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Original Article

Antifungal activity of terrestrial *Streptomyces rochei* strain HF391 against clinical azole -resistant *Aspergillus fumigatus*

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Abstract

Background and Purpose: Actinomycetes have been discovered as source of antifungal compounds that are currently in clinical use. Invasive aspergillosis (IA) due to *Aspergillus fumigatus* has been identified as individual drug-resistant *Aspergillus spp.* to be an emerging pathogen opportunities a global scale. This paper described the antifungal activity of one terrestrial actinomycete against the clinically isolated azole-resistant *A. fumigatus*.

Materials and Methods: Soil samples were collected from various locations of Kerman, Iran. Thereafter, the actinomycetes were isolated using starch-casein-nitrate-agar medium and the most efficient actinomycetes (capable of inhibiting *A. fumigatus*) were screened using agar block method. In the next step, the selected actinomycete was cultivated in starch-casein- broth medium and the inhibitory activity of the obtained culture broth was evaluated using agar well diffusion method.

Results: The selected actinomycete, identified as *Streptomyces rochei* strain HF391, could suppress the growth of *A. fumigatus* isolates which was isolated from the clinical samples of patients treated with azoles. This strain showed higher inhibition zones on agar diffusion assay which was more than 15 mm.

Conclusion: The obtained results of the present study introduced *Streptomyces rochei* strain HF391 as terrestrial actinomycete that can inhibit the growth of clinically isolated *A. fumigatus*.

Keywords: Actinomycetes, Antifungal Agents, *Aspergillus fumigatus*, Azoles resistant

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Introduction

Among the human pathogenic species of *Aspergillus*, *A. fumigatus* is perhaps the most devastating of *Aspergillus*-related diseases, followed by *A. flavus*, *A. terreus*, *A. niger*, and the model organism, *A. nidulans* [1-2]. *Aspergillus fumigatus* is a ubiquitous saprophytic mold that forms airborne spores (conidia). Humans inhale, on average, hundreds of these infectious propagules daily [3] .*A. fumigatus* pathogenesis and progression are the result of both fungal growth and the host response [4]. Invasive aspergillosis (IA) can cause a wide range of human ailments depending on host immune function [5]. Pathogenesis and virulence of aspergillus occurs when the host response is either too strong or too weak [6]. The types of hosts that are susceptible to invasive aspergillosis are the leukemic patients; hematopoietic stem cell transplant recipients, leukemia; patients on prolonged corticosteroid therapy, which is commonly utilized for the prevention and/or treatment of graft-versus-host disease in transplant patients; individuals with genetic immunodeficiencies such as chronic granulomatous disease (CGD); and individuals infected with human immunodeficiency virus [7]. In these patients, resistance is most commonly observed in *A.fumigatus*, and the isolates may be resistant to only itraconazole (ITZ) or exhibit a multi-azole or panazole–resistant phenotype. The phenotype depends on the underlying resistance mechanism, which commonly involves point mutations in the *cyp51A*-gene, the target for antifungal azoles [8].

In recent years the microorganisms have become important in the study of novel active compounds, secondary metabolites and chemical structure exhibiting antimicrobial may serve as model system in the discovery of new drugs [9]. The use of chemical fungicides has led to deteriorating human health and development of pathogen resistance to fungicide. Actinomycetes are the main source of antifungal. The antagonistic activity of actinomycetes is used for the bio-control of fungal diseases [10]. Actinomycetes produce about 75% of commercially and medically useful antibiotics [11-12]. Thus, the search for new antibiotics from these bacteria has gained importance. For example, it had been discovered in Egypt that a strain of *Streptomyc-es* spp., produced a strong antifungal antibiotics [13]. Furthermore, a research in Turkey for an antibacterial agent, producing *Streptomyces* spp. [14] and in China, a new strain of *Streptomyces* was discovered that kills certain pathogenic fungi [15].

