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Original Article

Antifungal and cytotoxic constituents from the endophytic fungus *Penicillium* sp.

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ABSTRACT

New mellein derivative; 4-methylmellein (**1**) along with five known compounds: 4-hydroxymellein (**2**) and 6-hydroxymellein (**3**), tyrosol (**4**), cervesterol (**5**), and stigmast-4-ene-3-one (**6**) were isolated from the ethyl acetate extract of the endophytic fungus *Penicillium* sp. isolated from the leaf of *Senecio flavus* (Asteraceae). Structures were established by 1D and 2D NMR, in addition to UV, IR and MS spectrometry. Compounds (**3**, **4** and **6**) were isolated for the first time from the genus *Penicillium*. Compounds **1**–**4** showed antifungal activity against *F. oxysporum* and variable activities against *A. flavus* and the yeast *C. albicans*. Compounds **1**–**3** showed cytotoxic activity against MCF-7 cell line.
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1. Introduction

Since the inspiring isolation of taxol from *Pestalotiopsis microspora*, the endophytic fungus of the yew tree *Taxus wallichiana*, many researches have been constructed to explore plant endophytes as a source of novel and biologically active natural products [1]. During the period from 1993 to 2001, literatures have reported 1500 compounds, more than half these compounds showed antibacterial, antifungal and antitumor activities [2]. Although the function of these products is not precisely known, they thought to play a role in the chemical defense and communication [3]. Many of them have been suggested to act as pheromones or repellents [4]. *Penicillium* is a large fungal genus, widely spread in most terrestrial and marine environments as well as food contaminants or food ingredients as cheese or sausage, it comprises more than 200 described species [5]. Vast array of active secondary metabolites have been isolated from *Penicillium* species, including naphthalenoids[6], alkaloids, mycotoxins, polyketides [7], steroids [8,9]. These metabolites have been reported to be allied with antioxidant, antiviral [10–12], antibacterial [13,14], antifungal [15], immune-suppressants, cholesterol-lowering activities [16]. New bioactive metabolites continue to be discovered from this genus nowadays, reflecting its current importance as a source of novel bioactive molecules [6].

The present work was constructed to investigate the metabolites of *Pencillium* sp. the endophyte secluded from the inner tissue of the leaf of *Senecio flavus* (Decne.) (Asteraceae), where the new 4-methylmellein have been isolated, as well as 4-hydroxymellein, 6-hydroxymellein, tyrosol, cervesterol, and stigmast-4-ene-3-one (Fig. 1).

2. Experimental

2.1. General

A Perkin-Elmer polarimeter 341 LC Model was utilized for the measurement of optical rotation (Perkin-Elmer, Waltham, MA, USA). UV spectra measurement was performed on a 1601 UV/VIS Shimadzu spectrophotometer using MeOH (Shimadzu, Kyoto, Japan). Infrared-400 Shimadzu spectrophotometer was utilized for IR measurement (Shimadzu, Kyoto, Japan). HRESIMS was carried out on an Orbitrap LTQ (ThermoFinnigan, Bremen, Germany). ESIMS spectra were achieved using LCQ DECA spectrometer (ThermoFinnigan, Bremen, Germany). A Bruker Avance DRX 400 MHz spectrometer was used for measuring 1D and 2D NMR spectra (Bruker BioSpin, Billerica, MA, USA). Chromatographic separations were achieved on sephadex LH-20 (0.25–0.1 mm) and silica gel 60 (0.04–0.063 mm). TLC analysis was carried out on silica

