Cytotoxic and Anti-metastatic Activities of Phenolic Compounds from *Dendrobium ellipsophyllum*

KASINEE TANAGORNMEATAR[1], CHATCHAI CHAOTHAM[2,3], BOONCHOO SRITULARAK[1,3], KITTISAK LIKHITWITAYAWUID[1,3] and PITHI CHANVORACHOTE[2,3]

Departments of [1]Pharmacognosy and Pharmaceutical Botany, and [2]Pharmacology and Physiology, and [3]Cell-Based Drug and Health Product Development Research Unit, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

Abstract. *Background/Aim: Phenolic compounds isolated from Dendrobium ellipsophyllum Tang & Wang (Orchidaceae) have been shown to possess potential pharmacological activity; however, their anticancer as well as anti-metastasis activities are largely unknown. The aim of the present study was to isolate active compounds from D. ellipsophyllum and to explore the possible effects of phenolic compounds isolated from the plant for cytotoxic as well as anti-metastatic properties. Materials and Methods: The compounds were isolated by using chromatographic techniques including silica gel and Sephadex LH20. Each of the isolates was evaluated for their cytotoxicity on H292 human lung cancer cell lines by 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide assay. The cytotoxic compounds were further evaluated for apoptosis-inducing and anoikis-sensitizing effects. Results: Ten phenolic compounds were isolated, 5,7-dihydroxy-chromen-4-one (**1**); 4,5-dihydroxy-2,3-dimethoxy-9,10-dihydrophenanthrene (**2**); moscatilin (**3**), 4,4'-dihydroxy-3,5-dimethoxybibenzyl (**4**); 4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl (**5**); (2S)-homoeriodictyol (**6**); (2S)-eriodictyol (**7**); chrysoeriol (**8**); phloretic acid (**9**); and luteolin (**10**). Compounds **4**, **5**, **8** and **10** exhibited appreciable cytotoxic activity with 50% inhibitory concentration values less than 250 μM. These compounds also showed potential apoptosis induction and anoikis-sensitizing effect at non-toxic concentrations. Conclusion: Compounds **4**, **5**, **8** and **10** are responsible for cytotoxic and anti-metastatic activities of D. ellipsophyllum.*

Correspondence to: Pithi Chanvorachote, Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand. Tel: +662 2188344, Fax: +662 2188340, e-mail: pithi.c@chula.ac.th; Boonchoo Sritularak, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand. Tel: +662 2188356, Fax: +662 2188357, e-mail: boonchoo.sr@chula.ac.th

Key Words: Dendrobium ellipsophyllum, bibenzyls, flavonoids, anti-metastasis, cytotoxicity, anoikis, lung cancer.

Lung cancer has been recognized as having a high mortality rate (1). It is now clear that the main causes of death from this type of cancer is due to metastasis of cancer cells to distant sites of the patient's body, as well as to resistance to chemotherapeutic drugs (2). Therefore, novel compounds that possess both anticancer and anti-metastatic activities are of great interest. Metastasis is the process of cancer cells spreading from the primary tumor to other sites through lymphatic or blood circulation (3). Although most of cells will die after detachment, metastatic cancer cells are able to resist programmed cell death induced by the loss of appropriate cell adhesion and are thus spread to secondary sites (4). This cell death after cell detachment is named anoikis and is considered the major obstacle to cancer cell metastasis (5). Much research has been conducted to search for potential compounds sensitizing cancer cells to anoikis (6-8).

Plants in the genus *Dendrobium* (Orchidaceae) have been found to have various kinds of biological activities, for example, antioxidative, antitumor, anti-angiogenesis, neuroprotective and immunomodulatory activities (9). These plant species have several known cytotoxic constituents, such as erianin, densiflorin and denchrysan A from *D. chrysotoxum* (10, 11), crepidatin and chrysotoxine from D. capillipes (12), and moscatilin, a cytotoxic compound previously reported from several species of this plant genus (13, 14). As part of our continuing studies on bioactive phenolics from *Dendrobium* spp. (15-17), we investigated the chemical constituents from the whole plant of *Dendrobium ellipsophyllum* Tang & Wang, (locally known in Thai as '*Ueang thong*'), a plant growing throughout Thailand with no previous record of chemical examination. In the present investigation, a methanolic extract prepared from the whole plant was found to exert significant cytotoxic effect against human lung cancer cells. Subsequent chemical investigation of the extract resulted in the isolation of 10 phenolic

compounds (**1-10**). Because the possible anti-metastatic activities of the compounds isolated from the plant have not been elucidated, the compounds were evaluated for cytotoxic and anti-metastatic effects on metastatic H292 human lung cancer cells. The knowledge gained from the present study may aid further investigation and development of these compounds for possible use in cancer therapy.

Materials and Methods

General experimental procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. Mass spectra were recorded on a UPLC mass spectrophotometer (Waters 2996-2695, ESI-MS; Waters Co., Milford, MA, U.S.A). NMR spectra were recorded on a Bruker Avance DPX-300 FT-NMR spectrometer or a Varian Unity INOVA-500 NMR spectrometer (Palo Alto, Santa Clara, CA, USA). Vacuum-liquid column chromatography (VLC) and column chromatography were performed on silica gel 60 (Kieselgel 60, 70-320 mesh; Merck, Darmstadt, Germany) silica gel 60 (Kieselgel 60, 230-400 mesh; Merck, Darmstadt, Germany) and Sephadex LH-20 (25-100 μm; Pharmacia Fine Chemical Co. Ltd., Uppsala, Sweden).

