CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The dry leaves of *Blumea balsamifera* DC. were purchased from a local herbal drug shop in Bangkok, Thailand, in May 1980. The plant materials were authenticated by comparison with herbarium specimens at the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand.

2. General Techniques

2.1 Thin Layer Chromatography (TLC)

Analytical

Technique : one way, ascending.

Adsorbent : silica gel G (E. Merck), calcium sulphate binder 13% ; 30 g / 60 ml of distilled water.

Plate size : 20 cm x 20 cm, 10 cm x 20 cm, 5 cm x 20 cm.

Layer thickness : 0.25 mm

Activation : air dried for 15 minutes and then heat at 105°C for 1 hour.

Solvent systems : a) chloroform : acetone (9:1)

b) anaesthetic ether

c) acetone : ethyl acetate (3:7)

Distance : 15 cm.

Laboratory temperature : 25-30°C.

Detection : a) The spots gave yellow fluorescense in ultraviolet light at 365 nm.

b) The spots gave red colourwith Vanillin-Sulphuric acid reagent after heating at 110°C for 5 minutes.

2.2 Column Chromatography (CC)

Adsorbent : silica gel 0.040-0.063 mm (E. Merck).

Packing : adsorbent packed dry into the column.

2.3 Melting Point

Melting points were determined by using a Kofler hot plate and were uncorrected.

2.4 Optical Rotation

Optical rotations were obtained in methanol with a Perkin Elmer model 241 polarimeter.

2.5 Circular Dichroism Spectra (CD)

CD spectra were obtained in methanol with a Jasco model J-40A automatic recording spectropolarimeter.

2.6 Ultraviolet Absorption Spectra

Ultraviolet absorption spectra were obtained with a Beckman model DB-G spectrophotometer.

2.7 Infrared Absorption Spectra

Infrared absorption spectra were determined with a Perkin Elmer model 283 spectrophotometer; absorption bands are reported in wave numbers (cm^{-1}).

2.8 Nuclear Magnetic Resonance (NMR) Spectra

The ^{1}H-NMR spectra were recorded with a Varian T-60A instrument operating at 60 MHz, with a Nicolet Model TT-7 Fourier Transform attachment. Tetramethylsilane was used as an internal standard and chemical shifts were reported in (ppm).

2.9 Mass Spectra

Mass spectra were obtained with a Varian MAT-112 S double-focusing spectrometer operating at 80 eV and 220

3. Isolation of Chamical Substances from Blumea balsamifera Leaves

3.1 Extraction

The dry pulverised leaves (4.5 kg) were macerated twice for 5 day-period each, with 95% ethanol (20 and 15L). The ethanol extracts were pooled, the alcohol removed under reduced pressure, and the residue suspended in 3 litres of 10% ethanol. After filtration, the filtrate was treated with a 10% aqueous lead acetate solution until no further precipitation occurred. Further filtration then afforded a clear solution which was extracted with chloroform (3 x 7 L.). The combined chloroform extract was dried (anhydrous $Na_2 SO_4$), evaporated under reduced pressure to yield 13.5 g of a brown syrupy mass.

3.2 Isolation of Chemical Compounds

The brown syrupy mass (13.5 g) was stirred with chloroform (90 ml) produced a pale yellow precipitate. After filtration and drying, a pale yellow precipitate, fraction A (977 mg) was obtained. The filtrate was evaporated under reduced pressure yielding a yellow syrupy mass, fraction B (12 g). No further study was carried on this fraction.

Seperation of fraction A

Thin layer chromatography of fraction A (silica gel G, chloroform: acetone (9 : 1)) indicated the presence of only two components (hRf = 17.3 and 35.5). The total fraction was divided into four portions, each portion was dissolved in chloroform (2 ml), adsorbed onto silica gel (5 g), dried, then placed on top of a dry silica gel column (2.5 x 40 cm). Elution with chloroform : acetone (9 : 1), collection of 20 ml fractions, comparison of fractions by thin layer chromatography, and combination of those having similar patterns yielded 3 major fractions as follows :-

1. Fractions 1-5 yielded no residue on evaporation.
2. Fractions 6-10 were homogeneous by thin layer chromatography. They were combined and evaporated to dryness under reduced pressure yielded a light yellow amorphous powder PS-2 (977 mg).
3. Fractions 11-19 were treated as fractions 6-10 and yielded a light yellow amorphous powder PS-1 (94 mg).

