Morphomolecular identification, metabolic profile, anticancer, and antioxidant capacities of *Penicillium* sp. NRC F1 and *Penicillium* sp. NRC F16 isolated from an Egyptian remote cave

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Background
There is a pressing need to screen for new sources of potent bioactive compounds to help in treating current widespread diseases. Fungi represent the perfect candidates that can fulfill this need owing to their ability to produce bioactive compounds.
Objective
To screen for fungi from a novel source, chemically analyze their extracts, and evaluate some of their bioactivities.
Materials and methods
Soil samples from El Shekh Sayed bat cave in Asyut, Egypt, were targeted as a novel source of fungi. Silylated ethyl acetate extracts were prepared from isolates of interest, and gas chromatography-mass spectrometer chemical analyses were performed on these extracts to identify existing metabolites. Moreover, the extracts were evaluated for their *in vitro* antioxidant and anticancer activities against human colon cancer (HCT116) and human breast cancer (MCF7) cell lines.
Results and conclusion
A total of 31 strains were isolated from the bat cave, and two of them were identified as *Penicillium* sp. NRC F1 and *Penicillium* sp. NRC F16. Chemical analyses of their silylated ethyl acetate extracts resulted in the detection of 114 compounds. *Penicillium* sp. NRC F1 and *Penicillium* sp. NRC F16 extracts have recorded antioxidant activities of 74.41±0.59 and 65.58±1.55%, respectively. The *Penicillium* sp. NRC F1 extract has exerted a cytotoxicity of 95.72±1.13 and 97.29±0.61% against HCT116 and MCF7 cell lines, respectively, whereas the *Penicillium* sp. NRC F16 extract has recorded 95.43±1.4 and 97.08±1.07%, respectively, against the same cell lines.
The results propose these strains as bioactive metabolite producers and encourage further *in vivo* investigations to confirm their potency.

Keywords:
anticancer, 2, 2-diphenyl-1-picrylhydrazyl, *Penicillium*, remote bat cave, silylated gas chromatography-mass spectrometer, soil fungi

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Introduction

Spreading of fatal diseases such as cancer, as well as the reported adverse effects, and shrinking repertoire of effective drugs have directed research studies toward screening for new sources for potent compounds having anticancer activities. Cancer diseases are responsible for a considerable number of mortalities worldwide. According to WHO reports, breast cancer is ranked as the second most common cause of death among the most common cancers (accounting for ~2.1 million cases in 2018 only). Colorectal cancer came in next at the third place, causing 1.80 million cases in the same year [1]. On the contrary, the search for natural sources rich in antioxidant compounds is attracting researchers' attention. Generally, free radicals and oxidants are harmful molecules that are induced by different factors such as smoking, pollution, radiation, and some medicines [2]. These molecules accumulate in body as a result of an imbalance between antioxidant defense mechanisms and reactive oxygen species generation, and hence become harmful, causing degradation and destruction of cell components. Moreover, the resulting oxidative stress plays a role in the pathogenesis of different chronic and degenerative diseases such as aging, cancer, inflammation, autoimmune disorders, rheumatoid arthritis, and neurodegenerative and cardiovascular diseases [3,4].

Fungi are eukaryotic microorganisms that are considered as a generous source of biologically active compounds. Many anticancer, antioxidant, anti-inflammatory, and antimicrobial secondary metabolites were previously reported from different fungal genera [5,6]. Among all fungi, Penicilli are the most famous as rich sources of bioactive compounds. Besides their production of hydrocarbons, different industrially important enzymes, and fatty acids, many Penicilli-originated secondary metabolites have been previously described. For example, the penicillin-producers *Penicillium camemberti*, *Penicillium rubens*, and *Penicillium roqueforti* are commonly used as cheese starters [7]. Moreover, *Penicillium nalgiovense* is used in the food industry for sausage fermentation [8]. On the contrary, bioactive compounds produced by different Penicilli such as orcinol, 3-oxoquinuclidine, 1,3,8-*p*-menthatriene, and limonene were also reported [7]. Interestingly, species belonging to the genus *Penicillium* act mysteriously as they can be toxic or beneficial [9]. Hence, isolation of new fungal isolates from novel remote sources is critically important to fortify and refresh arsenal of secondary metabolites in a trial to find promising compounds with potent biological activities.

In this study, a remote cave located in Asyut governorate, Egypt, was used as a source to screen for new fungal isolates. Moreover, two selected isolates were morphomolecularly identified through sequencing of their nuclear ribosomal internal transcribed spacer ITS1-5.8S-ITS2 regions. Furthermore, a gas chromatography-mass spectrometer (GC-MS) analysis was performed on the silylated extracts of these fungi to identify their metabolic profiles. Finally, the *in vitro* antioxidant and anticancer activities of both extracts were investigated against the HCT116 colorectal carcinoma, and MCF7 breast carcinoma human tumor cell lines.

Materials and methods

Sample collection

Cave soil samples were collected from different sites inside El Shekh Sayed bat cave that lies 44 km east to the Nile river in El Bayadya village at latitude 26°57' 34.8'N and longitude 31°27' 41.0'E, about 4 km south of El Badari, which is a famous archeological site in Asyut Governorate, Egypt (Fig. 1). The soil samples were from different sites inside the cave including soil from the entrance of the cave, rhizosphere soil at the entrance of the cave, soil from the middle of the cave (transition zone), soil from the wall of the cave, and soil from deep inside the cave (25 m inside the cave). Samples were kept in sterilized bags and transferred in a cool box (4°C) and processed within 24 h.

