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Characterization of an Antifungal Compound Produced by *Streptomyces paradoxus*

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Forty five *Streptomyces* strains were isolated from the soil of Riyadh region at Saudi Arabia. All these strains were screened for an antifungal activity against some unicellular and filamentous fungi. The results proved that one strain was found to possess broad spectrum antifungal activity, thus purification and characterization of an antifungal agent were taken place. The concerned strain was identified as *Streptomyces paradoxus*, where the nucleotide sequence of 16S RNA gene (1.5 Kb) of this strain evidenced a 99% similarity with *Streptomyces paradoxus*. Moreover, there is high matching between the isolated strain and *Streptomyces paradoxus* in the morphological, physiological and biochemical characteristics. The antifungal metabolite was extracted using n-Butanol (1:1, v/v) at pH 7.0. The separation and purification of an antifungal agent were performed using both thin layer chromatography (TLC) and column chromatography (CC) techniques. The physico-chemical characteristics of the purified antifungal agent have been investigated. The collected data revealed that, the suggested empirical formula of antifungal agent is $C_{24}H_{19}NO$. The minimum inhibitory concentrations "MICs" of the purified antifungal agent were also determined. Eventually, the results emphasized the fact that the purified antifungal agent was suggestive of being belonging to 4' phenyl-1-napthyl-phenyl acetamide antibiotic.

Key words: Antifungal agent; Streptomycetes; Antibiotic extraction and purification; PCR.

Although antifungal antibiotics are few worldwide comparisons with the other antimicrobial antibiotics, they are prevailing in the market due to their vital role in the control of mycotic diseases. The medical and pharmaceutical fields constantly require to new safe and more effective antifungal agents to overcome opportunistic fungal infections particularly in the immuno-compromised host. The history of new drug discovery processes shows that novel skeletons have come, in the majority of cases, from natural sources[1]. The search for new, greater potency has been progressing[2]. One reason for safer, broad-spectrum antifungal antibiotics with this is that when compared to antibacterial, fungi are like mammalian cells, eukaryotes and therefore agents that inhibit protein, RNA or DNA biosynthesis in fungi have greater potential for toxicity[3].

Streptomycetes group are considered the most producers for antibiotics, which using in human and veterinary medicine as well as agriculture and fishery industry. Many antibiotic substances were reported to be isolated from *Streptomyces fradiae* such as fradicin[4], fradicin-mycelin group substances[5], neomycin[6], mycelin, mukherjee, frenolicin[7], phosphonomycin[8], dekamycin[9], tylosin[10], urdamycins[11], and actinomycin Z complex[12]. The 4' phenyl-1-napthyl-phenyl acetamide antibiotic absorption maximum was at 230 nm in ethyl acetate and IR spectrum showed two absorption peaks in the region of 3399 and 2927 cm indicates that 4' phenyl-1-napthyl-phenyl acetamide compound. The absence of carboxylic acid (COOH), ester (COOR) and alkyne

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(C=C-), was confirmed by the lack of a band in the region of 1670- 1740, 1700-1750 and 2100-2260 cm^{-1} respectively[13]. The 1H NMR spectrum of purified 4' phenyl-1-napthyl phenyl acetamide the peaks of value at 7.08 to 8.5, 1H of C=C-H (m, 16H) due to aromatic protons including periprotons of napthalein ring 1.9 due to NH group. The molecular formula of the compound was established from the elemental analysis as $C_{24}H_{19}NO$[13].

In the present work, antifungal agent was obtained and characterized from *Streptomyces* strain, which isolated from the soil of Riyadh region at Saudi Arabia. This strain was identified based on morphological, physiological and biochemical characteristics, as well as profiling of 16S rRNA gene.

