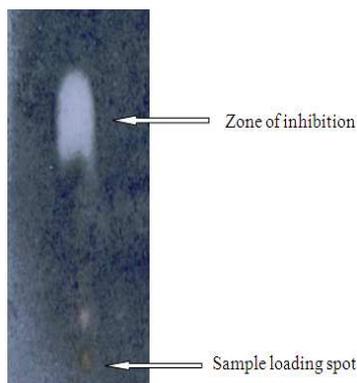


Fig. 7: Bioautography of ethyl acetate extract of *A. terreus* st.1 against *Curvularia* sp.

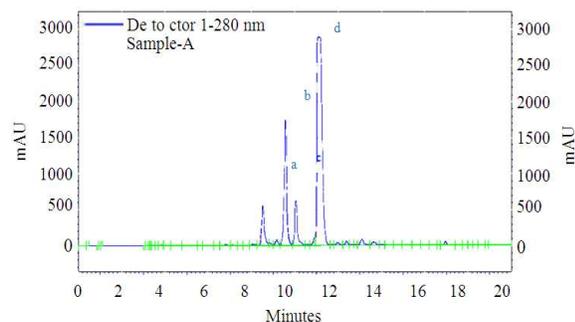


Fig. 8:HPLC chromatogram of active fraction obtained from TLC of *A. terreus* st. 1 extract

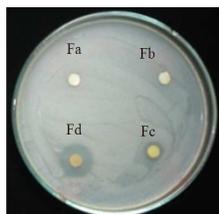


Fig. 9: Anticandidal activity of FPLC purified fractions (Fa-Fd) of the *A. terreus* st. 1 in disc diffusion assay against *C. albicans*

The composition of F3 fraction (fraction which showed antifungal activity) analyzed using HPLC with C4 column resulted in four subfractions Fa, Fb, Fc and Fd Fig. 8. Further, four subfractions from F3 were separated in FPLC resulted in 60% of fraction F3d. Bioassay of the purified compounds suggests that major fraction F3d possess anticandidal activity.

Table 3. Disc diffusion assay for FPLC fractions

Fraction	F3a	F3b	F3c	F3d
Activity in 0.25mg ml ⁻¹	0	0	8 mm	19 Mm

Table 4. Microbroth dilution test for FPLC fraction F3d

Concentration in mgml ⁻¹	Inhibition
0.1	-
0.2	+
0.3	+
0.4	+
0.5	+

+ inhibition of *Candida albicans*- absence of inhibition

The compound showed anticandidal activity in disc diffusion assay (19 mm dia inhibition zone) at 250 µg/disc of compound (Fig. 9 and Table 3). Broth microdilution assay indicated MFC of the Fd is 200 µg mL⁻¹ against *C. albicans* (Table 4). These results confirm the anticandidal activity of the partially purified ethyl acetate extract of *A. terreus* st. 1.

DISCUSSION

Fungi are rich source of biologically active metabolites that find wide-range of applications in pharmaceutical industry such as antibiotics, immunosuppressants, antiparasitics and anticancer agents. The soil fungi are known to produce diverse metabolites of such kind with diverse biological activities. Thus the current study is aimed at screening and identification of active metabolites from soil fungi with potential anti-candidal activity. Preliminary result of anti-candidal properties were identified by dual culture method using *Curvularia* and this test supported to opt the suitable fungus which producing antifungal compounds. Antifungal activity of *A. terreus* st. 1 was first evaluated with disc diffusion assay and further confirmed by broth dilution test. *A. terreus* st. 1 was identified to have efficient anti-candidal activity probably due to its significant production of unique secondary metabolites which is evident as *A. terreus* strains are reported to produce unique bioactive compounds (Almassi *et al.*, 1996; Li *et al.*, 2005). The *Candida albicans* activity was not observed in all other the fungal species screened might be due to lack of anticandidal metabolites. The present study also confirms that the anticandidal compounds from the *A. terreus* st. 1 were extractable using the organic solvent ethyl acetate.

