CHAPTER III

EXPERIMENTAL

1.Source of plant materials

The whole plant of *Dendrobium brymerianum* Rchb. f. was purchased from Jatujak market, Bangkok, in July 2011. Botanical identification was done by Associate Professor Thatree Phadungcharoen through comparison with the illustrations and information in *A Field Guide to the Wild Orchids of Thailand* (Vaddhanaphuti, 2005). A voucher specimen (BS-DB-092555) is deposited at the 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	:	Siliga 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 gracial 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	:	Siliga 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 examined by TLC under UV light at the wavelengths of 254 and 365 nm

2.2.2 Flash Column chromatography (FCC)

Adsorbent : Siliga 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 organic solvent, mixed with a small quantity of the adsorbent, triturated, dried and then gradually placed on top of the column.

Detection : Fractions were examined as described in section 2.2.1

2.2.3 Gel filtration chromatography

Adsorbent : Sephadex LH-20 (Pharmacia)

Packing method : The 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 let alone 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 examined in the same way as described in section 2.2.1

2.3 Spectroscopy

2.3.1 Mass spectra

Mass spectra were recorded on a micrOTOF BRUKER DALTONICS mass spectrometer (Department of chemistry, Faculty of Science, Mahidol University) and 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 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.

2.4 Physical property

Optical rotation was measured on a Perkin Elmer Polarimeter 341 (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.5 Solvents

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

3. Extraction and Isolation

3.1 Extraction

The dried whole plants (2.8 kg) were chopped, powdered and then macerated with methanol (3x5 L) for 72 hours three times. The methanol extract was concentrated under reduced pressure to give 100 g of a crude extract.

3.2 Separation of methanol extract

Crude extract (100 g) was initially separated by vacuum liquid chromatography (VLC). The procedure was performed as described in section 2.2.1. Silica gel (No.7734, 600 g) was used as the stationary phase and a step gradient of hexane-EtOAc (100:0 to 0:100) and EtOAc-MeOH (100:0 to 0:100) as the mobile phase. The eluates were collected about 500 mL per fraction and examined by TLC (silica gel, hexane-EtOAc 6:4) to yield eighty fractions. Fractions with similar chromatographic patterns were combined to give seven fractions, including Fractions

A (350 mg), B (5.34 g), C (10.46 g), D (10.01 g), E (3.47 g), F (16.90 g) and G (18.21 g). According to the method of bioassay-guided fractionation, fraction F, was selected for further study because of its potent cytotoxic effect against KB cell lines.

3.2.1 Isolation of compound DB1 (Moscatilin) and DB2 (Flavanthrinin)

Fraction F (16.90 g) was separated by FCC using silica gel (No. 9385) as the stationary phase with a step gradient mixture of hexane-EtOAc (100:0 to 0:100). Fifty fractions were obtained and combined according to the similarity of their TLC patterns (silica gel, CH_2Cl_2-EtOAc 9:1) to give eleven fractions: F-I (240 mg), F-II (1 g), F-III (145 mg), F-IV (650 mg), F-V (2.28 g), F-VI (4.80 g), F-VII (2.47 g), F-VIII (1.05 g), F-IX (568 mg), F-X (3.88 g), and F-XI (377 mg).

Fraction F-V (2.28 g) was then separated on a Sephadex LH-20 column, eluted with acetone, to give eight fractions: FV1 (50 mg), FV2 (20 mg), FV3 (40 mg), FV4 (400 mg), FV5 (1 g), FV6 (108 mg), FV7 (600 mg) and FV8 (60 mg).

Fraction FV4 (400 mg) was further purified on a Sephadex LH-20 column, eluted with acetone, to give compound DB1 as a brown amorphous solid (3.7 mg, R_f 0.42, silica gel, CH_2Cl_2-acetone = 9.5:0.5) which was later identified as moscatilin [**59**] and compound DB2 as a brown amorphous solid (47 mg, R_f 0.44, silica gel, CH_2Cl_2-acetone = 9.5:0.5) which was later identified as flavanthrinin [**176**].

3.2.2 Isolation of compound DB3 (Gigantol)

Fraction FV5 (1 g) was further purified on a Sephadex LH-20 column, eluted with acetone, to yield compound DB3 as a brown amorphous solid (50 mg, R_f 0.35, silica gel, CH_2Cl_2-acetone = 9.5:0.5). It was identified as gigantol [**50**].

