Short Communication:

Bioactive Secondary Metabolites from the Endophytic Fungi *Alternaria* sp.

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Abstract: *Shifting the drug discovery from plants to microorganisms due to minimizing the ecological effects has led to the increasing work on finding bioactive metabolites from endophytic fungi, especially those isolated from medicinal plants growing in arid and semi-arid land. An endophytic fungi* Alternaria *sp. isolated from dry land was cultivated on solid rice media and then extracted with ethyl acetate. From the fungal ethyl acetate crude extract of* Alternaria *sp., compounds altertoxin I (**1**) and II (**2**), and stemphyltoxin III (**3**), were isolated based on the bioactivity assay. The structures of compounds **1**-**3** were identified using UV, NMR, and mass spectral data and compared to those spectral data reported in the literature. The absolute configuration was determined to be the same as the previous report based on the comparison with the optical rotation value. The three compounds showed strong cytotoxic activity, with altertoxin II being the most active ones having the inhibition of growth observed at 94.4% against murine lymphoma L5178Y cell line at 10 µg/mL. The activity of altertoxin II (**2**) was probably contributed by the epoxytetralone moiety. The finding of this study still confirms the importance of endophytic fungi as sources of bioactive molecules.*

Keywords: *endophytic fungi; cytotoxic; altertoxin I and II; stemphyltoxin III*

■ INTRODUCTION

The increased emergence of multidrug-resistant requires new, effective, and less toxic drugs for curing the disease in humans, animals, and plants. Endophytic fungi have provided novel structure and important bioactive natural products and are constantly explored for their significant biological activity [1-2]. Various novel bioactive secondary metabolites having antibacterial, antiviral, cytotoxic, antifungal, and anticancer activities have been reported [3-8].

Several bioactive metabolites, including antibacterial and anticancer compounds, have been reported from endophytic fungi associated with plants that grow in the dry land. For example, diaporthemin A and B as well as flavomanin dimethyl ether were isolated from the endophytic fungi of *Diaporthe melonis* associated with medicinal plant of *Annona squamaosa* growing in the dryland of Timor [9]. In addition, a whitening agent kojic acid was also reported to be produced by endophytic fungi *Aspergillus flavus* from several medicinal plants growing in the dryland of Timor, including *Annona squamosa*, *Catharanthus roseus,* and *Curcuma domestica* [7]. Furthermore, antibacterial metabolites neosartorin and (-) palitantin were

produced by endophytic fungi *Aspergillus fumigatiaffinis* associated with the plant *Tribulus terrestris* growing in the arid land [10].

Although fungi have been known for producing antibiotic compounds such as penicillin, the isolation of anticancer compounds has been well documented in several reports [11-12]. The anticancer compound radicicol was produced by the endophytic fungus *Chaetomium Chiversii* (Chaetomiaceae) isolated from the stem tissue of Mormon tea (*Ephedra fasciculata* A. Nels) growing in the Sonoran desert [13]. PM181110, a depsipeptide isolated from *Phomopsis glabrae* associated with the leaves of *Pongamia pinnata* (family Fabaceae), had pronounced anticancer activity against 40 human cancer lines *in vitro* and *in vivo* [14]. Moreover, anticancer drugs originally from plants such as paclitaxel, vinca alkaloid vincristine and vinblastine, campthotecin, and podophyllotoxin were recently reported more common to be found in endophytic fungi as well. As a part of our studies on finding bioactive metabolites by endophytic fungi from arid land, we investigated the extract of fungal endophytes *Alternaria* sp. that showed cytotoxic activity when tested against L5178Y, a cell line of murine lymphoma. Further separation based on the bioactivity resulted in the isolation of cytotoxic compounds **1**-**3**.

■ EXPERIMENTAL SECTION

Materials

The endophytic fungus *A. fumigatiafinis* was isolated from fresh, healthy leaves of *Tribulus terrestris* (Zygophyllaceae). Malt agar medium containing chloramphenicol (medium composition: 15 g/L malt extract, 15 g/L agar, and 0.2 g/L chloramphenicol in distilled water, pH 7.4–7.8) was used for the cultivation of the endophytic fungi. Silica gel 60 M (230–400 mesh) was purchased from Macherey-Nagel GmbH & Co. KG, Dueren, Germany and while Sephadex LH-20 was ordered from Sigma. All solvents were freshly distilled.

