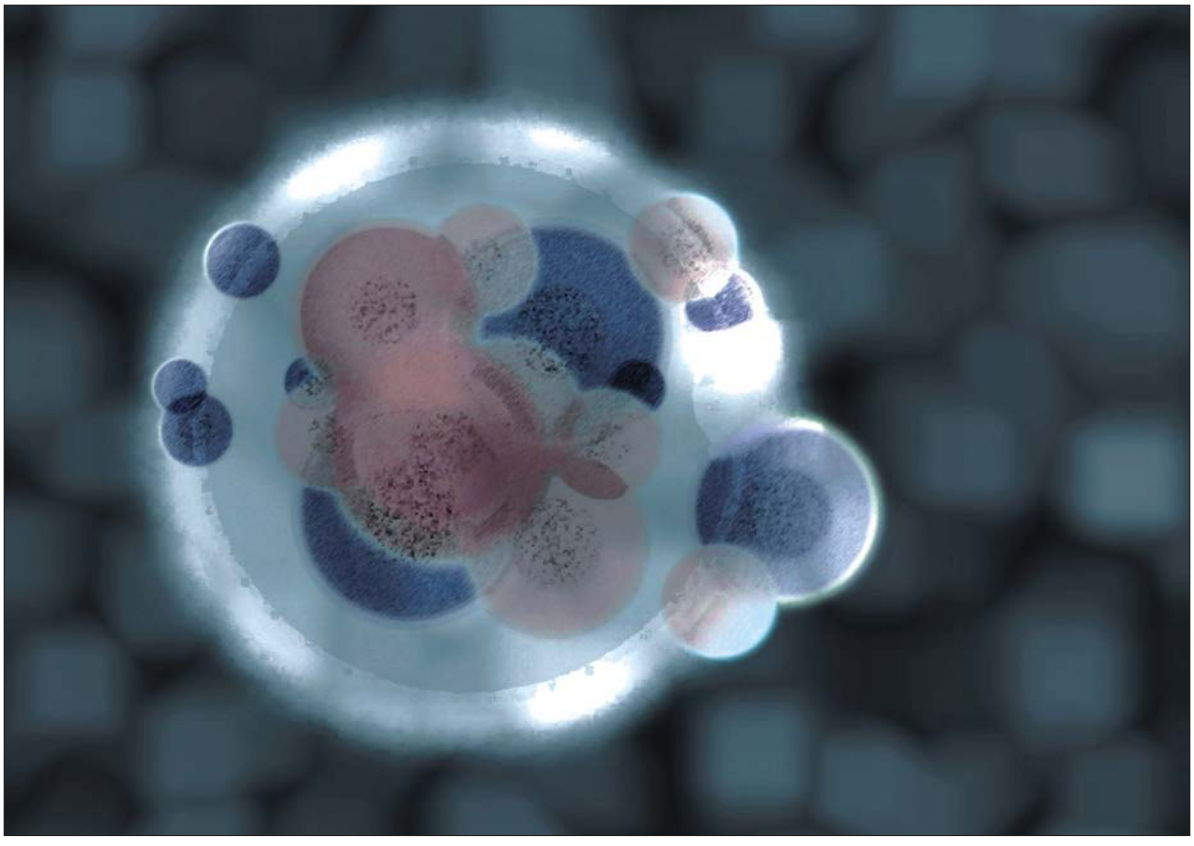

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Taxonomic Characterization and Chemical Study of the Antifungal Constituents of *Streptomyces* sp. KH-F12

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Abstract: An actinomycetes strain designated as KH-F12 showed a broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria and filamentous and unicellular fungi. It was isolated from soil sample collected from Adilam area, Kingdom of Saudi Arabia. Polyphasic taxonomic approaches were applied for the identification. Based on the cultural and biochemical characteristics of the isolate (KH-F12) was classified as a *Streptomyces*. Comparison with other *Streptomyces* species using the 16S rRNA gene sequence and the PCR-restriction enzyme Pattern Analysis (PRA) of strain KH-F12 developed a similarity level ranged from 97.2% with *Streptomyces albidoflavus*. The antifungal activity was stronger than the antibacterial one. An inhibition zone of (12 mm) with *B. subtilis* ATCC11774, (11 mm) *E. coli* ATCC8739, (30 mm) with *Candida albicans* ATCC66027 and *Candida albicans* ATCC2091, (32 mm) with *Saccharomyces cerevisiae* ATCC9763, (34 mm) with *Aspergillus niger* ATTC16404 and a zone of (14 mm) was recorded with *Aspergillus fumigatus* ATCC1022. The kinetics of antibiotic production, growth and pH were monitored in ISP2 broth culture. The antifungal activity against *A. niger* was stronger than that detected against *C. albicans*. The antimicrobial activity was found to be correlated with the cell growth, so the biomass increased from the first day to the 8th day. In order to isolate the active secondary metabolites, the lyophilized medium was subjected to various chromatographic purification steps guided by the antifungal assay. An active compound (F-12) with MIC of 0.5 μg mL^{-1} against the two *Candida* strains, 1.8 μg mL^{-1} against *A. fumigates* and 1.4 μg mL^{-1} against *A. niger* was isolated. Different spectroscopic techniques were applied to identify the isolated compound. However, the structure of F-12 is still under investigation.

