CHAPTER III

EXPERIMENTAL

1. Source of plant materials

The whole plant of *Dendrobium ellipsophyllum* Tang & Wang was purchased from Jatujak market, Bangkok, in May 2012. Botanical identification was performed by 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 Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2. General techniques

2.1 Analytical thin-layer chromatography (TLC)

Technique	:	One dimension ascending
Absorbent	:	Silica gel 60 F_{254} (E. Merck) precoated plate
Layer thickness	:	0.2 mm
Distance	:	6.5 cm
Temperature	:	Laboratory temperature (30-35°C)
Detection	:	1. Ultraviolet light at wavelengths of 254 and 365 nm 2. Spraying with anisaldehyde reagent (0.5 ml p-anisaldehyde in 50 ml glacial acetic acid and 1 ml conc. sulfuric acid) and heating at 105 °C for 10 min.

2.2 Column Chromatography

2.2.1 Vacuum liquid chromatography (VLC)

Adsorbent	:	Silica gel 60 (No. 7734) particle size 0.063-0.200 mm (E. Merck)
Packing method	:	Dry packing

Sample loading : The sample was dissolved in a small amount of organic solvent, mixed with a small quantity of the adsorbent, triturated, dried and then gradually placed on top of the column.

Detection : Each fraction was determined by TLC under UV light at the wavelengths of 254 and 365 nm.

2.2.2 Flash column chromatography (FCC)

Adsorbent : Silica gel 60 (No. 9385) particle size 0.040-0.063 mm (E. Merck)

Packing method : Wet packing

Sample loading : The sample was dissolved in a small amount of the organic solvent, mixed with a small quantity of the adsorbent, triturated, dried and then gradually applied on top of the column.

Detection : Fractions were examined by TLC under UV light at the wavelengths of 254 and 365 nm.

2.2.3 Medium pressure liquid chromatography (MPLC)

Adsorbent : Silica gel 60 (No.9385) particle size 0.040-0.063 mm (E. Merck)

Packing method : Dry packing

Sample loading : The sample was dissolved in a small amount of organic solvent, mixed with a small quantity of adsorbent, triturated, dried and then gradually placed on top of the column.

Detection : Fractions were examined by TLC under UV light at the wavelengths of 254 and 365 nm.

2.2.4 Gel filtration chromatography

Adsorbent	:	Sephadex LH-20 (Pharmacia)
Packing method	:	An appropriate organic solvent was used as the eluent. Gel filter was suspended in the eluent, left standing about 24 hours prior to use and then poured into the column and left to set tightly.
Sample loading	:	The sample was dissolved in a small amount of the eluent and then gradually distributed on top of the column.
Detection	:	Fractions were determined by TLC under UV light at the wavelengths of 254 and 365 nm.

2.3 Spectroscopy

2.3.1 Mass spectra

Mass spectra were recorded on a Bruker microTOF or a Micromass LCT mass spectrometer (Department of Chemistry, Factory of Science, Mahidol University) or a Water, Acquity ultra performance LC mass spectrophotometer (Department of Medical Sciences).

2.3.2 Ultraviolet (UV) absorption spectra

UV (in methanol) spectra were obtained on a Shimadzu UV-160A UV/VIS spectrophotometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.3.3 Infrared (IR) spectra

IR spectra were obtained on a Perkin-Elmer FT-IR 1760X spectrophotometer (Scientific and Technology Research Equipment Center, Chulalongkorn University).

2.3.4 Proton and carbon-13 nuclear magnetic resonance (^{1}H and ^{13}C-NMR) spectra

^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Bruker Avance DPX-300 FT-NMR spectrometer (Faculty of Pharmaceutical Sciences, Chulalongkorn University).

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Varian Unity INOVA-500 NMR spectrometer JEOL JMN-A 500 NMR spectrometer (500 MHz) (Scientific and Technology Research Equipment Center, Chulalongkorn University).

Deuterated solvents for NMR spectra were used, including deuterated chloroform ($CDCl_3$), deuterated acetone (acetone-d_6). Chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal.

