CHAPTER III
EXPERIMENTAL

1. Source of Plant Material

The flowers of *Melodorum fruticosum* Lour. were collected in Srisaket Province, Thailand in March 1998 and the voucher specimens are kept at the Herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2. General Techniques

2.1. Analytical Thin Layer Chromatography (TLC)

Technique	: One dimension, ascending
Adsorbent	: Silica gel 60 F_{254} (E. Merck) precoated plate
Layer thickness	: 0.2 mm
Distance	: 6 cm
Temperature	: Laboratory temperature (30-35°C)
Detection	: 1. Ultraviolet light at wavelengths of 254 and 365 nm
	2. 10% Sulfuric acid in ethanol and heated at 105° C for 10 min

2.2. Column Chromatography (CC)

2.2.1. Flash Column Chromatography

Adsorbent	: Silica gel 60 (No. 7734)
	particle size 0.063-0.200 nm
	Silica gel 60 (No. 9385)
	particle size 0.040-0.063 nm
Packing method	: Wet and dry packing

Sample loading : For wet packing, the sample was dissolved in a small amount of eluent, then apply gently on the top of the column

For dry packing, the sample was dissolved in a small volume of the optimal organic solvent, mixed with a small amount of adsorbent, triturated, dried, and then gently placed on top of the column.

Detection : TLC technique

2.2.2. Gel Filtration Chromatography

Gel filter : Sephadex LH-20 (Pharmacia)

MCI gel CHP20P (75~150μ) high porous polymer (Mitsubishi Chemical Corporation)

Packing method : The gel filter was dispersed in the eluent and left standing for gel initiation for about 24 hours before use.

Sample loading : The sample was dissolved in a small amount of eluent, then applied gently on top of the column

Detection : TLC technique

2.3. Preparative Centrifugal Thin Layer Chromatography

Instrument model : 1. Chromatotron Model 7924T

2. Lab Pump Model RH

Adsorbent : Silica gel 60 PF_{254} with Calcium Sulfate (E. Merck)

Layer thickness : 2 mm

Flow rate : 6 ml/min

Temperature : Laboratory temperature (30-35°C)

2.4. Spectroscopic Techniques

2.4.1. Ultraviolet (UV) Absorption Spectra

UV spectra were obtained on a Shimadzu UV-160A UV/vis spectrophotometer of the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.4.2. Infrared (IR) Absorption Spectra

IR spectra were recorded on a Perkin-Elmer Spectrum 2000 FT-IR spectrophotometer of the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Spectra of solid samples were recorded as KBr pellets and liquid sample was recorded as Nujol method.

2.4.3. Mass Spectra (MS)

EIMS and CIMS were obtained on a Micromass Platform II mass spectrometer of the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University, at 70 eV and 30 eV with methane gas, respectively.

2.4.4. Nuclear Magnetic Resonance (NMR) Spectra

^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, on a Bruker AVANCE DPX-300 FT-NMR spectrometer of the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Deuterated chloroform and deuterated dimethylsulfoxide were used as NMR solvents in this study. Spectral data were reported in ppm scale using the solvent chemical shifts as the reference frequencies.

2.4.4. Circular Dichroism Spectra

Circular dichroism (CD) spectra was measured on a Jasco J-715 Spectropolarimeter of the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University, with spectro-grade methanol.

2.5. Physical Properties Measurement Apparatus

2.5.1. Melting Point

Melting points were determined on a Gallenkamp Melting Point Apparatus.

2.5.2. Optical Rotation

Optical rotation was measured on a Perkin-Elmer Polarimeter model 341 of the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.5.3. Elemental Components

Elemental components of the compounds were analyzed on a Perkin-Elmer PE2400 Series II CHNS/O Analyzer of the Scientific and Technological Research Equipment Center, Chulalongkorn University: Option CHN, by the method of pyrolysis in high-purity oxygen (static-state oxidation) and quantitatively detected by thermal conductivity detector

2.6. Solvents

All commercial grade solvents were distilled prior to use.

3. Bioassay

Bioassay of cytotoxic activity against African green monkey kidney cell line (Vero cell line), human nasopharygeal carcinoma cell line (KB), and human breast cancer cell line (BC) in vitro were performed by SRB (Sulforhodamine B) colorimetic method[11]. The cytotoxic activity against murine lymphocytic leukemia (P388) cell line in vitro was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetic method.[12,13]

4. Extraction and Isolation

The dried flowers of *Melodorum fruticosum* (2.3 kg) were blended into small pieces. They were extracted with petroleum ether (3X3 L), ethyl acetate (3X3 L), and 70% aqueous acetone (3X3 L), successively. The obtained extracts were evaporated under reduced pressure to yield 58.44 g of petroleum ether extract, 58.04 g of ethyl acetate extract, and 24.78 g of 70% aqueous acetone extract.

