CHAPTER II
EXPERIMENT

2.1 Plant Materials

The bark of *Bombax malabaricum* (*Bombax ceiba* Linn.) (1) was obtained from Praputtabatt District, Saraburi Province Thailand, in March, 1996 and a voucher specimen of this plant (BKF No. 109239) is deposited at Forest Herbarium, Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok. They were air dried to avoid any chemical changes, ground and extrated with various solvents to separate and identify chemical constituents of the bark of *Bombax malabaricum* (*Bombax ceiba* Linn.)

2.2 Instruments and Equipments

2.2.1 Fourier Transform Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on a Perkin-Elmer Model IR 400 FT 1760 X. Fourier Tranform Infrared Spectrophotometer. Solid samples were generally examined by incorporating the sample with potassium bromide (KBr) to form a pellet. Liquid samples were dropped on sodium chloride cell.

2.2.2 Mass Spectrometer (MS)

The mass spectrometric analysis was performed on Mass Spectrometer Model Trio 2000.

2.2.3. ^{1}H and ^{13}C-Nuclear Magnetic Resonace Spectrometer

The ^{1}H-NMR and ^{13}C-NMR spectra were obtained by using a Bruker Model ACF 200 Spectrometer which operated at 200.13 MH_z. for ^{1}H and 50.32 MH_z for ^{13}C nuclei. The 500 MH_z. spectra were performed with a JNM 500 MH_z. from Jeol. The chemical shift δ (ppm) was assigned with

reference to the signal from the residual proton in deuterated solvent with respect to TMS.

2.2.4 Gas Chromatograph

The GC chromatograms were obtained from GC-7AG, HP 5890

2.2.5 Elemetal analyzer

The elemental analysis was performed on Perkin Elmer PE2400 SERIES II

2.2.6 Rotary Evaporator

Eyela rotary vacuum evaporator was used to evaporate the large amounts of volatile solvents.

2.2.7 Metting Points (m.p.)

The metting point was determined on a Fisher-John metting point apparatus and was uncorrected.

2.3 Chemical Reagents

2.3.1 Solvents

All solvents used in the research such as hexane, chloroform, methanol were commercial grade. They were purified prior to use by distillation. Reagent grade used in recrystallization were used as received.

2.3.2 Other chemicals

2.3.2.1 Merck's silica gel 60 Art. 7734 (70-230 mesh ASTM) was used as an adsorbent for column chromatography.

2.3.2.2 Merck's TLC aluminium sheets, silica gel 60F 254 precoated 25 sheets, 20 × 20 cm^2, layer 0.2 mm. was used to identify the identical fractions.

2.4 Physical Separation Techniques

2.4.1 Column Chromatography (CC) and flash Chromatography (FC)

A column chromatography was performed on a glass column using silica gel 60 Art. as an adsorbent. The size of the column used was depended on the amount (weight) of the sample, normally, a sample consisted of 1-4 % (wt./wt.) of the adsorbent and the amount of adsorbent was sufficient to make a maximum height to diameter ratio of 10:1

2.4.1.1 Preparation:

The stopcock of the column was closed and the glass rod was used to push the cotton wool to the bottom of column. The solvent was allowed to remain about half of the column. A slurry of the silica gel (25 % wt./v.) in a suitable solvent was mixed and added to the column. When the slurry of the silica gel was poured in, the glass rod was pulled out. The stopcock was opened to release the solvent pouring down slowly until remained about 5 cm. above the surface on the silica gel. The crude extracts were mixed with silica gel until friableness. Then carefully added to the column. A small amount of the solvent was added and passed through the column. The dried silica gel was sprinkled on top of the column and then a paper disc was placed on top of the silica gel column prior to the addition of the eluent. The column was developed by suitable solvents and the eluent was by monitored thin layer chromatography.

