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Bioactivity guided extraction of 5-(2, 4-dimethylbenzyl) pyrrolidin-2-one from marine *Streptomyces* VITSVK5 spp. and its anti-*aspergillus* activity against drug resistant clinical isolates

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ABSTRACT

Aspergillus is a major human pathogen responsible for the formation of aspergilloma in preformed lung cavity among pulmonary tuberculosis patients. Most of the strains causing Aspergillosis are drug resistant in nature. Hence looking for an effective antagonistic lead compound from natural sources are very much needed to tackle the drug resistance strains. Several antibiotics available in the market are from marine Streptomyces and being explored constantly for newer antibiotics. The aim of the present study was to isolate anti-Aspergillus compound from marine Streptomyces and assay for antifungal activity against drug resistant Aspergillus clinical isolates. In our systematic screening of actinomycetes for anti-Aspergillus activity resulted in isolation of a strain Streptomyces VITSVK5 spp. Bioactivity guided separation yielded a compound 5-(2, 4-dimethylbenzyl) pyrrolidin-2-one (DMBPO) and the anti-Aspergillus activity of the compound was tested against 11 multidrug resistant (MDR) Aspergillus clinical isolates. The compound showed the MIC_{50} value of 4 - 0.125µg/ml against MDR Aspergillus clinical isolates by NCCLS method and MIC_{50} of 3 - 0.125µg/ml by MTT assay. MTT assay at 50% cell viability was compared with NCCLS method and the former showed 95% agreement with NCCLS method. Based on the results it can be concluded that DMBPO has significant anti-Aspergillus activity against drug resistant clinical isolates.

Keywords: *Streptomyces* VITSVK5 spp.; 5-(2, 4-dimethylbenzyl) pyrrolidin-2-one; anti-*Aspergillus* activity; Extraction; MTT assay

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INTRODUCTION

Aspergillus species represents the second most common fungal pathogen in hospitals. *Aspergillus* is ubiquitous fungus that has three classic pulmonary presentations: invasive aspergillosis; allergic bronchopulmonary aspergillosis; and aspergilloma. Inhalation of *Aspergillus* spore by tuberculosis patients with the presence of preformed lung cavity resulted in the formation of a fungal ball called Aspergilloma. An aspergilloma may exist for many years asymptomatically with no therapy warranted. The antifungal agents currently available for the treatment of systemic fungal infections are amphotericin B, lipid formulations of amphotericin B, 5-fluorocytosine and azoles such as miconazole, ketoconazole, fluconazole and itraconazole. Currently, the criteria used for the selection of new drug candidates include inhibitors of fungal cell wall biosynthesis, potency comparable to amphotericin B, safety comparable to fluconazole, fungicidal activity *in vitro* and fungicidal activity *in vivo*. Evidence suggests that poor

response to antifungal agents is due to these drugs rarely achieve the minimal inhibitory concentrations within the lung cavities [1] including the administration of IV amphotericin B failed to show a satisfactory recovery in patients with aspergilloma [2]. On the other hand the surgical treatment of aspergilloma is associated with relatively high mortality rate [3,4].

Pharmacological screening and usage of natural products for the treatment of human diseases has had a long history from Ayurvedic medicine to modern drugs [5]. The majority of modern drugs reported are belongs to natural products [6]. The majority of clinically used antifungals have various drawbacks in terms of toxicity, efficacy and cost, and their frequent use has led to the emergence of resistant strains. Hence, there is a great demand for novel antifungals belonging to a wide range of structural classes, selectively acting on new targets with fewer side effects. One approach might be the testing of natural products for their antifungal activities as potential sources for drug development. As reported by Butler, 2005 [7] about seventy natural products or natural product derivatives are currently undergoing clinical trials in different parts of the world including United States, Europe, Japan, and Korea, out of which thirty were derived from microorganism and twelve were marine derived. Microorganisms from extreme environments have gained considerable attention in recent years because of its diversity and biological activities, mainly due to its ability to produce novel chemical compounds of high commercial value. At present there are sixteen compounds isolated from marine actinobacteria are in clinical trial against various diseases [8]. Actinomycetes are a group of prokaryotic organisms; they are gram-positive bacteria that grow extensively in soils rich with organic matter and are capable of producing several secondary metabolites [9]. The marine environment remains as a virtually untapped source for novel actinomycetes. The distribution and abundance of actinomycetes are generally depends on various ecological habitats which includes beach sand [10] and seawater [11].