Key words: Antifungal, *Streptomyces* sp. KH-F12, *Candida albicans*, *A. fumigatus*, *A. niger*

INTRODUCTION

Extensive use of antimicrobial agents since their introduction into the market make the utilization of existing antibiotics to be rapidly eroding and shifting the balance in favor of multi drug resistant pathogens (Drlica, 2001; Boubetraa *et al*., 2013). About two-thirds of discovered microbial bioactive substances originate from actinomycetes (Berdy, 2005; Demain and Sanchez, 2009). However, the number of these new antibiotics is noted to have decreased dramatically in recent years due to the high rediscovery rate of known compounds from actinomycetes (Stach, 2010; Subramani and Aalbersberg, 2013) and due to the exhaustion of common resources (Demain and Sanchez, 2009). The American Academy of Microbiology estimated that less than 1% of bacterial species are discovered and millions of microbes in the environment that are presently untouchable (Cragg and Newman, 2005).

Search for novel antibiotics has become more urgent as the result of the increasing prevalence of antibiotic resistant pathogens, the high incidence of fungal infections and increasing of opportunistic infections in the immunocompromised hosts. The need for new, more effective and safe antifungal compounds is a major challenge to the pharmaceutical industry (Dhanasekaran *et al*., 2008). For selectively isolating of rare and new genera of actinomycetes, techniques that enhance the growth of new desired species of actinomycetes (enrichment) or eliminate the undesirable *Streptomycete* species and other contaminants from the primary isolation plate (pretreatment), must be developed and employed (Tiwari and Gupta, 2013). In the course of our screening for new antibiotics, we found that

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Streptomyces sp. designated as KH-F12 recovered from a soil sample of Adilamm (KSA) is capable of producing an antifungal antibiotic.

MATERIALS AND METHODS

Sample collection and isolation of actinomycetes: Soil samples were collected as per the method given by (Muharram *et al*., 2013) from different locations at the middle area at KSA. They were collected at 2-3 m depth in sterile polypropylene bags and brought to the laboratory for isolation of actinomycetes. The locations and the nature of samples were recorded. Samples (2 g) were diluted ten times in sterile water and homogenized by vortexing for 15 min. Isolation was carried out on Starch Casein Agar medium (SCA), supplemented with 40 μg mL^{-1} actidione to inhibit the development of antagonist fungi and other soil eukaryotic microorganisms. The plates were incubated at 30°C for 7 days. Actinomycetes colonies were recognized on the basis of morphological characteristics by light microscopy. Most actinomycetes showed a vegetative mycelium and aerial hyphae, others showed only the substrate mycelium. The selected new strains were numbered as *Streptomyces* sp. (KH-1 to KH-34). Strains were maintained on ISP-2 agar medium by storage at 4°C for 2 months. Alternatively, strains were resuspended in 20% glycerol and stored at -80°C.

Culture characteristics and biochemical identification of *Streptomyces* sp. KH-F12: *Streptomyces* sp. KH-F12 was characterized morphologically and physiologically following the directions given by the International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966) and Bergey's Manual of Systematic Bacteriology (Locci, 1989). The morphological examination was carried out under the bright field of a phase contrast. For studying the morphological characteristics of the actinomycetes, the cover slip technique was used. Cover slip cultures were fixed using few drops of absolute methanol for 15 min and then washed with tap water. The cover slips were stained using 0.15 crystal violet for one minute, then washed by tap water, dried and examined by oil emersion lens. Actinomycetes were identified to the species level by comparing the morphology of spore bearing hyphae with entire spore chain and structure of spore as described in Bergey's manual. Micromorphology was observed by slide culture method (Williams and Cross, 1971). The ISCC-NBS color-name charts illustrated with centroid detection of the aerial, substrate mycelia and soluble pigments (Kenneth and Deane, 1955) was used. Cell wall composition was analyzed by the method of Lechevalier *et al*. (1989).

DNA extraction and amplification of 16S rDNA by PCR: Sambrook *et al*. (1989) stated that actinomycete strains were grown in 10 mL International *Streptomyces* Project Medium 1 (ISP 1)[18] with agitation at 30°C for 18-24 h and examined by Gram stain. Cells (4 mL) were harvested by centrifugation (7500 g for 2 min), washed once with 500 mL of 10 mM Tris-HCl/1 mM EDTA (TE) buffer (pH 7.7) and resuspended in 500 mL TE buffer (pH 7.7). The samples were heated in boiling water for 10 min, allowed to cool for 5 min and centrifuged (7500 g for 3 min). The supernatant (300 mL) was transferred to a clean tube and stored at 4°C. The PCR was carried out in 50 μL volumes containing 2 mM MgCl, 2 U *Taq* polymerase, 150 mM of each dNTP, 0.5 μM of each primer and 2 μL template DNA. Primers used in this study were F1 (5`-AGAGTTTGATCITGGCTCAG-3`; I = inosine), R1 (5`-ACGGITACCTTGTTACGACTT-3`) for the amplification of the 16S rDNA gene and AZF1 (5-AGCAACCAACGATGGTGTGTCCAT-3) and AZF2 (5-CAACTTGTCGAACCGCATACCCT-3) for the amplification of the heat shock protein gene (HSP-65). The PCR program used was an initial denaturation (96°C for 2 min), 30 cycles of denaturation (96°C for 45 sec), annealing (56°C for 30 sec) and extension (72°C for 2 min) and a final extension (72°C for 5 min). The PCR products were electrophoresed on 1% agarose gels containing ethidium bromide (10 μg mL^{-1}).