2.3.5 Optical rotation

Optical rotations were measured on a Perkin-Elmer 341 polarimeter (Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.4 Solvents

All organic solvents used throughout this work were of commercial grade and were redistilled prior to use.

3. Extraction and isolation

3.1 Extraction

Dried powered whole plant (4.8 kg) was extracted with methanol (3X10 L) at room temperature to give a crude extract (400 g) after evaporation of the solvent.

3.2 Separation of methanol extract

The crude extract (200 g) was separated by vacuum liquid chromatography (VLC) on silica gel (*n*-hexane-EtOAc gradient). The eluates were collected about 400 mL per fraction and examined by TLC (silica gel, *n*-hexane-EtOAc 1:1). Fractions with the same TLC pattern were combined to give 5 fractions

(A-E): fractions A (0.03 g), B (1.56 g), C (3.28 g), D (63.73 g) and E (95.03 g). Fraction D (63.73 g) was separated by VLC over silica gel, eluted with *n*-hexane-EtOAc gradient to give 7 fractions (D1-D7).

3.2.1 Isolation of compound DE1 (5,7-Dihydroxy-chromen-4-one)

Fraction D4 (2.39 g) was separated by Flash column chromatography (FCC) (silica gel; *n*-hexane-EtOAc gradient) and then further purified on Sephadex LH-20 (acetone) to give compound DE1 (4 mg).

3.2.2 Isolation of compound DE2 (4,5- Dihydroxy-2,3-dimethoxy-9,10-dihydrophenanthrene)

Fraction D5 (5.4 g) was subjected to medium pressure liquid column chromatography (MPLC) over silica gel, eluted with *n*-hexane-EtOAc gradient to give 14 fractions (D5a-D5n). Purification of fraction D5d (47 mg) on Sephadex LH-20 (acetone) gave compound DE2 (9 mg).

3.2.3 Isolation of compound DE3 (Moscatilin)

Fraction D5f (828 mg) was purified on Sephadex LH-20 (acetone) to give compound DE3 (188 mg).

3.2.4 Isolation of compound DE4 (4,4′-Dihydroxy-3,5-dimethoxybibenzyl) and compound DE5 (4,5,4′-Trihydroxy-3,3′-dimethoxybibenzyl)

Fraction D5g (954.3 mg) was separated on Sephadex LH-20 (acetone) to give 11 fractions (D5g1-D5g11). Fraction D5g8 (314 mg) was further purified by FCC (silica gel; *n*-hexane-EtOAc gradient) to give compound DE4 (5.7 mg) and compound DE5 (151 mg).

3.2.5 Isolation of compound DE6 ((2*S*)-Homoeriodictyol)

Fraction D5g9 (111.8 mg) was purified by FCC (silica gel; *n*-hexane-EtOAc gradient) to give compound DE6 (52.3 mg).

