CHAPTER III

EXPERIMENTATION

3.1 Plant material

Fresh tubers of *B. superba* Roxb. were collected from Lampang Province, Thailand, in 2002. It was cleaned, sliced into pieces, dried for 10-12 hrs in a hot air oven at 80°C, ground into fine powder by Associate Professor Dr. Wichai Cherdshewasart.

3.2 Instruments and Equipments

3.2.1 Nuclear Magnetic Resonance Spectrometry (NMR)

The ^{1}H-NMR, ^{13}C-NMR, gCOSY, gHSQC, gHMBC and NOESY spectra were recorded on the Varian Mercury+400 Spectrometer operated at 400 MHz for ^{1}H nuclei and at 100 MHz for ^{13}C nuclei. The chemical shift was expressed in parts per million (ppm) using residual protonated solvents as reference.

3.2.2 Mass Spectrometry (MS)

The mass spectrum was recorded on electrospray-time of flight spectrometer Mass Spectrometer Bruker Daltonic, Germany; Water Co.Ltd. Model LCT. The standard mass spectrums on high resolution mass were $C_{16}H_{14}O_5$ + Na (*m/z* 309.1314), C_{13} $H_{16}O_6$ + Na (*m/z* 291.0845) and $C_{15}H_{20}O_5Si$ + Na (*m/z* 331.0978).

3.2.3 Ultraviolet - Visible Spectrometry (UV-Vis)

The UV-Vis spectra were recorded on the Perkin Elmer Lambda 25 UV-VIS spectrophotometer, using chloroform ($CHCl_3$) and dichloromethane (CH_2Cl_2)

3.2.4. Optical Rotation

The specific optical rotation values were recorded on the LTD instruments model JASCO P-1030 polariemeter, Analytical Lab Science Company, made in Japan.

3.2.5. Electro-thermometer

The melting point was measured on a Electrothermal 9100.

3.3 Chemical Reagents

3.3.1 Solvents

The solvents used in this study, such as hexane, chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2) and methanol (MeOH) were commercial grade, and purified prior to use by distillation. The solvents in AR grade were used for crystallizations, and TLC systems.

3.3.2 Packing material

3.3.2.1. Scharlau's silica gels 60, 0.04-0.06 mm (230 - 400 mesh ASTM) were used as adsorbents for flash column chromatography.

3.3.2.2 Merck's silica gel 60 GF_{254}. 1.07731.1000 was applied as adsorbent for preparative TLC.

3.3.2.3 Merck's TLC aluminums sheet, silica gel $60F_{254}$ precoated 25 sheets, 20x20 cm^2, layer 0.2 mm were used for identification of the same fractions and preparative TLC.