2.4.2 Thin-Layer Chromatography (TLC)

In this research, Merck's TLC aluminium sheets, (silica gel 60F 254 precoated sheets, 20 × 20 cm^2, 0.2 mm. Thick) was used. Two lines were drawn on each plate, the first was 1.0 cm from the lower edged, the other line was 8.0 cm above and parallel to the first line. The solution of substances to be identified was applied as small spots on the lower line of the plate. After the solvent had evaporated, the plate was placed in a closed glass utensil which had the eluting solvent moved up the plate immediately. When the solvent reached the upper line, the plate was removed, allowed the solvent to evaporate and the plate was detected under UV light (254 mm) and I_2.

2.5 Color Test (18)

2.5.1 Liebermann-Burchard Test

This is a test for a steroidal or triterpenoidal nucleus. To a solution of solid or dry extract 2-3 mg in 0.5 cm^3 of chloroform, was added a few drops of acetic anlydride, followed by one drop of concentrated sulfuric acid. The color reaction obtained as blue green or blue indicated the presence of steroid compound and that obtained as purple or pink indicated the presence of triterpenoid compounds.

2.6 Extraction

Approximately 6 kg. of air-dried bark of *Bombax malabaricum* (*Bombax ceiba* Linn.) was mined and extracted with the methanol for about 2-3 days until the solvent was colorless by Soxhlet apparatus. The solvent was filtered and distiled by simple distillation. It was then filtered and the solvents were evaporated.

Then methanol crude extraced was extracted with hexane to sive hexane crude extract (Fraction I) which was a yellowish-brown material (59.50 g). The hexane insoluble part was extracted with chloroform in the same way and yielded the chloroform crude extract as a light brown material. (6.93 g) (Fraction II).

The insoluble was partition with n-butanol and gave the n-butanol crude extract a sticky pale brown material. (34.30 g). and the methanol residue as a dark brown material (160 g).

The weights of the extracts are shown in Table 2.1 and the extraction procedures are shown in Scheme 1.

Scheme 1 The extract of the bark of *Bombax malabaricum* .

