CHAPTER 3

EXPERIMENTAL

3.1 General methods

Melting points were measured on a Gallenkamp Electrothermal apparatus and are uncorrected. Infrared spectra were determined on a FT-IR model TENSER 27 (Bruker) spectrometer and absorption frequencies were reported in reciprocal centimeters (cm^{-1}). The ^{1}H and ^{13}C NMR spectra were recorded on Bruker DRX 400 MHz spectrometers and chemical shifts were given in ppm downfield from tetramethylsilane (TMS) as internal standard. All NMR spectra were measured in $CDCl_3$. Chemical shifts are reported as δ values in parts per million (ppm) and coupling constants (*J* values) are given in hertz (Hz). Mass spectra (electrospray ionization mode, ESI-MS) were measured on a micromass Q-TOF-2^{TM} (Waters) spectrometer. Optical rotations were obtained in $CHCl_3$ on an ADP 220 automatic polarimeter. Flash column chromatography was performed by employing Merck silica gel 60 and Merck silica gel 60H. Analytical and Preparative TLC were carried out using Merck silica gel 60 PF_{254} and visualized under visible and UV light (λ 254 nm)

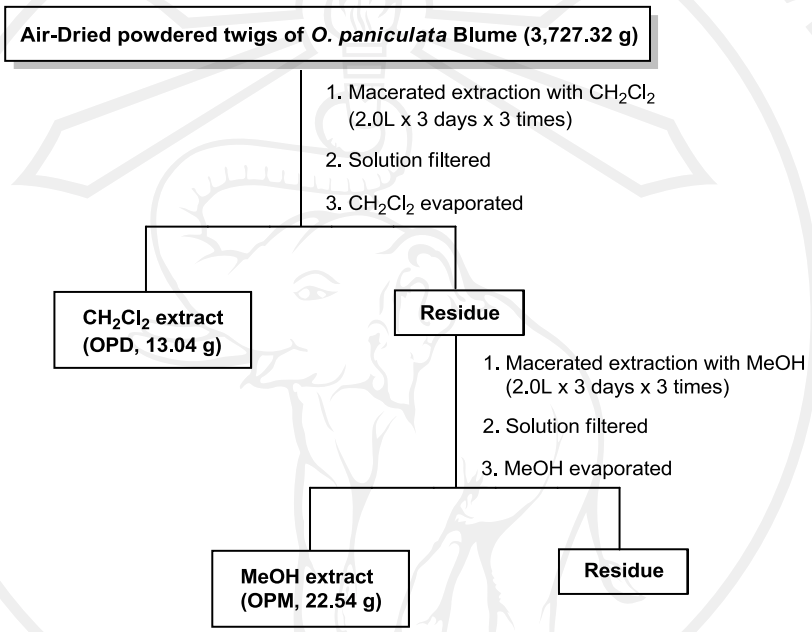
3.2 Plant materials

The twigs of *O. Paniculata* Blume was gathered from Doi Suthep-pui National park, Chiang Mai on Apirl 2008 in Thailand. A voucher specimen (MAXWELL 06-651) was deposited for future reference in the herbarium of Department of Biology, Faculty of Science, Chiang Mai University, Thailand.

3.3 Extraction and isolation

The air-dried powdered plant of the twigs of *O. paniculata* (3.70 kg) was extracted successively with dichloromethane (2 L x 3 days x 3 times), and followed by methanol (3 L x 3 days x 3 times) at room temperature. The extracts were

concentrated under reduced pressure by rotary evaporator to yield dichloromethane (OPD, 13.04 g) and methanol crude extracts (OPM, 22.54 g). Some parts of dichloromethane and methanol when subjected to *in vitro* screening tests, displayed several biological activities. Consequently, were further fractionated by column chromatography (Scheme 2).



Scheme 2 The extraction and fractionation procedures

3.4 Separation of the dichloromethane extract

The dichloromethane crude was loaded onto silica gel column chromatography and eluted with gradient system of *n*-hexane, dichloromethane, ethyl acetate and finally methanol. According to differences in composition, as indicated by thin layer chromatography (TLC), thirteen crude fractions were obtained (OPDF1-OPDF13) (Scheme 3).

Fraction OPDF1 and OPDF2

The residues fraction of OPDF1 (0.48 g) and OPDF2 (0.12 g) were subjected to silica gel column chromatography eluted with a gradient system of *n*-hexane and increasing polarity with dichloromethane, ethyl acetate and finally with methanol.

These fractions were collected and combined on the basis of TLC behavior but they were found to contain mainly waxes and were not further investigated (Scheme 3).