Figure 1

Location of El Shekh Sayed bat cave, Asyut governorate, Egypt, as illustrated by Google earth (a), and cave entrance (b).

Isolation and purification of fungi

One gram of soil samples from each site was placed into 9 ml of sterile distilled water. Ten-fold serial dilutions were prepared from the mixed solution. Isolation was conducted from suitable dilution of the soil samples by spreading over the surface of agar plates of potato dextrose medium (PDA; Sigma-Aldrich, Saint Louis, Missouri, USA). After incubation for 7 days at 30±2°C, the plates were checked for the growth of colonies, and single colonies were picked up and streaked onto the surface of agar plate of the same isolation medium and allowed to grow for 7 days. A touch of the terminal colonial growth of a single separate colony was transferred to pure slants of PDA medium to be preserved in a refrigerator by regular subculturing every 2 months.

Morphological and molecular identification of the selected isolates

Isolates were preliminary identified under a microscope following the description of Domsch *et al.* [10] and Moubasher [11].

The total fungal DNA was extracted from fungal hyphae and purified through an E.Z.N.A. Fungal DNA Mini Kit (D3390-01; Omega BIO-TEK, Norcross, Georgia, USA) following manufacturer's instructions. The obtained DNA was stored at −20°C until needed. For PCR amplification, DreamTaq Green PCR Master Mix (2X) (K1081; Thermo Fisher, Waltham, Massachusetts, USA) as well as the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4

(5′-TCCTCCGCTTATTGATATGC-3′) [12] were used for specific gene amplification according to the manufacturer's protocol using Creacon (Holland Inc.) using PCR system cycler (CreaCon, Technologies, The Netherlands). After that, the resulting PCR products were purified using an E.Z.N.A. Gel Extraction Kit (D2500-01; Omega BIO-TEK). The sequence analysis was employed using the ABI PRISM 3100 Genetic Analyzer (Micron-Corp., Seoul, South Korea).

Data analysis

A gel documentation system (Geldoc-it, UVP, England) was applied for data analysis using Totallab analysis software, ww.totallab.com (Ver.1.0.1). Aligned sequences were analyzed on the NCBI website (http://www.ncbi.nlm.nih.gov/webcite) using BLAST to confirm their identity. Genetic distances and MultiAlignments were computed by Pairwise Distance method using ClusteralW software analysis (http://www.ClusteralW.com). The nucleotide sequences were also compared with *Penicillium* isolate sequences available in the GenBank.

Fermentation and extraction of secondary metabolites

Erlenmeyer flasks containing potato dextrose broth medium (1 l each) were inoculated with 10 ml of fungal spore suspension. The flasks were then incubated aerobically at 30±2°C and 150 rpm for 7 days. Extraction of metabolites was conducted as described by Li *et al.* [13], with some modifications. In brief, the entire contents of each flask were transferred to Erlenmeyer flasks (of 2-l capacity) and extracted twice by mixing with ethyl acetate (AnalR, UK) (1 : 1, v/v), sonicated for 10 min with gentle warming, and the mixture was kept overnight at room temperature. Then, the organic layer was separated and the process was repeated till exhaustion. The ethyl acetate layers were collected and evaporated using a rotatory evaporator (Heidolph rotary evaporator; Schwabach, Germany) under reduced pressure at 45°C. The crude extract *Penicillium* sp. NRC F1 (0.089 g) and *Penicillium* sp. NRC F16 (0.136 g) were kept in the fridge for analysis.

Gas chromatography-mass spectrometer analysis and preparation of samples

The derivatization of samples for GC-MS analysis was carried out by silylation by keeping a 2.5-mg sample in a desiccator overnight to ensure complete dryness, and then 20 µl of pyridine supplemented with 30 µl of N,O-Bis-(trimethylsilyl) trifluoroacetamide was added, and the mixture was incubated for 30 min at 85°C for derivatization just before conducting the GC-MS analysis [1,14].

Mass spectrometer

A Finnigan MAT SSQ 7000 MS coupled with a Varian 3400 GC. DB-5 column, 30 m×0.32 mm (internal diameter), was employed with helium as carrier gas (He pressure, 20 Mpa/cm^2) and GC temperature program of 85–310°C at 3°C/min (10 min initial hold). The injector temperature was kept at 310°C. The mass spectra were recorded at 70 eV in the electron ionization mode [12,15]. The scan repetition rate was 0.5 s over a mass range of 39–650 atomic mass units.

To identify compounds in the ethyl acetate extract of the two fungal isolates, GC-MS analyses were conducted, and compounds were identified by comparing their retention times and mass fragmentation patterns with those of the database libraries [Wiley (Wiley Int. USA) and NIST (Nat. Inst. St. Technol., USA)]. Moreover, peaks were examined by single-ion chromatographic reconstruction to confirm their homogeneity.

Effect of extracts on human colon cancer (HCT116) and human breast carcinoma tumor (MCF7) cell lines

Cell cultures

HCT116 colon carcinoma and MCF7 breast carcinoma human tumor cell lines were cultured in 95% humidity, 5% CO_2, and 37°C. HCT116 was maintained in McCoy's 5A, whereas MCF7 in MEM media, supplemented with 10% fetal bovine serum and 1% antibiotic [1].

