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

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Research

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Abstract

Searching for remote locations to screen for microorganisms, identify their metabolites, and investigate their bioactivities against lethal diseases such as cancer is of critical importance. In the current study, two fungal strains where isolated from a remote cave in Asyut governorate, Egypt. These isolates were morphologically and molecularly identified through sequencing their ITS region as *Penicillium* sp. NRC F1, and *Penicillium* sp. NRC F16. Investigating the metabolic profiles of the silylated ethyl acetate extracts of these fungi through conducting GC-Ms analysis revealed presence of 114 compounds belonging to different chemical classes.

On the other hand, studying the *in vitro* bioactivity of both extracts showed moderate antioxidant activities. *Penicillium* sp. NRC F1 extract exhibited higher DPPH scavenging activity (74.41 ± 0.59%) at concentration of 200 µg/ml, in comparison with that exerted by *Penicillium* sp. NRC F16 extract at the same concentration (65.58 ± 1.55%). Moreover, studying the cytotoxicity of extracts against human colon cancer (HCT116), and human breast cancer (MCF7) cell lines revealed that cytotoxicity of both extracts was dose dependent. Promising cytotoxic effect was achieved against human colon cancer HCT116 using 200 µg/ml of *Penicillium* sp. NRC F1 extract (95.72 ± 1.13 % cytotoxicity), while *Penicillium* sp. NRC F16 ethyl acetate extract caused a cytotoxicity of 95.43 ± 1.4 %. Similarly, investigating the *in vitro* cytotoxicity of the extracts against human breast cancer MCF7 cell line resulted in observing promising activity of *Penicillium* sp. NRC F1 and *Penicillium* sp. NRC F16 extracts, and they exhibited 97.29 ± 0.61 %; and 97.08 ± 1.07 % cytotoxicity, respectively.

Results from this study nominate those strains as promising isolates and encourage for conducting further *in vivo* investigations to evaluate their potency.

1. Introduction

Spreading of fatal diseases as cancer, as well as the reported side effects and shrinking repertoire of effective drugs have directed researches toward screening for new sources for potent compounds having anticancer activities. Cancer diseases are responsible for a considerable number of mortalities worldwide. According to world health organization reports, breast cancer is ranked as the second cause of deaths among the most common cancers (accounting for about 2.1 million cases in 2018 only). Colorectal cancer came next in the third place causing 1.80 million cases in the same year (ElkhateebMohamedFayadEmamNafadyandDaba 2020). On the other hand, finding 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 (VitaleCoppolaPalmaEspositoBuonocoreAusuriTortorellaandDePascale2020). Those molecules accumulate in body as a result of imbalance between antioxidant defense mechanisms and reactive oxygen species (ROS) generation, and hence became harmful causing degradation and destruction of cells components. Moreover, resulting oxidative stress play role in the pathogenesis of different chronic and degenerative diseases as aging, cancer, inflammation, autoimmune disorders,
rheumatoid arthritis, neurodegenerative and cardiovascular diseases (Ahmad Yuan, Yuan Nawaz, Ze Zhuo, Talal Taleb Maisand, Qilong 2017; García-Sánchez, Miranda-Díaz and Cardona-Muñoz 2020).

Fungi are eukaryotic microorganisms that are considered as generous sources of biologically active compounds. Many anticancer, antioxidant, antiinflammatory, antimicrobial secondary metabolites were previously reported from different fungal genera (Bills and Gloer 2017; Bogner, Kamdem, Sichermann, Matthäus, Hölscher, Popp, Proksch, Grundler and Schouten 2017). Among all fungi, Penicilli are 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, P. rubens, and P. roqueforti) are commonly used as cheese starters (Kumar, Asthana, Gupta, Nigam, and Mahajan 2018). Also, P. nalgiovense, is used in food industry for sausages fermentation (Bernáldez Córdoba, Rodríguez, Cordero Polo, and Rodríguez 2013). On the other hand, bioactive compounds produced by different Penicilli such as orcinol; 3-oxoquinuclidine; 1,3,8-p-menthatriene; limonene were also reported (Kumar, Asthana, Gupta, Nigam, and Mahajan 2018). Interestingly, species belonging to the genus Penicillium act mysteriously as they can be toxic or beneficial (Kozlovskii, Zhelifonova, and Antipova 2013; Mady and Haggag 2020).

