Chromatographic Isolation and Structural Elucidation of Secondary Metabolites from the Soil-Inhabiting Fungus *Aspergillus fumigatus* 3T-EGY

Mohamed S. Abdel-Aziz¹, Mosad A. Ghareeb^{2*}, Amal M. Saad², Laila A. Refahy² and Ahmed A. Hamed¹

¹Microbial Chemistry Department, Genetic Engineering and Biotechnology Division, National Research Center, El-Bohouth Street 33, Dokki-Giza 12622, Egypt

²Medicinal Chemistry Department, Theodor Bilharz Research Institute, Kornaish El-Nile, Warrak El-Hadar 12411, Imbaba, Giza, Egypt

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Eight compounds were isolated and identified from the soil-inhabiting fungus *Aspergillus fumigatus* 3T-EGY, namely, stearic acid (1), α -linolenic acid (2), physcion (3), di-(2-ethylhexyl) phthalate (4), 2,4,5,17-tetramethoxy pradimicin lactone (5), 3,5-dihydroxy-7-*O*- α -rhamnopyranoyl-2*H*-chromen-2-one (6), juglanthraquinone A-5-*O*-D-rhodamine-(4'→1'')-2-deoxy-D-glucose (4'→1''')-cinerulose B (7), and micropeptin (8). Their structures were determined on the basis of one-dimensional (1D-) and two-dimensional nuclear magnetic resonance (2D-NMR) [¹H-, ¹³C-NMR, ¹H-¹³C COSY (COrelated SpectroscopY), and ¹H-¹³C HMBC (Heteronuclear Multiple Bond Correlation) spectroscopy]. Compound 7 showed moderate *in vitro* antimicrobial activity against three pathogenic strains with inhibition zones values were ranged from 9.0 to 10.66 mm compared to neomycin as a positive control with inhibition zones values were ranged from 14.0 to 19.0 mm.

Keywords: *Aspergillus fumigatus* 3T-EGY, phenolics, juglanthraquinone A triglycoside, VLC, *in vitro* antimicrobial activity

Introduction

Microbial secondary metabolites have low molecular weight, and they are not important for their growth and reproduction as well as cell growth [1]. Berdy (2005) reported that the largest microbial group producing bioactive secondary metabolites was filamentous bacteria (actinomycetes), representing about 45% of the total compounds discovered followed by fungi (38%), but unicellular bacteria possess about 17% only [2]. Fungi are common in nature and are considered as a fruitful source of many antibiotics [3, 4]. Fungi related to ascomycetes like *Aspergillus*, *Penicillium*, and *Fusarium* are the most famous producers of biologically active secondary metabolites in comparison to other fungal genera [2]. The majority of soil fungi were known for their potentiality to decompose organic matters and their contribution to nutrient cycling. In addition, they were considered as promising bioactive secondary metabolites producers as they have produced many bioactive compounds and chemically exceptional skeletal structures used as pharmaceuticals [5]. Recently, fungi have appeared as novel sources of antioxidants as a part of their bioactive secondary metabolites [6, 7]. Fungi are obviously a varied group having about 1.5 million species, which can give a broad diversity of metabolites such as alkaloids, benzoquinones, flavonoids, phenols, steroids, terpenoids, tetralones, and xanthenes [8]. In addition to antioxidants, fungi exhibit various bioactivities and functions. Fungi found different applications in medicine industry and considered to be possible sources of new therapeutic agents. Therefore, the aims of the current study were to isolate, identify the fungus, and evaluate the *in vitro* antimicrobial activity of different vacuum liquid chromatography (VLC) fractions from extract of the fungus *Aspergillus fumigatus* 3T-EGY, grown on rice medium. The chromatographic isolation and identification of its bioactive secondary metabolites were also studied.

Experimental

General Experimental Procedures. Melting point (uncorrected) was determined on an electrothermal apparatus. ¹H-, ¹³C-NMR, ¹H-¹³C COSY (COrelated SpectroscopY), and ¹H-¹³C HMBC (Heteronuclear Multiple Bond Correlation) spectra were obtained using a pulse sequence supplied from Varian Mecaury 300 MHz spectrometer (¹H, 300 MHz and ¹³C, 75 MHz, in deuterated dimethylsulphoxide [DMSO-*d*₆]). Chemical shifts (δ) were given in values (ppm) relative to trimethylsilane (TMS) as an internal reference and coupling constant (*J*) in Hertz. All solvents and reagents used were of analytical grade. Sephadex LH-20 (25–100 μ m, Pharmacia Fine Chemicals Inc., Uppsala, Sweden). Paper chromatography (PC) was carried out on Whatman No. 1 paper sheets (57 cm × 46 cm; Maidstone, England) (S₁, *n*-BuOH–AcOH–H₂O, 4:1:5 upper layer; S₂, H₂O–AcOH, 85:15). Spots were visualized under Vilber Lourmat UV lamp (VL-6LC France) at 254 and 365 nm and then sprayed with methanolic 1% FeCl₃ and/or 5% AlCl₃.

