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Short Communication

Antifungal metabolites from the fermentation broth of plant endophytic fungus *Pestalotiopsis photiniae*

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***Pestalotiopsis* genus, one of the most potential resources for biological active compounds, can produce most interesting compounds with broad activity spectrum. In the course of our research on bioactive metabolites of the genus *Pestalotiopsis* in China, the present study was undertaken to investigate the bioactive chemical constituents of the culture broth of *Pestalotiopsis photiniae* isolated from the branch of *Podocarpus macrophyllus* in Hainan, People's Republic of China, and this led to the isolation of three know compounds named guaidiol (1), ethyl everninate (2) and 10-norparvulenone (3). Compounds 1-3 displayed antifungal activity against three fungal strains including *Gibberelle zeae*, *Botrytis cinerea*, and *Phytophthora nicotianae*, with minimum inhibition concentration (MIC) values from 25.0 to 3.1 μg/ml (the positive control ketoconazole showed MIC values from 6.3 to 3.1 μg/ml).**

Key words: Entophytic fungi, *Pestalotiopsis photiniae*, antifungal activity.

INTRODUCTION

Pestalotiopsis species, belonging to the family of Amphisphaeriaceae, are broadly distributed in the world, occurring on a wide range of substrata (Wei et al., 2007; Li et al., 1996). Most of them are plant pathogens and some are saprobes in soil or in plant debris, which can produce different types of bioactive metabolites (Yang et al., 2012; Xu et al., 2011; Liu et al., 2011). During our ongoing chemical investigations of endophytic fungi as sources of new bioactive natural products, a subculture of *Pestalotiopsis photiniae* isolated from the Chinese podocarpaceae plant *Podocarpus macrophyllus* was grown in fermentation culture. Its ethyl acetate extract displayed significant antifungal activities against four fungal strains, *Gibberelle zeae*, *Botrytis cinerea*, *Phytophthora nicotianae* and *Monilia albican*. Bioassay-directed fractionation of this extract has led to the isolation of three known compounds guaidiol (**1**), ethyl everninate (**2**) and 10-norparvulenone (**3**). Details of the isolation, structural elucidation and antifungal activity of compounds **1-3** are reported herein (Figure 1).

EXPERIMENTAL SECTIONS

General

NMR spectra: Bruker AM-600 spectrometer; δ in ppm, J in Hz; Me_4Si as internal standard, measured in $CDCl_3$. FT-MS spectra: Bruker apex-ultra 7.0 T spectrometer in *m/z*. Column chromategraphy (CC): silica gel (200~300 mesh, Yantai Zhi Fu chemical Co., Ltd., P. R. China), Thin layer chromatography (TLC): silica gel GF_{254} plates (Yantai Zhi Fu chemical Co., Ltd, P. R. China) and Sephadex LH-20 gel (25~100 μm, GE Healthcare Co., Ltd., Sweden).

Material and cultivation conditions

Pestalotiopsis photiniae was isolated from the branch of *Podocarpus macrophyllus* in Hainan, P. R. China, in April, 2008, and identified by Prof. Jing-Ze Zhang, and assigned the accession number L328. The fungal strain was cultured on slants of potato dextrose agar (PDA) at 28°C for 7 days, and then inoculated into 500 ml Erlenmeyer flask containing 100 ml of PDA medium (20.0 g of glucose, 200.0 g of potato (peeled), 3.0 g of KH_2PO_4, 1.5 g of

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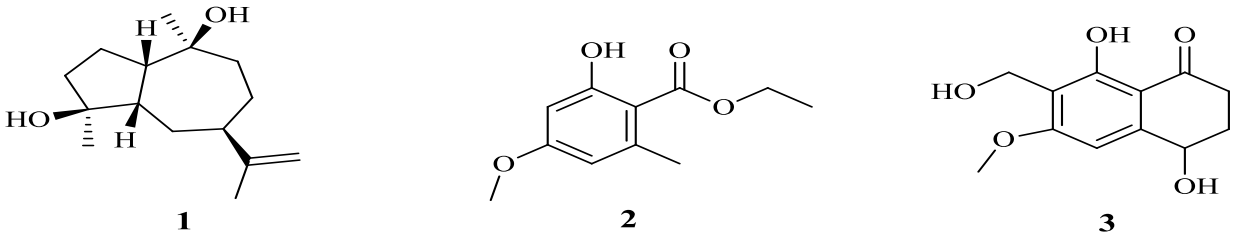

Figure 1. The structures of compounds **1-3**.

Table 1. Antifungal effect of compounds **1-3.**

Fungal strain	MIC (μg/ml)			
	1	**2**	**3**	**ketoconazole**
Monilia albican	>100	>100	>100	6.3
Gibberelle zeae	6.3	6.3	6.3	6.3
Botrytis cinerea	25.0	12.5	12.5	3.1
Phytophthora nicotianae	6.3	12.5	6.3	3.1

$MgSO_4$, 0.1 g of citric acid, and 10.0 mg of thiamin hydrochloride, in 1 liter of deionized H_2O). The final pH of the media was adjusted to 6.5 before sterilization. After 7 days of incubation at 28°C on rotary shakers at 150 rpm, 25 ml of culture liquid were transferred as seed into each 1000 ml Erlenmeyer flask containing 250 ml of PDA medium and static fermentation was carried out on a rotary shaker for 30 days.

