CHAPTER 3

EXPERIMENTAL

3.1 General Methods

Melting points were determined on a Gallenkamp Electrothermal apparatus and are incorrected. Infrared spectra were recorded 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). All NMR spectra were measured in $CDCl_3$ and chemical shifts were reported as δ-values in parts per million (ppm) relative to residue $CHCl_3$ as internal reference (^{1}H: δ 7.26, ^{13}C: δ 77.00) and coupling constants (*J* values) were reported in hertz (Hz). Mass spectra (electrospray ionization mode, ESI-MS) were measured on a micromass Q-TOF-2^{TM} (Waters) spectrometer. Optical rotations were measured in $CHCl_3$ on an Atago AP-300 automatic polarimeter. Flash column chromatography was performed by employing Merck silica gel 60 and Merck silica gel 60H. Preparative thin layer chromatography (PLC) plates were carried out using Merck silica gel 60 PF_{254}.

3.2 Plant material

The leaves of *V. parishii* were collected from Mae Jaem district, Chiang Mai, Thailand in 2008. A voucher specimen (MAXWELL 08-54) was deposited in the herbarium of Department of Biology, Faculty of Science, Chiang Mai University, Thailand.

3.3 Extraction and Isolation

The air-dried powdered leaves of *V. parishii* (1,265.06 g) were successively extracted with dichloromethane (VPD) (3.5 L x 3 days x 3 times), and followed by methanol (VPM) (3 L x 3 days x 3 times), respectively. The filtrates, crude extracts in

organic solvents, were evaporated under reduced pressure by a rotary evaporator to receive dichloromethane and methanol extracts, which were 57.79 and 43.33 g, respectively.

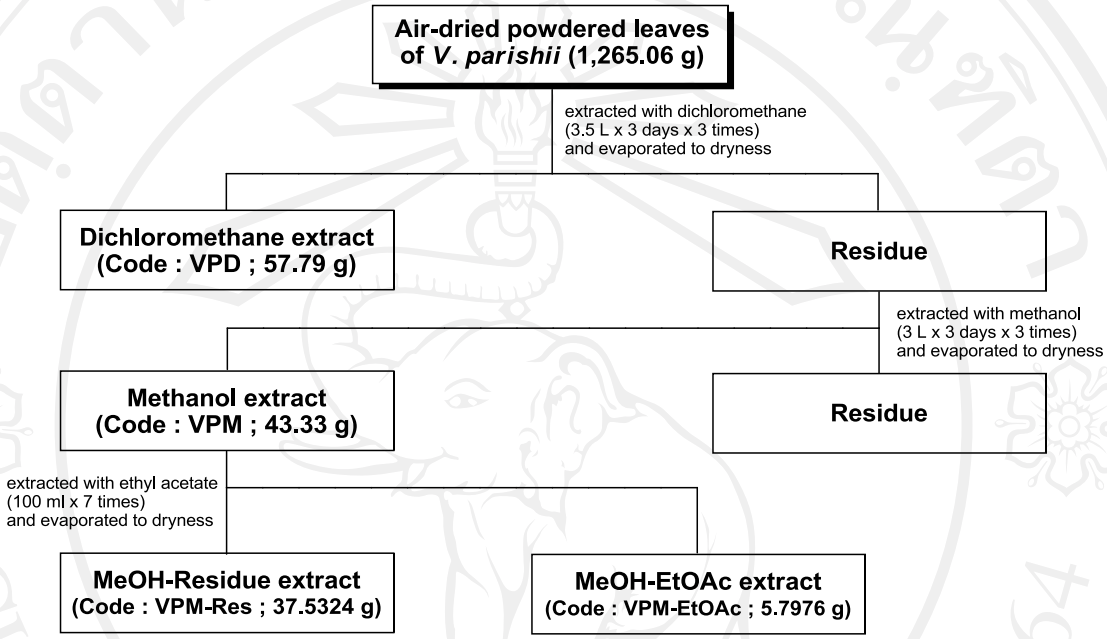

Figure 25 The extraction flowchart of *V. parishii* leaves

3.4 Separation of the dichloromethane extract

The dichloromethane extract from the leaves of *V. parishii* was isolated by column chromatography with silica gel 60 PF_{254} (Merck), then organic solvents were used as gradient elution, starting from *n*-hexane and increasing polarity of solvent by dichloromethane, ethyl acetate and methanol, respectively. The TLC results were used as the reference to combine the specific ten fractions from the first column separation. (Figure 24)

After the isolation of dichloromethane extract from *V. parishii* leaves, two fractions, VPD-F07 (1.29 g) and VPD-F08 (1.35 g), were subject to the investigation of constituents because this fraction had colorless crystal mixed with dark green oils. And then, the fraction was recrystallized with mixture of hexane : dichloromethane (1:1) to obtain a colorless crystal solid. This compound was identified as stigmasterol (**58**) (0.0599 g).