Among the different types of drugs, secondary metabolites of actinomycetes including antibiotics with diverse chemical structure and biological activities have occupied a prominent position in the pharmaceutical industry [16]. This study was explored for the isolation characterization of native actinomycetes for antifungal metabolites, to screen a new antifungal compound against drug resistant *A.fumigatus*.

Material and Methods

Fungal strains

In the current study, an azole-resistant strain (IFRC 500, Invasive fungi Research Center, Mazandaran University of Medical Sciences), previously isolated from Bronchoalveolar Lavage (BAL) and identified by molecular methods were used. The resistant strain harbored an L98H amino acid substitution and a 34-bp tandem repeat in the *cyp51A* gene promoter region, and exhibited an itraconazole minimum inhibitory concentration (MIC) of > 16 µg/ml. Stock cultures for the transient working collections were cultured on malt extract agar (MEA, Difco, Beckton, Dickinson, and Company, Franklin Lakes, NJ, USA) at 35°C for 48 h until use.

Collection of soil samples

100 soil samples were collected from different points of Kerman City, Iran. The samples were taken up to a depth of 20 cm after removing approximately 3 cm of the soil surface and the samples were placed in polyethylene bags to avoid external contamination and kept in 4°C until pretreatment.

Isolation of actinomycetes

For the isolation of actinomycetes, various methods were performed on the basis of different sources and media [17]. Soil samples were processed by serial dilution method and cultured by spread plate technique on starch-casein-agar (SCB) and incubated at 37°C for 2 weeks. Slants containing pure cultures were stored at 4°C until further examination [18].

Identification of active actinomycetes

Various levels for the identification of actinomycetes were used such as: i) Chemo-taxonomical level: identified based on chemical variation and characters in all genera of actinomyces. ii) Classical level: identified based on macroscopic and microscopic methods and other properties such as the color of colonies culture. iii) Molecular level: the 16S rRNA partial gene sequences obtained from active isolate compared with other bacterial sequences by using PubMed - NCBI BLAST search [17].

Screening of the antifungal activity

Spread-plate method

The antifungal activity of actinomycetes was tested by agar plug method [19]. For the actinomycetes grown on surface of SCB medium Petri dishes, agar discs were cut out and transferred to the surface of PDA plates seeded with azole-resistant *A.fumigatus*. The petri dishes were incubated at 25°C to allow the growth of test organisms.

Well Diffusion Method

The isolated strains were transferred into the CG (Casein-Glycerin) medium in a 250 ml flask and incubated at 25°C for 15 days. Wells were made in the center of PDA plates seeded with Azole-resistant *A. fumigatus*. 100 µl of

the test samples were transferred into the wells and plates were incubated at 25°C. The plates were then observed for zone of inhibition.

Assay for antifungal activity by minimum inhibitory concentration (MIC)

MIC was determined by the antimicrobial concentrations which were prepared as 1.25, 2.5, 5, 10, 20, 40 and 80 mg/ml in DMSO: MeOH (1:1, v/v) and tested in well-method technique against the pathogen. The lowest concentration which indicated growth inhibition was selected as MIC [18].

Results

Identification of Azole-resistant A. fumigatus

Of the 50 Aspergillus isolates, 40 (80%) were *Aspergillus fumigatus* , of which one A.

drugs by agar well diffusion method (CLSI M38-A2) (Figure 1).

Table 1. Identification of strains by chemotaxonomical level

Chemicals	Active strain
Citrate	+
SIM	+
MR	+
VP	+
lactase test	+
Proteases test	+
ketones test	-

Test for utilization of carbon sources	
Carbon sources	Active strain
Glucose	+
Sucrose	+
Maltose	+
Mannitol	+
Lactose	+
Starch	+

Growth in different temperature, osmolarity NaCl and pH					
Osmolarity NaCl	Growth	Temperature	Growth	pH	Growth
2/5	+	25	+	5	-
5	+	37	+	5/5	+
7/5	+	50	-	7/7	+
9/5	-	-		7/8	+
12/5	-	-		7/9	+
-		-		8	+
-		-		8/5	-

+, presence of growth; -, no growth

Identification of active strains

The active strain was identified by chemo-taxonomical level as well as the classical level. Results are shown in Tables 1 and 2.