3.2.3 Isolation of compound DB4 (Lusianthridin)

Fraction FV6 (108 mg) was purified on a Sephadex LH-20 column, eluted with acetone, to give compound DB4 as a brown amorphous solid (5 mg, R_f 0.40, silica gel, CH_2Cl_2-acetone = 9.5:0.5). It was identified as lusianthridin [**185**].

3.2.4 Isolation of compound DB5 (Nobilone) and DB6 (Dendroflorin)

Fraction F-VI (4.80 g) was further separated by FCC using silica gel (No. 9385) as the stationary phase with gradient elution [CH_2Cl_2-EtOAc (100:0 to 0:100)] to give eighteen fractions. After combination of the fractions with similar TLC patterns (silica gel, CH_2Cl_2-EtOAc 9:1), seven fractions were obtained: FVI1 (40 mg), FVI2 (2.70 g), FVI3 (13 mg), FVI4 (1.70 g), FVI5 (6 mg), FVI6 (93 mg) and FVI7 (15 mg).

Fraction FVI4 (1.70 g) was separated on a Sephadex LH-20 column, eluted with acetone, to give fifteen fractions. Fractions with similar TLC patterns (CH_2Cl_2-EtOAc 9:1) were combined to yield three fractions: FVI4A (864 mg), FVI4B (160 mg) and FVI4C (433 mg).

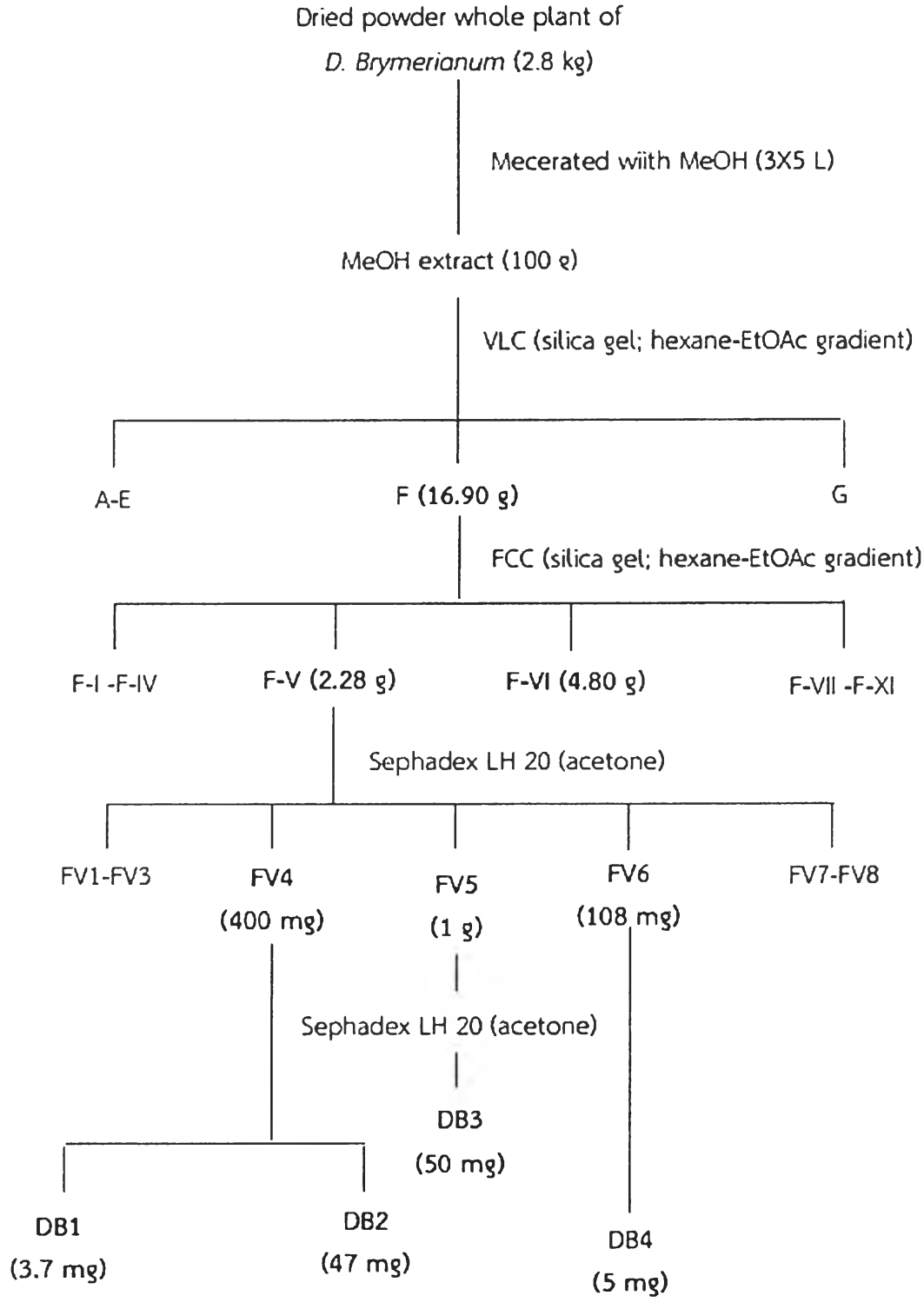
Fraction FVI4B (160 mg) was then separated by FCC using silica gel (No. 9385) as the stationary phase with gradient elution of CH_2Cl_2-EtOAc (10:0 to 0:1) to give compound DB5 as a red amorphous solid (6 mg, R_f 0.6, silica gel, CH_2Cl_2-EtOAc = 8:2) which was identified as nobilone [**112**], and compound DB6 as a red amorphous solid (74 mg, R_f 0.45, silica gel, CH_2Cl_2-EtOAc = 8:2) which was identified as dendroflorin [**110**].