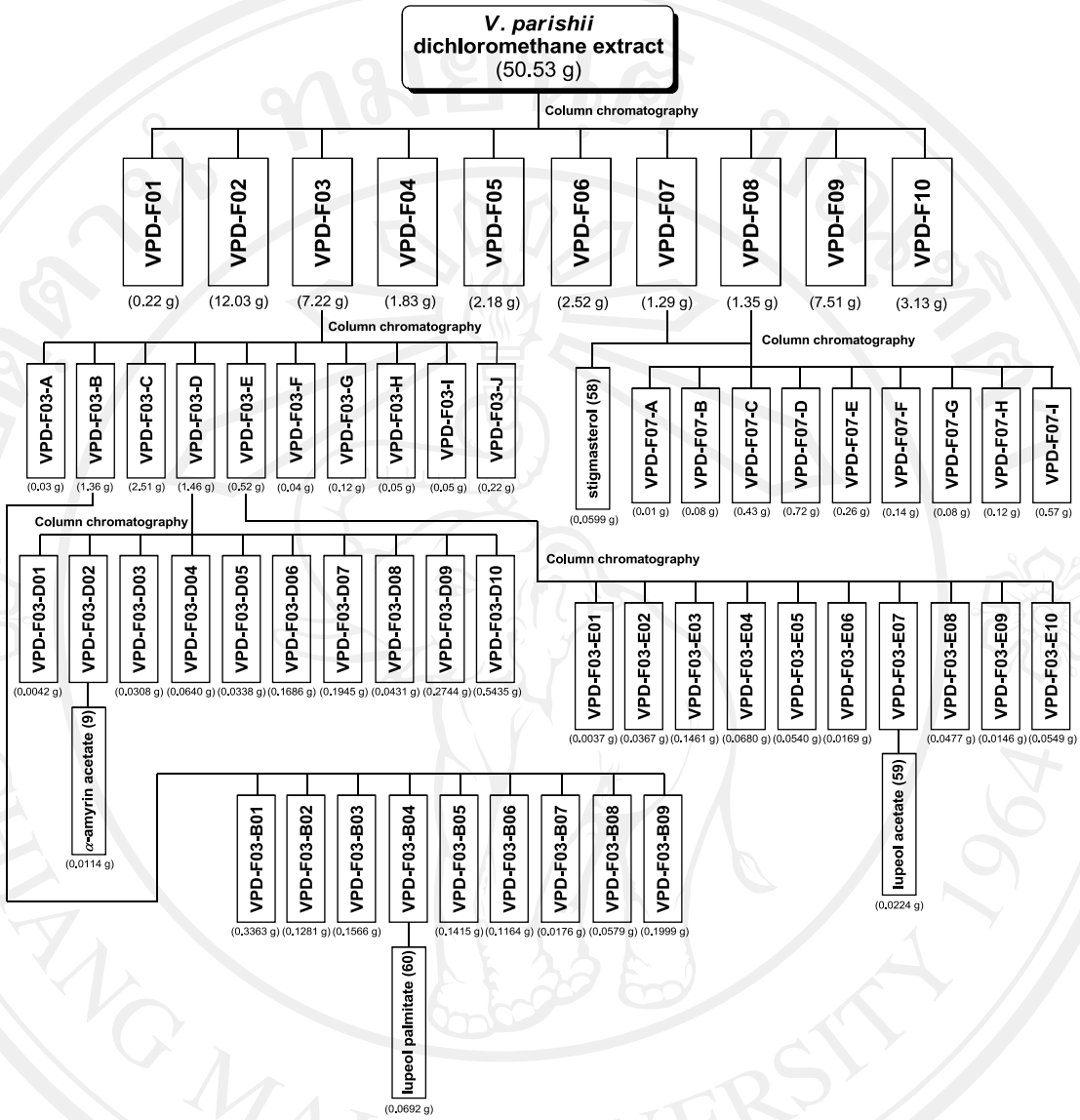

Figure 26 The isolating flowchart of dichloromethane extract of *V. parishii* leaves that showed separating of stigmasterol (**58**), lupeol palmitate (**60**), α-amyrin acetate (**9**), and lupeol acetate (**59**)

