CHAPTER III

RESEARCH METHODOLOGY

General Experimental Procedures

UV spectra were recorded on a Jasco V-650 spectrophotometer. Melting points were measured on a Stuart Scientific UK melting point apparatus. Optical rotations were measured on a ADP200 polarimeter. The FT-IR spectra were measured on a Perkin-Elmer Frontier spectrophotometer. ^{1}H and ^{13}C, DEPT (135), ^{1}H-^{1}H COSY, HMQC and HMBC experiments were carried out on a Bruker AV400 NMR spectrometer, operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. ESIQ-TOF mass spectra were obtained from a 6540 UHD Accurate-Mass Q-TOF LC/MS Agilent Technologies USA. Column chromatography (CC) was performed using Merck silica gel 60H for TLC (< 55μm; Darmstadt, Germany; 1.07736.1000) (CC equip with low pressure air pump), Sephadex LH-20 (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Thin-layer chromatography (TLC) was run using Merck silica gel F-254 (1.05554.0001).

The GC-MS analysis was performed on a 5973 Network MSD Agilent, a 6890N Network GC system Agilent and Agilent 19091S-433 HP-5MS capillary column (30 m × 0.25 mm, a film thickness of 0.25 μm). The GC was operated in the constant flow mode of 1.5 mL/min with helium as a carrier gas. The column oven was temperature programmed: 70°C (1 min), then 50°C/min to 240°C, 1°C/min to 320°C and held at 320°C for 10 min. The temperatures of ion source and quadrupole were 230°C and 150°C, respectively. The quadrupole was scanned in the 25-675 *m/z* range (Urbanova, et al., 2012).

Solvent

Hexane, dichloromethane, chloroform, ethyl acetate, acetone and methanol were obtained from Mallinckrodt Chemicals (USA). Toluene was obtained from RCI Labscan (Thailand). Chloroform-D and acetone-d_6 (99.9%D) were obtained from Wilmad LabGlass (USA). Methyl sulfoxide-d_6 (99.9%D) was obtained from ALDRICH (USA). Bis (trimethylsilyl)trifluoroacetamide with 1% trimethylsilyl chloride (BSTFA/TMSCl) was obtained from Acros Organics (USA).

Plant material

Lagerstroemia loudonii fresh fruits were collected in Naresuan University, Phitsanulok province, Thailand, during March to April 2013. Voucher specimens (003507) were deposited at Department of Biology, Faculty of Science, Naresuan University.

Extraction

The fresh fruits of *L. loudinii* (4 kg) were cracked and macerated in methanol (5.0 L × 2, each) at room temperature for one week. Then the solution was percolated, and the combined filtrates were evaporated under vacuum to produce a brown-gummy methanol extract. Then the solution was percolated, and the combined filtrates were evaporated under vacuum to produce a brown-gummy methanol extract (LFM). Partition LFM with ethyl acetate. The ethyl acetate fraction was evaporated under vacuum to produce a green-gummy ethyl acetate extract 6.87 g (0.17%). The residue was macerated in dichloromethane (DCM, 5.0 L × 2, each) at room temperature for another one week. Then the solution was percolated. After that the combined filtrates were evaporated under vacuum to produce 14.37 g (0.36%) of a green-powder DCM extract.

The DCM extract (LFD, 10.0 g) was suspended in non polar solvent to more polar solvent that was hexane (500 mL), DCM (250 mL), EtOAc (250 mL) and acetone (250 mL). Each soluble part was evaporated and labeled as fraction LFD-H (4.10 g), LFD-D (1.30 g), LFD-E (0.75 g) and LFD-A (0.61 g), respectively. The precipitate was suspended in MeOH (250 mL). The soluble part was evaporated but no

residue was found. Therefore, the precipitate was labeled as fraction LFD-M (2.93 g). Extraction procedure of fresh fruit of *L. loudinii* was shown in figure 1.

