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Antioxidant and Antitumor Activity of a New Sesquiterpene Isolated from Endophytic fungus Aspergillus glaucus

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Abstract: An endophytic fungus was isolated from the leaves of Ipomoea batatas. Based on morphological characteristics and fungus ribotyping, the fungus was identified as *Aspergillus glaucus.* By bioassay-guided fractionation, one potent antioxidant and anti-tumor secondary metabolite was isolated from ethyl acetate extract of the fungal culture filtrate. Structural elucidation of the yielded compound using intensive studies of their NMR (^{1}H, ^{13}C NMR) and HPLC ESI-MS spectrometry confirmed 2,14-dihydrox-7-drimen-12,11-olide. On the basis of spectroscopic analysis compound 1 was isolated from *Aspergillus glaucus* for the first time. *In-vitro* anti-tumor assay showed that the active compound has moderate cytotoxic effect against Hep-G2 cell IC_{50} value 61 µg/ml, While it affect strongly on growth of MCF-7 cells, $IC_{50,}$ 41.7 µg/ml.
Keyword*:* Endophytic Fungi; *Aspergillus glaucus*, Structural Elucidation; Bioactivity.

Introduction

There is high demand for innovative lead structures in order to discover and develop novel drugs for the treatment of cancer and other menacing diseases (1). Resistance of tumor cells, pathogenic bacteria and viruses is steadily increasing and many established drug therapies are not effective anymore. Natural products have been shown to possess the potential to be excellent lead structures and to serve as a basis for promising therapeutic agents in the area of anticancer treatment (2). Endophytes are the microorganisms that colonize the interior of the plant parts, without having any negative effect on the host (3), rather helping the plant by imparting resistance to host plant against biotic (4-5) and a biotic stresses (6-7). There are approximately 300,000 different plant species on earth. Generally, each individual plant is host to many endophytes, thus providing a rich reservoir of endophytic fungi (8 -11). Many endophytic fungi have proven to be a rich source of bioactive secondary metabolites which are of interest for proper medicinal or agrochemical applications (12). Fungi can be used not only as strong immunoceuticals but also as a source of potent metabolites, capable of penetrating cell membranes and interfering with particular signal transduction pathways linked to processes such as inflammation, cell differentiation and survival, carcinogenesis, and metastasis (13). In the present study, taxonomical characterizations of the strain together with the antitumor activities, structural elucidation of the secondary metabolite using NMR (^{1}H,^{13}C NMR) and mass (EI MS) were discussed.

Materials and Methods

Sample collection

Leaf samples healthy plant of sweat potato during October 2009. Immediately after the collection, plant parts were washed with tap water and processed for isolation of endophytic fungi.

Isolation and identification of endophytic fungi

Endophytic fungus was isolated from the healthy plant of sweat potato *Ipomoea batatas*, collected from the herbarium of Agriculture Research Center (ARC), Cairo. Leaves were cut into small segments and surface-sterilized by sequential washes in 95% ethanol for 30 sec, 5% sodium hypochlorite for 5 min, followed by 95% ethanol for 30 sec and rinsed with sterile water (14). The sample segments were then transferred to Potato dextrose agar (PDA) medium, supplemented with 200 µg/mL ampicillin and 200 µg/mL streptomycin to inhibit the bacterial growth until the mycelium or colony originating from the newly formed surface of the segments appeared. Plates were incubated at 30°C for 1 week. Furthermore, the endophytic nature of the isolated strain was checked every other day for 15 days, individual fungal colonies being transferred to Czapeks-Dox agar (CDA) with incubation at 30°C for 10 days. Fungal spore formation was encouraged by placing the endophytes onto autoclaved carnation leaves. The plates were continuously monitored for spore formation by stereo and light microscopy. The identifications of the endophytic fungi *Aspergillus* species was identified according to Raper and Fennell (15) and Klich (16).

Production, extraction and Fractionation of bioactive metabolites

Small pieces (1 cm^2) of well grown sub-cultures of *A. glaucus* were inoculated into seventeen Erlenmeyer flasks 1 liter, each containing 300 ml of sterilized Czapeck-Dox medium (g/L): Sucrose, 30; $NaNO_3$, 3; K_2HPO_4, 1; KCl, 0.5; $MgSO_4$, 0.5; $FeSO_4$, 0.01 and distilled water at pH 7.3. The inoculated flasks were incubated for 7 days at 30°C in static incubator. After harvesting, the fungal mate and supernatant were separated by filtration (17).

The culture filtrate (totaling 5 L) was extracted three times with equal volumes of ethylacetate (EtOAc). The EtOAc layer was evaporated to dryness (0.85 g), the crude extract, which was detected in antioxidant assay using DPPH (18), loaded on silica gel plates for fractionation. The mobile phase was chloroform: methanol (95:5 v/v). Five completely separated major fractions were detected by using UV lamp **F1**, **F2**, **F3**, **F4** and **F5** and eluted with EtOAc. The five fractions were subjected again to antioxidant assay**, F1** of the highest antioxidant activity, was subjected to further purification by TLC.