Cytotoxicity assay

Acid phosphatase assay was performed to evaluate cytotoxicity as described previously [16]. Briefly, human colon cancer cell line (HCT116) and human breast carcinoma cell line (MCF7) were used by seeding 10 000 cells per well in 96-well plates, left overnight till attaching, and then treated with different extracts for 3 days. For one plate, a substrate solution was prepared where a 20-mg tablet of pNPP (Sigma; cat. no. N2765) was dissolved in 10 ml of buffer solution (0.1 M sodium acetate, 0.1% triton X-100, pH 5.0). Cell monolayers were washed with 250 µl PBS. Then, 100 µl of pNPP substrate solution was added per well, and then plates were incubated for 4 h at 37°C. Then, 10 µl of 1 N sodium hydroxide stop solution was added per well. Absorbance was measured directly at a wavelength of 405 nm. All samples were tested in triplicate, and 0.5% DMSO was used as a negative control and 50 µM cisplatin was used as a positive control. Extracts were tested at serial dilutions with final concentrations of 200, 100, 50, and 25 µg/ml. Percent cytotoxicity=[1−(D/S)]×100, where D and

S denote the optical density of drug-treated and solvent-treated wells, respectively.

Antioxidant activity of extracts

The free radical scavenging activity of extracts was evaluated by using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay described previously [17]. Extracts were tested at final concentrations of 200, 100, 50, and 25 μg/ml using 0.1 mM DPPH dissolved in methanol. After incubation for 30 min in dark at room temperature, the absorbance was measured at 517 nm. Ascorbic acid (vitamin C) was used as a positive control at final concentrations of 20 μg/ml. The DPPH solutions treated with 0.5% DMSO were used as a negative control. The DPPH scavenging activity of extracts was calculated according to the following equation:

$$\text{Percentage reduction} = (1 - (X/\text{av}(NC)) \times 100.$$

Where X indicates the absorbance of fraction and av (NC) indicates the average absorbance of the negative control. EC50 values were calculated using probit analysis utilizing the SPSS computer program (SPSS for Windows, statistical analysis software package, version 9, 1989; SPSS Inc., Chicago, Illinois, USA).

Results

Isolation and morphological and molecular identification of fungi

Soil samples were collected from different sites inside El Shekh Sayed bat cave, Asyut governorate, Egypt. Different fungal strains belonging to specific genera were morphologically identified from samples collected from all sites. As shown in Table 1, *Aspergillus niger* and *Aspergillus flavus* were predominantly isolated from all sites. *Rhizopus stolonifer* and *Mucor circinelloides* came in the second place and were isolated four times each. *Alternaria alternata* was isolated three times, whereas *Aspergillus fumigatus* and *A*spergillus *versicolor* appeared twice. The richest site in fungal isolates was the rhizosphere soil at the entrance of the cave, where all isolates were isolated except for *A. fumigatus*. The most interesting isolates were two different *Penicillium* species, which were isolated from the rhizosphere soil at the entrance of the cave and showed characteristic antagonistic growth, which encouraged for studying both isolates. The surface of the colonies of *Penicillium* sp. NRC F1 appeared bluish-green and were velvety sulcate. The reverse side of colonies was brownish orange, and conidiophores appeared two-stage branched under a microscope. Conidia were bluish-green, smooth walled, and appeared globose to subglobose. On the contrary, the morphological and microscopic appearance of the second isolate suggested that it was also a *Penicillium* species. Colonies appeared velvety with whitish margin, green conidial heads, and the reverse side of colonies on PDA was yellowish in color. Conidiophores appeared under microscope smooth walled and asymmetrically terverticillate. Conidia were smooth walled, elliptical, globose to subglobose, and arranged in irregular columns. Molecular identification of both isolates through sequencing of their nuclear ribosomal internal transcribed spacer ITS1-5.8S-ITS2 regions came in accordance with morphological identification. Sequences showed high similarities to those of *Penicillium* sp., and sequences were deposited in the international Gene Bank as *Penicillium* sp. NRC F1, and *Penicillium* sp. NRC F16 under accession numbers MN382318 and MN382317, respectively. Phylogenetic tree was constructed based on the nuclear ribosomal ITS1-5.8S-ITS2 region related to *Penicillium* sp., as shown in Fig. 2.