3.2.5 Isolation of compound DB7 (Denchrysan B) and DB8 (Tristin)

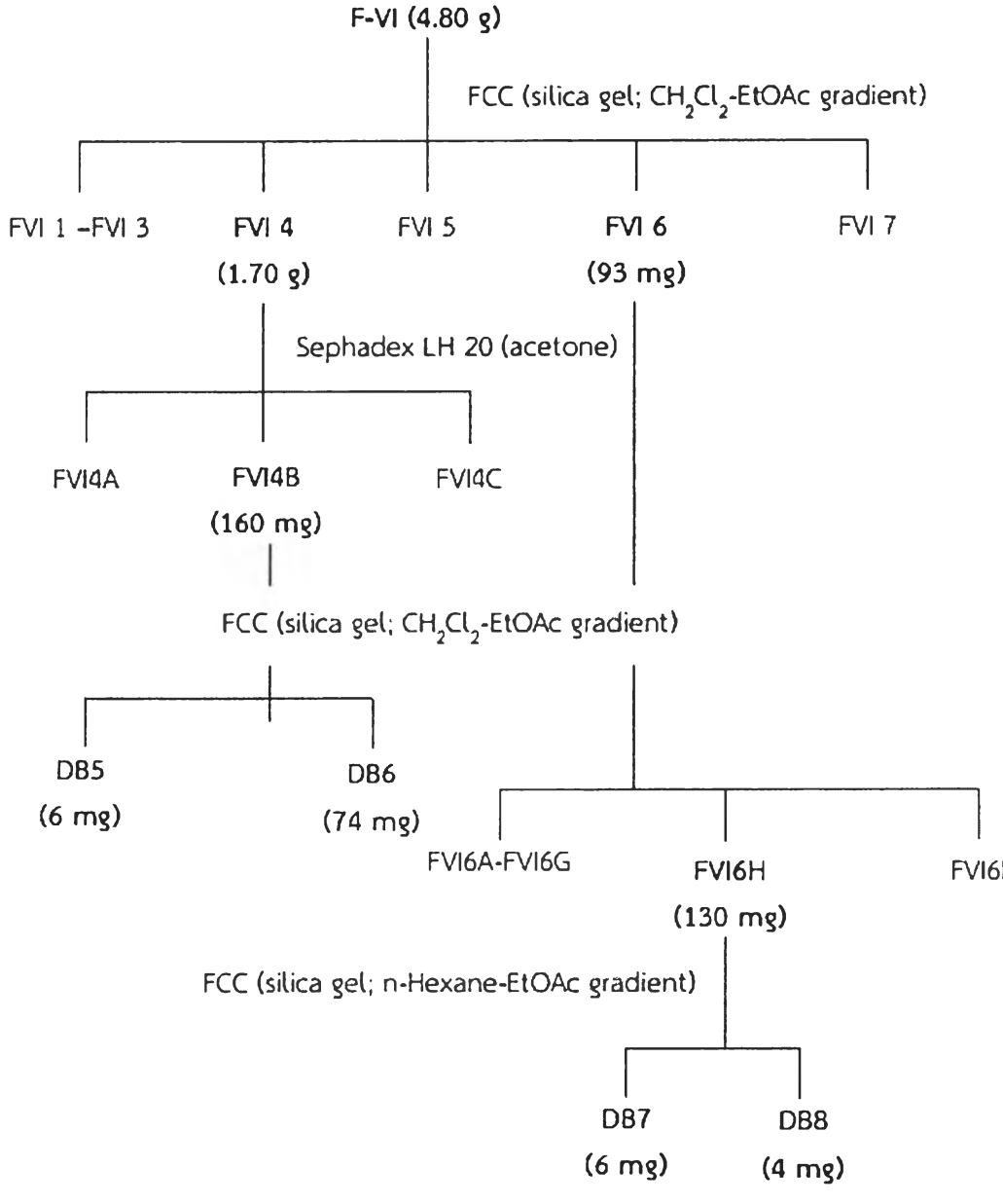
Fraction FVI6 (93 mg) was separated by FCC using silica gel (No. 9385) as the stationary phase with gradient elution of CH_2Cl_2-EtOAc (100:0 to 0:100) to yield sixty-two fractions. Fractions with similar TLC patterns (CH_2Cl_2-EtOAc 8.5:1.5)

