CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The bark of *Garcinia dulcis* (Roxb.) Kurz was collected from Ayuttaya Province, Thailand in August, 1994. The plant material was identified by Mrs. Nuchattra Chansuwanit of the Botanical Section, Division of Medicinal Plant Research and Development, Department of Medical Science, Ministry of Public Health, Nonthaburi, Thailand.

2. General Techniques

2.1 Analytical Thin-layer Chromatography

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 the wavelengths of 254 and 365 nm 2. 10% Sulphuric acid in ethanol

2.2 Preparative Thin-layer Chromatography

Technique	:	One dimension, ascending
Adsorbent	:	Silica gel 60 F_{254} (E. Merck) precoated plate
Layer thickness	:	0.2 mm
Distance	:	10 cm
Temperature	:	Laboratory temperature (30-35 °C)
Detection	:	Ultraviolet light at the wavelengths of 254 and 365 nm

2.3 Centrifugal Thin-layer Chromatography

Apparatus : Chromatotron (Model 7924 T, Harrison Research)

Adsorbent : Silica gel 6o GF_{254} (No. 7730) for thin-layer chromatography (E. Merck)

Layer thickness : 1 mm. The plates were prepared from a slurry of silica gel (50 g) and water (100 ml)

Flow rate : 2 ml/ min

Temperature : Laboratory temperature (30-35 °C)

Detection : Ultraviolet light at the wavelengths of 254 and 365 nm

2.4 Column Chromatography

2.4.1 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 and then pre-adsorbed on silica gel (equal amount), dried under the vacuum and placed gently on top of the silica gel column.

Monitoring of eluates: Fractions were monitored by TLC observing under ultraviolet light at the wavelengths of 254 and 365 nm and followed by spraying with 10% sulphuric acid in ethanol before being heated at 105 °C for 10 min. Fractions of similar chromato graphic pattern were combined.

2.4.2 Flash Column Chromatography

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

Packing method : Wet packing

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

Monitoring of eluates: Fractions were monitored in the same manner as described in section 2.4.1

2.4.3 Gel Filtration Chromatography

Gel filter : Sephadex LH-20 (Pharmacia)

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

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

Monitoring of eluates: Fractions were monitored in the same manner as described in section 2.4.1

2.5 Spectroscopy

2.5.1 Ultraviolet (UV) Absorption Spectra

UV spectra were obtained from Milton Roy Spectronic 3000 Array spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University). The samples were dissolved in methanol (Analytical Reagent Grade, J.T.Baker).

2.5.2 Infrared (IR) Absorption Spectra

IR spectra were obtained from a Perkin Elmer FT-IR 1760X spectrometer (Scientific and Technological Research Equipment Centre, Chulalongkorn University).

2.5.3 Mass Spectra (MS)

Electron Impact Mass Spectra (EIMS) of isolate GD-1and compounds GD-4, GD-5 and GD-7 were determined on a Kratos Profile spectrometer

(Chemistry Division, Department of Science Service, Ministry of Science, Technology and Energy). EI mass spectra of compounds GD-2, GD-3 and GD-6 were performed on a Finnigan MAT Incos 50 mass spectrometer (Department of Chemistry, Faculty of Science, Mahidol University).

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

500 MHz ^{1}H NMR and 125 MHz ^{13}C NMR spectra were obtained with a JEOL JMN-A500 spectrometer (the Scientific and Technological Research Equipment Centre, Chulalongkorn University).

The solvents for NMR measurement were deuterated chloroform ($CDCl_3$) , deuterated dimethylsulfoxide (DMSO-d_6) and deuterated acetone (acetone-d_6). The chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal.

2.6 Solvents

Throughout this work, all organic solvents were of commercial grade and were redistilled prior to use.

3. Extraction

The fresh bark of *Garcinia dulcis* (27 kg) was blended and macerated repeatedly with 95% ethanol for four 3-day periods. The filtrate from each maceration was pooled and evaporated to dryness *in vacuo* at temperature not exceeding 45 °C to give 2.5 kg of ethanol extract. This extract was suspended in water and then partitioned with chloroform (5 L). The chloroform fraction was concentrated *in vacuo* to yield a chloroform extract (216 g, 0.80 % based on wet weight of the bark). The aqueous fraction was further partitioned with butanol and evaporated to dryness *in vacuo* to yield a butanol extract and an aqueous extract (1655 g, 6.13 % and 617 g, 2.28 % based on wet weight of the bark, respectively).

