SUPPLEMENTARY MATERIAL

Uncommon secondary metabolites from *Etlingera pavieana* rhizomes

Sirichan Tachai and Nuchnipa Nuntawong*

Department of Chemistry and Centre for Innovation in Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, Thailand

*Corresponding author. Tel.: +66 88 0026124; Fax: +66 53 892277.

E-mail address: nuchnipa@hotmail.com; nuchnipa.n@cmu.ac.th

Abstract

From the rhizomes of *Etlingera pavieana* (Pierre ex Gagnep.) R.M. Sm., four phenylpropens, (*E*)-3-(4-methoxyphenyl)prop-2-en-1-amine (**1**), (*E*)-4-methoxycinamaldehyde (**2**), (*E*)-4-methoxycinamic acid (**3**) and (*E*)-1-methoxy-4-(3-methoxyprop-1-enyl)benzene (**4**), together with two other compounds, (*E*)-((*E*)-3-(4-methoxyphenyl)allyl)3-(4-hydroxyphenyl)acrylate (**5**) and 4-methoxybenzoic acid (**6**) were isolated. This is the first report on the presence of all compounds in *Etlingera*. Compounds **1** and **5** have been previously synthesised, but this is the first report of their isolation from a natural source. Compound **5** exhibited weak activity against *Mycobacterium tuberculosis* with MIC 50.00 µg/mL and cytotoxic activity against the KB, MCF7 and NCI-H187 cells with IC₅₀ values of 25.11, 20.16 and 34.83 µg/mL, respectively.

Keywords: *Etlingera pavieana*; Zingiberaceae; (*E*)-3-(4-methoxyphenyl)prop-2-en-1-amine; (*E*)-((*E*)-3-(4-methoxyphenyl)allyl)3-(4-hydroxyphenyl)acrylate; cytotoxic activity.

Experimental

Plant material

The rhizomes of *E. pavieana* were collected from Chanthaburi province of Thailand. The voucher specimen was authenticated J. F. Maxwell, Taxonomist of the CMU Herbarium and deposited at the CMU Herbarium, Department of Biology, Faculty of Science, Chiang Mai University, Thailand with specimen number CMU01– Sirichan Tachai.

General experimental procedures

Melting points were determined on an Electrothermal apparatus and are uncorrected. IR spectra were obtained using a Tensor 27 FT-IR Spectrophotometer. ^1H - and ^{13}C -NMR spectra were recorded on a Bruker AVANCE 400 NMR spectrometer, operating at 400 and 100 MHz, respectively. Electron impact mass spectra were recorded with Agilent-HP 5973 mass spectrometer. TLC was carried out on precoated silica gel 60 F₂₅₄ (0.2 mm thick, Merck) plate. The TLC spots were visualized under UV light and by heating the plates after spraying with anisaldehyde-H₂SO₄ reagent. Silica gel column chromatography was performed on Merck silica gel 60 (finer than 0.063 mm).

Extraction and Isolation

The air dried powdered rhizomes of *E. pavieana* (1.35 kg) were successively extracted with *n*-hexane, CH₂Cl₂ and MeOH (3x3L) at room temperature. The extracts were concentrated under reduced pressure to obtain the *n*-hexane extract (brownish viscous oil, 9.66 g), the CH₂Cl₂ extract (brownish viscous oil, 10.76 g) and the methanol extract (dark brownish gummy, 75.30 g). The CH₂Cl₂ extract showed cytotoxic activities and were therefore investigated for active compounds. The CH₂Cl₂ extract (10.5 g) was chromatographed over silica gel and eluted with binary mixture of *n*-hexane–CH₂Cl₂ (95:5 to 0:100), CH₂Cl₂–EtOAc (95:5 to 0:100) and EtOAc–MeOH (95:5 to 50:50) which afforded 13 fractions (C₁–C₁₃). Precipitate from fraction C₁ (0.27g) was recrystallised in *n*-hexane–EtOAc to yield compound **1** (257.9 mg). Precipitate from fraction C₂ (0.31g) was recrystallised in *n*-hexane–EtOAc to yield compound **1** (310.0 mg). Filtrate of fractions C₁ and C₂ was combined and rechromatographed and eluted with CH₂Cl₂ to afford compound **2** (1.8 mg). Fraction C₄ (1.64 g) was rechromatographed using *n*-hexane–CH₂Cl₂ (50:50) to give 5 fractions (C_{4a}–C_{4e}). Fraction C_{4b} was purified by was purified