Determination of Maximum UV Absorption

The absorption of UV or visible radiation corresponds to the excitation of outer electrons in the molecules. In many organic molecules, most absorption is based on transition of n or p electrons to the p* excited form. Experimentally, these transitions fall in spectrum of region 200-800 nm. Consequently, these transitions need an unsaturated group (called as chromophores) of the molecules to provide p electrons.

Spectral measurements

To elucidate the chemical structure of the separated active compound 1, it was analyzed using mass and nuclear magnetic resonance (NMR) spectrometry. The HPLC-ESI-MS system consisted of an HP1100 Series HPLC instrument (quaternary pump and degasser, column compartment, and auto sampler) and an LCQ ADVANTAGE MAX mass spectrometer from Thermo Finnigan, Xcalibure 1.4 software. ^1H and ^{13}C NMR spectra were recorded at 300°K pm Bruker DPX 300, ARX 400 spectrometers. All 1D and 2D spectra were obtained using the Bruker software. This sample is dissolved in DMSO-d_6, CD_3O. Solvent signals at 3.3 ppm and 49 ppm (CD_3OD) and at 2.49 ppm and 39.5 ppm (DMSO-d_6) were considered as internal standard. The observed chemical shift (δ) values were given in ppm and die coupling constants (*J*) in Hz.

Antioxidant activity

For the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay (18), aliquots of diluted extract (500 µl) were added to 3 ml of (DPPH) solution (0.1mM) in 95 % Ethanol. The reaction mixture was shaken well and incubated for 10 min at room temperature and the absorbance of the resulting solution was read at 517nm against a blank. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following equation:

$$\text{Scavenging ability (\%)} = [(\Delta A_{517 \text{ of control}} - \Delta A_{517 \text{ of sample}}) / \Delta A_{517 \text{ of control}}] \times 100.$$

Antitumor activity

The human hepatocarcinoma cell line (Hep G2), and human breast adenocarcinoma cell line (MCF-7), which were purchased from the American Type Culture Collection (ATCC) USA, were used to evaluate the cytotoxic effect of **Compound 1**. Cytotoxicity of tested sample was measured against different cell lines using

the MTT Cell Viability Assay (19). The extent of the reduction of MTT was quantified by measuring the absorbance at 570 nm. The percentage of relative viability was calculated using the following equation:

[Absorbance of treated cells/ Absorbance of control cells)] ×100.

Then, the half maximal inhibitory concentration (IC_{50}) values were expressed as the drug concentrations inhibiting cell growth by 50% setting the viability of untreated cells as 100 %. It was calculated from the equation of the dose response curve.

Results and Discussion

Taxonomical characterization of the endophytic fungal strain

The grown colonies of endophytic fungal strain, isolated from the *Ipomoea batatas*, were investigated on the basis of morphological characteristics and fungus ribotyping. The fungus produces surficial and submerged hyphae on rich medium like potato dextrose agar (PDA), however the growth being most prominent on CDA at 25–30°C. The colony on CDA is typically broadly, flat, dull green to grey-green. The mycelium is colorless and inconspicuous. Interestingly, the fungus has shown good rate of growth even up to 45°C on CDA. The final identification to species level was made using morphological, as shown in Figure (1), and physiological characteristics in addition to the molecular analysis. Microscopic studies of the fungus has shown the conidiophores 700-800x2-3 μm, hyaline, smooth-walled, Conidiogenous cells uniseriate, 5.0-7.5 x 3-4 μm. Conidia ovoidal or aculeate, echinulate, hyaline, 4.5-7.5 μm diameter. Ascomata covered with red hyphae, yellow, spherical to subspherical, 75-125 μm diam. Asci 8-spored, 10-12 μm diam. Ascospores smooth-walled or occasionally slightly roughened, with a pronounced equatorial furrow, hyaline to subhyaline, lenticular, 6-7 x 3.2-5.1 μm. Based on these typical features, the fungus has been identified as *Aspergillus glaucus* (15-16).