MATERIALS AND METHODS

Test microorganisms

The test microorganisms of fungi which used in this work to detect and determine an antifungal activity of *Streptomyces* strains were obtained from different culture collection laboratories. These fungi are: *Candida albicans* IMRU 3669, *Saccharomyces cervicea* ATCC 9763, *Aspergillus niger* IMI 31276, *Aspergillus fumigatus* ATCC 16424, *Aspergillus flavus* IMI 111023, *Fusarium oxysporum* ATCC 96285, *Rhizoctonia solani* ATCC 10183, and *Alternaria alternata* ATCC 66981. Bacterial strains which used as test microorganisms in this work were obtained from American Type Culture Collection Lab.; *Staphylococcus aureus* ATCC-33591 and *Bacillus sabtilis* ATCC-11774 as Gram-positive bacteria; *Pseudomonas aeruginosa* ATCC-19429 and *Escherichia coli* ATCC-13706 as Gram-negative bacteria.

Isolation of *Streptomyces* strains

Forty five *Streptomyces* strains were isolated from the soil of Riyadh region at Saudi Arabia using the serial dilution technique[14].

Screening of antifungal activity

The antifungal activity was determined by cup method assay[15].

Identification of *Streptomyces* strains

The morphological and cultural characteristics of *Streptomyces* isolate were detected according to the international key of streptomycetes identification[16, 17]. For electron micrograph, ISP4 agar medium was inoculated and incubated for seven days at 28°C. A plug of the culture was removed and fixed in glutaraldehyde (2.5% v/v), washed with water and post-fixed in osmium tetraoxide (1% w/v) for 1 h. The sample was washed twice with water and dehydrated in ascending ethanol before drying in a critical point drying apparatus (Polaron E3000) and finally coated in gold and examined in a JEOLISM 541OLV scanning electron microscope at 15 Kv.

DNA isolation and manipulation

The local isolated *Streptomyces* strain was grown for 6 days on a starch nitrate agar slant at 30°C. Two ml of a spore suspension were inoculated into the starch-nitrate broth and incubated for 3 days on a shaker incubator at 200 rpm and 30°C to form a pellet of vegetative cells (pre-sporulation). The preparation of total genomic DNA was conducted in accordance with 2 methods[18].

Amplification and sequencing of 16S rRNA gene

PCR amplification of 16S rRNA gene of the local *Streptomyces* strain was conducted using two primers, Strep F; 5.-ACGTGTGCAGC CCAAGACA-3., and Strep R; 5.ACAAGCCCT GGAAACGGGGT-3., in accordance with the method[19]. The PCR mixture consisted of 30 pmol of each primer, 100 ng of chromosomal DNA, 200 ¼M dNTPs and 2.5 units of Taq polymerase, in 50 ¼l of polymerase buffer. Amplification was conducted for 30 cycles of 1 min at 94°C, 1 min of annealing at 53°C and 2 min of extension at 72°C. The PCR reaction mixture was then analyzed via agarose gel electrophoresis and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA). The 16S rRNA gene was sequenced on both strands via the dideoxy chain termination method[20]. The 16S rRNA gene (1.5 Kb) sequence of the PCR product was acquired using a Terminator Cycle Sequencing kit (ABI Prism 310 Genetic Analyzer, Applied Biosystems, USA).

Fermentation

The *Streptomyces* strain was inoculated into 250 ml Erlenmeyer flasks containing 100 ml of liquid starch nitrate medium (20 flasks). The flasks were incubated on a rotary shaker (200 rpm) at 30°C for 6 days. A twenty liter total volume was filtered through Whatman No.1 filter paper and followed by centrifugation at 5000 rpm for 20 minutes.

Extraction and precipitation

The clear filtrate was adjusted at different pH values (4 to 9) and extraction process was carried out using different solvents separately at the level of 1:1 (v/v). The organic phase was concentrated to dryness under vacuum using a rotary evaporator. The precipitation of crude antifungal compound was carried out using acetone-diethyl ether 10:1 (v/v) and followed by centrifugation at 5000 rpm for 15 min.

