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ORIGINAL ARTICLE

Bioactive Secondary Metabolites from the Locally Isolated Terrestrial Fungus, *Penicillium* sp. SAM16-EGY

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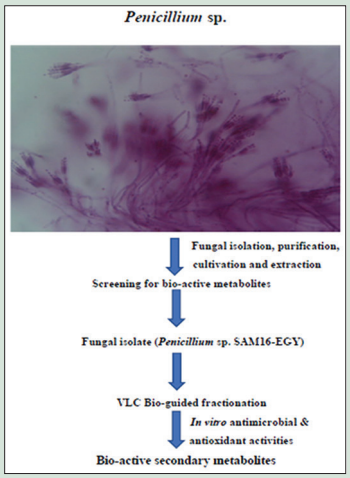
ABSTRACT

Background: *Penicillium* is a diverse genus occurring worldwide; its species are of major importance in the natural environment as decomposer of organic materials as well as food and drug production. **Objective:** Chromatographic isolation and identification of its bioactive secondary metabolites and their evaluation as antimicrobial agents. **Materials and Methods:** Disc agar plate method has been recognized to assess the antimicrobial activities. The antioxidant activity was determined using phosphomolybdenum method. The fungus strain SAM16-EGY was isolated from soil and was molecularly identified as *Penicillium* sp. SAM16-EGY using 18S ribosomal ribonucleic acid technique (acc. no., KP125952). **Results:** Seven compounds namely 3-*O*-docosyl-4-benzoyloxy methyl-3-oxobicyclo (4.1.0) heptane-1,5,6,7 tetrol (3-*O*-docosyl-3-debenzol rotepoxide) (1), (4bE, 6Z, 8E, 9aS, 10S)-1,4-dihydroxy-9a, 10-dihydro-10,12-epoxy-5-methylbenzo[a] azulen-12-one (2), 7α,9β,15β-triacetoxy-3-β-hydroxy jatropha-5E, 11E-diene (3), sesquiterpene I diol dihexoside malonate ester (4), piperogalone (5), (5R, 8Z, 11Z)-5 β-(6′-*O*-malonyl-β-glucopyranozyloxy-6-hyd roxy tetradeca-8, 11-dienoic acid (6), and n-trcosanyl-n-octaced-9-enoate (7) were isolated and identified from this fungus. Their structures were determined on the basis of proton nuclear magnetic resonance and carbon-13 nuclear magnetic resonance spectroscopy. Compounds 1, 2, 4, and 5 exhibited antimicrobial activities against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* only, whereas compound 3 exerted higher antimicrobial activity against *S. aureus* (9 mm), *P. aeruginosa* (9 mm), *C. albicans* (11 mm), and *Aspergillus niger* (13 mm) as compared to the other compounds. In the phosphomolybdenum assay, compound 5 showed high total antioxidant capacity value of 608.59 mg ascorbic acid equivalent/g compound, followed by compound 2 (443.66 mg) and compound 1 (332.16 mg). **Conclusion:** The isolated compounds showed promising antimicrobial and antioxidant activities.

Key words: 18S Ribosomal ribonucleic acid, antimicrobial, antioxidant, *Penicillium* sp. SAM16-EGY, secondary metabolites, vacuum liquid chromatography

SUMMARY

- The current research work concerned with isolation of fungi from the soil which were identified by the molecular techniques (18S ribosomal ribonucleic acid)
- The promising fungal extract underwent fractionation via vacuum liquid chromatography, and then, all resulting fractions were evaluated for their antimicrobial and antioxidant activities
- Chromatographic isolation and purification of the most active extract led to characterization of seven pure compounds which also were evaluated for their antimicrobial and antioxidant activities.



Abbreviations Used: 18SrRNA: 18S Ribosomal ribonucleic acid; TAC: Total antioxidant capacity; AAE: Ascorbic acid equivalent; ^{13}C-NMR: Carbon-13 nuclear magnetic resonance; ^{1}H-NMR: Proton nuclear magnetic resonance; VLC: Vacuum liquid chromatography; DMSO-*d*6: Deuterated dimethyl sulfoxide; MHz: Megahertz; CC: Column chromatography; PC: Paper chromatography; CD: Czapek–Dox; PCR: Polymerase chain reaction; DNA: Deoxyribonucleic acid; CFU: Colony forming units; Mo: Molybdenum; S.D.: Standard deviation; SPSS: Statistical Package for the Social Sciences; BLAST: Basic local alignment tool.

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INTRODUCTION

Fungi, along with bacteria, protozoa, small invertebrates, and plants, play an essential and significant role in the soil ecosystem. Soil fungi were also considered as very important producers for secondary metabolites. Fungi produced several skeletally unique compounds that were used as pharmaceuticals.[1] *Penicillium* genus in addition to *Aspergillus* comprises a large group of anamorphic as comycetes fungal genus. This genus *Penicillium* is widespread in occurrence in terrestrial environments. *Penicillium* genus constitutes more than 200 known species and most of them are soil inhabitant as well as in food, cheese, and sausages.[2-3] A wide range of bioactive secondary metabolites, including antibacterial, antifungal, immune suppressants, cholesterol-lowering agents, and mycotoxins were produced by *Penicillium* spp.[4] Secondary metabolites such as ergot alkaloids, diketopiperazines, quinolines, quinazolines,

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polyketides,[5] camazulene and azetidine,[6] viridicatol and kojic acid,[7] mycophenolic acid,[8] and compactins[9]are also known to be produced by *Penicillium*. *Penicillium* is also known to produce essential fatty acids and hydrocarbons and their therapeutically applications[10] by combating a number of human diseases.[11] Therefore, this research is undertaken with the aim of identifying locally isolated fungus and evaluates the *in vitro* antimicrobial activity of different vacuum liquid chromatography (VLC) fractions from extract of the fungus, *Penicillium* sp. SAM16-EGY, grown on rice medium. The chromatographic isolation and identification of its bioactive secondary metabolites were also studied.

MATERIALS AND METHODS

General experimental procedures

Proton nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were recorded using Varian Mcauley (^{1}H, 400 and ^{13}C, 100 MHz), in deuterated dimethyl sulfoxide (DMSO-*d*6). Melting point (uncorrected) was determined on an electrothermal apparatus. Sephadex LH-20 (25–100 μm, Pharmacia Fine Chemicals Inc., Uppsala, Sweden) was used for extra purification. Silica gel (70–230 mesh, Merck) was used for column chromatography (CC). Paper chromatography (PC) was carried out using Whatman No. 1 paper sheets (57 cm × 46 cm; Maidstone, England) and eluted via solvent systems S_1 (*n*-BuOH: AcOH: H_2O; 4:1:5 v/v/v; upper phase) and S_2 (H_2O: AcOH; 85:15 v/v).