Media. The following media were used in the study: nutrient agar medium (DSMZ1) (beef extract, 3; peptone, 10; agar, 18–20; distilled water, 1000 mL; pH 7), Czapek-Dox (CD) agar medium (DSMZ 130) (sucrose, 30; NaNO₃, 3; MgSO₄·7H₂O, 0.5; FeSO₄·7H₂O, 0.01; K₂HPO₄, 1; KCl, 0.5; distilled water, 1000 mL; agar, 18–20).

Isolation of Terrestrial Fungi. Soil samples were collected in the surrounding of Mansoura Governorate, Egypt during May 2012; soil was taken at 10 cm depth. Samples were sieved and air dried for 3–5 days at 28 °C. After drying, samples were kept at 10 °C until used. Fungal strains were isolated from soil samples. Enumeration of the microbes present in the soil was done by serial dilution-agar plating method. Serial dilution of soil suspension was prepared up to 10^{−6} dilution. Then, 0.1 mL of suspension from dilutions 10^{−3} to 10^{−6} was transferred to the petri dishes containing CD agar medium at 28 ± 2 °C for 6–8 days, and growth was observed after 2 days. The fungi

* Author for correspondence: m.ghareeb@tbri.gov.eg

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isolated on culture medium from soil were purified by spore suspension and streak method. The cultures were routinely transferred (every 6–8 days) onto fresh CD agar plates by streaking. Before fungal cultures were used for inoculation of liquid growth medium, the fungus was subjected to three transfers on CD agar plates by the direct agar transfer method [9].

Scale up Fermentation, and Extraction. Scale-up fermentation has been maintained using 15 Erlenmeyer flasks (1 L volume); each contains 100 g rice and 100 mL distilled water, sterilized at 121 °C (15 lb) for 20 min. Each flask was inoculated with spore suspension from 1 slant (10 days old). After incubation at 30 °C for 15 days, the medium was extracted with ethyl acetate several times till exhaustion. A reddish brown extract was produced (≈ 20 g).

Fungal Identification. Fungal isolate (3T) was identified by DNA isolation, amplification by polymerase chain reaction (PCR), and sequencing of the internal transcribed spacer (ITS) region. The primers ITS2 (GCTGCGTTCTTCATCGATGC) and ITS3 (GCATCGATGAAGAACGCAGC) were used for PCR amplification while ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were taken for sequencing. *Candida* sp. was tested as control. The sequence data were submitted to GenBank. The fungal strain (3T) culture was reserved in the Microbial Chemistry Department Culture Collection of Microorganisms.

In vitro Antimicrobial Activity. Disc agar plate method has been established to evaluate the antimicrobial activities of different fractions as well as compound 7 that dissolved in methanol (MeOH) [10]. Four different test microbes, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, and *Aspergillus niger*, were selected to evaluate the antimicrobial activities as representatives of Gram+ bacteria, Gram- bacteria, yeast, and fungal groups, respectively. The bacterial and yeast test microbes were grown on a nutrient agar medium. On the other hand, the fungal test microbe was cultivated on Czapek-Dox medium. The culture of each test microbe was diluted by distilled water (sterilized) to 10^7 to 10^8 colony forming units (CFUs)/mL, and then 1 mL of each was used to inoculate 1 L Erlenmeyer flask containing 250 mL of solidified agar media. These media were put onto previously sterilized Petri dishes (10 cm diameter having 25 mL of solidified media). Filter paper discs (5 mm Ø, Whatman No. 1 filter paper) loaded with 0.2 mg of each extract and 100 µg of pure sample. The discs were dried at room temperature under sterilized conditions. The paper discs were placed on agar plates seeded with test microbes and incubated for 24 h, at the appropriate temperature of each test organism. Antimicrobial activities were recorded as the diameter of the clear zones (including the disc itself) that appeared around the discs [11].