Sequencing of 16S rDNA: The amplified PCR products were purified by QIAquick PCR purification Kit (Qiagen Ltd., Crawley, UK). The PCR product was ligated into the pGEM-T cloning vector by following the instructions given by the manufacturer (Promega, Madison, WI, USA). Plasmids were transformed into *Escherichia coli* DH5a competent cells. Recombinant transformants were selected by blue/white colony selection. Individual white colonies were grown at 37°C overnight with shaking in 25 mL of LB medium containing ampicillin. After plasmid preparation, 2 μL (out of 50 μL) of each sample was amplified by PCR (Bio-Rad I cycler) using M13-F and M13-R primers to check for the presence of insert DNA. Only plasmids containing the expected 1500 bp inserts were sequenced and deposited in GenBank database under the accession number JQ283107. The obtained sequences were subjected to BLAST at http://www.ncbi.nlm.nih.gov/ search in NCBI database.

Antimicrobial activity: Antimicrobial activities of the actinomycetes strains were checked by growing the cells on Modified Nutrient Glucose Agar (MNGA) plates by single streak in the center. The plates were incubated at 30°C for 5 days. The test organisms were inoculated

perpendicular to the antagonist on the agar medium. Bacteria were incubated at 37°C for 18 h and fungi were incubated at 28°C for 48 h. The microbial inhibitions were observed by determining the diameter of the inhibition zones (Arasu *et al*., 2008). Study of the antimicrobial activity in the three used protocols was conducted against all of *Staphylococcus aureus* ATCC 29213; *Bacillus subtilis* ATCC 11774; *Streptococcus epidermidis* ATCC 12228; *Escherichia coli* ATCC 8739; *Candida albicans* ATCC 66027; *Candida albicans* ATCC 2091, *Aspergillus niger* ATTC 16404 and *A. fumigatus* ATCC 1022.

Time course of growth and antibiotic production: In order to select the culture time favorable for antibiotic production from isolate KH-F12, time course of growth and antibiotic production fermentation was carried out in ISP2 broth medium for 10 days. The 500 mL Erlenmeyer flasks containing 100 mL of medium were inoculated with 3 mL of a pre-culture broth of isolate KH-F12 prepared with the same medium and incubated at 30°C for 2 days. The cultures were incubated on a rotary shaker (200 rpm) at 30°C for 10 days. The antimicrobial activity in the culture broth was monitored by the conventional agar diffusion assay (well technique) using *Bacillus subtilis*, *Escherichia coli*, *Candida albicans* and *Aspergillus niger*. Each 10 mm diameter well was filled with 0.1 mL of supernatant. To purify the antibiotics, repeated fermentations were carried out to obtain a total of 7.0 L of culture broth.

Minimum Inhibitory Concentration (MIC): The minimum inhibitory concentration was quantified byincorporating known concentrations of antibiotic into solid growth medium using a conventional agar dilution method (Oki *et al*., 1990). Inocula of used test bacteria, fungi and yeasts were inoculated onto Mueller Hinton medium for bacteria and Sabouraud medium for fungi, containing different concentrations of active products (0.5, 1, 2, 5, 10, 20, 30, 50, 75 and 100 μg mL^{-1}). After a growth period of 24-48 h at 37°C for bacteria and 48-72 h at 28°C for fungi, the plates were examined for growth and the lowest antibiotic concentration that inhibited the growth of each orgamism was determined. Mueller Hinton and Sabouraud media, without active products and inoculated with target organisms, was used as a control treatment.

Cultivation and extraction of metabolites: *Streptomyces* sp. KH-F12 was cultivated at 30°C for 5 days in modified Nutrient Glucose Agar medium. At the end of the fermentation cycle the culture was filtered and the supernatant was separated by centrifuging at 8000 rpm for 15 min. The supernatant was lyophilized to give 2 g dried medium. The dried medium was subjected to Vacuum Liquid Chromatography (VLC) over RP18 (80 g, 7 cm i.d.) and elution was started with 100% water, then water/MeOH mixtures with gradual increase of MeOH content in a gradient system. All fractions were subjected to antifungal assay. Activity was traced to fractions 11 eluted with 90% MeOH (14 mg) and 12 eluted with 100% MeOH (40 mg). The two fractions were combined after TLC screening and subjected to centrifugal preparative TLC (CPTLC) using Chromatotron (Harrison Research Inc. model 7924), 2 mm silica gel P254 disc and 10% EtOAc in n-hexane as eluting system. Pure UV active zone was collected and dried to afford 12 mg of pure semisolid compound 1 with R_f value = 0.47 (Silica gel, 20% EtOAc in n-hexane).