Chemicals, media, and reagents

Nutrient agar medium (DSMZ1) composed of (g/l) beef extract (3), peptone (10), agar (18–20), and distilled water (1000 mL); Czapek–Dox (CD) agar medium (DSMZ 130) composed of the following composition (g/l): sucrose (30), $NaNO_3$ (3), $MgSO_4.7H_2O$ (0.5), $FeSO_4.7H_2O$ (0.01), K_2HPO_4 (1), KCl (0.5), distilled water (1000 mL), and agar (18–20) that were used for isolation (DSMZ30) and antimicrobial activity studies (DSMZ1 and DSMZ30).

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°C ± 2°C for 6–8 days and growth was observed after 2 days. The fungi isolated on culture medium from soil were purified by spore suspension and streak method. The cultures were routinely (every 6–8 days) transferred onto fresh CD agar plates by streaking. Before fungal cultures were used for inoculation of rice medium, the fungus was subjected to three transfers on CD agar plates by the direct agar transfer method.[12]

Screening, scale-up fermentation, and extraction

Erlenmeyer flasks (1000 mL volume), each containing 50 g rice medium in 50 mL distilled water, were inoculated by the fungal spores. Each two conical flasks were inoculated with one fungal slant (10 days old) and incubated at 30°C under static condition for 15 days. Scale-up fermentation has to be 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 (18 g).

Fungal identification

Fungal isolate (SAM16) 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 at PCR while ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used at sequencing. The purification of the PCR products was carried to remove unincorporated PCR primers and dNTPs from PCR products using Montage PCR Clean-up kit (Millipore). Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). *Candida* sp. was used as control. The fungal strain (SAM16) culture was reserved in the Microbial Chemistry Department Culture Collection of Microorganisms.

Antimicrobial activity evaluation

The antimicrobial activities of different fractions as well as pure compounds isolated from *Penicillium* sp. SAM16-EGY that grown on rice medium have been evaluated by disc agar diffusion method.[13] *Staphylococcus aureus* ATCC 6538 (Gram-positive bacterium), *Pseudomonas aeruginosa* ATCC 25416 (Gram-negative bacterium), *Candida albicans* ATCC 10231 (yeast), and *Aspergillus niger* NRRL A-326 (fungus) were selected to estimate the antimicrobial activities. Bacteria and yeast test microbes were cultivated on a DSMZ1, whereas the fungal test microbe was cultivated on CD medium (DSMZ130). 1 mL of spore suspension (10^6–10^8 CFU/ml) each test microbe was used to inoculate 1 L-Erlenmeyer flask containing 250 mL of solidified agar media. These media were poured in 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/or 100 μg of pure sample were dried at room temperature under sterilized conditions and placed on the agar plates seeded with test microbes and incubated for 24 and 48 h for bacteria and fungi, respectively, at 37°C and 30°C. Antimicrobial activities were measured as the diameter of the clear zones that appeared around the discs.[14]

Determination of total antioxidant capacity using ascorbic acid as standard

The antioxidant activity was determined via phosphomolybdenum assay. Basically, this assay depends on the reduction of molybdenum (Mo [VI] to Mo (V) via the interaction with the tested sample and consequent creation of a green-colored (phosphate = Mo [V]) complex at acidic medium with a maximal absorption at 695 nm. Briefly, 0.5 ml from tested sample (100 μg/ml) in methanol was pooled in dry bottles with 5 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The bottles were covered and incubated in water bath at 95°C for 90 min. After cooling, the absorbance was recorded at 695 nm against a blank (reagents and solvents without sample) under the same conditions. The antioxidant activity was expressed as the number of ascorbic acid equivalent (AAE), and all experiments were carried out in triplicate.[15]

Statistical analysis

All data were presented as mean ± standard deviation using SPSS 13.0 program (SPSS Inc. , Chicago, IL, USA).

Isolation and purification of secondary metabolites

The ethyl acetate (EtOAc) extract was evaporated to dryness to give a brownish mass (15 g) and then undergone 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 13 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; 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; finally, fraction (13) was eluted by 100% acetone. Among them, fractions 4, 5, 6, and 9 were subjected to further purification using Sephadex LH-20 column (30 cm × 2 cm) eluted with 100% MeOH to afford five pure isolates. Briefly, fraction 4 (1.0 g) was subjected to Sephadex LH-20, eluted with gradient mix elution system; CH_2Cl_2-MeOH till 100% MeOH to afford two compounds 1 and 2. However, fraction 5 (2.5 g) was subjected to silica gel CC., eluted with CH_2Cl_2:MeOH via gradient mix elution system to afford two compounds 3 (CH_2Cl_2:MeOH; 60:40, v/v) and 4 (CH_2Cl_2:MeOH; 20:80, v/v). Fraction 6 (1.25 g) was subjected to Sephadex LH-20 eluted with 100% MeOH to afford compound 5. Finally, fraction 9 (2.0 g) was subjected to silica gel CC., eluted with CH_2Cl_2:MeOH via gradient mix elution system to afford two compounds 6 and 7.

RESULTS AND DISCUSSION

Identification of the fungal isolate SAM16-EGY

The basic local alignment tool (BLAST) search for the DNA sequence (590 bp) of fungal isolate SAM16 revealed 99% similarity to *Penicillium* sp. strain 19 (acc. no.: KY401064.1). Figure 1 shows the aligned sequence data of 18S ribosomal ribonucleic acid (18SrRNA) amplified from strain SAM16 while Figure 2 shows the AB1 chromatogram of DNA sequencing of the isolate SAM16. The phylogenic tree of this fungal isolate was also constructed [Figure 3]. Based on the above identification techniques, our local soil fungal isolate was identified as *Penicillium* sp. SAM16-EGY with the GeneBank accession number KP125952 (http://www.ncbi.nlm.nih.gov/nuccore/KP125952). Traditional methods of fungal identification including the study of their morphology, growth on diverse media, kind of spores as well as biochemical performance such as production of pigments have been generally used, and several new species are identified according to these methods until now.[16] These old methods are considered as time-consuming, low sensitive, not easy to manage, and nonspecific.[16,17] Targeting specific regions within the ribosomal RNA gene clusters using universal primers through PCR amplification is another selective method for the identification of fungi to the species level and also used for analyzing fungal variety.[18] In this work, ITS regions (ITS1–ITS5) of (rRNA gene clusters are used. Primers routinely used for the amplification of ITS regions of ribosomal DNA are ITS1 and ITS4.[19]