In the present study the anti-*Aspergillus* activity of 5-(2, 4-dimethylbenzyl) pyrrolidin-2-one extracted from marine *Streptomyces* VITSVK5 spp**.** was tested against drug resistant *Aspergillus* clinical isolates.

MATERIALS AND METHODS

Isolation of actinomycetes:
The strain *Streptomyces* VITSVK5 spp. was isolated from the salt terns of Marrakanam coast of Bay of Bengal, southern India. The strain was selectively isolated on Starch casein agar, ISP No.1 medium and the nutritional and cultural conditions for the growth were optimized. Isolation and characterization of the strain was previously reported [12].

Extraction and purification of compound:
Well grown slant culture of the potential isolate was used for preparation of seed culture. The seed culture was inoculated in 50 ml medium containing the optimized production medium prepared with sea water 50%, distilled water 50%, pH 8.2 and incubated for 2 days in rotary shaker (200 rpm) at 30°C. The inoculums (10%) were transferred into 200 ml production medium in 1 liter Erlenmeyer flasks and kept for fermentation for a week. After fermentation, the broth was centrifuged at 4000 rpm for 10 min at 10°C and the supernatant was separated by filtering in 0.2 µm pore-size membrane filters. The supernatant was extracted twice with n- Butanol (400 ml) and washed with 500 ml water then the culture was harvested by centrifugation for 10 min at 4000 rpm at 10^{o}C and the filtrate was separated by centrifugation (16000*x*g, Remi High speed centrifuge). After separation, the organic phase was dried over Na_2SO_4 (anhydrous). The extract was then concentrated in rotary vacuum and lyophilized using a freeze drier (Thermo, USA) at 5^{o}C for 5 h. The crude extracts were stored at – 20°C. The butanol layer was concentrated and the residual suspension (750 mg) was chromatographed over silica gel column and eluted with chloroform: methyl alcohol (9:1, 8.5:1.5, 8:2, 7.5:2.5, 7:3). The active fractions were collected, concentrated and further separated by preparative TLC on silica gel with chloroform: methyl alcohol (8:2) and the purity of the compound was analyzed.

Structure elucidation:
The UV spectra of the compound were measured using UV-Visible spectrophotometer (Techcomp, Hong Kong). In order to investigate the presence of various functional groups in bioactive compound, the sample was lyophilized and mixed with KBr (1:20; 0.02 g of sample with KBr at a final weight of 0.4 g). The pure compound was then grounded, desorbed at 60°C for 24 h and pressed to obtain IR-transparent pellets. Infrared spectra of the compound were obtained using a Fourier Transform Infrared Spectrometer (FT/IR-AVATAR 330). The spectra were collected within a scanning range of 400-4000/cm. The FT-IR was first calibrated for background signal scanning with a

control sample of pure KBr, and then the experimental sample was scanned. The spectra obtained were analyzed for various functional groups. The proton NMR (^{1}H NMR) and carbon NMR (^{13}C NMR, V Bruker Avance III 500 MHz (AV 500)) spectra of the compound were obtained by using a dimethyl sulfoxide d6 (DMSO-d6) as solvent. It was further evaluated with DEPT-135. It was further confirmed by mass spectroscopy (HR-MS, Jeol GCMATE II). The structure of the compound was established with the help of spectral data obtained from various spectroscopic techniques. The 3D structure of the compound was obtained by using ChemDraw software (Ultra 8.0).

Collection of sputum sample:
Sputum samples were selected for the isolation of *Aspergillus* sp. as it is the most common specimen for the diagnosis of fungal pneumonia and chronic pulmonary disease. Sputum samples (exporated morning) were collected in a sterile container on two consecutive days from pulmonary TB patients admitted in the Government Vellore Medical College and Hospital (GVMCH), Vellore, Tamil Nadu, India after obtaining informal consent from them. The best specimen was obtained when the patient first awakens from sleep, after the oral cavity was cleansed. At least two expectorated morning sputum samples on two consecutive days were collected from each patient in a sterile container and were transported to the laboratory within one hour and were processed immediately.