Instrumentation

The NMR analysis was performed using Bruker NMR spectrometer ARX 500. Agilent Finnigan Thermoquest LCMS HP1100 LCQ Deca XP was used for obtaining the mass spectral data, while the value of optically active rotation was measured with MC polarimeter of Perkin-Elmer-241. The HPLC data were obtained from Dionex P580 with PDA detector monitoring at 235 and 254 nm. Malt agar medium added with chloramphenicol was used to isolate and cultivate endophytic fungi.

Procedure

Fungi isolation and cultivation

The endophytic fungus was isolated from fresh leaves of *Tribulus terrestris*, identified using internal transcribed spacer (ITS) DNA with specific primer pairs of fungi, and then cultivated on solid rice media following the previous procedure reported [5-6,10]. The fungi were grown in solid rice media using three Erlenmeyer flasks. Rice (100 g) was put into an Erlenmeyer flask (1 L) and then added with 110 mL of distilled water. The media were kept overnight and autoclaved. The endophytic fungi from the Petri dish were cut into small segments and then added aseptically to Erlenmeyer flasks that already contain the solid rice media. The culture was then kept at room temperature for about 3–4 weeks. After the optimum growth, 300 mL of solvent (EtOAc) were transferred to the cultures and then were shaken overnight at 140 rpm. The mixture of cultures was then filtered with a Buchner funnel, and the solvent was removed with a Buchi rotary evaporator.

Fractionation and separation

The fungal extract obtained from ethyl acetate extraction (510 mg) was fractionated with Sephadex LH-20 using the mixture of CH_2Cl_2 and MeOH (1:1) as the mobile phase. The fraction VI (19 mg) was then separated by HPLC using a semi-preparative column (C-18 phenomenex) and the mixture of MeOH and H_2O (8:2) as the mobile phase (8:2) to yield compounds **1** (5 mg), **2** (3 mg) and **3** (4 mg).

Altertoxin (1). Reddish purple powder; $[\alpha]^{22}_D$ + 120.2 (c. 0.1, Acetone); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ = 12.7 (s, 1H), 12.3 (s, 1H), 8.06 (d, J = 8.85 Hz, 1H), 8.00 (d, J = 8.7 Hz, 1H), 7.04 (d, J = 8.8 Hz, 1H), 6.94 (d, J = 8.7 Hz, 1H), 4.51 (m, 1H), 3.01 (m, 1H), 3.00 (m, 1H), 2.98 (dd, J = 15.1, 6.0 Hz, 1H), 2.95 (d, J = 8.3 Hz), 2.85

(dd, *J* = 15.8, 4.08 Hz, 1H), 2.59 (m, 1H), 2.30 (d, *J* = 3.6 Hz, 1H). ^{13}C-NMR (DMSO-d_6, 125 MHz): δ = 204.9, 204.8, 161.5,160.7, 141.0, 138.9, 133.1, 132.7,125.0, 124.0, 118.3, 116.8, 115.9, 113.8, 69.4, 64.9, 51.4, 47.7, 34.9, 33.7; UV λ_{max} (PDA) (MeOH) 212.8, 257.8, 284.9 and 358.0 nm; ESIMS *m/z* ESI-MS m/z 351.2 $[M-H]^-$.

Altertoxin II (2). Dark brownish solid; $[\alpha]^{22}_D$ + 380.2 (c. 0.2, Acetone); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ = 12.7 (s, 1H), 11.8 (s, 1H), 8.17 (d, *J* = 8.8 Hz, 1H), 8.08 (d, *J* = 8.7 Hz, 1H), 7.07 (d, *J* = 8.8 Hz, 1H), 7.00 (d, *J* = 8.7 Hz, 1H), 5.6 (s, 1H), 4.38 (d, *J* = 3.6 Hz, 1H), 3.78 (d, *J* = 3.6 Hz, 1H), 3.59 (s, 1H), 3.13 (ddd, *J* = 18.2, 14.5, 5.0 Hz, 1H), 2.75 (m, 1H), 2.70 (m, 1H). ^{13}C-NMR (DMSO-d_6, 125 MHz): δ = 198.9, 195.2, 163.4,161.8, 137.3, 134.0, 133.0, 125.8, 124.1, 118.9, 115.9, 114.8, 114.0, 67.8, 56.2, 52.0, 44.7, 33.3, 31.1; UV λ_{max} (PDA) (MeOH) 214.9, 262.1 and 362.3 nm; ESIMS *m/z* 372.6 $[M+Na]^+$.