Molecular level

Blast search for the 16S rRNA gene sequences of the isolates KP137826.1 in the NCBI data bank showed a maximum similarity of 86% with *Streptomyces rochei* strain HF391.

Actinomyces spp. kp137826 alone showed significant strong antifungal activity against the azoles-resistant *A.fumigatus*. The diameter of the zone of complete inhibition was measured to the nearest millimeter. Antibiotic production was not detected in 7 days culture filtrate, but that showed maximum antibiotic production after 9 days of incubation (Figure 2).

Minimum Inhibitory Concentration (MIC) determination

The best concentrations of the pure antifungal compounds from the *Streptomyces rochei strain HF391* against azole-resistant *A. fumigatus* was 80 mg/ml. Furthermore, the inhibition zone (35mm) was measured as well (Figure 3).

Table 2. Identification of strains by Classical level

Morphological Characteristics	Active strain
Color of aerial mycelium	Grey
Reverse side colour	Pale Grey
Colony surface	Smooth
Growth	Good

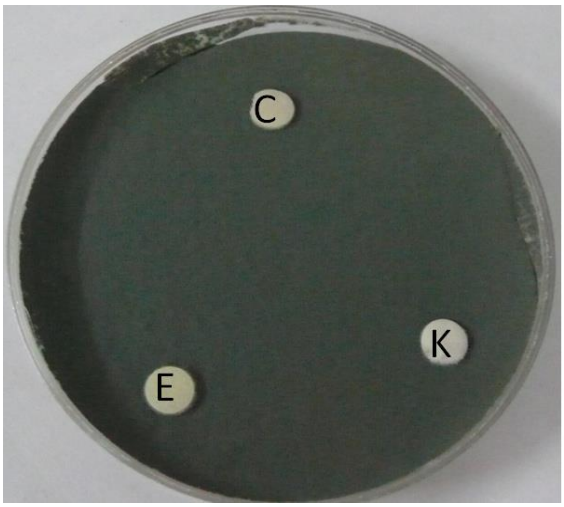

Figure 1. Colony of azole -resistant *A. fumigatus* on PDA medium C: Clotrimazole; E: Itraconazole and K:Ketoconazole

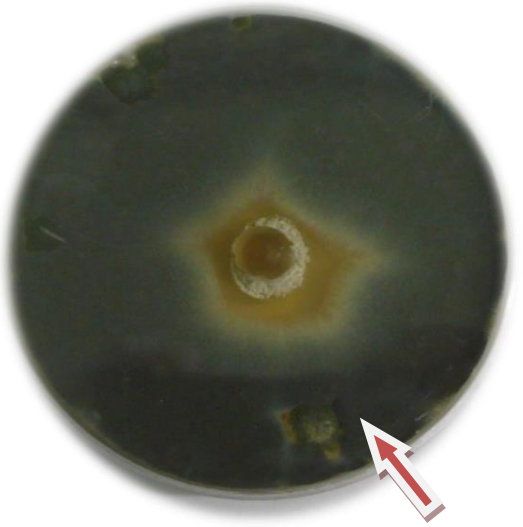

Figure 2. Zone of inhibition of *Actinomyces* spp. kp137826 (mm) against *A.fumigatus*

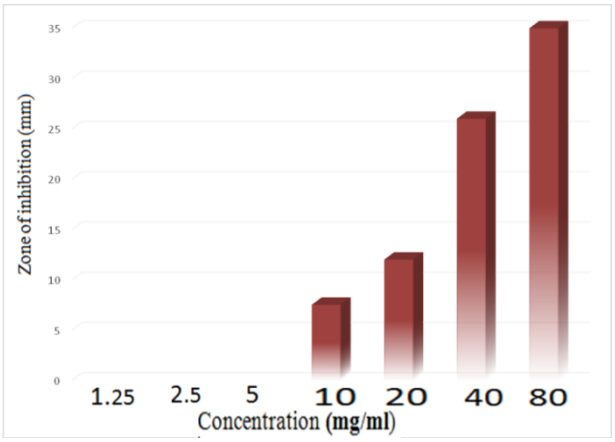

Figure 3. Minimum inhibitory concentration (MIC) values of the culture supernatant of *Actinomyces* spp. *kp137826* against *A. fumigatus*