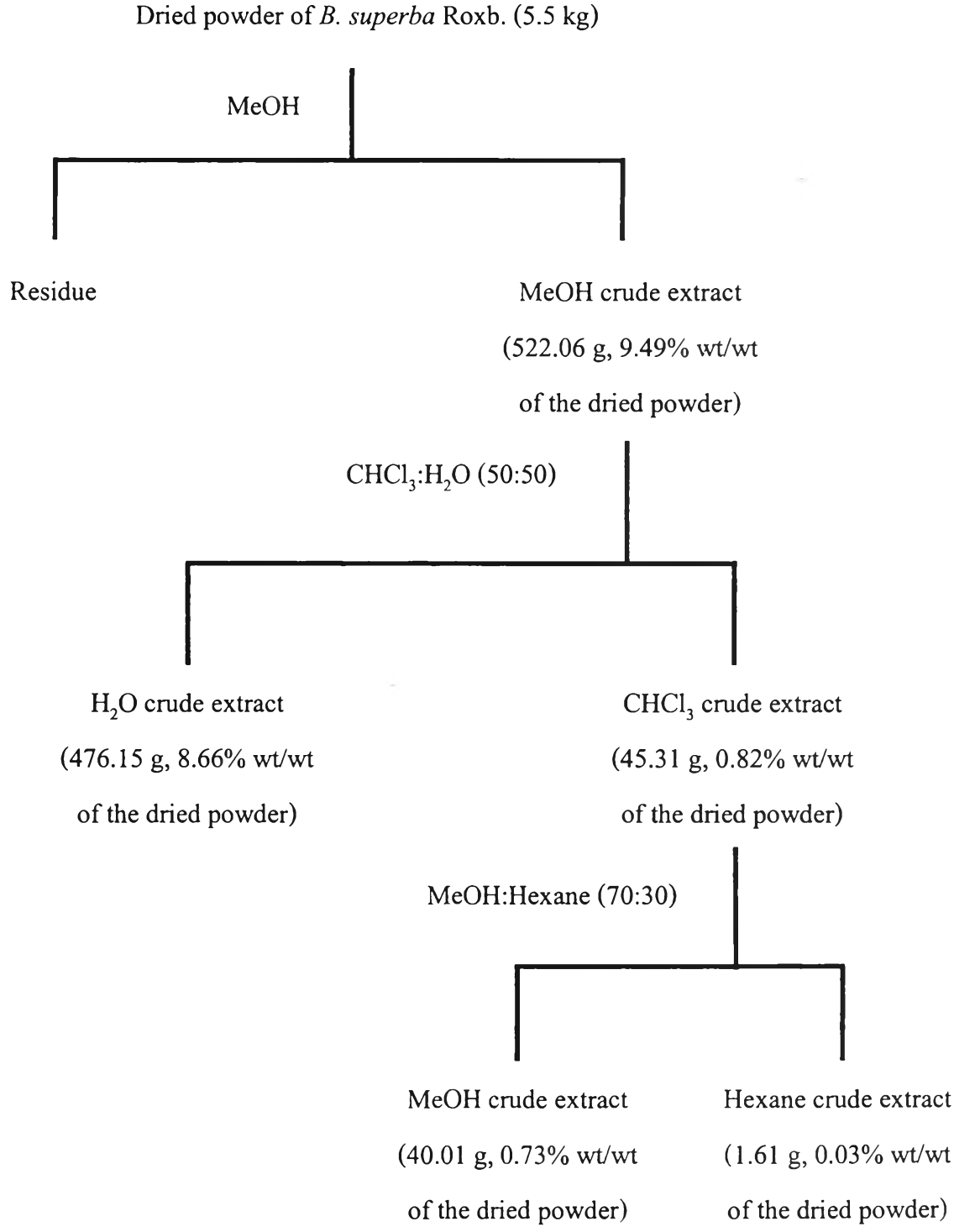
3.4 Extraction procedures

The root powder of *B. superba* Roxb. at the amount of 5.5 kg was extracted with MeOH (3x15 liters), for 7 days at room temperature. The obtained supernatant was filtered and evaporated under reduced pressure to dryness, exhibiting MeOH crude extract (522.06 g). The MeOH crude extract was partitioned with $CHCl_3$:H_2O (50:50) and evaporated, exhibiting H_2O crude extract (476.15 g) and $CHCl_3$ crude extract (45.31 g), respectively. The $CHCl_3$ crude extract (45.31 g) was partitioned with MeOH:hexane (70:30), evaporated, exhibiting MeOH crude extract (40.01 g) and hexane crude extract (1.61 g), respectively.

These crude extracts of the tuberous roots of *B. superba* Roxb. are shown in Table 1 and the procedure of extraction are shown in Scheme 1.

Table 1 The crude extracts of the tuberous roots of *B. superb* Roxb.

Solvent extract	Appearance	Weight (g)	% wt/wt of the dried powder
Hexane	Pale yellow oil	1.16	0.03
Methanol	Dark brown oil	40.01	0.73
Water	Dark brown oil	476.15	8.66



Scheme 1 The procedure of extraction of *B. superba* Roxb. (5.5 kg)