3.2.6 Isolation of compound DE7 ((2*S*)-Eriodictyol)

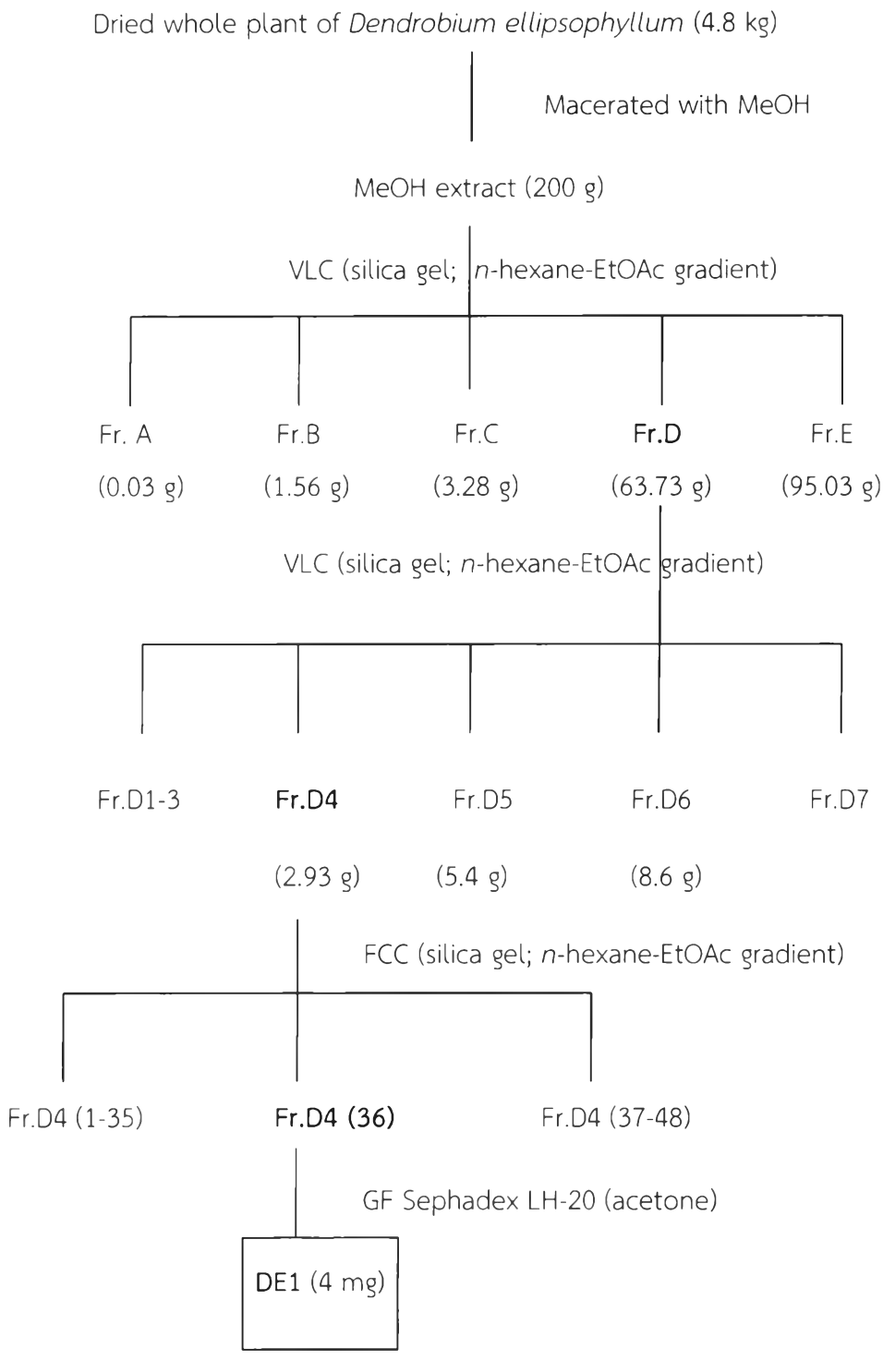
Fraction D5i (1.0 g) was separated by FCC (silica gel; *n*-hexane-EtOAc gradient) to give compound DE7 (364 mg).

