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Bioactive Compounds Produced by Strain of *Penicillium* sp.

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Abstract: During our search for bioactive compounds from fungi, the terrestrial *Penicillium*sp.KH Link 1809 isolate KHMM was fermented on large scale using solid rice medium. After harvesting, working up and purification of the afforded extract using different chromatographic techniques, the bioactive metabolites viridicatol (**1**) and kojic acid (**2**) were isolated. The chemical structures of **1** and **2** were confirmed by extensive 1D and 2D NMR and mass measurements, and by comparison with literature data. The antimicrobial activity of the strain extract was studied using a panel of pathogenic microorganisms.

Keywords: *Penicillium* sp., Bioactive metabolites, Biological activity, Taxonomy.

1. INTRODUCTION

Fungi represent strongly rich producers of potent bioactive secondary metabolites [1], acting as antibiotics [2], antimycotics [3], antiviral [4], and anticancer agents [5], or are pharmacologically active in other ways [6]. The genus of *Penicillium* is one of the most prolific sources of bioactive drugs and large range of compounds including polyketides, alkaloids, terpenoids, and peptides, including a large huge number of cytotoxic compounds [7,8]. Based on the taxonomical characterization, the genus *Penicillium* is representing one of the most complex of the fungal world, with 225 species approximately and a continued discovery of new species [9]. Traditionally, species in the genus *Penicillium*, which are fundamentally saprophytic and ubiquitous, have been regarded as a fruitful investigational ground for the finding of novel bioactive compounds, leading to the discovery of blockbuster drugs, such as penicillin [10] and the anticholesterolemic agent compactin [11], miscellaneous antitumor products [12], and mycotoxins contaminating food [13]. Most of these fundamental studies were carried out on strainsfrom soil and food commodities [14].However, the search for further new bioactive compounds from fungi isolated from untouched habitats is still strongly recommended and needed to overcome the huge gap between the currently inadequate drugs and needed new ones to treat the recently discovered diseases [15,16].

In our continual program for searching of bioactive compounds from the genus of *Penicillium*, the *Penicillium*sp. KHMM isolated from Egyptian habitats [17], has been applied to biological and chemical assays, announcing potent antimicrobial activity against different pathogenic microorganism, mainly Gram positive and Gram negative bacteria, and yeast (Table 1). Chemically, the strain extract showed several middle-polar zones during TLC visualized by UV light and spraying with anisaldehyde/sulphuric acid. A large scale fermentation of the strain on rice-solid medium followed by working up and purification using a series of different chromatographic techniques afforded the bioactive compounds viridicatol (**1**) and kojic acid (**2**). The chemical structures of the isolated compounds were confirmed by extensive 1D and 2D NMR and ESI HR mass measurements, and by comparison with literature data. The antimicrobial activity of the strain was studied using a set of microorganisms. The isolation and taxonomical characterization of the fungal strain is reported as well.

2. EXPERIMENTAL

2.1 General

NMR spectra (^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMQC and HMBC) were measured on Bruker Avance DRX 500 and DRX 600 MHz

spectrometers [Q] using standard pulse sequences and referenced to residual solvent signals. HR-EI-MS was determined using GCT Premier Spectrometer. The ultraviolet and visible (UV–Vis) spectra were measured on Spectro UV–Vis Double Beam PC8 scanning auto Cell UVD-3200, LABOMED, INC [Q].Column chromatography was carried out on silica gel 60 (0.040–0.063 mm, Merck [Q]) and Sephadex LH-20 as the stationary phases. Preparative TLC (0.5 mm thick) and analytical TLC were performed with pre-coated Merck silica gel 60 $PF_{254+366}$. R_f values and Visualisation of chromatograms was carried out under UV light (254 and 366 nm) and further by spraying withanisaldehyde/sulphuric acid followed by heating.

2.1. Isolation and Taxonomy of the producing strain

The fungus *Penicillium* sp. KHMM was isolated from a soil sample collected from Tag elezz agricultural research station, Dakahyia region, Egypt, at a depth of 5 cm under sterile conditions. The fungus was isolated by conventional dilution plate technique [18]. One gram of the sample was suspended in 10 mL of sterilized water and shaked for 2 h. The sediments were then left to settle down for few minutes. The supernatant was then applied to successive dilution up to 10^{-6}. An aliquot (1 mL) of the afforded suspension was spread over Czapek-Dox agar medium (gL^{-1}: 30 sucrose, 3 $NaNO_3$, 1 K_2HPO_4, 0.5 KCl, 0.5 $MgSO_4$, 0.01 $FeSO_4$, 20 agar-agar, and distilled water (1 L) at pH 7.3]), and incubated at 28°C for 14 days, the growing colonies isolated in slants of the same medium and stored in refrigerator at 4°C until use.

