CHAPTER III

METERIALS AND METHODS

1. Plant Materials

Two *Ophiorrhiza* species for instant *Ophioriza trichocarpon* Blume and *Ophioriza rugosa* Wall. were obtained from Queen Sirikit Botanic garden, Amphur Mae Rim, Chiang Mai Province, the herbarium voucher specimens (QBE Nichakan No.1, 2) were indentified and deposited at Queen Sirikit Botanic Garden Herbarium. Another *Ophiorrhiza* aff. *nutans* Cl. *ex* Hk. f. were collected from Doitung mountain forest Amphur Maephaloung, Chiang Rai Province the voucher specimens of herbarium (No. 06-355 CMU) were indentified by J. F. Maxwell and deposited at Chiang Mai University (CMU) Herbarium, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand.

2. Chemical

Commercial grade organic solvents were redistilled prior to use for extraction, as eluents for thin layer chromatography and column chromatography. Organic solvent used for antioxidant activity assessment were analytical grade including 2, 2′-diphenyl-1- picrylhydrazyl ($DPPH^{\bullet}$) and ascorbic acid were purchased from Sigma (St. Louis, USA). Trolox and quercetin were obtained from Aldrich (Milwaukee, USA). Absolute ethanol (99%) was purchased from Merck (Damstadt, Germany). All the other chemicals and solvents used were analytical grades.

3. Instruments and Apparatus

3.1 Analytical Thin Layer Chromatography: Silica gel 60GF precoated aluminium plates (0.25 mm.) were purchased from Merck (Damstadt, Germany). Detection of spots were carried out by observation under UV lamp at 254 and 365 nm.

3.2 Column Chromatography: Open column chromatography was carried out using Silica gel 60 (0.200-0.500 mm), Silica gel (0.063-0.200 mm) were purchased from Merck (Damstadt, Germany) and Celite 545 coarse from Fluka, Buchs, Switzerland.

3.3 Medium pressure liquid chromatograph (MPLC): It is consisted of a pump with 3-way valve, Buchi (B-681), Switzerland, Spectrophotometer variable wave lengh (190-590 nm), Knauer variable wavelength monitor (version 293), Germany, Peak detector, Pharmacia (LKB-REC101) USA and Silica (SiO_2) column.

3.4 Proton and Carbon nuclear magnetic resonance spectrometers (^{1}H-NMR and ^{13}C-NMR spectrometer)

4. Phytochemical screening

Alkaloids: Crude extract 0.3 g. of each plant was dissolved in 15 mL 2 N hydrochloric acid and then heated on a boiling water bath at 60-70 °C. After cooling, the mixture was filtered and treated with a few drop of Kraut's reagent, Dragendroff's reagent, Mayer's reagent, Valser's reagent and Wagner's reagent. The sample was then observed for the present of turbidity of yellow or white precipitation (113-115).

Flavonoids: Crude extract 0.3 g. of each plant was extracted with petroleum ether (10 mL. x2). The precipitate was dissolved in 10 mL of 50% ethanol and filtered. The filtrate was transferred into two separated test tubes. To each test tube magnesium metal was added followed by 5-6 drops of concentrated hydrochloric acid. Red color was observed for flavoniods and orange color for flavones (113-115).

Coumarins: A few drop of water was added into a test tube containing 0.3 g of crude extract. Then the test tube was covered with a cork attached to a strip of filter paper which was moistened with dilute sodium hydroxide solution. The test tube was warmed on a boiling water bath for about 3-5 min. The filter paper was removed and observed under UV at 365 nm for about 1 min. If there are coumarins, a bright fluorescence will be observed (114).

Antraquinone glycosides: The solution containing 0.3 g of crude extract was boiling with dilute hydrochloric acid on a water bath for about 15 min., cooled and filtered. Then the filtrate was extracted with 10x2 mL of chloroform. The chloroform layer was treated with dilute sodium hydroxide solution and mixed well. Red colour will be observed in alkali layer (115).