3.2.7 Isolation of compound DE8 (Chrysoeriol) and compound DE9 (Phloretic acid)

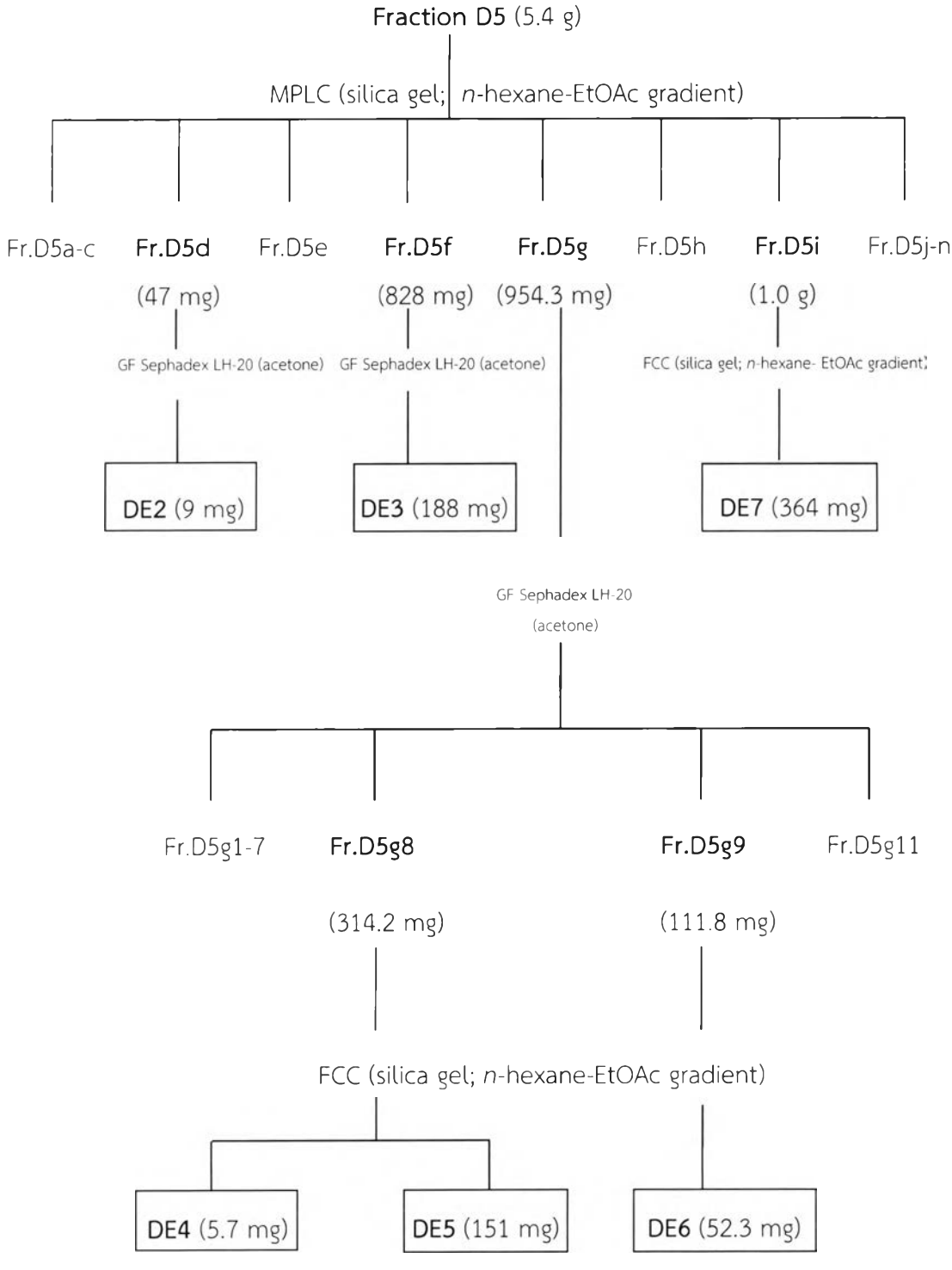
Fraction D6 (8.6 g) was separated by FCC (silica gel; *n*-hexane-EtOAc gradient) to give 23 fractions (D6a-D6w). Fraction D6i (1.0 g) was further separated on Sephadex LH-20 (acetone) to give 10 fractions (D6i1-D6i10). Fractions D6i4 to D6i6 were combined and then separated by FCC (silica gel; *n*-hexane-EtOAc gradient) to give 10 fractions (D6i4-6.a-D6i4-6.j). Fractions D6i4-6.a to D6i4-6.c were combined and purified on Sephadex LH-20 (acetone) to give compound DE8 (8 mg). Compound DE9 (18 mg) was obtained from fraction D6i4d-f after purification on Sephadex LH-20 (acetone).