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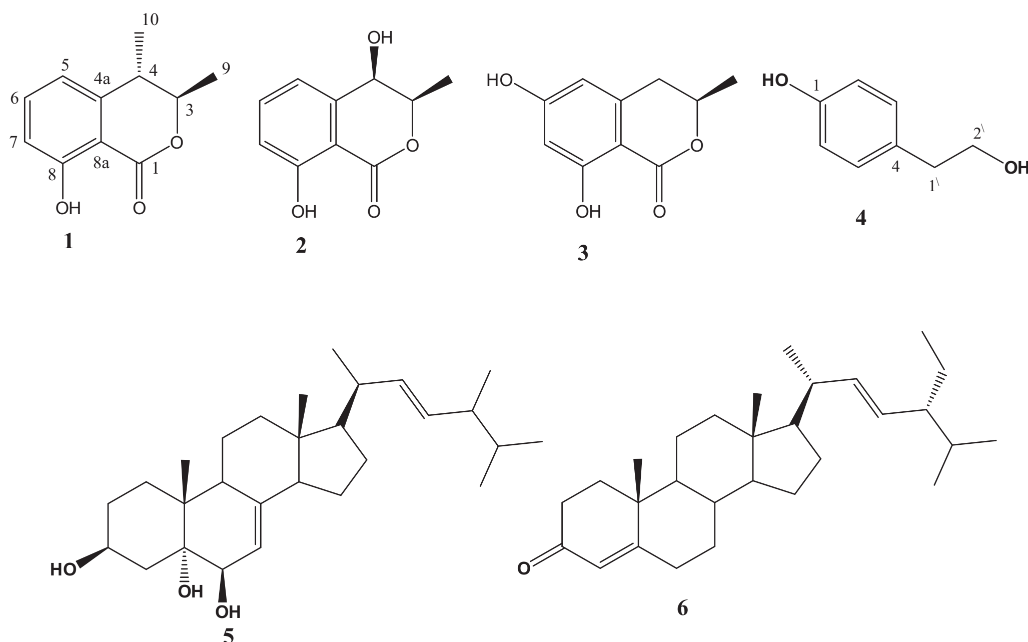

Fig. 1. Structure of the isolated compounds **1**–**6**.

60 F_{254} TLC pre-coated plates (0.2 mm) (Merck, Darmstadt, Germany).

2.2. Plant material

Senecio flavus (Decne) (Asteraceae) was collected in April 2015 from the plant wildly growing at campus of Al-Azhar University. The plant sample was authenticated by Prof. Dr. A.A. Fayed, Prof. of Plant Taxonomy, Faculty of Science, Assiut University, Assiut, Egypt. A herbarium specimen was kept at the Department of Pharmacognosy herbarium, Al-Azhar University (SF/4/2015).

2.3. Isolation of fungal material

The endophytic fungus *Penicillium* sp. was isolated from the healthy surface sterilized leaves of *S. flavus*. The leaves were first washed with water, then immersed in 70% aqueous ethanol for 1 min, and rinsed in sterile water. Finally they were sliced into 2×2 cm pieces, with 3 to 4 pieces deposited on petri dish containing potato dextrose agar plates (PDA, Difco), having gentamicin sulfate (200 mg/L) as antibacterial agent to prohibit bacterial growth and plates were incubated at room temperature. The fungus under investigation was found to grow exclusively out of the leaf tissue. From the growing cultures pure strains of *Penicillium* sp. were isolated by repeated re-inoculation on PDA plates [8].

2.4. Identification of the fungal strain

The fungus was identified according to the characteristics of the culture morphology and spores features using light microscopy (CX31RBSF, Olympus). The fungi were identified by Ph.D. Mohamad Taha, Department of Microbiology, Al-Azhar University, Assiut, Egypt, and deposited with the culture code (PES/4/2015).

2.5. Cultivation

Mass growth of the fungus for the isolation and identification of metabolites was carried out in 12 Erlenmeyer flasks (1 L). The fungus was grown on rice solid medium (100 mL of distilled water added to 100 g commercially available rice and kept overnight prior autoclaving) at room temperature under static conditions for 30 days.