Purification by TLC

Separation of the antifungal compound into its individual components was conducted by thin layer chromatography using n-butanol-ethyl acetate-water (9: 9: 1). After ascending the plate was taken out and dried, then sprayed with ninhydrin[13, 21].

Purification by column chromatography

Purification of an antifungal compound was carried out using silica gel column chromatography (2.5 × 50 cm). Mixture of Chloroform and Methanol 95:5 (v/v) was used as an eluting solvent. The column was left overnight until the silica gel (Prolabo) was completely settled. One-ml crude extract to be fractionated was added on the silica gel column surface and the extract was adsorbed on top of silica gel. Fifty fractions were collected (5 ml/fraction) and tested for their antifungal activity[13].

Elemental and spectroscopic analyses

The elemental analyses for C, H, O, N and S elements as well as IR, UV, Mass spectrum and NMR spectrum were carried out in analytical chemistry laboratory at Pharmacy College, King Saud University, Riyadh, Saudi Arabia.

Determination of MICs for an antifungal agent

The minimum inhibitory concentration (MIC) has been determined by the paper method assay[15].

Characterization of an antifungal agent

The antibiotic which produced by *Streptomyces paradoxus* was characterized according to the recommended international references[22].

RESULTS AND DISCUSSION

There soil samples were collected from different locations in Riyadh region at Saudi Arabia. These samples were dried at room temperature to be ripe for actinomycetes isolation purpose. There forty five *Streptomyces* strains were isolated from these soil samples on starch nitrate agar plates by using serial dilution method. All these strains were screened to produce antifungal activity against some unicellular and filamentous fungi. The antifungal activity was assayed by measurement of inhibition zone diameter (mm). Notable, the screening test revealed that only one strain gave promising and highest antifungal activities Table (1) comparison with the others. Another study stated that, certain strain of *Streptomyces* bacteria which isolated from the soil was found has an effective antifungal activity against some pathogenic and saprophytic fungi[13]. Subsequently, the most potent *Streptomyces* strain

Table 1. Antifungal activities of *Streptomyces* strain against unicellular and filamentous fungi using inhibition zone method

	Fungi	Mean value of inhibition zone (mm)
Unicellular	*Candida albicans* IMRU 3669	30
	Saccharomyces cervicea ATCC 9763	40
Filamentous	*Aspergillus niger* IMI 31276	35
	Aspergillus fumigatus ATCC 16424	35
	Aspergillus flavus IMI 111023	40
	Fusarium oxysporum ATCC 96285	42
	Rhizoctonia solani ATCC 10183	30
	Alternaria alternata ATCC 66981	30

IMRU: Waksman Institute of Microbiology, Rutgers, The State University of New Jersey, Piscataway, NJ, USA.; ATCC: American Type Culture Collection, Manassas, VA, USA.; IMI: CABI Bioscience, Egham, UK (formerly International Mycological Institute; same as CMI).

Table 2. Morphological, physiological, and biochemical characteristics of most potent *Streptomyces* strain

Characteristics	Results
Spore mass	Gray
Spore surface	Smooth
Spore chain	Spiral
Color of substrate mycelia	YB
Color of diffusible pigment	NF
Diaminopimelic acid (DAP)	LL-DAP
Sugar pattern	ND
Amylase, protease, lipase, catalase, urease	+
Lecithinase, chitinase, pectinase	-
Melanoid pigment, H_2S	+
Nitrate reduction, Citrate utilization	-
Coagulation of skim milk	+
Degradation of esculin and xanthine	+
Growth with sodium azide and thallus acetate	-
Maximum growth temperature (°C)	37
Growth at 7 % NaCl	-
Resistance to ampicillin (10 µg)	-
Resistance to erythromycin (15 µg)	-
Staphylococcus aureus ATCC-33591	-
Pseudomonas aeruginosa ATCC-19429	-
Escherichia coli ATCC-13706	-
Bacillus sabtilis ATCC-11774	-
L-Arabinose, D-fructose, D-Glucose	+
D-Galactose, *meso*-Inositol, lactose	+
Mannitol, D-Mannose, rhamnose	+
Raffinose, sucrose, trehalose, starch, maltose	+
D-Xylose	-
L-Cysteine, L-arginine, L-Histidine	+
L-proline, L-methionine, L-Serine	+
L-Valine, L-Tyrosine, potassium nitrate	+
L-Phenylalanine, L-Lysine	-