Isolation and Purification of Secondary Metabolites. The ethyl acetate (EtOAc) extract was evaporated to dryness to give a brownish mass (20 g) and then underwent fractionation using VLC on silica gel 60 using solvents in a gradient of increasing polarity; *n*-hexane-ethyl acetate, dichloromethane-methanol (CH_2Cl_2 -MeOH), and 100% acetone step gradient elution to afford thirteen fractions eluted from the VLC as follows: fractions 1–6 were eluted by *n*-hexane-EtOAc; 100:0–80:20–60:40–40:60–20:80–80:20–0:100 (%v/v), respectively, for the fractions 1–6; also, fractions 7–12 were eluted by CH_2Cl_2 -MeOH; 100:0–80:20–60:40–40:60–20:80–80:20–0:100 (%v/v), respectively, for the fractions 7–12, finally fraction (13) was eluted by 100% acetone. The *in vitro* antimicrobial activity of these fractions was evaluated; among them, the most promising fraction was undergoing further chromatographic isolation and purification via size exclusion chromatography using Sephadex LH-20 column (30 × 2 cm) eluted with 100% MeOH to afford eight pure compounds (Figure 1). In details, fraction 3 (1.5 g)

was subjected to Sephadex LH-20 eluted with gradient mix elution system, CH_2Cl_2 -MeOH till 100% MeOH; each sub-fraction was eluted as a single band to afford four compounds 1–4. On the other hand, fraction 4 (1.25 g) was subjected to Sephadex LH-20 column eluted with 100% MeOH to afford two compounds 5 and 6. Also, fraction 5 (1 g) was subjected to Sephadex LH-20 column eluted with 100% MeOH to afford compound 7. Finally, fraction 6 (0.75 g) was subjected to Sephadex LH-20 column eluted with 100% MeOH to afford compound 8.

Results and Discussions

Identification of the Fungal Isolate 3T-EGY. Basic Local Alignment Search Tool (BLAST) search for the fungus isolate revealed 99% similarity to *A. fumigatus*. The phylogenetic tree of this fungal isolate was also constructed (Figure 2). Based on the above identification techniques, our local soil fungal isolate was identified as *A. fumigatus* 3T-EGY with the GeneBank accession number KP140961 (<http://www.ncbi.nlm.nih.gov/nuccore/KP140961.1>). Conventional fungal identification protocols including morphological characteristics, growth on different media, and type of spores as well as biochemical behavior such as pigment production, etc. have been commonly applied, and several new species still now are identified according to this methods [12]. However, these methods take long time, have low

Figure 1. Flow chart representing the cultivation, extraction, and purification of bioactive metabolites from fungus *Aspergillus fumigatus* 3T-EGY

Figure 2. The phylogenetic tree of *Aspergillus fumigatus* 3T-EGY isolated from soil

sensitivity, difficult to control, and non-specific [13, 14]. Targeting specific regions within the ribosomal RNA gene clusters using universal primers through PCR amplification is another optional method for the fungal identification to the species level and also for analyzing fungal diversity [15]. In this context, internal transcribed spacer (ITS) regions (ITS-1 to ITS-5) of ribosomal DNA (rDNA) gene clusters are used. Primers routinely used for the amplification of ITS regions of ribosomal DNA are ITS-1 and ITS-4 [16].

In vitro Antimicrobial Activity of the Fungal Extract and Resulting Fractions (1–13) from VLC Column. The *in vitro* antimicrobial activity of the soil inhabiting fungus extract was evaluated against four pathogenic microbial strains, i.e., *S. aureus*, *Pseudomonas aeruginosa*, *C. albicans*, and *A. niger*. The results revealed that the extract showed strong activity against *P. aeruginosa* and *C. albicans* with equal inhibition zones of 15 mm, and it was also showed a moderate activity against *S. aureus* and *A. niger* with inhibition zones of 10 and 9 mm, respectively. Penicillin G was used as positive control at concentration of 100 µg/disc with inhibition zones (*S. aureus*, 27 mm; *P. aeruginosa*, 20 mm; *C. albicans*, 25 mm; and *A. niger*, 0 mm) (Table 1). On the other hand, the resulting fractions (1–13) from the VLC column of the ethyl acetate extract were subjected to *in vitro* antimicrobial screening. Fractions 3, 4, and 5 exhibited the highest antimicrobial activity against all test microbes with inhibition zones were ranged from 8.0 to 18.5 mm. Moreover, fractions 6 and 8 showed a moderate activity with inhibition zones were ranged from 8.0 to 18.5 mm. Furthermore, fraction 7 showed a weak activity against only two test microbes, *P. aeruginosa* (6.5 mm) and *C. albicans* (6 mm).