4. Isolation

4.1 Isolation of Chemical Compounds from Chloroform Extract by Quick Column Chromatographic Technique

The chloroform extract (55 g) was dissolved in a small amount of chloroform, triturated with silica gel 60 (No. 9385) and dried under the vacuum. It was then fractionated by the quick column chromatographic technique using a sintered glass filter column of silica gel (650 g, 4 x 19.8 cm). Eluation was performed in a polarity-gradient manner with hexane, ethylacetate and methanol as the solvents.

The eluates were examined by TLC using 20 % ethylacetate in hexane and 5 % methanol in chloroform as developing solvents. Fractions with similar chromatographic pattern were combined to yield eight fractions : V-1 (0.60 g), V-2 (0.21 g), V-3 (2.26 g), V-4 (0.80 g), V-5 (1.24 g), V-6 (3.00 g), V-7 (6.70 g), V-8 (5.50 g) and V-9 (13.11 g).

4.1.1 Isolation of Isolate GD-1

Isolate GD-1 (23 mg) was obtained as colorless needles from fraction V-2 through recrystallization from methanol. The yield was 8.51×10^{-5} % based on fresh weight of the bark. The isolate was identified as a mixture of β-sitosterol and stigmasterol (139 and 140, respectively).

4.1.2 Isolation of Compound GD-2

Fraction V-3 (2.26 g) was divided into four 500-mg portions. Each was fractionated by gel filtration chromatography using a column of Sephadex LH-20 (100 g, 2 x 100 cm) with methanol as the eluent. Seven fractions, approximately 20 ml each, were collected based on the color bands. The eluates were monitored by TLC using chloroform as the developing solvent.

Fraction 7 (0.067 g), a yellow mass, was purified by preparative TLC (precoated silica gel 60 F_{254} 0.2 mm, 10 x 20 cm) developed with $CHCl_3$ (3 times) and recrystallized from acetone to give compound GD-2 as yellow needles (7 mg, 2.59 x 10^{-5}.% based on fresh weight of the bark). It was identified as 1,7-dihydroxyxanthone [10].

4.1.3 Isolation of Compound GD-3

Fraction V-4 (0.80 mg) was fractionated by gel filtration technique, using a column of Sephadex LH-20 (100g, 2 x 80 cm) with a mixture of chloroform and methanol (1:1) as the eluent. Twenty-ml fractions were collected based on the color band. The TLC chromatogram using various developing solvent systems showed only one compound. Evaporation of this fraction under reduced pressure gave 22.4 mg of compound GD-3 as golden-yellow needles (8.29 x 10^{-5} % based on fresh weight of the bark). It was identified as 12b-Hydroxy-des-D-garcigerrin A [19].

4.1.4 Isolation of Compound GD-4

Fraction V-5 (1.24 g) was equally divided into two portions. Each was fractionated on a column chromatography using silica gel 60 (4 x 20 cm). The eluent was a mixture of chloroform and methanol. Thirty fractions, approximately 30 ml each were collected. The eluates were examined by TLC using 1 % methanol in chloroform as the developing solvent. Fractions showing similar chromatographic pattern were combined.

Fractions 13-18(0.2 g) were combined and rechromatographed on a silica gel 60 (3 x 18 cm) column. The column was eluted with a mixture of chloroform and methanol in a polarity gradient manner. The eluates were collected approximately 20 ml per fraction and were examined by TLC. Fraction 10 was recrystallized from solvent to yield compound GD-4 (8 mg). This compound was identified as oleanolic acid [179].

4.2 Isolation of Chemical compounds from Chloroform Extract by Gel Filtration Technique

Crude chloroform extract (50 g) was divided into 10-g portions. Each was fractionated by gel filtration chromatography using a column of Sephadex LH-20 (100 g, 2 x 80 cm) with methanol as the eluent. The eluates were collected 50 ml per fraction and examined by TLC using 10 % methanol in chloroform as the developing solvent. Fractions with similar chromatographic pattern were combined to give three fractions namely, S-1 (21.96 g), S-2 (24.73 g) and S-3 (3.31 g).

