CHAPTER III

EXPERIMENTAL

1 . Source of Plant Material

The stem bark of *Garcinia atroviridis* Griff. was collected from Amphor Lungsuan, Chumphorn, Thailand in December, 1995. The plant material was authenticated by comparison with the herbarium specimen in the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Co-operative. A voucher specimen was deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn Universitsy, Bangkok, Thailand.

2 . General Techniques

2.1 Thin-layer Chromatography (TLC)

Technique : One way, ascending

Adsorbent : Silica gel 60 F_{254} (E. Merck) precoated plate

Layer thickness : 0.2 mm

Distance : 5 cm

Temperature : Laboratory temperature (30-35 ^{o}C)

Detection : 1. Ultraviolet light at the wavelength of 254 and 365 nm

: 2. 10% Sulfuric acid in ethanol

: 3. Anisaldehyde-sulfuric acid spraying reagent

(0.5% ethanol solution of anisaldehyde with 5% sulfuric acid)

2.2 Column Chromatography

2.2.1 Quick Column Chromatography

Adsorbent : Silica gel 60 (No. 9385) particle size 0.040-0.063 nm (230-400 mesh ASTM, E. Merck)

Packing method : Dry packing

Sample loading : The sample was dissolved in a small amount of organic solvent, mixed with a small quantity of adsorbent, triturated, then dried and added gently to the top of the column.

Examination of eluate : Fractions were examined by TLC observing under ultraviolet light at the wavelength of 254 and 365 nm and followed by spraying with 10% sulfuric acid in ethanol before being heated at 105 °C for 10 min. Those fractions of similar pattern were combined.

2.2.2 Flash Column Chromatography

Adsorbent : Silica gel 60 (No. 9385) particle size 0.040-0.063 nm (230-400 mesh ASTM, E. Merck)

Packing method : Dry packing

Sample loading : The sample was dissolved in a small amount of organic solvent, mixed with a small quantity of adsorbent, triturated, then dried and added gently to the top of the column.

Examination of eluate : Fractions were examined in the same manner as described in the section 2.2.1.

2.2.3 Gel Filtration Chromatography

Gel filter : Sephadex LH-20 (Pharmacia)

Packing method : Gel was suspended in the eluent and left standing to swell for 24 hours prior to use then poured into the column and was allowed to be set tightly.

Sample loading : The sample was dissolved in a small volume of eluent and loaded on the top of the column.

Examination of eluate : Fractions were examined in the same manner as described in the section 2.2.1.

2.3 Spectroscopy

2.3.1 Ultraviolet (UV) Absorption Spectrum

The spectrum was obtained on a UVIDEC-610C double-beam spectrophotometer (JASCO) (Department of Medicinal Chemistry, Faculty of Pharmacy, Meijo University, Japan).

2.3.2 Infrared (IR) Absorption Spectra

The spectra were obtained from a Perkin Elmer FT-IR spectrometer 1760x (Scientific and Technological Research Equipment Center, Chulalongkorn University) in potassium bromide disc.

2.3.3 Mass Spectra (MS)

The Electron Impact Mass Spectra (EIMS) and High Resolution Mass Spectrum (HRMS) were performed on a Hitachi M-80 spectrometer (Department of Medicinal Chemistry, Faculty of Pharmacy, Meijo University,

Japan) and a Fisons VG Trio 2000 quadrupole mass spectrometer (Department of Chemistry, Faculty of Science, Chulalongkorn University).

2.3.4 Proton and Carbon-13 Nuclear Magnetic Resonance (^{1}H and ^{13}C NMR) spectra

The 500 MHz ^{1}H-NMR and 125 MHz ^{13}C-NMR spectra were obtained by a JEOL JMN-A 500 spectrometer (Scientific and Technological Research Equipment Center, Chulalongkorn University) and the 600 MHz ^{1}H NMR and 150 MHz ^{13}C NMR were measured by a JEOL JMN-A-600 NMR spectrometer (Department of Medicinal Chemistry, Faculty of Pharmacy, Meijo University, Japan).

The solvents for NMR spectra were deuterated chloroform ($CDCl_3$) and deuterated dimethyl sulfoxide (DMSO-d_6). The chemical shifts were reported in ppm scale using the chemical shift of tetramethylsilane (TMS) at 0 ppm as the reference signal.