Fraction OPDF3

Fraction OPDF3 (0.58 g) was submitted to column chromatography using, organic solvents of increasing polarity; *n*-hexane, dichloromethane, ethyl acetate and methanol as eluents. After TLC monitoring, seven sub-fractions (OPDF3.1-OPDF3.7) were obtained. Moreover, the purification of the OPDF3.3 was performed by preparative TLC (100% CH_2Cl_2) to give 0.008 g of eugenol (**22**) (Scheme 3).

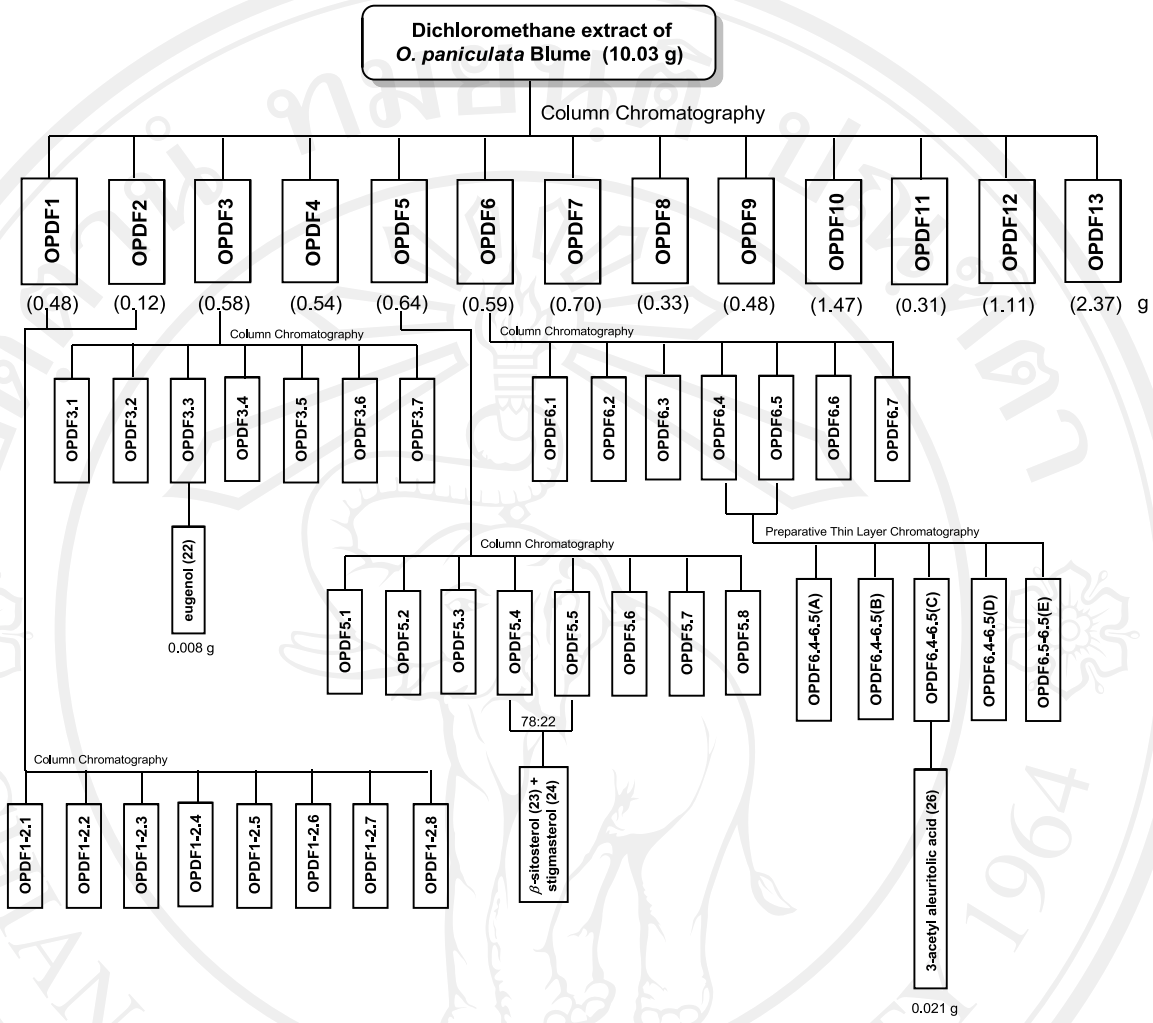
Fraction OPDF5

The fraction OPDF5 (0.64 g) which eluted by gradient elution was started from *n*-hexane gradually enriched with dichloromethane in hexane additive to ethyl acetate and finally methanol was obtained into eight sub-fractions (OPDF5.1-OPDF5.8). Accordingly, their TLC pattern, OPDF5.4 and OPDF5.5 with similar TLC profile were pooled and further recrystallized twice from dichloromethane : *n*-hexane (0.15:0.35) to afford 78:22 of the phytosterol mixtures as, *β*-sitosterol (**23**) and stigmasterol (**24**) (Scheme 3).