4. Characterisation of PS-1 and PS-2

PS-1 and PS-2 were characterised by studies on colour reactions, melting points, ultraviolet, infrared, nuclear magnetic resonance and mass

spectra. The hRf values given are those obtained with the following solvent systems :-

a) silica gel G / chloroform : acetone ((9 : 1)

b) silica gel G / anaesthetic ether

c) silica gel G / acetone : ethyl acetate (3 : 7)

4.1 Characterisation of PS-1

PS-1 was obtained as light yellow amorphous powder, soluble in ethanol and in acetone, moderately soluble in chloroform and anaesthetic ether and insoluble in petroleum ether.

hRf values

a) 17.3 (Fig. XVIII) b) 56.6 (Fig. XIX) c) 60 (Fig. XX)

Colour reaction

PS-1 gave pink colour with "Shinoda test" (magnesium-hydrochroric acid reaction), and violet colour with 1% ferric chloride solution.

Melting point

173-174°C.

Optical rotation

$[\alpha]_D^{24} = + 14.9°$

Circuler dichroism (MeOH)

$[\theta]_{328} = + 3.35 \times 10^5$ deg. cm^2 / d mole

$[\theta]_{293.5} = - 1.31 \times 10^6$ deg. cm^2 / d mole

$[\theta]_{253.5}$ = + 1.95 x 10^5 deg. cm^2 / d mole

$[\theta]_{220}$ = + 1.44 x 10^6 deg. cm^2 / d mole

(Fig. XXI)

Molecular weight

318 (mass spectrometry)

Ultraviolet absorption spectra

MeOH λ (nm)	327 sh	(log ε = 3.89)	indicates characteristic of
	290	(4.48)	flavonoid nucleus.
	235 sh	(4.54)	
	206	(4.82)	
	(Fig. XXIII)		
MeOH+$NaOCH_3$	327	(4.62)	indicates free phenolic OH.
	290 sh	(4.03)	
	250 sh	(4.09)	
	206	(4.82)	
	(Fig. XXV)		
MeOH+NaOAc	327	(4.58)	indicates presence of free OH
	294 sh	(4.19)	at 7 position of flavonoid nucleus.
	(Fig. XXIV)		
MeOH+NaOAc + H_3BO_3	327 sh	(3.89)	lacks of change, indicates
	290	(4.48)	absence of 6,7 di-OH.
	(Fig. XXIV)		

MeOH+$AlCl_3$ λ (nm)	381	(3.92)	indicates the presence of a free OH
	315	(4.55)	at the 5-position.
	282	(4.11)	
	223	(4.64)	
	206	(4.76)	
	(Fig. XXVI)		
MeOH+$AlCl_3$+HCl	377	(4.01)	persistence of shift in presence of
	315	(4.55)	HCl indicates lack of any *ortho* di-OH.
	282	(4.25)	
	223	(4.64)	
	206	(4.77)	
	(Fig. XXVI)		

Infrared absorption spectrum (Potassium bromide)

ν_{max}	3400 cm^{-1}	a polymeric OH stretching vibration.
	2950 cm^{-1}	absorption due to CH_3 group.
	1645 cm^{-1}	the characteristic of the stretching vibration of a γ-pyrone-$\overset{O}{\overset{\|}{C}}$-.
	1600 cm^{-1}	aromatic double bonds.
	1360 cm^{-1}	phenolic hydroxyl group.
	1160 cm^{-1}	*meta* hydroxy substitution; 5,7-di-OH.
	(Fig. XXXI)	

NMR spectra

In deuterodimethyl sulfoxide (DMSO-d_6) at 60 MHz in δ value (ppm) from tetramethylsilane (T.M.S.)

Chemical shift (δ)	Proton	Multiplicity	Coupling Constants
3.78	3H ($Ar-OCH_3$(4'))	s	
4.48	1H (3)	d	J = 11 Hz
3.81	3H (3OH at (3)(5)(7)	m	
5.03	1H (2)	d	J = 11 Hz
5.87	2H (6)(8)	dd (AB system)	
6.91	3H (2')(5')(6')	s	
11.88	1H (OH at (7))	bs	

(Fig. XXXIII)

In deuteroacetone at 60 MHz in δ value (ppm) from T.M.S.

Chemical shift (δ)	Proton	Multiplicity	Coupling Constants
3.88	3H ($Ar-OCH_3$(4'))	s	
4.61	1H (3)	d	J = 11.5 Hz
5.08	1H (2)	d	J = 11.5 Hz
5.98	2H (6)(8)	dd (AB system)	
7.00-7.10	3H (2')(5')(6')	m	
11.68	1H (OH (7))	bs	

(Fig. XXXIV)

Protons are are identified by the labelling scheme shown for the structure (Figure XIV). Integrated signal areas are in accordance with the number of protons assigned to them. Coupling constants derived from observed signal multiplicifies are reported.