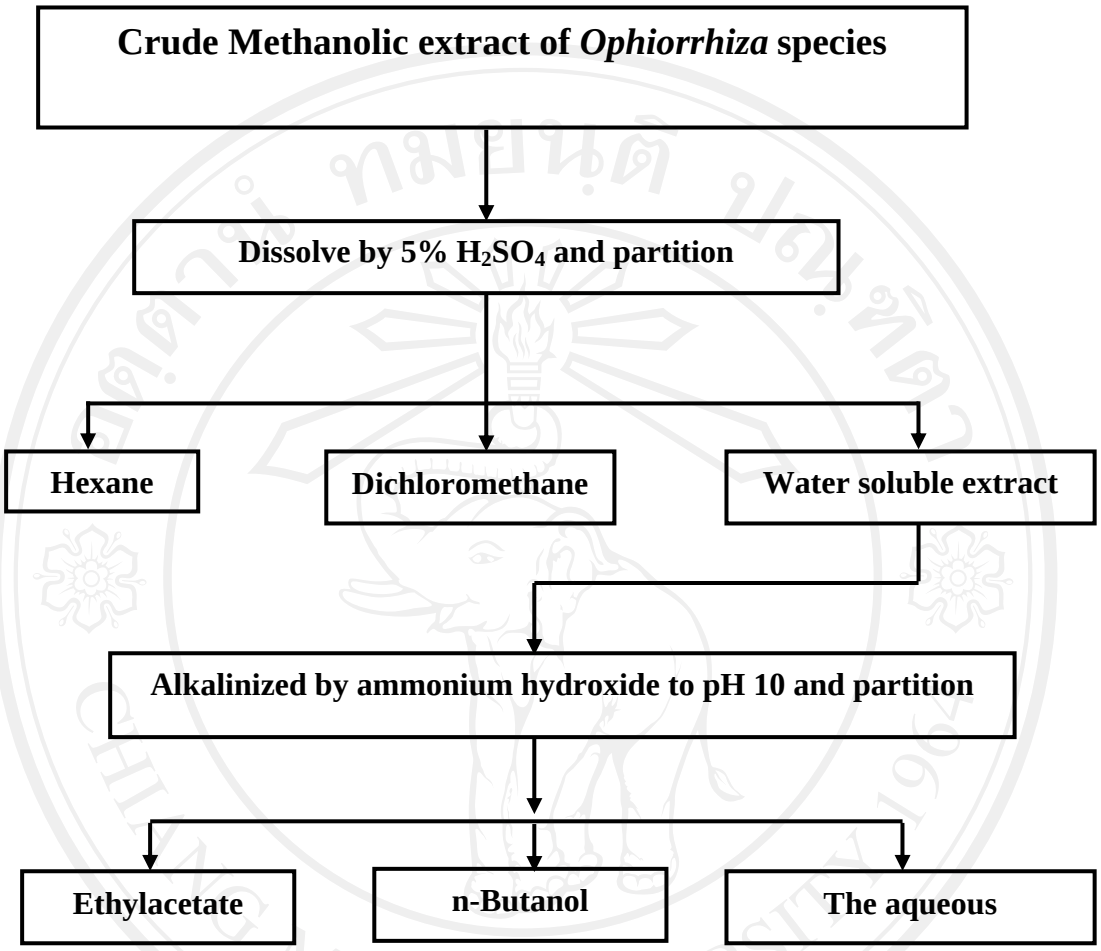
Scopoletin: Crude extract 0.1 g was dissolved in 3 mL dichloromethane and warmed on a water bath, filtered and evaporated to dryness. The residue was dissolved with a few drops of toluene. The sample solution was applied on a silica gel 60G plate (previously activated at 105º C for about 2 hours). Development was performed in a chromatographic tank containing ethyl acetate: methanol: water 100:6:4. After development, the TLC chromatogram was viewed under long wavelength UV at 365 nm. If there is scopoletin, blue colour spot will be observed (114).

Saponins: Crude extract 0.3 g was dissolved in 10 mL of water and boiled then filtered. The filtrate was transferred into two separated test tubes, the first test tube was shaken vigorously for 1 min., beehive bubble lasting for 30 seconds will be observed. To the second test tube, 1 mL of 10% sulfuric acid was added and boiled on a water bath. Then the tube was shaken vigorously until 1-2 cm beehive bubble lasting for 10 min was observed (113-115).

5. Extraction of *O. trichocarpon* Bl., *O. rugosa* Wall. and *O.* aff. *nutans* Cl. *ex* Hk. f.

Fresh whole plants of *O. trichocarpon* Bl., *O. rugosa* Wall. and *O.* aff. *nutans* Cl. *ex* Hk. f. were extracted with hot methanol for the combined methanolic extracts were evaporated to dryness under reduced pressure, brownish sticky extracts were obtained.