Isolation of *Aspergillus* strains:
Specimens obtained were subjected to a series of routine direct procedures. The sputum samples were cultured on Sabouraud's Dextrose agar (Hi Media, India) plates with chloramphenicol (0.5 g/l) with or without cycloheximide (0.5 g/l) at 27°C and 37°C and were examined every day for 7 days. Cultures were scrapped with mycelium and were examined by wet mount preparation with or without the addition of 10% potassium hydroxide (KOH), which aids in the visualization of hyphal elements through the partial digestion and clearing of proteinaceous material while leaving the fungal cell wall intact [13]. Direct microscopic examination of the specimens was considered positive if typical septate hyphae were observed. Aspergillus strains were distinguished on the basis of colony morphological features and microscopic structures obtained with lactophenol cotton blue stain. Isolates were stored in glycerol 20% at -70°C until needed.

Determination of MIC_{50} value by NCCLS method:
Stock solution of different antifungal drugs amphotericin B (AMB), itraconazole (ITC), ketoconazole (KET), fluconazole (FLU) and lead compound were prepared by dissolving in dimethyl sulfoxide (DMSO) and then diluted with RPMI-1640 medium to have the concentration range of 0.03–32 µg/ml. The anti-*Aspergillus* activity against clinically isolated MDR *Aspergillus* sp. [12,14] was determined using broth dilution method using standard protocol (CLSI M38–A).

Determination of MIC_{50} value by MTT Method:
The MIC values were also determined by a colorimetric method using the dye MTT (Sigma Chemical, St. Louis, Mo.) [15]. After the MIC values were visually determined on each of the microtitre plates, 25 ml of RPMI 1640 containing 5 mg of MTT/ml was added to each well. Incubation was continued at 37°C for 3 h. The content of each well was removed (centrifugation is not required for filamentous fungi due to their adherence to the wells), and 200 ml of isopropanol containing 5% HCl (1 M) was added to extract the dye. After 30 min of incubation at room temperature and gentle agitation, the optical density (OD) was measured with a microtitre plate reader spectrophotometer at 550 nm. The OD of the blank, which consisted of an uninoculated plate incubated together with the inoculated plates, was subtracted from the OD values of the inoculated plates. The percentage of MTT conversion to its formazan derivative for each well was calculated by comparing the OD at 550 nm of the wells with that of the drug-free control based on the following equation: (A550 of wells that contained the drug/A550 of the drug-free well) x 3. MIC_{50} were considered to be the lowest concentrations of drug showing at least 50% reductions in the OD value compared with that of the drug-free well.

Statistical Analysis:
All the experiments were performed in triplicates and the data obtained were expressed as mean ± standard error. *P*-Values <0.05 were considered as statistically significant. The MIC_{50} values and their respective 95% confidence intervals were calculated by non-linear regression analysis using the data analysis software (Prism).

RESULTS AND DISCUSSION

In the course of our systematic screening programme for antifungal potential of marine actinomycetes from southern coast of India, the strain *Streptomyces* VITSVK5 spp. was isolated from sediment samples of the Marakkanam coast Tamil Nadu, India. The strain was identified by molecular taxonomic characterization using 16S rRNA partial gene sequence analysis and 16S rDNA sequence analysis. The 16S rDNA sequence (1424 base pairs) of *Streptomyces* VITSVK5 spp. has been submitted to NCBI under the accession no. GQ848482. The strain was found to be novel and crude extract showed significant antifungal activity against drug resistant strains of *Aspergillus* clinical isolates [12]. Since the butanolic extract of *Streptomyces* VITSVK5 sp. showed the maximum inhibitory activity against drug resistant *Aspergillus* clinical isolates, it was subjected to bioactive guided extraction and purification of active principle. Extraction and purification of 10 L of the culture broth yielded 112.3 mg of pure compound. TLC separation of the compound yielded a single spot with R_f value of 0.43 (Chloroform-Methanol, 8:2). The band was visualized by iodine reagent and sulphuric acid. The purified compound was subjected to UV, FT-IR, ^{1}H NMR, ^{13}C NMR, DEPT, HRMS spectroscopic studies in order to establish the chemical structure of the active compound. The spectral data obtained for the compound was used to establish the structure of the compound. UV/vis (MeOH) λmax 290, FT-IR (cm^{-1}) 3436 (-NH), 2928, 1729 (C=O), ^{1}H-NMR (DMSO-d6 500 MHz) : 0.871 (s, J=64 Hz, -CH_3), 1.23 – 1.39 (m, J=44 Hz, 2xCH_2), 3.37 (t, 1H), 4.29 (s, 2H), 7.25 (s, 1H), 7.65-7.69 (t, 2H), 8.17 (s, 1H); ^{13}C-NMR (DMSO-d6 500 MHz) : 13.80 (CH_3), 18.60 (CH_3), 34.65(2x CH_2), 60.34(CH_2), 64.98 (CH), 128.62, 131.4, 133.6, 134.3, 136.7, 144.7, 166.9. DEPT, HRMS, m/z (found/cal.): 203.1325/203.1310. Based on the spectral data the structure of the compound was identified as 5-(2, 4-dimethylbenzyl) pyrrolidin-2-one (DMBPO) and the molecular formula was determined as $C_{13}H_{17}NO$. The structure of the compound is illustrated in Fig. 1A. The 3D structure of the compound (Fig. 1B) was modeled using Chem3D Ultra software (Version 8). The compound is fluorescent in colour, soluble in methanol, chloroform, and DMSO. The physico-chemical properties of DMBPO are given in Table 1.