Characterization of F-12: Ultraviolet absorption spectra were obtained in methanol on a Unicum Heyios α UV-Visible spectrophotometer. Optical rotations were recorded on a Jasco P-2000 Polarimeter. CD was measured using J-815 CD Spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a UltraShield Plus 500 MHz (Bruker) (NMR Unite at the College of Pharmacy, Salman Bin Abdulaziz University) spectrometer operating at 500 MHz for proton and 125 MHz for carbon, respectively. The chemical shift values are reported in δ (ppm) relative to the residual solvent peak, the coupling constants (J) are reported in Hertz. 2D-NMR experiments (COSY, HSQC and HMBC) were obtained using standard Bruker program.

RESULTS

Isolation, Characterization and Taxonomy of *Streptomyces* sp.: KH-F12.The isolate KH-F12 was recovered by soil serial dilution plating method on starch casein agar medium agar. The cultural, microscopic (spore chainmorphology) characteristics and biochemical characteristics of the isolate (KH-F12) are depicted in Table 1. The isolate KH-F12 was identified as *Streptomyces albidoflavus*. Identification was performed according to the recommended international Keys (Buchanan and Gibsons, 1974; Williams, 1989; Hensyl, 1994). PCR-restriction enzyme Pattern Analysis (PRA) was used to confirm this identification. In PRA analysis, a set of specific restriction endonucleases were used to digest the DNA of the 16S rRNA gene. Figure 1 shows adoublet band in the size range 540-650 bp as a result of a Sau3AI digestion performed on the 16S rDNA of the isolate KH-F12 (Lane, 3). The size of the doublet band indicates that the isolate is most likely to belong to the genus *Streptomyces*. *Asn*I (Lane, 4) and *Sph*I (Lane, 6) endonucleases did not restrict 16S rDNAs

Table 1: Morphological and biochemical characteristics of *Streptomyces* KH-F12

Characteristic	KH-F12	Characteristic	KH-F12
Morphological characteristic		**Utilization of different amino acids**	
Aerial hyphae	Rectiflexibiles	L-Cycteine	+
Spore mass	Grey	L-Valine	+
Spore surface	Smooth	L-Histidine	+
Color of substrate ycelium	Red/orange	L-Phenylalanine	+
Diffusible pigment	Red/orange	L-Arginine	+
Cell wall hydrolysate:		L-Lysine and L-Hydroxproline	-
Diaminopimelic acid (DAP)	*meso*-DAP	L-Glutamic acid	+
Protein; starch, egg-yolk (lecithin) and cellulose	+	**Growth inhibitors:**	
Pectin and lipid	+	Thallous acetate (0.001)	-
		Sodium azide (0.01)	+
		Phenol (0.1)	+
Catalase test	+	**Growth at different temperatures (°C):**	
Resistance of different antibiotics		10	+
Gentamycin (100)	-	20	+
Neomycin (50)	-	30-45	+
Streptomycin (100)	+	50	-
Tobramycin (50)	-	**Growth at different pH values:**	
Rifampicin (50)	+	4	±
		5-9	+
Cephaoridine (100)e	+	10	
Vancomycine (50)	-	**Growth at different concentrations of NaCl (%)**	
Production of melanin pigment on:		4	+
Peptone yeast-extract iron agar (ISP-6)	+	7	+
Tyrosine agar medium (ISP-7)	+	12	-
Tryptone-yeast extract broth (ISP-1)	+		
Degradation of:		**Utilization of diff. carbon sources**	
Xanthin	+	Rhamnose	+
Aesculin	+	Raffinose	-
H_2S production	+	Mannitol	+
Nitrate reduction	+	L-Arabinose	+
Citrate utilization	+	*meso*-Inositol	-
Urea test	+	Lactose	+
Coagulation of milk	+	Maltose	+
Utilization of: diff. carbon sources		Trehalose	+
D-Xylose	+	L-Melizitose	-
D-Mannose	+	D-fructose	+
D-Glucose	+	Sodium citrate	+
D-Galactose	+		
Sucrose	+		

+: Positive, -: Negative, ±: Doubtful result

while the *Kpn*I enzyme restricted the 16S rDNA producing two bands between 410-470 and 1000-1100 bp (Lane, 5). Based on the generated PRApattern (Fig. 1), the published filamentous actinomycete 16S rRNA gene sequences in the GenBank database and the *in silico* analyses results of Cook and Meyers (2003) and Muharram *et al*. (2010). The identification of the isolate KH-F12 was confirmed as *S. albidoflavus*.

Investigation of *Streptomyces* KH-F12 for antimicrobial metabolite: The potential activity of the KH-F12 isolate against various microorganisms (bacteria, filamentous fungi and yeasts) was evaluated on ISP2 medium by disc methodat 30°C for 24 h for bacteria and yeasts and 36 h for filamentous fungi. KH-F12 showed a broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria and filamentous and umicellular fungi. An Inhibition zone of (12 mm) with *B. subtilis* ATCC11774, (11 mm) *E. coli* ATCC8739, (30 mm) with *C. albicans* ATCC66027 and *C. albicans* ATCC2091, (32 mm) with *S. cerevisiae* ATCC9763, (34 mm) with *A. niger* ATTC16404 and a zone of (18 mm) was recorded with *A. fumigatus* ATCC1022. Antimicrobial activity pattern showed strong to very strong activity against fungi as given in Table 2. The isolate KH-F12 was further cultivated in large scale and bioactive metabolite was extracted.