2.6. Metabolites isolation

The culture was extracted using EtOAc and concentrated under vacuum. The obtained extract was suspended in distilled water (200 mL) and fractionated between *n*-hexane and 90% MeOH. The MeOH extract (6.1 g) was separated by VLC (silica gel 60), eluted successively with *n*-hexane, $CHCl_3$, EtOAc and MeOH, which were independently concentrated to get FV-1 (0.6 g), FV-2 (1.3 g), FV-3 (2.4 g) and FV-4 (1.4 g). FV-2 fraction was chromatographed over silica using *n*-hexane:EtOAc gradient, 100 mL fractions were gathered and checked by TLC to get seven sub-fractions: FV2-1 to FV2-7. Subfraction FV2-2 (70 mg) was chromatographed on silica gel using *n*-hexane:EtOAc (9:1) as an eluent to yield compounds (**5**) and (**6**) (2.8 and 2.4 mg, respectively). Fraction FV-3 was chromatographed on sephadex eluted with MeOH to get 5 subfractions FV3-1 to FV3-5. The FV3-2 (23 mg) was chromatographed on silica gel eluted with $CHCl_3$:MeOH gradient, where compound (**4)** (3.6 mg) was obtained. FV3-3 (30 mg) was chromatographed on silica gel eluted with $CHCl_3$:MeOH gradient to yield compounds (**2**) and (**3**) which were individually purified on silica ($CHCl_3$: MeOH, 8:2) to yield (**2**) (2.7 mg) and (**3**) (3.1 mg). FV3-4 (21 mg), was handled similar to FV3-3 to yield (**1**) (2.6 mg). The remaining fractions were kept for further study.

*4-Methylmellein (**1**):* White amorphous solid. $[\alpha]_D^{20}$ −15.6 (MeOH). UV (MeOH): λ_{max} 218, 245, 315 nm. IR(KBr): 3436, 2978, 1729, 1651, 1056 cm^{-1}. NMR data ($CDCl_3$, 400 and 100 MHz) see Tables 1 and 2. HRESIMS *m/z* 193.0861 (calcd for 193.0865 $[M+H]^+$, $C_{11}H_{12}O_3$).

*4-Hydroxymellein (**2**):* White amorphous solid. $[\alpha]_D^{20}$ −39.6 (MeOH). EIMS *m/z* 195 $[M+H]^+$. UV (MeOH): λ_{max} 215, 265, 320 nm. IR(KBr): 3465, 2981, 1731, 1665, 1610, 1516 cm^{-1}. NMR data ($CDCl_3$, 400 and 100 MHz), Tables 1 and 2.

*6-Hydroxymellein (**3**):* White amorphous solid. $[\alpha]_D^{20}$ −18.4 (MeOH). EIMS *m/z* 194 $[M]^+$, 176, 150, 148 and 130. UV (MeOH):

Table 1
^{1}H NMR of compounds **1–3** ($CDCl_3$, 400 MHz).

No.	**1** δ_H (m, J in Hz)	**2** δ_H (m, J in Hz)	**3** δ_H (m, J in Hz)
1	–	–	–
3	4.53 (m, 1 H)	4.61 (m, 1 H)	4.63 (m, 1 H)
4α	3.18 (m, 1 H)	4.47 (br.s, 1 H)	2.79 (br.s, 1 H)
4β			2.81 (m, 1 H)
4a	–	–	–
5	6.53 (dd, J = 7.7, 2.1 Hz, 1 H)	6.85 (d, J = 7.3 Hz, 1 H)	6.27 (d, J = 2.1 Hz, 1 H)
6	7.26 (dd, J = 7.7, 7.5 Hz, 1 H)	7.43 (dd, J = 7.3,7.5 Hz, 1 H)	–
7	6.21 (dd, J = 7.5, 2.1 Hz, 1 H)	6.95 (d, J = 7.5 Hz, 1 H)	6.10 (d, J = 2.1 Hz, 1 H)
8	–	–	–
8a	–	–	–
Me-9	1.39 (d, J = 6.5 Hz, 3 H)	1.50 (d, J = 6.5 Hz, 3 H)	1.49 (d, J = 6.6 Hz, 3 H)
Me-10	1.28 (d, J = 6.1 Hz, 3 H)	–	–
8-OH	11.22 (br.s)	11.10 (br.s)	11.30 (br.s)

Table 2
^{13}C NMR of compounds **1–3** ($CDCl_3$, 100 MHz).

No.	δ_C **1**	δ_C **2**	δ_C **3**
1	170.1	169.2	169.5
3	80.1	78.3	76.1
4	37.6	67.3	34.1
4a	144.2	140.4	142.3
5	117.1	118.7	106.7
6	136.1	136.8	164.6
7	113.7	101.3	101.5
8	161.4	162.3	164.3
8a	108.1	107.0	101.0
Me-9	19.2	16.1	19.7
Me-10	17.2	–	–

λ_{max} 218, 270, 305 nm. IR(KBr): 3430, 1655, 1587, 1475 cm^{-1}. NMR data ($CDCl_3$, 400 and 100 MHz), Tables 1 and 2.