Fraction VPD-F03 (7.22 g) was selected to separate the constituents by column chromatography. Initially, this fraction was eluted by organic solvents, starting from 100% hexane and increasing the polarity by adding dichloromethane, ethyl acetate and methanol, respectively, to give 10 sub-fractions of VPD-F03 (VPD-F03-A–VPD-F03-J). Additionally, three sub-fractions, VPD-F03-B, VPD-F03-D, and VPD-F03E, were chosen to isolate the compounds by column chromatography. Three triterpenes,

containing lupeol palmitate (**60**) (0.0692 g), *α*-amyrin acetate (**9**) (0.0114 g), and lupeol acetate (**59**) (0.0224 g), were obtained from sub-fractions VPD-F03-B, VPD-F03-D, and VPD-F03-E, respectively.

Fraction VPD-F04 was also chosen to investigate its compositions with column chromatography like the previous fractions. The fraction was treated as same as fraction VPD-F03 to donate lupenone (**61**) (0.0073 g), a triterpene that was isolated from sub-fraction VPD-F04-I.

In the same strategy, fraction VPD-F05 was purified by chromatographic technique. Two known triterpenes, *α*-amyrin (**13**) (0.0102 g) and lupeol (**41**) (0.0077 g), were found in sub-fraction VPD-F05-H and VPD-F05-I respectively.

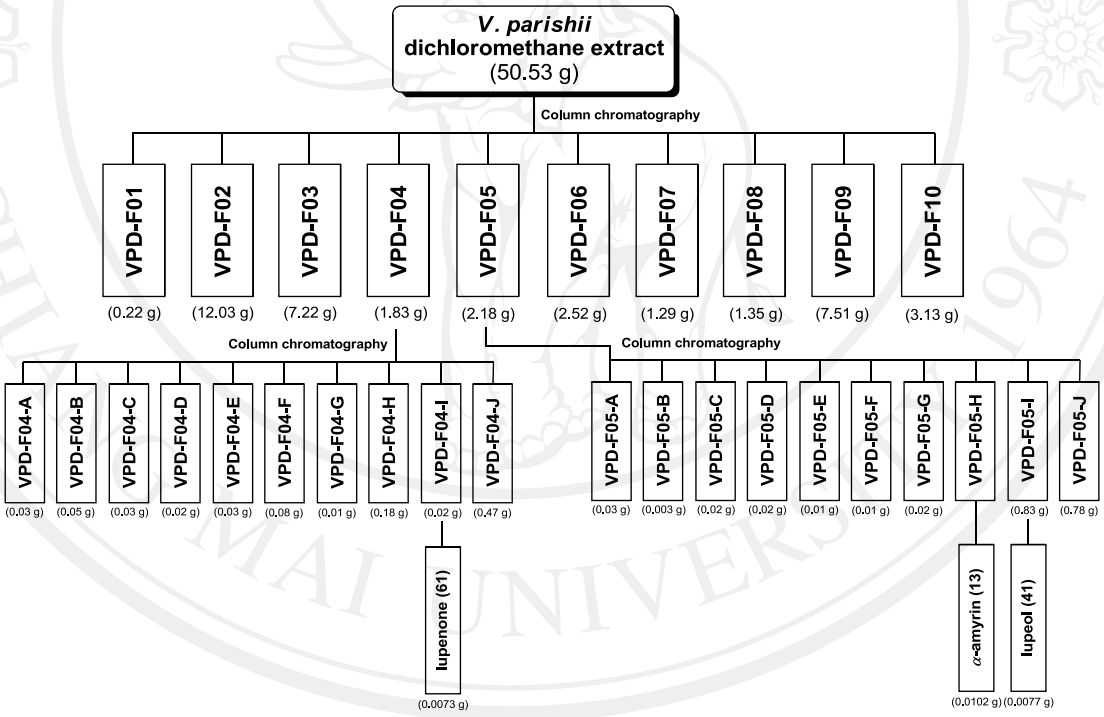

Figure 27 The isolating flowchart of dichloromethane extract of *V. parishii* leaves that showed separating of lupenone (**61**), *α*-amyrin (**13**) and lupeol (**41**)

Lastly, fraction VPD-F01 and sub-fraction VPD-F07-F were recrystallized from dichloromethane for the former and mixture of hexane : dichloromethane (1:1) for the latter. From this method, hentriacontane (**62**) (0.0099 g) and palmitic acid (**63**)

(0.0014 g) were discovered in the fraction VPD-F01 and sub-fraction VPD-F07-F of dichloromethane extract of *V. parishii*, respectively.