were combined to give nine fractions: FVI6A (4.2 mg), FVI6B (3.7 mg), FVI6C (5.6 g), FVI6D (12.6 mg), FVI6E (34.4 mg), FVI6F (26.2 mg), FVI6G (41.1 mg), FVI6H (130 mg) and FVI6I (600 mg)

Fraction FVI6H (130 mg) was then purified by FCC using silica gel (No. 9385) as the stationary phase with gradient elution of n-hexane-CH_2Cl_2(100:0 to 0:100) to give compound DB7 as a red amorphous solid (6 mg, R_f 0.24, silica gel, hexane-EtOAc = 1:1) which was identified as denchrysan B [**109**]. This chromatographic separation also gave compound DB8 as a brown amorphous solid (4 mg, R_f 0.20, silica gel, hexane-EtOAc = 1:1) which was identified as tristin [**70**].



Scheme 1 Separation of the MeOH extract of *Dendrobium brymerianum*



Scheme 1 Separation of the MeOH extract of *Dendrobium brymerianum*

4. Physical and spectral data of isolated compounds

4.1 Compound DB1 (Moscatilin)

Compound DB1 was obtained as a brown amorphous solid, soluble in acetone (3.7 mg, 1.32×10^{-4} % based on dried weight of whole plant).

ESI-MS : $[M+H]^+$ ion at *m/z* 305 ($C_{17}H_{20}O_5$); Figure 3

FT-IR : ν cm^{-1} (KBr) : 3410, 1609, 1517, 1463, 1227, 1115; Figure 4

UV : λ_{max} nm (log ε), in methanol: 220 (3.30), 281 (2.68); Figure 5

^{1}H NMR : δ ppm, 500 MHz, in acetone-d_6; see Table 2, Figure 6

^{13}C NMR : δ ppm, 125 MHz, in acetone-d_6; see Table 2, Figure 8

4.2 Compound DB2 (Flavanthrinin)

Compound DB2 was obtained as a brown amorphous solid, soluble in acetone (47 mg, 1.67×10^{-4} % based on dried weight of whole plant).