Stemphyltoxin III (3). Reddish brown amorph powder; $[\alpha]^{22}_D$ + 250.2 (c. 0.1, Acetone); ^{1}H-NMR (DMSO, 500 MHz-d_6): δ = 12.4 (s, 1H), 12.2 (s, 1H), 8.13 (d, *J* = 8.7 Hz, 1H), 8.13 (d, *J* = 8.7 Hz, 1H), 7.85 (d, *J* = 10.4 Hz, 1H), 7.08 (d, *J* = 8.7 Hz, 1H), 7.01 (d, *J* = 8.7 Hz, 1H), 6.53 (d, *J* = 10.4 Hz, 1H), 4.59 (d, *J* = 3.6 Hz, 1H), 3.75 (d, *J* = 3.6 Hz, 1H). ^{13}C-NMR (Acetone-d_6, 125 MHz): δ = 198.6, 191.1, 163.7, 161.9, 148.0, 140.9, 136.7, 133.8, 132.8, 129.4, 125.8, 125.1, 118.9, 117.5, 115.3, 113.3, 66.8, 57.4, 53.6, 43.5; UV λ_{max} (PDA) (MeOH) 214.9, 262.1 and 362.3 nm UV λ_{max} (PDA) (MeOH) 214.7, 261.7 and 361.3 nm; ESIMS *m/z* 347.0 $[M-H]^-$.

Bioassay

A microplate 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay was used to evaluate the cytotoxic property of the isolated compounds against the murine lymphoma (L5178Y) cell line as previously described [11-12]. Compounds **1**-**3** were tested at a standard concentration of 10μg/mL with kahalalide F (IC_{50} 4.3 μM) as the positive control. The culture media was brought into concentration contain 0.7 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated at 37 °C for 2 h. The cells were lysed with 5% of formic acid in isopropanol. The cell treated only with DMSO as vehicle control was 100% viable. In contrast, the reduction of cell viability from MTT as a viability marker was measured at 560 nm with a Wallac Victor2 multilabel counter.

■ RESULTS AND DISCUSSION

The fungal ethyl acetate extract from solid fermentation using rice was subjected to fractionation using column chromatography with sephadex LH-20. Fraction VI (16 mg) was further separated with HPLC to afford altertoxin I (**1**) and II (**2**) together with stemphyltoxin III (**3**). Altertoxin I (**1**) was isolated as reddish-purple powder having molecular ion peaks at 351.2 (base peak) from electrospray ionization LCMS negative mode indicating **1** has a weight of 352. Its UV absorbances showed λ_{max} at 212.8, 257.8, 284.9, and 357.9 nm in methanol. The molecular formula was determined to be $C_{20}H_{16}O_6$ confirmed from the spectroscopic data of mass and ^{1}H-NMR spectrum as well as supported by the presence of twenty carbons from the ^{13}C-NMR spectrum.

The ^{1}H-NMR spectrum showed two *ortho* aromatic signals resonating at δ_H 8.06 and 7.04 (H-1 and H-2, respectively) and δ_H 6.94 and 8.00 (H-11 and H-12, respectively). Two hydroxyl signals on aromatic rings were observed at δ_H 12.7 (3-OH) and at δ_H 12.3 (10-OH). Three methylene groups were observed at δ_H 2.59 and 3.00 (H-5), at δ_H 2.3 and 3.01 (H-6), as well as at δ_H 2.97 and 2.87 (H-8). Analysis of the COSY spectrum led to the identification of the spin systems C1–C2 and C6b–C7 (OH)–C8. HMBC signals from H-1, H-2, and OH-3 to C-3 and correlations from H-11, H-12, and OH-10 to C-10 confirmed the position of two aromatic hydroxyl groups located at C-3 and C-10, respectively. The ^{13}C-NMR spectrum revealed two carbonyl carbon atoms, two tetra-substituted aromatic rings, three carbons of methylene groups, and two carbons of methine group, including one bearing oxygen atom. The isolated compound **1** was confirmed as altertoxin I by comparing the UV, NMR data, and mass spectra with $[\alpha]_D$ value from the metabolite earlier reported from several *Alternaria* species [7,15-16].