Hence, isolation of new fungal isolates from novel remote sources is critically important in order 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 morpho-molecularly identified through sequencing of their nuclear ribosomal internal transcribed spacer ITS1-5.8S-ITS2 regions. Furthermore, a GC-Ms chemical analysis was performed on the silylated extracts of those fungi in order to identify their metabolic profiles. Finally, the \textit{in vitro} antioxidant and anticancer activities of both extracts was investigated against the HCT116 colorectal carcinoma, and MCF7 breast carcinoma human tumor cell lines.

\section*{2. Materials And Methods}

\subsection*{2.1 Isolation of Fungi}

\subsubsection*{2.1.1 Sample collection}

Cave soil samples were collected from different sites inside El Shekh Sayed cave that lies 44 Km east to 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 archaeological site in Asyut Governorate, Egypt (Figure 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 cool box (4°C) and processed within 24h.

2.1.2 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, USA). After incubation for seven days at 30±2°C, the plates were checked for colonies growth and single colonies were picked-up and streaked onto the surface of agar plate of the same isolation medium, and allowed to grow for seven 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 refrigerator by regular sub-culturing every two months.

2.2 Morphological and Molecular identification of the selected isolates

2.2.1 Morphological Identification

Isolates were preliminary identified under microscope following the description of (DomschGamsandAnderson 1980) and (Moubasher 1993).

2.2.2 Molecular identification of isolates

The total fungal DNA was extracted from fungal hyphae and purified through E.Z.N.A.® Fungal DNA Mini Kit (D3390-01, Omega BIO-TEK, USA) following manufacturer instructions. Obtained DNA were stored at -20°C until needed. For PCR amplification, DreamTaq Green PCR Master Mix (2X) (K1081, Thermo Fisher, USA) using the universal primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´); and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) (DemirelSariozluandİlhan 2013) for specific gene amplification according to manufacturer protocol using Creacon (Holland, Inc) Polymerase Chain Reaction (PCR) system cycler. After that, resulting PCR products were purified using E.Z.N.A.®Gel Extraction Kit, (D2500-01, Omega BIO-TEK, USA). The sequence analysis was employed using the ABI PRISM® 3100 Genetic Analyzer (Micron-Corp., Korea).

2.3 Data analysis

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 NCBI website (http://www.ncbi.nlm.nih.gov/website) using BLAST to confirm their identity. Genetic distances and MultiAlignments were computed by Pairwise Distance method using ClusteralW software analysis (www.ClusteralW.com). The nucleotide sequences were also compared with Penicillium isolates sequences available in the GenBank.

2.4 Fermentation and Extraction of secondary metabolites
Erlenmeyer flasks containing potato dextrose broth medium (one liter each) were inoculated with 10 ml 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 (LiZhouZhuChangYuanGaoZhangZhaoandLou 2015) with some modifications. Briefly, the whole contents of each flask was transferred to Erlenmeyer flasks (of 2 liter capacity) and extracted twice by mixing with ethyl acetate (AnaIR, UK) (1:1, v/v), sonicated for 10 min. with gentle warming, and the mixture was kept overnight at room temp. Then, the organic layer was separated and the process was repeated till exhaustion. The ethyl acetate layers were collected and evaporated using rotatory evaporator (heidolph rotary evaporator, Schwabach, Germany) under reduced pressure at 45°C. The crude extract Penicillium sp. NRC F1 (0.089 g) Penicillium sp. NRC F16 (0.136 g) were kept in the fridge for analysis.