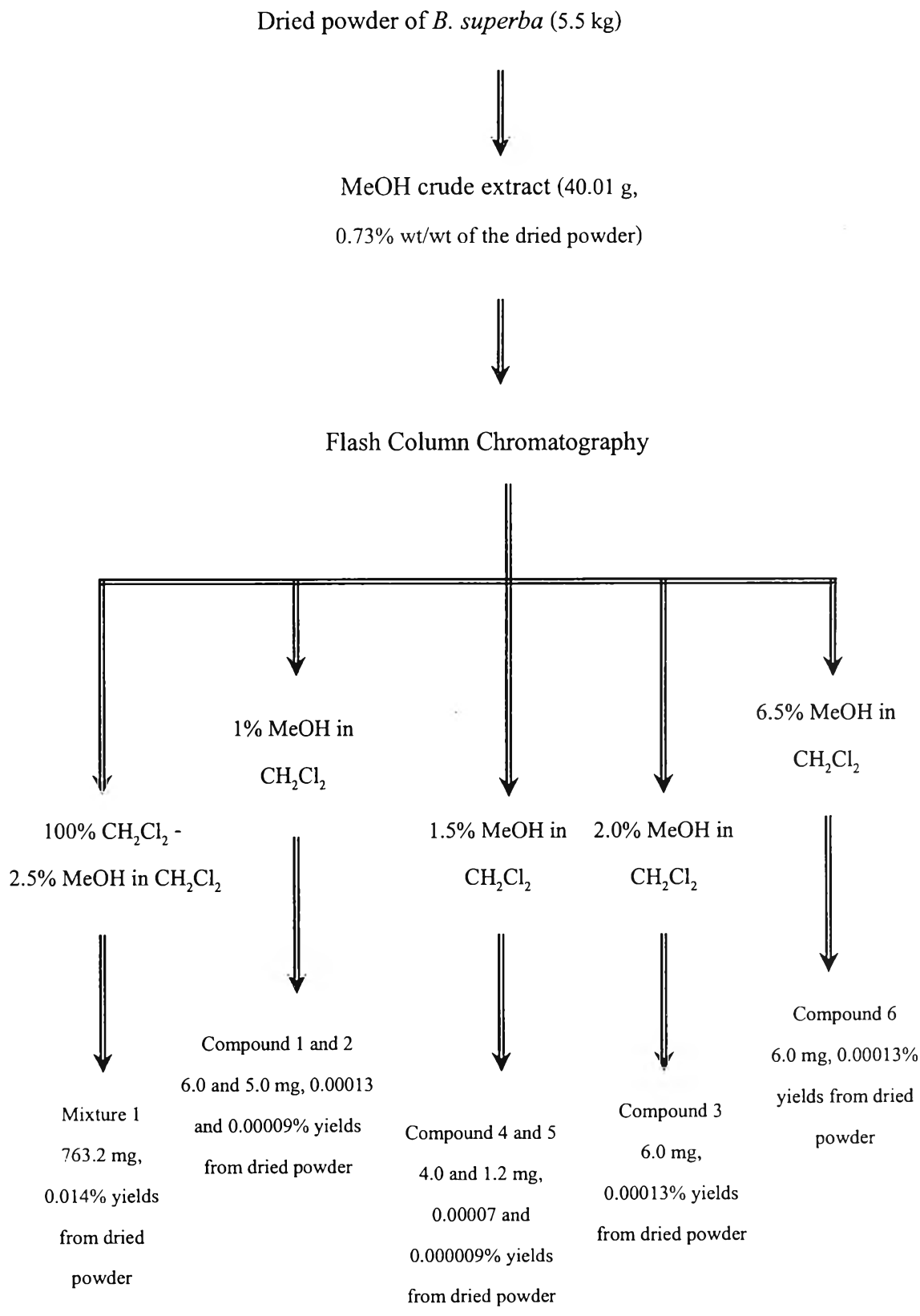
3.5 Isolation procedures

-Separation of MeOH crude extract

The MeOH crude extract (40.01 g) was separated by flash column chromatography, 12 cm of height and 12.5 cm of diameter. Firstly, MeOH crude extract (40.01 g) was dissolved in a small amount of suitable solvent and mixed with silica gel 60, 0.04-0.06 mm (230 - 400 mesh ASTM), in 1:1. Secondly, cut filter paper into circle, 12.2 cm in diameters, and then put in the column. Then, silica gel (800 g) was packed in the column, 9.0 cm in high. Thirdly, this MeOH crude extract was put on top of column, 1.0 cm in high. Then, cut filter paper into circle, 12.2 cm in diameters, in V-shape around the circle, and place on top of the column. Fourthly, column was eluted with 100% of CH_2Cl_2, CH_2Cl_2- MeOH gradient; the gradual concentration has increase in a stepwise of 1% MeOH, together with to applying on TLC plate of each fraction. Finally, each fraction was collected in 500 ml, and then checked fraction by TLC plate to these fractions how same or not same components. Results; from separation by flash column chromatography of MeOH crude extract, are summarized in Table 2. Then, crystallization or preparative TLC further purified fractions. The isolation of compounds form MeOH crude extract is shown in Scheme 2. The results of purified compounds are shown in Table 3.

-Separation of water crude extract

The water crude extract (476.15 g) was obtained as sugar (209.50 g; 3.81% wt/ wt of dried powder) by precipitation of with MeOH.