Altertoxin II (**2**), obtained as a dark brownish solid, had a molecular ion base peak of 372.6 $[M+Na]^+$ from ESI-LCMS in positive mode, suggesting a molecular weight of 350 g/mol. The UV_{max} (MeOH) displayed at

214.9, 262.1, and 362.3 nm. Its molecular formula was determined as $C_{20}H_{14}O_6$ based on analysis of ESI-MS and ^{13}C-NMR spectrum and thus, having a molecular weight of two amu less than that of **1**. The ^{1}H NMR data of compound **2** (Table 1) was very similar to compound **1**, except that compound **2** possessed more additional signals resonating at δ 4.38/56.2 (CH-7) and 3.78/52.0 (CH-8), which were attributable to an epoxy ring. In addition, the ^{13}C-NMR signal observed at 67.8 (C-6a) suggested that **2** also contained an aliphatic carbon-bearing OH group. Compound **2** was identified as altertoxin II by comparing the spectroscopic data of NMR, mass spectra, and UV_{max} absorption with those early reported [7,16].

Stemphyltoxin III (**3**) was isolated as a reddish-brown amorph powder and displayed UV_{max} at 214.7, 261.7, and 361.3 nm. Electrospray ionization MS in negative mode revealed a molecular ion peak of 347.0 $[M-H]^-$ suggesting a molecular weight of 348, two amu less than that of **2**. The ^{13}C-NMR spectrum contained twenty carbons and thus offering the molecular formula of 3 as $C_{20}H_{12}O_6$. The ^{1}H-NMR spectrum of **3** (Table 1) had two more signals on olefinic protons at δ 6.53 (H-5) and 7.53 (H-6) compared with the ^{1}H-NMR spectrum of compound **2**. The NMR data of **3** possessed the signals containing an epoxy ring at δ 4.59/57.4 (C-7) and 3.75/53.6 (H-8) together with two AX *ortho* aromatic spin systems with firsts system observed at δ_H 8.13 and 7.08 (H-1 and H-2, respectively), while the second system is appearing at δ_H 7.01 and 8.13 (H-11 and H-12, respectively). In addition,

Table 1. ^{1}H and ^{13}C-NMR data of compounds **1**-**3**

No	Altertoxin I[a]		Altertoxin II[b]		Stemphyltoxin III[b]	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	8.06 d 8.85	133.1	8.17 d 8.8	134	8.13 d 8.7	132.8
2	7.04 d 8.8	118.3	7.07 d 8.8	118.9	7.08 d 8.7	118.9
3		161.5		163.4		161.9
3a		113.8		114		113.3
4		204.9		198.9		198.6
5eq	2.59 m	33.7	2.75 m	31.1	6.53 d 10.4	129.4
5ax	3.01 m		3.13 ddd 5.0, 14.5, 18.2			
6ax	2.3 d 3.6	34.9	2.7 m	33.3	7.85 d 10.4	148
6eq	3.0 m					
6a		69.4		67.8		66.8
6b	2.95 d 8.3	51.4	3.59 s	44.7		43.5
7	4.51 m	64.9	4.38 d 3.6	56.2	4.59 d 3.6	57.4
8	2.98 dd 15.1, 6	47.7	3.78 d 3.6	52	3.75 d 3.6	53.6
	2.85 dd 15.8, 4.8					
9		204.8		195.2		191.1
9a		116.8		114.8		115.3
9b		138.9		137.3		140.9
10		160.7		161.8		163.7
11	6.94 d 8.7	115.9	7.00 d 8.7	115.9	7.01 d 8.7	117.5
12	8.00 d 8.7	132.7	8.08 d 8.7	133	8.13 d 8.7	133.8
12a		125		125.8		125.1
12b		124		124.1		125.8
12c		141		134		136.7
3-OH	12.7 s		11.8 s		12.2 s	
10-OH	12.3 s		12.7 s		12.4 s	
6a-OH			5.6 s			