2.5 GC-Ms analysis

2.5.1 Preparation of samples

Samples derivatization for GC-Ms analysis was carried out by Silylation by keeping 2.5 mg sample in desiccator overnight to ensure complete dryness then 20μl pyridine supplemented with 30μl N,O-Bis-(trimethylsilyl) trifluoroacetamide (BSTFA) were added and the mixture was incubated for 30 min. at 85 °C for derivatization just before conducting the GC-Ms analysis (ChristovBankovaHegaziAbd El HadyandPopov 1998); (ElkhateebMohamedFayadEmamNafadyandDaba 2020).

2.5.2 Mass spectrometer

A Finnigan MAT SSQ 7000 mass spectrometer coupled with a Varian 3400 gas chromatograph. DB-5 column, 30 m x 0.32 mm (internal diameter), was employed with helium as carrier gas (He pressure, 20 Mpa/cm2) and GC temperature program, 85 – 310 °C at 3 °C/ min (10 min. intial hold). The injector temperature was kept at 310°C. The mass spectra were recorded at 70 eV in electron ionization mode (Li et al., 2015). The scan repetition rate was 0.5 s over a mass range of 39 – 650 atomic mass units (amu).

To identify compounds in the ethyl acetate extract of the two fungal isolates, GCMs 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.

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

2.6.1 Cell cultures

HCT116 colon carcinoma, and MCF7 breast carcinoma human tumor cell lines were cultured in 95% humidity, 5% CO2 and 37°C. HCT116 was maintained in McCoy’s 5A, while MCF7 in MEM media,
supplemented with 10% fetal bovine serum and 1% antibiotic (ElkhateebMohamedFayadEmamNafadyandDaba 2020).

2.6.2 Cytotoxicity assay

Acid phosphatase assay was conducted to evaluate cytotoxicity as described previously (YangSinaiandKain 1996). Briefly, human colon cancer cell line (HCT116), and human breast carcinoma cell line (MCF7) were used by seeding 10000 cell per well in 96 well plates, left overnight till attach, and then treated with different extracts for three days. For one plate, a substrate solution was prepared where 20 mg tablet of pNPP (Sigma; cat. no. N2765) was dissolved in 10 ml buffer solution (0.1 M sodium acetate, 0.1% triton X-100, pH 5.0). Cell monolayers were washed with 250 µl PBS. 100 µl of pNPP substrate solution were added per well, then plates were incubated for 4 hours at 37°C. 10 µl of 1N sodium hydroxide stop solution were added per well. Absorbance was measured directly at wavelength 405 nm. All samples were tested in triplicates, and 0.5% DMSO was used as negative control and 50 µM cisplatin was used as positive control. Extracts were tested at serial dilutions with nal concentration of 200, 100, 50, and 25 µg/ml. Percent cytotoxicity = [1-(D/S)] × 100, where D and S denote the optical density of drug and solvent treated wells, respectively.

2.7 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 (Blois 1958). Extracts were tested at nal concentrations of 200, 100, 50, and 25 µg/ml using 0.1mM 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 positive control at nal concentrations of 20 µg/ml. The DPPH solutions treated with 0.5% DMSO used as a negative control. The DPPH scavenging activity of extracts was calculated according to the equation:

Percentage reduction = (1-(X/ av(NC)) ×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, USA).

3. Results

3.1 Isolation and morphological and molecular identification of fungi

Soil samples were collected from different sites inside El Shekh Sayed 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 A. flavus were predominantly isolated from all sites. Rhizopusstoloniferand Mucor circinelloides came in the second place and were isolated 4 times each. Alternaria alternatawas isolated 3 times, while A. fumigatus, and A. versicolor appear twice. The richest site in fungal isolates was the rhizosphere soil at 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 rhizosphere soil at entrance of the cave and showed characteristic antagonistic growth which encouraged for studying both isolates.

The colonies surface of *Penicillium* sp. NRC F1 appeared in bluish-green color and were velvety sulcate. Colonies reverse was brownish orange in color, and conidiophores appeared two-stage branched under microscope. Conidia were bluish-green, smooth-walled, and appeared globose to subglobose. On the other hand, the morphological and microscopic appearance of the second isolate suggest that it is also a *Penicillium* species. Colonies appeared velvety with whitish margin, green conidial heads, and colonies reves 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 nuclear ribosomal ITS1-5.8S-ITS2 region related to *Penicillium* sp. was shown in figure 2.