Fraction OPDF6

The more polar fraction OPDF6 (0.59 g) was fractionated by silica gel column chromatography carried out and using organic solvents as gradient elution starting from *n*-hexane and increasing polarity including, dichloromethane, ethyl acetate and methanol, respectively to afford six sub-fractions (OPDF6.1-OPDF6.7). After TLC monitoring, two similar sub-fractions OPDF6.4 (1.29 g) and OPDF6.5 (1.35 g) were associated and combined together for separation.

The OPDF6.4-6.5 (2.58 g) was purified by repeated column chromatography to give five sub-fractions [OPDF6.4-6.5(A)]-[OPDF6.4-6.5(E)]. The five sub-fractions were further submitted to preparative TLC, using mixture of *n*-hexane : ethyl acetate : acetone (92:6:2) as eluents to afford compound **26**; 3-acetyl aleuritolic acid (0.09 g) (Scheme 3).



Scheme 3 The isolating flowchart of dichloromethane extract of *O. paniculata* Blume

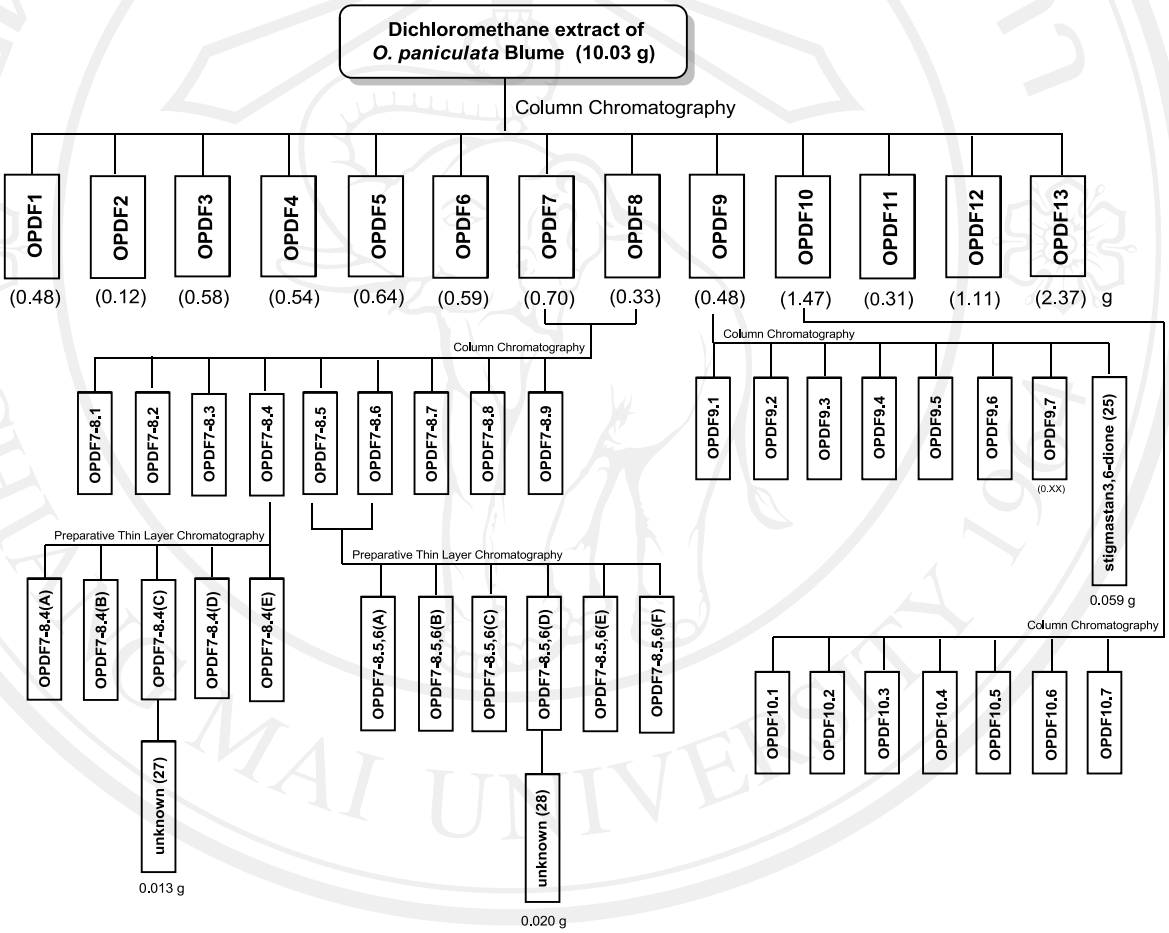
Fraction OPDF7 and OPDF8

The two residues of fraction OPDF7 (0.70 g) and OPDF8 (0.33 g), were subjected to column chromatography over silica gel and eluted with a gradient system of *n*-hexane, dichloromethane, ethyl acetate and methanol, respectively. Nine sub-fractions of OPDF7-8 including, (OPDF7-8.1)-(OPDF7-8.9), were collected and combined according to their TLC pattern.