Table 1. *In vitro* antimicrobial activity of the ethyl acetate extract of *Aspergillus fumigatus* 3T-EGY grown on rice medium

Test microbe	Microbial group	Clear zone (φ mm) ^a	
		3T-EGY	Penicillin G ^b
<i>S. aureus</i>	G+ bacteria	10	27
<i>P. aeruginosa</i>	G- bacteria	15	20
<i>C. albicans</i>	Yeast	15	25
<i>A. niger</i>	Fungus	9	0

^aInhibition zones diameter (mm).

^bPenicillin G was used as a positive control (100 µg/disc).

In addition, there is no any activity recorded with fractions 1, 2, 9, 10, 11, 12, and 13 (Table 2).

Reviewing the literature, it was revealed that the endophytic isolate *A. fumigatus* R7 exhibited strong *in vitro* antibacterial activity against Gram+ *B. subtilis* (16 mm) and *S. aureus* (15 mm), and Gram- bacteria *P. aeruginosa* (19 mm) and *E. coli* (16 mm), and there is no any antifungal activity against *A. niger*, *A. flavus*, and *C. albicans* [17]. Accordingly, our results are in agreement with the finding of previous studies. Also, the crude extract of *A. fumigatus* BTMF9 exhibited *in vitro* antimicrobial activity against Gram-positive bacteria *B. circulans* [18].

Structure Elucidation. The promising resulting fractions from VLC column which underwent further purification upon Sephadex LH-20 column to afford eight pure isolates, these compounds were identified on the basis of their one-dimensional (1D-) and two-dimensional nuclear magnetic resonance (2D-NMR) [¹H-, ¹³C-NMR, ¹H-¹H COSY, and ¹H-¹³C HMBC analyses] (Figure 3).

Table 2. *In vitro* antimicrobial activity of the different fractions from VLC column of ethyl acetate extract of *Aspergillus fumigatus* 3T-EGY grown on rice medium

Fraction no.	Clear zone (φ mm) ^a			
	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>	<i>A. niger</i>
1	0	0	0	0
2	0	0	0	0
3	11.50 ± 0.70 ^b	14.50 ± 0.65	13.50 ± 0.75	8.50 ± 0.80
4	14.0 ± 1.41	16.0 ± 0.75	18.50 ± 2.12	9.50 ± 0.90
5	11.50 ± 1.21	10.0 ± 2.15	9.50 ± 1.30	8.0 ± 1.23
6	6.50 ± 0.72	6.50 ± 0.84	6.0 ± 0.0	9.0 ± 1.43
7	0.0–0.0	8.50 ± 0.79	6.0 ± 0.0	0.0–0.0
8	6.50 ± 0.92	0	6.50 ± 1.45	12.0 ± 1.25
9	0	0	0	0
10	0	0	0	0
11	0	0	0	0
12	0	0	0	0
13	0	0	0	0
Penicillin G ^c	27	20	25	0
Streptomycin ^d	13	21	0	0

^aInhibition zones diameter (mm).

^bMean ± SD, n = 3.

^cPenicillin G was used as a positive control (100 µg/disc).

^dStreptomycin was used as a positive control (100 µg/disc).

Compound 1 was isolated as light yellow wax, infrared (IR): λ_{max} (cm^{-1}): 722, 787, 1075, 1170, 1225, 1380, 1470, 1550, 1682 (carbonyl group), 1730, 2975 (CH-aliphatic), and 3475 (hydroxyl group). $^1\text{H-NMR}$ (300 MHz, $\text{DMSO-}d_6$) spectrum showed a broad singlet signal observed at δ_{H} 0.78 ppm which indicated aliphatic methyl group. A large singlet appeared at δ_{C} 1.33 ppm indicative for thirty protons of fifteen consecutive methylene groups. A broad singlet signal appeared at δ_{H} 2.46 ppm (2H, br s, H-2) of methylene group vicinal to carboxyl group. $^{13}\text{C-NMR}$ (75 MHz, $\text{DMSO-}d_6$) spectra showed peaks at δ_{C} 175.01 ppm (C-1) indicative to carbonyl group of carboxylic acid and at δ_{C} 39.33 ppm (C-2) indicative to oxy methylene group. The consecutive methylenes were detected at δ_{C} 25.02 (C-3), 29.17–29.67 (C-4 & C-15), 34.15 (C-16), and 22.65 (C-17); the last free methyl group appeared at δ_{C} 14.31 (C-18) ppm. All peaks in the $^1\text{H-}$ and $^{13}\text{C-NMR}$ spectrum exist in aliphatic region, and this is an indication for aliphatic nature of compound 1. According to previous data and via comparison with physical and spectral data from the literature [19–21], compound 1 was identified as stearic acid.