Metabolic profiles of selected *Penicillium* isolates

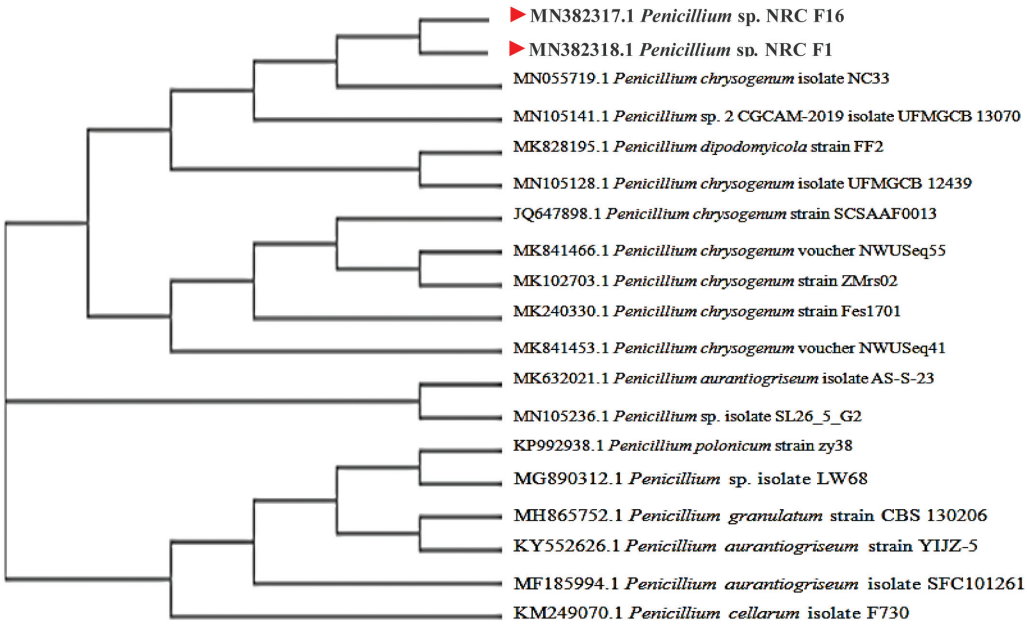
Sialylation of the ethyl acetate extracts was conducted to facilitate detection of as much as possible polar and nonpolar compounds contained in those two fungal extracts. GC-MS analyses were then conducted to identify compounds in these extracts. As shown in

Table 1 Fungal strains isolated from different sites inside El Shekh Sayed bat cave

Isolated species	Site collected from	Number of isolates
Aspergillus niger	From all sites	7
Aspergillus fumigatus	Deep inside the cave, wall of cave	2
Aspergillus *versicolor*	Rhizosphere soil at entrance of the cave	2
Aspergillus flavus	From all sites	7
Rhizopus stolonifer	Rhizosphere soil at entrance of the cave; deep inside the cave; wall of cave	4
Mucor circinelloides	Rhizosphere soil at entrance of the cave; deep inside the cave; wall of cave	4
Penicillium sp. F1	Rhizosphere soil at entrance of the cave	1
Penicillium sp. F16	Rhizosphere soil at entrance of the cave	1
Alternaria alternata	Rhizosphere soil at entrance of the cave	3
Total number of isolates		31

Potato dextrose medium was used for isolation of fungi, and plates were incubated for 7 days at 30±2°C.

Figure 2



Phylogenetic tree for nuclear ribosomal ITS1-5.8S-ITS2 region for *Penicillium* sp. NRC F1 and *Penicillium* sp. NRC F16.

Tables 2 and 3, analyses revealed the identification of 114 compounds from different chemical classes. Most compounds were common in both *Penicillium* sp. NRC F1 and *Penicillium* sp. NRC F16 extracts. Relatively close concentrations (8.23 and 9.56%) of organic acids were detected in *Penicillium* sp. NRC F1 and *Penicillium* sp. NRC F16 extracts, respectively. Sorbic acid represented the highest concentration in *Penicillium* sp. NRC F1 extract (4.23%), whereas in case of *Penicillium* sp. NRC F16 extract, galactonic acid was the highest (3.83%). On the contrary, the concentration of monocarboxylic acids in both extracts was nearly the same (Table 3), whereas di-carboxylic acids were only detected in the *Penicillium* sp. NRC F16 extract (0.75%) and were not detected in the *Penicillium* sp. NRC F1 extract. Lipid compounds were also detected in both extracts, but their existence in the *Penicillium* sp. NRC F16 extract was in general two times higher (7.6%). Palmitic acid was the most abundant saturated fatty acid in both *Penicillium* sp. NRC F1 (1.97%) and *Penicillium* sp. NRC F16 (5.11%). Linoleic acid (omega-6) was the most abundant unsaturated fatty acid in *Penicillium* sp. NRC F16 (3.79%) followed by oleic acid (2.8%). On the contrary, phenolic compounds detected in the *Penicillium* sp. NRC F1 extract (5.24%) was higher than that detected in the *Penicillium* sp. NRC F16 extract (2.35%). The concentration of carbohydrates in both samples was the same 9.05%, and D-(−)-fructofuranose was detected in the *Penicillium* sp. NRC F1 extract (2.95%), whereas it was absent in the *Penicillium* sp. NRC F16 extract. Ribose was the main sugar in the *Penicillium* sp. NRC F16 extract (2.76%) and appeared as traces in the *Penicillium* sp. NRC F1 extract. The GC-MS analyses revealed also the abundance of sugar alcohol in the *Penicillium* sp. NRC F16 extract at concentrations as twice as that detected in the *Penicillium* sp. NRC F1 extract (Table 3). Mannitol was the main alcohol detected in the *Penicillium* sp. NRC F1 extract (3.18%), whereas sorbitol was the main alcohol detected in the *Penicillium* sp. NRC F16 extract (7.7%). On the contrary, the nitrogenous and sulfur compounds detected in the *Penicillium* sp. NRC F1 extract (6.66 and 1.7%, respectively) was as twice as that detected in the *Penicillium* sp. NRC F16 extract (3.75 and 0.26%, respectively).