The sub-fractions, (OPDF7-8.5) to (OPDF7-8.6), with similar chromatographic patterns were pooled and further separated by preparative TLC using mobile phase

n-hexane : ethyl acetate : acetone (4.7:0.1:0.2) as mobile phase 0.013 g of unknown (**27**) which was combined with [OPDF7-8.5(D)]-[OPDF7-8.6(D)].

According to TLC appearance, sub-fractions OPDF7-8.4 showed the presence of spot with strong absorption at 254 nm, which was separated by preparative TLC using mixtures of *n*-hexane : ethyl acetate : acetone (4.7:0.2:0.1) as eluents to obtain 0.020 g of unknown (**28**) in OPDF7-8.4(C) as depicted in Scheme 4.



Scheme 4 The isolating flowchart of dichloromethane extract of *O. paniculata* Blume

Fraction OPDF9

The fraction OPDF9 was submitted to silica gel column chromatography. Fractions eluted starting with, *n*-hexane and increasing the polarity to give seven sub-fraction. The mother-liquors showed the presence of a spot with a strong absorption at 254 nm and further purified by crystallization with *n*-hexane : ethyl acetate : acetone (4.2:0.5:0.3) affording a white amorphous crystals. This compound was concluded as 0.059 g of earlier isolated compound as stigmastan-3,6-dione (**25**) (Scheme 4).

Fraction OPDF10

The residue fraction of OPDF10 was chromatographed over column of silica gel eluted as gradient system with organic solvent, *n*-hexane and increasing the polarity by adding on dichloromethane, ethyl acetate and methanol, respectively. After TLC monitoring, the behavior chromatographic fractions were combined into seven sub-fractions (OPDF10.1-OPDF10.7) (Scheme 4).

The structural elucidations of all the isolated compounds (**22**-**26**) were accomplished by spectral data which were IR, EIMS, ^{1}H and ^{13}C NMR spectra including, DEPT, COSY, HMQC and HMBC as well as comparison with previously literature vales.

3.5 Biological assay

Determination of small lung cancer cell (NCI-H187)

NCI-H187 (human small cell lungcarcinoma, ATCC CRL-5804) were diluted to 105 cellsmL^{-1}. Test compounds were diluted in distilled water and added to micro liter plates in total volume 100 mL. Plates were incubated at 37°C, 5% CO_2 for 5 days. Fifty micro liters of 2 mgmL^{-1} MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; Thiazolyl blue) was added to each well of the plate. Plates were wrapped with aluminium foil and incubated for 4 h. After incubation period, the micro plates were spinned down at200g for 5 min. MTT was then removed from the wells and the formazan crystals were dissolved in 200 mL of 100% DMSO and 25 mL of Sorensen's glycine buffer. Optical density (OD) was read in micro titer plate reader at wavelength of 510 nm.[18-21]

Determination of anti-cancer (KB and Brest cancer)

KB (human epidermoid carcinoma of cavity, ATCC CCL-17) and BC (breast cancer cell lines) were determined by colorimetric cytotoxicity assay that measured cell growth from cellular protein content according to Skehan et al.[18-21]. Elliptine and doxorubicin were used as positive control. DMSO was used as negative control. Briefly, cells at a logarithmic growth phase were harvested and diluted to 10^5 cellmL^{-1} with fresh medium and gently mixed. Test compounds were diluted in distill water and put into micro titer plates in total volume 200 mL Plates were incubated at 37°C, 5% CO_2 for 72 h. After incubation period, cells were fixed by 50% trichloroacetic acid. The plates were incubated at 4°C for 30 min, washed with tap water and air-dried at room temperature. The plates were stained with 0.05% sulforhodamine B (SRB) dissolved in 1% acetic acid 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. Optical density was read in micro liter plate reader at wavelength of 510 nm.

Determination of antituberculosis assay

Antituberculosis assay against Mycobacterium tuberculosis H37Ra was performed in duplicate using the micro plate Alamar blue assay.[19, 21] The reference compounds were isoniazid and kanamycin sulfate. The minimum inhibitory concentrations (MICs) of no more than 200 mg/ml were considered active.[18, 21]

Determination of cytotoxicity (Vero cells)

The growth of host cells, Vero cell lines (ATCCCCL-81), infected with virus and treated with compounds was compared with control cells, infected with virus only. Acyclovir and DMSO were used as positive and negative controls, respectively. The extracts were tested at non cytotoxic concentrations (inhibit cell growth < 50%). Compounds that inhibit virus more than 50% were further tested to determine the concentrations that inhibit viral activity by 50% (IC_{50}).[18, 21]