Mass spactrum

m/e 318 (M^+, 37%, $C_{16}H_{14}O_7$), 289 (44), 166 (50), 165 (26), 164 (36), 153 (100), and 137 (39). (Fig. XXXVII)

Oxidation of PS-1

A sample (15 mg) of PS-1 was suspended in 2N H_2SO_4 (5 ml) and heated on a steam bath under a gentle stream of air for 24 hours. After cooling to room temperature and extraction with ethyl acetate (4 x 5 ml), partition was effected against a saturated aqueous solution of sodium bicarbonate to remove residual acid, and the organic phase was dried. (anhydrous Na_2SO_4) and evaporated under reduced pressure to yield 14.2 mg (95%) of a dark yellow, waxy solid, tamarixetin; ultraviolet absorption spectra, λ_{max} (MeOH) 369, 292, 270 sh, 255 and 205 nm; λ_{max} (MeOH + $NaOCH_3$), 388, 326, 277, 205 nm, with no change for at least 24 hours.

Trimethylsilylation of tamarixetin: A sample (14 mg) of the oxidation product, tamarixetin, was treated with TRI-SIL (3 ml) for 15 minutes. Solvent and excess reagent were removed under reduced pressure (oil pump) at room temperature to yield 22 mg (82%) of a crude product which displayed ^{1}H-NMR (CCl_4) , 3.87 (3H, s, $ArOCH_3$), 6.13 (1H, d, J = 2 Hz, 6-H), 6.29 (1H, d, J = 2 Hz, 8-H), 6.84 (1H, d, J = 8.5 Hz, 5'-H) and 7.57-7.75 (2H, m, 2'-H and 6'-H).

From the spectral data obtained and the oxidation product study, PS-1 was characterised as (2R:3R)-dihydroquercetin-4'-methyl ether. The structure of which is

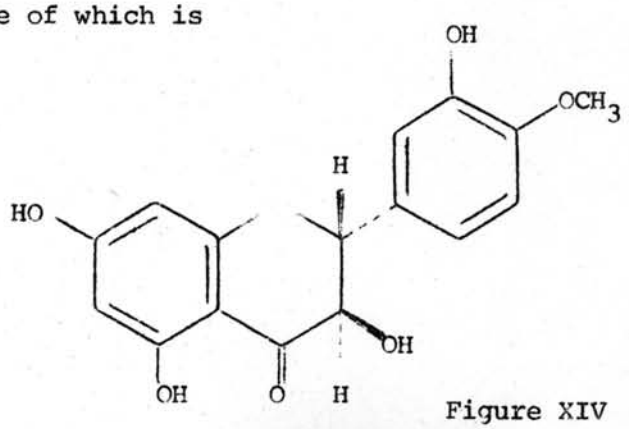

Figure XIV

4.2 Characterisation of PS-2

PS-2 was obtained as light yellow amorphous powder, soluble in ethanol, chloroform and acetone, moderately soluble in anaesthetic ether, insoluble in petroleum ether. It tasted slightly sweet.

hRf values

a) 35.5 b) 70 c) 70

Colour reaction

PS-2 gave pink colour with "Shinoda test" and violet colour with 1 % ferric chloride solution.

Melting point

164 - 167° C.

Optical rotation

$[\alpha]_D^{24} = + 14.8°$

Circular dichroism (MeOH)

$[\theta]_{331.5} = + 3.59 \times 10^5$ deg.dm^2/ d mole

$[\theta]_{294} = - 1.26 \times 10^6$ " "

$[\theta]_{253.5} = + 1.71 \times 10^5$ " "

$[\theta]_{221.5} = + 1.28 \times 10^5$ " "

(Fig. XXII)

Molecular weight

332 (mass spectrometry)

Ultraviolet absorption spectra

MeOH : λ (nm)	327 sh	(log ε = 3.76)	indicates characteristic of
	287	(4.49)	flavonoid nucleus.
	230 sh	(4.57)	
	216 sh	(4.68)	
	205	(4.81)	
	(Fig. XXVII)		
MeOH+$NaOCH_3$:	327 sh	(3.76)	indicates no free phenolic OH in
	290	(4.47)	a position to extend conjugation.
	230 sh	(4.57)	
	217 sh	(4.70)	
	207	(4.79)	
MeOH+NaOAc :	327 sh	(3.76)	indicates absence of a free OH at
	287	(4.49)	the 7 position of the flavonoid
			nucleus.
	(Fig. XXVIII)		
MeOH+NaOAc+H_3BO_3 :	327 sh	(3.76)	lack of change supports absence
	287	(4.49)	of 6,7 di-OH.
	(Fig. XXIX)		
MeOH+$AlCl_3$:	384	(3.92)	indicates the presence of a free
	315	(4.57)	5-OH.
	287 sh	(4.11)	
	225	(4.70	
	206	(4.92)	
	(Fig. XXX)		