The ethyl acetate extract, after evaporation by rotary evaporator, gave 8.22 g of a residue and 49.81 g of a liquor. The residue could be crystallized and recrystallized from ethyl acetate to yield 5.89 g of colorless silky crystals of compound MF-1. The liquor of this extract was dissolved in ethyl acetate, then fractionated by extensive repeated column chromatography, gel filtration (Sephadex LH-20), Silica gel 60 (art. 7734.100, 70-230 mesh ASTM), and Silica gel 60 (1.09385.100, 230-400 mesh ASTM).

Gel filtration technique was carried out on a Sephadex LH-20 column and eluted with ethyl acetate to obtain 7 fractions. The fractions with similar TLC pattern were combined to yield 5 fractions, ES1 (2.519 g), ES2 (7.441 g), ES3 (24.520 g), ES4 (6.835 g), and ES5 (3.866 g). The third (ES3) and fourth (ES4) fractions gave interesting TLC pattern and they were subjected to further study.

The ES3 fraction was subjected to a silica gel 60 column eluted with a mixture of hexane and ethyl acetate gradient in a stepwise fashion. Thirteen fractions were collected, then combined according to their TLC patterns (using 2% methanol in chloroform as developing solvent) to give 5 fractions codenamed ES3-1 (1.568 g), ES3-2 (2.844 g), ES3-3 (2.645 g), ES3-4 (3.470 g), and ES3-5 (10.667 g). Fraction ES3-1 was separated by column chromatography using a silica gel 60 column eluted with hexane and increasing gradient of ethyl acetate. The collected fractions were combined according to their TLC pattern developed by 2% methanol in chloroform to furnish 4 fractions. The first fraction was crystallized in ethylacetate to yield 602.5 mg of compound MF-5.

The ES3-4 fraction was subjected to a silica gel 60 column eluted with mixture of chloroform and methanol in the gradient pattern. Those with the same TLC pattern (using 1% methanol in chloroform as developing solvents) were combined to yield 5 fractions. The first fraction displayed a blue fluorescent spot when observed under UV light at 325 nm. This fraction was, then separated by reverse phase gel filtration technique using a column of MCI gel and eluted with ethyl acetate. Five fractions were collected, then combined according to their TLC patterns using 2% methanol in chloroform as developing solvent to furnish 3 fractions. The third fraction (31.1 mg) showed only one fluorescent spot on TLC chromatogram that developed by various solvent systems. This fraction gave compound MF-4.

Fraction ES4 was rechromatographed over a Sephadex column to give four fractions: ES4-1 (0.135 g), ES4-2 (3.372 g), ES4-3 (1.568 g), and ES4-4 (0.784 g). The third (ES4-3) fraction exhibited only a single spot on TLC chromatogram in various developing solvent systems, established a pure compound which was assigned MF-3.

The 70% aqueous acetone extract after evaporation under reduced pressure furnished 140.2 mg of a residue and 24.66 g of a liquor part. This residue was

crystallized and recrystallized from ethyl acetate to yield colorless bulky crystals of compound MF-2 (60.4 mg).

The petroleum ether extract (58.44 g) was fractionated by a silica gel column eluted with mixture of hexane. and ethyl acetate, to give 19 fractions. They were combined, according to the same TLC pattern using 25% ethyl acetate in hexane as developing solvent, into 4 fractions. The second fraction (3.61 g) was separated by Silica gel column eluted with mixture of hexane and ethyl acetate gradient in stepwise fashion. Seven fractions were collected and combined according to their TLC pattern to give 2 combined fractions. The first fraction (1.68 g) was further chromatographed over a silica gel column eluted with gradient of hexane and ethyl acetate. Twenty one fractions were collected and combined according to their TLC pattern into five fractions, of which the second fraction (958.8 mg) was subjected to chromatotron eluted by hexane-ethyl acetate (1:1) and 6 fractions were collected. The second fraction (432.1 mg) was further purified by a column of MCI gel, eluted by ethyl acetate. Fractions were collected and combined according to their TLC pattern using hexane-ethylacetate (1:1) as developing solvent to give 3 fractions. Compound MF-6 (149.6 mg) was obtained as yellow oil from the second fraction.