3.2.8 Isolation of compound DE10 (Luteolin)

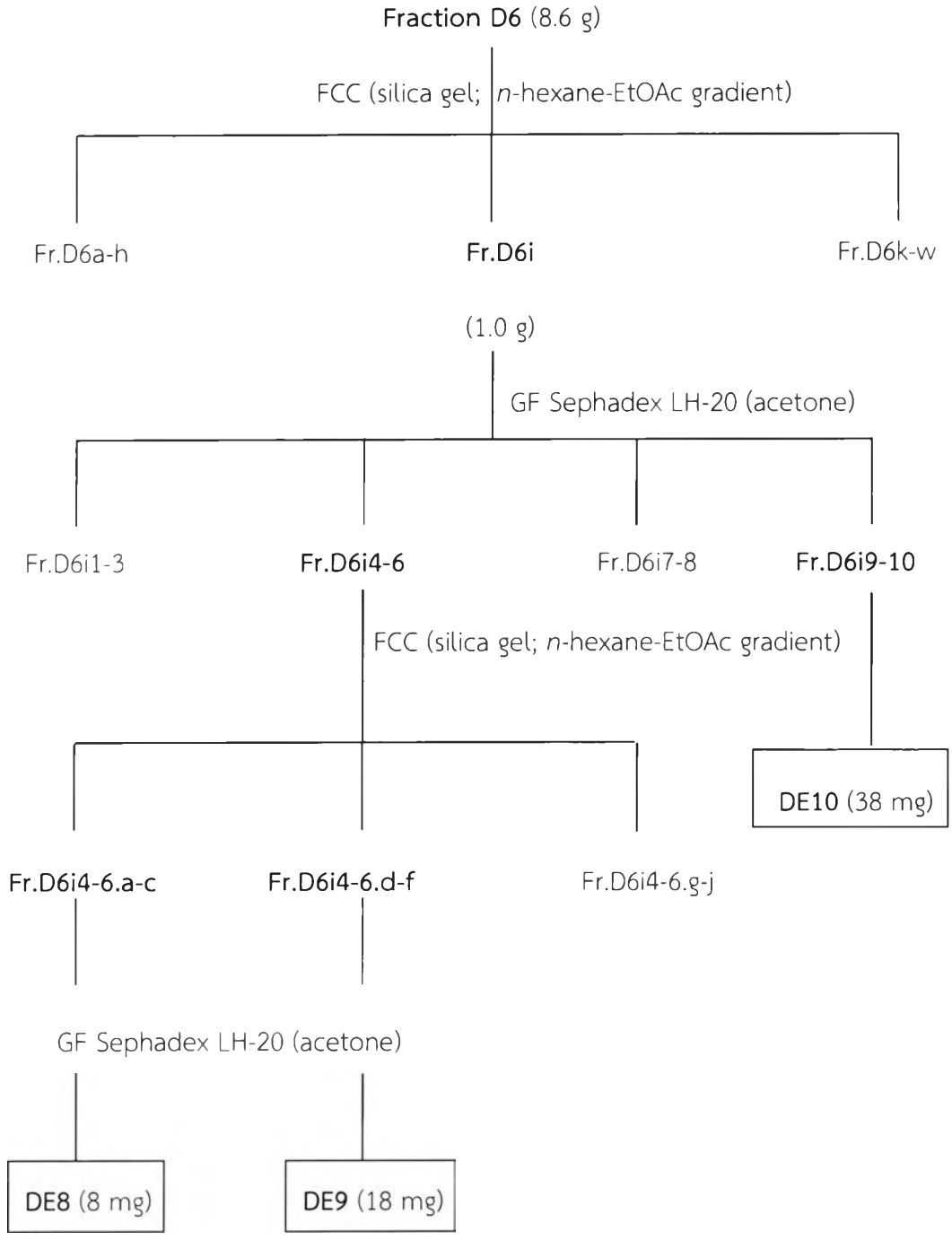
Fraction D6i9-10 was purified on Sephadex LH-20 (acetone) to give compound DE10 (38 mg).



Sheme 1 Separation of the MeOH extract of *Dendrobium ellipsophyllum*



Scheme 1 Seperation of the MeOH extract of *Dendrobium ellipsophyllum* (continued)



Scheme 1 Separation of the MeOH extract of *Dendrobium ellipsophyllum* (continued)

4. Physical and spectral data of isolated compounds

4.1 Compound DE1 (5,7-Dihydroxy-chromen-4-one)

Compound DE1 was obtained as colorless needles, soluble in acetone (4 mg, 8.33×10^{-2} % based on dried weight of whole plants).