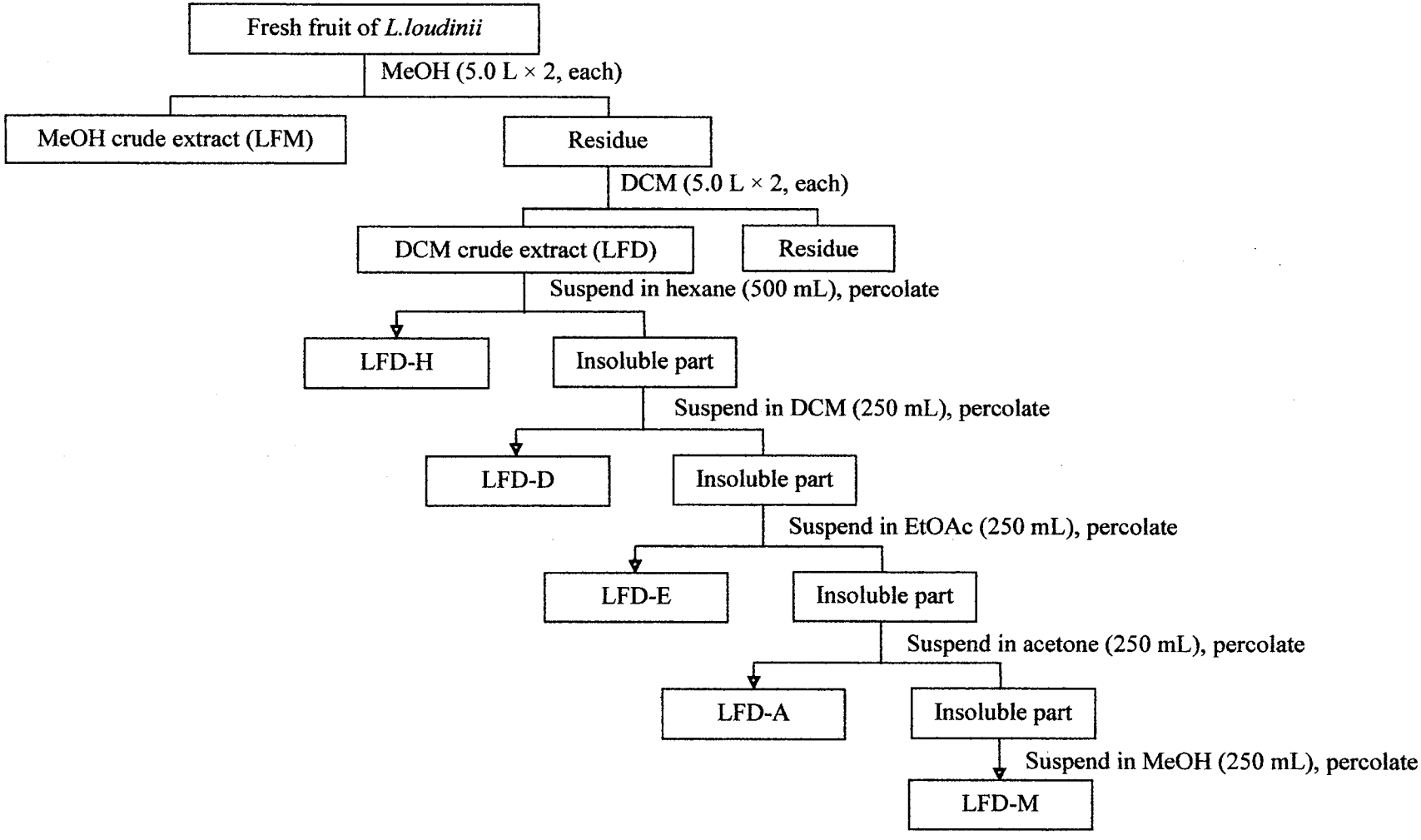

Figure 1 Extraction procedure of fresh fruit of *L. loudinii*

Isolation by column chromatography

Fraction LFD-H (4.10 g) was separated by silica gel CC (3 cm × 10 cm) with a solvent gradient of 100% hexane to 15% acetone-DCM to afford twenty-six subfractions (h1 - h26). **LFD-H1** (0.72 g, 17.6% of fraction) was obtained from subfraction h3. **LFD-H2** (0.88 g, 21.5% of fraction) was obtained from subfraction h8. Mix of subfraction h11 to h18 (0.38 g) was fractionated by CC on silica gel (2 cm × 10 cm) and eluted with 100% hexane to 50% DCM-hexane to afford sixty-one subfractions (hm1 - hm61). **LFD-H3** (86.0 mg, 2.1% of fraction) was obtained from hm24. **LFD-H4** (7.3 mg, 0.18% of fraction) was obtained from hm38. Isolation procedure of fraction LFD-H was shown in Figure 2

Fraction LFD-D (0.89 g) was separated by silica CC (3 cm × 10 cm) with a solvent gradient of 20% DCM-hexane to 100% DCM, 1% EtOAc-DCM to 10% EtOAc-DCM to afford twenty-five subfractions (d1 - d25). **LFD-D1** was