In vitro anticancer activities of the extracts

The *in vitro* anticancer activity of the two fungal ethyl acetate extracts were investigated against human colon cancer HCT116 and human breast cancer MCF7 cell lines at different concentrations (25, 50, 100, and 200 µg/ml). As shown in Fig. 3a, and b, the highest cytotoxic effect against HCT116 cell line was obtained after treatment with 200 µg/ml of *Penicillium* sp. NRC F1 extract, exhibiting 95.72 ±1.13% cytotoxicity, whereas a cytotoxicity of 95.43 ±1.4% was achieved using the same concentration of the *Penicillium* sp. NRC F16 ethyl acetate extract (Fig. 3a). On the contrary, the *in vitro* cytotoxicity of the extracts against MCF7 cell line (Fig. 3b) resulted in observing promising activities of *Penicillium* sp. NRC F1 and *Penicillium* sp. NRC

Table 2 Gas chromatography-mass spectrometer analysis of *Penicillium* sp. NRC F1 and *Penicillium* sp. NRC F16 ethyl acetate crude extracts

Peak no.	RT	Compounds	*Penicillium* sp. NRC F1 area %	*Penicillium* sp. NRC F16 area %
Organic acids				
Monocarboxylic acids				
1	5.41	D-lactic acid, bis-TMS	0.71	0.68
2	6.01	Hexanoic acid (caproic acid), TMS	–	0.25
3	6.22	Acetic acid, bis-TMS	–	0.12
4	7.2	Sorbic acid, TMS	4.23	0.45
5	10.05	Heptanoic acid (Enanthic acid), TMS	–	0.55
6	15.65, 18.14	4-Hydroxybutyric acid, bis-TMS	–	0.24
7	17.1	3-Hydroxy-3-butenoic acid, bis-TMS	–	0.31
8	18.9	2,3-Dihydroxyisobutanoic acid, tris-TMS	–	0.13
9	24.71	Decanoic acid (capric acid), tris-TMS	–	0.22
10	28.79	Undecanoic acid, TMS	–	0.16
11	42.8, 43, 36.03, 36.54, 40.32, 41.61, 46.57	2-Deoxy-erythro-pentonic acid, tetrakis-TMS organic acid	0.71	1.68
12	45.7	Galactonic acid, hexakis-TMS	1.61	3.83
13	55.8	2-Deoxyribonic acid, tetrakis-TMS	0.61	0.19
14	57.3	2-Keto-d-gluconic acid, pentakis-TMS	0.36	–
Total	8.23	8.81		
Di-carboxylic acids				
15	4.03	Malonic acid, bis- TMS	–	0.34
16	19.96	Maleic acid, bis-TMS	–	0.09
17	21.34	Methylmaleic acid (Citraconic acid), bis-TMS	–	0.07
18	24.80	3-Methylglutaconic acid, bis-TMS	–	0.07
19	26.88	Malic acid, tri-TMS	–	0.18
Total	–	0.75		
Alcohols, esters, and ketones				
20	3.16	Prenol, TMS	0.2	–
21	5.49	10-Undecyn-1-ol, TBDMS	–	0.11
22	8.19	Exo-Norborneol, TMS derivative	0.33	–
23	13.46	Dihydroxyacetone, bis-TMS	–	0.14
24	20.83	2-hydroxypropane, bis-TMS isopropyl alcohol	–	0.1
25	22.14	1,4-Butanediol, bis-TMS	–	0.12
26	41.8	Gluconolactone (3R,4S,5R,6R)-, tetrakis-TMS	0.61	2.21
27	42.19	Gulonic acid γ-lactone, tetrakis-TMS	–	2.49
Total			1.14	5.17
Lipid compounds				
Fatty acids: saturated fatty acids				
28	15.40	Octanoic acid (Caprylic acid),TMS	–	0.59
29	20.28	Nonanoic acid (Pelargonic acid), tris-TMS	–	0.31
30	45.946.11	Hexadecanoic acid (Palmitic Acid), tri-TMS	1.97	5.11
31	49.6	Methyl tetracosanoate (Methyl lignocerate), bis-TMS	0.28	–
32	50.9, 51.2	9-Octadecanoic acid (Stearic acid)	1.62	1.05
33	51.91	Hexacosanoic acid (Cerotic acid), TMS	–	2.01
Total	3.87	9.07		
Unsaturated fatty acid				
34	50.7	9,12-Octadecadienoic acid, TMS (Linoleic acid) omega-6	2.64	3.79
35	51.15	Oleic acid, TMS, omega-9	–	2.8
36	56.07	α-Linolenic acid, TBDMS omega-3	–	0.36
37	56.27,57.00	13-Eicosenoic acid, TMS, omega-7	–	0.38
Total	2.64	7.33		
Triglyceride				
38	47.17	Monoolein, bis-TMS	–	0.72
39	61.1	1-Monopalmitin, bis-TMS	0.24	–
40	83.04	1-Monolinolein, bis-TMS	–	0.07

(*Continued*)

Table2 (*Continued*)