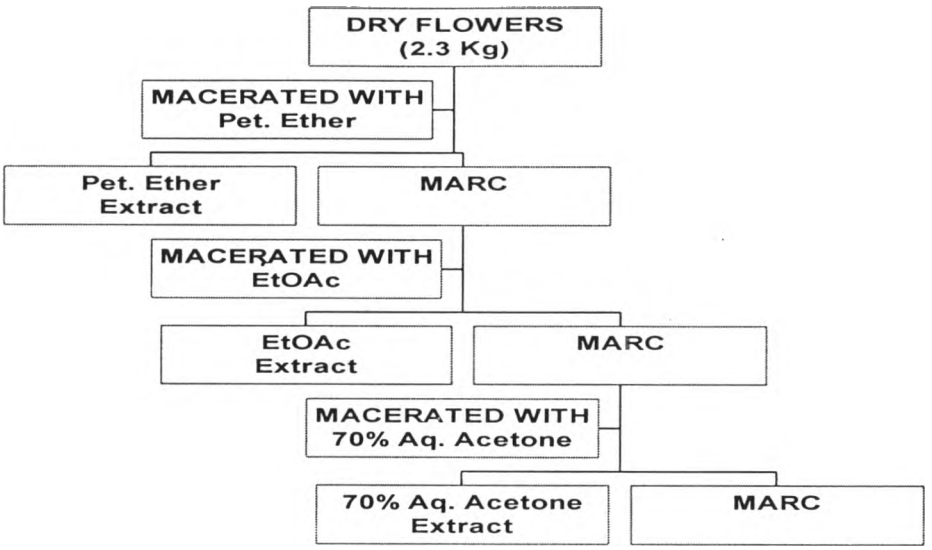

Figure 3: Extraction scheme of *Melodorum fruticosum* flowers

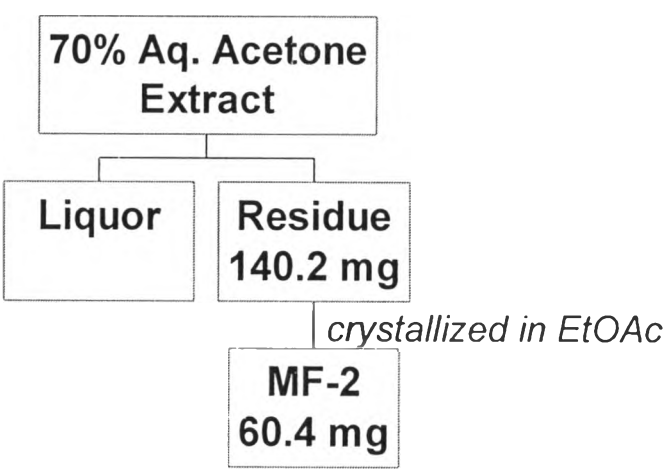

Figure 4: Isolation scheme of 70% aqueous acetone extract

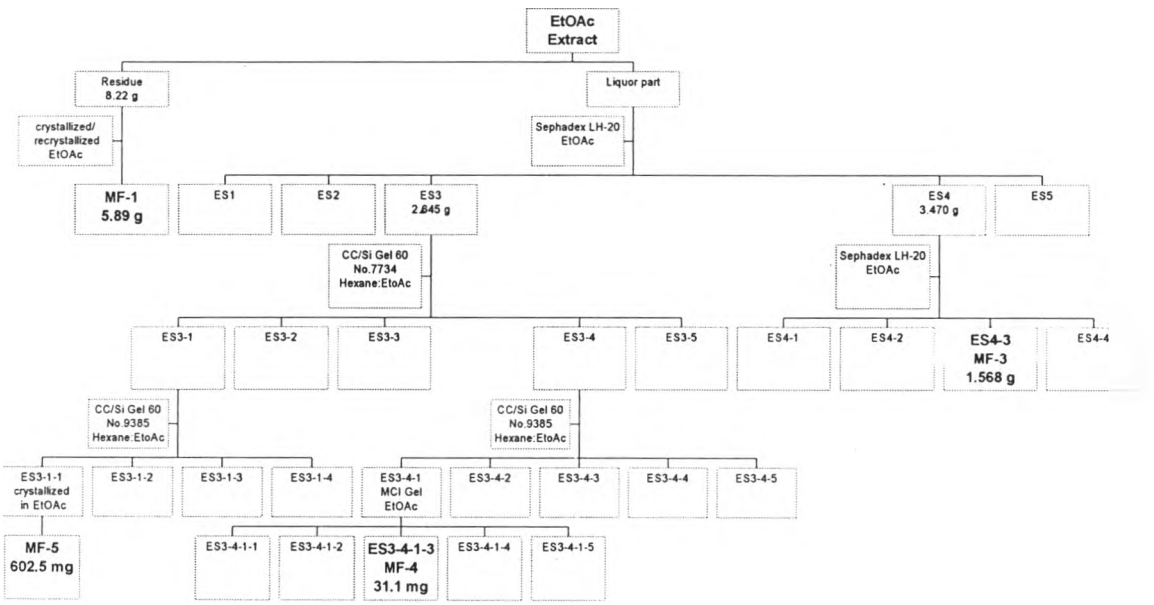

Figure 5: Isolation scheme of ethyl acetate extract

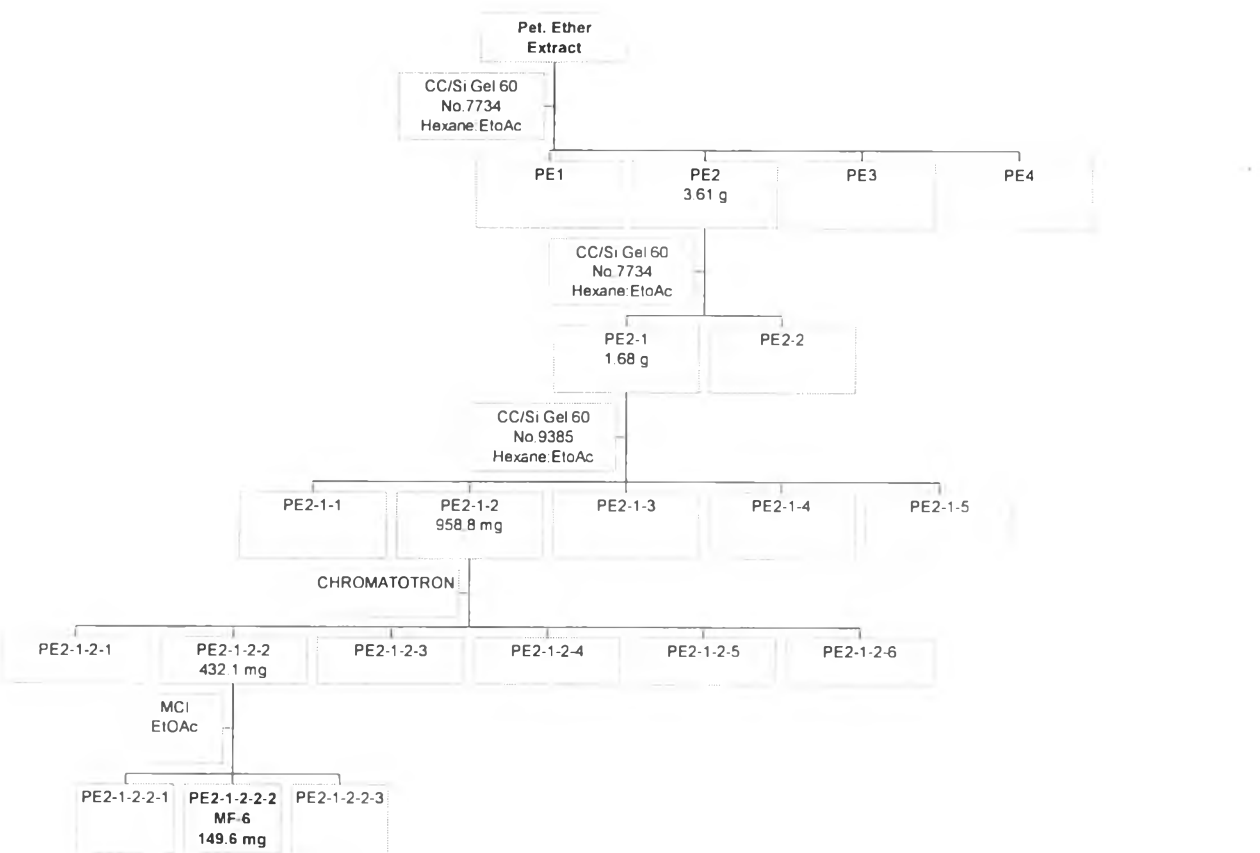

Figure 6: Isolation scheme of petroleum ether extract

Physical and Spectral Data of Isolated Compounds

1. Compound MF-1

Colorless crystals (5.89 g); mp 136-138°C; UV ($CHCl_3$) λ_{max} 283 (log ε 4.52) nm; IR ν_{max} 1790 (C=O), 1731 (C=O), 1704(C=O), 1649 (C=C) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$), see Table 2; ^{13}C NMR (75 MHz, $CDCl_3$), see Table 2; CIMS *m/z* 259 [MH^+] (0.88); EIMS *m/z* 258 [M^+] (0.34), 228 (5.41), 136 (23.54), 123 (30.99), 105 (100), 95 (13.41), 77 (62.57), 69 (16.52), 51 (35.38); anal. C 65.15%, H 3.87%, calcd for $C_{14}H_{10}O_5$, C 65.12%, H 3.88%