Plant material. *D. ellipsophyllum* was purchased from Jatujak market, Bangkok, in May 2012. Botanical identification was performed in comparison with herbarium specimens at the Department of National Park, Wildlife and Plant Conservation, Ministry of National Resources and Environment. A voucher specimen (BS-DE-052555) is on deposit at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Extraction and isolation. Dried powdered whole plant of *D. ellipsophyllum* (4.8 kg) was extracted with MeOH (3×10 l) at room temperature to give a viscous mass of dried extract (400 g) after evaporation of the solvent. This material (200 g) was subjected to VLC on silica gel (*n*-hexane-EtOAc gradient) to give five fractions (A-E). Fraction D (63 g) was separated by VLC over silica gel, eluted with *n*-hexane-EtOAc gradient to give seven fractions (D1-D7). Fraction D4 (2.3 g) was separated by column chromatography (silica gel; *n*-hexane-EtOAc, gradient), and further purified on Sephadex LH-20 (acetone) to give **1** (4 mg). Fraction D5 (5.4 g) was subjected to medium pressure liquid chromatography over silica gel, eluted with *n*-hexane-EtOAc gradient to give 14 fractions (D5A-D5N). Purification of fraction D5D (47 mg) and D5F (828 mg) on Sephadex LH20 (acetone) gave **2** (9 mg) and **3** (188 mg), respectively. Fraction D5G (954.3 mg) was separated by column chromatography over silica gel (n-hexane-EtOAc, gradient), and further purified on Sephadex LH20 (acetone) to yield **4** (5 mg), **5** (151 mg) and **6** (52 mg), respectively. Separation of fraction D5I (1.0 g) by column chromatography (silica gel; *n*-hexane-EtOAc , gradient) gave **7** (364 mg). Fraction D6 (8.6 g) was separated by column chromatography (silica gel; *n*-hexane-EtOAc, gradient) to give 12 fractions (D6A-D6L). Fraction D6I (1.0 g) was subjected to repeated column chromatography over silica gel, eluted with *n*-hexane-EtOAc (gradient), and further purified on Sephadex LH-20 (acetone) to yield **8** (8 mg), **9** (18 mg) and **10** (38 mg), respectively.

*Identification of compounds **1-10***. The structures of the isolates were determined through analysis of their spectroscopic data in comparison with reported values, and were identified as 5,7-dihydroxy-chromen-4-one (**1**) (19); 4,5-dihydroxy-2,3-dimethoxy-9,10-dihydrophenanthrene (**2**) (20); moscatilin (**3**) (21), 4,4'-dihydroxy-3,5-dimethoxybibenzyl (**4**) (22); 4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl (**5**) (16); (2S)-homoeriodictyol (**6**) (23); (2S)-eriodictyol (**7**) (24); chrysoeriol (**8**) (25); phloretic acid (**9**) (26); and luteolin (**10**) (27).

5,7-Dihydroxy-chromen-4-one (1): colorless needles; $C_9H_6O_4$; ESI-MS *m/z* 179 $[M+H]^+$; ^{1}H-NMR (300 MHz, acetone-d_6) δ: 6.21 (1H, d, *J*=6.0 Hz, H-3), 6.26 (1H, d, *J*=2.1 Hz, H-6), 6.39 (1H, d, *J*=2.1 Hz, H-8), 8.05 (1H, d, *J*=6.0 Hz, H-2), 12.76 (1H, s, HO-5); ^{13}C-NMR (75 MHz, acetone-d_6) δ: 94.7 (C-8), 99.8 (C-6), 106.4 (C-10), 111.5 (C-3), 157.6 (C-2), 159.2 (C-9), 163.4 (C-5), 165.1 (C-7), 182.5 (C-4).

4,5-Dihydroxy-2,3-dimethoxy-9,10-dihydrophenanthrene (**2**): brown amorphous solid; $C_{16}H_{16}O_4$; ESI-MS *m/z* 273 $[M+H]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 2.72 (4H, m, H_2-9, H2-10), 3.93 (3H, s, MeO-2), 3.99 (3H, s, MeO-3), 6.56 (1H, s, H-1), 6.87 (1H, d, *J*=8.0 Hz, H-8), 6.98 (1H, d, *J*=8.0 Hz, H-6), 7.16 (1H, t, *J*=8.0 Hz, H-7); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 30.9 (C-9, C-10), 55.9 (MeO-2), 61.2 (MeO-3), 105.0 (C-1), 113.1 (C-4a), 118.0 (C-8), 120.0 (C-6), 120.4 (C-4b), 128.0 (C-7), 134.0 (C-3), 136.7 (C-10a), 140.2 (C-8a), 143.7 (C-4), 150.4 (C-2), 153.2 (C-5).

Moscatilin (**3**): brown amorphous solid; $C_{17}H_{20}O_5$; ESI-MS *m/z* 305 $[M+H]^+$; ^{1}H-NMR (500 MHz, acetone-d_6) δ: 2.78 (4H, m, H_2-α ,H_2-α'), 3.75 (6H, s, MeO-3, MeO-5), 3.76 (3H, s, MeO-3'), 6.48 (2H,s, H-2,6), 6.64 (1H, dd, *J*=8.0, 2.0 Hz, H-6'), 6.75 (1H, d, *J*=8.0 Hz, H-5'), 6.78 (1H, d, *J*=2.0 Hz, H-2'); ^{13}C-NMR (125 MHz, acetone-d_6) δ: 38.3 (C-α'), 38.8 (C-α), 56.1 (MeO-3'), 56.5 (MeO-3, MeO-5), 106.7 (C-2, C-6), 112.9 (C-2'), 115.4 (C-5'), 121.6 (C-6'), 133.1 (C-1), 134.1 (C-1'), 134.8 (C-4), 145.3 (C-4'), 147.9 (C-3'), 148.3 (C-3,5).

4,4'-Dihydroxy-3,5-dimethoxybibenzyl (**4**): brown amorphous solid; $C_{16}H_{18}O_4$; ESI-MS *m/z* 275 $[M+H]^+$; ^{1}H-NMR (500 MHz, acetone-d_6) δ: 2.76 (4H, m, H_2-α ,H_2-α'), 3.76 (6H, s, MeO-3,5), 6.46 (2H, s, H-2,6), 6.72 (2H, d, *J*=8.5 Hz, H-3', H-5'), 7.00 (2H, d, *J*=8.5 Hz, H-2', H-6'); ^{13}C-NMR (125 MHz, acetone-d_6) δ: 38.0 (C-α'), 39.0 (C-α), 56.4 (MeO-3, MeO-5), 106.7 (C-2, C-6), 115.8 (C-3', C-5'), 130.1 (C-2', C-6'), 133.1 (C-1), 133.5 (C-1'), 134.9 (C-4), 148.4 (C-3, C-5), 156.3 (C-4').