Peak no.	RT	Compounds	*Penicillium* sp. NRC F1 area %	*Penicillium* sp. NRC F16 area %
Total	0.24	0.79		
Other lipid compound				
41	56.5	Prostaglandin D(2), tetrakis-TMS	0.31	–
42	71.5	O-methylox,19-Norpregna-1,3,5,7,9-pentaen-21-al, bis-TMS	0.35	–
43	76.7,78.2	α-(+)-Prostaglandin F2, tetrakis-TMS	0.19	0.28
Total	0.85	0.28		
Phenolic compounds				
44	37.1,41.17	Magnolol, mono- TMS ether	0.27	0.5
45	37.2	Tert-Butylhydroquinone, bis-TMS	0.59	–
46	41.13	Magnolol, bis-TMS ether	–	0.26
47	47.1	4-Hydroxyanthraquinone-2-carboxylic acid, bis-TMS	1.32	–
48	74.2	1,2-Diphosphacyclohex-3-ene, 4-methyl-5-phenyl-6-(1-phenyl-1-propen-2-yl)- tetra-TMS	0.52	–
Total	2.7	0.76		
Phenolic acids				
49	14.2	Benzoic acid, TMS	0.37	0.51
50	23.4	Fumaric acid, 2-methyl, bis-TMS	0.67	–
51	28.53	3-(2-Hydroxyethyl)phenol, bis-TMS	–	0.12
52	31.7	p-Hydroxybenzoic acid, bis-TMS	0.25	0.07
53	38.7	Isoferulic acid, bis-TMS	–	0.57
54	38.9	Protocatechoic acid (3,4-dihydroxy-benzoic acid), tri-TMS	0.26	–
55	39.87	Isophthalic acid, bis-TMS	–	0.29
56	56.1	4-Coumaric acid, 2TBDMS	0.99	–
Total	2.54	1.56		
Carbohydrates				
57	19.68	Glyceric acid, tris-TMS	–	0.21
58	23.46	2-deoxy-D-erythro-pentopyranose, tris-TMS	–	0.18
59	39.4, 42.6,45.3	β-D-Allopyranose, pentakis-TMS	0.88	0.51
60	39.6	D-Fructofuranose pentakis-TMS	0.51	0.51
61	40.07, 40.8	D-Allofuranose, pentakis-TMS	–	0.35
62	41.1	D-Fructopyranose, pentakis-TMS	0.2	0.51
63	41.19	α-D-galactoside, O-methyl, tetrakis-TMS	–	0.37
64	42.14	D-Ribopyranose, tetrakis-TMS	–	1.37
65	42.1,42.57	D-Ribose, tetrakis-TMS	0.87	2.76
66	45.41	β-D-(+)-Mannopyranose, pentakis-TMS	–	2.13
67	63.6	Sucrose, octakis –TMS	1.14	
68	66, 66.4, 66.5,66.6, 66.8	D-(–)-Fructofuranose, pentakis-TMS (isomer 2)	2.95	–
69	68.8	D-(+)-Trehalose, octakis-TMS	0.87	–
70	69.1	Lactulose, octakis-TMS	0.39	–
71	69.7	Myo-Inositol, dimethyl phosphate-pentakis-TMS	1.26	–
72	86.88	Lactose, octakis-TMS	–	0.15
Total	9.07	9.05		
Sugar alcohol				
73	16.6,26.47	Glycerol, tris-TMS ether	1.03	0.08
74	28.10	Erythritol, tetrakis-TMS	0.26	1.41
75	36.4	Xylitol, pentakis-TMS	1.52	1.47
76	41.3 32.65	1-Deoxypentitol, tetrakis-TMS	0.22	0.28
77	41.41,42.88	L-Fucitol, pentakis-TMS	–	1.65
78	43.5	Mannitol, hexakis-TMS	3.18	0.1
79	44.01	D-Glucitol, hexakis-TMS (sorbitol)	0.35	7.7
Total	6.56	12.69		
Nitrogenous compounds				
80	3.37	Ethylamine	0.52	–
81	3.46	Carbodiimide, bis-TMS	0.32	0.38
82	4.76	Carbamate, Tris-TMS	1.18	–

(*Continued*)

Table2 (*Continued*)

Peak no.	RT	Compounds	*Penicillium* sp. NRC F1 area %	*Penicillium* sp. NRC F16 area %
83	5.06	Formamide, bis-TMS	–	0.09
84	12.7	5-Hydroxy-2-methylpyridine, TMS derivative	0.42	–
85	19.4	Uracil, bis-TMS	0.19	–
86	25.96	Methyl thiouracil, bis-TMS	–	0.15
87	27.6	Pyroglutamic acid, bis-TMS	0.31	0.18
88	32.6, 33.8	Methyl 2-(acetylamino)-4-O,6-O-(methylboranediyl)-3-O-(trimethylsilyl)-2-deoxy-alpha-D-glucopyranoside	0.66	1.1
89	35.50	D-gluco-hexodialdose, 2,3,4,5-tetrakis-o-(TMS)-, bis(o-methyloxime)	–	0.11
90	40.1	5-allyl-1,3-dimethyl-5-(3-hydroxy-1-methylbutyl) barbituric acid o-tris-TMS	0.75	–
91	40.2	Adenine, bis-TMS	0.92	–
92	44.19,44.31	N-acetyl-Glucosamine, o-methyloxime, tetrakis-o-TMS-	–	0.19
93	44.47, 56.63, 57.76. 57.52	α-D-Glucopyranosiduronic acid, 3-(5-ethylhexahydro-2,4,6-trioxo-5-pyrimidinyl)-1,1-dimethylpropyl 2,3,4-tris-O-(TMS)-, methyl ester	–	0.86
94	47.3	3-tert-Butyl-N'-(3-phenyl-2-propenylidene)-1H-pyrazole-5-carbohydrazide, bis-TMS	0.77	–
95	49.2	Lorazepam, bis-TMS	0.62	–
96	53.68	1H-Indole-3-acetonitrile, TMS	–	0.34
97	55.08	d-Fructopyranose, 1-deoxy-1-(methylphenylamino)-2,3,4,5-tetrakis-O-TMS	–	0.35
Total	6.66	3.75		
Sulfur compound				
98	3.98, 4.02	2,2'-Sulfonyldiethanol, bis-TMS	*1.48*	–
99	35.70	Thiosalicylic acid, bis-TMS	–	0.26
100	56.6	D-Galactofuranose, 1-C-(2-heptyl-1,3-dithian-2-yl)-2,3,5,6-tetra-TMS	0.22	–
Total	1.7	0.26		
Miscellaneous				
101	3.25, 3.40	Trifluoromethyl-bis-(TMS)methyl ketone	0.65	0.33
102	16.5	Phosphoric acid, tris-TMS	1.31	3.55
103	28.98 35.15	α- D-Mannopyranoside, methyl 2,3-bis-O-(TMS)-, cyclic butylboronate	–	0.5
104	37.8	α-Glycerophosphoric acid, tetrakis-TMS	0.68	1.01
105	40.44	1,5-Anhydrohexitol, tetrakis-TMS	–	0.26
106	49.1, 49.2	1-Cyclohexene-3,5-dione, hexakis-TMS	0.7	–
107	49.69	1,4-Diboracyclohexane, 1,4-diethyl-2,3,5,6-tetrakis- TMS	–	0.3
108	71.38, 75.09, 76.88	1-Cyclopentene-1-propanoic acid, 2-[3,7-bis[(trimethylsilyl)oxy]-1-octenyl]-5-ox	–	0.77
Total	3.34	6.72		
Unidentified				
109	54.628	Unidentified	0.89	–
110	55.188	Unidentified	2.46	–
111	57.85	Unidentified	1.37	–
Total	4.72	–		