Extraction and isolation

The fermented material was extracted with ethyl acetate. Evaporation of the solvent *in vacuo* gave a brown oily residue (18.0 g), which was subjected to a silica gel column chromatography employing a step gradient of petroleum ether/acetone (100:0, 98:2, 95:5, 90:10, 80:20, 50:50 (v/v)) to obtain six fractions *Frs. 1-6*. The antifungal activities of *Frs.1-6* were evaluated and the results showed that *Fr.5* exhibited significant antifungal activity. Then, *Fr. 5* (3.0 g) eluted with petroleum ether/acetone (80:20) was further fractionated by silica gel column chromatography using petroleum ether/ acetone gradient elution (from 20:1 to 1:1, v/v) to obtain seven fractions. *Fr. 5-2* (210 mg) eluted with petroleum ether/ acetone (10:1, v/v) was further purified by repeated CC (silica gel; petroleum ether/ethyl acetate 15:1 (v/v)) and Sephadex LH-20 (acetone) to afford compound **2** (9 mg). Compounds **1** (4 mg) and **3** (6 mg) were obtained from *Fr. 5-3* (150 mg) eluted with petroleum ether/acetone (8:1, v/v) after repeated CC (silica gel; chloroform/ acetone 20:1 (v/v)), Sephadex LH-20 ($CHCl_3/CH_3OH$, 1:1) and preparative TLC (chloroform/acetone, 6:1).

Antifungal assays

Antifungal assays were conducted in triplicate by following the National Center for Clinical Laboratory Standards (NCCLS) recommendations. The fungal strains, *Monilia albican*, *Gibberelle zeae*, *Botrytis cinerea*, and *Phytophthora nicotianae*, were preserved in our laboratory and were grown on potato dextrose agar. Targeted microbes (3-4 colonies) were prepared from broth culture (28°C for 72 h), and the final spore suspensions of fungal (in PDA medium) were 10^4 mycelial fragments/ml. Test samples (1 mg/ml as stock solution in DMSO and serial dilutions) were transferred to 96-well clear plate in triplicate, and the suspension of the test organisms was added to each well, achieving a final volume of 120 μl (ketoconazole was used as the positive control). After incubation, the minimum inhibitory concentration (MIC) was defined as the lowest test concentration that completely inhibited the growth of the test organisms (Ding et al., 2008).

RESULTS AND DISCUSSION

A phytochemical investigation on the fermentation broth of plant endophytic fungus *Pestalotiopsis photiniae* led to the isolation of three known compounds (Figure 1), their structures were elucidated by comparison of the spectral data with those reported data. To our knowledge, compounds 1-3 were isolated for the first time from this fungus, which displayed significant antifungal activity (Table 1) against three fungal strains including *Gibberelle zeae*, *Botrytis cinerea*, and *Phytophthora nicotianae*, with MIC values from 25.0 to 3.1 μg/ml (the positive control ketoconazole showed MIC values from 6.3 to 3.1 μg/ml). But those compounds were inactive against *Monilia albican* (MIC >100 μg/ml).

IDENTIFICATION

Guaidiol (**1**): $C_{15}H_{26}O_2$, White powder; ^{1}H NMR (600 MHz, Acetone-d_6): 2.00 (1H, m, H-1), 1.36, 1.80 (2H, m, H-2), 1.51, 1.69 (2H, m, H-3), 2.73 (1H, m, H-5), 1.40, 1.80 (2H, m s, H-6), 2.05 (1H, m, H-7), 1.55, 1.86 (2H, m, H-8), 1.60 (2H, m, H-9), 4.53, 4.63 (2H, s, H-12), 1.63 (3H, s, H-13), 1.11 (3H, s, H-14), 1.16 (3H, s, H-15); ^{13}C NMR (125 MHz, Acetone-d_6): 50.3 (d, C-1), 30.8 (t, C-2), 37.7 (t, C-3), 74.9 (s, C-4), 53.1 (d, C-5), 27.4 (t, C-6),

44.7 (d, C-7), 33.4 (d, C-8), 40.6 (t, C-9), 83.4 (s, C-10), 153.0 (s, C-11), 108.7 (t, **C**-12), 21.2 (q, C-13), 33.3 (q, C-14), 26.1 (q, C-15). It was identified as guaidiol by comparison of the spectral data with the literature (Syu et al., 1998).

Ethyl everninate (**2**): $C_{11}H_{14}O_4$, white powder; ^{1}H NMR (600 MHz, CD_3OD): 6.46 (1H, s, H-3), 6.64 (1H, s, H-5), 4.28 (2H, q, H-8), 1.21 (3H, t, H-9), 2.46 (3H, s, H-10), 3.68 (3H, s, H-11); ^{13}C NMR (125 MHz, CD_3OD): 115.0 (s, C-1), 158.5 (s, C-2), 108.6 (d, C-3), 159.5 (s, C-4), 96.3 (d, C-5), 137.7 (s, C-6), 169.3 (s, C-7), 60.6 (t, C-8), 13.1 (q, C-9), 18.3 (q, C-10), 54.8 (q, C-11). It was identified as ethyl everninate by comparison of the spectral data with the literature and with the standard sample by TLC (Sun et al., 1986).

10-Norparvulenone (**3**): $C_{12}H_{14}O_5$, White powder; ^{1}H NMR (600 MHz, CD_3OD): 2.65, 2.81 (2H, m, H-2), 2.04, 2.81 (2H, m, H-3), 4.80 (1H, dd, J = 3.9, 9.1Hz, H-4), 6.87 (1H, s, H-5), 4.66 (2H, s, H-9), 3.95 (3H, s, -OCH_3); ^{13}C NMR (125 MHz, CD_3OD): 204.6 (s, C-1), 36.3 (t, C-2), 33.0 (t, C-3), 68.9 (d, C-4), 151.4 (s, C-4a), 102.1 (d, C-5), 166.1 (s, C-6), 116.0 (s, C-7), 164.0 (s, C-8), 53.1 (t, C-9), 111.4 (s, C-8a), 56.8 (q, OCH_3). It was identified as 10-norparvulenone by comparison of the spectral data with the literature (Fukami et al., 2000).

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