HR-ESI-MS : $[M+Na]^+$ ion at *m/z* 201.0153 (calcd for $C_9H_6O_4Na$ 201.0163); **Figure 5**

FT-IR : υ_{max} cm^{-1} (KBr): 3434, 3083, 1644, 1616, 1463; **Figure 6**

UV : λ_{max} nm (log ε), in MeOH: 295 (2.92), 256 (3.35), 216 (3.22); **Figure 7**

^{1}H NMR : δ ppm, 300 MHz, in acetone- d_6; see **Table 2, Figure 8**

^{13}C NMR : δ ppm, 75 MHz, in acetone- d_6; see **Table 2, Figure 9**

4.2 Compound DE2 (4,5- Dihydroxy-2,3-dimethoxy-9,10-dihydrophenanthrene)

Compound DE2 was obtained as a brown amorphous solid, soluble in acetone (9 mg, 0.19 % based on dried weight of whole plants).

HR-ESI-MS : $[M+Na]^+$ ion at *m/z* 295.0901 (calcd $C_{16}H_{16}O_4Na$ 295.0946); **Figure 12**

FT-IR : υ_{max} cm^{-1} (KBr): 3434, 3025, 1615, 1458; **Figure 13**

UV : λ_{max} nm (log ε), in MeOH: 275 (3.12), 221 (3.37); **Figure 14**

^{1}H NMR : δ ppm, 300 MHz, in $CDCl_3$; see **Table 3, Figure 15**

^{13}C NMR : δ ppm, 75 MHz, in $CDCl_3$; see **Table 3, Figure 16**

4.3 Compound DE3 (Moscatilin)

Compound DE3 was obtained as a brown amorphous solid, soluble in acetone (188 mg, 3.92 % based on dried weight of whole plants).

ESI-MS : $[M+H]^+$ ion at *m/z* 305 ($C_{17}H_{20}O_5$); **Figure 20**

FT-IR : υ_{max} cm^{-1} (KBr): 3437, 3022, 1611, 1455; **Figure 21**

UV : λ_{max} nm (log ε), in MeOH: 286 (3.45), 228 (3.47); **Figure 22**

^{1}H NMR : δ ppm, 500 MHz, in acetone- d_6; see **Table 4, Figures 23-25**

^{13}C NMR : δ ppm, 125 MHz, in acetone- d_6; see **Table 4, Figures 26-28**

4.4 Compound DE4 (4,4′-Dihydroxy-3,5-dimethoxybibenzyl)

Compound DE4 was obtained as a brown amorphous solid, soluble in acetone (5.7 mg, 0.12 % based on dried weight of whole plants).

ESI-MS : $[M+H]^+$ ion at *m/z* 275 ($C_{16}H_{18}O_4$); **Figure 29**

FT-IR : υ_{max} cm^{-1} (KBr): 3399, 3016, 1614, 1458; **Figure 30**

UV : λ_{max} nm (log ε), in MeOH: 279 (2.81), 228 (3.42); **Figure 31**

^{1}H NMR : δ ppm, 500 MHz, in acetone- d_6; see **Table 5, Figures 32-34**

^{13}C NMR : δ ppm, 125 MHz, in acetone- d_6; see **Table 5, Figure 35**

4.5 Compound DE5 (4,5,4′-Trihydroxy-3,3′-dimethoxybibenzyl)

Compound DE5 was obtained as a brown amorphous solid, soluble in acetone (151 mg, 3.15 % based on dried weight of whole plants).

ESI-MS : $[M+H]^+$ ion at *m/z* 291 ($C_{16}H_{18}O_5$); **Figure 40**

FT-IR : υ_{max} cm^{-1} (KBr): 3434, 2923, 1615, 1464; **Figure 41**

UV : λ_{max} nm (log ε), in MeOH: 281 (2.89), 227 (3.40); **Figure 42**

^{1}H NMR : δ ppm, 500 MHz, in acetone- d_6; see **Table 6, Figures 43-45**

^{13}C NMR : δ ppm, 125 MHz, in acetone- d_6; see **Table 6, Figures 46-48**

4.6 Compound DE6 ((2*S*)-Homoeriodictyol)

Compound DE6 was obtained as a colorless needles, soluble in acetone (52.3 mg, 1.09 % based on dried weight of whole plants).