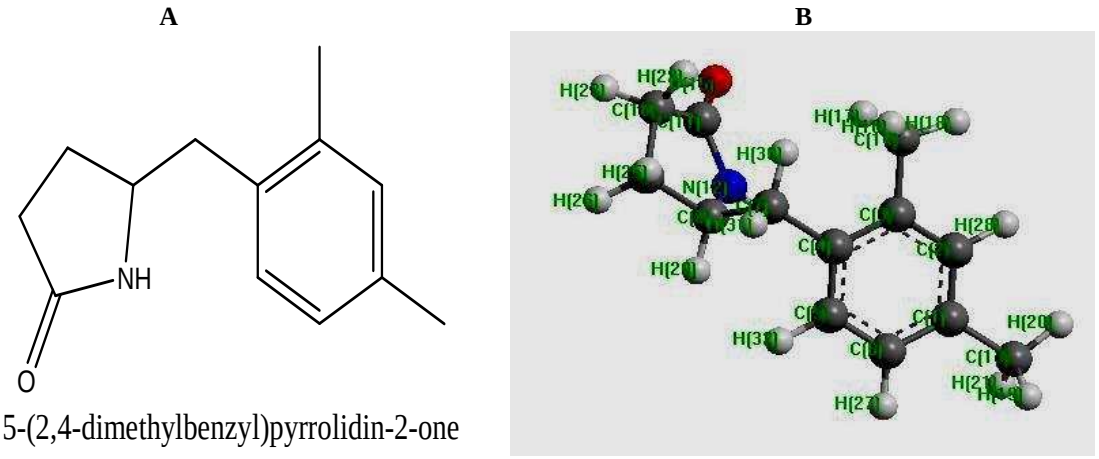

Fig. 1. A) 2D Structure of 5-(2, 4-dimethylbenzyl) pyrrolidin-2-one ($C_{13}H_{17}NO$) and B) 3D structure of 5-(2, 4-dimethylbenzyl) pyrrolidin-2-one ($C_{13}H_{17}NO$) modeled in Chem3D Ultra 8.0

Table 1. Physicochemical properties of DMBPO

Exact Mass: 203.13	Mol. Wt.: 203.28
m/e: 203.13 (100.0%), 204.13 (14.4%), 205.14 (1.2%)	El.analysis: C, 76.81; H, 8.43; N, 6.89; O, 7.87
Boiling Point: 644.46 [K]	Melting Point: 407.76 [K]
Critical Temp: 857.99 [K]	Critical Pres: 26.06 [Bar]
Critical Vol: 653.5 [cm3/mol]	Gibbs Energy: 172.95 [kJ/mol]
Log P: 2.39	MR: 62.81 [cm3/mol]
Henry's Law: 7.11	Heat of Form: -84.37 [kJ/mol]
CLogP: 2.062	CMR: 6.1622

The antifungal activity of the purified compound 5-(2, 4-dimethylbenzyl) pyrrolidin-2-one (DMBPO) was tested against all the MDR strains of *Aspergillus* sp. isolated from sputum samples of pulmonary tuberculosis patients and

compared with standard drugs. The pure compound showed the maximum MIC_{50} value of 4 µg/ml (MDR6 and MDR9) whereas least value of 0.125 µg/ml was observed for MDR3, 4 and 5. The MIC_{50} values of selected drugs and DMBPO against MDR *Aspergillus* clinical isolates are given in Table 2. Gendy and Bondkly (2010) [16] have reported the isolation of novel antimycotic agents, saadamycin and 5, 7-dimethoxy -4-p-methoxy phenylcaumarin from *Streptomyces* sp. Both compounds exhibited significant antimycotic activity against dermatophytes and other clinical fungi.