[a]DMSO [b]Acetone

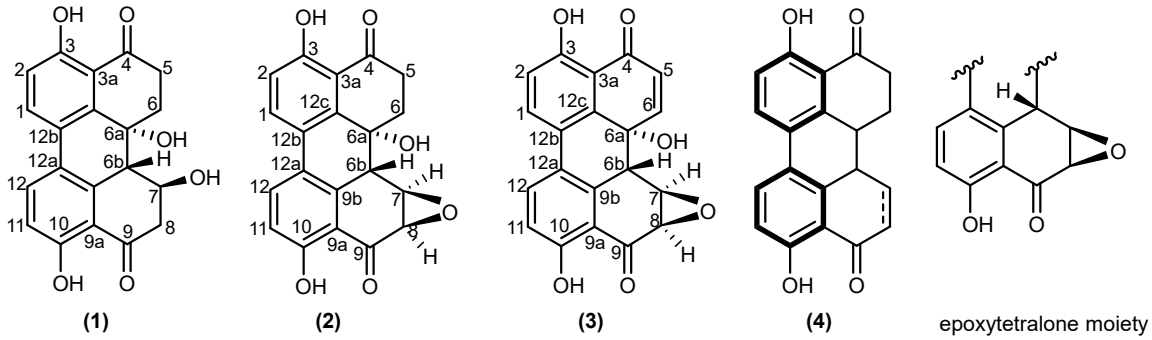

Fig 1. Structure of altertoxin I (**1**), alteroxin II (**2**), stemphyltoxin III (**3**), biphenyl scaffold (**4**)

Table 2. Cytotoxicity assay of compound **1-3** from the fungus *Alternaria* sp.

No	Compound (10 µg/mL)	Percentage (%) of L5178Y growth inhibition
1	Altertoxin I	63.8
2	Altertoxin II	94.4
3	Stemphyltoxin III	85.9
Control	Kahalide F	IC_{50} 4.3 µM

two signals related to aromatic hydroxyl groups were observed at δ_H 12.2 for 3-OH and at δ_H 12.4 for 10-OH. All the spectroscopic data of **3** were identical to those previously reported for stemphyltoxin III (**3**) isolated from the fungus *Alternaria alternata* [17-18].

The three isolated metabolites (**1**-**3**) were derived from the biphenyl scaffold (**4**), with their absolute configuration has been determined using ECD calculation [19]. The high positive value of optical rotation observed here was in agreement with those previously reported, and thus the structures of compounds **1**-**3** were depicted in Fig. 1. Altertoxin II, together with Altertoxin IV had previously been reported from an endophytic fungus *Alternaria tenuisima,* which is also isolated from *Tribulus terestris*. This medicinal plant is usually found in the semi-arid region [20]. Moreover, endophytic fungi *Alternaria* species had also been isolated from the medicinal plant *Tinospora cordifolia* (Willd.) Miers growing in semi-arid land and *Quercus emoryi* (Emory oak) from arid land [8]. This finding might suggest that endophytic fungus *Alternaria* might assist plants growing in dry and warm regions by producing cytotoxic metabolites (mycotoxin) as chemical defenses for their adaptation.

Compounds **1**-**3** were tested for their cytotoxic properties against L5178Y mouse lymphoma cells. All the tested compounds **1**-**3** could inhibit the growth of L5178Y cancer cells up to 94.4% at the concentration of 10 µg/mL (Table 2). Compound (**2**) was the most active compound. Altertoxin II (**2**) isolated from *Alternari tenuisima* associated with the medicinal plant *Tribulus terestris* had been known to have moderate anticancer activity when tested against PC-3 cancer cell line [20]. Interestingly, altertoxin II was found highly effective against Ewing sarcoma, while altertoxin I was reported not active recently [21]. Thus, the anticancer activity of altertoxin II found in this study and also supported by the specific activity reported in the literature may provide altertoxin II as a valuable chemical probe for lead development.

■ CONCLUSION

The endophytic fungus *Alternaria* sp. was cultivated on solid rice media and then extracted with ethyl acetate. Three fungal metabolites were isolated from the ethyl acetate extracts, including altertoxin I, II, and stemphyltoxin III. Altertoxin II was the most active compound against L5178Y mouse lymphoma cell, followed by altertoxin III, while altertoxin I was the least active compound in this study. The higher cytotoxic activity of compounds **2** and **3** was probably due to the presence of the epoxy ring. Thus, this SAR information

may provide valuable information for the development of altertoxin II as a lead compound. The finding of this study still confirms the importance of endophytic fungi as sources of bioactive molecules.

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