4,5,4'-Trihydroxy-3,3'-dimethoxybibenzyl (**5**): brown amorphous solid; $C_{16}H_{18}O_5$; ESI-MS *m/z* 291 $[M+H]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 2.80 (4H, m, H_2-α ,H2-α'), 3.84 (3H, s, MeO-3), 3.86 (3H, s, MeO-3'), 6.25 (1H, br s, H-2), 6.47 (1H, br s, H-6), 6.65 (1H, br s, H-2'), 6.70 (1H, br d, *J*=8.0 Hz, H-6'), 6.85 (1H, d, *J*=8.0 Hz, H-5'); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 37.7 (C-α'), 38.2 (C-α), 55.9 (MeO-3'), 56.2 (MeO-3), 103.6 (C-2), 108.6 (C-6), 111.2 (C-2'), 114.2 (C-5'), 121.0 (C-6'), 130.5 (C-1), 133.7 (C-4), 133.8 (C-1'), 143.7 (C-5, C-4'), 146.3 (C-3'), 146.7 (C-3).

(2*S*)-Homoeriodictyol (**6**): colorless needles; $C_{16}H_{14}O_6$; ESI-MS *m/z* 303 $[M+H]^+$; $[\alpha]^{20}{}_D$ -18.6 (*c*=0.2, MeOH); ^{1}H-NMR (500 MHz, acetone-d_6) δ: 2.72 (1H, dd, *J*=17.0, 3.0 Hz, H-3$_{cis}$), 3.19 (1H, dd, *J*=17.0, 13.0 Hz, H-3$_{trans}$), 3.87 (3H, s, MeO-3'), 5.41 (1H, dd, *J*=13.0, 3.0 Hz, H-2), 5.94 (1H, d, *J*=2.5 Hz, H-6), 5.96 (1H, d, *J*=2.5 Hz, H-8), 6.86 (1H, d, *J*=8.0 Hz, H-5'), 6.98 (1H, dd, *J*=8.0, 2.0 Hz, H-6'), 7.17 (1H, d, *J*=2.0 Hz, H-2'), 12.17 (1H, s, HO-5); ^{13}C-NMR (125 MHz, acetone-d_6) δ: 43.5 (C-3), 56.2 (MeO-3'), 80.1 (C-2), 95.8 (C-8), 96.7 (C-6), 103.1 (C-10), 111.1 (C-2'), 115.6 (C-5'), 120.4 (C-6'), 131.2 (C-1'), 147.8 (C-4'), 148.3 (C-3'), 164.3 (C-9), 165.2 (C-5), 167.3 (C-7), 197.2 (C-4).

(2*S*)-Erodictyol (**7**): colorless needles; $C_{15}H_{12}O_6$; ESI-MS *m/z* 289 $[M+H]^+$; $[\alpha]^{20}{}_D$-18.7 (*c*=0.2, MeOH); ^{1}H-NMR (500 MHz, acetone-d_6) δ: 2.72 (1H, dd, *J*=17.0, 3.0 Hz, H-3$_{cis}$), 3.11 (1H, dd, *J*=17.0, 12.5 Hz, H-3$_{trans}$), 5.37 (1H, dd, *J*=12.5, 3.0 Hz, H-2), 5.93

(1H, d, *J*=2.0 Hz, H-6), 5.95 (1H, d, *J*=2.0 Hz, H-8), 6.86 (2H, d, *J*=1.5 Hz, H-5', H-6'), 7.02 (1H, d, *J*=1.5 Hz, H-2'), 12.16 (1H, s, HO-5); ^{13}C-NMR (125 MHz, acetone-d_6) δ: 43.4 (C-3), 79.9 (C-2), 95.8 (C-8), 96.7 (C-6), 103.1 (C-10), 114.6 (C-2'), 115.9 (C-5'), 119.2 (C-6'), 131.5 (C-1'), 145.9 (C-3'), 146.3 (C-4'), 164.2 (C-5), 165.2 (C-9), 167.2 (C-7), 197.1 (C-4).

Chrysoeriol (8): yellow powder; $C_{16}H_{12}O_6$; ESI-MS *m/z* 301 $[M+H]^+$; ^{1}H-NMR (500 MHz, acetone-d_6) δ: 3.98 (3H, s, MeO-3'), 6.24 (1H, d, *J*=2.0 Hz, H-6), 6.54 (1H, d, *J*=2.0 Hz, H-8), 6.69 (1H, s, H-3), 7.00 (1H, d, *J*=8.4 Hz, H-5'), 7.59 (1H, dd, J=8.4, 2.0 Hz, H-6'), 7.63 (1H, d, *J*=2.0 Hz, H-2'), 13.01 (1H, s, HO-5); ^{13}C-NMR (125 MHz, acetone-d_6) δ: 56.5 (MeO-3'), 94.7 (C-8), 99.7 (C-6), 104.4 (C-3), 105.1 (C-10), 110.5 (C-2'), 116.3 (C-5'), 121.3 (C-6'), 123.6 (C-1'), 148.8 (C-3'), 151.4 (C-4'), 157.8 (C-9), 159.0 (C-5), 163.3 (C-2), 164.9 (C-7), 183.1 (C-4).

