CHAPTER III

EXPERIMENTAL

1. Source of Plant material

The stem bark of *Polyalthia jucunda* (Pierre) Finet & Gagnep. was collected from Nakhon Ratchasima Province, Thailand, in November 2004. It was identified by comparison with herbarium specimen (N. Koonkhunthod No.121063) at the Royal Forest Department, Bangkok, Thailand.

2. Phytochemical Techniques

2.1 Chromatographic Techniques

2.1.1 Thin Layer Chromatography (TLC)

Techniques	:	one way ascending
Stationary phase	:	TLC aluminium sheet silica gel 60F 254, Layer thickness 0.2 mm
Distance	:	5 cm
Temperature	:	28-35°C (room temperature)
Detection	:	1) UV light at the wavelengths of 254 and 356 nm 2) 10% sulfuric acid in ethanol and heating at 110 °C 3) Dragendorff Reagent
Solvent	:	Various solvent systems depending on materials

2.1.2 Column Chromatography (CC)

Column	:	Flat bottom glass column (various diameter)
Stationary phase	:	Silica gel 60 (No. 9385, E. Merck) particle size 0.040-0.063 mm (230-400 mesh ASTM)
Packing method	:	Dry and wet packing
Sample loading	:	1) Dry packing: The sample was dissolved in a small amount of suitable organic solvent, mixed with a small quantity of adsorbent, triturated, dried and then loaded on top of the column. 2) Wet packing: The sample was dissolved in a small amount of the eluent, then loaded on top of the column

Solvent system : Various solvent systems depending on materials.

Detection : Fractions were examined by TLC observing under UV light at the wave-lengths of 254 and 356 nm, then the TLC plate was sprayed with 10% sulfuric acid in ethanol and heated at 110 °C or sprayed with Dragendorff's reagent. The fractions of similar TLC pattern were combined.

2.1.3 Gel filtration Chromatography

Gel filter : Sephadex ™LH-20

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 set tightly.

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

Solvent : Methanol 100%

Methanol-dichloromethane (1:1)

2.1.4 Preparative Thin Layer Chromatography (PTLC)

Stationary phase : Kieselgel 60F 254, Layer thickness 1 mm

Distance : 15 cm

Temperature : 28-35 °C (room temperature)

Detection : UV light (254 and 365 nm)

Solvent : Dichloromethane-acetone (9:1)

2.2 Spectroscopy

2.2.1 Mass Spectra (MS)

Electron impact mass spectrum (EIMS) were measured on a Hitachi Perkin Elmer-RMV-6M instrument and Fast Atom Bombardment Mass Spectrum (FABMS) (Department of Chemistry, Leicester University, UK)

2.2.2 Nuclear Magnetic Resonance (NMR) spectra

The NMR spectra were recorded at ambient temperature in $CDCl_3$, with a Bruker AMC instrument operating at 300 MHz. (Departamento de Química, Universidade de Aveiro, 4810-1933 Aveiro, Portugal) or a Bruker Avance DPX-300 NMR spectrometer (Faculty of Pharmaceutical Sciences, Chulalongkorn University). NMR solvents used in this study was deuterated chloroform ($CDCl_3$). Chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal.

2.3 Physical property measurement apparatus

2.3.1 Melting Points

Melting points were obtained on a Fisher-John Melting Point Apparatus (Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University)

2.3.2 Optical Rotations

Optical Rotations were measured on a Polax-2 L instrument (Fundação para a Ciência e Tecnologia (Unidade de I&D 226/96), POCTI (QA III), FEDER and CIIMAR Plurianual.)