Time course of growth, pH and antimicrobial activity: The kinetics of antibiotic production, growth and pH were monitored in ISP2 broth cultures, as shown in Table 3. The activities were tested on the second day against *C. albicans* and *A. niger*, reaching a maximum on the 8th day and on the 9th day for *C. albicans* and *A. niger*, respectively. The antifungal activity against *A. niger* was stronger than that detected against *C. albicans*. The antimicrobial activity was found to be correlated with the cell growth, so the biomass increased from the first day to the 8th day and then decreased after the 9th day. The pH increased gradually to an

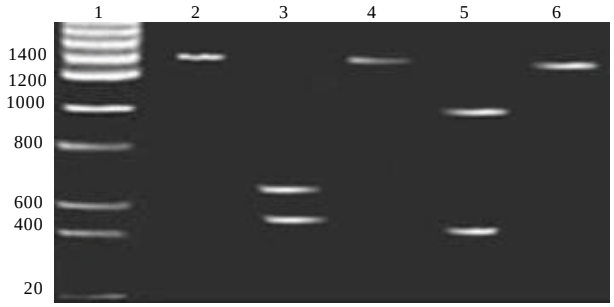

Fig. 1: Restriction analysis of the 16S rDNA of the isolate KH-F12. Lane 1: DNA fragments of 200 bp DNA ladder, Lane 2: Non-digested 16S rDNA (1.496 bp), Lane 3: Restriction analysis of the 16S rDNA by *Sau*3AI endonuclease, Lane 4: Restriction of 16S rDNA by *Asn*I, Lane 5: Restriction of 16S rDNA by *Kpn*I and Lane 6: Restriction of 16S rDNA by *Sph*I

Table 2: Screening for the antimicrobial activity of the actinomycete isolate KH-F12

Test organism	Inhibition zone produced by isolate KH-F12 (mm)
Staphylococcus aureus ATCC29213	9
Bacillus subtilis ATCC11774	12
Streptococcus epidermidis ATCC12228	13
Escherichia coil, ATCC8739	11
M. luteus ATCC 9341	14
K. pneumonia ATCC 700603	12
Candida albicans ATCC66027	30
Candida albicans ATCC2091	30
Saccharomyces cerevisiae ATCC9763	32
Aspergillus niger ATTC16404	34
Aspergillus fumigatus ATCC1022	14

Table 3: Time course of growth, pH and antimicrobial activity against *B. subtilis*, *E. coli* on *C. albicans* and *A. niger* on ISP2 medium

			Inhibition zone (mm)	
Days	Dry weight (g L^{-1})	pH	*C. albicans* ATCC66027	*A. niger*
2	6.20	8.51	15.70	18.70
3	7.60	8.43	16.00	21.00
4	7.92	8.40	16.43	21.01
5	8.22	7.88	17.40	22.00
6	8.41	7.79	18.00	26.00
7	10.18	7.76	21.23	27.23
8	10.00	8.29	30.00	28.00
9	9.78	8.41	28.00	34.00
10	7.77	7.69	24.00	31.00

alkaline state with a maximum (8.51) recorded on the second day and small variations were recorded thereafter.

Minimum inhibitory concentration: To determine the minimum inhibitory concentration of the compound, inocula of test organisms were inoculated Sabouraud-medium containing different concentrations of the active product (0.5, 1, 2, 5, 10, 20, 30, 50, 75 and 100 μg mL^{-1}). The MIC for the two *Candida* strains was 0.5, 1.8 μg mL^{-1} *A. fumigatus* and 1.4 μg mL^{-1} for *A. niger* (Fig. 2).

Spectral data of F-12: Compound (F-12): $[\alpha]_D$ = 16.8 (c = 0.0004, $CHCl_3$). UV λ_{max} ($CHCl_3$) 295 (sh), 284, 274, 240 nm. ^{1}H- and ^{13}C-NMR ($CDCl_3$): Table 4.

DISCUSSION

The finding of new bioactive compounds is a never ending process to meet the everlasting demand for novel biomolecules with antimicrobial properties in order to combat human pathogens. Microbial natural products represent an important route to the discovery of novel therapeutic agents. About 45% of more than 22,000 microbiologically active compounds have been obtained from actinomycetes, especially the excellent producers in the genus *Streptomyces* (Berdy, 2005).

On the basis of the morphological and chemical characteristics described above, isolate KH-F12 was classified in the genus *Streptomyces*. Comparison with

Table 4: ^{1}H-NMR and ^{13}C-NMR data of F-12 in CD_3OD* in ppm (multiplicity, J in Hz).