Phloretic acid (**9**): brown amorphous solid; $C_9H_{10}O_3$; ESI-MS *m/z* 167 $[M+H]^+$; ^{1}H-NMR (300 MHz, acetone-d6) δ: 2.53 (2H, t, *J*=7.8 Hz, H2-8), 2.79 (2H, t, *J*=7.8 Hz, H_2-7), 6.74 (2H, d, *J*=8.1 Hz, H-3, H-5), 7.05 (2H, d, *J*=8.1 Hz, H-2, H-6); ^{13}C-NMR (75 MHz, acetone-d_6) δ: 30.7 (C-7), 36.3 (C-8), 115.8 (C-3, C-5), 130.1 (C-2, C-6), 132.5 (C-1), 156.4 (C-4), 173.9 (C-9).

Luteolin (**10**): yellow powder; $C_{15}H_{10}O_6$; ESI-MS *m/z* 287 $[M+H]^+$; ^{1}H-NMR (500 MHz, acetone-d_6) δ: 6.24 (1H, d, *J*=2.0 Hz, H-6), 6.51 (1H, d, *J*=2.0 Hz, H-8), 6.57 (1H, s, H-3), 7.00 (1H, d, *J*=8.4 Hz, H-5'), 7.47 (1H, dd, J=8.4, 2.1 Hz, H-6'), 7.49 (1H, d, *J*=2.1 Hz, H-2'); ^{13}C-NMR (125 MHz, acetone-d6) δ: 94.6 (C-8), 99.7 (C-6), 104.2 (C-3), 105.3 (C-10), 114.1 (C-2'), 116.6 (C-5'), 120.1 (C-6'), 123.8 (C-1'), 146.4 (C-3'), 150.0 (C-4'), 158.8 (C-9), 163.4 (C-5), 164.8 (C-2), 165.1 (C-7), 183.0 (C-4).

Cells and reagents. Human lung cancer H292 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 containing 5% fetal bovine serum, 2 mM L-glutamine, and 100 units/mL penicillin/streptomycin in a 5% CO_2 environment at 37˚C. 2,3-*Bis*(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT), and other chemicals were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Hoechst 33342 was obtained from Molecular Probes, Inc (Eugene, OR, USA).

Anoikis and cell viability. To prevent cell adhesion, tissue culture 6-well plates were coated with 200 μl (6 mg/ml in 95% ethanol) of poly (2-hydroxyethyl methacrylate) (poly-HEMA; Sigma), and left dry overnight in a laminar flow hood at room temperature. Adherent H292 cells in the culture plate were trypsinized into a single-cell suspension in RPMI medium, and then seeded in poly-HEMA-coated plates at a density of 1×10^5 cells/ml. Suspended cells were incubated at 37˚C for different times up to 24 h. For cell viability assay, cells were harvested, washed, and incubated with 20 μM of XTT for 4 h at 37˚C. Optical density was then determined with a V-max photometer (Molecular Devices Inc., Menlopark, CA, USA) at a wavelength of 450 nm as previously described (18).

Apoptosis nuclear staining assay. Apoptotic and necrotic cell death were determined by Hoechst 33342 and propidium iodide (PI) co-staining. After specific treatments, the cells were incubated with 10 μM of Hoechst 33342 and 5 μg/ml of PI for 30 min at 37˚C. Nuclear condensation and DNA fragmentation of apoptotic cells and PI-positive necrotic cells were visualized and scored under a fluorescence microscope (Olympus IX51 with DP70; Olympus, Center Valley, PA, USA) as previously described (28).

Table I. *The 50% inhibitory concentration (IC_{50}) values for cytotoxicity of compounds **1-10** against H292 human lung cancer cells*.

Compound	IC_{50} (μM)
5,7-Dihydroxy-chromen-4-one (**1**)	>250
4,5-Dihydroxy-2,3-dimethoxy-9,10 -dihydrophenanthrene (**2**)	>250
Moscatilin (**3**)	226.09±5.67
4,4'-Dihydroxy-3,5-dimethoxybibenzyl (**4**)	197.74±0.78
4,5,4'-Trihydroxy-3,3'-dimethoxybibenzyl (**5**)	96.56±0.22
(2*S*)-Homoeriodictyol (**6**)	>250
(2*S*)-Eriodictyol (**7**)	>250
Chrysoeriol (**8**)	217.74±3.08
Phloretic acid (**9**)	>250
Luteolin (**10**)	202.57±0.48

Data are mean±SD of three independent experiments.

Statistical analysis. The data are presented as the mean±SD from at three independent experiments, and analyzed by one-way ANOVA and *post hoc* at a significance level of $p<0.05$.

Results

Phytochemical study of the MeOH extract of the whole plant of *D. ellipsophyllum* led to the isolation of 10 phenolic compounds (Figure 1) which included 5,7-dihydroxy-chromen-4-one (**1**); 4,5-dihydroxy-2,3-dimethoxy-9,10-dihydrophenanthrene (**2**); moscatilin (**3**); 4,4'-dihydroxy-3,5-dimethoxybibenzyl (**4**); 4,5,4'-trihydroxy-3,3'-dimethoxy-bibenzyl (**5**); (2*S*)-homoeriodictyol (**6**); (2*S*)-eriodictyol (**7**); chrysoeriol (**8**); phloretic acid (**9**); and luteolin (**10**). Each of these isolates was then investigated for its cytotoxicity. The cytotoxic compounds were evaluated for apoptosis induction and anti-metastatic effects.

Cytotoxicity on lung cancer cells. The compounds isolated from *D. ellipsophyllum* were further investigated for the cytotoxicity on human lung cancer cells. Subconfluent (80%-90%) monolayer H292 cells were treated with these compounds at a concentration of 100 μM for 24 h, and cell viability was evaluated by the XTT assay. Two bibenzyls, **4** and **5**, and two flavonoids, namely, **8** and **10** exhibited appreciable cytotoxic effect against H292 cells. The 50% inhibitory concentration (IC_{50}) of all compounds was determined (Table I). It is worth noting that compound **5** exhibited the strongest cytotoxic activity with an IC_{50} of 96.5 μM. Because the major concern for the treatment of lung cancer besides cancer metastasis is the possibility of the cells being resistant to drugs, we aimed to elucidate the possible dual cytotoxic and anti-metastasis effects of the compounds. As we were interested in the compounds that have both actions, the compounds possessing potent cytotoxic activity with IC_{50} value less than 250 μM were selected for further elucidation.