2.4 Solvents

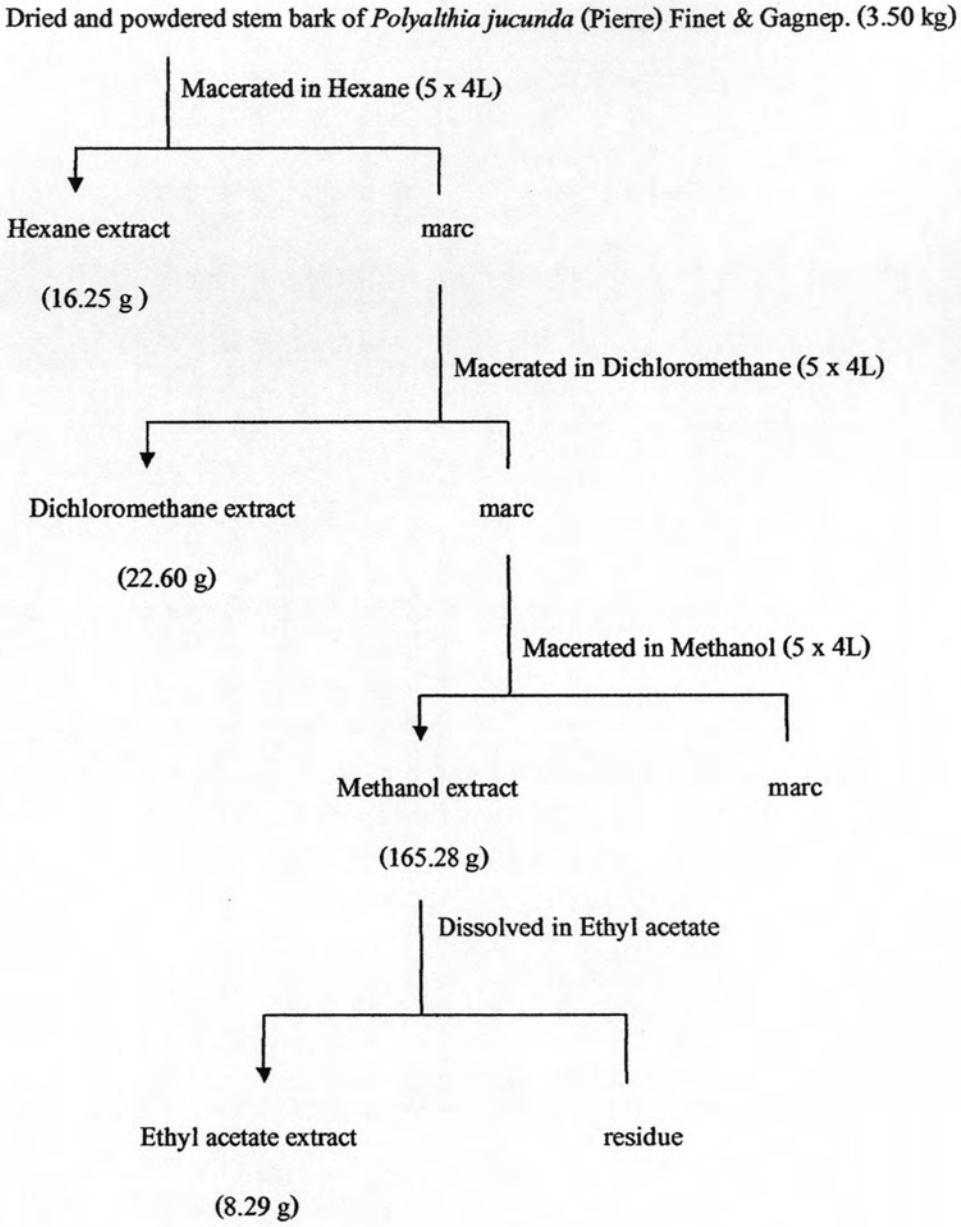
Organic solvents used in extraction were commercial grade. For column chromatography, solvents were redistilled prior to use.

3. Extraction and Isolation

3.1 Extraction of the stem bark of *Polyalthia jucunda* (Pierre) Finet & Gagnep.

Dried and powdered stem bark of *Polyalthia jucunda* (Pierre) Finet & Gagnep. (3.50 kg) was macerated by hexane (5 x 4 L) at room temperature. The hexane solution was evaporated under reduced pressure to give a hexane extract 16.25 g (0.46% of dried weight). The marc was macerated by dichloromethane (5 x 4 L) at room temperature and the CH_2Cl_2 solution was evaporated under reduced pressure to give a crude dichloromethane extract 22.60 g (0.65% of dried weight). The marc was then macerated by methanol to exhaustion (5 x 4 L) at room temperature and the methanolic solution was evaporated under reduced pressure to give a crude methanol extract 165.28 g (4.72% of dried weight), which was then dissolved in ethyl acetate (5 x 4 L). The ethyl acetate solutions were combined and evaporated under reduced pressure to give an ethyl acetate extract 8.29 g (0.24% of dried weight). These extracts were subjected to column chromatography for future purification.

Extraction of stem bark of *Polyalthia jucunda* (Pierre) Finet & Gagnep. is summerized in Scheme 1.



Scheme 1. Extraction of the stem bark of *Polyalthia jucunda* (Pierre) Finet & Gagnep.