$MeOH+AlCl_3+HCl$:	384	(3.89)	persistence of change in presence
	312	(4.50)	of HCl indicates lack of any *ortho*
	287 sh	(4.22)	di-OH system.
	225	(4.68)	
	206	(4.91)	

(Fig. XXX)

Infrared absorption spectram (Potassium bromide)

ν_{max} 3480 cm^{-1} a polymeric OH stretching vibration.

2930 cm^{-1} absorption due to CH_3 group.

1630 cm^{-1} the characteristic of the stretching vibration of a γ-pyrone $-\overset{O}{\overset{\|}{C}}-$

1510 cm^{-1} aromatic double bonds.

1360, 1200 cm^{-1} phenolic hydroxyl group.

(Fig. XXXII)

NMR spectra

In DMSO-d_6 at 60 MHz in δ value (ppm) from T.M.S.

Chemical shift (δ)	Proton	Multiplicity	Coupling Constants
3.79	6H (2-OCH_3(7)(4'))	bs	
4.55	1H (3)	m	
5.09	1H (2)	d	J = 11 Hz
6.10	2H (6)(8)	dd (AB system)	
6.95	3H (2')(5')(6')	s	

(Fig. XXXV)

In dueteroacetone at 60 MHz in δ value (ppm) from T.M.S.

Chemical shift (δ)	Proton	Multiplicity	Coupling Constants
2.78	3H (30H at (3)(5)(3'))	bm	
3.86	3H ($ArOCH_3$ (4'))	s	
3.87	3H ($ArOCH_3$(7))	s	
4.62	1H (3)	d	J = 11.6 Hz
5.11	1H (2)	d	J = 11.6 Hz
6.07	2H (6)(8)	dd (AB sytem)	
7.00-7.10	3H (2')(5')(6')	m	

(Fig. XXXVI)

Protons are identified by the labelling scheme shown for the structure (Figure XV). Integrated signal areas are in accordance with the number of protons assigned to them. Coupling constants derived from observed signal multiplicities are reported.

Mass spectrum

m/e 332 (M^+, 25% $C_{17}H_{16}O_7$), 303 (36), 179 (16), 167 (100), 166 (29), 164 (35), 151 (20) and 137 (23)

(Fig. XXXVIII)

Oxidation of PS-2

A sample of PS-2 (15 mg) was suspended in 2N.H_2SO_4 (5 ml) and heated on a steam bath under a gentle stream of air for 48 hours. After cooling to room temperature and extraction with ethyl acetate (4 x 5 ml), partition was effected against a saturated aqueous solution of sodium bicarbonate to remove residual acid, and the organic phase was dried

(anhydrous Na_2SO_4) and evaporated under reduced pressure to yield 12.4 mg (83%) of a yellow waxy solid, PS-2b (Figure XVII p.107); ultraviolet absorption spectra, λ_{max} (MeOH), 364, 288, 260, 213 and 208 nm, λ_{max} (MeOH + $NaOCH_3$) 413, 333, 290, 230, and 211 nm with no change for at least 24 hours.

From the spectral data obtained and the oxidation product study, PS-2 was characterised as (2R:3R)-dihydroquercetin 4',7-dimethyl ether. The structure of which is

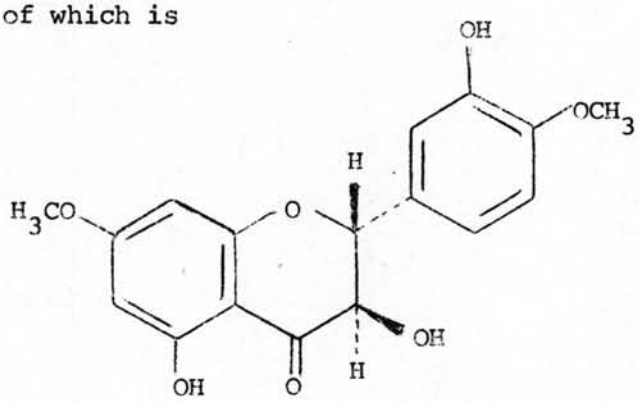

Figure XV