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

| Isolate                  | Site                                                                 | Number of isolates |
|--------------------------|----------------------------------------------------------------------|--------------------|
| *Aspergillus niger*      | From all sites                                                      | 7                  |
| *A. fumigatus*           | Deep inside the cave, wall of cave                                  | 2                  |
| *A. versicolor*          | Rhizosphere soil at entrance of the cave                            | 2                  |
| *A. 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**             |

3.2 Metabolic profiles of selected *Penicillium* isolates
Sialylation of the ethyl acetate extracts was conducted to facilitate detection of as much as possible polar and non-polar compounds contained in those two fungal extracts. GC-Ms analyses were then conducted in order to identify compounds in these extracts. As shown in table 2 and 3, analyses revealed the identification of 114 compounds from different chemical classes. Majority of compounds were common in both Penicillium sp. NRC F1, and Penicillium sp. NRC F16 extract. 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%), while in case of Penicillium sp. NRC F16 extract galactonic acid was the highest (3.83%). On the other hand, the concentration of monocarboxylic acids in both extracts was nearly the same (table 3), while di-carboxylic acids were only detected in Penicillium sp. NRC F16 (0.75%) and was not detected in Penicillium sp. NRC F1 extract. Lipid compounds were also detected in both extracts but their existence in Penicillium sp. NRC F16 extract was in general twice 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 other hand, phenolic compounds detected in Penicillium sp. NRC F1 extract (5.24%) was higher than that detected in 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 Penicillium sp. NRC F1 extract (2.95%) while it was absent in Penicillium sp. NRC F16 extract. Ribose was the main sugar in Penicillium sp. NRC F16 extract (2.76%) and appear as traces in Penicillium sp. NRC F1 extract. The GC-Ms analyses revealed also the abundance of sugar alcohol in Penicillium sp. NRC F16 extract at concentration as twice as that detected in Penicillium sp. NRC F1 extract (table 3). Mannitol was the main alcohol detected in Penicillium sp. NRC F1 extract (3.18%), while sorbitol was the main alcohol detected in Penicillium sp. NRC F16 extract (7.7%). On the contrary, the nitrogenous and sulphur compounds detected in Penicillium sp. NRC F1 extract (6.66%, 1.7%, respectively) was as twice as that detected in Penicillium sp. NRC F16 extract (3.75%, 0.26%, respectively).

Table 2: GC-Ms 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 (Citroconic 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

|    |    |                                  |     |      |
|----|----|----------------------------------|-----|------|
| 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 | a-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   |
| **Total**        |                |               | **0.24** | **0.79** |
| **- Other lipid compound** |            |               |        |        |
| 41.              | 56.5           | Prostaglandin D(2), tetrakis-TMS | 0.31   | —      |
| 42.              | 71.5           | 0-methylloex,19-Norpregna-1,3,5,7,9-pentaen-21-al, bis-TMS | 0.35   | —      |
| 43.              | 76.7,78.2      | a-(+)-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                                             |
| 58. 23.46 2-deoxy-D-erythro-pentopyranose, tris-TMS                           |
| 59. 39.4, 42.6, 45.3 b-D-Allopyranose, pentakis TMS                           |
| 60. 39.6 D-Fructofuranose pentakis TMS                                        |
| 61. 40.07, D-Allofuranose, pentakis TMS                                       |
| 62. 41.1 D-Fructopyranose, pentakis-TMS                                       |
| 63. 41.19 a-D-galactoside, O-methyl, tetrakis-TMS                             |
| 64. 42.14 D-Ribopyranose, tetrakis-TMS                                        |
| 65. 42.1, 42.57 D-Ribose, tetrakis-TMS                                        |
| 66. 45.41 b-D-(+)-Mannopyranose, pentakis-TMS                                |
| 67. 63.6 Sucrose, octakis -TMS                                                |
| 68. 66, 66.4, 66.5, 66.6, 66.8 D(-)-Fructofuranose, pentakis-TMS (isomer 2) |
| 69. 68.8 D-(+)-Trehalose, octakis -TMS                                        |
| 70. 69.1 Lactulose, octakis –TMS                                              |
| 71. 69.7 Myo-Inositol, dimethyl phosphate -pentakis-TMS                       |
| 72. 86.88 Lactose, octakis –TMS                                               |
| Total                                                                        |
|                                                                              |
| Sugar alcohol                                                                |
| 73. 16.6, 26.47 Glycerol, tris-TMS ether                                      |
| 74. 28.10 Erythritol, tetrakis-TMS                                           |
| 75. 36.4 Xylitol, pentakis-TMS                                               |
| 76. 41.3 1-Deoxypentitol, tetrakis-TMS                                       |
| 77. 41.41, 42.88 L-Fucitol, pentakis-TMS                                      |
| 78. 43.5 Mannitol, hexakis-TMS                                               |
| 79. 44.01 D-Glucitol, hexakis-TMS(sorbitol)                                   |
|                                                                              |
|                                                                              |
|                                                                              |
|                                                                              |
|                                                                              |