3.2 Isolation

3.2.1 Fractionation of the dichloromethane extract

The dichloromethane extract (22.60 g) was applied to a silica gel column (300 g, 5 x 40 cm) and eluted with pet. ether-CH_2Cl_2, CH_2Cl_2 and CH_2Cl_2-MeOH, with 250 ml fractions being collected as follows: Fractions 1-22 (pet. ether-CH_2Cl_2, 1:1), 23-82 (pet. ether -CH_2Cl_2, 3:7), 83-112 (pet. ether - CH_2Cl_2, 1:9), 113-143 (CH_2Cl_2), 144-189 (CH_2Cl_2-MeOH, 19:1), 190-208 (CH_2Cl_2-MeOH, 9.3:0.7), 209-239 (CH_2Cl_2-MeOH, 9:1). Two hundred and thirty-nine fractions (250 ml each) were collected and combined according to their TLC pattern into twenty-one major fractions (C1-C21) as shown in Table 3. Finally, the column was washed down with methanol to give fraction C22

Table 3. Combined fractions from the dichloromethane extract.

Fractions	Number of eluates	Weight (g)
C1	1-7	3.63
C2	8-9	0.09
C3	10-15	0.49
C4	16-22	0.43
C5	23-24	0.13
C6	25-31	0.55
C7	32-42	0.73
C8	43-55	0.34
C9	56-76	0.28
C10	77-82	0.15
C11	83-91	0.17
C12	92-98	0.15
C13	99-112	0.10
C14	113-143	0.42
C15	144-185	0.15
C16	186-189	7.21
C17	190-195	1.54
C18	196-197	0.19
C19	198-208	0.57
C20	209-231	0.97

Table 3. Combined fractions from the dichloromethane extract. (continued)

Fractions	Number of eluates	Weight (g)
C21	232-239	1.25
C22	methanol eluate	3.01

3.2.1.1 Isolation of compound PJ1

Fraction C14 (0.42 g) was applied to a silica gel column chromatography (20 g, 1 x 20 cm) and eluted with pet. ether-CH_2Cl_2, CH_2Cl_2 and CH_2Cl_2-MeOH, with 5 ml subfractions being collected as follows: Subfractions 1-112 (pet. ether -CH_2Cl_2, 1:4), 113-209 (pet. ether -CH_2Cl_2, 1:9), 210-243 (CH_2Cl_2), 244-311 (CH_2Cl_2-MeOH, 9.9:0.1). Three hundred and eleven subfractions (5 ml each) were collected and combined according to their TLC pattern into eleven major fractions (C14.1-C14.11) as shown in Table 4. Finally, the column was washed down with methanol to give fraction C14.12

Table 4. Combined fractions from fraction C14

Fractions	Number of eluates	Weight (mg)
C14.1	1-11	22.30
C14.2	12-21	8.90
C14.3	22-30	7.00
C14.4	31-54	5.10
C14.5	55-112	15.80
C14.6	113-185	15.40
C14.7	186-209	9.90
C14.8	210-226	7.80
C14.9	227-243	14.10
C14.10	244-306	61.20
C14.11	307-311	121.30
C14.12	methanol eluate	129.70

Subfractions C14.10 (61.20 mg) was applied to a Sephadex LH-20 column (1 x 40 cm) and eluted with CH_2Cl_2-MeOH (1:1) . Thirty-three subfractions (3 ml each) were collected and combined according to their TLC pattern into three major subfractions (C14.10.1-C14.10.3) as shown in Table 5.