ESI-MS : $[M+H]^+$ ion at *m/z* 303 ($C_{16}H_{14}O_6$); **Figure 50**

FT-IR : υ_{max} cm^{-1} (KBr): 3464, 3046, 1635, 1614, 1468; **Figure 51**

UV : λ_{max} nm (log ε), in MeOH: 280 (2.84), 214 (3.58); **Figure 52**

^{1}H NMR : δ ppm, 500 MHz, in acetone- d_6; see **Table 7, Figures 53-57**

^{13}C NMR : δ ppm, 125 MHz, in acetone- d_6; see **Table7, Figure 58**

Specifc rotation: $[\alpha]^{25}_{D}$ -18.67 (*c* 0.1, MeOH)

4.7 Compound DE7 ((2*S*)-Eriodictyol)

Compound DE7 was obtained as a colorless needles, soluble in acetone (364 mg, 7.58 % based on dried weight of whole plants).

HR-ESI-MS : $[M+Na]^+$ ion at *m/z* 311.0505 (calcd for $C_{15}H_{12}O_6Na$ 311.0531); **Figure 60**

FT-IR : υ_{max} cm^{-1} (KBr): 3366, 2922, 1636, 1604, 1451; **Figure 61**

UV : λ_{max} nm (log ε), in MeOH: 288 (3.67), 227 (3.68); **Figure 62**

^{1}H NMR : δ ppm, 500 MHz, in acetone- d_6; see **Table 8, Figure 63**

^{13}C NMR : δ ppm, 125 MHz, in acetone- d_6; see **Table 8, Figure 64**

Specifc rotation: $[\alpha]^{25}_{D}$ -18.66 (*c* 0.1, MeOH)

4.8 Compound DE8 (Chrysoeriol)

Compound DE8 was obtained as a yellow powder, soluble in acetone (8 mg, 0.17 % based on dried weight of whole plants).

ESI-MS : $[M+H]^+$ ion at *m/z* 301 ($C_{16}H_{12}O_6$); **Figure 70**

FT-IR : υ_{max} cm^{-1} (KBr): 3411, 3087, 1599, 1652, 1435; **Figure 71**

UV : λ_{max} nm (log ε), in MeOH: 286 (3.54), 226 (3.70); **Figure 72**

^{1}H NMR : δ ppm, 500 MHz, in acetone- d_6; see **Table 9, Figures 73-74**

^{13}C NMR : δ ppm, 125 MHz, in acetone- d_6; see **Table 9, Figure 75**

4.9 Compound DE9 (Phloretic acid)

Compound DE9 was obtained as a brown amorphous solid, soluble in acetone (18 mg, 0.38 % based on dried weight of whole plants).

HR-ESI-MS : $[M+Na]^+$ ion at *m/z* 189.0442 (calcd for $C_9H_{10}O_3Na$ 189.0527); **Figure 79**

FT-IR : υ_{max} cm^{-1} (KBr): 3421, 3015, 1713, 1602, 1456; **Figure 80**

UV : λ_{max} nm (log ε), in MeOH: 265 (2.99), 223 (3.35); **Figure 81**

^{1}H NMR : δ ppm, 300 MHz, in acetone- d_6; see **Table 10, Figure 82**

^{13}C NMR : δ ppm, 75 MHz, in acetone- d_6; see **Table 10, Figure 83**

4.10 Compound DE10 (Luteolin)

Compound DE10 was obtained as a yellow powder, soluble in acetone (38 mg, 0.79 % based on dried weight of whole plants).