1 accccaaaggcccctccatcgggccctttccccgggggggggtatgcggaaggatcatta
61 ccgagtgagggccctctgggtccaacctcccacccgtgtttattttaccttgttgcttcg
121 gcgggcccgccttaactggccgccgggggggcttacgccccgggcccgcgcccgccgaag
181 acaccctcgaactctgtctgaagattgtagtctgagtgaaaatataaattatttaaaact
241 ttcaacaacggatctcttggttccggcatcgatgaagaacgcagcgaaatgcgatacgta
301 atgtgaattgcaaattcagtgaatcatcgagtctttgaacgcacattgcgccccctggta
361 ttccgggggggcatgcctgtccgagcgtcatttctgccctcaagcacggcttgtgtgttgg
421 gccccgtcctccgatcccgggggacgggcccgaaaggcagcggcggcaccgcgtccggtc
481 ctcgagcgtatggggctttgtcacccgctctgtaggcccggccgcgcgcttgccgatcaac
541 ccaaatttttatccaggtgacctcgatcagggtagggatccccagttcc

Figure 1: Aligned sequence data (590 bp) of 18S ribosomal ribonucleic acid amplified from strain SAM16

The antimicrobial activity of different vacuum liquid chromatography fractions from the ethyl acetate extract of *Penicillium* sp. SAM16-EGY against different groups of test microbes

The ethyl acetate extract from *Penicillium* sp. SAM16-EGY, grown on rice medium, was fractionated via VLC into 13 fractions and these fractions were subjected to *in vitro* antimicrobial activity test against four test micro-organisms, i.e., *S. aureus* (Gram-positive bacterium), *Pseudomonas aeruginosa* (Gram-negative bacterium), *C. albicans* (yeast), and *Aspergillus niger* (fungus). Results postulated in Table 1 revealed the antimicrobial activity of the VLC fractions. It has been found that fractions 4, 5, and 6 showed considerable activity against all test microbes except the fungus, *A. niger*. Fraction 5 showed almost the highest antimicrobial activity against *C. albicans* (14.5 mm), *S. aureus* (10 mm) and *P. aeruginosa* (10 mm). On the other hand, fractions 9 and 10 exhibited weak activity against *P. aeruginosa* (6/6 mm), *C. albicans* (6/6 mm), and *A. niger* (6/6 mm), and no antimicrobial activity was noticed with *S. aureus*. *Penicillium* species are known with their antimicrobial potentials.[20-22] The antimicrobial activity of the methanolic extract of *Penicillium* species isolated from Iranian agricultural soil was evaluated against five microbial strains including; *C. albicans*, *Bacillus subtilis*, *S. aureus*, *Salmonella typhi*, and *Escherichia coli* with inhibition zones were ranged from 10 to 30 mm.[21] Moreover, Shaaban *et al.* have been reported on the antimicrobial activity of the crud extract of the terrestrial fungus *Penicillium* sp. KH Link 1809 against five microbial strains, i.e., *B. subtilis* (27 mm), *S. aureus* (26 mm), *P. aeruginosa* (31 mm), and *C. albicans* (25 mm), and there is no activity was recorded against *A. niger*.[7]

Structural elucidation of the isolated compounds

The promising antimicrobial fractions resulted from VLC were undergo further purification to afford seven compounds were identified on the basis of their one-dimensional (^{1}H and ^{13}C-NMR analyses). The chemical structures of the isolated compounds were illustrated in Figure 4.

Compound 1 was isolated as a yellow powder, R_f: 0.88 (S_1, PC). The ^{1}H and ^{13}C-NMR spectral data indicated that compound 1 belong to polyoxygenated cyclohexane, it was similar to those known compounds rotepoxide A,[23] and 3-debenzoylrotepoxide A, which previously isolated from *Kaemphera rotunda* L.,[24] except the presence of long alcoholic chain at position 3. Complete assignments of all protons and carbons are summarized in Table 2. ^{1}H-NMR showed a characteristic signal for benzoate moiety at δ_H 7.45–7.48 ppm (5H, m, H-2'-6') which confirmed by aromatic carbons signals in ^{13}C spectra at δ_C 130.17 (C-1'), 129.09 (C-2', 6'), 128.19 (C-3', 5'), 132.18 (C-4').[25] ^{1}H-NMR spectra showed an AB quartet at δ_H 3.91 and 4.38 ppm was attributed to oxy-methylene protons (2H, q, H2-7) and at δc 65.93 (C-7). Moreover, it showed two doublets resonated at δ_H 3.91 and 5.08 ppm (*d*, *J* = 3.0 Hz) were assigned to H-2 and H-6, respectively, and three doublet of doublet signals at δ_H 3.89 (*dd*, H-3), 3.91 (*dd*, H-4) and 4.61 (dd, H-5). ^{1}H-NMR spectra showed presence of long chain alcohol residue which observed at δ_H 4.38 (2H, t, H2-1'), 1.7 (4H, m, H_2-2' and 3'), 1.0 (32 H, brs, H-4.-21'), and terminal methyl at δ_H 0.62 (3H, t, *J* = 6 Hz, Me-22').[26] Thirty-six carbon atoms were observed in the ^{13}C-NMR spectra which were classified as; two quaternaries at δ_C 63.53 (C-1) and 130.17 (C-1'), five oxygenated methines at δ_C 67.84 (C-2), 74.0 (C-3), 63.08 (C-4), 69.75 (C-5), and 61.0 (C-6), carbonyl carbon at δ_C 167.43 ppm in addition to six aromatic carbons. 21 methylene carbons (C-1" to C-21") belong to long alcoholic chain showed signals at δ_C 31.26–22.85 ppm and one methyl carbon at δ_C 14.31 ppm.[27] According to the above-mentioned data, compound 1 was identified as 3-*O*-docosyl-1-benzoyloxy methyl-6-epoxy-cyclohexane-2, 3, 4, 5 tetrol.