Fraction S-3 (3.31 g) was further separated by gel filtration chromatography. The eluates were examined by TLC, and fractions giving the same chromatogaphic pattern were combined to yield seven Fractions, S-31 (1.69 g), S-32 (0.23 g), S-33 (0.88 g), S-34 (0.26 g), S-35 (0.44 g), S-36 (0.07 g) and S-37 (0.25 g).

4.2.1 Isolation of Compound GD-5

Fraction S-32 (0.2310 g) was separated by centrifugal thin-layer chromatography on a chromatotron with a silica gel plate, eluting with 2% methanol in chloroform. The eluates were collected 5 ml per fraction and monitored by TLC using chloroform : methanol (95 : 5) as the developing solvent. This gave fractions S-321 (44 mg) and S-322 (16 mg).

Fraction S-321 (44 mg) was purified by preparative TLC on a precoated silica gel 60 F_{254} (0.2 mm, 10 x 20 mm) plate developed with 5 % methanol in chloroform. A yellow band with R_f 0.25 ($CHCl_3$: MeOH 95 : 5) was scraped and extracted with a mixture of chloroform and methanol (7 : 3) to give compound GD-5 as yellow needles (9 mg, 3.3 x 10^{-5} % based on fresh weight of the bark). This compound was identified as 1-*O*-methylsymphoxanthone [92].

4.2.2 Isolation of Compound GD-6

Fraction S-33 (0.8863 g) was fractionated on a chromatotron with a silica gel plate, eluting with 2 % methanol in chloroform. Five-ml fractions were collected and combined after monitoring with TLC, using chloroform : methanol (95 : 5) as the developing solvent, to give fractions S-331 (5 mg), S-332 (140 mg) and S-333 (211 mg).

Fraction S-333 (211 mg) was separated by preparative TLC using precoated silica gel 60 F_{254} (0.2 mm, 10 x 20 cm) plate and 5 % methanol in chloroform as the developing solvent. The less polar fraction gave a yellow compound which on recrystallization from chloroform-methanol furnished compound GD-6 (29 mg) as yellow needles. The yield was 1.1×10^{-4} % based on fresh weight. This compound was subsequently identified as symphoxanthone [102].

4.2.3 Isolation of Compound GD-7

Fraction S-334 (96 mg) was purified by gel filtration chromatographic technique, using a column of Sephadex LH-20 (2 x 80 cm) with methanol as the eluent. The eluates were collected 10 ml per fraction and examined by TLC, using 5 % methanol in chloroform as the developing solvent. Fractions 1 - 6 were combined and concentrated under reduced pressure. A yellow compound was crystallized from the combined fractions. It was recrystallized from chloroform-methanol to furnish compound GD-7 (54 mg, 2.0×10^{-4} % based on fresh weight of the bark). This compound was identified as garciniaxanthone E [90].

5. Characterization of Isolated Compounds

5.1 Characterization of Isolate GD-1

Isolate GD-1 was obtained as colorless needles (23 mg). It was soluble in chloroform.

EIMS *m/z* (% relative intensity); Figure 2
414 (M^+, 11), 412 (M^+, 13), 399 (5), 396 (8), 394 (4), 381 (5), 329 (4)
273 (9), 255 (24), 231 (8), 213 (16), 173 (10), 147 (20)

IR ν cm^{-1}, film; Figure 3
3300, 2960, 1460, 1380, 1060

^{1}H NMR δ ppm, 500 MHz in $CDCl_3$; Figures 4a-4c
See Table 8

^{13}C NMR δ ppm, 125 MHz in $CDCl_3$; Figures 5a-5b
See Table 9

5.2 Characterization of Compound GD-2

Compound GD-2 was obtained as bright yellow needles from acetone (7 mg). It was soluble in chloroform.

EIMS *m/z* (% relative intensity); Figure 8
228 (M^+, 100), 200 (18), 172 (5), 171 (12), 144 (17), 115 (42),
107 (15), 89 (14), 63 (41), 53 (24)

UV λ_{max} nm, in methanol; Figure 9
385, 287, 259, 234

IR ν cm^{-1}, film; Figure 10
3500, 2960, 1630, 1460, 1220

^{1}H NMR δ ppm, 500 MHz in DMSO-d_6; Figures 11a-11b
See Table 10

^{13}C NMR δ ppm, 125 MHz in DMSO-d_6; Figure 15
See Table 10

melting point 238 - 240°C (uncorrected)

5.3 Characterization of Compound GD-3

Compound GD-3 was obtained as golden yellow needles from methanol (22.4 mg). It was soluble in chloroform and acetone.