ESI-MS : $[M+H]^+$ ion at *m/z* 287 ($C_{15}H_{10}O_6$); **Figure 85**

FT-IR : υ_{max} cm^{-1} (KBr): 3425, 2919, 1653, 1616, 1447; **Figure 86**

UV : λ_{max} nm (log ε), in MeOH: 349 (3.61), 223 (3.59); **Figure 87**

^{1}H NMR : δ ppm, 500 MHz, in acetone- d_6; see **Table 11, Figures 88-89**

^{13}C NMR : δ ppm, 125 MHz, in acetone- d_6; see **Table 11, Figures 90-91**

5. Determination of cytotoxicity

The cytotoxicity assay against two cancerous human-cell lines, including KB (oral human epidermal carcinoma) and MCF-7 (breast cancer) cells, was done in this study by the Bioassay Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC). The test was performed using resazurin microplate assay method (REMA) (O'Brien *et al.*, 2000). The protocols according to bioassay laboratory guideline (Bioassay laboratory protocol 01, 2009) are as follows:

Assay	Cancer cell growth inhibition
Method	Resazurin microplate assay (REMA)
Positive control	Doxorubicin, ellipticine, and tamoxifen
Negative control	0.5% DMSO
Maximum final test concentration	50 µg/mL

Description

Two cancerous human-cell lines are available for this assay:

1. KB cell line (epidermoid carcinoma of oral cavity, ATCC CCL-17)
2. MCF-7 cell line (breast adrenocarcinoma, ATCC HTB-22)

This assay was performed as follows:

1. Cells at a logarithmic growth phase were harvested and diluted to 7×10^4 cells/mL for KB and 9×10^4 cells/mL for MCF-7 in fresh medium.
2. Consecutively, 5 µL of test sample was diluted in 5% DMSO, and 45 µL of cell suspension was added to 384-well plates then incubated at 37°C in 5% CO_2 incubator.
3. After incubation period for 3 days, 12.5 µL of 62.5 µg/mL resazurin solution was added to each well, then incubated at 37°C for 4 hours.

4. SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA) was used to measure the fluorescence signal at the excitation and emission wavelengths of 530 nm and 590 nm, respectively. The percent inhibition of cell growth was calculated using the following equation:

$$\% \text{ Inhibition} = [1-(FU_T/FU_C) \times 100]$$

whereas, FU_T and FU_C are the mean fluorescent unit from treated and untreated conditions, respectively.

Dose response curves were plotted from 6 concentrations of 2-fold serially diluted test compounds and the sample concentrations that inhibit cell growth by 50% (IC_{50}) can be acquired using the SOFTMax Pro software (Molecular Devices, USA).

6. Determination of anti-metastatic activity

6.1 Cells and reagents

Human lung cancer H292 cells were obtained from the American Type Culture Collection (Manassas, VA). 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)-2H-tetrazolium-5-carboxani lide (XTT), and other chemicals were obtained from Sigma Chemical, Inc. (St. Louis, MO). Hoechst 33342 were obtained from Molecular Probes, Inc (Eugene, OR).

6.2 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 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 various times up to 24 h.

For cell viability assay, cells were harvested, washed, and incubated with 20 μM of 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) for 4 h at 37°C. Optical density was then determined with a V-max photometer (Molecular Devices Inc., Menlopark, CA) at a wavelength of 450 nm.

6.3 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. Nuclei 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).

6.4 Statistical analysis

Mean densitometry data from independent experiments were normalized to result in cells in the control. The data are presented as the mean ± SD from 3 or more independent experiments, and analyzed by the Student's *t* test at a significance level of $p < 0.05$.

7. Determination of anti-herpes simplex virus activity

7.1 Viruses and cells

HSV strains used in this study were HSV-1 (KOS) and HSV-2 (Baylor186). Vero cells (ATCC CCL81) were grown and maintained in Eagle's minimum medium supplemented with 10% fetal bovine serum.

7.2 Plaque reduction assay

Anti-HSV activity of the compound was determined by the plaque reduction assay modified from the previously reported method (Chansriniyom *et al.*, 2009; Lipipun *et al.*, 2003). In the inactivation assay, each of 30 plaque forming units of HSV-1 or HSV-2 was mixed with various concentrations of compound and incubated for 1 hour, and then the mixture was added to Vero cells in 96-well tissue culture plate. After 1 hour incubation for virus adsorption, the overlay media were added. The infected cultures were incubated at 37 °C for 2 days. The infected cells were fixed, stained, and the plaques were counted. The 50% effective concentration (EC_{50}) was determined.