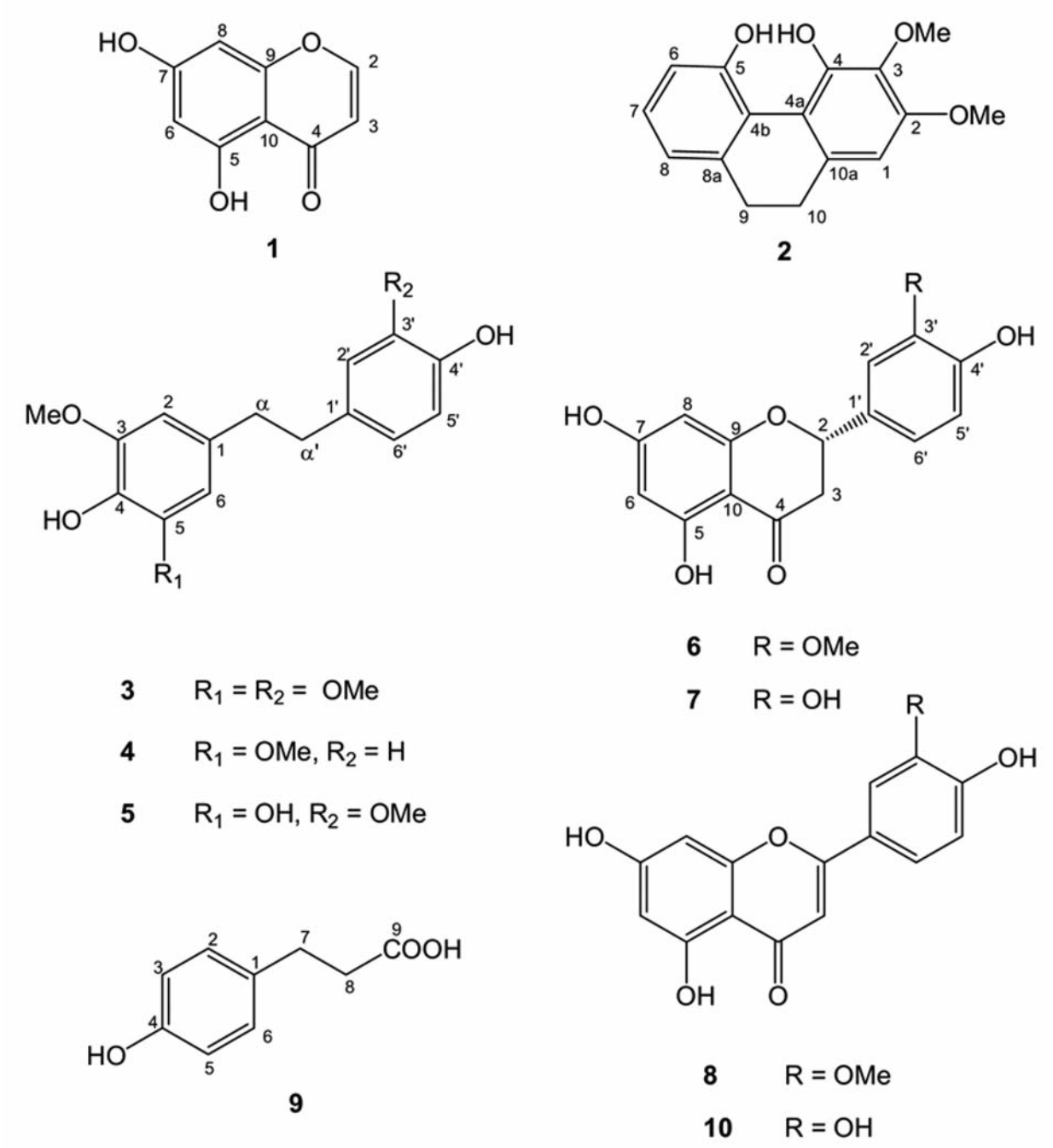

Figure 1. *Structures of compounds **1-10** isolated from Dendrobium ellipsophyllum Tang & Wang (names are given in Table I).*

Apoptosis induction effect of the compounds. Lung cancer cells were exposed to compounds **4**, **5**, **8**, and **10** for 24 h, and apoptosis as well as necrosis cell death were determined by Hoechst33342 and PI co-staining assay. Figure 2 shows that all compounds at 100 and 200 μM significantly induced apoptosis of cells, as indicated by an increase in cells possessing condensed or fragmented nuclei. The results are also consistent with the above finding that compound **5** has the highest anticancer activity.

Anoikis-sensitizing activity. Because the anoikis-sensitizing activity and anti-metastasic potential of compounds **4** and **5**, and chrysoeriol (**8**) have not been investigated to date, the present study attempted to examine their ability at enhancing anoikis response of metastatic lung cancer cells. Although an