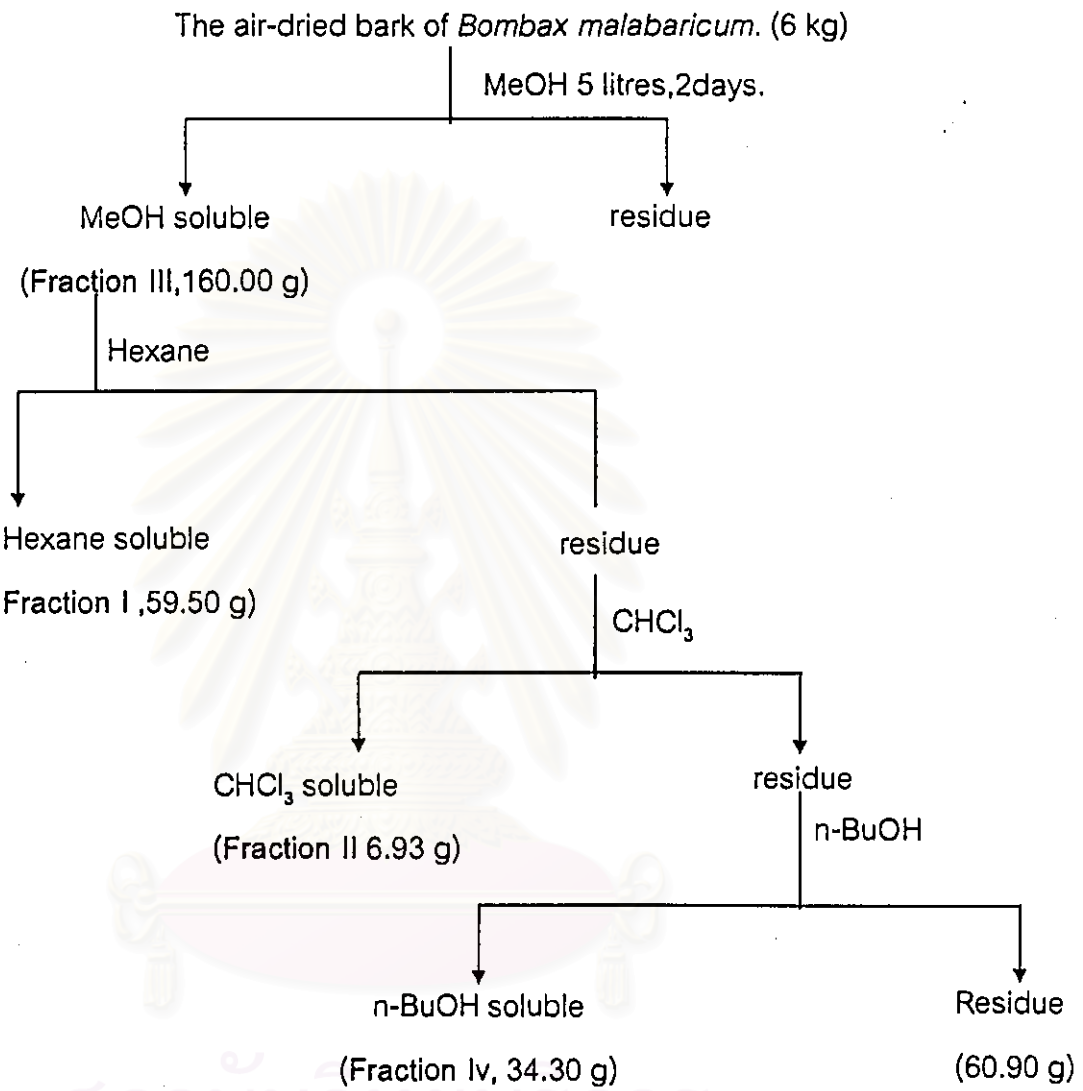

Table 2.1 The weight of the extracts

Weight of the bark of *Bombax malabaricum* in methanol extracts (160 g)	Weight and % Yield (%wt./wt.)		
	Hexane crude extracts (g)	Chloroform crude extracts (g)	n-BuOH crude extracts (g)
	Yellowish-brown oil 59.50 g (1.19)	light brown 6.93 g (0.14)	sticky pale brown 34.30 g (0.69)

2.7 Isolation of Chemical Constituents of the bark of *Bombax malabaricum* (*Bombax ceiba* Linn.)

2.7.1 Separation of hexane crude extract by column hromatography.

The hexane crude extract (Fraction I, 59.50 g) was chromatographed on a silica gel (700 g) column packed in hexane. The eluent was stepwisely changed from hexane, a gradient of hexane-chloroform, chloroform, and gradient of chloroform-methanol, respectively. The eluted solution was collected approximately 500 cm^3. The solution in each fraction was concentrated by rotary evaporator and distilled to the volume of 50 cm^3. They were then transfered into a small flask. The elution was monitored by TLC. The identical fractions were combined together. The results of the column chromatographic separation of hexane crude extract (Fraction I) are presented in Table 2.2.

Table 2.2 The results of the column chromatographic separation of crude hexane extract (Fraction I)

Eluents	Fraction No.	Remarks
100 % hexane	1-15	light yellow oil
100 % hexane	31-32	solid yellow oil
10 % $CHCl_3$ / hexane	33-35	solid in brown oil (Mixture I)
20 % $CHCl_3$ / hexane	36-39	solid in brown oil
20 % $CHCl_3$ / hexane	40-45	solid in brown oil
20 % $CHCl_3$ / hexane	46-50	solid in brown oil (Compound II)
20 % $CHCl_3$ / hexane	51-56	solid in yellow-brown oil
20 % $CHCl_3$ / hexane	57-60	solid in yellow-brown oil
30-40%$CHCl_3$/ hexane	61-65	solid in yellow-brown oil
50 % $CHCl_3$ / hexane	66-70	solid in yellow
50 % $CHCl_3$ / hexane	71-75	solid in brown oil (Compound IV)
50 % $CHCl_3$ / hexane	76-80	solid in yellow-brown oil
70 % $CHCl_3$ / hexane	81-85	greenish oil
100% $CHCl_3$	86-89	brown oil
5% MeOH/$CHCl_3$	90-95	brown oil
10% MeOH/$CHCl_3$	96-105	brown oil

2.7.2 Separation of chloroform crude extract by column chromatography.

The chloroform crude extract (Fraction II, 6.93 g) was chromatographed on a silica gel (70 g) column packed in hexane the eluent was changed stepwise from hexane,a gradient of hexane-chloroform, chloroform,and a gradient of chloroform-methanol, respectively. The chromatographic separation conditions were the same as described in the separation of Fraction I. The results of the column chromatographic separation of chloroform crude extract (Fraction II) are shown in table 2.3.