Scheme 2 Isolation procedure of MeOH crude extract

3.6 Purification and properties of the compounds from *B. superba* Roxb.

3.6.1 Purification and properties of mixture 1

The methanol crude extract was separated by 100% dichloromethane - 2.5% methanol in dichloromethane. Similar fractions were combined and then the solvents were removed by evaporation to obtain the fraction number 1-7. The sediment of the mixture 1 has been placed within 1 day of these fractions. Filter and wash the mixture 1 by methanol, and then the waste solution was re-crystallized in dichloromethane. The crystal after re-crystallizing was washing with methanol.

The mixture 1 was white crystal; weight 762.3 mg. (1.910% yields from methanol crude extract and 0.014% yields from dried powder) and melting point 138-139°C. This compound was dissolved in hexane, CH_2Cl_2 and $CHCl_3$. The R_f value was found to be 0.33 in CH_2Cl_2.

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Figure 26 and Table 4) δ (ppm): 0.68 - 2.31, 3.51, 5.11 and 5.37.

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Figure 27 and Table 5) δ (ppm): 32 carbons of 11.89 – 56.78 (C), 71.81 (C), 121.74 (CH), 129.27 (CH), 138.31 (CH) and 140.77 (s).

3.6.2 Purification and properties of compound 1

The methanol crude extract was separated by 1% methanol in dichloromethane. Similar fractions were combined and then the solvents were removed by evaporation to obtain the fraction number 3 and 4. The sediments of the compound 1 and mixture 1 have been placed within 2 day of fraction number 3. Filter and wash the compound 1 by methanol, and then the waste solution was re-crystallized in dichloromethane and methanol (5:1). The crystalline solid after re-crystallizing was purified by preparative TLC (Merck's TLC aluminium sheets 20x20 cm silica gel 60 F_{254}) of 1% methanol in dichloromethane to system solvent.

The compound 1 was pale brown crystalline solid; weight 6.0 mg. (0.015% yields from methanol crude extract and 0.00013% yields from dried powder) and melting point 239-240 °C. This compound was dissolved in $CHCl_3$: MeOH (5:1) and DMSO. The R_f value was found to be 0.42 (1% MeOH in CH_2Cl_2), UV ($CHCl_3$) λ_{max} 287.94 nm (log ε 3.21) at 27°C, $[\alpha]_D^{26.5}$ -19.90 (*c* 0.04 g/100ml, $CHCl_3$).

^{1}H-NMR spectrum ($CDCl_3$:CD_3OD (5:1), 400 MHz) (Figure 28 and Table 6) δ (ppm): 3.81 (3H, s, 7-OCH_3), 6.32 (2H, d; *J*= 2.4 Hz, 6-H), 6.35 (2H, d; *J*= 2.0 Hz, 8-H), 6.89 (2H, d; *J*=8.8 Hz, 3′-H and 5′-H), 7.33 (2H , d; *J*=8.0 Hz, 2′-H and 6′-H) and 7.80 (1H, s).

^{13}C-NMR spectrum ($CDCl_3$:CD_3OD (5:1), 100 MHz) (Figure 29 and Table 6) δ (ppm): 55.78 (q), 92.43 (d), 98.18 (d), 106.18 (s), 115.53 (d), 121.72 (s), 124.05 (s), 130.15 (d), 152.81(d), 157.19 (s), 158.00 (s), 162.75 (s), 165.50 (s) and 181.02 (s).

TOF-MS (Figure 34): *m/z* 307.0583 $[M + Na]^+$

3.6.3 Purification and properties of compound 2

The methanol crude extract was separated by 1% methanol in dichloromethane. Similar fractions were combined and then the solvents were removed by evaporation to obtain the fraction number 3 and 4. From the purification of compound 1 by preparative TLC of 1% methanol in dichloromethane, the fraction number 3/D was repeatedly preparative TLC (Merck' s TLC aluminium sheets 20x20 cm silica gel 60 F_{254}) of 1% methanol in dichloromethane and 100% dichloromethane, respectively and then wash the crystalline by hexane.

The compound 2 was white crystalline solid; weight 5.0 mg. (0.013% yields from methanol crude extract and 0.00009% yields from dried powder) and melting point 127-129°C **[26]**. This compound was dissolved in CH_2Cl_2, $CHCl_3$, EtOAc and EtOH. The R_f value was found to be 0.51 (1% MeOH in CH_2Cl_2), UV($CHCl_3$) λ_{max} 236.06 and 265.93 nm (log ε 4.14 and 4.34) at 27°C, $[\alpha]_D^{26.5}$ -223.60 (*c* 0.01 g/100ml, CH_2Cl_2) in literature $[\alpha]_D^{22}$ -230 ($CHCl_3$) **[26]**.