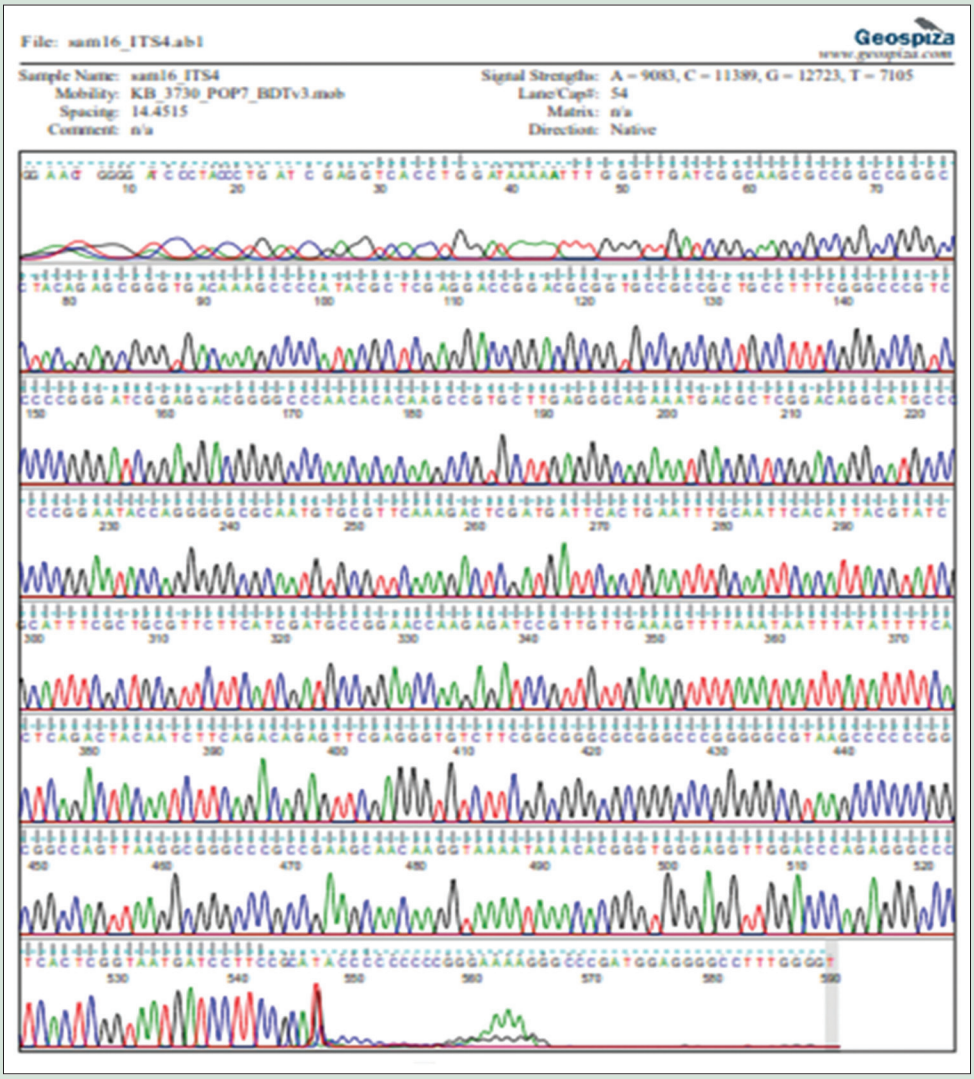

Figure 2: AB1 chromatogram of DNA sequencing of the isolate SAM16

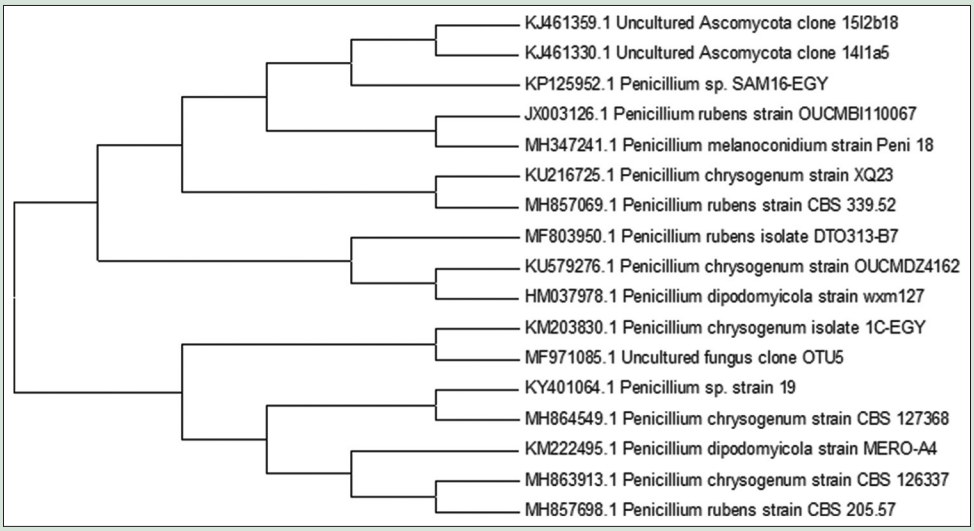

Figure 3: Phylogenetic tree showing relationship of strain SAM16 with other related fungal species retrieved from GenBank based on their sequence homologies of 18S ribosomal ribonucleic acid

Table 1: The antimicrobial activity of different vacuum liquid chromatography fractions from the ethyl acetate extract of *Penicillium* spp. SAM16-EGY against different groups of test microbes

	Clear zone (Θ mm)[a]			
	Candida albicans	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Aspergillus niger***
Fraction				
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	9.50±0.70[b]	8.0±0.0	9.0±1.41	6.0±0.0
5	14.50±0.70	10.0±0.0	10.0±0.0	0
6	11.0±0.0	8.5±0.70	11.0±0.0	0
7	0	0	0	0
8	0	0	0	0
9	6.0±0.0	0	6.0±0.0	6.0±0.0
10	6.0±0.0	0	6.0±0.0	6.0±0.0
11	0	0	0	0
12	0	0	0	0
13	0	0	0	0
Antibiotic				
Streptomycin[c]	0	15	22	0
Penicillin G[d]	25	26	21	0

[a]Inhibition zones diameter (mm); [b]Mean±SD, (*n*=2); [c]Streptomycin was used as a positive control (100 µg/disc); [d]Penicillin G was used as a positive control (100 µg/disc). SD: Standard deviation

Table 2: Proton and carbon-13 nuclear magnetic resonance spectral data (400/100 MHz-DMSO-d_6) of compounds 1 and 2