2.7. Antifungal activity

Sterile filter paper discs (6 mm diameter), were impregnated with 50 μg of the samples using methanol as the carrier solvent. The impregnated discs were then placed on agar plates previously inoculated with *Aspergillus flavus*, *Fusarium oxysporum* and *Candida albicans*. Solvent controls were run against each organism. After incubation at 37 °C for 24 h, the antifungal activity was recorded as clear zones of inhibition surrounding the disc (mm). Nystatin (10 μg/disc) was used as standard antifungal. All experiments were performed in triplicate and the activity was expressed as the mean of inhibition zone diameters (Table 3). Fungal strains were secured by Assiut University Mycology Center, Assiut, Egypt.

2.8. Cytotoxicity assay

The cytotoxicity was evaluated by the [3H] thymidine assay using breast cancer (MCF-7) and colon cancer (COLO-205) cell lines [17]. Doxorubicin (10 μg), was used as a positive control. The cell lines were obtained from Al-Azhar University Centre for Viral Research, Cairo, Egypt.

3. Results and discussion

Compound **1** was isolated as white amorphous solid. Its molecular formula was defined as $C_{11}H_{12}O_3$ by HRESIMS pseudo-molecular ion peak at m/z 193.0861 $[M+H]^+$ (calcd. for 193.0865), requiring 6 degrees of unsaturation, four of which were attributed to aromatic ring. The IR spectrum showed absorption bands at ν_{max} cm^{-1}; 3436 (hydrogen bonded phenolic hydroxyl), 2978 (aromatic) and 1651 (ester/lactone carbonyl). The ^{1}H NMR spectrum (Table 1), suggested the presence of trisubstituted aromatic ring as three ABX proton signals at δ_H 6.53 (dd, J = 7.7, 2.1 Hz, H-5), 7.26 (dd, J = 7.7, 7.5 Hz, H-6) and 6.21 (dd, J = 7.5, 2.1 Hz, H-7), and two secondary methyls at δ_H 1.39 (d, J = 6.5 Hz, CH_3-9) and 1.28 (d, J = 6.1 Hz, CH_3-10), in addition to a hydrogen bonded phenolic hydroxyl at δ_H 11.22 (br.s, 8-OH). The COSY (Fig. 2) and HSQC experiments have identified two spin systems; the first is the aromatic ABX protons, and $(CH_3)CH–CH(CH_3)$, as the two methyls δ_H 1.39/δ_C 19.2 and 1.28/17.2, being connected to the two multiplet methines at δ_H 4.53/δ_C 80.1 and 3.18/37.6, respectively. The ^{13}C-NMR/HSQC (Table 1), exhibited presence of 11 carbons assigned to four quaternary, two methyls, five methines. Further analysis of the ^{13}C spectrum identified a carbonyl δ_c 170.1 (C-1), six phenyls δ_c 108.1 (C-8a), 161.4 (C-8), 113.7 (C-7), 136.1 (C-6), 117.1 (C-5) and 144.2 (C-4a), in addition to an oxymethine δ_C 80.1 (C-3) and aliphatic methine δ_C 37.6 (C-4). The HMBC (Fig. 2), correlations of CH_3-10/C-3, H-3/C-1 and C-4, CH_3-9/C-4, H-4/C-4a, H-5/C-4a, C-4 and C-8a identified a lactone moiety connected with the aromatic ring at the quaternary carbons C-4a and 8a. HMBC correlations of the aromatic proton at δ_H 7.26 (H-6) with the oxygenated C-8 confirmed its position. These findings identified (**1**) as 4-methylmellein. The sign of optical rotation of **1** was the same as 3*R*-mellein [18], implying the stereochemistry at C-3 to be *R*

Table 3
Antifungal and cytotoxic activities of the isolated compounds (**1–4**).