Compound 2 was obtained as a light yellow wax; the IR spectrum showed a broad band at 3410.26 cm^{-1} and 1716.70 cm^{-1} , indicating the presence of OH group and a carbonyl (C=O) group, and also showed peaks at 2929.97 cm^{-1} , 1458.23 cm^{-1} , and 1408.08 cm^{-1} indicating the presence of aliphatic bonds. The $^1\text{H-NMR}$ spectrum showed a characteristic signal at δ_{H} 0.81 ppm (3H, s, Me-18) for methyl group and multiplets at δ_{C} 1.33–1.61 ppm (methylene hump) for 20 aliphatic protons, and at δ_{H} 2.13 ppm (2H, t, $J = 7.2\text{ Hz}$, H-2), δ_{H} 2.46 ppm (4H, H-8, and H-17), and δ_{H} 2.22 ppm (2H, t, $J = 7.2\text{ Hz}$, H-14), it also showed broad multiple peaks at δ_{H} 4.99–5.29 ppm for olefinic protons. Furthermore, $^{13}\text{C-NMR}$ spectrum showed a peak at δ_{C} 170 ppm indicating the presence of carbonyl (C=O) carbon and peaks at δ_{C} 130.22 and 128.27 ppm for four olefinic carbons. All the previous data coincide with the spectral data of α -linolenic acid which was previously isolated from *Rhodiola rosea* [22, 23]. Therefore, compound 2 was identified as α -linolenic acid.

Compound 3 was obtained as orange crystal (m.p. 208–210 °C; R_f upon PC in 15% AcOH (0.05) & BAW (0.87) and

Figure 3. Chemical structures of the isolated compounds from *Aspergillus fumigatus* 3T-EGY

upon TLC in CH_2Cl_2 –MeOH, 9:1 (0.76)). ^1H -NMR spectrum showed signals in aromatic region at δ_{H} 6.75 ppm (1H, br s, H-7), δ_{H} 7.06 ppm (1H, br s, H-2), δ_{H} 7.36 ppm (1H, d, $J = 2.5$ Hz, H-5), and δ_{H} 7.66 ppm (1H, d, $J = 2.5$ Hz, H-4), it also showed two singlets at δ_{H} 2.46 ppm (3H, s, Me) and δ_{H} 3.84 ppm (3H, s, O-Me), and a characteristic signal at δ_{H} 13.29 ppm for hydroxyl groups. These data agreed with ^{13}C -NMR spectra which showed sixteen carbons; among them, fourteen carbons appeared in the aromatic region and two carbons showed signals at δ_{C} 21.8 (Me) and δ_{C} 56.7 ppm (OMe). The above mentioned spectral data agree with that reported in literature for physcion from genus *Aspergillus* [24, 25], lichen *Xanthoria* [26], and higher plant species [27]. Moreover, physcion has been widely isolated and characterized from both terrestrial and marine sources [24, 28]. Therefore, compound 3 was identified as physcion (1,8-Dihydroxy-3-methoxy-6-methyl-antraquinone or Emodin-3-methyl ether).