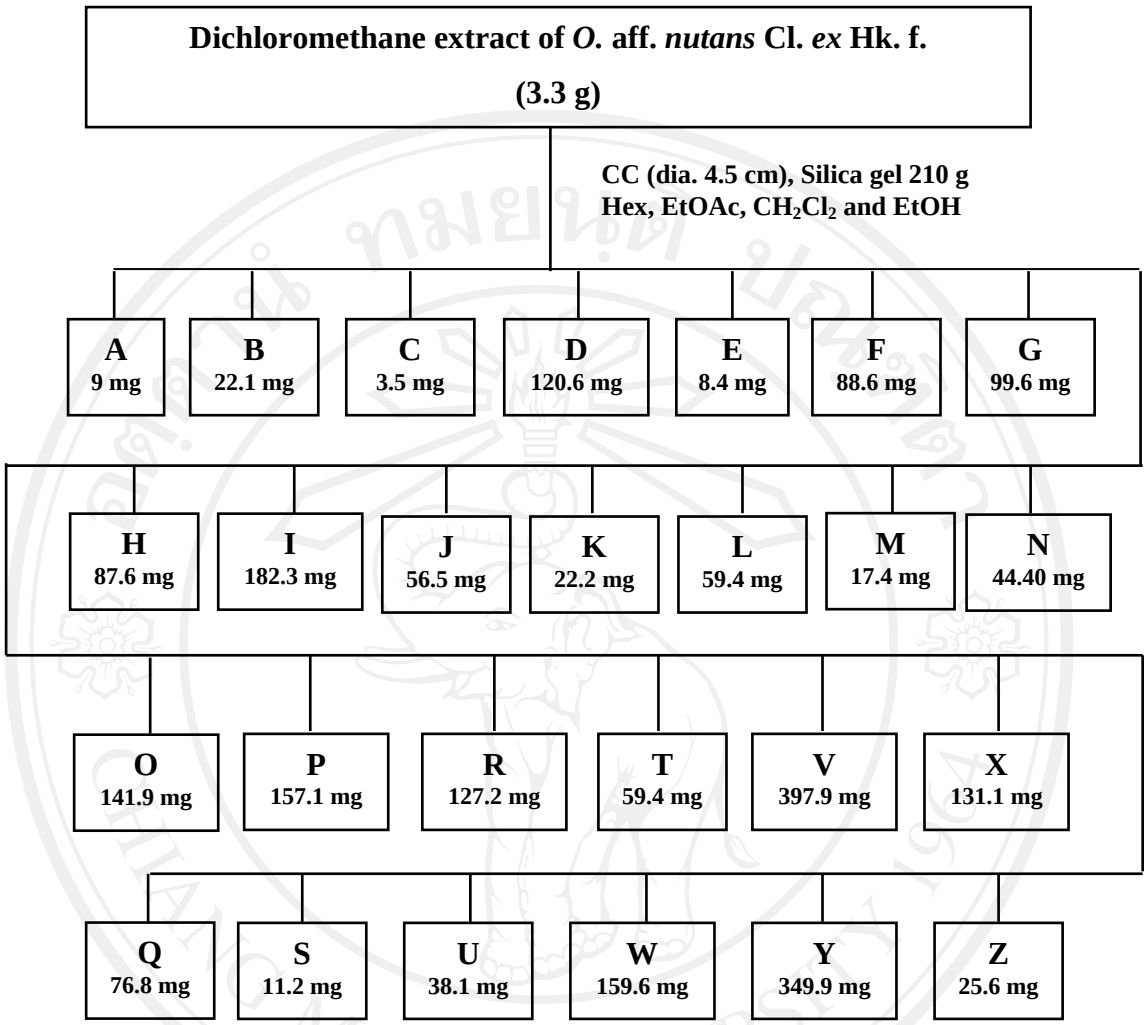
The crude methanolic extract of *O.* aff. *nutans* Cl. *ex* Hk. f. was dissolved in 5% sulfuric acid and partitioned by hexane and dichloromethane. Then the acid soluble extract was made alkaline to pH 10 by using ammonium hydroxide solution and partitioned by ethyl acetate and n-butanol respectively. Each fraction was evaporated to dryness under reduced pressure which gave a crude extract for bioactivity tested and purification. The procedure is shown in Scheme 1.3



Scheme 3.1 Extraction of whole powdered plant of *Ophiorrhiza* species

5.1 Isolation of the dichloromethane extract of *O.* aff. *nutans* Cl. *ex* Hk. f.

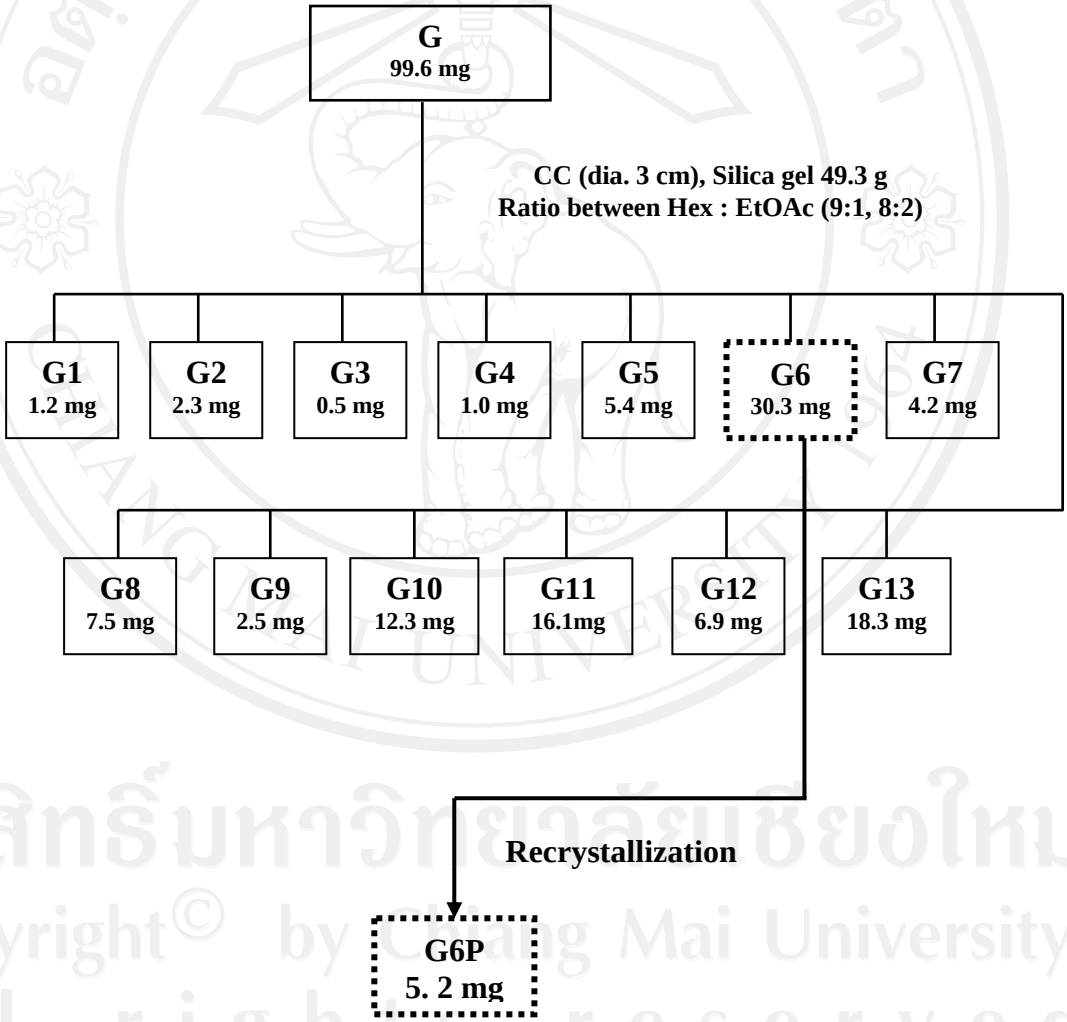
The dichloromethane extract 3.3 g was fractionation by column chromatography, using silica gel 60 column (i.d. 4.5 cm). Twenty six fraction (A to Z) were obtained by using the eluent (50-125 ml each) as follows; hexane: ethyl acetate, 8:2,7:3, 6:4, 1:1, 4:6, 2:8; 100% ethyl acetate; ethyl acetate: dichloromethane, 9:1, 8:2, 6:4, 4:6, 2:8, 100% dichloromethane and dichloromethane: ethanol, 9:1, 8:2, 7:3, 6:4, 1:1 respectively. (**Scheme 3.2**)