On Czapeck-Dox agar medium, Colonies of the fungal isolate showed vegetative mycelium abundant, colorless or pale- or brightly-colored, Colonies low to moderately deep, plane to very faintly sulcate; margins low, narrow to wide (1–5 mm), sporulation highly dense, conidia dull to greyish green exudate clear to almost a hazy yellow, sometimes absent; soluble pigment mostly yellowish orange.

In micromorphology, conidiophores typically borne from green mycelia embedded in medium, stipes mostly hyaline but sometimes green, mostly biverticillate, infrequently terverticillate, monoverticillate side branches sometimes present; Stipes very short to very long, typically rough, a minor proportion smooth to finely rough. Branches two when present, etulae 3–5 per stipe, Phialides ampulliform, 6–9 per etula, Conidia smooth, globose to subglobose.According to theses cultural and morphological features, and according to Raper and Thom (1949) [19], the terrestrial fungal strain KHMM is belonging to *Penicillium* genus. An authentic isolate of the strain is deposited at collection of Dr M. M. El Metwally, Botany and Microbiology Department, Faculty of Science, Damanhour University, Egypt.

Fermentation, working up and isolation

The *Penicillium* sp. KHMM was inoculated from well grown agar plates with dark green sporulating colonies into 0.1L sterilized glass bottles each containing modified rice medium composition: 8 g commercial rice; 10 mL distilled water. The bottles were incubated for 15 days at 30°C.

After harvesting, 50 mL of 1:1 DCM/MeOH was added to each bottle, followed by aggressive shaking for two hrs, and the afforded organic extract was decanted, filtered, and then concentrated *in vacuo* till dryness, affording (9.5 g) as brown crude extract. The crude extract was then suspended in water and applied to successive extraction in separating funnel (0.5 L), starting with pet. ether, dichloromethane (DCM), ethyl acetate and n-butanol, consequently. The corresponding fractionated extracts were concentrated to dryness affording KH1 (0.3 g), KH2 (0.4 g), KH3 (3.2 g) and KH4 (2.4 g), respectively, as reddish brown fractions. Fractions KH1 and KH2 showed undesired components according to TLC visualization and excluded.

The ethyl acetate fraction KH3 (3.2 g) was subjected to purification on silica gel column (3 × 60 cm), eluted with DCM-MeOH gradient (0.5 L DCM/3%MeOH [97:3], 0.5 L DCM-MeOH [95:5], 0.5 L DCM-MeOH [90:10], 0.5 L DCM-MeOH [80:20] , 0.5 L DCM-MeOH [1:1], 0.2 L MeOH. Three fractions were afforded according to TLC monitoring: FI (3.2 g), FII (1.2 g), FIII (0.3 g). Fraction FI is mostly containing fatsand phthalate and discarded. Purification of fraction FII using Sephadex LH-20 (MeOH), followed by silica gel column eluted by DCM-MeOH and finally with Sephadex LH-20 (MeOH) afforded a colorless solid of viridicatol (**1**, 5 mg). As the same for the ethyl acetate fraction, purification of the n-butanol fraction KH4 (2.4 g) using silica gel column followed by Sephadex LH-20 afforded a colourless solid of kojic acid (2, 8 mg).

Viridicatol (1):

Colorless solid, UV absorbing showing brown coloration on spraying with anisaldehyde/sulphuric acid: R_f = 0.45 (DCM/7% MeOH).^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) see Table 1.
(+)-ESI MS: *m/z* (%) 276 ($[M+Na]^+$, 100), 529 ($[2M+Na]^+$, 70); (-)-ESI MS: *m/z* (%) 252 ($[M-H]^-$, 100).

Kojic acid (2):

Colorless solid, UV absorbing showing no color staining on spraying with anisaldehyde/sulphuric acid. R_f = 0.22 (DCM/10% MeOH). ^{1}HNMR (DMSO-*d6*, 500 MHz):δ8.99 (br s, 1H, OH), 5.69 (br s, 1H, OH), 7.97 (d, 1H, *J*=7.97, H-2), 6.34 (s, 1H, H-5), 4.28 (s, 2H, H_2-7). ^{13}CNMR (DMSO-*d6*, 125 MHz): δ174.2 (C_q-4), 168.2 (C_q-6), 153.0 (C_q-3), 138.9 (CH-2), 110.0 (CH-5), 59.7 (CH_2-7).