YB: Yellowish brown; NF: Not found; ND: Not detected; +: Produced or growth; -: Not produced or no growth

for production of antifungal activity was identified as *Streptomyces paradoxus*. The latter was found has high similarity with the reference strain upon genetic, morphological, physiological and biochemical levels. The scanning electron micrograph (Figure 1) illustrated that; the spore chain is spiral and consisting of ellipsoidal spores with smooth surface. As shown in Table (2) the spore mass or aerial mycelia seem gray on starch nitrate agar medium with yellowish brown color for substrate mycelia, and no formation for diffusible pigments. The cell wall analysis proved that presence of LL-Diaminopimelic acid and not detected sugar pattern. Moreover, the strain could be produced amylase, protease, lipase, catalase and urease enzymes. However, lecithinase, chitinase and pectinase enzymes could not be produced. Melanin pigment is produced on peptone yeast-extract iron agar and tyrosine agar media. Hydrogen sulfide is produced while nitrate is not reduced and citrate is not utilized. The strain of *Streptomyces* was found has the ability to coagulate the skim milk and degrade both of esculin and xanthine. This strain can't grow on the medium contained sodium azide and/or thallus acetate. Notable, the strain under study can grow up to 37°C but the growth is completely absent at 45°C and also at 7% NaCl. The growth of strain is completely inhibited on the growth medium contained ampicillin (10 µg) or erythromycin (15 µg). The results of antibacterial test proved that, this strain does not have antibacterial effect either Gram-positive or Gram-negative bacteria. The strain can be utilized L-Arabinose, D-fructose, D-Glucose, D-Galactose, *meso*-Inositol, lactose, mannitol, D-Mannose, rhamnose, raffinose, sucrose, trehalose, starch and maltose as a sole

Table 3. The MICs of an antifungal agent against unicellular and filamentous fungi

	Fungi	MIC (mg/ml)
Unicellular	*Candida albicans* IMRU 3669	33.7
	Saccharomyces cervicea ATCC 9763	25.4
Filamentous	*Aspergillus niger* IMI 31276	33.7
	Aspergillus fumigatus ATCC 16424	40.8
	Aspergillus flavus IMI 111023	42.3
	Fusarium oxysporum ATCC 96285	35.5
	Rhizoctonia solani ATCC 10183	30.7
	Alternaria alternata ATCC 66981	30.4

Table 4. Comparative study of the physicochemical properties of an antifungal agent and reference antibiotic (4' phenyl-1-napthylphenyl acetamide)

Characteristic	Purified Antifungal agent	4' phenyl-1-napthyl- phenyl acetamide
Melting point	240°C	240°C
Molecular weight	337.30	337
Chemical analysis:		
C	84.98	ND
H	5.70	ND
N	4.33	ND
O	4.99	ND
S	0.0	ND
UV	225 and 321	225 and 321
Formula	$C_{24}H_{19}NO$	$C_{24}H_{19}NO$
Active against	Unicellular and filamentous fungi	Unicellular and filamentous fungi