|   |   | Total |   |
|---|---|-------|---|
|   |   |       |   |
| **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 | — |
| 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 | a-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-carboxydrazide, 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** |   |       |   |
| 98. | 3.98, 4.02 | 2,2'-Sulfonyldiethanol, bis-TMS | 1.48 | — |
| 99. | 35.70 | Thiosalicylic acid, bis-TMS | — | 0.26 |

|   |   | Total |   |
|---|---|-------|---|
|   |   |       |   |
| **Sulphur 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  | 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| —   |

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                                   |
| Sulphur compound        | 1.7                                  | 0.26                                   |
| Miscellaneous           | 3.34                                 | 6.72                                   |

### 3.3 In vitro anticancer activities of extracts against Human colon cancer (HCT116) and Human breast carcinoma tumor (MCF7) cell lines

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, 200 μg/ml). As shown in Fig. 3 (a, and b), promising cytotoxic activities were exerted by *Penicillium* sp. NRC F1 and *Penicillium* sp. NRC F16 ethyl acetate extracts. The highest cytotoxic effect against human colon cancer HCT116 was obtained after treatment with 200 μg/ml of *Penicillium* sp. NRC F1 extract exhibiting 95.72±1.13 % cytotoxicity, while treatment using the same concentration of *Penicillium* sp. NRC F16 ethyl acetate extract resulted in a cytotoxicity of 95.43±1.4 % (Fig 3 a). On the other hand, investigating the *in vitro* cytotoxicity of the extracts against human breast cancer MCF7 cell line (Fig. 3 b) resulted in observing promising activity of *Penicillium* sp. NRC F1 and *Penicillium* sp. NRC F16 ethyl acetate extracts, and they exhibited 97.29±0.61 %; and 97.08±1.07 % cytotoxicity, respectively as shown in Fig. 3 (a, and b).
3.4 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 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 concentration of 200 µg/ml showed a higher DPPH scavenging activity (74.41±0.59%) in comparison with that recorded by *Penicillium* sp. NRC F16 ethyl acetate extract using the same concentration (65.58± 1.55%).

4. Discussion

Screening for new microorganisms capable of producing biologically active compounds is attracting continuous research attention. Mainly, due to the high pressure caused by the spread of many life-threaten 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, 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. Out of the 31 obtained fungal isolates, two strains (were isolated from rhizosphere soil at the entrance of the cave) have been chosen in order to investigate their metabolic profile, and study their potential biological activities. Morphological identification of samples suggested that both belong 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. Total of 114 compounds were detected in both extracts, and higher concentrations of majority of compounds were found in *Penicillium* sp. NRC F16 extract except for phenolic compounds and nitrogenous compounds which were present in relatively higher concentrations in *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 *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 *Penicillium* sp. NRC F1 extract.

