CHAPTER II

EXPERIMENTAL

2.1 Plant Material

The seed kernels of *Caesalpinia major* (Medik.) Dandy & Exell. were collected from Sanamchaikate, Chachaengsao, Thailand in November, 1996. This specimen was identified by comparison with the herbarium No. 55398 at the Herbarium Section in the Department of Royal Forestry, Ministry of Agricultural and Cooperative, Bangkok, Thailand.

2.2 Equipment

2.2.1 Fourier Transform-Infrared Spectrophotometer (FT-IR) and Infrared Spectrophotometer (IR)

The FT-IR spectra were recorded on a Perkin-Elmer model 1760 x Fourier Transform Infrared Spectrophotometer and the IR spectra were recorded on a Perkin Elmer Model IR 718 Infrared Spectrophotometer. Solid samples were examined by incorporating the sample into a pellet of potassium bromide.

2.2.2 ^{1}H and ^{13}C-Nuclear magnetic Resonance Spectrometer

Routine ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker NMR Model ACF 200 spectrometer operated at 200.13 MHz for ^{1}H and 50.26 MHz for ^{13}C-nuclei. The chemical shift (δ) in ppm were referenced to the signal from the residual proton in deuterated solvents. Assignments of carbon spectra were assisted by a Distortionless Enhancement by Polarization Transfer (DEPT) experiment. Specialized NMR experiments (COSY, NOESY, HMBC, HMQC, etc.) were performed at the Chiba University, in Japan.

2.2.3 Gas Chromatography (GC)

The GC analysis was performed on a Shimadzu Gas Chromatograph using

OV-1 column, column temperature 255° C, injection temperature 290°C, N_2 flow rate 50 ml/min with FID detector.

2.2.4 Mass Spectrometer (MS)

The MS analysis was performed on a Fison instruments Model Trio-2000 Mass Spectrometer in EI mode

2.2.5 Melting Point Apparatus (m.p.)

The melting point were recorded on a Ficher-John melting point apparatus and are uncorrected.

2.3 Solvents and Chromatographic Media

2.3.1 All solvents, except when they were reagent grade were purified by distillation.

2.3.2 Merck's silica gel 60 Art 7734.1000 (70-230 mesh ASTM) was used as adsorbents for column chromatography.

2.3.3 Merck's TLC aluminium sheets, silica gel 60 F254 pre-coated plates, 20 x20 cm^2 , layer thickness 0.2 mm were used for checking the fractions.

2.4 Extraction and Fractionation

The fresh seeds of *C. major* (Medik.) Dandy & Exell. (3kg) were peeled, crushed and extracted three times with MeOH (4 l) at room temperature and the combined extracts were evaporated to dryness under reduced pressure. the precipitate was filtered to get a white precipitate (23.0 g, Fraction I). The residue greenish brown syrup was dissolved in 90% methanol and partitioned with hexane. The hexane layer was concentrated under reduced pressure to give the hexane crude extract (10.9 g, Fraction II). The methanol layer was concentrated under reduced pressure and partitioned with chloroform and water. The chloroform layer was concentrated under reduced pressure to give the chloroform crude extract (26.0 g, Fraction III). The water layer was partitioned with isobutanol to give the isobutanol crude extract (40.6 g,

Fraction IV) and the water crude extract (91.1 g, Fraction V) remained after evaporation as shown in Table 2.1

Scheme 2.1 Extraction of fresh seeds of *Caesalpinia major* (Medik.) Dandy &Exell.