Table 2.3 The results of the column chormatographic separation of chloroform crude extract (Fraction II)

Eluents	Fraction No.	Remarks
100% Hexane	1-5	solid in brown oil
20%$CHCl_3$ /Hexane	6-7	solid in brown oil
50%$CHCl_3$ /Hexane	8-10	solid in brown oil
50%$CHCl_3$ /Hexane	11-17	solid in brown oil
50%$CHCl_3$ /Hexane	18-20	solid in light yellow oil
60%$CHCl_3$ /Hexane	21-26	solid in light yellow oil
60%$CHCl_3$ /Hexane	27-29	solid in brown oil
60%$CHCl_3$ /Hexane	30-32	solid in brown oil (Compound IV)
70%$CHCl_3$ /Hexane	33-35	brown oil
70%$CHCl_3$ /Hexane	36-38	brown oil
80%$CHCl_3$ /Hexane	39-40	brown oil
80%$CHCl_3$ /Hexane	41-43	brown oil
80%$CHCl_3$ /Hexane	44-46	solid in brown oil
90%$CHCl_3$ /Hexane	47-48	solid in brown oil
90%$CHCl_3$ /Hexane	49-52	solid in red-brown oil
90%$CHCl_3$ /Hexane	53-56	solid in red-brown oil
100% $CHCl_3$	57-58	solid in red-brown oil
100% $CHCl_3$	59-60	solid in brown
5% MeOH / $CHCl_3$	61-64	solid in red-brown (Compound II)
5% MeOH / $CHCl_3$	65-71	solid in red-brown
10%MeOH / $CHCl_3$	72	solid in red-brown

Table 2.3 (continued)

Eluents	Fraction No.	Remarks
10%MeOH / $CHCl_3$	73-74	solid in red-brown
15%MeOH / $CHCl_3$	75-76	solid in red-brown
15%MeOH / $CHCl_3$	77-80	solid in red-brown
20%MeOH / $CHCl_3$	81-85	solid in red-brown (Compound III)

2.7.3 Separation of n-BuOH crude extract by column chromatography.

The n-BuOH crude extract (Fraction IV, 34.30 g) was chromatographed on a silica gel (300 g) column packed in hexane the eluent was changed stepwise from hexane, a gradient of hexane-chloroform, chloroform, a gradient of chloroform-methanol and methanol, respectively. The chromatographic separation conditions were the same as described in the separation of Fraction I. The results of the column chromatographic separation of n-BuOH crude extract (Fraction IV) are shown in table 2.4.

Table 2.4 The results of the column chromatographic separation of n-BuOH crude extract (Fraction IV)

Eluents	Fraction No.	Remarks
100% Hexane	1-10	light yellow oil
20% $CHCl_3$	11-12	brawn oil
50%$CHCl_3$ /Hexane	22-23	solid in brawn oil
50% $CHCl_3$ /Hexane	24-25	solid in brown oil (Compound V)
50% $CHCl_3$ /Hexane	26-27	sticky oil, dark brawn
60% $CHCl_3$ /Hexane	28-29	sticky oil, dark brawn
70% $CHCl_3$ /Hexane	30-31	sticky oil, dark brawn
80% $CHCl_3$ /Hexane	32-33	sticky oil, brawn
100% $CHCl_3$	34-35	brown oil
2% MeOH/$CHCl_3$	36-38	brown oil
5% MeOH/$CHCl_3$	39-40	brown oil
10% MeOH/$CHCl_3$	44-51	dark brawn oil
10% MeOH/$CHCl_3$	52-59	dark brawn oil
15% MeOH/$CHCl_3$	60-65	solid in brown oil
20% MeOH/$CHCl_3$	66-71	solid in brown oil
25% MeOH/$CHCl_3$	86-90	solid in greenish oil
50% MeOH/$CHCl_3$	91-100	solid in brown oil
100% MeOH	101-110	solid in brown oil