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Figure 36 and Table 8) δ (ppm): 3.53 (1H, m, 6a-H), 3.62 (1H, t, 6-H), 3.77 (3H, s, 9-OCH_3), 4.24(1H, dd; *J*= 4.4, 10.4 Hz, 6-H), 5.49 (1H , d; *J*= 6.4 Hz, 11a-H), 6.43 (2H, d; *J*= 8.8 Hz, 10-H), 6.45 (1H, s, 4-H), 6.46 (1H, s, 8-H), 6.56 (1H, d; *J*= 8.4 Hz, 2-H), 7.13 (1H, d; *J*= 8.8 Hz, 7-H) and 7.39 (1H, d; *J*= 8.4 Hz, 1-H).

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Figure 37 and Table 8) δ (ppm): 39.46 (d), 55.51 (q), 66.53 (t), 78.51 (d), 96.91 (d), 103.65 (d), 106.37 (d), 109.74 (d), 112.50 (s), 119.15(s), 124.76 (d), 132.20 (d), 156.65 (s), 157.21 (s), 160.40 (s) and 161.27 (s).

TOF-MS (Figure 42): *m/z* 293.0782$[M + Na]^+$

3.6.4 Purification and properties of compound 3

The methanol crude extract was separated by 2% methanol in dichloromethane. Similar fractions were combined and then the solvents were removed by evaporation to obtain the fraction number 6. The sediments of the compound 3 and mixture 1 have been placed within 2 day of fraction number 6. Filter and wash the compound 1 by hexane, and then the waste solution was re-crystallized in 1% methanol in dichloromethane. The crystal after re-crystallizing was purified by preparative TLC (Merck' s TLC aluminium sheets 20x20 cm silica gel 60 F_{254}) of 2% methanol in dichloromethane to system solvent.

The compound 3 was pale orange crystalline solid; weight 6.0 mg. (0.015% yields from methanol crude extract and 0.00013% yields from dried powder) and melting point 261-262 °C. This compound was dissolved in $CHCl_3$: MeOH (3:1) and DMSO. The R_f value was found to be 0.24 (5% MeOH in CH_2Cl_2), UV($CHCl_3$) λ_{max} 273.00 and 295.01 nm (log ε 3.19 and 3.34) at 27°C, $[\alpha]_D^{26.5}$-32.85 (*c* 0.01 g/100ml, $CHCl_3$).

^{1}H-NMR spectrum ($CDCl_3$:CD_3OD (3:1), 400 MHz) (Figure 44 and Table 10) δ (ppm): 3.81 (3H, s, 4′-OCH_3), 6.79 (1H, d; *J*= 2.4 Hz, 8-H), 6.88 (1H, dd; *J*=2.0 and 9.2 Hz, 6-H), 6.93 (2H, d; *J*= 9.2 Hz, 3′-H and 5′-H), 7.43 (2H , d; *J*=9.2 Hz, 2′-H and 6′-H), 7.87 (1H, s, 5-H) and 8.09 (1H, d; *J*=9.2 Hz, 2-H).

^{13}C-NMR spectrum ($CDCl_3$:CD_3OD (3:1), 100 MHz) (Figure 45 and Table 10) δ (ppm): 55.30 (q), 102.41 (d), 113.88 (d), 115.09 (d), 117.44 (s), 124.13 (s), 124.52 (s), 127.81 (d), 130.15 (d), 158.21 (d), 159.60 (s), 162.43 (s) and 176.50 (s).

TOF-MS (Figure 50): *m/z* 291.0627 $[M + Na]^+$

3.6.5 Purification and properties of compound 4

The methanol crude extract was separated by 1.5% methanol in dichloromethane. Similar fractions were combined and then the solvents were removed by evaporation to obtain the fraction number 5. The purification obtain compound 4 through preparative TLC technique (Merck' s TLC aluminium sheets 20x20 cm silica gel 60 F_{254}) of 70% ethyl acetate in hexane, 2% methanol in dichloromethane and 1% methanol in dichloromethane, respectively.