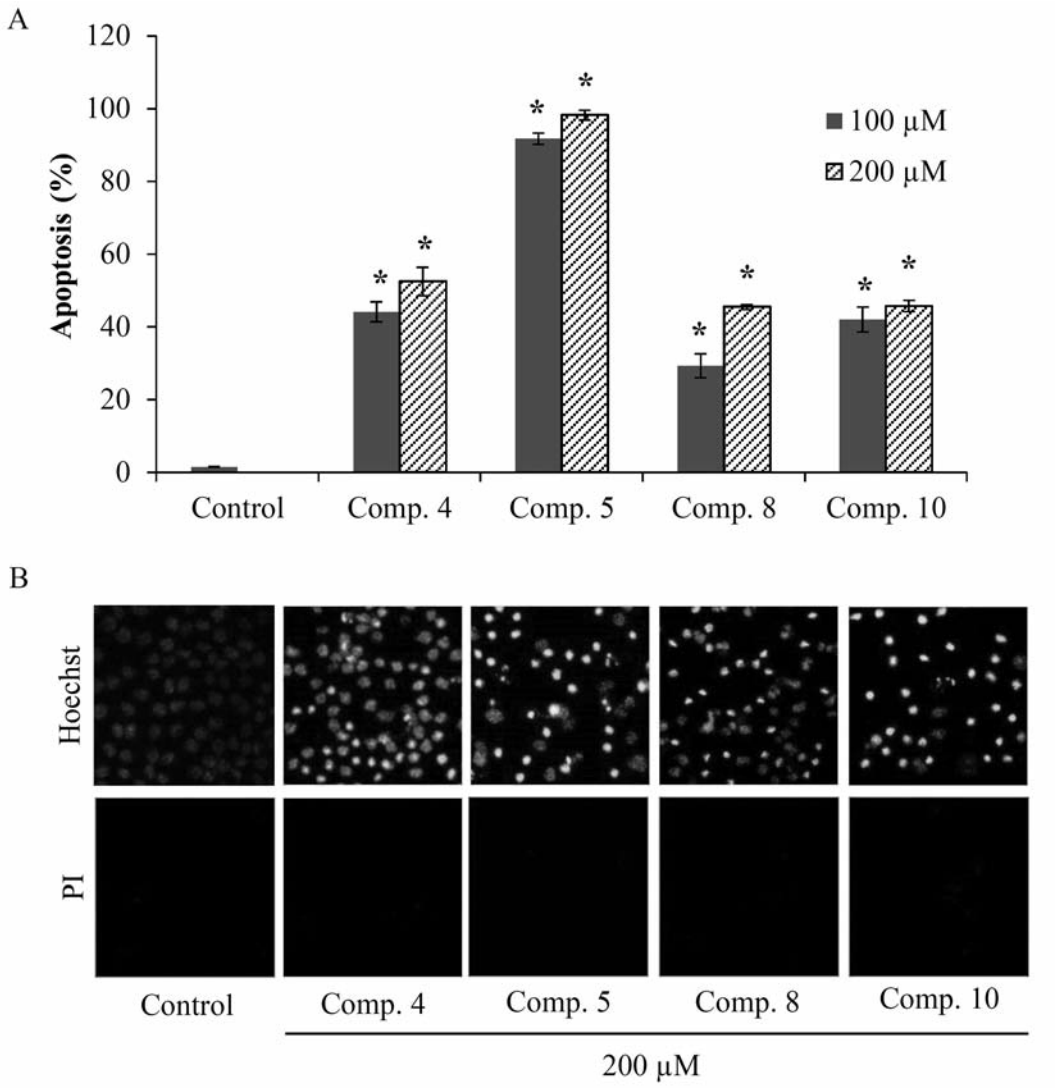

Figure 2. *A: Percentage of H292 cell apoptosis induced by compounds **4**, **5**, **8** and **10** was obtained from Hoechst 33342/propidium iodide (PI) assays. Data represent the means±SD (n=3). *p<0.05 versus non-treated control cells. B: Morphology of apoptotic nuclei stained with Hoechst 33342 and propidium iodide.*

anti-metastatic effect of luteolin (**10**) has been reported, there is no record of its anoikis-sensitizing activity. The results indicated that all compounds at 1-5 μM had no effect on cell viability of adhered H292 cells (data not shown). The cells were then treated with 0-5 μM in detached condition and cell viability over time was evaluated as described in Materials and Methods. Interestingly, our results indicated that the metastatic lung cancer cells had high anoikis resistance (Figure 3) as indicated by approximately 60% of the cells surviving after 24-h detachment. Importantly, the treatment of the cells with compounds at these non-toxic concentrations significantly enhanced cell anoikis. These results suggest the possible anoikis-sensitizing effect of these compounds. It is interesting that compound **5**, possessing highest cytotoxic activity, also had the fastest action in sensitizing the cells to anoikis. A significant effect was detected as early as 6 h of treatment, however, the overall effect at 24 h of treatment with compound **5** was comparable to that of other tested compounds.

Discussion

In the quest for safe and effective chemotherapeutic drugs, compounds obtained from natural resources have a significant interest. In terms of anti-metastasis drugs, the compounds should not only exhibit cytotoxic effect but also possess inhibitory activity on cancer metastasis (6-8). This study is the first to report the anti-metastasis potentials of compounds which are major compositions of *D. ellipsophyllum*.