Table 5. Combined subfractions from fraction C14.10

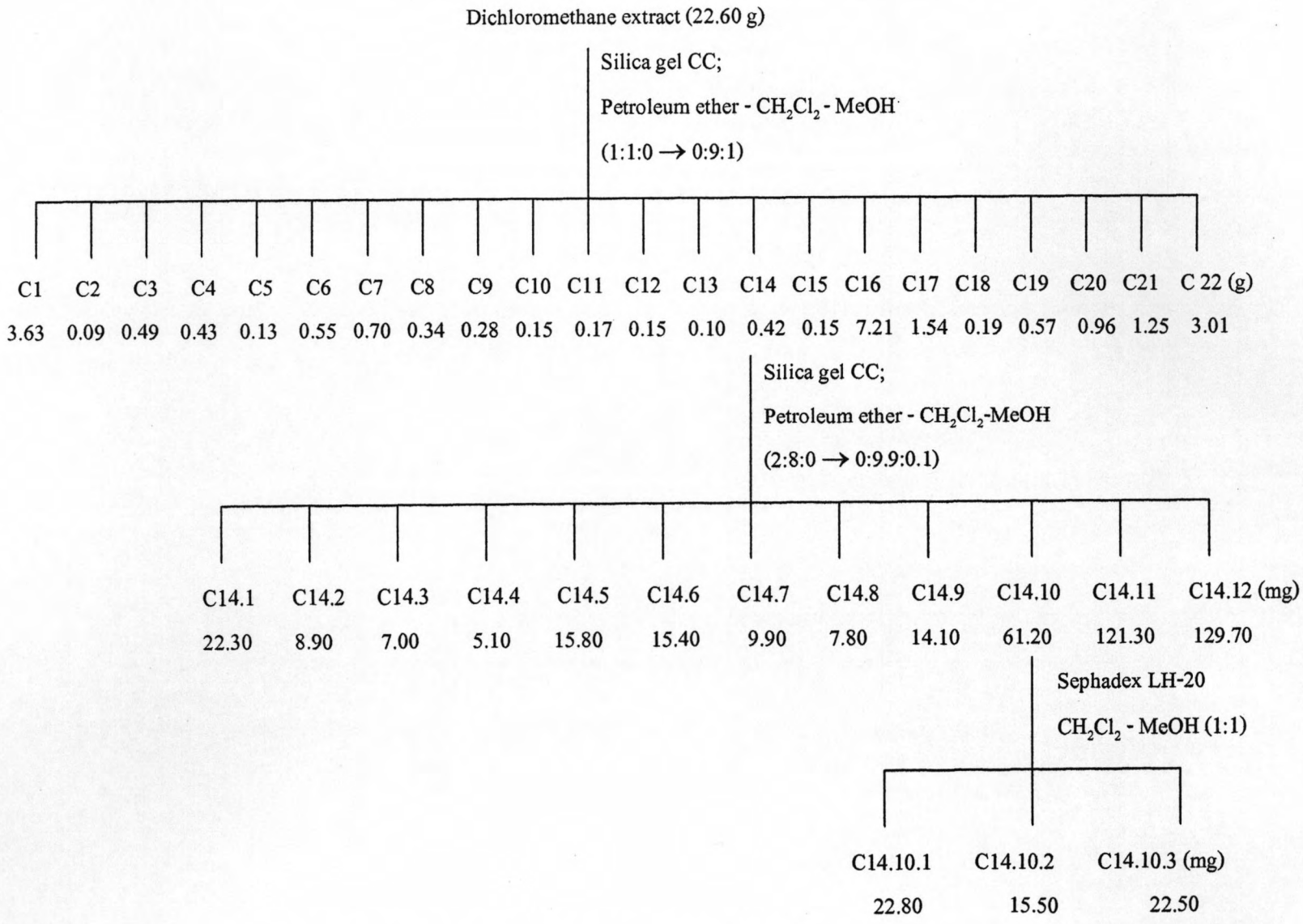
Fractions	Number of eluates	Weight (mg)
C14.10.1	1-12	22.80
C14.10.2	13-16	15.50
C14.10.3	17-33	22.50

Subfractions C14.10.3 (22.50 mg) was applied to a Sephadex LH-20 column (1 x 40 cm) and eluted with CH_2Cl_2-MeOH (1:1). Seventy subfractions (1 ml each) were collected and combined according to their TLC pattern into three major subfractions (C14.10.3.1-C14.10.3.3) as shown in Table 6.

Table 6. Combined subfractions from fraction C14.10.3

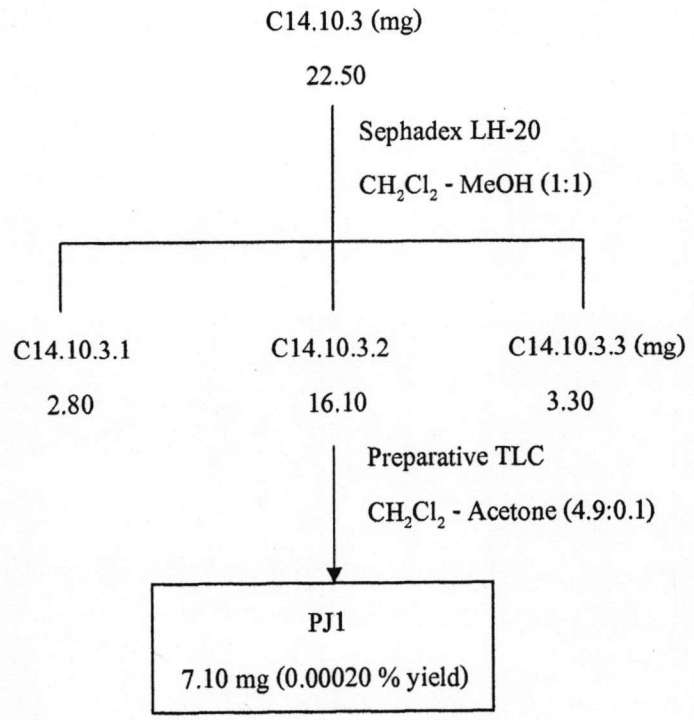
Fractions	Number of eluates	Weight (mg)
C14.10.3.1	1-30	2.80
C14.10.3.2	31-51	16.10
C14.10.3.3	52-70	3.30