Comp.	Antifungal activity (Zone of inhibition in mm)			Cytotoxicity assay against MCF-7 (ED_{50})
	A. flavus	*F. oxysporum*	*C. albicans*	
1	–	6	5	>10 μg/mL
2	7	7	6	6.1 μg/mL
3	8	6	–	8.3 μg/mL
4	–	6	–	–
Nystatin	12	17	–	–
Doxorubicin	–	–	–	1.8 μg/mL

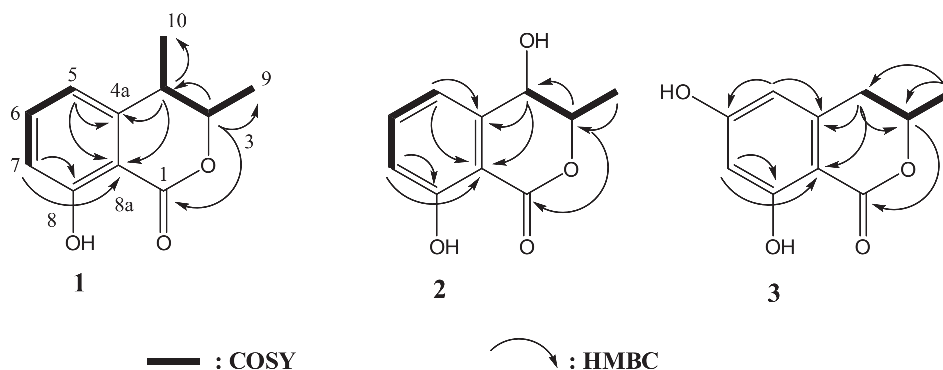

Fig. 2. Diagnostic COSY and HMBC correlations of compounds (**1**–**3**).

configuration. Absence of NEOSY correlations between CH_3-3 and CH_3-4, indicated that these methyls were in opposite directions to each other, which indicated that the relative configurations to be 3*R*,4*S* [19]. Comparison with the published data of gamahorin, previously isolated from the phytopathogenic fungus *Epichloe typhina* [20], revealed that both compounds only differed in the substituent at C-7, where **1** is lacking the hydroxymethylene moiety at C-7. Accordingly, compound **1** was identified as (3*R*,4*S*) 4-methylmellein, it was previously described as a catalytic hydrogenation derivative of oospolactone (3,4-dimethyl-8-hydroxyiso coumarin), the fungal metabolite isolated from the mycelium of *Oospora* sp. [21], but this is the first isolation as a natural product.

Compound **2** was isolated as white amorphous solid and its EIMS analysis revealed molecular ion peak at *m/z* 195 $[M+H]^+$. Except absence of the aliphatic methine (δ_H 3.18) and the presence of two oxymethines at δ_H 4.61 and 4.47, the ^1H NMR (Table 1) spectrum was comparable to **1**. The mellein nucleus was evident as presence of the ABX three doublets protons at δ_H 6.85 (d, *J* = 7.3 Hz, H-5), 6.95 (d, *J* = 7.5 Hz, H-7) and 7.43 (dd, *J* = 7.5, 7.3 Hz, H-6), phenolic hydroxy at δ_H 11.16 and the secondary methyl at δ_H 1.50 (d, *J* = 6.5 Hz, CH_3-9). The COSY experiment (Fig. 2), confirmed a spin system of $OHCH\text{-}CH\text{-}CH_3$ as the cross peaks of the C$\underline{H}_3$-9/C$\underline{H}$-3, which in turn coupled to a hydroxymethine (HOC$\underline{H}$-4), identifying 1-methyl-2-hydroxy-ethyl moiety. The ^{13}C NMR/HSQC (Table 2) showed resonance of ten carbons: one methyl, two oxymethines and six aromatic carbons, and a carbonyl. The HMBC experiment (Fig. 2), confirmed the mellein structure as the cross peaks between H-3 (δ_H 4.61) with the carbonyl (δ_C 169.2, C-1), the cross peaks between the hydroxymethine (H-4) with the 2 aromatic quaternary carbons δ_C 107.0 (C-8a) and 140.4 (C-4a), and cross peak of the doublet proton at δ_H 6.95 (H-7) with the oxygenated aromatic carbon δ_C 162.3 (C-8). Based on the previous data and comparison of the spectral data with literatures, **2** was identified as (3*R*,4*R*)-4-hydroxymellein [22]. It was previously isolated from *Pencillium* sp. secluded from *Alibertia macrophylla* (Rubiaceae) [23], and from the marine fungus *Microsphaeropsis* sp. secluded from the marine sponge *Myxilla incrustans (H)* [24].