by preparative layer chromatography using *n*-hexane-acetone (95:5) as eluent, to afford compound **3** (1.9 mg). Fraction C_{4c} was rechromatographed and eluted with *n*-hexane-CH₂Cl₂-acetone (60:20:20) to give 6 fractions (C_{4c1}-C_{4c6}). Fraction C_{4c6} was purified by silica gel column and eluted with *n*-hexane-CH₂Cl₂-MeOH (38:58:4) to afford compounds **4** (6.70 mg) and **5** (49.9 mg). Fraction C₅ was rechromatographed on silica gel and eluted with CH₂Cl₂-EtOAc (95:5 to 0:100) to give 9 fractions (C_{5a}-C_{5g}). Fraction C_{5e} was rechromatographed and eluted with CH₂Cl₂-EtOAc (95:5) to yield compound **6** (3.6 mg). All compounds **1-6** were identified by interpretation of their spectral data including EIMS, ¹H and ¹³C NMR (including DEPT135, COSY, HMQC and HMBC experiments), as well as by comparison of their spectral data with those reported in the literature. Placement of the methoxy group at the 4'''-OCH₃ of compound **5** was confirmed by HMBC correlation of the methoxy protons with C-3''' (δ 114.03), C-4''' (δ 159.65) and C-5''' (δ 114.03) as shown in Figure S1 and Table S1.

Spectroscopic data of isolated compounds

***(E)*-3-(4-methoxyphenyl)prop-2-en-1-amine (**1**):**

white crystal; m.p= 155.2-156.4 °C; EIMS: *m/z* 147 (100), 132 (5), 121 (2), 115 (11), 91 (12), 77 (4); ¹H NMR (400 MHz, CDCl₃): δ 7.28 (2H, d, *J*=8.7 Hz, H-2,6), 6.85 (2H, d, *J*=8.7 Hz, H-3,5), 6.42 (1H, d, *J*=15.6 Hz, H-3'), 6.04 (1H, dt, *J*=15.6, 7.7 Hz, H-2'), 3.81 (s, 3H, OCH₃), 3.48 (2H, d, *J*=7.7 Hz, H-1'); ¹³C NMR (100 MHz, CDCl₃): δ 159.3 (C-4), 133.1 (C-3'), 129.5 (C-1), 127.6 (C-2,6), 122.30 (C-2'), 114.0 (C-3,5), 55.3 (OCH₃), 42.7 (C-1'); FTIR (neat): ν = 3427, 2916, 2848, 1603, 1574, 1173, 1510, 1244, 1024, 830, 804 cm⁻¹.

***(E)*-4-methoxycinnamaldehyde (**2**):**

pale yellow amorphous solid; m.p= 57.0-59.2 °C; EIMS: *m/z* 162 (100), 161 (80), 133 (22), 131 (61), 91 (37), 77 (19); ¹H NMR (400 MHz, CDCl₃): δ 9.65 (1H, d, *J*=7.8 Hz, H-1'), 7.53 (2H, d, *J*=8.8 Hz, H-2,6), 7.45 (1H, d, *J*=15.8 Hz, H-3'), 6.95 (2H, d, *J*=8.8 Hz, H-3,5), 6.62 (1H, dd, *J*=15.8, 7.8 Hz, H-2'), 3.85 (s, 3H, OCH₃); ¹³C NMR (100 MHz, CDCl₃): δ 193.7 (C-1'), 162.0 (C-4), 152.7 (C-3'), 130.3 (C-2,6), 126.6 (C-2'), 126.5 (C-1), 114.6 (C-3,5), 55.4 (OCH₃); FTIR (neat): ν = 2926, 2847, 1674, 1599, 1510, 1255, 969, 821 cm⁻¹.

(E)-4-methoxycinnamic acid (3):

colourless crystals; m.p= 172.7-174.6 °C; EIMS: m/z 178 (100), 177 (13), 161 (32), 133 (20), 132 (15), 91 (7), 77 (19); ^1H NMR (400 MHz, CDCl_3): δ 7.74 (1H, d, $J=15.9$ Hz, H-3'), 7.51 (2H, d, $J=8.7$ Hz, H-2,6), 6.92 (2H, d, $J=8.7$ Hz, H-3,5), 6.32 (1H, d, $J=15.9$ Hz, H-2'), 3.85 (s, 3H, 4-OCH₃); ^{13}C NMR (100 MHz, CDCl_3): δ 171.3 (C-1'), 161.7 (C-4), 146.7 (C-3'), 130.1 (C-2,6), 126.8 (C-1), 115.0 (C-2'), 114.4 (C-3,5), 55.4 (OCH₃); FTIR (neat): ν = 3456, 2937, 1678, 1600, 1513, 1257, 825 cm^{-1} .