2.8 Purification and Properties of Substances from Chromatography

2.8.1 Purification and properties of Mixture I

The white solid in yellow oil was obtained in fraction no 33-35 from the hexane crude extract (10% $CHCl_3$ / Hexane) (Table 2.2). Tt was purified by recrystallization from hexane for several times to give Compound I as a white solid 0.04g (0.06% wt. by wt. of hexane crude extract) , mp. 57-58 $^{\circ}$ C . This solid was soluble in hexane , dichloromethane but chloroform and insoluble in methanol and acetone.

ν_{max} (Cm^{-1}) : 2925 (s) , 2860 (s) , 1470 (m) , 1380 (w) , 805 (w) and 725 (w) (Fig.4)

Gas chromatogram (Conditions: Column OV-1 , column temp. 250°C, injection temp. 290°C, carrier gas N_2 50 ml/min) The chromatogram showed seven peaks at retention times 6.26, 8.01, 10.22, 13.30, 17.12, 22.19, and 28.59 min, respectively. (Fig.5)

2.8.2 Purfication and properties of Compound II

Compound II was abtained as white needle like solid in solid red-brown from fraction 46-50 (Fraction I) and from fraction 61-64 (Fraction II) which were eluted from 20% Chlorofrom in hexane and 5% Methanol in chlorofrom , respectively. It was purified by recrytallization from hexane for several times to give Compound II as white solid 0.007 g (0.10% wt. by wt. of hexane crude extracts) and 0.002 g (0.02% wt. by wt. of chloroform crude extract), m.p. 137-138 $^{\circ}$C and R_f value 0.72 (Silicagel : 5% MeOH in chloroform) . This compound was soluble in chloroform, dichloromethane , slightly soluble in hexane and insoluble in methanol . Compound II gave green color with Liebermann - Burchard's reagent.

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ν_{max} (KBr, cm^{-1}) 3590-3066 (S,br) , 2935 (s) , 2869 (s) , 1716 (br), 1590 (s) , 1460(m) , 1059 (w) , 800 (w) ; (Fig.10)

m/z (EI) 414 (M^+, 50%) , 396 (18) , 381 (12) , 329(18) , 303(21) m 273(15), 255(22), 231(14) , 213(28), 145(46), 107(62), 95(54), 81(56), 55(78),43 (100); (Fig.14)

δ_H(500MH_z , $CDCL_3$) 0.66-2.30(m), 3.52(m), 5.34-5.36 (m) ; (Fig. 11)

δ_C(125.65MH_z , $CDCL_3$) 11.85. 11.98, 18.78, 19.06, 19.39, 19.80, 21.10, 23.11, 24.31, 26.18, 28.24, 29.23, 29.69, 31.69, 31.92, 33.99, 36.15, 36.53, 37..29, 39.80, 40.43, 42.32, 45.89, 50.18, 51.24, 56.11, 56.80, 71.82,121.70, 129.33, 138.28, 140.78 (Fig. 12)

GLC analysis of Compound II showed one peak at retention time at 21.60 min (Condition of GLC analysis : column OV-1 , column temperature 255 °C , injection temperature 290°C , N_2 flow rate 50 mL / min) . Mixture of standard steroid: campesterol , stigmasterol and β-sitosterol showed peak at retention time 17.58 , 18.32 and 20.73 min , respectively. (Fig.13)

2.8.3. Purification and properties of Compound III

Compound III was obtained as solid in red-brown from Fraction II in fraction No. 81-85 which was eluted by 20% methanol in chloroform. It was purified by recrystallitation from hot ethanol to give Compound III as white amorphous solid , 0.003g (0.04% wt. by wt. of Chloroform crude extract) , m.p 256-258°C and R_f value was 0.44 (silica gel,

10% methanol in chloroform). This compound was insoluble in hexane and slightly soluble in methanol.

