Chemistry of Natural Compounds, Vol. 50, No. 3, July, 2014 [Russian original No. 3, May–June, 2014]

ANTIMICROBIAL METABOLITES FROM THE ENDOPHYTIC FUNGUS *Aspergillus* sp. OF *Eucommia ulmoides*

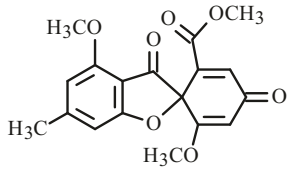
Hongchi Zhang,[1*] Rui Liu,[1] Feng Zhou,[1] Runmei Wang,[1] Xiaocui Liu,[1] and Hangtao Zhang[2]

Endophytic fungi are eukaryotic organisms that live inside plant tissues and are usually specific at the host species level [1]. They have proven to be a rich source of novel organic compounds with interesting biological activities and a high level of biodiversity [2]. In the course of our search for biologically active metabolites from endophytic fungi from Chinese medicinal plants, a subculture of an isolate of *Aspergillus* sp., obtained from roots of *Eucommia ulmoides*, was cultivated on potato dextrose agar (PDA). An ethyl acetate extract of the culture showed significant antimicrobial activity. This prompted us to carry out secondary metabolites studies on this fungus, which resulted in the isolation of seven compounds. Herein we describe the isolation, structural elucidation, and antimicrobial activities of its secondary metabolites.

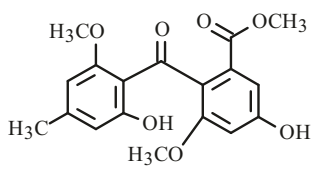
The fungal strain ER15 was isolated from the roots of *Eucommia ulmoides*, collected in the Qinling Mountains, Shaanxi Province, China, on September 10, 2010. The fungus was identified as a member of the genus *Aspergillus* by DNA amplification and sequencing of the ITS region. The fungal strain has been preserved at the Shanxi Datong University, Shanxi Province, China.

Starter cultures were maintained on PDA medium at 28°C for 7 days. Plugs of agar supporting mycelial growth were cut and transferred aseptically to 1000 mL Erlenmeyer flasks containing 400 mL of liquid Czapek medium at 28°C on a rotary shaker set to 120 r/min for 15 days. The fungal culture (60 L) was filtered through cheesecloth. The filtrate was concentrated to 10 L below 60°C and then extracted five times with ethyl acetate (15 L). The dried mycelium (55°C, 95 g) was extracted three times with methanol (4 L). All extracts were concentrated at reduced pressure to afford 12.1 g of a crude extract. The crude extract was separated into eight fractions (A–H) on silica gel (300 g) using gradients of ethyl acetate–methanol (1:0, 100:1, 50:1, 20:1, 10:1, 5:1, 2:1, 0:1). Fraction A was separated by silica gel column chromatography eluting with petroleum ether–ethyl acetate (1:2) to give pure compound **1** (87 mg), together with crude compound **2**. The crude compound **2** was then recrystallized from ethyl acetate–methanol to give the pure compound **2** (11 mg). Fraction B was separated by column chromatography on silica gel with a gradient of ethyl acetate in petroleum ether to give five subfractions (B1-5). Fraction B3 was further separated by silica gel column chromatography and eluted with a gradient of ethyl acetate in petroleum ether and Sephadex LH-20 with ethyl acetate–methanol (1:1) to give compounds **3** (118 mg), **4** (27 mg), and **5** (19 mg). Similarly, compounds **6** (10 mg) and **7** (37 mg) were isolated from fraction B4 after elution with a gradient of ethyl acetate in petroleum ether and Sephadex LH-20 with ethyl acetate–methanol (1:1).

The secondary metabolites were identified as ergosterol (**1**) [3], cerevisterol (**2**) [4], 5-hydroxymethylfuran-3-carboxylic acid (**3**) [5], 5-methoxymethylfuran-3-carboxylic acid (**4**) [6], allantoin (**5**) [7], trypacidin (**6**) [8], and monomethylsulochrin (**7**) [9] by comparison of their spectral data with the reported data in the literature.



6



7

1) College of Life Science, Shanxi Datong University, 037009, Datong Shanxi, P. R. China, e-mail: zhanghclw@163.com; 2) Institute of Health in Ordnance Industry, 710065, Shaanxi, P. R. China. Published in *Khimiya Prirodnykh Soedinenii*, No. 3, May–June, 2014, pp. 454–455. Original article submitted November 21, 2012.