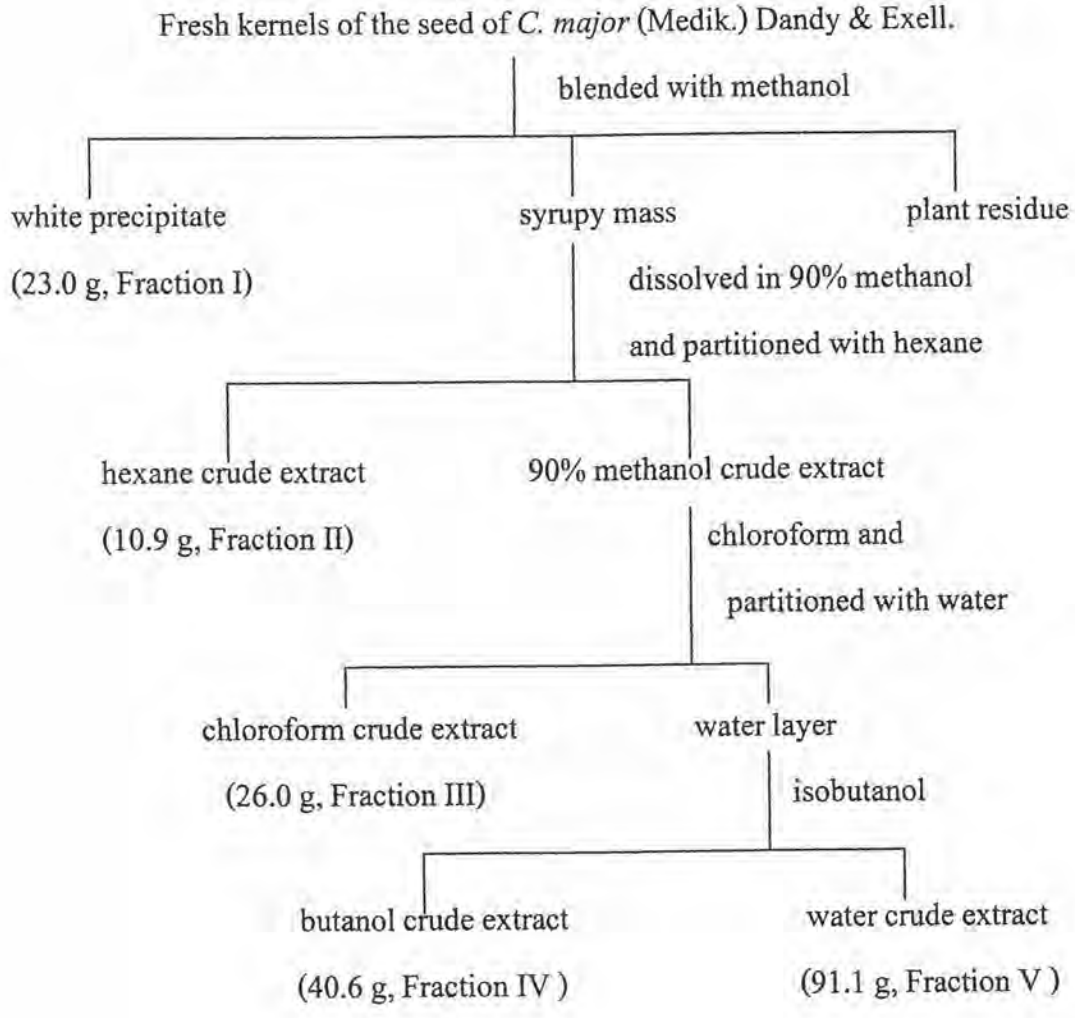

Table 2.1 The result of fresh seed kernels extraction of *C. major* (Medik.) Dandy & Exell.

Fraction	Remark	Weight (g)	% wt. By wt. of fresh seeds
Fraction I	White solid	23.0	0.8
Fraction II	yellow oil	10.9	0.4
Fraction III	greenish brown syrup	26.0	0.9
Fraction IV	greenish brown syrup	40.6	1.4
Fraction V	dark brown syrup	91.1	3.0

2.5 Isolation of the chemical constituents of *Caesalpinia major* (Medik.) Dandy & Exell.

2.5.1 Separation of Fraction I

The white precipitate (23.0g) was subjected to a silica gel column chromatography (345 g). The column was eluted with chloroform and the polarity of eluent was gradually increased by10% every 1000 ml until 100% methanol was reached. The eluted fractions were collected to approximately 200 ml for each fraction and this was subsequently concentrated to 20 ml. Each fraction was monitored by TLC using chloroform and 4% methanol in chloroform. In addition, fractions with similar composition were combined and left for crystallization.