Antimicrobial Assay Using Agar Diffusion Test

Antimicrobial activity testing of the crude extract of the fungal isolate KH was carried out against a set of microorganisms using the agar diffusion technique. Paper-disk diffusion assay [20] with

some modifications has been followed to measure the antimicrobial activity. 20 mL of medium seeded with test organism were poured into 9 cm sterile Petri dishes. After solidification, the paper disks were placed on inoculated agar plates and allowed to diffuse the loaded substances into refrigerator at 4 °C for 2 h. The plates were incubated for 24 h at 35 °C. Both bacteria and yeasts were grown on nutrient agar medium: 3g/L beef extract, 10g/L peptone, and 20g/L agar. The pH was adjusted to 7.2. Fungal strain was grown on potato dextrose agar medium (g/L): Potato extract, 4; Dextrose, 20; Agar No. 1, 15 (pH 6). The samples were dissolved in DCM/10% MeOH. Aliquots of 50 µL (= 50 μg) were soaked on filter paper discs (9 mm) and dried at room temperature under sterilized conditions. The paper discs were placed on inoculated agar plats and incubated for 24 h at 37°C for bacteria and 48 h (30°C) for the fungi. After incubation, the diameters of inhibition zones were measured with a wide panel of test microorganisms comprising Gram positive bacteria (*Bacillus subtilis* ATCC6633 and *Staphylococcus aureus* ATCC6538-P), Gram negative bacteria (*Pseudomonas areuginosa* ATCC 27853), yeast (*Candida albicans* ATCC 10231, and the fungus *Aspergillus niger* NRRL A-326.

3. RESULTS AND DISCUSSIONS

3.1. Fermentation and Structure Elucidation

The *Penicillium* sp. KH Link 1809 was cultured on rice solid medium. Biologically, the afforded extract of the strain exhibited high activity against Gram positive, Gram negative bacteria, and yeast, namely: *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* ATCC6538-P), *Pseudomonas areuginosa* ATCC 27853, and *Candida albicans* ATCC 10231 (Table 1). In the chemical screening monitored by TLC, the fungal extract exhibited two major UV absorbing bands with a narrow polarity range, one of them exhibited a brown staining with anisaldehyde/sulphuric acid. Some other unpolar bands were detected as violet-blue on spraying with the same reagent, which might be attributed to some fats, phthalates or sterols. Separation of the strain produced middle polar bioactive metabolites using a series of chromatographic techniques (see experimental section) afforded Viridicatol (**1**) and kojic acid (**2**).

Viridicatol (1)

As Colorless solid, compound **1** was obtained showing UV absorbance at 254 nm during TLC, which stained as brown on spraying with anisaldehyde/sulphuric acid. The molecular weight of **1** was deduced as 253 Dalton according to ESI MS modes, such that two *pseudo* ion peaks were exhibited at *m/z* 276 [M+Na] and 529 [2M+Na] were exhibited, and one ion peak was visible at *m/z* 252 [M-H] in the ESI negative mode. HRESI MS of 1 revealed the corresponding molecular formula as $C_{15}H_{11}NO_3$, bearing eleven double bond equivalents.

Based on the ^{1}H, ^{13}C and HMQC NMR spectra (CD_3OD) (Table 2), the numbers of hydrogen and carbon atoms were in agreement with the molecular formula. Based on the ^{1}H NMR spectrum, H,H COSY experiment, and coupling constants, eight proton signals (δ8.15, 7.36 [2H], 7.34, 7.26, 6.88, 6.83, 6.82),were observed in the aromatic region, indicating the presence for *ortho*- and *meta*-disubstituted benzene systems. In the ^{13}C NMR spectrum and HMQC experiment, one carbonyl carbon signal (δ159.1) and 14 sp^2carbon signals, including eight methine signals (δ129.1, 124.9, 122.4, 121.6, 120.7, 116.5, 115.0, 114.5), were observed. According to the above data, followed by study the structure using HMBC experiment (Fig.1), the structure of the title alkaloid was assigned as viridicatol (**1**) [21,22]. Viridicatol was isolated previously along with viridicatin from a strain of *P*. viridicatin [23]. Functionalized 4-arylquinolin-2(1*H*)-ones constitute generally, a valuable class of biologically active molecules, including several fungal metabolites such as viridicatin [24] and 3-*O*-methylviridicatin [25]. Viridicatol was reported to show cytotoxicity toward KB, KBv200, A549, hepG2, MCF7, K562, SMMC7721, and SGC7901 tumor cell lines with IC_{50} values of 25.0, 16.5, 60.0, 85.0, 45.0, 25.0, 80.2, and 80.0 g/mL, respectively.