ν_{max} (KBr, cm^{-1}) 3400 (m,br) , 2945 (s) , 2870 (s) , 1650(m) , 1460 (m) , 1380(m) , 1050(m) , 1030(w) ; (Fig.15)

δ_H (200MH_z , DMSO) 0.66-2.50 (m) , 2.95-3.70(m), 4.25(d),5.32 (d,= 5.1) ; (Fig.16)

δ_c (50.26 MH_z, DMSO) 11.65 . 11.76 , 18.60 , 19.00 , 19.63 , 20.26 , 22.76 , 23.83 , 25.85 , 27.69 , 28.95 , 29.30 , 31.41 , 31.51 , 35.44 , 36.27 , 36.88 , 38.48 - 41.00 overlapped with the solvent peak) , 41.90 , 45.34 , 49.75 , 55.60 , 56.25 , 61.40 , 70.38 , 73.59 , 76.70 , 76.92 , 77.20 , 100.90 , 121.10 , 140.60 (Fig.17)

m/z (EI) 414 (M^+, 24%), 396(42) , 381(17) , 329(11) , 303(19) , 273 (11) , 255(21) , 231(10) , 213(25) , 145(56) , 107 (44) , 95(52), 81(54) , 55(100) ; (Fig.18)

2.8.4. Purification and properties of Compound IV

Compound IV was obtained as solid in brown oil from Fraction I in fraction no. 71-75 which was eluted by 50% chloroform in hexane and was obtained from Fraction II in fraction no. 30-32 eluted by the same eluent. It was purified by recrystallitation from hot ethanol to afford compound IV as a pale purple amorphous solid, 0.06 g (0.1% wt. by wt. of crude hexane extract) and 0.007 g (0.01% wt. by wt. of crude chloroform extract) m.p. 215-216 ^oC and the R_f value was 0.37 (silica gel : 5% methanol in chloroform). This compound was insoluble in hexane and slightly soluble in methanol. Compound IV gave green color with Lieermann-Burchard's reagent.

ν_{max} (KBr, cm^{-1}) 3336-3060 (s,br), 2953 (s), 2871 (s), 1614 (w), 1455 (s), 1382 (s), 1070 (w), 826 (w) ; (Fig.19).

m/z (EI) (M^+,10%), 411 (4), 315 (6), 218 (38), 207 (66), 189 (81), 161 (28), 121 (57), 109 (80), 95 (100), 81 (82), 55 (75) and 43 (85) ; (Fig.27).

δ_H (500 MH_z, $CDCl_3$) 0.76-2.01 (m). 3.20 (m), 4.68 (d) ; (Fig.20).

δ_C (125.65 MH_z, $CDCl_3$) 14.55, 15.36, 15.98, 16.10, 18.00, 18.33, 19.31, 20.95, 25.18, 27.42, 27.46, 27.99, 29.67, 29.87, 34.31, 35.59, 3.19, 38.08, 38.73, 38.85, 40.00, 40.86, 42.85, 42.99, 47.98, 48.32, 50.46, 55.33, 78.99, 109.31, 150.90 (Fig.23).

2.8.5. Purification and properties of Compound V

Compound V was obtained as solid in brown oil from Fraction IV in fraction no. 24-25 which was eluted by 50% chloroform in hexane. It was purified by hexane to afford Compound V as a yellow oil, 0.004 g (1.16×10^{-2}% wt. by wt. of crude n-BuOH extract). It had a melting point of 208-210°C and R_f value of 0.52 (silica gel : 5% methanol in chloroform. This compound was soluble in methanol, chloroform and slightly soluble in chloroform.

ν_{max} (KBr, cm^{-1}) 3445 (mbr), 2950 (s), 2850 (m), 1775 (s), 1485 (w), 1090 (s) ; (Fig.29).

δ_H (500 MH_z, $CDCl_3$) 1.24-1.27 (t), 2.04 (s), 4.10-4.14 (q) ; (Fig.30).

δ_C(125.65 MH_z) 14.06, 20.88, 60.28, 76.73-77.23 (overlapped with the svent peak), 171.07 (Fig.31).

m/z (EI) 132 (M^+, 2%), 73 (100), 57 (86) ;(Fig.33).