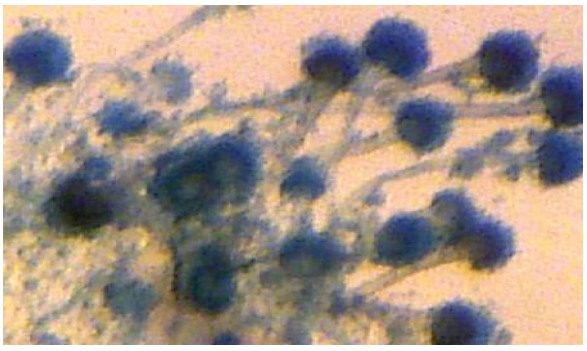


Figure1: Microscopic characterization of the endophytic *Aspergillus glaucus.*

Isolation and identification of the active constituents

In this study**,** 0.85 g crude extracellular extract was obtained from 5 liters of Czapeck-Dox medium of the endophytic fungus, *Aspergillus glaucus***.** The crude extract was subjected to the antioxidant assay. As shown in Table 1, it showed a high scavenging effect where after 1 hr it was 82%. The EtOAc extract was fractionated and purified repeatedly by TLC on silica gel to yield four major compounds: compound 1, compound 2, compound 3 and compound 4, as well as a minor negligible overlapped fraction. The four major compounds were subjected to antioxidant assay. The Compound 1 was the compound of choice for further study where it showed a highest scavenging activity, 89%, while the others were less. The further purification of compound 1 was followed by using UV-2401PC visible spectrophotometer (Shimadzu, Kyoto, Japan), where the pure compound showed a single sharp peak at λ= 284 nm as shown in figure (2).

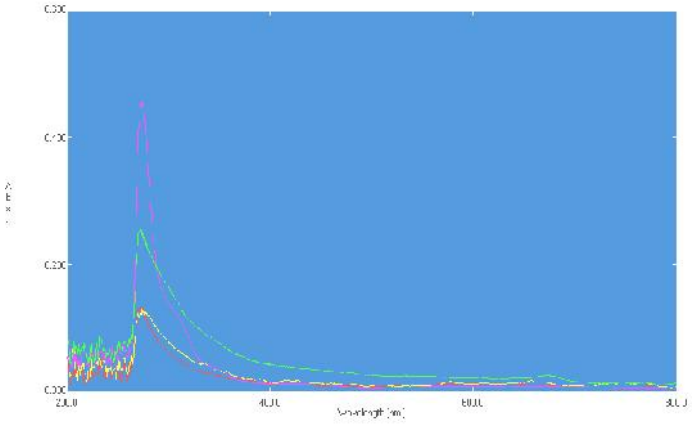


Figure 2: UV-Scanning of compound **1**

Chemical characterization of compound 1

Compound **1** is a yellow solid, showing UV absorbance on TLC, which turned yellow with anisaldehyde/sulphuric acid and heating. The molecular weight was deduced as 266 g/mol by ESIMS; and the HRESIMS established the molecular formula as $C_{15}H_{22}O_4$.

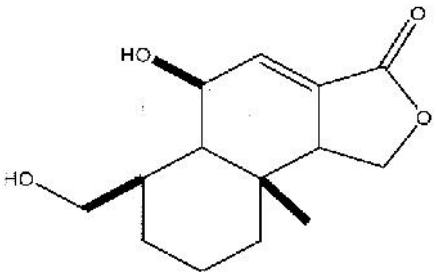
Compared to the other isolated sesquiterpene lactones, the ^1H NMR spectrum of this compound did not show the presence of any aromatic protons. The presence of three methylene groups, H_2-1, H_2-2 and H_2-3 forming a contiguous COSY spin system was also observed for this compound. The ^1H NMR showed the presence of one olefinic, three methane and two methylene protons, as well as one hydroxyl group. As in other sesquiterpene lactones, the olefinic proton at d 6.60 coupled to H-6 and further to H-5 in the COSY spectrum. An allylic (*4JHH*) coupling of 2.5 Hz between H-7 and H-9 suggested the presence of a double bond in between. The ROESY correlation of H-5 with H-9 as well as H-6 and H_3-13 indicated the close spatial proximity between H-5, H-6, H-9, and H_3-13 on the α-face of the molecule Table (1 & 2). Consequently, 6-OH was present in β-orientation, together with H_3-15 and H_2-14 as shown by the ROESY correlations of H_3-15 with H_2-14. The structure of compound 1 was established as 2, 14-dihydrox-7-drimen-12, 11-olide **(1)**.

Table 1: ^1H and ^{13}C NMR spectroscopic data of **compound 1** in CD_3OD

Position	CD_3OD at 500 MHz (^1H) and 125 MHz (^{13}C)	
	^1H	^{13}C
	δ (ppm) J (Hz)	δ_H (ppm)
1	Aβ 1.67 dm (12.6); Bα 1.33 m	43.0
2	Aβ 1.58 m; Bα 1.42 m	19.5
3	Aβ 1.78 dm (14.6); Bα 1.25 m	41.0
4	-	39.5
5	1.45 d (4.1)	56.5
6	4.63 dd (6.6; 4.1)	64.5
7	6.74 dd (6.6; 2.8)	137.0
8	-	128.0
9	2.85 ttt (9.1; 9.1; 2.8)	53.0
10	-	35.0
11	A 4.48 t (9.1); B 4.13 t (9.1)	69.5
12	-	173.5
13	1.13 s	27.0
14	A 4.26 d (11.8)	68.5