	^{1}H	^{13}C
CH_3×3	1.07 (s, 9 H)	31.68
CH_3	1.95 (s, 3 H)	42.64
CH_2	3.62 (s, 2 H)	60.46
CH_2	2.97 (d, 6.8 Hz)	60.67
C	-	78.48
C	-	99.44
CH	5.56 (d, 15.8 Hz)	114.94
CH	8.01 (d, 8 Hz)	125.68
CH	7.18 (m)	126.23
CH	7.30 (m)	126.80
CH	7.30 (m)	127.00
CH	7.18 (m)	128.97
CH	7.58 (dd, 1.1, 7.8 Hz)	129.37
CH	7.64 (dd, 1.1, 7.8 Hz)	129.61
C	-	133.77
C	-	135.27
C	-	135.43
CH	5.90 (dt, 6.8, 15.8 Hz)	139.38

*Assignments were done based on DEPT, COSY, HSQC and HMBC experiments

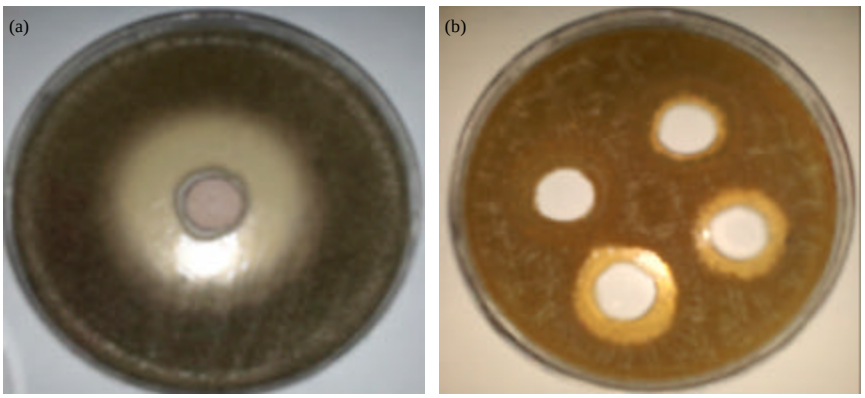

Fig. 2(a-b): (a) Disc diffusion plate showing zone of inhibition on fungal culture of *Aspergillus fumigatus* ATCC1022, (b) Determination of the MIC against *Aspergillus niger* ATTC16404. It was quantified by incorporating known concentration of KF-12 antibiotic into solid plate using a conventional agar dilution method. Inocula of used test fungi were inoculated onto Sabouraud medium for fungi, containing different concentrations of active products (0.5, 1, 2, 5, 10, 20, 30, 50, 75 and 100 μg mL^{-1}). After a growth period of 48-72 h at 28°C for fungi, the plates were examined for growth and the lowest antibiotic concentration that inhibited the growth of each organism was determined. F-12 showed an MIC of 1.4 μg mL^{-1} against *A. niger*

other *Streptomyces* species using the 16S rRNA gene sequence and the PCR-restriction enzyme pattern analysis (PRA) of strain KH-F12 developed a similarity level ranged from 97.2-98.8% with *S. albidoflavus* (Drancourt *et al*., 2000; Cook and Meyers, 2003; Mignard and Flandrois, 2006).

Our results showed that the antifungal activity was stronger than the antibacterial one. The same observations were announced by Igarashi *et al*. (1997). These authors reported that the formamicin produced by a strain of *Saccharothrix* sp. exhibited broad, strong antifungal activity against phytopathogenic fungi and moderate antibacterial activities against Gram-positive bacteria. It was very interesting to note that the activity results of our compound was in agreement with those of Andriole (1999), Lass-Florl *et al*. (2008) on many antifungal compounds tested against a very large number of strains of yeasts and filamentous fungi. In contrast, the studies showing activity against filamentous fungi, with no activity against yeasts, are very rare (Hosoe *et al*., 2004) or almost non-existent.