 0009-3130/14/5003-0526

TABLE 1. Antimicrobial Activity of Secondary Metabolites of Endophytic Fungus ER15 from *Eucommia ulmoides*

Strains	Compound							Positive control	
	1	**2**	**3**	**4**	**5**	**6**	**7**	A	B
Gram Positive Bacteria									
Bacillus subtilis	64	64	2	8	–	16	–	0.5	
Staphylococcus aureus	32	64	1	4	256	16	256	1	
Staphylococcus faecalis	32	32	2	4	–	8	–	1	
Gram Negative Bacteria									
Escherichia coli	32	64	2	4	256	16	256	0.5	
Pseudomonas aeruginosa	128	32	1	2	–	32	–	0.5	
Salmonella typhimurium	128	428	1	2	–	32	–	1	
Fungi									
Candida albicans	128	256	4	8	16	128	–		4
Candida krusei	256	256	2	8	32	128	–		4
Fusarium solani	–	–	4	4	64	256	–		8
Penicillium chrysogenum	–	–	4	4	64	256	–		4
Aspergillus niger	–	–	2	8	16	–	–		4

Negative control A: DMSO; negative control B: the blank MHB – not active at concentration up to 256 μg/mL.
Positive control A: gentamicin as positive control for the antibacterial assay; positive control B: nystatin as positive control for the antifungal assay.

Ergosterol (1). Colorless needle, mp 155–157°C. ESI-MS *m/z* 397.2 $[M + H]^+$.

Cerevisterol (2). Colorless needle, mp 240–242°C. ESI-MS *m/z* 431.4 $[M + H]^+$.

5-Hydroxymethylfuran-3-carboxylic Acid (3). Colorless powder, mp 151–153°C. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 8.03 (1H, s, H-2), 6.35 (1H, s, H-4), 4.30 (2H, d, J = 6.0, H-7), 9.08 (1H, s, 3-COOH), 5.69 (1H, t, J = 6.0, 6.0, 7-OH). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 139.71 (C-2), 146.16 (C-3), 110.28 (C-4), 168.54 (C-5), 174.37 (C-6), 59.91 (C-7).

5-Methoxymethylfuran-3-carboxylic Acid (4). Colorless powder, mp 156–160°C. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 7.98 (1H, s, H-2), 6.29 (1H, s, H-4), 4.24 (2H, d, J = 6.0, H-7), 3.71 (3H, s, H-8), 9.03 (1H, s, 3-COOH). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 139.21 (C-2), 147.65 (C-3), 111.18 (C-4), 168.54 (C-5), 174.38 (C-6), 59.92 (C-7), 56.14 (C-8).

Allantoin (5). White powder, mp 228–230°C. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 8.07 (1H, s, 1-NH), 10.55 (1H, s, 3-NH), 6.89 (1H, d, J = 8.2, 4-NH), 5.80 (2H, s, 6-NH_2), 5.25 (1H, d, J = 8.2, H-4). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 157.21 (C-2), 62.86 (C-4), 174.06 (C-5), 157.80 (C-6).

Trypacidin (6). White powder, mp 197–198°C. ESI-MS *m/z* 345.2 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 6.46 (1H, s, H-5), 2.64 (3H, s, H-6a), 5.81 (1H, s, H-7), 7.20 (1H, d, J = 2.2, H-2′), 6.60 (1H, d, J = 2.2, H-4′), 3.91 (3H, s, 4-OCH_3), 3.69 (6H, s, 6′-OCH_3 and 5′-OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 83.96 (C-2), 190.44 (C-3), 107.95 (C-3a), 158.31 (C-4), 105.50 (C-5), 152.45 (C-6), 23.32 (C-6a), 105.77 (C-7), 173.85 (C-7a), 138.52 (C-1′), 137.34 (C-2′), 185.00 (C-3′), 103.20 (C-4′), 168.83 (C-5′), 163.11 (C-6′), 56.29 (4-OCH_3), 52.87 (6′-OCH_3), 56.87 (5′-OCH_3).