Compound 1			Compound 2		
Position	**δ_H ppm[a]**	**δ_C ppm**	**Position**	**δ_H ppm**	**δ_C ppm**
1	-	63.53	1	-	149.88
2	4.38 (d, J=3 Hz)[b]	67.84	2	6.87 (d, J=9.0 Hz)	120.64
3	3.89 (dd)	74.0	3	6.66 (d, J=9.0 Hz)	117.0
4	3.91 (dd)	62.08	4	-	147.0
5	4.61 (dd)	69.75	4a	-	135.56
6	3.90 (d, J=3 Hz)	61.0	4b	-	129.0
7	5.09	65.93	5	-	128.94
1’		130.17	6	7.0 (d, J=12.0 Hz)	144.88
2’,6’	7.48–7.45 (5 H, m)	129.09	7	6.47 (dd, J=12.0,6.0 Hz)	128.0
3’,5’		128.19	8	7.34 (dd, J=6.0, 3.0 Hz)	127.0
4’		132.18	9	-	126.5
C=O-	-	167.43	9a	1.71 (d, J=8.0 Hz)	48.33
1”	4.2	49.05	10	6.17 (d, J=8.0 Hz)	61.24
2”	1.79 m	30.26	10a	-	127.0
3”	1.77 m	28.83	11’	1.24, s	16.78
4” to 3”	1.0–22.7	20.85-31.91	12	-	169.44
-CH3	0.62, t	14.31			

[a]H: Chemical shifts δ in (ppm); [b]Coupling constants J in (Hz)

Compound 2 was isolated as a yellow powder, R_f: 0.86 (S_1, PC), ^{1}H-NMR spectra [Table 2] exhibited a characteristic signal for aromatic ring, it showed signals at δ_H 6.87 (*d*, *J* = 9.0 Hz, H-2), 6.66 (d, *J* = 9.0 Hz, H-3), indicating AB system similar to those of a 1,4 hydroquinone moieties.[28] It also showed signals at δ_H7.34 (*dd*, *J* = 6.0, 3.0 Hz, H-8), 7.0 (*J* = 12.0 Hz, H-6), and 6.47 (*dd*, *J* = 12.0, 6.0 Hz, H-7), which related to coupling system of olefinic protons, there are signals at δ_H 6.17 (*d*, *J* = 8.0 Hz, H-10) and at δ_H 1.71 (*d*, *J* = 8.0 Hz, H-9a) which associated with methines, one of which corresponding to oxymethine proton. Also, a characteristic signal for methyl group was recorded at δ_H 1.24 ppm. ^{13}C-NMR spectrum [Table 2] displayed signals for 16 carbon atoms, 13 of which corresponding to sp2 hybridized carbons; it showed aromatic carbon signals at δ_C 149.88 (C-1), 120.64 (C-2), 117.0 (C-3), 147.6 (C-4), 135.56 (C-4a), and 127.0 (C-10a), one methine carbon at δ_C 48.33 (C-9a), oxymethine at δ_C 61.24 (C-10), and methyl group at δ_C 16.78 ppm. Moreover, ^{13}C-NMR revealed five monohydrogenated and eight nonhydrogenated carbon atoms; one characteristic carbon of γ-lactone carboxyl at δ_C 169.49 (C-12), oxygenated carbon at δ_C 149.88 (C-1), and oxygenated carbons of 1,4-hydroquinone at δ_C 147.0 (C-4); these spectral data confirmed its type-like structure as monoterpenoid hydroquinone which by comparison with literature data; compound 2 was previously isolated from *Cordia globosa* and identified as (4bE, 6Z, 8E, 9aS, 10S)-1,4-dihydroxy-9a, 10-dihydro-10,12-epoxy-5-methylbenzo[a] azulen-12-one.[28]

Compound 3 was isolated as white fine crystal, R_f: 0.76 ($CHCl_3$: MeOH: H_2O; 7: 3: 0.5, v/v/v, TLC). ^{1}H-NMR showed [Table 3] the presence of eight methyl signals at δ_H 0.87 (3H, s, H-18), 0.88 (3H, s, H-19), 0.90 (3H, d, *J* = 5 Hz, H-16), 0.91 (3H, s 7-OAc), 1.24 (3H, *d*, *J* = 8 Hz, H-20), 1.71 (3H, s, H-17), 1.80 (3H, s, 9-OAc), and 2.09 (3H, s, 15-OAc) ppm. Five aromatic protons were resonated at δ_H 7.48 (2H, *d*, *J* = 9 Hz, H-2’ and H-6’), 7.69 (2H, m), and 7.13 (H, *d*, *J* = 6 Hz, H-4’). Three olefinic protons showed signals at δ_H 6.15 (H, *d*, *J* = 10 Hz, H-5), 5.95 (H, *d*, *J* = 15 Hz, H-11), and 5.15 (H, *d*, *J* = 15 Hz, H-12). ^{13}C-NMR spectra [Table 3] exhibited 33 carbon resonances including four