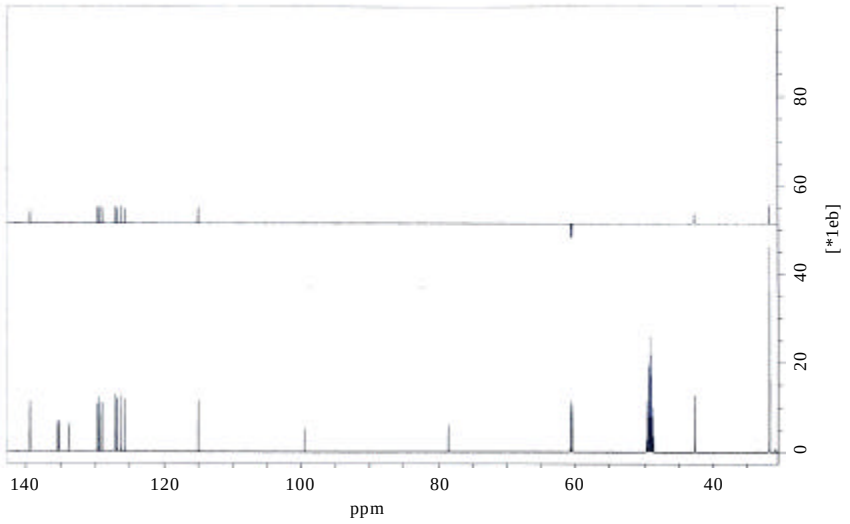

Fig. 3: ^{13}C NMR and DEPT135 spectra of F-12

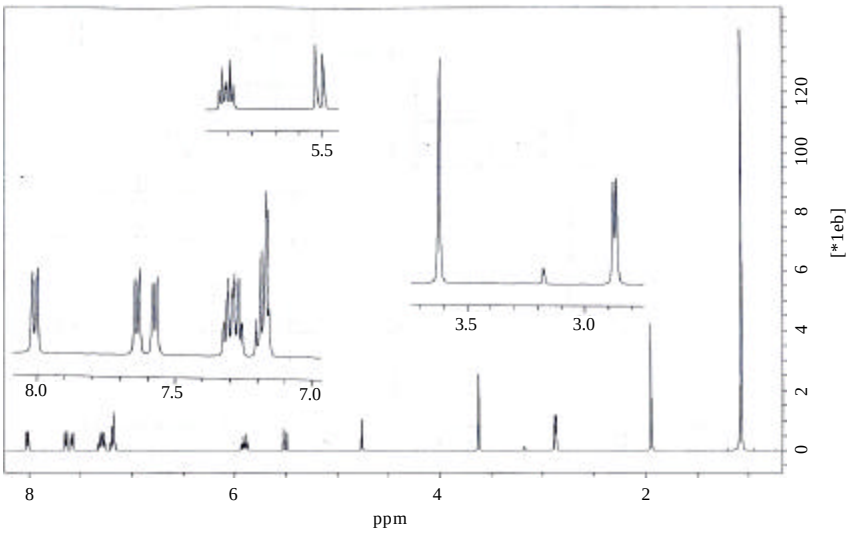

Fig. 4: ^{1}H NMR spectrum of F-12

The ^{13}C-NMR spectrum of F-12 showed (Fig. 3) 18 carbon signals. The carbon signals were sorted by DEPT 135 experiment into two methyl signals one of them was assigned for 3 magnetically equivalent methyl groups as Indicated from ^{1}H NMR and HSQC experiments, two OCH_2, nine CH and five quaternary carbons. Comprehensive study of both 1D- and 2D-NMR spectra enable the identification of some systems as parts of F-12. The first identified system is a cinnamyl amine moiety as indicated from the CH = CH-CH_2-N moiety in the ^{1}H-NMR (Fig. 4) and COSY experiment (Fig. 5). The signal at δ_H 2.97 (d, J = 6.8 Hz) correlated with the CH_2 carbon signal at δ_C 60.67 in HSQC experiment (Fig. 6) was assigned for the CH_2-N coupled with the olefinic CH at δ_H 5.90 (dt, J = 6.8, 15.8 Hz) and δ_C 139.38 ppm. The second olefinic CH appears at δ_H 5.56 (d, J = 15.8 Hz), δ_C 114.94 ppm. The signals for the aromatic protons are part of the 7 CH aromatic signals clear in the ^{1}H NMR and ^{13}C NMR. HMBC experiment (Fig. 7) showed three bonds correlations between CH_3 at δ_H 1.95 (s), δ_C 42.64,

CH_2 at δ_H 2.97 (d, J = 6.8 Hz), δ_C 60.67 and δ_H 3.62 (s), δ_C 60.46 ppm. These data were diagnostic for fragment A (Fig. 8) as part of F-12. The other system identified is a tert-butanol moiety (fragment B). The three methyls are