Scheme 3.2 Isolation of dichloromethane extract of *O.* aff. *nutans* Cl. *ex* Hk. f.

5.1.1 Isolation of fraction G

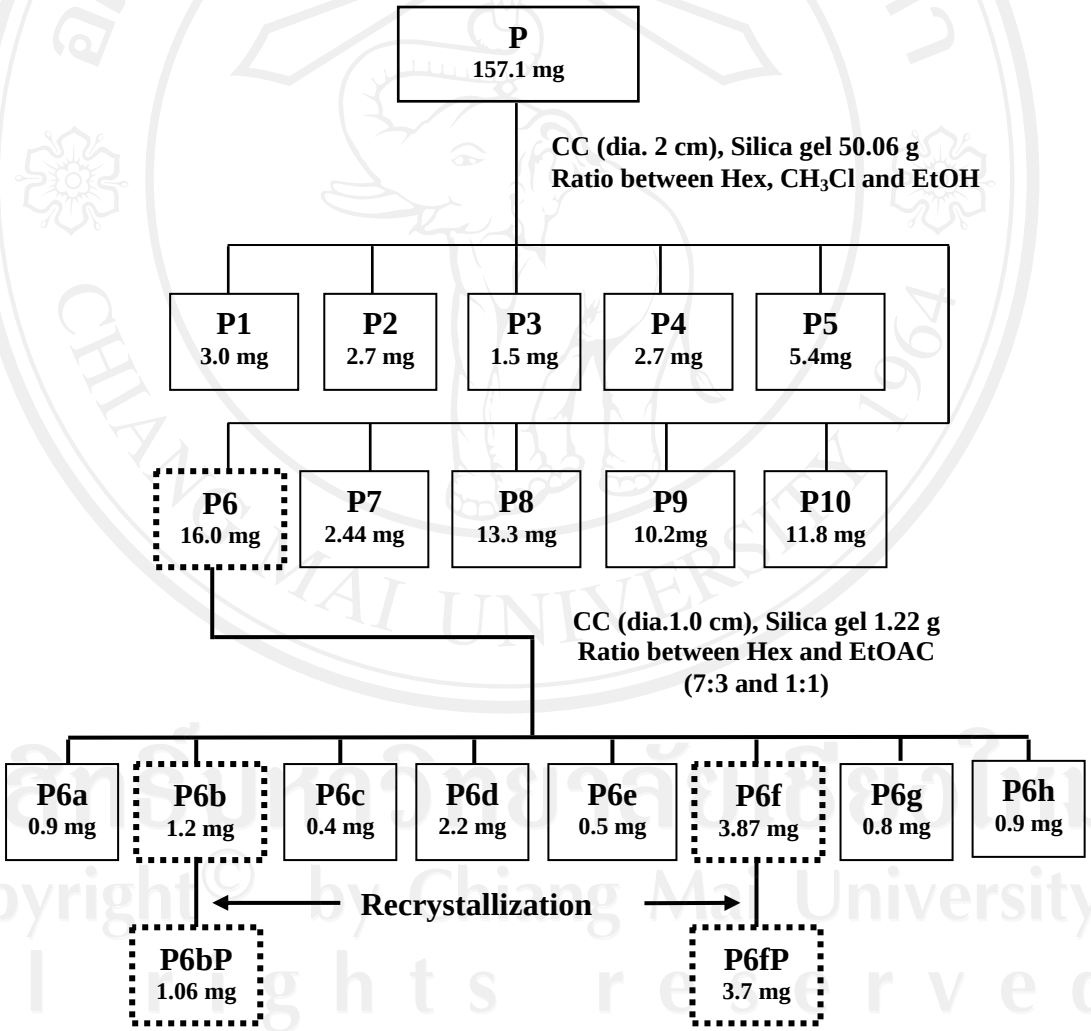
The fraction G (99.6 mg) was selected for further purification. This fraction was separated by using silica gel 60 (49.3 g) column. Hexane and ethyl acetate were used as eluents respectively. Thirteen fractions were obtained. A portion of fraction G6 was recrystallized with hexane followed by ethyl acetate to obtain white needle crystal (G6P) as shown in **Scheme 3.3**.