Subfractions C14.10.3.2 (16.10 mg) displayed one major spot (R_f = 0.48) and one minor spot (R_f = 0.42) on TLC plate when detected under UV light. Purification of this subfraction by preparative TLC (Silica gel, CH_2Cl_2-acetone, 4.9:0.1) gave **compound PJ1** as whitish amorphous powder (7.10 mg, 0.00020 % yield) from the major spot.



Scheme 2a. Fractionation of the dichloromethane extract.

Dichloromethane extract (continued)



Scheme 2b. Fractionation of the dichloromethane extract. (continued)

3.2.2 Fractionation of the ethyl acetate extract

The ethyl acetate extract (8.29 g) was applied to a silica gel column (250 g, 5 x 40 cm) and eluted with CH_2Cl_2, CH_2Cl_2-acetone, acetone and acetone-MeOH, with 200 ml fractions being collected as follows: Fractions 1-36 (CH_2Cl_2), 37-62 (CH_2Cl_2-acetone, 19:1), 63-72 (CH_2Cl_2-acetone, 9:1), 73-93 (CH_2Cl_2-acetone, 7:3), 94-110 (CH_2Cl_2-acetone, 1:1), 111-139 (CH_2Cl_2-acetone, 3:7), 140-156 (CH_2Cl_2-acetone, 1:9), 157-179 (acetone), 180-204 (acetone-MeOH, 4:1), 205-213 (acetone-MeOH, 3:2). Two hundred and thirteen fractions (200 ml each) were collected and combined according to their TLC pattern into eighteen major fractions (EA1-EA18) as shown in Table 7. Finally, the column was washed down with methanol to give fraction EA19

Table 7. Combined fractions from the ethyl acetate extract.

Fractions	Number of eluates	Weight (g)
EA1	1-6	0.23
EA2	7-10	0.04
EA3	11-14	0.16
EA4	15-19	0.10
EA5	20-36	0.16
EA6	37-55	0.11
EA7	56-62	0.10
EA8	63-65	0.07
EA9	66-72	0.10
EA10	73-81	0.11
EA11	82-93	0.14
EA12	94-110	0.28
EA13	111-139	0.62
EA14	140-147	0.11
EA15	148-156	0.18
EA16	157-179	0.85
EA17	180-204	1.92
EA18	205-213	0.20
EA19	methanol eluate	2.75

3.2.2.1 Isolation of compound PJ2

Fraction EA10 (0.11 g) was applied to a silica gel column (20 g, 1 x 20 cm) and eluted with CH_2Cl_2-acetone (9:1). Forty-eight subfractions (5 ml each) were collected and combined according to their TLC pattern into three major subfractions (EA10.1-EA10.3) as shown in Table 8. Finally, the column was washed down with methanol to give fraction EA10.4

Table 8. Combined fractions from fraction EA10

Fractions	Number of eluates	Weight (mg)
EA10.1	1-3	2.60
EA10.2	4-10	10.70
EA10.3	11-48	80.10
EA10.4	methanol eluate	11.30

Subfractions EA10.3 (80.10 mg) was applied to a Sephadex LH-20 column (1 x 40 cm) and eluted with CH_2Cl_2-MeOH (1:1). Twenty-five subfractions (3 ml each) were collected and combined according to their TLC pattern into six major subfractions (EA10.3.1-EA10.3.6) as shown in Table 9.

Table 9. Combined fractions from subfraction EA10.3

Fractions	Number of eluates	Weight (mg)
EA10.3.1	1-4	4.10
EA10.3.2	5-8	10.60
EA10.3.3	9-13	35.00
EA10.3.4	14-20	27.10
EA10.3.5	21-22	1.90
EA10.3.6	23-25	1.10

Subfractions EA10.3.3 (35.00 mg) displayed one major spot (R_f = 0.47) and one minor spot (R_f = 0.41) on TLC plate when detected under UV light. Purification of this subfraction by preparative TLC (Silica gel, CH_2Cl_2-acetone, 4.5:0.5) gave **compound PJ2** as pale yellow amorphous powder (6.40 mg, 0.00018 % yield) from the major spot.