2.4 Solvents

Throughout this work, all organic solvents were commercial grade and had to be redistillated prior to use.

3. Extraction Procedure

The minced fresh stem bark of *Garcinia atroviridis* Griff. (12 kg) was repeatedly macerated thrice for 3-day periods with chloroform (10 lit.) and then 95% ethanol (10 lit.). The filtrates from each maceration were pooled and concentrated under reduced pressure at temperature not exceeding 45 ^{o}C. The ethanol extract was further partitioned with ethyl

acetate and water. Both ethyl acetate and water extract were separately evaporated to dryness under reduced pressure to yield 116.61 g and 213.80 g (0.97% and 1.78% based on fresh weight, respectively)

4. Isolation Procedure

4.1 The Isolation of GA-1

The chloroform extract (14.28 g) was dissolved in a small volume of the chloroform/ethanol mixture, triturated with silica gel 60 (No. 9385) and dried under reduced pressure. It was then fractionated by quick column chromatographic technique using a sintered glass filter column of silica gel (20x4cm). The eluents were used in the order as shown below:

-Hexane	3000 ml	fractions # 1-10
-Chloroform in hexane (5%)	1800 ml	fractions # 11-17
-Chloroform in hexane (10%)	1000 ml	fractions # 18-20
-Chloroform in hexane (15%)	600 ml	fractions # 21-22
-Chloroform in hexane (20%)	2000 ml	fractions # 23-31
-Chloroform in hexane (30%)	1600 ml	fractions # 32-40
-Chloroform in hexane (40%)	1200 ml	fractions # 41-46
-Chloroform in hexane (50%)	1000 ml	fractions # 47-52
-Chloroform in hexane (60%)	800 ml	fractions # 53-56
-Chloroform in hexane (70%)	2000 ml	fractions # 57-66
-Chloroform	6000 ml	fractions # 67-95
-Ethanol in chloroform (5%)	4000 ml	fractions # 96-114
-Ethanol in chloroform (10%)	2000 ml	fractions # 115-125
-Ethanol in chloroform (15%)	1200 ml	fractions # 126-133

-Ethanol in chloroform (20%)	800 ml	fractions # 134-138
-Ethanol in chloroform (30%)	600 ml	fractions # 139-142
-Ethanol in chloroform (40%)	600 ml	fractions # 143-144

Ethanol was used to wash the column until the eluates were diluted and clear compared to previous ones. The eluates were examined by TLC using 25% ethyl acetate in hexane and 5% ethanol in chloroform as developing solvents. Fractions with similar chromatographic pattern were combined.

Table 6 The combined fractions from the crude chloroform extract

Fraction	Number of Eluate	Weight (g)
F-01	1-17	1.6784
F-02	18-36	0.9640
F-03	37-50	0.9480
F-04	51-70	0.3248
F-05	71-78	0.8264
F-06	79-85	1.0879
F-07	86-139	0.9156
F-08	140-144	0.7240
F-09	Ethanol eluted	1.8204

Fraction F-04 (0.3248 g) was dissolved in a small volume of ethanol further purified by using a column of Sephadex LH-20 (2x80 cm) with ethanol as eluent. Fifteen-ml fractions were collected based on the colour band. A white precipitated was obtained during evaporation. It was

recrystallized from a mixture of chloroform and ethanol as colorless needles to yield 14 mg ($9.8x10^{-5}$ % based on fresh weight) and was designated as GA-1. This compound was identified as β-sitosterol.

4.2 The Isolation of GA-2

F-07 (0.9156 g) was dissolved in a small volume of chloroform and triturated with silica gel 60 (No. 9385) (2g). This mixture was dried under reduced pressure. It was fractionated by the column chromatographic technique using a column of silica gel (4.5x15 cm) with a gradient system of 1-50% ethanol in chloroform as an eluent. Forty-ml fractions were collected based on the color band. The eluates were examined by TLC using 5% ethanol in chloroform as developing solvent. The fractions showing the same pattern were combined. The result was shown in table 7.