ESI-MS : $[M+H]^+$ ion at *m/z* 241 ($C_{15}H_{12}O_3$); Figure 9

FT-IR : ν_{max} cm^{-1} (KBr) : 3300, 1613, 1433, 1261, 1203, 1161, 99, 847; Figure 10

UV : λ_{max} nm (log ε), in methanol: 256 (4.55); Figure 11

^{1}H NMR : δ ppm, 300 MHz, in $CDCl_3$; see Table 3, Figure 12

^{13}C NMR : δ ppm, 75 MHz, in $CDCl_3$; see Table 3, Figure 14

4.3 Compound DB3 (Gigantol)

Compound DB3 was obtained as a brown amorphous solid, soluble in acetone (50 mg, 1.78×10^{-3} % based on dried weight of whole plant).

ESI-MS : $[M+H]^+$ ion at *m/z* 275 ($C_{16}H_{18}O_4$); Figure 15

FT-IR : ν cm^{-1} (KBr): 3400, 1613, 1598, 1514, 1272, 1233, 1150, 836; Figure 16

UV : λ_{max} nm (log ε), in methanol: 222 (5.52), 281 (5.00); Figure 17

^{1}H NMR : δ ppm, 500 MHz, in acetone-d_6; see Table 4, Figure 18

^{13}C NMR : δ ppm, 125 MHz, in acetone-d_6; see Table 4, Figure 21

4.4 Compound DB4 (Lusianthridin)

Compound DB4 was obtained as a brown amorphous solid, soluble in acetone (5 mg, 1.78×10^{-4} % based on dried weight of whole plant).

HRESI-MS : $[M+Na]^+$ ion at *m/z* 265.0852 ($C_{15}H_{14}O_3Na$); Figure 22

FT-IR : ν cm^{-1} (KBr) : 3367, 2924, 1657, 1611, 1454, 1229, 1146; Figure 23

UV : λ_{max} nm (log ε), in methanol: 221 (4.40), 278 (4.33); Figure 24

^{1}H NMR : δ ppm, 500 MHz, in acetone- d_6; see Table 5, Figure 25

^{13}C NMR : δ ppm, 125 MHz, in acetone- d_6; see Table 5, Figure 27

4.5 Compound DB5 (Nobilone)

Compound DB5 was obtained as a red amorphous solid, soluble in acetone (6 mg, 2.14×10^{-4} % based on dried weight of whole plant).

HRESI-MS : $[M+Na]^+$ ion at *m/z* 265.0479 ($C_{14}H_{10}O_4Na$); Figure 28

FT-IR : ν_{max} cm^{-1} (KBr) : 3403, 3288, 2924, 1699, 1448, 1323, 1263, 1143, 963, 738; Figure 29

UV : λ_{max} nm (log ε), in methanol: 274 (3.35); Figure 30

^{1}H NMR : δ ppm, 500 MHz, in acetone-d_6; see Table 6, Figure 31

^{13}C NMR : δ ppm, 125 MHz, in acetone-d_6; see Table 6, Figure 32

4.6 Compound DB6 (Dendroflorin)

Compound DB6 was obtained as a red amorphous solid, soluble in acetone (74 mg, 2.64×10^{-3} % based on dried weight of whole plant).

HRESI-MS : $[M+Na]^+$ ion at *m/z* 281.0445 ($C_{14}H_{10}O_5Na$); Figure 36

FT-IR : ν cm^{-1} (KBr) : 3276, 2924, 1682, 1608, 1494, 1320, 1157, 961, 634;

Figure 37

UV : λ_{max} nm (log ε), in methanol: 258 (4.33); Figure 38

^{1}H NMR : δ ppm, 500 MHz, in acetone- d_6; see Table 7, Figure 39

^{13}C NMR : δ ppm, 125 MHz, in acetone- d_6; see Table 7, Figure 41

4.7 Compound DB7 (Denchrysan B)

Compound DB7 was obtained as a red amorphous solid, soluble in acetone (6 mg, 2.14×10^{-4} % based on dried weight of whole plant).