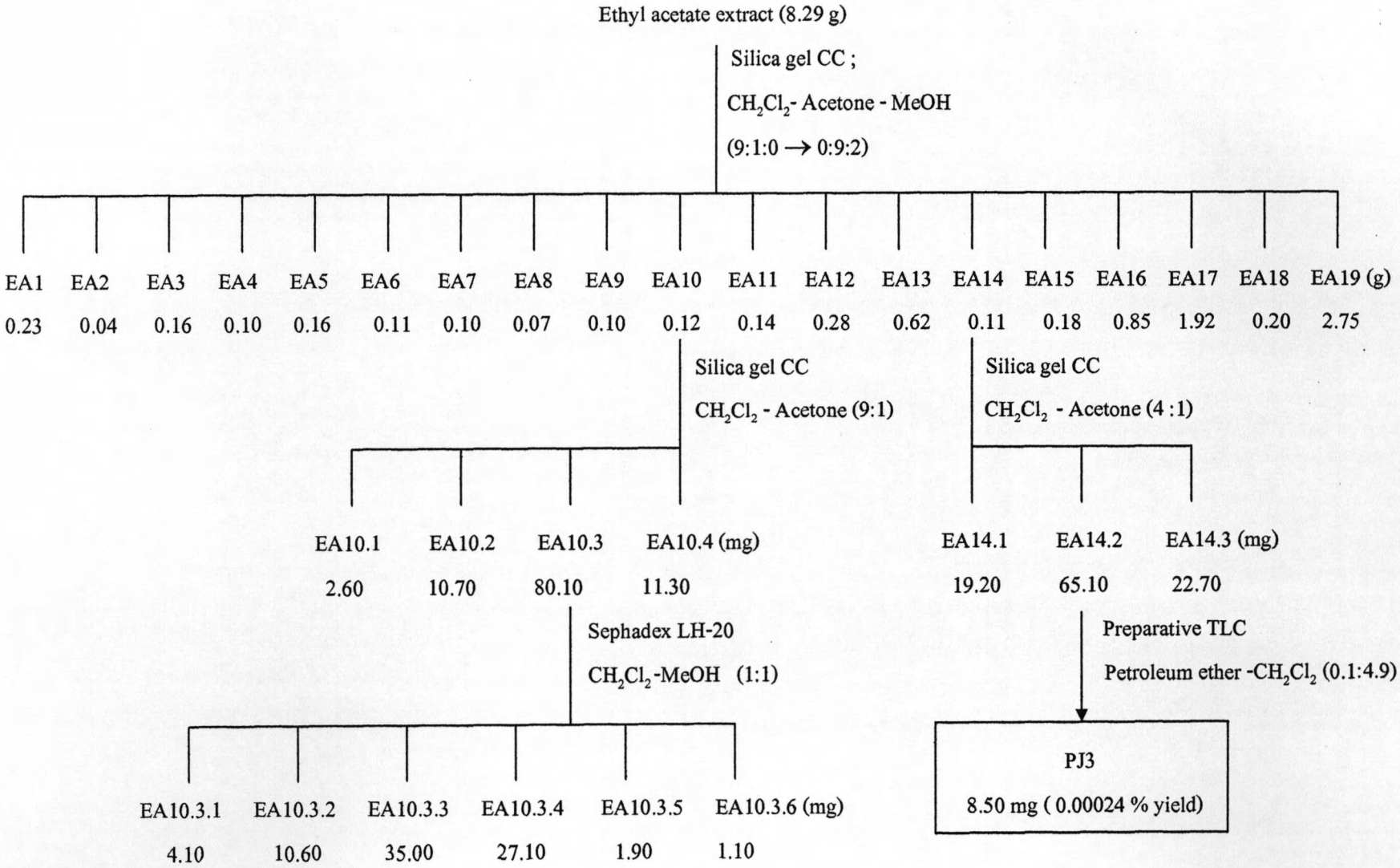
3.2.2.2 Isolation of compound PJ3

Fraction EA14 (0.11 g) was applied to a silica gel column (20 g, 1 x 20 cm) and eluted with CH_2Cl_2-acetone (4 :1). Twenty-one subfractions (5 ml, each) were collected and combined according to their TLC pattern into two major subfractions (EA14.1-EA14.2) as shown in Table 10. Finally, the column was washed down with methanol to give fraction EA14.3