2.5.2 Separation of Fraction II

Hexane crude extract (10.9 g) was subjected to a silica gel column chromatography (150 g). The column was initially eluted with 50% chloroform in hexane. The polarity of eluent was gradually increased from chloroform in hexane to chloroform, ethyl acetate in chloroform, ethyl acetate and then 20% methanol in ethyl acetate. The eluted fractions were collected and concentrated to 20 ml. Each fraction was monitored by TLC using 50% chloroform in hexane, chloroform, 10%, 20% ethyl acetate in chloroform and 3% methanol in chloroform. Furthermore, fractions with similar composition were combined together.

2.5.3 Separation of Fraction III

Chloroform crude extract (26.0 g) was subjected to a silica gel column chromatography (480 g). The column was initially eluted with 50% ethyl acetate in hexane. The polarity of eluent was gradually increased from ethyl acetate in hexane to ethyl acetate and 20% methanol in ethyl acetate. The eluted fraction were collected to approximately 150 ml for each fraction and the solvent was, then, evaporated to

20 ml. Each fraction was monitored by TLC using 30%, 40%, 50%, 70%, 80% ethyl acetate in hexane, ethyl acetate, 20% and 40% methanol in ethyl acetate. Moreover, fractions with similar composition were combined together.

2.5.4 Separation of Fraction IV

Butanol crude extract (40.6 g) was chromatographed on Sephadex LH 20 column. The column was eluted with methanol. The eluted fractions were collected and concentrated to 20 ml and checked by TLC using mixture of chloroform, methanol and water (65:35:10 by volume). The fractions with similar composition were combined together.

2.5.5 Separation of Fraction V

Water crude extract (91.1 g) was chromatographed on Sephadex LH 20 column. The column was eluted with water and 50% methanol in water. The eluted fractions were collected and concentrated to 20 ml and checked by TLC using mixture of chloroform, methanol and water (65:35:10 by volume). The fractions with similar composition were combined together.

2.6 Purification and properties of the eluted compounds

2.6.1 Purification and properties of Compound I

Compound I was obtained as solid in yellow oil from Fraction I in fraction No. 2-5 which were eluted with 100% chloroform. Recrystallization from hot methanol afforded Compound I as colorless needle-like crystals, 504 mg (0.017% wt. by wt. of fresh seeds), m.p. 202-203 $^{\circ}$C and Rf value 0.33 (SiO_2 : $CHCl_3$). This compound was soluble in chloroform, slightly soluble in ethyl acetate and methanol and insoluble in hexane.

ν_{max} (KBr, cm^{-1}) 3590 (m), 2970 (m), 2945 (m), 2879 (m), 2865 (m), 1738 (s), 1369 (m),1254 (s), 1228 (s), 1035 (m) ; m/z (EI, 30 eV) 418 (M^+, 12%) , 400 (16), 358

(10), 340 (37), 298 (12), 280 (34) , 265 (31), 158 (35), 146 (100) ; m/z (FAB) 419 $[M+H]^+$, 97%) , 401 (46) , 341 (75) , 281 (66) , 173 (37) , 154 (100), 136 (79),107 (30.8), 43 (51.2) ; Found C 69.07% H 8.28% Calcd for $C_{24}H_{34}O_6$ C 68.90% H 8.13%; ^1H NMR (500MHz, $CDCl_3$) : see Table 3.1 and Figure 6-7;^{13}C NMR (500 MHz, $CDCl_3$) : see Table 3.1 and Figure 8.