The compound 4 was yellow crystalline solid; weight 4.0 mg. (0.010% yields from methanol crude extract and 0.00007% yields from dried powder) and melting point 228-229 °C. This compound was dissolved in $CHCl_3$:MeOH (5:1) and DMSO. The R_f value was found to be 0.65 (5% MeOH in CH_2Cl_2), UV($CHCl_3$) λ_{max} 240.93, 270.97 and 315.07 nm (log ε 5.04, 4.99 and 5.34) at 27°C, $[\alpha]_D^{26.5}$ -5.65 (*c* 0.08 g/100ml, $CHCl_3$).

^{1}H-NMR spectrum ($CDCl_3$:CD_3OD (5:1), 400 MHz) (Figure 52 and Table 12) δ (ppm): 3.78 (3H, s, 6′-OCH_3), 3.95 (3H, s, 6-OCH_3), 6.91 (2H, d; *J*= 8.8 Hz, 3′-H and 5′-H), 6.92 (1H, s, 8-H), 7.43 (2H, dd; *J*=8.8 Hz, 2′-H and 6′-H), 7.59 (1H ,s, 5-H) and 7.86 (1H , s, 2-H).

^{13}C-NMR spectrum ($CDCl_3$:CD_3OD (5:1), 100 MHz) (Figure 53 and Table 12) δ (ppm): 55.31 (q), 56.54 (q), 102.66 (d), 104.79(d), 113.98 (d), 117.92 (s), 122.51 (s), 124.44 (s), 130.19 (d), 145.39 (s), 151.30 (s), 152.10 (d), 152.58 (s), 159.82 (s) and 175.75 (s).

TOF-MS (Figure 58): *m/z* 299.0930 $[M + H]^+$

3.6.6 Purification and properties of compound 5

The methanol crude extract was separated by 1.5% methanol in dichloromethane. Similar fractions were combined and then the solvents were removed by evaporation to obtain the fraction number 5. The purification obtain compound 5 through preparative TLC technique (Merck' s TLC aluminium sheets 20x20 cm silica gel 60 F_{254}) of 70% ethyl acetate in hexane, 1% methanol in dichloromethane and 100% dichloromethane, respectively.

The compound 5 was pale yellow crystalline solid; weight 1.2 mg. (0.0013% yields from methanol crude extract and 0.000009% yields from dried powder) and melting point 162-163 °C. This compound was dissolved in CH_2Cl_2, $CHCl_3$, EtOAc, EtOH and DMSO. The R_f value was found to be 0.43 ($CHCl_3$), UV($CHCl_3$) λ_{max} 239.05 and 300.97 nm (log ε 5.24 and 5.20) at 27°C, $[\alpha]_D^{26.5}$-19.68 (*c* 0.05 g/100ml, $CHCl_3$).

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Figure 60 and Table 14) δ (ppm): 3.84 (3H, s, 4′-OCH_3), 3.92 (3H, s, 7-OCH_3), 6.85 (1H, d; *J*= 2.2 Hz, 8-H), 6.97 (1H, d; *J*= 8.8 Hz, 3′-H and 5′-H), 6.99 (1H, dd; *J*=8.8 and 2.2 Hz, 6-H), 7.50 (2H , d; *J*=8.4 Hz, 2′-H and 6′-H), 7.92 (1H , s, 2-H) and 8.21 (1H, s, 5-H).

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Figure 61 and Table 14) δ (ppm): 55.35 (q), 55.84 (q), 100.10 (d), 113.95 (d), 114.56 (d), 118.58 (s), 124.32 (s), 124.88 (s), 127.81 (s), 130.14 (d), 152.08 (d), 158.01 (s), 159.65 (s), 163.96 (s) and 175.95 (s).

TOF-MS (Figure 65) : *m/z* 305.0790 $[M + Na]^+$

3.6.7 Purification and properties of compound 6

The methanol crude extract was separated by 6.5% methanol in dichloromethane. Similar fractions were combined and then the solvents were removed by evaporation to obtain the fraction number 15. The sediments of the compound 6 have been placed within 2 day of fraction number 15. Filter and wash the compound 6 by methanol, and then the waste solution was re-crystallized in 6% methanol in dichloromethane.

The compound 6 was white crystalline solid; weight 6.0 mg. (0.015% yields from methanol crude extract and 0.00013% yields from dried powder) and melting point 78-79°C. This compound was found to dissolve $CHCl_3$, EtOAc and EtOH. The R_f value was found to be 0.49 (5% MeOH in CH_2Cl_2), UV($CHCl_3$) λ_{max} 231.04 nm (log ε 5.29) at 27°C, $[\alpha]_D^{26.5}$-85.90 (*c* 0.022 g/100ml, $CHCl_3$).