The use of defined medium (Czepekdox medium) in the current study was successful in the generation of biomass with adequate production of active metabolites and also the study optimized the efficient extraction of active metabolites from *A. terreus* st. 1 without interruption of other cellular components. Further, anticandidal activity of these fungal metabolites was confirmed to have germ tube inhibitory activity against *C. albicans*, which is a unique characteristic feature for an anti-fungal agent. The germ tube inhibitory property can be very effective in controlling yeast-mold shift in dimorphic fungi. Identification of novel compound with germ tube inhibition activity lead to develop drug which prevent in entry level or prevent colonization in host tissue.

Interestingly, proteinase an important virulence factor of *Candida* sp. was found to be inhibited by the metabolites extracted from *A. terreus* st. 1. This has been confirmed by both plate assay and SDS-PAGE assay. These unique features of this compound(s), anticandidal, germ tube and proteinase inhibitory activity, might be exploited in the anti-fungal drug development to treat invasive infections of dimorphic fungi especially infections caused by *Candida* spp., (Naglik *et al*, 2004). Therefore, active compound(s) of *A. terreus* st. 1 may serve as lead compounds to control the colonization and establishment of *Candida*. These unique features of this compound(s) from of *A. terreus* st. 1 might be exploited in the anti-fungal drug development to treat invasive infections of dimorphic fungi especially infections caused by *Candida* spp., particularly immunosuppressed patients.

Fractionation of extract for identification of active anticandidal compound(s) was efficiently achieved using TLC, HPLC and FPLC techniques Bioautography was very useful in tracking the antifungal compound(s) of ethyl acetate extract on thin layer chromatograms. Wedge and Nagle (2000) used the 2D bioautography for the detection of antifungal compounds from the aquatic/marine cyanobacteria, algae and plants by screening against plant pathogenic fungi such as *Colletotrichum fragariae*, *C. gloeosporioides* and *Phomopsis* sp. Similarly single dimensional bioautography method used in this study was found to be most simple, rapid and efficient method for direct tracing of anti-fungal fractions from the crude extracts. HPLC analysis of TLC purified biologically active fractions showed four subfractions and one fraction which is recovered relatively higher quantity found to have antifungal activity. Structural elucidation of compounds is under the progress.

Species of genus *Aspergillus* reported as prolific producer of variety of bioactive compounds. Number of antifungal compounds was reported earlier from the species of *Aspergillus*. Brefeldin A, a compound with

antifungal, antiviral and anticancer activity was isolated from the *A. clavatus* (Wang *et al.*, 2002) and fumigaclavine with antifungal activity and cytotoxicity was isolated from *A. fumigatus* (Liu *et al.*, 2004). Song *et al.*, (2004) reported that the production of compound fonsecinone A with antifungal activity and xanthine oxidase inhibition activity from *A. niger*. Though number of such reports describes the diverse biological roles of metabolites isolated from different fungal sources, however, none of the report evidenced isolation of anticandidal compounds from *A. terreus*. This study is the first of this kind to report the anti-candidal activity of *A. terreus* and fractionate its active components to partially purify and characterize the extracts.

CONCLUSION

The investigation of soil fungi resulted in identification of potential soil fungi *A. terreus* st. 1 and confirmed to produce antifungal compounds with germ tube and proteinase inhibitory properties. Bioautography is ascertained to be a most simple, effective and useful for direct identification of antifungal compound(s) from crude extracts on TLC chromatograms. This shall certainly reduce the time and cost involved in the battery of bioassay guided extract purification processes. Fractionation and bioassay guided purification studies confirmed the anti-candidal potentials of compound(s) isolated from *A. terreus* st. 1, which thus can be studied further for the developed of anti-candidal drug. Thus with all these it can be concluded that, this active fraction from *A. terreus* st. 1, has the potential to be developed as an anti-candidal drug.

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