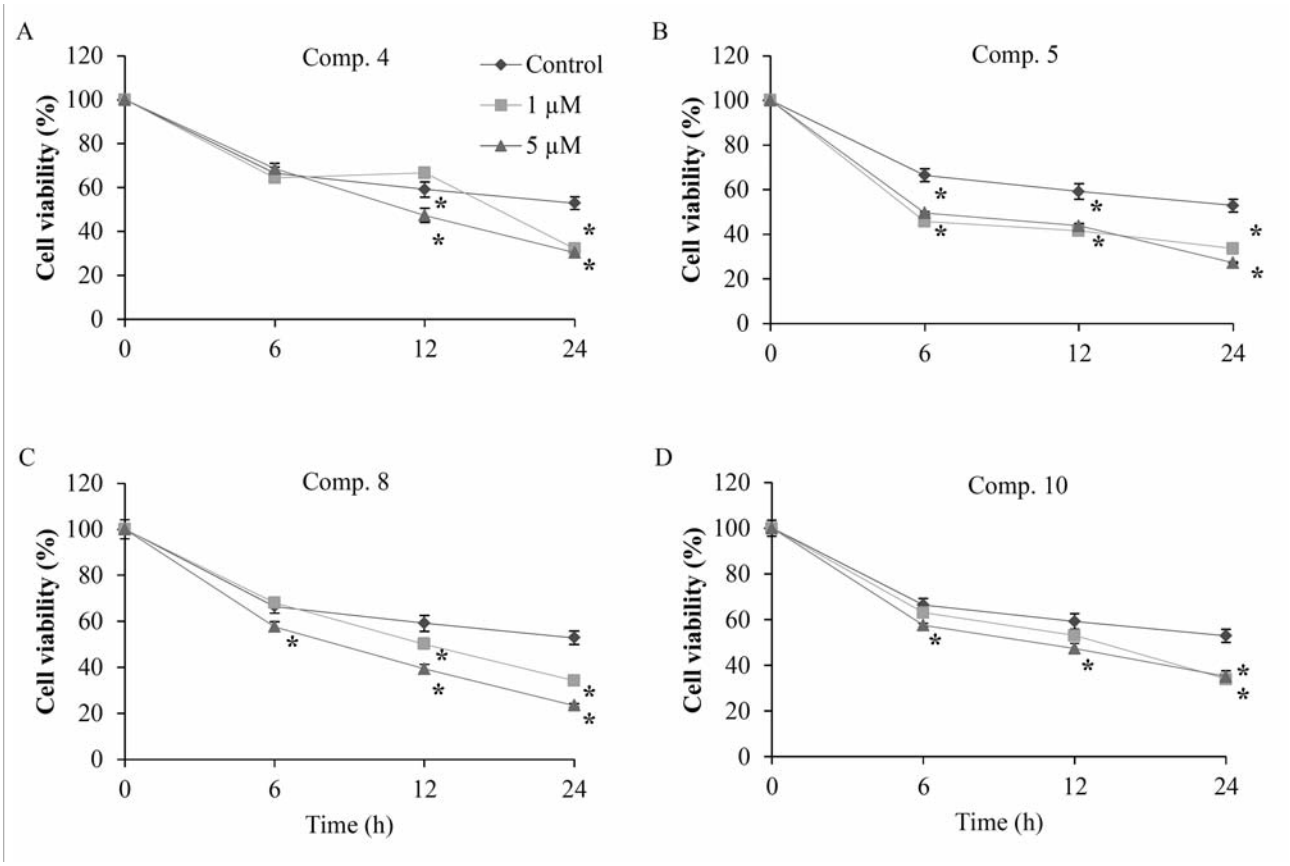

Figure 3. *Anoikis-sensitizing activity of compounds **4** (A), **5** (B), **8** (C) and **10** (D) as assessed by anoikis assay. H292 cells were exposed to different concentrations of each compound (0-5 μM) and cell viability was determined by the XTT assay at the indicated time. Data represent the mean±SD (n=3). *p<0.05 versus non-treated control cells.*

Anoikis-resistant cells also known as circulating tumor cells are considered as critical potentiating factor of cancer metastasis which leads to low survival rate of patients (2, 5). The potential anti-metastatic compounds from *D. ellipsophyllum* are demonstrated herein to have the anoikis sensitizing effect in detached lung cancer cells (Figure 3). The induction of anoikis was significantly enhanced by compounds **4** and **5** which corresponds to anti-metastatic activity of compound **3** (moscatilin), a similar bibenzyl (13, 14). Compound **5** should be considered as the most potent compound from *D. ellipsophyllum* due to the lowest IC_{50} of anticancer activity and the earliest and highest anoikis-sensitizing in human lung cancer cells. Previously, we and others have shown that several naturally-derived compounds, such as renieramycin M, a marine tetrahydroisoquinoline alkaloid, has a potent anti-metastasis activity against H460 lung cancer cells (29). Additionally, we have provided that curcumin sensitizes lung cancer cells anoikis through its ability to reduce anti-apoptotic B-cell lymphoma 2 (BCL2) protein (30). Likewise, compounds such as artonin E (8) or ecteinascidin 770 (31) can trigger cancer anoikis through the down-regulation of BCL2 or myeloid cell leukemia 1(MCL1). It is interesting to note that all tested compounds used for anoikis sensitization here only required low concentrations in comparison with the concentrations generally required for anticancer agents.

In summary, our results demonstrated the possible anticancer and anti-metastatic effects of bibenzyls and flavonoids isolated from *D. ellipsophyllum* that may encourage further investigation and development of these compounds for use in cancer treatment.

Acknowledgements

This work was supported by a research grant from Ratchadaphiseksomphot Endowment Fund of Chulalongkorn University. K.T. is grateful to the Graduate School of Chulalongkorn University for a research grant. C.C. would like to thank the Rachadapisaek Sompote Post-Doctoral Fund, Chulalongkorn University. We thank the Research Instrument Center of Faculty of Pharmaceutical Sciences, Chulalongkorn University, for providing research facilities, and Mr. Krich Rajprasit, a proofreader.

References

1 Siegel R, Jemal A, Ward E and Naishadham D: Cancer statistics 2013. CA Cancer J Clin *62*: 11-29, 2013.

2 Ray MR and Jablons DM: Lung cancer metastasis novel biological mechanisms and impact on clinical practice. *In*: Hallmarks of Metastasis. Keshamouni V, Arenberg D, Kalemkerian G (eds.). Springer, New York, pp. 29-46, 2009.

3 Hanahan D and Weinberg RA: The hallmarks of cancer. Cell *100*: 57-70, 2000.

4 Harlozinska A: Progress in molecular mechanisms of tumor metastasis and angiogenesis. Anticancer Res *25*: 3327-3333, 2005.

5 Chiarugi P and Giannoni E: Anoikis a necessary death program for anchorage-dependent cells. Biochem Pharmacol *76*: 393-401, 2008.

6 Mawji IA, Simpson CD, Gronda M,Williams MA, Hurren R, Henderson CJ, Datti A, Wrana JL and Schimmer AD: A chemical screen identifies anisomycin as an anoikis sensitizer that functions by decreasing FLIP protein synthesis. Cancer Res *67*: 8307-8315, 2007.