Compound 4 was obtained as very light yellow oil, dissolved in most organic solvents but insoluble in water (R_{f} upon TLC [n-hexane– CH_2Cl_2 ; 8:2, v/v], 0.66 [n-hexane–ethyl acetate; 8.5:1.5, v/v]). It gave purple color with concentrated H_2SO_4 . The IR spectrum showed a characteristic carbonyl band at 1655 cm^{-1} , aromatic (Ar–C–H) at 3050 cm^{-1} , aliphatic (–C–H) band at 2932 cm^{-1} , strong band for etheric bond (–C–O) at 1021 cm^{-1} , and methyl vibration band from 1410 to 1319 cm^{-1} . ^1H -NMR (300 MHz, $\text{DMSO}-d_6$): δ_{H} 7.67 (2H, dd, $J = 6$ Hz, 2.7 Hz, H-3, H-6), δ_{H} 7.59 (2H, dd, $J = 6$ Hz, 2.7 Hz, H-4, H-5), δ_{H} 4.12 (4H, m, H-1'), δ_{H} 1.60 (2H, m, H-2'), δ_{H} 1.21–1.33 (12H, m, H-3'-H-4', H-5'), δ_{H} 0.88 (6H, t, $J = 5.7$ Hz, H-6'), δ_{H} 1.49 (4H, m, H-7'), δ_{H} 0.80 (6H, t, $J = 5.1$ Hz, H-8'). ^{13}C -NMR (75 MHz, $\text{DMSO}-d_6$): δ_{C} 166.7 (–C=O), δ_{C} 131.8 (C-1, C-2), δ_{C} 131.1 (C-3, C-6), δ_{C} 129.3 (C-4, C-5), δ_{C} 67.2 (C-1'), δ_{C} 38.2 (C-2'), δ_{C} 31.4 (C-3'), δ_{C} 28.8 (C-4'), δ_{C} 22.4 (C-5'), δ_{C} 10.6 (C-6'), δ_{C} 23.3 (C-7'), and δ_{C} 13.7 (C-8'), on the basis of its spectral data compound 4 was identified as di-(2-ethylhexyl) phthalate [29, 30].

Compound 5 was obtained as fine powder (R_{f} upon PC in 15% AcOH [0.81]). It showed a characteristic violet spot on PC, which indicated its phenolic nature. It showed a characteristic spectral data of poly-aromatic nucleus compounds. ^{13}C -NMR spectrum showed presence of 31 carbon atoms. Two quinone carbonyl groups are deduced from two carbon signals appeared at δ_{C} 189.0 and 184.7 ppm. Aromatic carbons showed numerous signals at δ_{C} 103.46–144.94 ppm, which proved poly-aromatic ring structures, supported by ^1H -NMR spectral data at δ_{H} 6.96 and 6.61 ppm. Four methoxy groups attached to aromatic ring showed a characteristic signals at δ_{C} 62.60, 65.40, 57.30, and 55.9 ppm in ^{13}C -NMR spectrum and four singlets at δ_{H} 3.88, 3.76, 3.67, and 3.63 ppm in ^1H -NMR spectrum. Moreover, the presence of OH signal at δ_{H} 12.94 ppm indicated presence of phenolic hydroxyl group. Also, the carbon signal appeared at δ_{C} 168.09 ppm suggested the presence of unsaturated lactone structure of methylated isocoumarin moiety. Four methine (–CH–) groups showed characteristic signals at δ_{C} 120.6, 108.5, 107.2, and 103.4 ppm. Two methylene groups appeared at δ_{C} 21.83 & 28.94 ppm and δ_{C} 2.7–2.8 & 2.4–2.5 ppm in ^1H -NMR spectrum. Furthermore, the carboxylic carbon attached to aromatic ring gave a signal at 173.5 ppm in ^{13}C -NMR spectrum. These spectral data were compared to that of polyketide compounds, i.e., griseorhodin A, collinone, precollinone, pradimicin lactone, polyketide KS-619-1, and anthraquinone compounds; it showed high similarities to these compounds [31]. Therefore, compound 5 could be identified as 2,4,5,17-tetramethoxy pradimicin lactone.

Compound 6 was obtained as orange powder (R_{f} upon PC in 15% AcOH [0.14]). Chemical and physical data suggested that compound 6 is coumarin glycoside. The chemical

structure of the compound was interpreted via ^1H -NMR analysis. It showed three aromatic singlet signals at δ_{H} 7.38, 7.06, and 6.66 ppm attributed to H-4, H-6, and H-8, respectively. It also showed singlet at δ_{H} 13.62 ppm characteristic to aromatic hydroxyl proton. The anomeric proton appears as doublet at δ_{H} 4.13 ppm (d, $J = 5.7$ Hz, H-1 Rha). It showed also a characteristic rhamnose methyl at δ_{C} 1.21 ppm. Therefore, compound 6 could be identified as 3,5-dihydroxy-7-O- α -rhamnopyranoyl-2H-chromen-2-one [32].