Compound **3** was isolated as white amorphous powder, its ESIMS analysis revealed pseudomolecular ion peak at *m/z* 195 $[M+H]^+$. The ^{13}C NMR (Table 2)/HSQC spectra showed the presence of ten carbons associated with the mellein nucleus. The ^1H NMR spectrum (Table 1) was similar to **2** except the absence of the hydroxymethine at δ_H 4.47, instead there was an additional aliphatic methylene at δ_H 2.79 (br.s, CH_2-4α) and 2.81 (1H, m, CH_2-4β). In addition to the presence of two *meta*-coupled aromatic protons at δ_H 6.10 and 6.27 (1H, d, *J* = 2.1 Hz, each) suggesting a hydroxyl group in between. These data proposed **3** to be 6-hydroxymellein. The COSY and HMBC experiments (Fig. 2), confirmed 6-hydroxymellein structure. Comparing the spectral data with literatures, confirmed compound **3** to be 6-hydroxymellein, that was previously isolated from *Aspergillus terreus* secluded from *Arabidopsis thaliana* [25], this is the first isolation from the genus *Penicillium*.

Compound **4** was isolated as yellowish oily residue. The IR showed absorption band assigned to a hydroxyl group at 3675 cm^{-1}. Its ESIMS spectrum showed pseudomolecular peak at *m/z* 139 $[M+H]^+$. The ^{13}C NMR spectrum indicated the presence of an oxygenated aromatic carbon, four aromatic methines, quaternary aromatic, aliphatic methylene, in addition to aliphatic oxygenated meythylene. The ^1H NMR spectrum confirmed the presence of *p*-substituted aromatic ring [two doublets at δ_H 6.91 (2H, m) and 6.65 (2H, m)], and a hydroxyl-ethyl moiety as the methylene 2.68 (2H, m) and oxymethylene δ_H 3.68 (2H, m). Comparison of the spectroscopic data with literatures has identified **4** as tyrosol, which was isolated from the marine fungus *Penicillium brevicompactum* [26], and it is widespread in fungi [27], but this is the first report from terrestrial *Penicillium* sp.

According to the spectral data and comparison with literatures compound **5** was identified as cerevisterol [28], which was previously isolated from *Penicillium* sp. secluded from *Mauritia flexuosa* [8]. Compound **6** was identified as stigmast-4-ene-3-one [29], which was previously isolated from *Aspergillus terrus* [30], but this is the first isolation from genus *Penicillium*.

Evaluating the antifungal activity of the compounds was carried out against *A. flavus*, *F. oxysporum* and *C. albicans* at concentration of 50 µg per disc, compounds **1**–**4** showed inhibition zones ranged from 6 to 8 mm (Table 3). Compound **2** was active against all the tested organisms, **1** showed activity against *F. oxysporum* and *C. albicans*. **3** was active against *A. flavus* and *F. oxysporum*. Finally, **4** was only active against *F. oxysporum*, while **5** and **6** were inactive against all the tested organisms. On evaluating the cytotoxic activity towards MCF-7 and COLO-205 cells, none of the tested compounds were active against COLO-205. Meanwhile **1**–**3** were active towards MCF-7, with the highest activity attributed for **2** followed by **3**, while **1** was the least active (Table 3).

4. Conclusions

From the rice solid culture medium of the endophyte *Penicillium* sp. secluded from the *Senecio flavus* (Asteraceae) fresh healthy leaf; three mellein derivatives (**1**–**3**) were isolated, of which 4-methylmellein **1** was a new natural product, in addition to three other compounds. Compounds **3**, **4** and **6** were isolated for the first time from the genus *Penicillium*. Structures were elucidated by extensive spectroscopic analysis. Biological evaluation conferred antifungal activity for compounds **1**–**4**, and cytotoxic activity for compounds **1**–**3**.

Conflict of interest

None.

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