ND: No data

carbon source, but D-Xylose is not. L-Cysteine, L-Arginine, L-Histidine, L-Proline, L-Methionine, L-Serine, L-Valine, L-Tyrosine and potassium nitrate could be utilized as a sole nitrogen source, but L-Phenylalanine and L-Lysine are not. The profiling of 16S rDNA gene was carried out by using PCR using the universal primers. The product of the PCR was analyzed on 1.5% ethidium bromide gel. The fermentation was taken place at 30°C for six days. *Streptomyces paradoxus* strain was grown on 20 liters of starch nitrate broth medium (fermentation batch). Subsequently, the culture broth was filtered through Whatman No.1 filter paper to exclude the mycelia, and then followed by centrifugation at 5000 rpm for 20 minutes. The cell free extract containing an active metabolite was polled for extraction process by using n-butanol 1:1 (v/v). The organic phase was collected and evaporated under reduced pressure using rotary evaporator. The crude extract was dissolved in a small amount of methanol, then filtrated and precipitated with acetone: diethyl ether 10:1 (v/v) and followed by centrifugation at 5000 rpm for 20 minute. Its color is grayish yellow. An antifungal agent was separated by using TLC plates and mobile phase that composed of n-butanol: ethyl

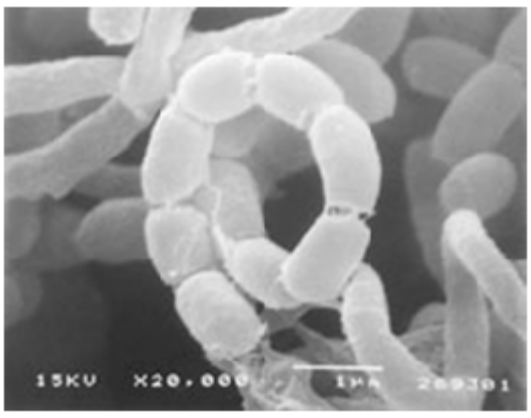

Fig. 1. Scanning electron micrograph of most potent *Streptomyces* strain after growth on Yeast-Malt extract agar medium for 14 days