Scheme 3.3 Isolation scheme of compound G6P

5.1.2 Isolation of fraction P

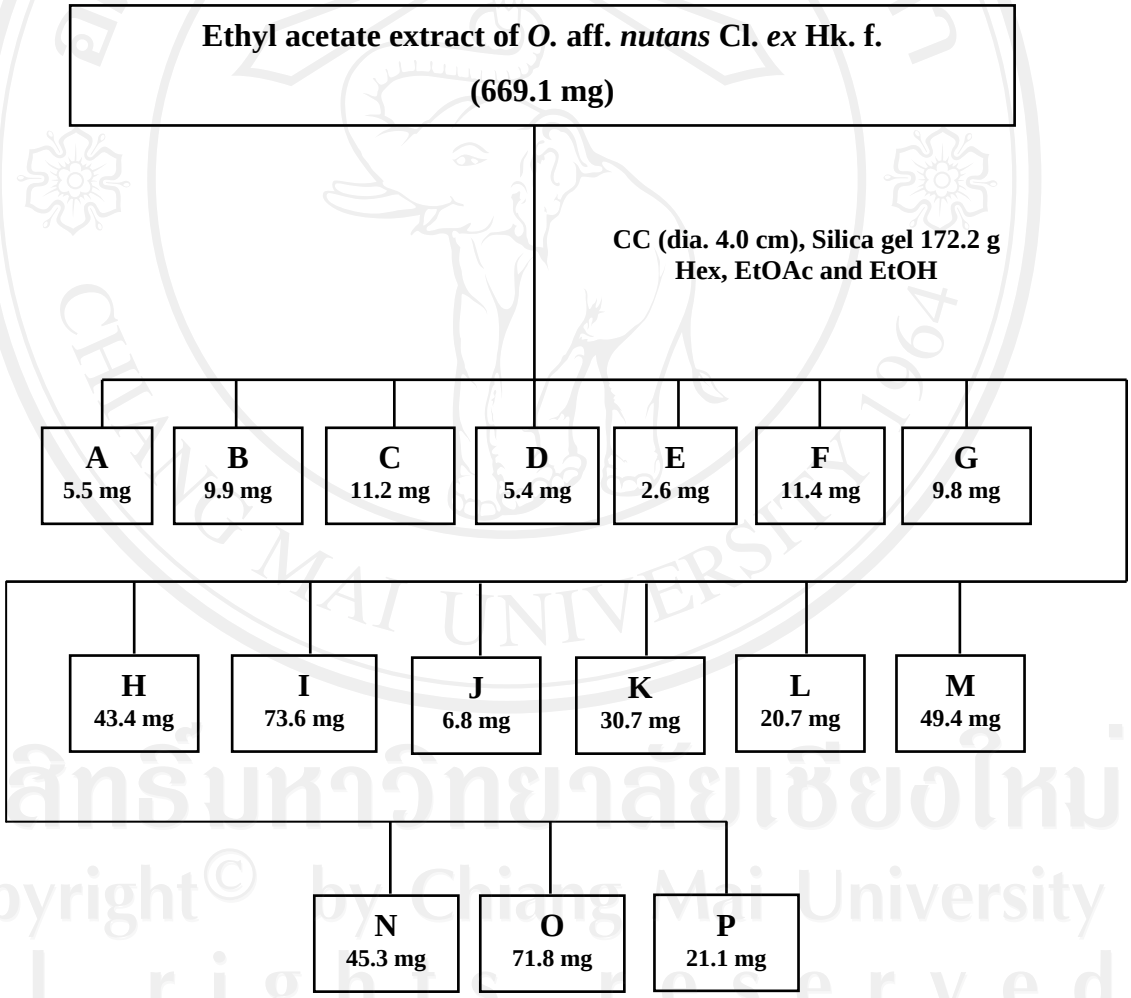
A portion of fraction P (157.1 mg) was fractionated by Silica gel 60 column, using hexane $CHCl_3$ and EtOH as eluents (Hex: $CHCl_3$, 2:8,1:9; 100% $CHCl_3$; $CHCl_3$:EtOH, 9.5:0.5, 9:1, 7:3 and 1:1). Ten fractions were obtained. Fraction P6 was taken for further purification, using silica column. Hexane and ethyl acetate were used as eluents. Eight fractions were obtained (P6a-P6h). Fractions P6b and P6f were taken for recrystallization, using hexane followed by ethyl acetate yielded white crystal (P6bP) and yellow crystal (P6fP) as shown in **Scheme 3.4**.