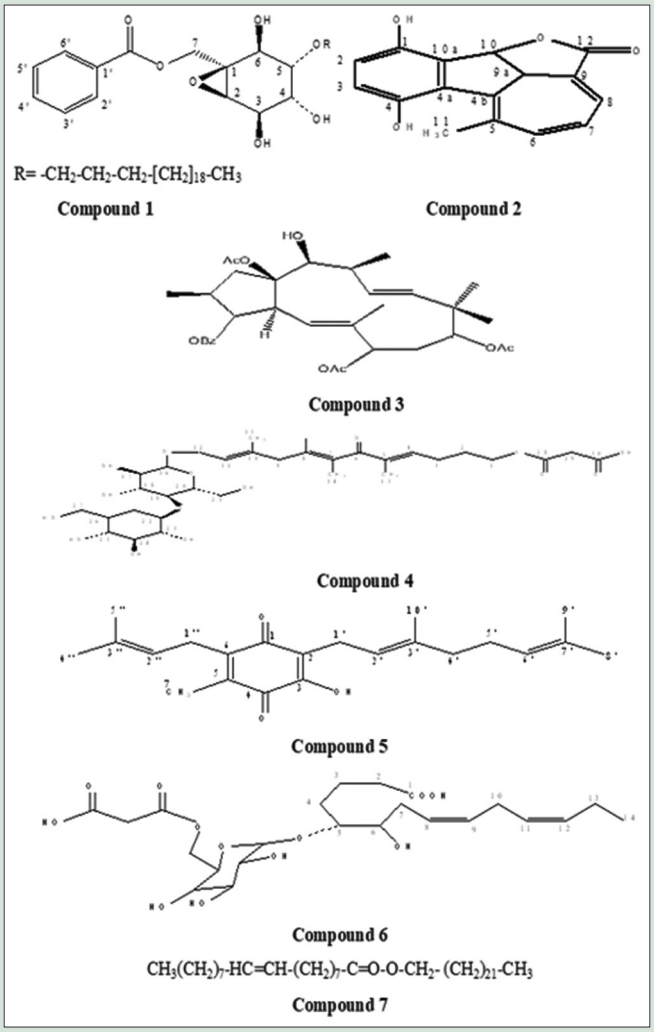

Figure 4: Chemical structures of the isolated compounds

Table 3: Proton and carbon-13 nuclear magnetic resonance spectral data (400/100 MHz-DMSO-d_6) of compounds 3 and 4

Compound 3			Compound 4		
Position	δ_H ppm	δ_C ppm	Position	δ_H ppm	δ_C ppm
1α	2.51 (dd)	40.36	1	3.99	66.0
1β	2.22 (t)		2	2.42	20.74
2	2.30 (m)	39.7	3	2.51	29.02
3	4.80 (t)	83.0	4	-	147.0
4	3.33 (m)	49.06	5	-	128.92
5	6.16 (d)	124.0	6	-	111.43
6	-	132.8	7	-	133.91
7	4.49 (d)	67.88	8	5.20	127.0
8α	2.11 (dd)	30.25	9	2.00	35.0
8β'	1.83 (m)		10	-	149.0
9	4.22 (m)	67.88	11	5.41	120.0
10	-	40.16	12	4.22	70.0
11	5.95 (d)	132.18	13	0.9	12.0
12	5.15 (d)	132.06	14	1.22	24.0
13	2.45 (m)	40.06	15	1.86	23.0
14	3.36 (d)	84.00	16	4.31	105.0
15	-	90.10	17	3.18	75.0
16	0.90 (d)	11.26	18	3.31	78.80
17	1.71 (s)	14.35	19	3.49	81.11
18	0.87 (s)	16.11	20	3.61	78.80
19	0.88 (s)	20.15	21	3.0	57.27
20	1.24 (d)	22.50	22	5.56	93.78
3-OBz	-	-	23	3.41	74.0
C=O	-	174.40	24	3.72	64.0
1'	-	126.99	25	3.25	71.50
2'/6'	7.48 (d)	129.13	26	3.45	77.81
3'/5'	7.69 (m)	125.00	27	3.67	65.0
4'	7.13 (d)	125.55	28	3.65	165.83
7-OAc	0.91 (s)	19.55, 172.74	29	2.17	25.50
9-OAc	1.80 (s)	23.71, 169.82	30	-	182.77
15-OAc	2.09 (s)	22.85, 166.83	-	-	-

carbonyl, one benzoyl carbonyl resonated at δ_C174.40 (Bz C = O), and three acetate carbonyl at δ_C 172.74, 169.82, and 166.83 ppm. In addition, to the presence of different type of resonating carbons including five methyls at δ_C 11.26 (C-16), 14.35 (C-17), 16.11 (C-18), 20.15 (C-19), and at 22.5 (C-20), two methylene resonated at δ_C 40.36 (C-1) and 30.25 (C-8), ten methines at δ_C 39.7 (C-2), 83.0 (C-3), 49.06 (C-4), 67.88 (C-7, C-9), 40.06 (C-13), and 84.0 (C-14) including three olefinic at δ_C 124.0 (C-5), 132.18 (C-11), and 132,06 (C-12) in addition to three quaternary carbons at δ_C132.8 (C-6), 40.16 (C-10), and 90.0 (C-15). According to the above data and comparison to published literature, compound 3 was identified as 7α, 9β, 15β-triacetoxy-3-β-hydroxy jatropha-5E, 11E-diene (Jatrophane diterpenoid), which was previously isolated from *Euphorbia helioscopia*.[29]

Compound 4 was isolated as white amorphous, R_f: 0.58 ($CHCl_3$: MeOH: H_2O; 7: 3: 0.5, v/v/v, TLC).^{1}H-NMR spectra [Table 3] showed two anomeric protons corresponding to two glucose moieties were resonated at δ_H 4.31 (d, J = 8.5 Hz) and 5.50 (*d*, J = 7.8 Hz) as two doublet which supported and anchored two hexose moieties supported by two anomeric carbons at δ_C 105.0 and 96.7 ppm. Three methyls singlet signals were resonated at δ_H 0.9 (3H, s, Me-13), 1.22 (3H, s, Me-14), and 1.86 (3H, s, Me-15). ^{1}H-NMR spectra showed resonance of six methylene groups, two oxygenated methylens at δ_H/δ_C4.22 (2H, brs, H_2-12)/70.0 and at δ_H/δ_C 3.99 (2H, *d*, J = 4.0 Hz, H_2-1)/66.01, respectively; two singlet methylens were resonated at δ_H 2.00 (2H, s, H_2-9) and 2.27 (2H, s, H2-29 malonate methylene) and two methylens were resonated at δ_H 2.51 (2H, *d*, J = 0.4 Hz, H_2-3) and 2.42 (2H, m, H_2-2). ^{13}C-NMR showed 30 carbon atoms including three carbonyl groups were resonated at δ_C 171.43, 165.35, and 183.55 ppm of malonate moieties, 12 carbon of two hexose sugars, 15 carbons of terpene core, and three of malonate moieties [Table 3]. Comparison with literature spectral data, compound 4 showed data similar to that previously isolated from *Solanum habrocnaites*[30] and identified as Sesquiterpene I diol dihexoside malonate ester.