(E)-1-methoxy-4-(3-methoxyprop-1-enyl)benzene (4):

yellow solid; m.p= 114.3-115.8 °C; EIMS: m/z 147 (100), 132 (9), 91 (20), and 71 (8); ^1H NMR (400 MHz, CDCl_3): δ 7.36 (2H, d, $J=8.7$ Hz, H-2,6), 6.88 (2H, d, $J=8.7$ Hz, H-3,5), 6.65 (1H, d, $J=15.8$ Hz, H-1'), 6.13 (1H, dt, $J=15.8, 7.6$ Hz, H-2'), 3.86 (1H, d, $J=7.6$ Hz, H-3'), 3.82 (s, 3H, 4-OCH₃), 2.88 (s, 3H, 3'-OCH₃); ^{13}C NMR (100 MHz, CDCl_3): δ 160.3 (C-4), 138.7 (C-1'), 128.3 (C-1), 128.2 (C-2,6), 114.3 (C-3,5), 113.2 (C-2'), 59.4 (C-3'), 55.5 (C4-OCH₃), 39.2 (C1'-OCH₃); FTIR (neat): ν = 2937, 1600, 1511, 1245, 1124, 975, 814 cm^{-1} .

(E)-((E)-3-(4-methoxyphenyl)allyl)3-(4-hydroxyphenyl)acrylate (5):

pale yellow solid; m.p= 109.7-110.5 °C; EIMS: m/z 310 (6), 309 (22), 279 (10), 173 (24), 147 (100), 121 (14); ^1H NMR (400 MHz, CDCl_3): δ 7.67 (1H, d, $J=16.0$ Hz, H-3'), 7.43 (2H, d, $J=8.6$ Hz, H-2,6), 7.35 (2H, d, $J=8.7$ Hz, 2''',6'''), 6.86 (4H, t, $J=8.6$ Hz, H-3,5, 3''',5'''), 6.65 (1H, d, $J=15.8$ Hz, H-3''), 6.34 (1H, d, $J=16.0$ Hz, H-2'), 6.22 (1H, dt, $J=15.8, 6.6$ Hz, H-2''), 5.37 (s, 1H, 4'-OH), 4.84 (1H, d, $J=6.6$ Hz, H-1''), 3.81 (s, 3H, 4'''-OCH₃); ^{13}C NMR (100 MHz, CDCl_3): δ 167.1 (C-1'), 159.6 (C-4'''), 157.6 (C-4), 144.6 (C-3'), 133.9 (C-3''), 130.0 (C-2,6), 127.9 (C-2''',6'''), 128.6 (C-1'''), 127.2 (C-1), 121.1 (C-2''), 115.8 (C-3,5), 115.5 (C-2'), 114.0 (C-3''',5'''), 65.3 (C-1''), 55.3 (OCH₃); FTIR (neat): ν = 3338, 1680, 1600, 1511, 1250, 1168, 971, 832 cm^{-1} .

4-methoxybenzoic acid (6):

white solid; m.p= 184.2-184.6 °C; EIMS: m/z 152 (M^+ , 88), 135 (100), 107 (11) and 77 (20); ^1H NMR (400 MHz, CDCl_3): δ 8.07 (2H, d, $J=8.8$ Hz, H-2,6), 6.95 (2H, d, $J=8.8$ Hz, H-3,5), 3.88

(s, 3H, 4-OCH₃); ¹³C NMR (100 MHz, CDCl₃): δ 171.3 (C-1'), 164.0 (C-4), 132.3 (C-2,6), 121.6 (C-1), 113.7 (C-3,5), 55.5 (OCH₃); FTIR (neat): ν = 3464, 1686, 1603, 1427, 1261, 845 cm⁻¹.