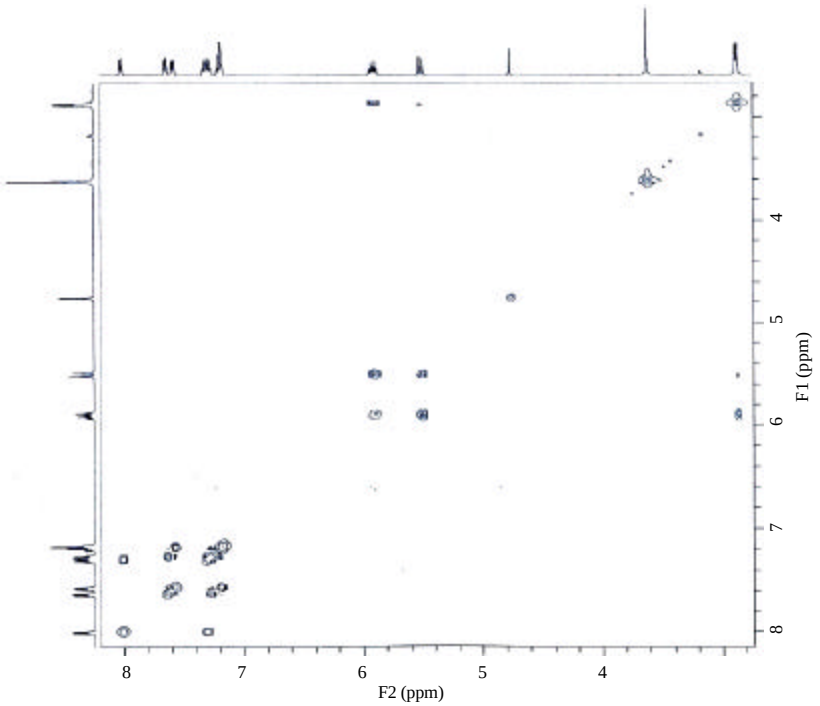

Fig. 5: COSY spectrum of F-12

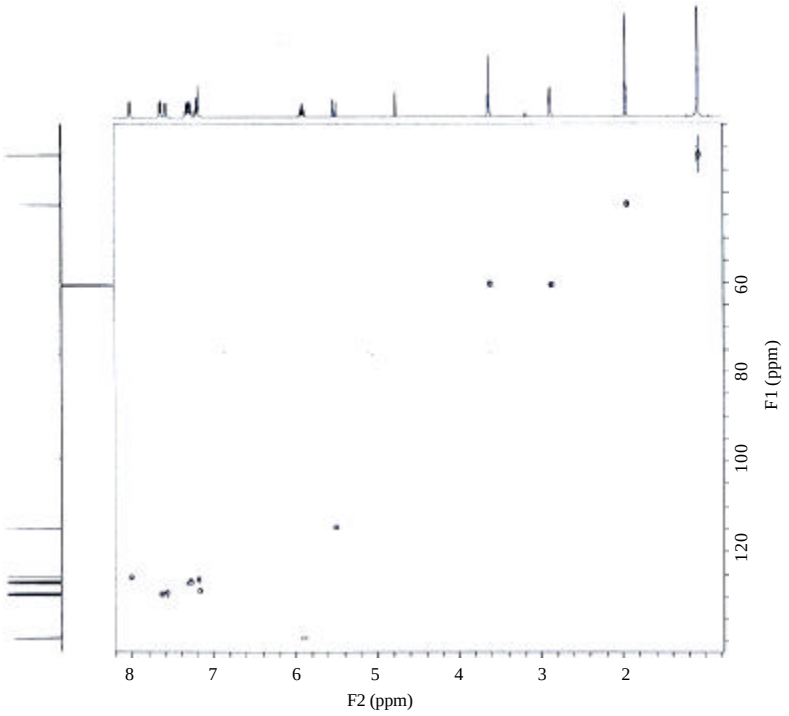

Fig. 6: HSQC spectrum of F-12

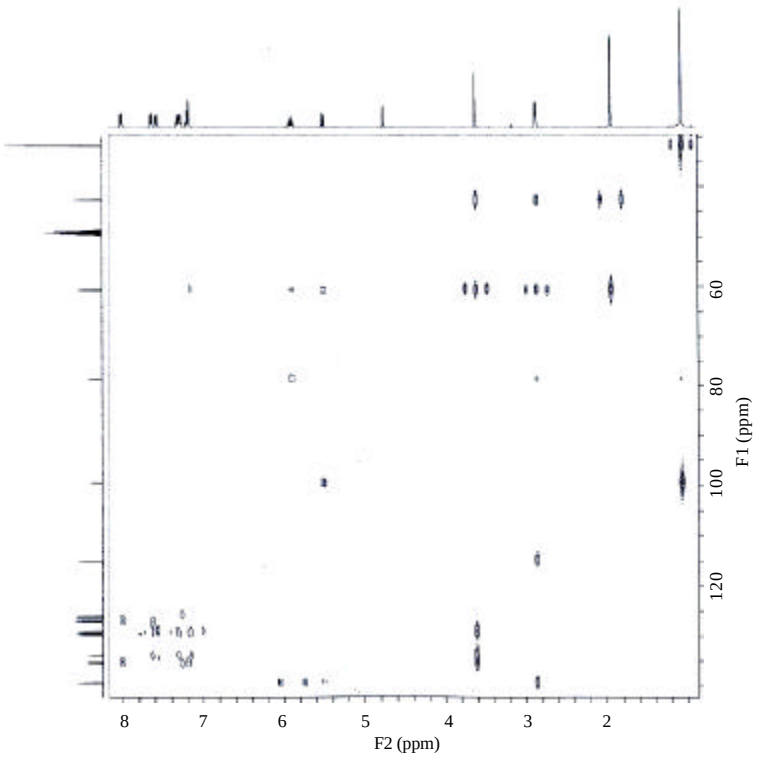

Fig. 7: HMBC spectrum of F-12

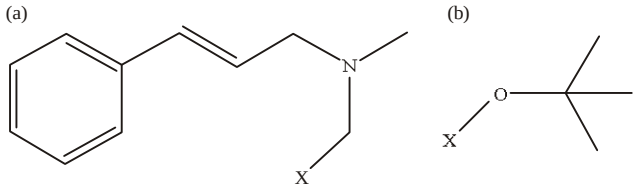

Fig. 8(a-b): Structure of identified fragments of the compound F-12, (a) Fragment A and (b) Fragment B

magnetically equivalent appears as singlet at δ_H 1.07 integrated for 9 protons correlated with carbon signal at δ_C 31.68 ppm. The quaternary carbon appears at δ_C 78.48 ppm. As the ^{13}C NMR indicated the presence of 20 carbons in F-12, however, yet MS obtained did not comply with the NMR data. Further data will be acquired after purification of F-12 from the culture media of *Streptomyces* KH-F12.

CONCLUSION

Taking into account the growing population of fungal strains resistant to azoles (Cannon *et al*., 2009) and the fact that amphotericin B shows severe toxic effects toward mammalian cells (Rajasingham *et al*., 2012; Laniado-Laborin and Cabrales-Vargas, 2009), the results of this antifungal compound deserve to be investigated for its cytotoxicity towards healthy mammalian cells. Further *in vivo* studies are necessary to fully determine the potential of this compound especially against the human fungal pathogens. By the results of these incoming investigations, this compound can be a subject of patent notification.

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