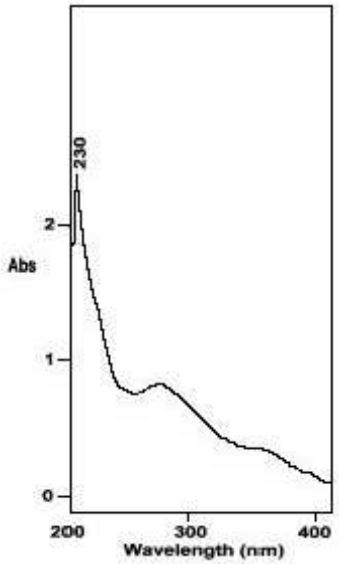

Fig. 2. UV absorbance of an antifungal agent

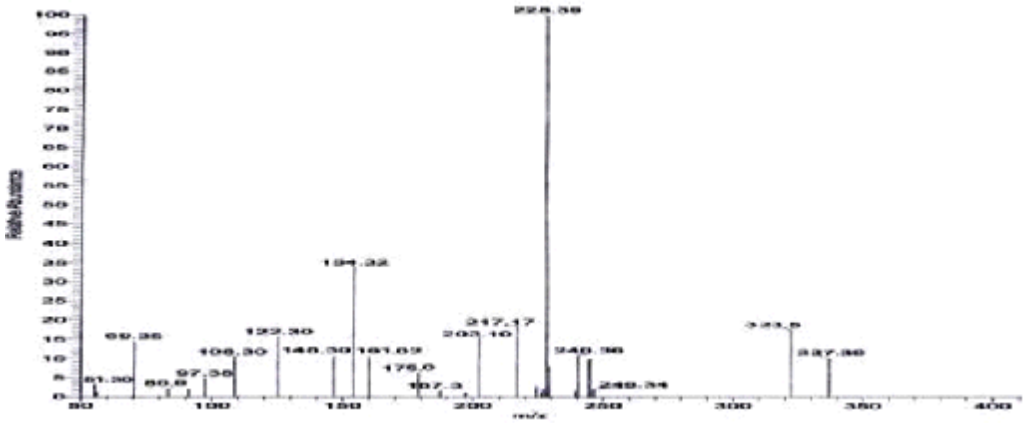

Fig. 3. Mass spectrum of an antifungal agent

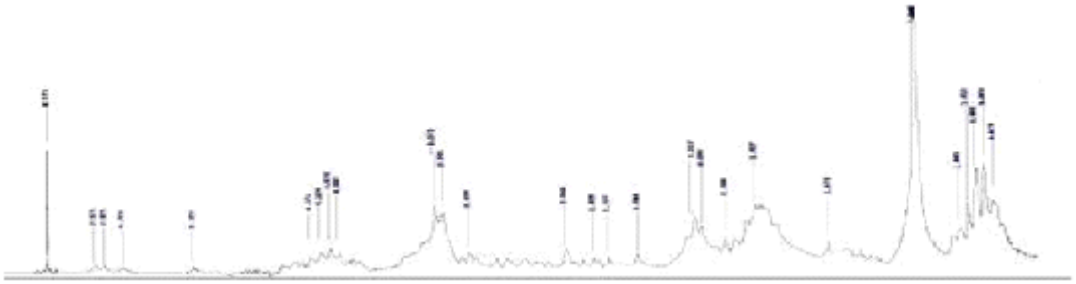

Fig. 4. NMR spectrum of an antifungal agent

acetate: water (9:9:1) at ascending direction. The plates were taken out and dried, then sprayed with ninhydrin. Only one yellowish brown band appeared. The purification of an antifungal agent was conducted through silica gel column chromatography which eluted with a mixture of chloroform and methanol 95:5 (v/v). The results revealed that, the antifungal activities were present in successive nine fractions. Another study reported that, the antifungal metabolites were extracted by n-butanol 1:1 (v/v) at pH 7 [13, 23, 24].

The physicochemical properties study revealed that, the purified antifungal agent had a characteristic odor with melting point at 240°C. The compound is high soluble in ethyl acetate, butanol and methanol, but it could not be dissolved in chloroform, acetone, diethyl ether, petroleum ether, hexane and benzene. These results were found similar to that stated by other studies[25, 26]. The elemental analytical data of an antifungal agent were showed as the following: C = 84.98; H = 5.70; N = 4.33; O = 4.99 and S = 0.0. This analysis indicates a suggested empirical formula of $C_{24}H_{19}NO$. These results are similar to other studies[13, 26]. The spectroscopic analysis of the purified of an antifungal compound proved that, maximal IR spectra showed two absorption peaks in the region of 3399 and 2927 cm^{-1}. As well as, the UV absorption spectrum recorded a maximum absorption peak at 225 and 321 nm (Figure 2). The mass spectrum showed that, the molecular weight at 337.30 (Figure 3). The NMR spectrum showed that, the peaks of value at 7.08 to 8.5, 1H of C=C-H due to aromatic protons including periprotons of napthalein ring 1.9 due to NH_2 group (Figure 4). These results were found similar to that recorded by other works[13, 26].

The minimum inhibitory concentrations (MICs) of an antifungal compound against unicellular and filamentous fungi were determined by paper method assay (Table 3). The antifungal activity produced by *Streptomyces paradoxus* showed maximum inhibitory effect against *Aspergillus flavus* IMI 111023 (42.3), *Aspergillus fumigatus* ATCC 16424 (40.8), *Fusarium oxysporum* ATCC 96285 (35.5), *Candida albicans* IMRU 3669

and *Aspergillus niger* IMI 31276 (33.7), *Rhizoctonia solani* ATCC 10183 (30.7), *Alternaria alternata* ATCC 66981 (30.4), and *Saccharomyces cervicea* ATCC 9763 (25.4). These results were found similar to that stated by other studies[13, 27]. On the basis of the recommended keys for the identification of antibiotics and in view of the comparative study of the recorded properties of the antifungal agent, it could be stated that the antifungal compound may be belonging to 4' phenyl-1-napthyl-phenyl acetamide antibiotic (Table 4). These results were supported by other similar results[13, 26, 27].

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