Table 2: ^{1}H and ^{13}C NMR spectroscopic data of **compound 1** in DMSO-d_6

Position	In DMSO-d_6 at 500 MHz (^{1}H) and 125 MHz (^{13}C)	
	^{1}H	^{13}C
	δ (ppm) J (Hz)	δ (ppm)
1	1.55 m; 1.15 m	42.0
2	1.45 m; 1.25 m	-
3	1.80 bd (14.2); 1.05 m	-
4	-	39.0
5	1.20 d (4.0)	56.0
6	4.52 dd (6.5; 4.0)	-
7	6.60 dd (6.5; 2.5)	137.0
8	-	128.0
9	2.75 ttt (9/0; 9.0; 2.5)	53.0
10	-	34.0
11	4.40 t (9.0); 4.05°	68.0
12	-	170.0
13	1.10 s	27.0
14	4.05°	68.5



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Antitumor activity of 2, 14-dihydrox-7-drimen-12, 11-olide

MTT(3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay was based on the ability of active mitochondrial dehydrogenase enzyme of living cells to cleave the tetrazolium rings of the yellow MTT and formed dark blue insoluble formazan crystals which were largely impermeable to cell membranes, resulting in its accumulation within healthy cells. Solubilization of the cells resulted in the liberation of crystals. The number of viable cells was directly proportional to the level of soluble formazan dark blue color. By using MTT assay, the effect of the 2, 14-dihydrox-7-drimen-12, 11-olide on the proliferation of both Hep-G2 and MCF-7 cells after 48 h of incubation, was shown in Figures (3 & 4). The treatment of Hep-G2 cells with the 2, 14-dihydrox-7-drimen-12, 11-olide to a marginal inhibition in the cell growth as concluded from IC_{50} value 61 µg/mL, which revealed no moderate cytotoxic effect of the extract against hepatic carcinoma. While, the effect of the 2, 14-dihydrox-7-drimen-12, 11-olide on the growth of MCF-7 cells revealed that the 2, 14-dihydrox-7-drimen-12, 11-olid*e* had a strong cytotoxic effect on these cells as indicated by its low $IC_{50,}$ 41.7 µg/ml.

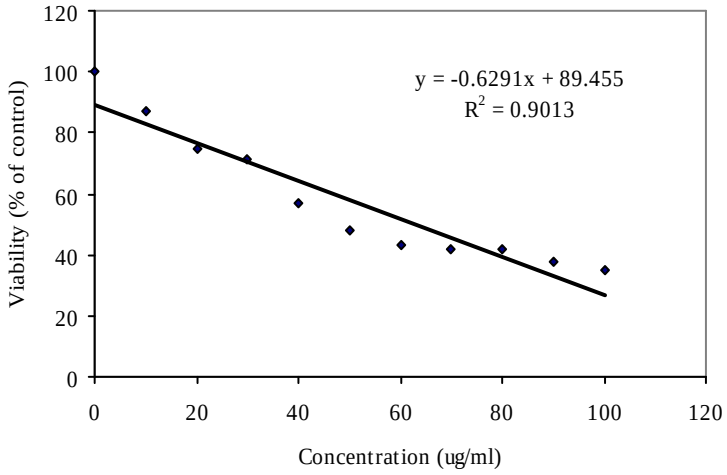

Figure 3: Cytotoxic effect of 2, 14-dihydrox-7-drimen-12, 11-olid*e* from *A. glaucus* against Hep-G2 cells using MTT assay

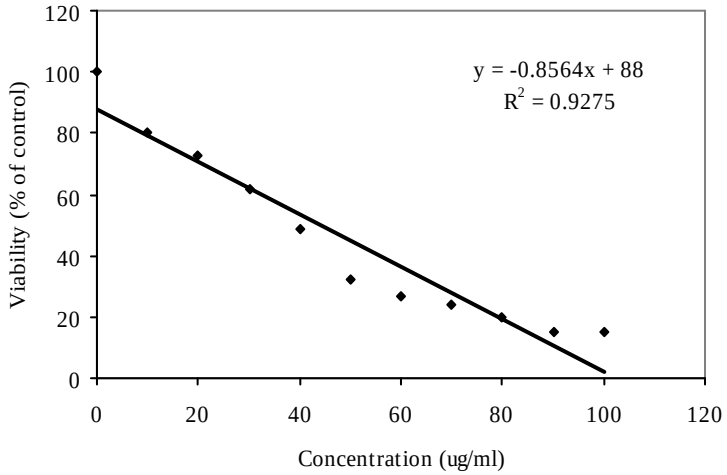

Figure 4: Cytotoxic effect of 2, 14-dihydrox-7-drimen-12, 11-olid*e* from *A. glaucus* against MCF-7 cells using MTT assay

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