Compound 5 was isolated as pale yellow fine crystals, R_f: 0.62 (S_2, PC).^{13}C-NMR spectra suggesting the presence of aromatic moiety showed signals in the range of 153.40–135.0 ppm; ^{1}H-NMR spectra [Table 4] showed singlet of aromatic methyl at δ_H 2.01 (3H, s, Me-7), δ_C 10.43 (Me-7). ^{13}C-NMR also showed that the presence of conjugated carbonyl groups deduced from two signals at δ_C 187.70 and 176.45 ppm indicate the presence of a quinone moiety. ^{1}H and ^{13}CNMR spectra showed characteristic signals of prenyl moieties as methylene group at δ_H 3.17 (2H, *d*, J = 6 Hz, H_2-1") δ_C 27.6, ethylene proton was appeared at δ_H 4.13 (H, m, H-2") and δ_C 122.0, 131.94 (C-2"and 3"), and two methyls were resonated at δ_H 1.24 (3H, brs, H_3-4") and δ_C 23.00 (Me-4") and δ_H 1.29 (3H, brs, H3-5") and δ_C 21.70 (Me-5");[31] they also showed characteristic signals of geranyl moiety, two ethylene protons at δ_H 5.72 (H, t, H-2') and δ_C 114.75, 135.0 (C-2' and C-3') and δ_H 6.2 (H, m, H-6') and δ_C 128.64, 131.94 (C-6'and C-7'), methylene protons were resonated at δ_H/δ_C 3.3 (2H, *d*, J = 7 Hz, H_2-1')/21.74 and 1.29 (2H, t, J = 7 Hz, H_2-4')/40.4 and 2.25 (2H, m, H_2-5')/27.68. Two methyls were resonated at δ_H/δ_C 0.87 (3H, brs, H_3-9')/13.74 (Me-9') and 0.88 (3H, brs, H_3-10')/15.0 (Me-10').[32] The absence of aromatic protons

indicated that quinone nucleus is completely substituted by two alkenyl moieties, geranyl and prenyl chains, and methyl and hydroxyl groups. Comparison with literature, spectral data of compound 5 was identified as Piperogalone, which was previously isolated from *Peperomia galioides*.[33]

Compound 6 isolated as colorless oil. ^{1}H- and ^{13}C-NMR spectrum of compound 6 [Table 4] showed characteristic signals of long chain of an unsaturated aliphatic compound, it showed a triplet signal at δ_H 0.85 (6H, t, J = 4.0 Hz, Me-14) indicative to terminal methyl supported by signal at δ_C 14.34, and it also showed methylene signals at δ_H 1.17-1.24 (brs) and at δ_H 1.4 (brs) indicative of chain of methylene groups confirmed by signals at δ_C 31.1-22.53. Four olefinic protons appeared as four multiples at δ_H 5.7, 6.5, 7.00 and 7.11 (4H, m, H-8,-9,-11 and-12) as well as at δ_C 126.51 (C-8), 129.12 (C-9), 127.14 (C-11), and 132.08 (C-12). In addition to two oxymethine protons, it showed signals at δ_H 72.89 and 72.38 (C-6 and C-5). ^{1}H and ^{13}C-NMR spectrum data indicated presence of sugar moiety at δ_H 4.27 (H, d, J = 8.0 Hz, anomeric H-1') and at δ_C 97.31 (anomeric carbon). Terminal carboxylic carbon gave signal at δ_C 177.64 (C-1), in addition to two carbonyl of malonyl moiety at δ_C 174.89 and 171.49 ppm. The previous data show similarities to C-14 oxylipin glucosides isolated from *Lemna paucicostata*. Therefore, according to these data compound 6 can identified as (5R, 8Z, 11Z)-5 β-(6'-*O*-malonyl-β-glucopyranozyloxy-6-hydroxy tetradeca-8, 11-dienoic acid.[34]

Compound 7 is a colorless crystal, melting point 79°C – 80°C. Both ^{1}H-NMR and ^{13}C-NMR spectral data [Table 4] of this compound characteristic features of unsaturated long chain aliphatic compounds. ^{13}C-NMR displayed signals for ester carbon at δ_C 167.46 (C-1), two vinylic carbon at δ_C 132.16 (C-9) and 129.09 (C-10), oxygenated methylene at δ_C 67.85 (C-1"). The other methylene groups carbon appears in the range of δ_C 31.61–22.48 ppm and two terminal methyl showed signal at δ_C 14.30 (C-18) and 11.21 (C-25'). ^{1}H-NMR spectrum showed multiplet at δ_H 5.0-5.45 assigned to vinyl H-9 and H-10, two triplets at δ_H 3.99 was ascribed to oxygenated methylene (2H, t, J = 8.0 and4.0 Hz, H-1') and at δ_H 2.26 (2H, m, H_2-11). It showed two terminal methyl signals at δ_H 0.89 (3H, t, J = 4.0 Hz, Me-18) and 0.63 (3H, t, J = 4.0 Hz, Me-23'). Compound 7 was previously isolated from *Albizzia lebbeck* and *Cuminum cyminum*. On the basis of the above data, compound 7 was identified as *n*-trcosanyl-*n*-octaced-9-enoate.[35-37]

Table 4: Proton and carbon-13 nuclear magnetic resonance spectral data (400/100 MHz-DMSO-d_6) of compounds 5, 6, and 7

Position	Compound 5		Compound 6		Compound 7	
	δ_H ppm	δ_C ppm	Position	δ_H ppm	Position	δ_C ppm
1	-	187.75	1	177.64	1	167.46
2	-	122.0	2	31.72	1'	67.46
3	-	153.46	3	22.53	9	132.16
4	-	176.45	4	30.24	10	129.09
5	-	153.18	5	75.89	CH2	30.25–22.48
6	-	135.0	6	72.38	Me-18	14.30
7	2.0, s	10.43	7	29.52	Me-23'	11.21
1'	3.3 d	21.74	8	126.51	-	-
2'	5.72, t	114.75	9	129.12	-	-
3'	-	135.0	10	29.43	-	-
4'	1.29, t	40.4	11	127.14	-	-
5'	2.25, m	27.68	12	132.08	-	-
6'	6.2, m	128.64	13	22.84	-	-
7'	-	131.94	14	14.34	-	-
8'	1.24, brs	23.0	1'	97.31	-	-
9'	0.88	13.74	2'	75.27	-	-
10'	0.87	15.0	3'	77.17	-	-
1"	3.17	27.6	4'	71.01	-	-
2"	4.13 m	122.0	5'	77.17	-	-
3"	-	131.94	6'	63.75	-	-
4"	1.24	23.0	1"	174.89	-	-
5"	1.29	21.74	2"	55.24	-	-
			3"	171.79	-	-