Antimycobacterial Assay

The antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H₃₇Ra using the green fluorescent protein microplate assay (GFPMA) (Changsen et al., 2003). The lowest drug concentration effecting and inhibition of ≥ 90 % was considered the MIC. The standard drugs, rifampicine, streptomycin, isoniazid, ofloxacin and ethambutol showed MIC values of 0.00312-0.025, 0.156-0.313, 0.0234-0.0468, 0.391-0.781 and 0.234-0.469 μg/mL, respectively.

Cytotoxic activity

The cytotoxicity against human oral cavity cancer (KB), human breast cancer (MCF7) and human small cell lung cancer (NCI-H187) cells was determined by resazurin microplate assay (REMA) which was a modified method of the use of a fluorescent dye for mammalian cell cytotoxicity (O'Brien et al., 2000). Briefly, cells at a logarithmic growth phase were harvested and diluted to 9 × 10⁴ cells/ml in fresh medium. Successively, 5 μl of test sample, diluted in 10% DMSO and 45 μl of cells suspension were added to 384-well plates. Plates were incubated at 37 °C in 5% CO₂ incubator for 5 days. After that, 12.5 μl of 62.5 μg/ml resazurin solution was added to each well and the plates were then incubated at 37 °C for 4 h. Fluorescence signal was measured using SpectraMax M5 multidetection microplate reader at dual wavelengths of 530 and 590 nm. IC₅₀ values were derived from dose-response curves, using six concentrations of 2-fold serially diluted samples, by the SOFTMax Pro software (Molecular device). The standard drugs ellipticine exhibited IC₅₀ values against KB and NCI-H187 at 1.10, 0.220 and 1.21 μg/mL and tamoxifen exhibited IC₅₀ values against MCF7 at 5.83 μg/mL.

References

Changsen C, Franzblan S, Palittapongarnpim P. 2003. Improved green fluorescent protein reporter gene-based microplate screening for antituberculosis compounds by utilizing an acetamidase promoter. *Antimicrob Agents Chemother.* 47:3682–3687.

Hu LH, Zou HB, Gong JX, Li HB, Yang LX, Cheng W, Zhou CX, Bai H, Guéritte F, Zhao Y. 2005. Synthesis and biological evaluation of a natural ester sintenin and its synthetic analogues. *J Nat Prod.* 68:342-348.

O'Brien J, Wilson I, Orton T, Pognan F. 2000. Investigation of the *Alamar Blue* (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem.* 269:5421-5426.

Table S1. ^1H -, ^{13}C -NMR and HMBC data of compound **5**

Positions	$\delta^1\text{H}$ (Mult., J in Hz)		$\delta^{13}\text{C}$	HMBC
	Compound 5	Ref. (Hu et al., 2005)	Compound 5	
1'			167.13	-
1''	4.84 (<i>d</i> , 6.6)	4.87 (<i>d</i> , 6.4)	65.32	C-1', 2'', 3''
2'	6.34 (<i>d</i> , 16.0)	6.37 (<i>d</i> , 16.0)	115.57	C-1', 3'
2''	6.22 (<i>dt</i> , 15.8, 6.6)	6.36 (<i>dt</i> , 16.0, 6.4)	121.10	C-1'', 1'''
3'	7.67 (<i>d</i> , 16.0)	7.67 (<i>d</i> , 16.0)	144.62	C- 1', 2, 3, 5, 6
3''	6.65 (<i>d</i> , 15.8)	6.71 (<i>d</i> , 16.0)	133.98	C-1'', 2''', 6'''
1			127.27	-
1'''			128.59	-
2, 6	7.43 (<i>d</i> , 8.6)	6.94-7.43 (<i>m</i>)	130.00	C-3', 4
2''', 6'''	7.35 (<i>d</i> , 8.7)	6.94-7.43 (<i>m</i>)	127.90	C-3'', 4'''
3, 5	6.85 (<i>t</i> , 8.5)	6.94-7.43 (<i>m</i>)	115.84	C-1, 4
3''', 5'''	6.87 (<i>t</i> , 8.6)	6.94-7.43 (<i>m</i>)	114.03	C-1''', 4'''
4			157.59	-
4'''			159.65	-
4-OH	5.37 (<i>s</i>)	5.90 (<i>s</i>)	115.84	-
4'''-OCH ₃	3.81 (<i>s</i>)	3.93 (<i>s</i>)	55.30	C-3''', 4''', 5'''

Figure S1. Selected HMBC correlations of compound **5**