Kojic acid (2)

As polar Colorless solid, exhibiting UV absorbance at 254 nm during TLC, compound 2 was obtained, which showed no color staining on spraying with anisaldehyde/sulphuric acid. Based on EI MS, the molecular weight of **2** was deduced as 142 Daltons, and the corresponding molecular formula was established by EI HRMS as $C_6H_6O_4$, containing four double bond equivalents. The ^{1}H NMR spectrum (DMSO-d_6) of **2** displayed two broad signals at δ 8.99 and δ5.69, which could be attributed to exchangeable protons of phenolic and aliphatic hydroxyl groups, respectively. Two further proton singlets each of 1H were visible at δ7.97 and 6.34, in addition to anoxy-methylene signal as singlet at δ4.28. Based on the ^{13}C and APT NMR spectra, three quaternary sp^2 carbon signals were displayed at δ174.2, 168.3 and 153.0, representing a lactone carbonyl (δ174.2), oxygenated sp^2 carbon (168.3) in β-position with respect to a lactone carbonyl, and phenolic one (δ153.0). Two sp^2methine carbons were exhibited at δ138.9, and 110.0, along with an oxy-methylene carbon (δ59.7) as well. Based on the revealed chromatographic features, spectroscopic data, and search in the corresponding data bases (AntiBase [1], Dictionary of Natural Products (DNP) [26], and Scifinder [27], compound **2** was confirmed as kojic acid. Biologically, kojic acid is showing high toxicity and high antibiotic activity against Gram-Positive and Gram negative bacteria, and antifungal properties [28]. Kojic acid is a well-known as tyrosinase inhibitor, and hence it has been used as whitening or anti-hyperpigment agent because of its ability to suppress dermal-melanin production [29].

3.2. Biological activity studies

Antimicrobial activity testing of the stain extract of the terrestrial fungus *Penicillium* sp. KH Link 1809

was carried out against five microorganisms using the agar diffusion technique. The extract showed high antimicrobial activity (50 μg per disk) against Gram-positive bacteria (*Bacillus subtilis* ATCC6633 [27 mm], *Staphylococcus aureus* ATCC6538-P [26 mm]), Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853 [31 mm]), and *Candida albicans* ATCC 10231 [25 mm]. Nevertheless, the extract showed no activity against *Aspergillus niger* NRRL A-326. (Table 1)

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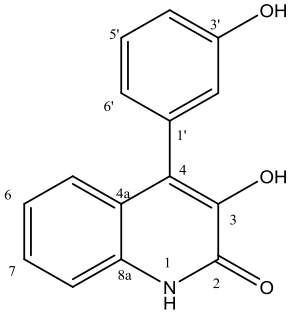

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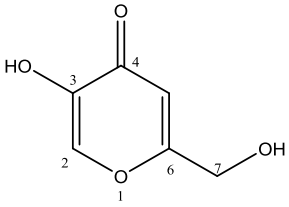

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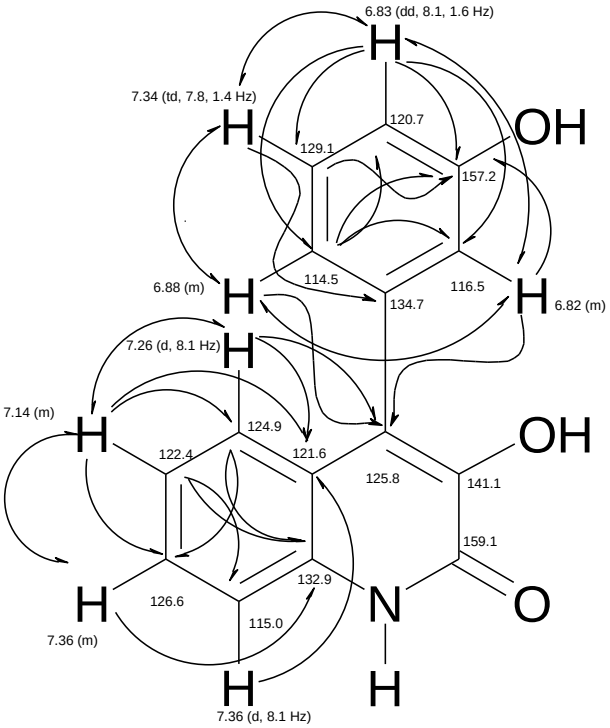

Fig.1: H,H COSY (↔) and HMBC (→) correlations of Viridicatol (**1**)

Table 1: Antimicrobial activities of *Penicillium sp.*KHMM Link 1809 extract

Extract	Diameter of zone inhibition (mm)			
	P. aeruginosa	*St. aureus*	*B. subtilis*	*C. albicans*
KH	31	26	27	25

Table 2: ^{1}H (CD_3OD, 500 MHz) and ^{13}C (CD_3OD, 125 MHz) NMR data of Viridicatol (**1**)

Nr.	δ_C	δ_H
2	159.1	
3	141.1	
4	125.8	
4a	121.6	8.15 (dd, 8.0, 1.5)
5	124.9	7.26 (d, 8.1)
6	122.4	7.14 (m)
7	126.6	7.36 (m)
8	115.0	7.36 (d, 8.1)
8a	132.9	
1’	134.7	
2’	116.5	6.82 (m)
3’	157.2	
4’	120.7	6.83 (dd, 8.1, 1.6)
5’	129.1	7.34 (td, 7.8, 1.4
6’	114.5	6.88 (m)

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