Similarly, Thorne and Alder (2002) [17] have reported the *in vitro* antibacterial activity of daptomycin, a natural product derived from the fermentation of *S. roseosporus* against methicillin-resistant *Staphylococcus aureus*, methicillin-resistant *Staphylococcus epidermidis*, vancomycin resistant enterococci (VRE), and penicillin-resistant *Streptococcus pneumoniae.* Recently the antifungal (*C.albicans*) bioactivity of actinomycetes isolated from marine sponges collected from offshore Ras Mohamed (Egypt) and from Rovinj (Croatia) was evaluated and found to be potent inhibitor, which has open a new face of isolating a novel strain residing symbiotically with potent bioactivity in marine microorganisms [18]. Several earlier reports supports our result that offshore sediments collected has been shown to possess antimicrobial activity against a group of bacterial and fungal pathogens with moderate antagonistic activity against *Aspergillus* species such as *Streptomyces* spp. PM-32 [19].

Table 2. MIC_{50} values of DMBPO and drugs against *Aspergillus* clinical isolates (MDR1-11)

MDR Clinical isolates	AMB (µg/ml)	ITR (µg/ml)	FLU (µg/ml)	KET (µg/ml)	DMBPO (µg/ml)
MDR 1	8±0.33	2±0.23	4±0.33	16±0.62	2±0.33
MDR 2	0.25±0.23	8±0.33	8±0.256	8±0.33	0.5±0.12
MDR 3	8±0.115	4±0.33	2±0.23	16±0.75	0.125±0.04
MDR 4	16±0.33	2±0.256	0.2568±0.23	4±0.33	0.125±0.11
MDR 5	8±0.33	8±0.33	1±0.23	2±0.23	0.125±0.27
MDR 6	2±0.256	4±0.23	1±0.115	8±0.256	4±0.66
MDR 7	16±0.62	8±0.33	8±0.115	8±0.33	0.25±0.25
MDR 8	1±0.256	32±0.62	4±0.23	16±0.62	2±0.33
MDR 9	0.125±0.115	8±0.62	0.5±0.115	4±0.23	4±0.22
MDR 10	0.25±0.115	4±0.33	4±0.33	4±0.115	0.25±0.33
MDR 11	0.25±0.23	2±0.23	8±0.33	4±0.256	2±0.66

AMB: Amphotericin B; ITR: Itraconazole; FLU: Fluconazole; KET: Ketokonazole; DMBPO:- 5-(2, 4-dimethylbenzyl) pyrrolidin-2-one; MDR:- Multidrug Resistant strain

Antagonistic activity of different *Streptomyces* species isolated from soil samples has already been reported earlier [20]. Anti-*Aspergillus* (*A. niger*) activity of *Streptomyces* species isolated from coastal water of Dhanushkodi, Tamil Nadu India has been reported earlier [21].

Determination of MICs for filamentous fungi can be facilitated with a method which overcomes observer bias and quantifies the hyphal growth of molds. Because turbidity measurements and colony counts are not useful in the case of filamentous fungi, colorimetric methods based on the measurement of metabolic activity may facilitate better determination of MIC [22]. The colorimetric method using the dye MTT has been investigated as an alternative method for the NCCLS method for in vitro susceptibility testing of fungi. MIC values of DMBPO against MDR strains are presented in Fig. 2. In our study the level of agreement between the MTT assay and NCCLS method was 95% similar. All the samples was found to be statistically significant at $p<0.05$. The MIC_{50} values for all the drug resistant strains were calculated at 50% cell viability. Cell viability was directly proportional to the percentage of MTT conversion. The MTT method relies on metabolic activity of mycelia and not directly on growth [23,24]. Any factor which influences the metabolic rate might have an effect on reduction of MTT even if the biomass remains the same. The high level of agreement with the NCCLS method might indicate that growth corresponds directly with the metabolic status of fungi. Several problems have been reported in the determination of the MICs of antifungal agents, such as trailing effect caused by partial inhibition of fungal growth [25] and the subjectivity