2.6.2 Purification and properties of Compound II

Compound II was obtained as solid in yellow oil from Fraction II in fraction No. 80 which was eluted with 30% ethyl acetate in chloroform. It was purified by recrystallization from methanol to afford compound II as colorless crystals , 10 mg (3×10^{-4}% wt. by wt. of fresh seeds). The melting point was 149-151 °C and the Rf value was 0.48 (SiO_2 : 10% methanol in chloroform). This compound was soluble in methanol, slightly soluble in chloroform and insoluble in hexane.

ν_{max} (KBr, cm^{-1}) 3573 (m), 3500 (m), 2986 (m), 2943 (m), 1739 (s), 1715 (s), 1654 (w), 1375 (m), 1266 (s), 1243 (s), 1176 (m), 1036 (m), 933 (m) ; m/z (EI, 30eV) 450 (M^+, could not be detected), 432 (could not by detected), 414 (25%), 390 (26), 372 (8), 354 (26), 330 (100), 315 (70), 312 (57), 297 (15), 294 (69), 279 (19), 275 (47), 161 (51), 98 (45) ; m/z (FAB) 451 ($[M+H]^+$22%), 345 (16), 307 (24), 289 (13), 192 (84), 154 (100), 136 (72) ; ^1H NMR (500 MHz, $CDCl_3$) : see Table 3.2, Figure 27-28 ; ^{13}C NMR (500 MHz, $CDCl_3$) : see Table 4.2, Figure 29-31.

2.6.3 Purification and properties of Mixture III

Mixture III was obtained as white needle-like solid in orange oil from Fraction II in fraction No. 19-28 which was eluted with 80% chloroform in hexane. It was purified by recrystallization from hot hexane to afford compound III as colorless needles, 6.9 mg (2×10^{-4}% wt. by wt. of fresh seeds), m.p. 135-136°C and Rf value 0.44 (SiO_2 : 10% EtOAc in $CHCl_3$). This mixture was soluble in chloroform, slightly soluble in hexane, ethyl acetate and insoluble in methanol.

ν_{max} (KBr, cm^{-1}) 3695-3048 (m, br) 2959 (s), 2937 (s), 1651 (w), 1464 (m), 1380 (m), 1059 (m), 1025 (w), 802 (w) ; m/z (EI) 414 (M^+, 28%), 396 (10), 381 (7), 329 (15), 303 (16), 273 (13), 255 (20), 213 (25), 145 (48), 107 (71), 95 (70), 91 (57), 81 (73), 69 (57), 55(89), 43 (100) ; ^{1}H NMR δ_H (200MHz, $CDCl_3$) : 0.7-2.3 (m), 3.53 (m), 5.35 (d), see Figure 35 ; ^{13}C NMR (50.26 MHz, $CDCl_3$) : see Table 3.3 , Figure 36.

2.6.4 Purification and properties of Compound IV

Compound IV was obtained as semi solid from Fraction III in fraction No. 1-3 which was eluted with 50% ethyl acetate in hexane. The material obtained (2 g) was further fractionated on a silica gel column and eluted with 70-80% chloroform in hexane. After solvent evaporation, an oil was obtained from this step in fraction No. 186-208 . The fraction 186-208 were applied to prep. TLC plates (developed with chloroform- methanol, 98:2) to afford Compound IV as an oil, 7.2 mg ($2x10^{-4}$ wt. by wt. of fresh seeds). This compound was soluble in chloroform , methanol and insoluble in hexane.

m/z (EI, 30 eV) 418 (1%), 400 (23), 358 (49), 340 (9), 298 (23), 280 (55), 265 (30), 199 (33), 147 (68), 135 (32), 134 (45), 109 (51) ; ^{1}H NMR (200 MHz,$CDCl_3$):see Table 3.4 , Figure 39 ; ^{13}C NMR (50.26 MHz, $CDCl_3$) : see Table 3.4 , Figure 40-45.