The free radical scavenging activity recorded by *Penicillium* sp. NRC F1 extract was higher than that reported for the ethyl acetate extract of *Penicillium chrysogenum* hPc.var.c (73±0.34 %) (CanturkArtaganandDikmen 2016); and that achieved by the ethanolic extract of *Penicillium fumiculosum* (51.34 %) (JakovljevicMilićevićStojanovicSolujicandVrvic 2014). On the other hand, *Penicillium* sp. NRC F1, and *Penicillium* sp. NRC F16 extracts exerted promising anticancer activities against tested cancer cell lines, which may be due to presence of fatty acids such as stearic acid that has anti-breast cancer effects and which is capable of inhibiting breast tumorigenesis, inducing apoptosis and preventing human breast
cancer cell proliferation \cite{EvansCoweySiegalandHardy2009,EvansTolineDesmondSiegalHashimandHardy2009}. It should be noted that stearic acid was also used as a protecting agent in many epidemiological investigations to treat and prevent breast cancer \cite{SaadatianElahiNoratGoudableandRiboli2004}. The Monocarboxylic acid (caproic acid) detected in \textit{Penicillium} sp. NRC F16 extract has been also reported to have anticancer activity \cite{NarayananBaskaranAmalaradjouandVenkitanarayanan2015,WidiyartiSundowoMegawatiandErnawati2019}. On the other hand, presence of many unsaturated fatty acids such as omega 6 in both extracts, and omegas 3, 6, 7, 9 in extract of \textit{Penicillium} sp. NRC F16 contributed in the antioxidant and anticancer activities as reported by numerous studies \cite{WendelandHeller2009,CockbainToogoodandHull2012,ElkhateebEl-SayedFayadAlKolaibeEmamandDaba2020}. Both extracts showed promising anticancer activities against both tested cancer cell lines but activity was observed to a higher instance upon starting treatment with lower concentrations of \textit{Penicillium} sp. NRC F16 extract (50 $\mu$g/ml of extract resulted in 93.78±0.6 % cytotoxicity) where unsaturated fatty acids occupied 7.3% of total detected compounds peak area. The ability of different \textit{Penicillium} species to exert anticancer activities has been reported previously. The ethyl acetate extract of \textit{Penicillium chrysogenum} hPc.var.c exerted anticancer activity against colorectal adenocarcinoma cells (Caco-2) \cite{CanturkArtaganandDikmen2016}; \textit{P. citrinum} showed activity against human breast cancer cell line (MDA-MB-231) \cite{MadyWaelAbdouHaggagandElSayed2017}; \textit{P. janthinellum} KTMT5 exhibited promising anticancer activity against glioblastoma human cancer cell lines (UMG87) \cite{TapfumaSebolaUche-OkereaforKoopmanHussanMakatiniMekutoandMavumengwana2020}.

## 5. 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. \textit{Penicillium} species recovered in this study from a remote cave in Asyut governorate, Egypt showed promising \textit{in vitro} bioactivities as antioxidant, and anticancer activities against tested human colon cancer, and human breast cancer cell lines. Further studies are encouraged to investigate the \textit{in vivo} potential of these promising strains and evaluate the possibility of employing such fungi as sources of bioactive compounds.

## Declarations

### 6.1 Ethics approval and consent to participate

Not applicable

### 6.2 Consent for publication

Not applicable

### 6.3 Availability of data and materials
6.4 Competing interests
The authors declare that they have no competing interests

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6.6 Authors' contributions
All authors read and approved the final manuscript.

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Not applicable

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Figures
Figure 1

description of El shekh Sayed cave, Asyut governorate, Egypt as illustrated by google earth (a), and cave entrance (b).
Figure 2

Phylogenetic tree for nuclear ribosomal ITS1-5.8S-ITS2 region for Penicillium sp. NRC F1, and Penicillium sp. NRC F16.
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 ± standard deviation (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 ± standard deviation (error bars) for three independent experiments.

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