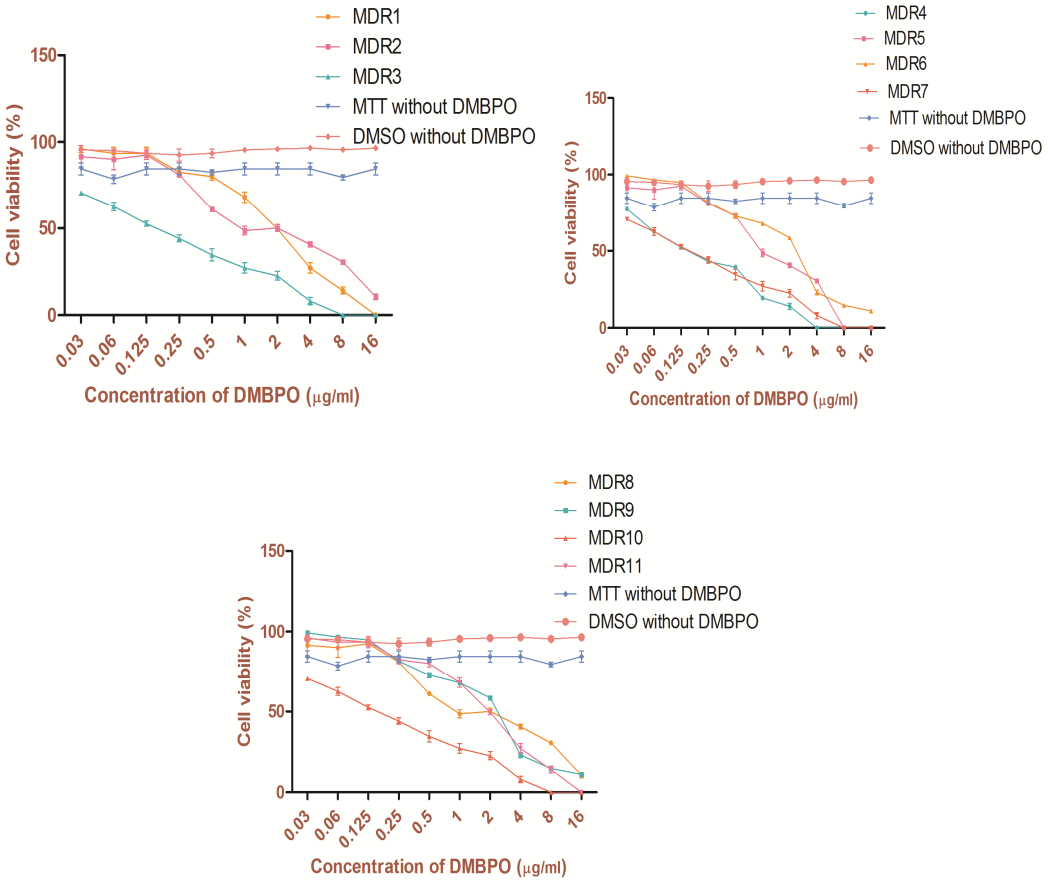

Fig. 2. Effect of DMBPO on cell viability of MDR *Aspergillus* clinical isolates A) MDR1, 2 and 3, B) MDR 4, 5, 6 and 7 and C) MDR 8, 9, 10, and 11

of visual reading with the aid of a magnifying mirror [26]. These shortcomings are more in the case of filamentous fungi, because they produce hyphae thereby quantification of the growth is very difficult. Moreover, the unequal growth, clumping, and adherence [27] of molds in assay tubes makes very difficult in establishing a clear endpoint both by visual reading as well as by spectrophotometer. For filamentous fungi, *A. fumigatus* the MTT method has been used for interpretation of data. Although previous studies have shown that the MTT method produced in interpretable dose-response curves with both amphotericin B and itraconazole against *A. fumigatus* [22,28]. The applicability of the MTT method for the antifungal susceptibility testing of various filamentous fungi against different antifungal drugs and the comparison of this method with the NCCLS method (M-38P) had not been done.

CONCLUSION

Screening of marine sediment samples yielded a novel *Streptomyces* sp. VITSVK5. It exhibits a strong antagonistic activity against the *Aspergillus* MDR strains isolated from chronic pulmonary disease patients. Out of 11 MDR strains isolated 8 are belongs to *A.niger* and 3 are *A.fumigatus*, which shows the prevalence of *A.niger* in chronic pulmonary disease patients of Vellore region, Tamil Nadu, India. Bioactive guided extraction and purification yielded an anti-*Aspergillus* compound DMBPO and it exhibited strong antagonistic activity (MIC 0.125µg/ml) against MDR strains. Thus DMBPO is the potential bioactive compound from marine *Streptomyces* sp. VITSVK5 having anti-*Aspergillus* activity against drug resistant *Aspergillus* clinical isolates.

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