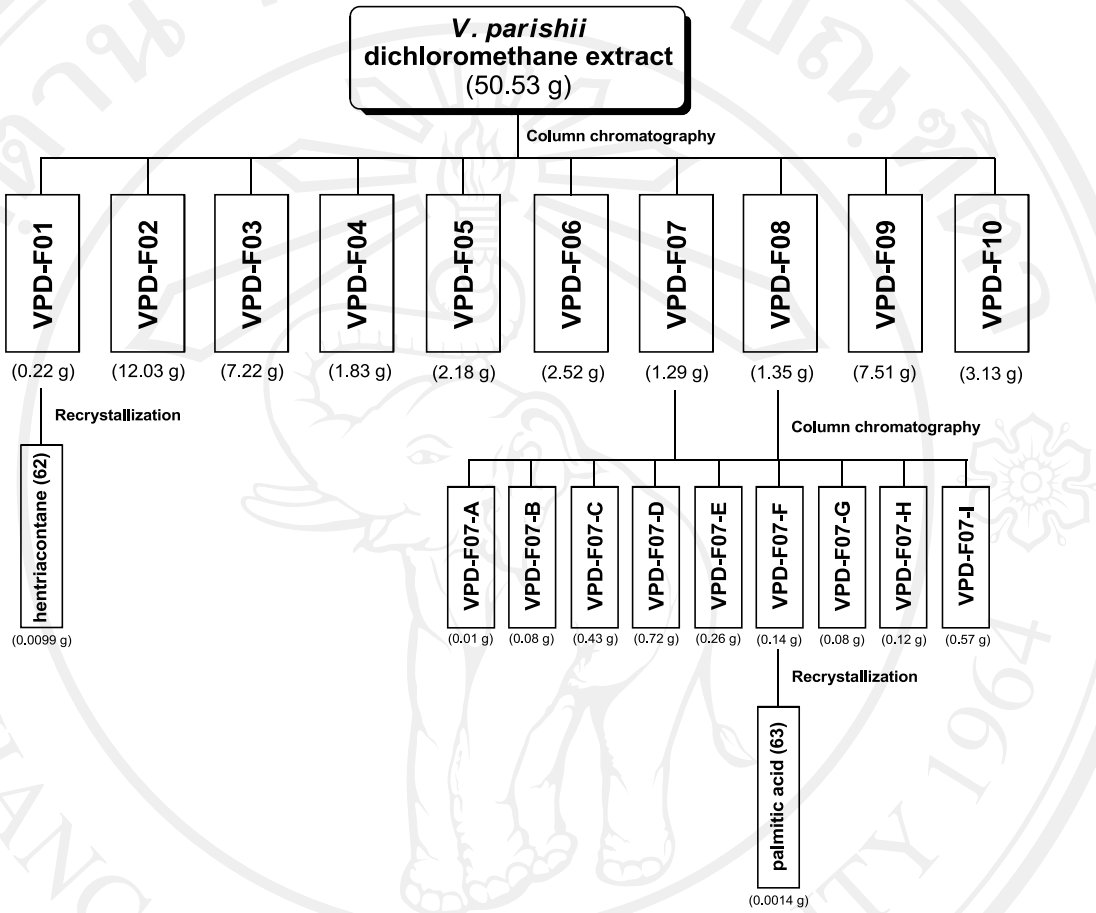

Figure 28 The isolating flowchart of dichloromethane extract of *V. parishii* leaves that showed separating of hentriacontane (**62**) and palmitic acid (**63**)

To identify the structures of isolated compounds, spectroscopic data such as ^{1}H NMR, ^{13}C NMR, COSY, DEPT, HMQC, HMBC, mass spectrum, and IR spectrum, were used as the main evidences for characterizing separated compounds and confirmed with physical properties. Moreover, all data of each compound from this experiment were compared with other previous reports.

3.5 Biological assay

Assay for activity against *P. falciparum* (K1, multidrug-resistant strain) was performed using the microculture radioisotope technique described by Desjardins.[66] IC_{50} represents the concentration that causes 50% reduction of parasite growth as indicated by the in vitro uptake of [3H]-hypoxanthine by *P. falciparum*. A standard antimalarial compound, dihydroartemisinin, showed an IC_{50} value of 1.8 ng/mL in the same assay system. The antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA).[67] Standard drugs, isoniazid and kanamycin sulfate, the reference compounds for the antimycobacterial assay, showed minimum inhibitory concentrations (MICs) of 0.040-0.090 and 2.0-5.0 μg/mL, respectively. Cytotoxicity against African green monkey kidney fibroblast (Vero) was evaluated using the colorimetric method.[68]