Discussion

In our study, among fifty BAL samples, only one azoles (Clotrimazole, Itraconazole and Ketoconazole) -resistant *A. fumigatus* was found. In another study investigated the prevalence of azole-resistant *Aspergillus* spp. Only 4 azole-resistant isolates were found, which corresponds with a prevalence of 1.9% [20]. Another study showed the prevalence of 12.8% among *A. fumigatus* isolates that had been sent to hospitals in the Netherlands [21]. For patients with aspergillosis affected by azoles resistance *A. fumigatus* treated with voriconazole, the propor-tion of death was 48% [22]. Another study investigated the prevalence of azole-resistant *Aspergillus* spp, described the emergence of acquired resistance of *A. fumigatus* to azole compounds [23].

In our research, among the 100 actinomycete isolates, *Actinomyces* spp. kp137826, exhibited strong antifungal activity against azole-resistant *A. fumigatus*. The rate of antifungal metabolite production correlated with the growth rate of the *Actinomyces* spp. kp137826. Among the bacteria, actinomycetes are the important source of bioactive compounds and many clinically relevant antibiotics in use today and may continue to be so. The other study performed on 153 isolates showed broad spectrum antifungal activity [24]. Augustine reported that out of 335 isolates, 230 (69 %) isolates were active against bacteria, fungi and yeast [25]. Of the 312 Actinomycete strains from different regions, of which, 22% exhibited antifungal activity against fungi [26]. Michael *et al*. and Gomes*et al*. isolated chitinolytic actinomycetes and found its antifungal activity [27-28].

Our study shows that only *Actinomyces* spp. kp137826 exhibited antifungal activity against azoles-resistance *A. fumigatus*. The MIC of the antifungal compound was determined as 80 mg/ml and showed the highest zone of inhibition *A. fumigatus* (35 mm). The other study used also different concentrations e.g. 2, 4, 6, and 10% of extract were used to check antifungal activity and the minimum inhibitory concentration [29].

Streptomyces spp and *Nocardia* spp. also showed anti-*Aspergillus* activity because of observed in Netherlands in 1999 [30].Screened287 isolates from various habitats and recorded166, 164, 134, and 132 actinomycete isolates active against *C. albicans*, *A. niger*, *M. gypseum* and *T. rubrum*,respectively [33]. In another research, among 316 actinomycetes, 19, 67, 42, 37, 18 and 25 isolates showed activity against *C. albicans*, *T. rubrum*, *M. canis*, *M. gyseum*, *A. flavus*, *A. fumigatus*, respectively [34]. *Streptomyces rochei* AK 39 also exhibited antifungal activity against dermatophytes when grown on starch-casein agar (SCA) medium with pH 7 and 37°C [35]. Several 32 [36]. In our study, we observed the antifungal activity of *Actinomyces* spp. actinomycetes were reported to possess anti-*Aspergillus* activity, e.g. *Streptomyces* spp. PM- kp137826 against

A.fumigatus, enabling the discovery of new antibiotics and hence, merit future studies.

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Authors' contributions

S.H. in charge of data collection, sampling, doing biochemical tests and help to write the manuscript, H.F. presented the basic theme of the biochemical articles and help to do the most of the lab tests, GH.S. help to collect the actinomycetes and their recognition and edition of manuscript, M.F. presented the method of collecting the *Aspergillus* resistance to Azoles and testing them all, A. KR.: help to collect the articles and write the manuscript and SA. AM. presented the basic theme of the article, writing the paper and supervised the study.

Conflicts of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Financial Disclosure

No financial interests related to the material of this manuscript have been declared.

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