Compound 7 was obtained as pale yellow fine crystal (m.p. 264–266; R_{f} upon PC in 15% AcOH [0.75]). It showed a characteristic yellow spot on PC. In the ^1H -NMR spectrum two meta coupled aromatic protons at δ_{H} 8.21 ppm (d) and 9.10 ppm (brs) were appeared in addition to three aromatic protons at δ_{H} 7.42, 7.63, and 7.80 ppm, which indicate the aromatic nature of the compound (Table 3). ^{13}C -NMR spectrum showed two quinone (–C=O) groups at δ_{C} 187.1 and 197.1 ppm, in addition to carboxylic group at δ_{C} 166.7 and acetate group signal at δ_{C} 170.7 ppm. EtO– group was observed at δ_{H} 0.80 ppm (d, $J = 7.5$ Hz) and δ_{H} 4.36 (d, $J = 6.6$ Hz). Moreover, long-range correlation from δ_{H} 4.36 to 170.70 was observed. Three anomeric carbons were observed at ^{13}C -NMR spectrum at δ_{C} 94.4, 92.6, and 91.4 ppm indicating the glycosidic nature of the compound. The oxymethine and methylene signals appeared between δ_{H} 3.0–5.45 ppm suggested the presence of glycosidic side chain attached to carbon-5 through O-linkage indicated by a characteristic signal at δ_{C} 128.5 in ^{13}C -NMR and HMBC spectrums. Also, ^1H -NMR revealed many overlapping methyl and methylene signals in the up field region at δ_{H} 2.6–0.80 ppm and at δ_{C} 94.4–43.6 ppm, the two signals at δ_{C} 43.6 and 44.6 indicating the presence of two carbons attached to nitrogen atom.

Table 3. HMBC assignments, ^1H (300 MHz) and ^{13}C (75 MHz) NMR spectral data of compound 7 in $\text{DMSO}-d_6$ (δ in ppm)

Position	δ_{C} ppm	δ_{H} ppm	HMBC
Aglycone			
1	128.54		
2	133.90	8.21 (d)	
3	134.08		
4	129.10	9.1 (brs)	
4a	130.40		
5	128.45		
6	127.80	7.42	
7	–	7.62	
8	120.75	7.8	
8a	131.63		
9	187.14		
9a	133.58		
10	197.14		
10a	111.8		
Sugar moieties			
1'	94.46	5.45	5, 3'
2'	29.2	2.4	4'
3'	57.4	–	1', 5'
4'	72.06	3.25	2', 6'
5'	65.86	3.55	3'
6'	20.55	1.66	4'
3'-N(CH ₃) ₂	43.66/44.65	2.17	
1''	92.65	4.92	4', 3''
2''	24.65	1.9	4''
3''	25.34	–	5'', 1''
4''	75.11	3.17	2'', 6''
5''	66.98	4.44	3''
6''	22.2	1.9 (d, 6.6)	4''
1'''	91.42	5.15	4'', 3'''
2'''	28.71	1.6	4'''
3'''	33.85	2.41	1''', 5'''
4'''	–	4.1	2''', 6'''
5'''	68.39	4.35 (6.6)	3'''
6'''	14.23	1.20 (5.1)	4'''
3-COOH	166.79		
EtOCO–	170.70		
–CH ₂ –	65.8	4.36 (d, $J = 6.6$ Hz)	
–CH ₃	14.0	0.8 (d, $J = 7.5$ Hz)	

Complete assignments and connectivities of the compound were determined from H-H COSY, HMQC (Heteronuclear Multiple-Quantum Correlation), and HMBC spectra. By comparing ^1H - and ^{13}C -NMR data with literature, it was shown that compound 7 is quinone connected to side chain at C-5 of three sugar moieties, viz., rhodosamine, 2-deoxy-D-glucose, and cinerulose B. Three anomeric proton signals were assigned at δ_{H} 5.45 ppm (rhodosamine), 4.92 ppm (deoxy glucose), and 5.15 ppm (cinerulose), in the ^1H -NMR spectrum through their direct one bond coupling in the HMQC spectrum with their own anomeric carbon signals at δ_{C} 94.4, 92.6, and 91.4 ppm, respectively. The interglycosidic and sugar aglycone linkages were deduced from the long-range three-bond HMBC correlations. The HMBC exhibited correlations between H-1' [δ_{H} 5.45 (rhodosamine)] and C-5 [δ_{C} 128.4] aglycone, H-1'' [δ_{H} 4.92] deoxy glucose] and C-4' [δ_{C} 72.06] rhodosamine], and H1''' [δ_{H} 5.15] cinerulose] and C-4'' [δ_{C} 75.11] deoxy glucose (Table 3). All ^1H - and ^{13}C -resonances were assigned with the aid of HMQC and HMBC correlation peaks and comparison with the corresponding data of structurally related compounds [33]. Accordingly, compound 7 was identified as Juglanthraquinone A-5-*O*-rhodosamine-(4'→1'')-2-deoxy-D-glucose (4''→1''')-cinerulose B.