^{1}H-NMR spectrum ($CDCl_3$:CD_3OD (5:1), 400 MHz) (Figure 67 and Table 16) δ (ppm): 0.81(-CH_3, t), 1.21 (42H, s), 1.55 (2H, t), 2.00 (1H, s), 2.27 (2H, s), 2.30 (2H, s), 3.50 (1H, dd; *J*=6.0 and 11.2 Hz, 3-H), 3.60 (1H, dd; *J*= 3.2 and 11.6 Hz, 3-H), 3.83 (1H, t, 2-H) and 4.07 (1H, dd; *J*=3.2 and 5.6 Hz, 1-H).

^{13}C-NMR spectrum ($CDCl_3$:CD_3OD (5:1), 100 MHz) (Figure 68 and Table 16) δ (ppm): 14.12 (s), 22.70 (s), 24.89 (s), 29.70 (s), 31.93 (s), 34.18 (s), 63.26 (s), 65.12 (s), 70.08 (s) and 174.51(s).

TOF-MS (Figure 69) : *m/z* 493.79 $[M + Na]^+$

3.7 Biological assay

3.7.1 Anti-cancer

Bioassay of anti-cancer activity against three cell line, including KB (Human epidermoid carcinoma of cavity, ATCC CCL-17), BC (Breast cancer cell line) and NCI-H 187 (Human small cell lung carcinoma, ATCC CRL-5804) cancer, in *vitro* was performed by MTT solution (3-[4,5-Dimethylhiazol-2-yl]-2,5-diphenyltetra-zolium bromide; Thiazolyl blue) In principle, the viable cell number per well is directly proportional to the production of formazan, which following solubilization, can be measured spectrophotometrically.

The isolated compounds were tested for anti-cancer activity toward three cell line types with method as follow.

Determination of anti-cancer assay (KB, BC)

KB (Human epidermoid carcinoma of cavity, ATCC CCL-17) and BC (Breast cancer cell line) were determined by colorimetric cytotoxicity assay that measured cell growth from cellular protein content according to Skehan P. (1990) **[27]**. Elliptine and doxorubicin were used as positive control. DMSO was used as negative control. Briefly, Cells at a logarithimic growth phase were harvested and diluted to105 cells/ml with fresh medium and gently mixed. Test compounds were diluted in distill water and put into microtiter plates in total volume 200 µl. Plates were incubated at 37°C, 5% CO_2 for 72 hrs. After incubation period, cells were fixed by 50% trichloroacetic acid. The plates were incubated at 4°C for 30 min, washed plates with tap water and air-dried at room temperature. The plates were stained with 0.05% sulforhodamine B dissolved in 1% acetic for 30 min. After staining period, SRB was removed with 1% acetic acid. Plates were air-dried before bound dye was solubilized

with 10 mM Tris base for 5 min on shaker. OD was in microtiter plate reader at wavelength of 510 nm.

Determination of anti-cancer assay (NCI-H 187)

NCI-H 187 (Human small cell lung carcinoma, ATCC CRL-5804) were determined by MTT assay previously described in detail by Plumnb J.A. (1989) **[28],** cells were diluted to 105 cells/ml. Test compounds were diluted in distilled water an added to microtiter plates in total volume of 200 µl. Plates were incubated at 37°C, 5% CO_2 for 5 days. 50 µl of 2 mg/ml MTT solution (3-[4,5-Dimethylhiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) was added to each well of the plate the plate. Plates were wrapped with aluminium foil and incubated for 4 hrs. After incubation period, the micro plates were spinned down at 200x g for 5 min. MTT was then removed from the wells and the formazan crystals were dissolved in 200 µl for 100% DMSO and 25 µl of Sorensen' glycine buffer. OD was read in microtiter plate reader at wavelength of 510 nm.

Cell lines growth and growth inhibition were expressed in terms of mean (±1 of SD) absorbance units and/ or percentage of control absorbance (±1 of SD%) following subtraction of mean "background" absorbance. In addition, the IC_{50} was expressed ad the sample concentration in ug/ml that caused a 50% inhibition of growth compared with controls. Doxorubincin or Adiblastina was used as a positive control in very experiment.