Scheme 3.4 Isolation of compound P and repeats separation for purified P6bP and P6fP

5.2. Isolation the ethyl acetate extract of *O.* aff. *nutans* Cl. *ex* Hk. f.

The ethyl acetate extract 669.1 mg was fractionated by column chromatography (diameter 4.0 cm) on Silica gel 60 (174.2 g) using hexane, ethyl acetate and ethanol as eluents (100% Hex; Hex: EtOAc, 9:1, 7:3, 6:4, 1:1, 3:7, 1:9; 100% EtOAc; EtOAc: EtOH, 9:1, 7:3, 1:1, 3:7, 1:9 and 100% EtOH). Sixteen fractions were obtained (fraction A-P) as shown in **Scheme 3.5**.

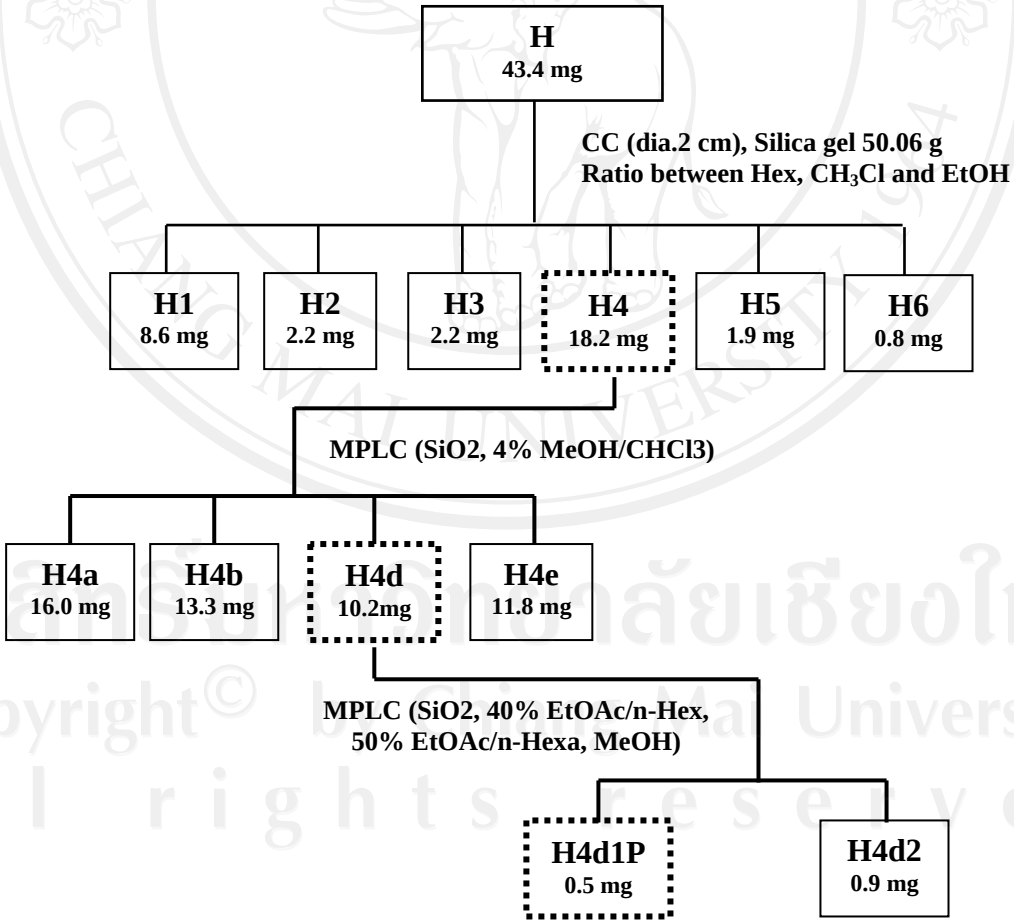


Scheme 3.5 Isolation of ethyl acetate extract of *O.* aff. *nutans* Cl. *ex* Hk. f.

5.2.1 Isolation of fraction H

The fraction H (43.4 mg) was rechromatographed using silica gel 60 (15 mg) column. The ratio between Hex: EtOAc, 2:8, 1.5:8.5, 1:9; 100% EtOAc; and EtOAc: EtOH, 9:1 and 8:2 were used as eluents respectively. Each 2 ml of 60 fractions were collected and combined according to their TLC patterns giving 6 fractions H1 to H6. Among these, a portion of H4 was separated by MPLC using Silica column. 4% Methanol in chloroform was used as mobile phase giving 5 fractions, H4a to H4e.