Compound 8 was obtained as colorless amorphous solid (R_f upon PC in 15% AcOH [0.9]; UV [MeOH] 280 nm). Both ^1H and ^{13}C spectra (DMSO- d_6) showed a characteristic signals for peptide nucleus. ^{13}C -NMR spectrum showed nine signals for the amide carbonyl at δ_{C} 173.5, 173.3, 173.0, 170.4, 169.0, 166.4, 165.3, 156.3, and 156.1 ppm. Resonances present in the aromatic region of ^{13}C -NMR at δ_{C} 136.8, 130.2, 128.8, 127.1, 128.3, and 129.1 ppm, supporting the presence of phenyl alanine moiety, and also resonances at δ_{C} 128.1, 130.7, 114.9, 156.1, 114.9, and 130.3 ppm, suggesting the presence of tyrosin moiety. These data are in agreement with ^1H -NMR spectra at δ_{H} 7.43–6.37 ppm. Moreover, butyric acid side chain was identified by methyl triplet at δ_{H} 0.87 and δ_{C} 14.0 ppm. Compound 8 was also recognized by presence of 3-amino-6-hydroxy-2-piperdone (Ahp) moiety (–C=O) at δ_{C} 169.0 and –CH₂–OH at δ_{C} 71.8 ppm. Therefore, compound 8 was identified as micropeptin via comparison its spectral and physical data with that of literature [34].

In vitro Antimicrobial Activity of Compound 7. Compound 7 showed a moderate *in vitro* antimicrobial activity against three pathogenic strains including *S. aureus*, *P. aeruginosa*, and

Table 4. *In vitro* antimicrobial activity of compound 7 (50 $\mu\text{g}/\text{disc}$) against four pathogenic microbial strains, compared to neomycin as a positive control

Test microbe	Clear zone (ϕ mm) ^a	
	Compound 7	Neomycin ^c
<i>S. aureus</i>	9.0 $\pm$ 1.0 ^b	14.0 $\pm$ 1.0
<i>P. aeruginosa</i>	10.0 $\pm$ 1.0	17.3 $\pm$ 0.57
<i>C. albicans</i>	10.66 $\pm$ 1.15	19.0 $\pm$ 1.0
<i>A. niger</i>	0–0	0–0

^aInhibition zones diameter (mm).

^bMean $\pm$ SD, $n = 2$.

^cNeomycin was used as a positive control (50 $\mu\text{g}/\text{disc}$).

C. albicans with inhibition zones values ranging from 9.0 to 10.66 mm, compared to neomycin as a positive control with inhibition zones values ranging from 14.0 to 19.0 mm (Table 4 and Figure 4).

Literature review revealed that the *in vitro* antifungal activities of sixteen pure isolates from *A. fumigatus* were evaluated against *B. cinerea*, *A. solani*, *A. alternata*, *C. gloeosporioides*, *F. solani*, *F. oxysporum* f. sp. niveum, *F. oxysporum* f. sp. vasinfectum, and *G. saubinetii*, with minimum inhibitory concentration (MIC) values of 6.25–50 $\mu\text{g}/\text{mL}$ [35]. Moreover, the antibacterial and antifungal activities of some isolates (asperfomoid, fumigaclavine C, fumigaclavine E, fumigaclavine G, 3b-hydroxy-5a,8a-epidioxo-ergosta-6,22-diene, monomethylsulochrin, ergosterol, fumitremorgin C, and helvolic acid) from *A. fumigatus* were evaluated against *C. albicans*, *P. anaerobius*, *B. distasonis*, *E. coli*, *H. pylori*, *S. aureus*, and others [36].

Conclusions

Fungi play an essential role in the production of several bioactive secondary metabolites. Fungal strain (3T) was isolated from the Egyptian local agricultural soil and was tested for its ability to produce bioactive metabolites by cultivating it on solid rice medium. The produced bioactive extract was fractionated using VLC (13 fraction), and the produced fractions were biologically evaluated by measuring their antimicrobial activities. The highly bioactive fractions (3–6) were further purified via using the Sephadex LH-20 column. One bioactive compound, Juglanthraquinone A-5-*O*-D-rhodosamine-(4'→1'')-2-deoxy-D-glucose (4''→1''')-cinerulose B, and other seven compounds were elucidated and characterized.

Conflicts of Interest

The authors declare no conflict of interest.

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Figure 4. *In vitro* antimicrobial activity of compound 7 against four pathogenic microbial test strains

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