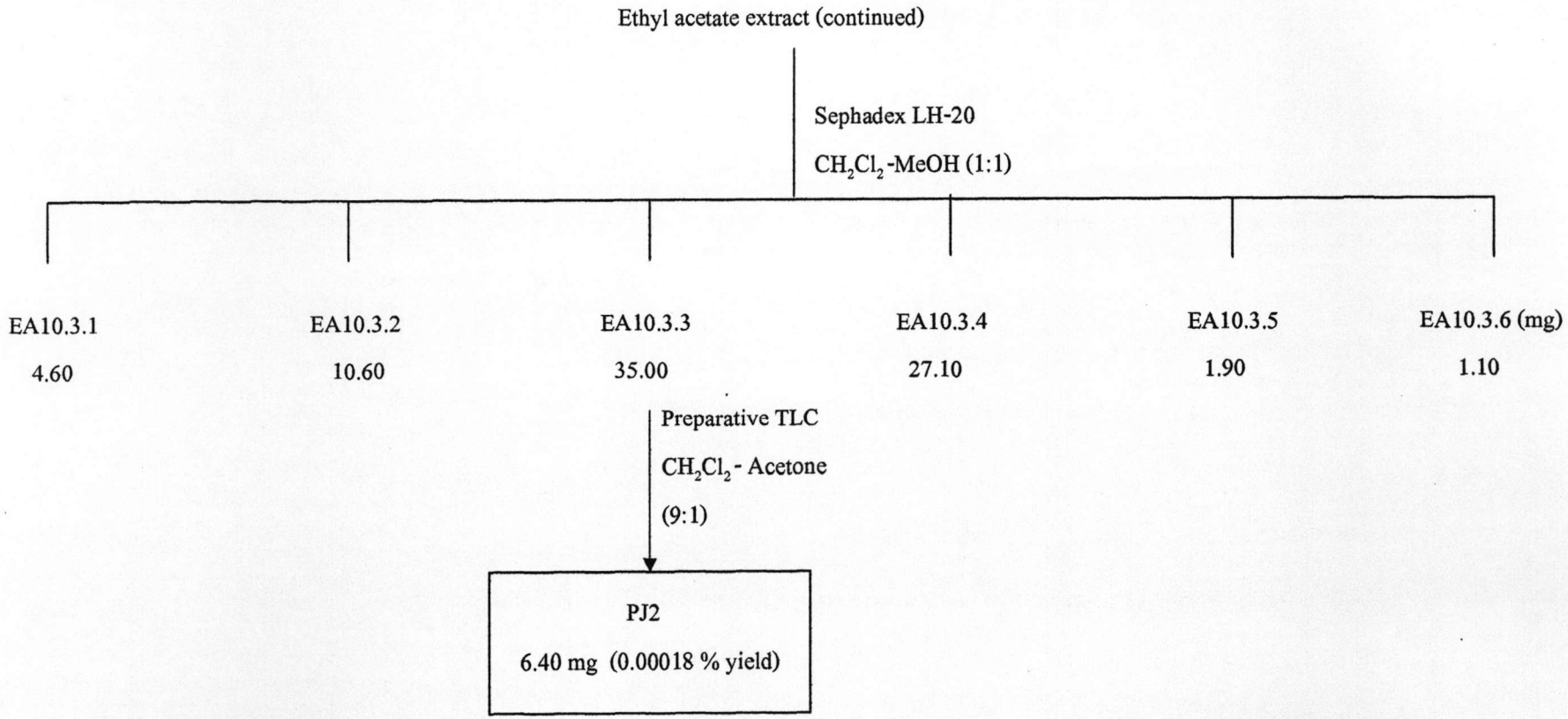
Table 10. Combined fractions from fraction EA14

Fractions	Number of eluates	Weight (mg)
EA14.1	1-8	19.20
EA14.2	9-21	65.10
EA14.3	methanol eluate	22.70

Subfractions EA14.2 (65.10 mg) displayed one major spot (R_f = 0.49) and one minor spot (R_f = 0.57) on TLC plate when detected under UV light. Purification of this subfraction by preparative TLC (Silica gel, CH_2Cl_2- petrol, 4.9:0.1) gave **compound PJ3** as white powder (8.50 mg, 0.00024 % yield) from the major spot.



Scheme 3a. Fractionation of the ethyl acetate extract.