F16 extracts, exhibiting a cytotoxicity of 97.29±0.61 and 97.08±1.07%, respectively.

Antioxidant activity of *Penicillium* sp. NRC F1 and *Penicillium* sp. Nrc F16 ethyl acetate extracts

The *in vitro* free radical scavenging activity of the ethyl acetate extracts of *Penicillium* sp. NRC F1 and *Penicillium* sp. NRC F16 was tested using DPPH as a reagent. As shown in Fig. 4, both extracts exhibited moderate antioxidant effects as a dose-dependent scavenging of DPPH. The ethyl acetate extract of *Penicillium* sp. NRC F1 at a concentration of 200 µg/ml showed a higher DPPH scavenging activity (74.41±0.59%) in comparison with that recorded by the *Penicillium* sp. NRC F16 ethyl acetate extract using the same concentration (65.58 ±1.55%).

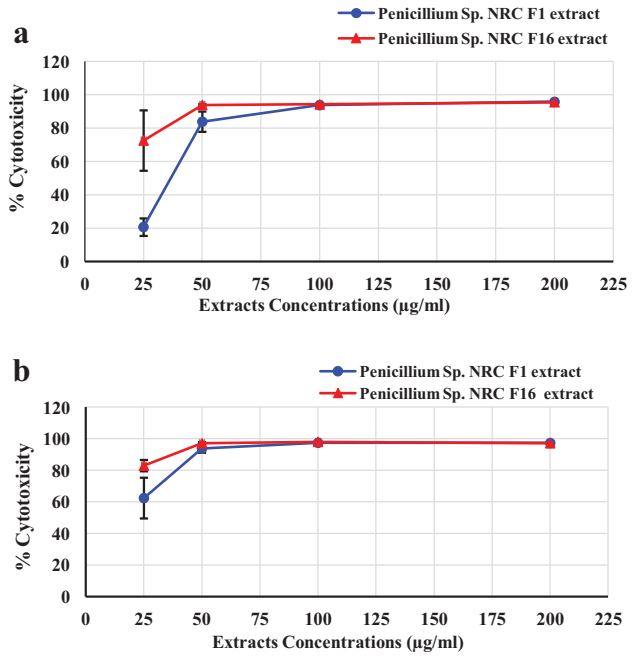
Discussion

Screening for new microorganisms capable of producing biologically active compounds is attracting continuous

Table 3 Relative concentration % of different chemical groups detected in *Penicillium* sp. NRC F1 and *Penicillium* sp. NRC F16 ethyl acetate extracts

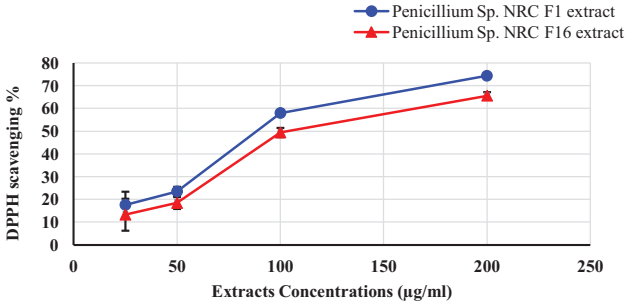
Chemical groups	*Penicillium* sp. NRC F1 extract (%)	*Penicillium* sp. NRC F16 extract (%)
Organic acids	8.23	9.56
Monocarboxylic acids	8.23	8.81
Di-carboxylic acids	–	0.75
Alcohols, esters, and ketones	1.14	5.17
Lipid compounds	7.6	17.47
Saturated fatty acids	3.87	9.07
Unsaturated fatty acids	2.64	7.33
Triglycerides	0.24	0.79
Others	0.85	0.28
Phenolic compounds	5.24	2.35
Phenolic acids	2.54	1.56
Others	2.7	0.76
Carbohydrates	9.07	9.05
Sugar alcohol	6.56	12.69
Nitrogenous compounds	6.66	3.75
Sulfur compound	1.7	0.26
Miscellaneous	3.34	6.72

Figure 3



Cytotoxicity % of ethyl acetate extracts of *Penicillium* sp. NRC F1 (represented by closed circles) and *Penicillium* sp. NRC F16 ethyl acetate extracts (represented by closed triangles) against human colon cancer cell line HCT116 (a) and human breast cancer cell line MCF7 (b). Values are presented as the means±SD (error bars) for three independent experiments.