In vitro antimicrobial activity of the isolated compounds 1–7

Seven compounds were isolated from ethyl acetate extract of *Penicillium* sp. SAM16-EGY; these compounds were subjected to *in vitro* antimicrobial activity test against four pathogenic microbial strains, i.e., *S. aureus* (G-positive bacterium), *P. aeruginosa* (Gram-negative bacterium), *C. albicans* (yeast), and *A. niger* (fungus). Results postulated in Table 5 and Figure 5 revealed the antimicrobial activity of these compounds. It has been found that compounds 1, 2, 4, and 5 showed a noticeable activity against all test microbes except the fungus, *A. niger*. However, compound 3 showed almost the highest antimicrobial activity against *C. albicans* (11 mm), *S. aureus* (9 mm), *P. aeruginosa* (9 mm), and *A. niger* (13 mm). From the obtained data, it is clear that compound 3 (Jatrophane diterpenoid) possess higher antifungal activity against *A. niger* and no antifungal activities were recorded with the rest of the tested compounds. Our findings are in agreement with the study done by El-Bassuony, who reported on the antibacterial activity of two Jatrophane diterpenoids against Gram-positive bacteria *Bacillus cereus* (11 mm) and *S. aureus* (6 mm).[38]

Table 5: Antimicrobial activity of pure isolated compounds (1–7) from the ethyl acetate extract of *Penicillium* spp. SAM16-EGY against different groups of test microbes

	Clear zone (Θ mm)[a]			
	Candida albicans	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Aspergillus niger*
Compound				
1	7	7	7	0
2	7	8	7	0
3	11	9	9	13
4	7	7	7	0
5	7	7	7	0
6	6	8	9	0
7	ND[b]	ND	ND	ND
Antibiotic				
Streptomycin[c]	0	15	22	0
Penicillin G[d]	25	26	21	0

[a]Inhibition zones diameter (mm); [b]ND: Not detected; [c]Streptomycin was used as a positive control (100 µg/disc); [d]Penicillin G was used as a positive control (100 µg/disc)

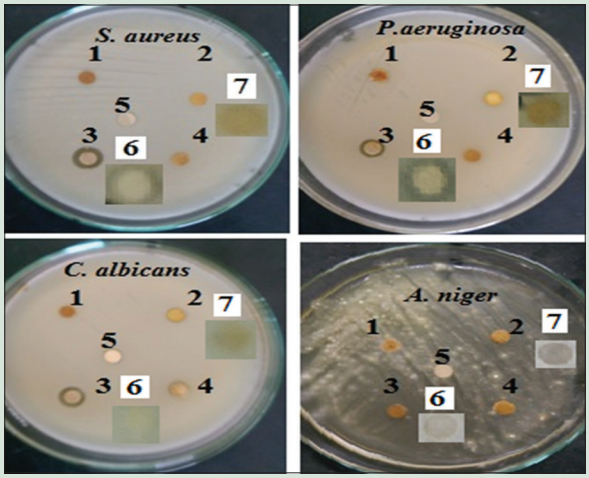

Figure 5: *In vitro* antimicrobial activities of the isolated compounds against four pathogenic microbes

Total antioxidant capacity of different vacuum liquid chromatography fractions and isolated compounds

Oxidative stress is considered a great health issue leading to several health disorders. This phenomenon is due to an overproduction of free radicals and consequent accumulation of reactive species. Several studies have demonstrated that natural compounds derived from both medicinal plants or fungal extracts have a great ability to eliminate hazards of such reactive species and thus are considered promising naturally occurring antioxidant agents.[39-42]

In the present study, 13 fractions resulting from VLC of the ethyl acetate extract of *Penicillium* sp. SAM16-EGY and seven isolated compounds were investigated for their total antioxidant capacities using phosphomolybdenum method. In phosphomolybdenum assay, total antioxidant capacity (TAC) values for the tested fractions were ranged from 212.53 to 687.56 mg AAE/g fraction. Fraction 3 showed the highest TAC of 687.56, while fraction 9 showed the lowest TAC of 212.53 mg AAE/g fraction [Table 6]. On the other hand, the TAC values for the tested compounds were ranged from 332.16 to 608.59 mg AAE/g compound. The results are in the order: Compound 5 > 2 > 1, and no any activities were detected with compounds 3, 4, 6, and 7 [Table 7]. *Penicillium* species are known by their numerous biological activities;[43,44] among them is the antioxidant potential. These species have arisen as the new sources of naturally occurring antioxidant secondary metabolites.[45,46] Hulikere *et al*. reported on the antioxidant activity of the ethyl acetate extract of *Penicillium citrinum*, which may be returned to the presence of certain phenolic compounds in such extract.[47] In this context, the TAC of ethyl acetate extract of *Penicillium* sp. was evaluated via phosphomolybdenum method and the results revealed that it has antioxidant capacity of 325.76 mg equivalent to ascorbic acid. Moreover, Yuan *et al*. reported on the free radical scavenging activity of adenosine isolated from *Penicillium* sp. YY-20.[48] Accordingly, the current study implies that the *Penicillium* sp. could be used as a vital source of natural antioxidant agents.

Table 6: Total antioxidant capacity values of different vacuum liquid chromatography fractions

VLC fraction	Total antioxidant capacity (mg AAE/g fraction)[a,b]
1	429.73±2.0
2	596.97±5.32
3	687.56±4.02
4	542.38±2.01
5	325.19±4.02
6	327.52±6.03
7	416.95±2.0
8	434.37±5.32
9	212.53±3.48
10	394.88±2.01
11	351.91±9.21
12	279.93±5.37
13	282.25±3.53

[a]Results are (means±SD) (n=3); [b]AAE. AAE: Ascorbic acid equivalent; SD: Standard deviation; VLC: Vacuum liquid chromatograph

Table 7: Total antioxidant capacity values of the isolated compounds

Compound	Total antioxidant capacity (mg AAE/g compound)[a]
1	332.16±4.02
2	443.66±5.30
3	ND
4	ND
5	608.59±3.48
6	ND
7	ND

[a]ND=Not detected; AAE: Ascorbic acid equivalent

CONCLUSION

Soil-inhabiting fungi were considered as a prolific source for the isolation of several bioactive secondary metabolites. Fungi isolating from soil were identified by the molecular techniques (18SrRNA) because these techniques surpass the manual one in their accuracy and saving time. VLC was used as a fast system for fractionating the extract. The fractions which exhibit antimicrobial activities were selected for furtherer studies including isolation, purification, and structure elucidation of the pure compounds obtained. The antimicrobial and antioxidant activities of the produced compounds were also studied. The continuous work in that field could result in the discovery of new compounds with unexpected biological activity.

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Conflicts of interest

There are no conflicts of interest.

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