HRESI-MS : $[M+Na]^+$ ion at *m/z* 267.0634 ($C_{14}H_{12}O_4Na$); Figure 42

FT-IR : ν cm^{-1} (KBr) : 3330, 3204, 2922, 1612, 1601, 1464, 1317, 1240, 1149, 1035, 957, 827; Figure 43

UV : λ_{max} nm (log ε), in methanol: 220 (3.38), 277 (3.19); Figure 44

$[\alpha]_D^{20}$: -7.9 (*c* = 0.1, MeOH)

^{1}H NMR : δ ppm, 300 MHz, in acetone- d_6; see Table 8, Figure 45

^{13}C NMR : δ ppm, 75 MHz, in acetone- d_6; see Table 8, Figure 46

4.8 Compound DB8 (Tristin)

Compound DB8 was obtained as a brown amorphous solid, soluble in acetone (4 mg, 1.42×10^{-4} % based on dried weight of whole plant).

HRESI-MS : $[M+Na]^+$ ion at *m/z* 283.0943 ($C_{15}H_{16}O_4Na$); Figure 49

FT-IR : ν cm^{-1} (KBr) : 3419, 2924, 1607, 1514, 1279, 1153, 1031, 828; Figure 50

UV : λ_{max} nm (log ε), in methanol: 220 (3.27), 277 (3.00); Figure 51

^{1}H NMR : δ ppm, 300 MHz, in acetone- d_6; see Table 9, Figure 52

^{13}C NMR : δ ppm, 75 MHz, in acetone- d_6; see Table 9, Figure 53

5. Determination of cytotoxicity

The cytotoxic evaluations in this study were conducted by The Bioassay Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC) and Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

5.1 Cancer cell growth inhibitor

The cytotoxicity assay against KB cell line (epidermoid carcinoma of oral cavity) was performed using Resazurin Microplate Assay (REMA) method (O'Brien *et al.*, 2000), with Ellipticine and Doxorubicin as positive controls, and 0.5% DMSO as a negative control. The samples were diluted to 50 µg/mL for final test concentration. The protocols are as follows :

1. Cells at a logarithmic growth phase were harvested and diluted to $2.2x10^4$ cells/mL in fresh medium.
2. Successively, 5 µL of test sample diluted in 5% DMSO, and 45 µL of cell suspension were added to 384-well plates, incubated at 37 °C in 5% CO_2 incubator.
3. After the 3 days of incubation period, 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 hours.
4. Fluorescence signal was measured using a SpectraMax M5 multi-detection microplate reader (Molecular Device, USA) at the excitation and emission wavelengths of 530 nm and 590 nm.
5. 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 3-fold serially diluted test compounds and the sample concentrations that inhibit cell growth by 50% (IC_{50}) could be derived using the SOFTMax Pro software (Molecular Device, USA)

The criteria of interpretation are shown as follows :

Inactive = % inhibition < 50%

Active (IC_{50} included) = % inhibition > 50%

5.2 Cell viability and Anti-migration assay

5.2.1 Cell viability assay

To determine cytotoxicity, cell viability was determined by MTT assay which measured cellular capacity to reduce MTT (yellow) to purple formazan crystal by mitochondrial dehydrogenase. Details of the test methods are as follows :

1. Lung cancer H460 cells, which were obtained from the American Type Culture Collection (Manassas, VA), 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. The cells, at a density of $1x10^4$ cells/well, were seeded in a 96-well plate and allowed to attach for 12 h, treated with different concentrations of compounds for 24 h, and incubated with 100 µL of 500 µg/mL MTT solution for 4 h at 37°C. Then, MTT solution was removed, and 100 µL of 99.9% DMSO was added to dissolve the formazan crystal. The intensity of formazan product was measured at 570 nm using a microplate reader. All

analyses were performed in at least three independent replicate cultures. The percentage of cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{A_{570} \text{ of treatment} \times 100}{A_{570} \text{ of untreated control}}$$

5.2.2 Anti-migration assay

The most active cytotoxic compounds were chosen for further investigation of their anti-migration activity by wound-healing assay. The methods are as follows :

1. A monolayer of cells, at a density of 1.5×10^4 cells/well, was cultured in 96-well plates, and a wound space was created by a 1-mm width tip.
2. After rinsing with PBS, the cell monolayers were treated with non-toxic concentrations of compounds (0.1 μg/mL), and allowed to migrate for 0-48 hours.
3. Micrographs were taken under a phase contrast microscope (×100; Olympus IX51 with DP70), and wound spaces were measured from 10 random fields of view using Olympus DP controller software.
4. Quantitative analysis of cell migration was performed using an average wound space from random fields of view, and the relative migration was calculated.