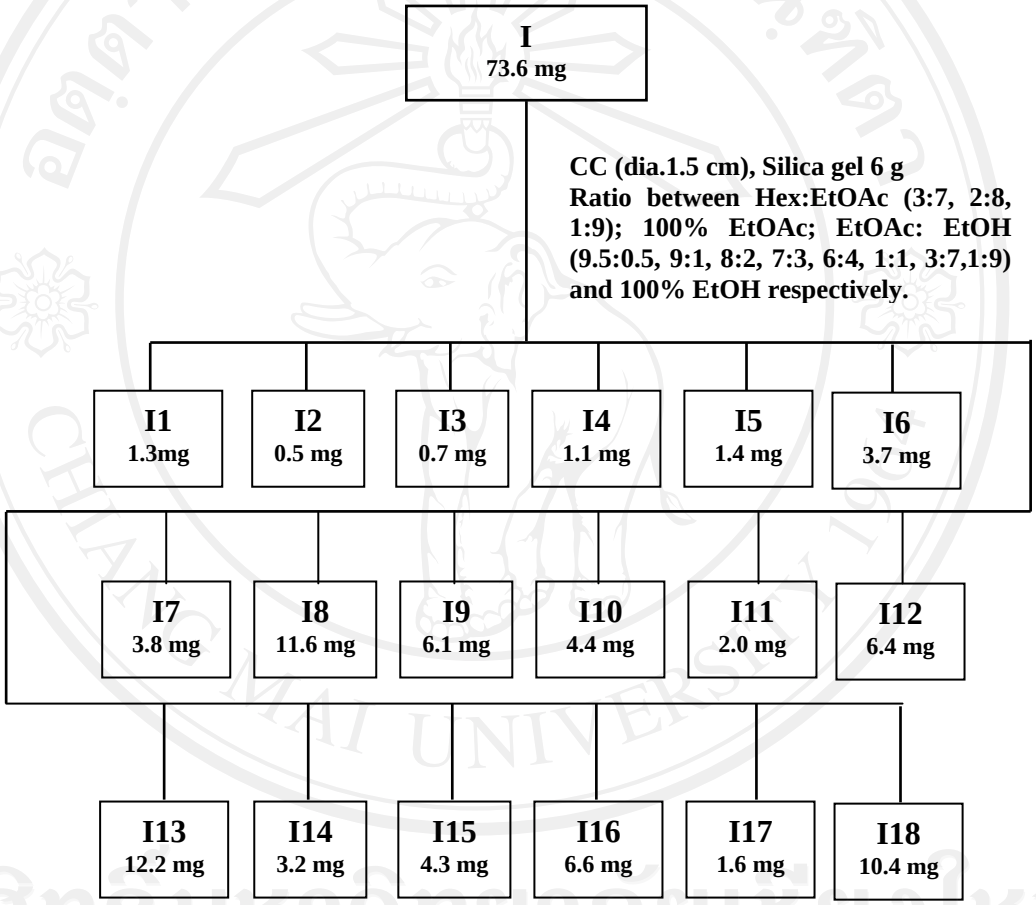
The fraction H4d (4.9 mg) was separated by MPLC again. 50% Ethyl acetate in n-hexane and methanol were used as mobile phases respectively. Two fractions were obtained (H4d1P and H4d2). Fraction H4d1P may be pure compound. (**Scheme 3.6**)



Scheme 3.6 Isolation of fraction H for purified of H4d1P

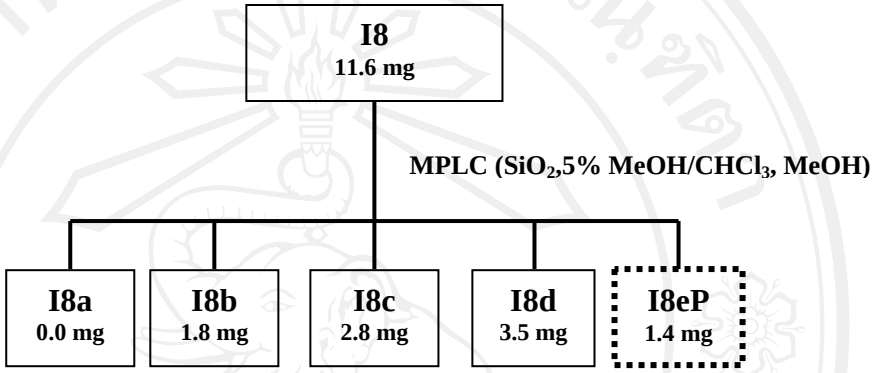
5.2.2 Isolation of fraction I

A portion of fraction I (73.6 mg) was fractionated by Silica gel 60 column. Hexane, EtOAc and EtOH were used as eluents. Eighteen fractions were obtained (I1-I18) as shown in **Scheme 3.7**.



Scheme 3.7 Isolation of compound I

A portion of fraction was I8 (11.6 mg) separated by MPLC using Silica column, 5% methanol in chloroform and methanol were used as eluents. Five fraction (I8a to I8e). Fraction I8e may be pure compound (**Scheme 3.8**).



Scheme 3.8 Isolation of fraction I8 for purified of I8eP

6. Bioactivity Determination

6.1 Antioxidant activity Assessment by DPPH• method

The antioxidant activity of the crude extracts and isolated compounds were performed by DPPH method as follows:-

A stock solution (5.0×10^{-4} mol L^{-1}) of DPPH• was prepared by dissolving the appropriate amount in ethanol. This solution was kept at 4 ºC and protected from light. It was stable and can be used for a week. The DPPH• working solution containing 1.0×10^{-4} mol L^{-1} was prepared by measuring 50 mL of the stock solution. The volume was made up to 200 mL with ethanol. This working solution was prepared daily and protected from light. Ascorbic acid, Trolox and quercetin stock solutions were prepared by dissolving the appropriate amounts of the respective solids in ethanol. Working standard solutions containing either ascorbic acid, Trolox and quercetin in the concentration range of 0.5-5.0 µg mL^{-1} were prepared by dilution of

the respective stock solution with ethanol.