Monomethylsulochrin (7). Colorless powder, mp 179–182°C. ESI-MS *m/z* 369.2 $[M + Na]^+$. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 6.62 (1H, d, J = 2.2, H-4), 7.04 (1H, d, J = 2.2, H-6), 3.71 (6H, s, 7-OCH_3 and H-8), 6.48 (1H, s, H-3′), 6.08 (1H, s, H-5′), 2.31 (3H, s, H-7′), 3.39 (3H, s, H-9′), 13.01 (1H, s, 2′-OH). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 128.35 (C-1), 127.94 (C-2), 156.27 (C-3), 102.92 (C-4), 157.02 (C-5), 107.98 (C-6), 166.24 (C-7), 56.19 (C-8), 111.00 (C-1′), 164.28 (C-2′), 110.37 (C-3′), 148.18 (C-4′), 103.23 (C-5′), 160.94 (C-6′), 22.54 (C-7′), 199.76 (C-8′), 55.69 (C-9′), 52.31 (7-OCH_3).

The isolated compounds **1–7** were tested for their antifungal and antibacterial activity towards the tested microorganisms (Table 1). 5-Hydroxymethylfuran-3-carboxylic acid (**3**) and 5-methoxymethylfuran-3-carboxylic acid (**4**) showed potent antibacterial and antifungal activities. Moreover, ergosterol (**1**), cerevisterol (**2**), and trypacidin (**6**) inhibited growth of bacteria and fungi. Allantoin (**5**) showed mild antifungal activity but weak antibacterial activity. Monomethylsulochrin (**7**) showed only weak antibacterial activity and no antifungal activity. It is noteworthy that the activity of 5-hydroxymethylfuran-3-carboxylic acid is close to that of gentamicin, an antibacterial drug, and is stronger than that of nystatin, an antifungal drug.

The minimum inhibitory concentration (MIC) values of the microorganisms were determined as sensitivity to the compounds in biological evaluation. This experiment was performed by the method of serial dilutions [10]. These compounds were dissolved in DMSO (1.28 mg/mL) and then diluted in sterile Mueller–Hinton broth (MHB) to achieve concentrations ranging from 512 to 0.25 μg/mL. All the microorganisms grown in MHB for 18 h (bacteria) or 12 h (fungi) were adjusted to 10^7 CFU/mL for bacteria and 10^6 CFU/mL for fungi. One hundred microliters of each concentration of compound was mixed with 100 μL of each suspension and incubated at 37°C for 24 h (bacteria) or 28°C for 48 h (fungi). All determinations were performed in duplicate using gentamicin (in DMSO) as a positive control in antibacterial assays, and nystatin (in DMSO) in antifungal assays. DMSO and the blank MHB were used as negative control. The growth was observed both visually and by measuring the O.D. at 620 nm. The lowest concentration of test sample showing no visible growth was recorded as the minimum inhibitory concentration. For each compound, the assay was performed in triplicate to ensure reproducible results.

In the present study on bioactive secondary metabolites, seven compounds were isolated from endophytic fungus ER15 of *E. ulmoides*. The above findings demonstrate that the endophytic fungus ER15 of *E. ulmoides* is a promising source for antimicrobial secondary metabolites.

REFERENCES

1. G. A. Strobel, *Microbes Infect.*, **5**, 535 (2003).
2. G. Strobel and B. Daisy, *Microbiol. Mol. Biol. Rev.*, **67**, 491 (2003).
3. F. De Simone, F. Senatore, D. Sica, and F. Zollo, *Phytochemistry*, **18**, 1572 (1979).
4. S. Gupta, C. Montllor, and Y. S. Hwang, *J. Nat. Prod.*, **58**, 733 (1995).
5. A. Evidente, G. Cristinzio, B. Punzo, A. Andolfi, A. Testa, and D. Melck, *Chem. Biodiv.*, **6**, 328 (2009).
6. G. M. Cabrera, M. Julia Roberti, J. E. Wright, and A. M. Seldes, *Phytochemistry*, **61**, 189 (2002).
7. F. Yin, L. Hu, and R. Pan, *Chem. Pharm. Bull.*, **52**, 1440 (2004).
8. W. B. Turner, *J. Chem. Soc. Perkin Trans. 1*, **9**, 6658 (1965).
9. J. Y. Liu, Y. C. Song, Z. Zhang, L. Wang, Z. J. Guo, W. X. Zou, and R. X. Tan, *J. Biotechnol.*, **114**, 279 (2004).
10. J. M. Andrews, *J. Antimicrob. Chemother.*, **48**, 5 (2001).