obtained from subfraction d3 (8.80 mg, 0.10% of fraction). **LFD-D2** was obtained from subfraction d17 (4.20 mg, 0.05% of fraction). Isolation procedure of fraction LFD-D was shown in Figure 3

Fraction LFD-E (0.75 g) and LFD-A (0.61 g) were combined and separate by silica CC (3 cm × 10 cm) with a solvent gradient of 1% acetone-$CHCl_3$ to 100% acetone to afford twenty-five subfractions (e1 - e25). **LFD-E1** was obtained from subfraction e20 (0.14 g, 10.3% of fraction) Isolation procedure of fraction LFD-E and LFD-A was shown in Figure 4

Fraction LFD-M (2.93 g) was washed with hexane several times. **LFD-M1** (2.92 g, 99.6% of fraction) was obtained from the precipitate.

Analytical procedure

The structure of LFD-H1, LFD-H2, LFD-H3, LFD-H4, LFD-D1, LFD-D2, LFD-E1 and LFD-M1 were determined by 1D nuclear magnetic resonance (NMR) spectroscopy. Structures of LFD-E1 and LFD-M1 were confirmed by 2D NMR spectroscopy, melting point, optical rotations, IR spectroscopy, UV-Vis spectrophotometry and ESIQ-TOF mass spectrometry.