The test sample (20 µL) was added to 180 µL of 10 µM DPPH• solution in a 96-well microtiter plate. The reaction mixture was incubated at 37 ºC for 30 min, and then the absorbance of each well was measured at 540 nm. The DPPH• solution was used as negative control. Trolox, ascorbic acid and quercetin were used as reference standards. For 50% inhibitory concentration (IC_{50}) evaluation of crude medicinal plant extracts, a graph showing concentration versus %DPPH• reduction was plotted. The IC_{50} was then calculated from the calibration curve and the activity was expressed as the percentage DPPH• scavenging relative to control using the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

6.2. Antibacterial and antifungal activity

The antimicrobial activity of the crude extracts were determined in accordance with the agar-well diffusion method described by Irobi et al. (1994). The antibacterial activity against include *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 (Gram positive bacteria) was tested and *Pseudomonas aeruginosa* ATCC 27853 (Gram negative bacteria). The antifungal activity of the sample was tested against *Candida albican* and fungus *Aspergillus flavus* and *Trichophyton mentagrophyte*,

The bacterial isolates were first grown in a nutrient broth for 18 h before use and standardized to 0.5 McFarland standards (106 cfuml-1). Two hundred microliter of the standardized cell suspensions were spread on a Mueller-Hinton agar (Oxoid). Wells were then bored into the agar using a sterile 6 mm diameter cork borer. The crude extract of sample was diluted with ethanol as following; One hundred micro liters of sample solution were transferred to the well (8 mm in diameter) and the same volume of ethanol was used as a negative control then allowed to stand at room temperature for about 2 h and then inoculated plates were incubated for 24 h at 37°C. After incubation, the diameter of the inhibition zone was measured. The

measurements were done basically from the edge of the zone to the edge of the well. The inhibition zones were compared with those of 75 μg mL^{-1} gentamycin standard.

The fungal isolates were allowed to grow on a Sabouraud dextrose agar (SDA) (Oxoid) at 25°C until they sporulated. The fungal spores were harvested after sporulation by pouring a mixture of sterile glycerol and distilled water to the surface of the plate and later scraped the spores with a sterile glass rod. The harvested fungal spores and bacterial isolates were standardized to an OD 600 nm of 0.1 before use. One hundred microliter of the standardized fungal spore suspension was evenly spread on the SDA (Oxoid) using a glass spreader. Wells were then bored into the agar media using a sterile 6 mm cork borer and the wells filled with the solution of the extract taking care not to allow spillage of the solution to the surface of the agar medium. The plates were allowed to stand on the laboratory bench for 1 h to allow for proper diffusion of the extract into the media. Plates were incubated at 25 °C for 96 h and later observed for zones of inhibition. The effect of the extract on fungal isolates was compared with 0.25 mg mL^{-1} ketoconazole standard was used as positive control and methanol was used as negative control.

6.3. Cytotoxicity and Anticancer studies

The cytotoxic effect tests were studied on normal African green monkey kidney (Vero) cells. The crude extracts were dissolved in dimethyl sulphoxide (DMSO) and the volume was made up to 10 mL with Dulbecco's Modified Eagle Medium (DMEM) to obtain 1 mg mL^{-1} concentration. Stock cells were cultured in minimum essential medium with 2% fetal calf serum supplemented with glutamine at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. The medium was changed every 3 days. Monolayer cultures of Vero cells were trypsinzed and the cells were plated out at 6×103 cells/well in 96-well microtitre plate. The cell growth was found to be exponential during 2–3 days in the medium. The cultured cells were incubated with crude extracts at the concentration rang of 0-50 μg mL^{-1}. Control cells were incubated with DMSO (final concentration, 0.2%) at 37 °C, 5% CO2, cell viability counts were made by sulforhodamine B assay (SRB) (118). The percentage viability was calculated and plotted against concentration to get the IC_{50} values.

The anticancer activities of crude extracts against NCI-H187-Small cell lung cancer, MCF7-Breast cancer and KB-Oral cavity cancer were determined using the resazurin microplate assay (REMA) method (119). Ellipticine and doxorubicin were used as positive controls. DMSO and sterile distilled water were used as negative controls. Cells at a logarithmic growth phase were harvested and diluted to105 cells/ml in fresh medium and gently mixed. Test compounds were diluted in culture medium in a ratio of 1:2 giving 8 concentrations. Five µl of the test sample and 45 µl of cells were put into 96-well microtiter plates with a total volume of 50 µl/well. Plates were incubated at 37 ˚C, 5% CO_2, for 72 h for KB and MCF7 and 5 days for NCI-H187. After the incubation periods, 12.5 µl of resazurin solution was added to each well and the plates were incubated at 37 ˚C for 4 h. The plates were then processed for optical density absorbance analysis 538 using a Victor 3 Microplate reader at dual wavelengths of 530 and 590 nm.

7. Identification

Proton and Carbon nuclear magnetic resonance spectra ^{1}H-NMR and ^{13}C-NMR: ^{1}H-NMR and ^{13}C-NMR spectra were measured on JEOL-600 MHz spectrometer; 600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C-NMR in deuterated chloroform (CDCl3) and hexadeuteron-DMSO (DMSO-d_6), Faculty of Pharmaceutical Science Chiba University, Japan. The chemical shifts were record in ppm with reference to TMS signal.