2. Compound MF-2

Colorless crystals (60.4 mg); mp 139-140°C; UV ($CHCl_3$) λ_{max} 292 (log ε 4.17) nm; IR ν_{max} 1785 (C=O), 1731 (C=O), 1703(C=O), 1628 (C=C) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$), see Table 4; ^{13}C NMR (75 MHz, $CDCl_3$), see Table 4; CIMS *m/z* 287 [$M+C_2H_5^+$] (0.72), 259[MH^+] (11.54); EIMS *m/z* 258 [M^+] (0.13), 228 (2.82), 136 (9.28), 123 (19.98), 105 (100), 95 (8.41), 77 (36.68), 69 (8.62), 51 (14.30); anal. C 65.11%, H 3.87%, calcd for $C_{14}H_{10}O_5$, C 65.12%, H 3.88%

3. Compound MF-3

Yellow powder (1.568 g); mp 282-283°C; UV (MeOH) λ_{max} 314 (log ε 4.03) nm, 269 (log ε 4.42) nm; IR ν_{max} 3400-2400 (OH), 1650 (C=O), 1614 (C=C) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6), see Table 5; ^{13}C NMR (75 MHz, DMSO-d_6), see Table 6; EIMS *m/z* 254 [M^+] (100), 226 (30), 152 (33), 124 (43), 113 (26), 105 (10), 102 (24), 96 (23), 77 (26), 69 (40), 66 (15), 55 (10), 51 (25), 50 (22)

4. Compound MF-4

Colorless crystals (31.1 mg); mp 130-133°C; UV ($CHCl_3$) λ_{max} 303 (log ε 4.12) nm, 264 (log ε 4.36) nm; IR ν_{max} 1649 (C=O), 1607 (C=C) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$), see Table 8; ^{13}C NMR (75 MHz, $CDCl_3$), see Table 8; EIMS *m/z* 282 [M^+] (100), 281 (65), 265 (7), 253 (54), 251 (32), 236 (72), 224 (27), 209 (51), 165 (8), 150 (28), 137 (13), 122 (30), 105 (23), 102 (29), 77 (57), 69 (68)

5. Compound MF-5

White crystalline solid (602.5 mg); mp 116-118°C; UV ($CHCl_3$) λ_{max} 317 (log ε 4.40) nm; IR ν_{max} 3500-2700 (OH), 1724 (C=O), 1658 (C=C), 1587 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$), see Table 9; ^{13}C NMR (75 MHz, $CDCl_3$), see Table 9; EIMS *m/z* 291 [MH^+] (8), 290 [M^+] (2), 273 (3), 259 (1), 231 (30), 168 (37), 155 (64), 136 (7), 126 (7), 123 (52), 113 (53), 106 (14), 105 (100), 95 (20), 85 (8), 77 (82), 69 (17), 59 (15), 55 (20), 51 (59); anal. C 62.06%, H 4.85%, calcd for $C_{15}H_{14}O_6$, C 62.07%, H 4.86%

6. Compound MF-6

Yellow oil (149.6 mg); $[\alpha]^{25}_{589}$ +21.13 (c 17.5 mg/ml, $CHCl_3$) {lit. +209 (c=1, $CHCl_3$)}; CD (c 0.62 mg/ml, MeOH) $[\theta]^{25}$ (nm) +0.13 (387.4), +0.99 (302.4), +6.32 (271.4), +0.12 (242.6), -0.02 (242.4), -7.78 (225) {lit. CD (c 0.62 mg/ml, MeOH) $[\theta]^{25}$ (nm) +0.01 (387.8), +0.99 (339.2), +1.36 (328.6), 0.00 (310.6), -1.49 (302.8), +0.04 (300.2), +17.06 (281.6)}; UV ($CHCl_3$) λ_{max} 270 (log ε 4.32) nm (lit. 287 nm in MeOH); IR ν_{max} 1781 (C=O), 1742 (C=O), 1723 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$), see Table 10; ^{13}C NMR (75 MHz, $CDCl_3$), see Table 10; EIMS *m/z* 302 [M^+] (8), 272 (13), 243 (32), 242 (39), 230 (11), 180 (77), 138 (90), 125 (82), 123 (12), 122 (21), 110 (40), 106 (49), 105 (100), 97 (60), 82 (34), 77 (94)0, 69 (48), 55 (77), 51 (76), 50 (53)