7 Yamaguchi H, Kidachi Y, Kamiie K, Noshita T, Umetsu H and Ryoyama K: Glycyrrhetinic acid induces anoikis-like death and cytoskeletal disruption in the central nervous system tumorigenic cells. Biol Pharml Bull *33*: 321-324, 2010.

8 Wongpankam E, Chunhacha P, Pongrakhananon V, Sritularak B and Chanvorachote P: Artonin E mediates MCL down-regulation and sensitizes lung cancer cells to anoikis. Anticancer Res *32*: 5343-5352, 2012.

9 Ng TB, Liu J, Ho J, Ye X, Wing Sze SC, Tong Y and Zhang KY: Review of research on *Dendrobium*, a prized folk medicine. Appl Microbiol Biotechnol *93*: 1795-1803, 2012.

10 Li YM, Wang HY and Liu GO: Erianin induces apoptosis in human leukemia HL-60 cells. Acta Pharmacol Sin *22*: 1018-1022, 2001.

11 Chen Y, Li Y, Qing C, Zhang Y, Wang L and Liu Y: 1,4,5-Trihydroxy-7-methoxy-9*H*-fluoren-9-one, a new cytotoxic compound from *Dendrobium* chrysotoxum. Food Chem *108*: 973-976, 2008.

12 Phechrmeekha T, Sritularak B and Likhitwitayawuid K: New phenolic compounds from *Dendrobium* capillipes and *Dendrobium secundum*. J Asian Nat Prod Res *14*: 748-754, 2012.

13 Ho CK and Chen CC: Moscatilin from the orchid *Dendrobium* loddigesii is a potential anticancer agent. Cancer Invest *21*: 729-736, 2003.

14 Kowitdamrong A, Chanvorachote P, Sritularak B and Pongrakhanon V: Moscatilin inhibits lung cancer cell motility and invasion *via* suppression of endogenous reactive oxygen species. BioMed Res Intl Article ID *765894*: 1-11, 2013.

15 Sritularak B and Likhitwitayawuid K: New bisbibenzyls from *Dendrobium* falconeri. Helv Chim Acta *92*: 740-744, 2009.

16 Sritularak B, Anuwat M and Likhitwitayawuid K: A new phenanthrenequinone from *Dendrobium* draconis. J Asian Nat Prod Res *13*: 251-255, 2011.

17 Sritularak B, Duangrak N and Likhitwitayawuid K: A new bibenzyl from *Dendrobium secundum*. Z Nat Forsch *66c*: 205-208, 2011.

18 Chanvorachote P, Kowitdamrong T, Ruanghirun B, Sritularak B, Mungmee C and Likhitwitayawuid K: Anti-metastatic activities of bibenzyls from *Dendrobium* pulchellum. Nat Prod Commun *8*: 115-118, 2013.

19 Du Q, Jerz G and Winterhalter P: Preparation of three flavonoids from the bark of Salix alba by high-speed countercurrent chromatographic separation. J Liq Chrom Rel Technol *27*: 3257-3264, 2004.

20 Wollenweber E, Doerr M, Stelzer R and Arriaga-Giner FJ: Lipophilic phenolics from the leaves of *Empetrum nigrum* - chemical structures and exudate localization. Bot Acta *105*: 300-305, 1992.

21 Majumder PL and Sen RC: Moscatilin, a bibenzyl derivative from the orchid *Dendrobium* moscatum. Phytochemistry *26*: 2121-2124, 1987.

22 Katerere DR, Gray AI, Nash RJ and Waigh RD: Phytochemical and antimicrobial investigations of stilbenoids and flavonoids isolated from three species of Combretaceae. Fitoterapia *83*: 932-940, 2012.

23 Liu YL, Ho DK and Cassady JM: Isolation of potential cancer chemopreventive agents from *Eriodictyon californicum*. J Nat Prod 55: 357-363, 1992.

24 Encarnación DR, Nogueiras CL, Salinas VHA, Anthoni U, Nielsen PH, Christophersen C, Wang H, Yao X, Tuchagues J.-P. and Ögren M: Isolation of eriodictyol identical with huazhongilexone from Solanum hindsianum. Acta Chem Scand *53*: 375-377, 1999.

25 Park Y, Moon BH, Yang H, Lee Y, Lee E and Lim Y: Complete assignments of-NMR data of 13 hydroxymethoxyflavones. Magn Res Chem *45*: 1072-1075, 2007.

26 Owen, RW, Haubner R, Mier W, Giacosa A, Hull WE, Spiegelhalder B and Bartsch H: Isolation, structure elucidation and antioxidant potential of the major phenolic and flavonoid compounds in brined olive drupes. Food Chem Toxicol *41*: 703-717, 2003.

27 Park Y, Moon BH, Lee E, Lee Y, Yoon Y, Ahn JH and Lim Y: ^{1}H and ^{13}C-NMR data of hydroxyflavone derivatives. Magn Res Chem *45*: 674-679, 2007.

28 Chanvorachote P and Pongrakhananon V: Ouabain down-regulates MCL-1 and sensitizes lung cancer cells to TRAIL-induced apoptosis. Am J Phys Cell Phys *304*: 263-272, 2013.

29 Halim H, Chunhacha P, Suwanborirux K and Chanvorachote P: Anti-cancer and antimetastatic activities of renieramycin M, a marine tetrahydroisoquinoline alkaloid, in human non-small cell lung cancer cells. Anticancer Res *31*: 193-201, 2011.

30 Pongrakhananon V, Nimmannit U, Luanpitpong S, Rojanasakul Y and Chanvorachote P: Curcumin sensitizes non-small cell lung cancer cell anoikis through reactive oxygen species-mediated Bcl-2 down-regulation. Apoptosis *15*: 574-585, 2010.

31 Powan P, Saito N, Suwanborirux K and Chanvorachote P: Ecteinascidin 770, a tetrahydroisoquinoline alkaloid, sensitizes human lung cancer cells to anoikis. Anticancer Res *33*: 505-512, 2013.

Received June 2, 2014
Revised July 15, 2014
Accepted July 16, 2014