Scheme 3b. Fractionation of the ethyl acetate extract. (continued)

3.3 Characterization of isolated compounds

3.3.1. Compound PJ1

Appearance : Whitish amorphous powder

Solubility : Soluble in chloroform

Melting Point : 132-134 °C

EIMS *m/z* (% relative intensity) : 190 (M^+, 48), 175 (100), 162 (39), 119 (30)

$[\alpha]_D^{24}$: +100.2 ° ($CDCl_3$, c = 0.02 g/100 ml)

^{1}H-NMR (δ ppm, 300 MHz, $CDCl_3$) (Figures 4a-4c, Table 11)

^{13}C-NMR (δ ppm, 300 MHz, $CDCl_3$) (Figures 5a-5b, Table 11)

3.3.2. Compound PJ2

Appearance : Pale yellow amorphous powder

Solubility : Soluble in chloroform

Melting Point : 122-124 °C

EIMS *m/z* (% relative intensity) : 226 (M^+, 40), 208 (22), 177 (65), 128 (62), 109 (57), 71 (100)

$[\alpha]_D^{24}$: +12.6° ($CDCl_3$, c = 0.33 g/100 ml).

^{1}H-NMR (δ ppm, 300 MHz, $CDCl_3$) (Figures 10a-10b, Table 12)

^{13}C-NMR (δ ppm, 300 MHz, $CDCl_3$) (Figures 11a-11b, Table 12)

3.3.3. Compound PJ3

Appearance : White powder

Solubility : Soluble in chloroform

Melting Point : 142-144 °C

FABMS *m/z* (%) : 454 (M^+, 100), 437 (50), 367, 327

$[\alpha]_D^{24}$: +56° ($CDCl_3$, c = 0.27 g/100 ml).

^{1}H-NMR (δ ppm, 300 MHz, $CDCl_3$) (Figures 18a-18c, Table 13)

^{13}C-NMR (δ ppm, 300 MHz, $CDCl_3$) (Figures 19a-19b, Table 13)

4. Cytotoxic activity

4.1 Material and Methods

4.1.1 Samples

Stock solutions of compounds were prepared in DMSO (Dimethylsulfoxide) (Sigma Chemical Co.) and stored at -20 °C. The frozen samples were freshly diluted with culture medium prior to the assays. Final concentrations of DMSO (0.25%) did not interfere with the growth of cell lines.

4.1.2 Cell growth assay

The effects of compounds on the growth of the human tumor and non-tumor cell lines were evaluated according to the procedure adopted by the National Cancer Institute (NCI, USA) for the *in vitro* anticancer drug discovery screen which uses the protein-binding dye sulforhodamine B (SRB) (Sigma Chemical Co.) to assess cell growth inhibition (Skehan *et al*, 1990, Monks *et al.*, 1991). Four human tumor cell lines were used, namely the estrogen-dependent ER(+) MCF-7 (breast adenocarcinoma), the estrogen-independent ER(-) MDA-MB-231 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and SF-268 (CNS cancer). The non-tumor cell line MRC-5 (human fetal lung) was also used. NCI-H460 and SF-268 cell lines were provided by the National Cancer Institute (NCI, Bethesda, U.S.A.) and MCF-7, MDA-MB-231 and MRC-5 cell lines were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, U.K.). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium (Gibco BRL) supplemented with 5% heat-inactivated fetal bovine serum (Gibco BRL) for MCF-7 and 10% for MDA-MB-231 and MRC-5, 2 mM glutamine (Sigma Chemical Co), 100 U/mL penicillin (Gibco BRL), 100μg/mL streptomycin (Gibco BRL) and trypsin (Gibco BRL) at 37°C in humidified atmosphere containing 5% CO_2. The optimal plating density of each cell line that ensure exponential growth throughout all the experimental period was the same as originally published (Monks *et al*, 1991) and was respectively $1.5x10^5$ cells/ml to MCF-7, MDA-MB-231, MRC-5 and SF-268 and $7.5x10^4$ cells/ml to NCI-H460. Cells in 96-well plates were allowed to attach overnight and then exposed for 48 hr. to five concentrations of compounds, starting from a maximum concentration of 150 mM. Following this incubation period the adherent cells were fixed in situ, washed and dyed with SRB. The bound stain was solubilized and the absorbance was measured at 492 nm in a plate reader (EAR 400, STL-Lab instruments). For each compound tested a dose-response curve was generated and the growth, inhibition of 50% (GI_{50}), corresponding to the concentration of compound that inhibits 50% of the net cell growth was determined as described (Monks *et al*, 1991). Doxorubicin (Sigma Chemical Co.), used as a positive control, was tested in the same manner.