Table 7 The combined fractions from F-07

Fraction	Number of Eluate	Weight (g)
FA-01	1-3	0.0224
FA-02	4-6	0.3649
FA-03	7-9	0.1043
FA-04	10-15	0.1477
FA-05	16-30	0.2224

FA-02 (0.3649 g) was dissolved in a small volume of ethanol and further purified by using a column of Sephadex LH-20 (2x80 cm) with ethanol as eluent. Fifteen-ml fractions were collected based on the color band.

Table 8 The combined fraction from FA-02

Fraction	Number of Eluate	Weight (g)
FB-01	1-5	0.0195
FB-02	6-11	0.0209
FB-03	12-15	0.0459
FB-04	16-21	0.0623
FB-05	22-28	0.0897
FB-06	29-35	0.0423

FB-05 (0.0897 g) was separated using a column of silica gel 60 (No. 9385) (2x15 cm) with a gradient system of 25-100% ethyl acetate in hexane as an eluent. Twenty-ml fractions were collected and combined after examining with TLC using 25% ethyl acetate in hexane as developing solvent. The eluates No. 8-12 showed one yellow spot on TLC. The yellow powder was crystallized from hexane to yield 25 mg (2.08×10^{-4} % based on fresh weight) and was designated as GA-2 and was identified as 5,8,12-trihydroxy-2,2-dimethyl-2H,6H-pyrano[3,2-b]xanthene-6-one

5. Characterization of the Isolated Compounds

5.1 Characterization of GA-1

GA-1 was crystallized as colorless needles from mixture of chlorofrom and ethanol. It was soluble in chloroform.

Melting point: 136-138 °C (Uncorrected)

EIMS : *m/z* (% relative intensity) ; Figure 1

414(32), 399(14), 396(17), 381(14), 329(21), 273(15), 255(22), 231(16), 213(29), 173(15), 163(22), 161(24),

159(28), 147(24), 145(39), 135(22), 133(29), 131(20), 121(23), 119(23), 109(23), 107(43), 105(37), 95(51), 83(86), 81(57), 69(100), 57(94)

IR : υ cm^{-1}, KBr disc ; Figure 2

3500-3200, 2960-2860, 1642, 1465, 1381, 1062, 840, 802

^{1}H-NMR : δ ppm, 500 MHz, in $CDCl_3$; Figures 3-5

0.67-2.3, 3.52(m), 5.34(m)

^{13}C-NMR : δ ppm, 125 MHz, in $CDCl_3$; Figures 6-8

11.87, 11.99, 18.78, 19.05, 19.40, 19.82, 21.10, 23.08, 24.31, 26.09, 28.25, 29.16, 31.67, 31.91, 33.95, 36.15, 36.51, 37.27, 39.78, 42.31, 45.84, 50.14, 56.07, 56.78, 71.80, 121.71, 140.77

5.2 Characterization of GA-2

GA-2 was obtained as yellow amorphous powder. It was soluble in ethyl acetate and DMSO-d_6.

Melting point : 257-259 °C (Uncorrected)

EIMS : *m/z* (% relative intensity) ; Figure 9

326(47), 311(100), 282(3), 189(4), 163(3), 138(13), 105(6), 83(8), 56(16)

HRMS : found 326.0781, calcd 326.0789

IR : υ cm^{-1}, KBr disc ; Figure 10

3590-3100, 1655, 1579, 1482, 1381, 1356, 1232, 1171

UV : λmax nm (logε), in methanol ; Figure 11

205(4.48), 235(4.46), 262(4.41), 288sh(4.71), 298(4.75), 356(3.80), 404(3.83)

^{1}H-NMR : δ ppm, 600 MHz, in DMSO-d_6; Figures 12-13

1.44(s), 5.76(d, *J*=10.25 Hz), 6.62(d. *J*=10.25 Hz), 7.29(dd, *J*=2.93, 9.16 Hz), 7.40(d, *J*=2.93 Hz), 7.50(d, *J*=9.16 Hz), 8.73(s), 9.99(s), 12.70(s)

^{13}C-NMR : δ ppm, 150 MHz, in DMSO-d_6; Figure 14

27.76, 77.94, 102.28, 103.48, 107.90, 114.91, 119.11, 120.13, 124.68, 125.35, 128.54, 145.10, 148.46, 148.74, 149.08, 153.91, 180.38