EIMS *m/z* (% relative intensity); Figure 21

312 (M^+, 66), 297 (100), 279 (22), 271 (22), 269 (16), 257 (31), 229 (3), 189 (7), 141 (12), 137 (11), 121 (8), 107 (8), 91 (4), 77 (10), 65 (10), 53 (9)

UV λ_{max} nm, in methanol; Figure 22

406, 316, 265, 248

IR ν cm^{-1}, film; Figure 23

3300, 2960, 1585, 1460, 1290

^{1}H NMR δ ppm, 500 MHz in DMSO-d_6; Figures 24a-24b

See Table 13

^{13}C NMR δ ppm, 125 MHz in DMSO-d_6; Figure 25

See Table 13

^{1}H NMR δ ppm, 500 MHz in acetone-d_6; Figures 34a-34b

See Table 14

^{13}C NMR δ ppm, 125 MHz in acetone-d_6; Figure 35

See Table 14

^{1}H NMR δ ppm, 500 MHz in $CDCl_3$; Figures 36a-36b

See Table 15

melting point 220 - 222°C (uncorrected)

5.4 Characterization of Compound GD-4

Compound GD-4 was obtained as colorless needles (8 mg). It was soluble in chloroform.

EIMS *m/z* (% relative intensity); Figure 37

411 (1), 377 (1), 285 (1), 248 (50), 208 (3), 207 (10), 203 (41), 91 (31), 69 (56), 55 (79), 43 (100)

IR ν cm^{-1}, film ; Figure 38

3400, 2900, 1700, 1490, 1060, 790

^{1}H NMR δ ppm, 500 MHz in $CDCl_3$; Figures 39a-39c

See page 142

^{13}C NMR δ ppm, 125 MHz in $CDCl_3$; Figure 40

See Table 16

melting point 305 - 307°C (uncorrected)

5.5 Characterization of Compound GD-5

Compound GD-5 was obtained as yellow needles (9 mg). It was soluble in methanol.

EIMS *m/z* (% relative intensity); Figure 43

342 (M^+, 11), 324 (4), 295 (11), 281 (15), 267 (18), 255 (9), 239 (6), 211 (3), 181 (5), 152 (20), 115 (27), 91 (21), 77 (65), 63 (39), 53 (79), 41 (100), 31 (18)

UV λ_{max} nm, in methanol; Figure 44

257, 320

IR ν cm^{-1}, film ; Figure 45

3300 (br), 1600, 1490, 1200

^{1}H NMR δ ppm, 500 MHz in DMSO-d_6; Figures 47a-47b

See Table 18

^{13}C NMR δ ppm, 125 MHz in DMSO-d_6; Figure 51

See Table 18

5.6 Characterization of Compound GD-6

Compound GD-6 was obtained as yellow needles (29.7 mg). It was soluble in methanol.

EIMS *m/z* (% relative intensity); Figure 56

328 (86), 313 (41), 295 (100), 287 (43), 267 (65), 189 (26), 170 (27), 153 (46), 91 (31), 77 (51)

UV λ_{max} nm, in methanol; Figure 57

260, 330

IR ν cm^{-1}, film; Figure 58

3300, 1650, 1490, 1260

^{1}H NMR δ ppm, 500 MHz in DMSO-d_6; Figures 59a-59b

See Table 20

^{13}C NMR δ ppm, 125 MHz in DMSO-d_6; Figure 60

See Table 20

5.7 Characterization of Compound GD-7

Compound GD-7 was obtained as a yellow powder (54 mg). It was soluble in methanol.

EIMS *m/z* (% relative intensity); Figure 69

464 (M^+, 4), 421 (8), 395 (3), 339 (17), 325 (13), 299 (7), 273 (2), 153 (4), 109 (7), 83 (10), 69 (48), 55 (33), 44 (100)

UV λ_{max} nm, in methanol; Figure 70

255, 325

IR ν cm^{-1}, film; Figure 71

3500 (br), 1650, 1450, 1270

^{1}H NMR δ ppm, 500 MHz in DMSO-d_6; Figures 72a-72c

See Table 22

^{13}C NMR δ ppm, 125 MHz in DMSO-d_6; Figure 73

See Table 22