Figure 4



DPPH radical scavenging activity of *Penicillium* sp. NRC F1 (represented by closed circles) and *Penicillium* sp. NRC F16 ethyl acetate extracts (represented by closed triangles at different concentrations). Values are presented as the means±SD (error bars) for three independent experiments. DPPH, 2, 2-diphenyl-1-picrylhydrazyl.

research attention, mainly owing to the increased strain caused by the spread of many life-threatening diseases such as cancer. For this purpose, new and uncommon environments were screened as promising sources of microbes having unique potentials. In this study, soil samples recovered from a remote cave located in Asyut governorate, Egypt, was investigated as a source of fungal strains. Generally, fungi are abundantly isolated from soil samples. However, the total number of isolated fungal strains in this study was relatively small (31 isolates), which may be due to the nature of the cave environment that affected presence of nutrients and hence number of microbes. Of the 31 obtained fungal isolates, two strains (were isolated from rhizosphere soil at the entrance of the cave) have been chosen to investigate their metabolic profile and study their potential biological activities. Morphological identification of samples suggested that both belonged to the genus *Penicillium*. This finding was confirmed after sequencing their ITS regions. Hence, isolates were identified as *Penicillium* sp. NRC F1 and *Penicillium* sp. NRC F16.

Extraction and GC-MS chemical analyses were performed to identify metabolites present in the silylated ethyl acetate extracts of the two isolates. A total of 114 compounds were detected in both extracts, and higher concentrations of most compounds were found in the *Penicillium* sp. NRC F16 extract except for phenolic compounds and nitrogenous compounds, which were present in relatively higher concentrations in the *Penicillium* sp. NRC F1 extract (Table 3). Studying the *in vitro* biological activities of both extracts as antioxidant agents revealed promising activities. *Penicillium* sp. NRC F1 showed higher DPPH scavenging activity (74.41±0.59%) in comparison with that recorded by the *Penicillium* sp. NRC F16 extract (65.58±1.55%).

This can be attributed to the presence of higher concentrations of phenolic compounds and other compounds known for their antioxidant effect in the *Penicillium* sp. NRC F1 extract. The free radical scavenging activity recorded by the *Penicillium* sp. NRC F1 extract was higher than that reported for the ethyl acetate extract of *Penicillium chrysogenum* hPc.var.c (73±0.34%) [18] and that achieved by the ethanolic extract of *Penicillium fumiculosum* (51.34%) [19]. On the contrary, *Penicillium* sp. NRC F1 and *Penicillium* sp. NRC F16 extracts exerted promising anticancer activities against tested cancer cell lines, which may be owing to the presence of fatty acids such as stearic acid, which has anti-breast cancer effects and which is capable of inhibiting breast tumorigenesis, inducing apoptosis, and preventing human breast cancer cell proliferation [18,19]. It should be noted that stearic acid was also used as a protecting agent in many epidemiological investigations to treat and prevent breast cancer [20]. The monocarboxylic acid (caproic acid) detected in *Penicillium* sp. NRC F16 extract has been also reported to have anticancer activity [20,21]. On the contrary, presence of many unsaturated fatty acids such as omega-6 in both extracts and omegas 3, 6, 7, and 9 in the extract of *Penicillium* sp. NRC F16 contributed to the antioxidant and anticancer activities as reported by numerous studies [22–24]. Both extracts showed promising anticancer activities against tested cancer cell lines. Higher activity (93.78±0.6% cytotoxicity) was recorded using 50 μg/ml of Penicillium sp. NRC F16 extract which contains unsaturated fatty acids that reached 7.3% of the total detected compounds' peak area. The ability of different *Penicillium* species to exert anticancer activities has been reported previously. The ethyl acetate extract of *P. chrysogenum* hPc.var.c exerted anticancer activity against colorectal adenocarcinoma cells (Caco-2) [18]. *Penicillium citrinum* showed activity against human breast cancer cell line (MDA-MB-231) [25]. *Penicillium janthinellum* KTMT5 exhibited promising anticancer activity against glioblastoma human cancer cell lines (UMG87) [26,27].

Conclusion

Finding novel sources to screen for microbes having promising biological activities is of critical need, and cave environment is an attractive source for such microbes. *Penicillium* species recovered in this study from a remote cave in Asyut governorate, Egypt, showed promising *in vitro* bioactivities such as antioxidant and anticancer activities against tested human colon cancer and human breast cancer cell lines. Further studies are encouraged to investigate the *in vivo* potential of these promising strains and evaluate the possibility of employing such fungi as sources of bioactive compounds.

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Conflict of interest

There are no conflicts of interest.

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