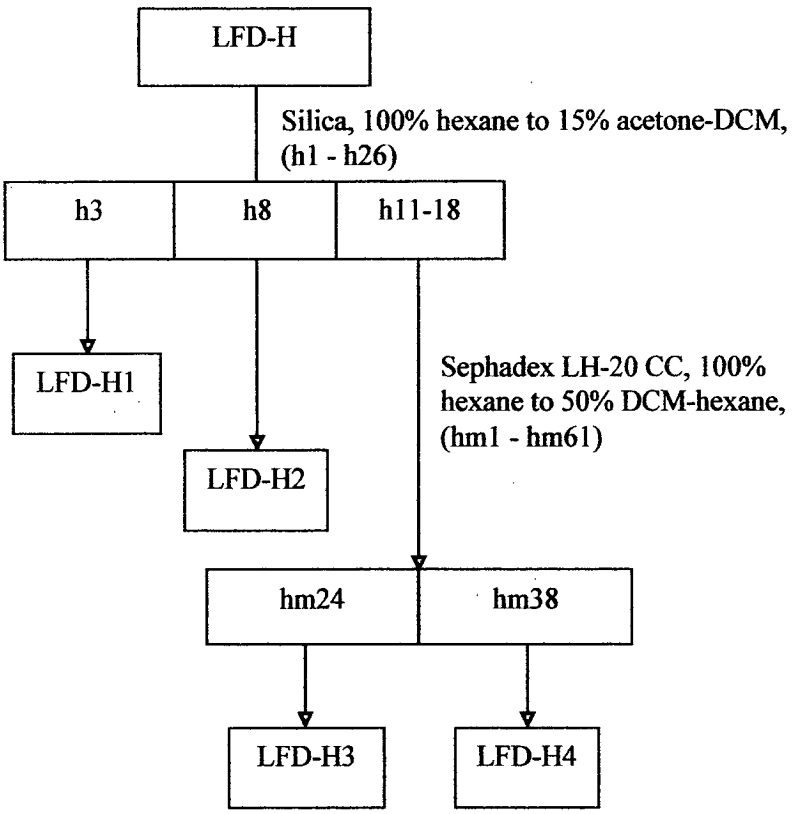

Figure 2 Isolation procedure of fraction LFD-H

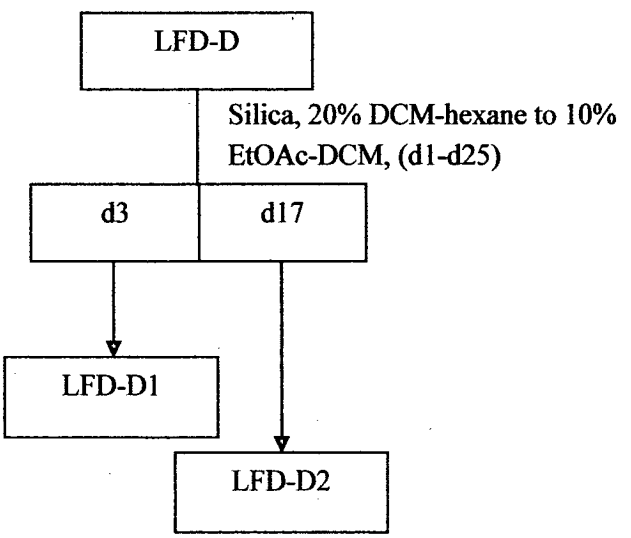

Figure 3 Isolation procedure of fraction LFD-D

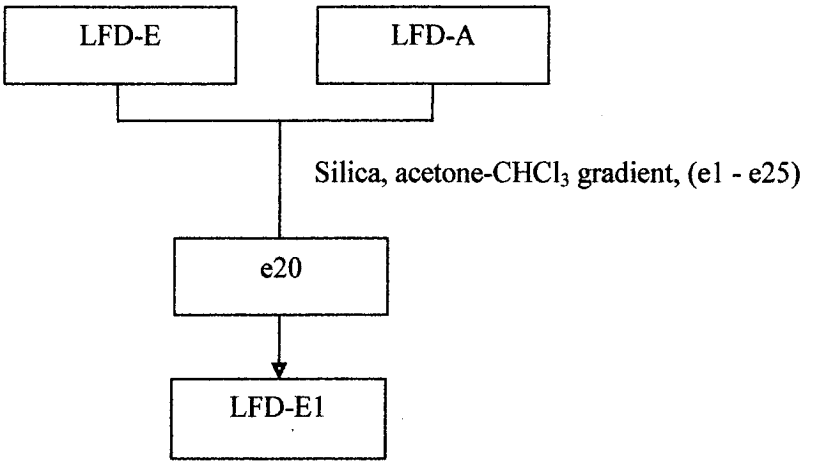

Figure 4 Isolation procedure of fraction LFD-E and LFD-A

Silylation

The silylation of LFD-D2 was carried out based on Inarkar and Lele report (Inarkar and Lele, 2012). LFD-D2 was trimethylsilylated using 200 μL of BSTFA/ TMSCl and 100 μL of toluene. The mixture was stirred at 75°C for 30 min. The reaction mixture was cooled and concentrated under nitrogen flush. The residue was redissolved in hexane, and the mixture was labeled as **LFD-D2-Silyl**.

Gas chromatography-mass spectrometry analysis

LFD-H1, LFD-H2, LFD-H4, LFD-D1 and LFD-D2-Silyl were analyzed and identified using GC-MS. The compounds were identified by comparison of fragmentation patterns in mass spectra with those of Wiley7n.1 library.