CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The entire plants of *Bidens biternata* Merr. & Sherff were obtained from Dansai, Loei, Thailand in October, 1992. The plant material were authenticated by comparison with the herbarium specimen in the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperative.

2. General Techniques

2.1 Analytical Thin-layer Chromatography(TLC)

Technique : One way, ascending

Adsorbent : Silica gel 60 F254 (E. Merck) precoated plate.

Layer thickness : 0.2 μm

Distance : 5.5 cm

Temperature : Laboratory temperature (30-35^{o}C)

Detection :
1. Visual detection under day light
2. Visual detection under ultraviolet light at the wavelength of 254 and 365 nm.
3 Anisaldehyde-sulphuric acid spraying reagent (0.5% ethanolic solution of anisaldehyde with 5% sulphuric acid)

2.2 Column Chromatography (CC)

Adsorbent : Silica gel 60 (number 7734) particle size 0.063-0.200 mm(70-230 mesh ASTM)

Packing method : Dry packing

Sample loading : The sample was dissolved in small amount of organic solvent, mixed with a small quantity of adsorbent, then dried, triturated and added gently on the top of the column

Examination of eluates : Fractions were examined by TLC using visual detection under day light, ultraviolet light at wavelength of 254 and 365 nm

and sprayed with anisaldehyde-sulphuric acid spraying reagent.

2.3 Gel Filtration Chromatography

Gel filter : Sephadex LH-20 (Pharmazia)

Packing method : Gel filter was suspended in the eluant and left standing to swell prior to use for 24 hours. Then poured it into the column and allowed it to be settled tightly.

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

Examination of eluates : Fractions were examined in the same manner as described in section 2.2.

2.4 Gas Chromatography

Model : Shimadzu GC R1A

Column : OV-1

For BB1;

Column temperature : 250°C

Injection temperature : 290°C

N_2 flow rate : 50 ml/min

For BB2;

Column temperature : 260°C

Injection temperature : 290°C

N_2 flow rate : 45 ml/min

2.5 Spectroscopy

2.5.1 Ultraviolet (UV) Absorption Spectra

The spectra were obtained on a Hitashi UV-220A spectrometer (Scientific and Technological Research Equipment Center, Chulalongkorn University). The samples were dissolved and adjusted the concentrations using methanol and chloroform (garantee grade, E. Merck) as solvent.

2.5.2 Infrared (IR) Absorption Spectra

The spectra were obtained on a Shimatsu IR-440 infrared spectrometer (The Scientific and Technological Research Equipment Center, Chulalongkorn University) in potassium bromide discs to determined the spectra.

2.5.3 Mass Spectra (MS)

The Electron Impact Mass Spectra (EIMS) and Atmospheric Pressure Chemical Ionization Mass Spectra (APCI) were determined on a Fisons VG Trio 2000 quadrupole mass spectrometer (Department of Chemistry, Faculty of Sciences, Chulalongkorn University).

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

The 200 MHz ^{1}H NMR spectra were obtained with a Bruker ACF 200 spectrometer (Department of Chemistry, Faculty of Sciences, Chulalongkorn University).

The 500 MHz ^{1}H NMR spectra and 125 MHz ^{13}C NMR spectra were obtained with a JEOL JMN-A 500 spectrometer (The Scientific and Technological Research Equibment Center, Chulalongkorn University).

The operating solvent for NMR spectra were deuterated chloroform ($CDCl_3$), deuterated methanol (4d-CD_3OD) and deuterated pyridine (5d-C_6D_5N). The chemical shifts were reported in ppm scale using the chemical shift of trimethylsilane (TMS) at 0 ppm as the reference signal.

2.6 Solvents

Throughout this work, all organic solvents were commercial grade and had to be redistillated prior to use.

3. The Extraction

The dried powder of the entire plant of *Bidens biternata* Merr. & Sherff (10 kg) was repeatedly marcerated for four times in methanol (each, for 7,3,2,2 day) and then filtered. The filtrate of each marceration was concentrated to remove methanol under reduced pressure at temperature not over 50°C. Remaining volume of this fraction, called F-001, was approximately 5 litre. The fraction F-001was partitioned with chloroform (10 litre). The chloroform extract (F-002) was seperated and evaporated to dryness under reduced pressure to yield 400 g of brown mass (4% based on dry weight of *B. biternata* 's powder). The water extract (F-003) was evaporated on water bath yeilded 1 kg (10% based on dry weight of *B. biternata* 's powder).

Fraction F-002 was dissolved in methanol (1.5 litre) and add water to make a solution of 80% methanol. It was partitioned with hexane (4 litre). Hexane extract and methanol extract were evaporaed to dryness under reduced pressure to yield 150 and 80 g. (1.5 and 0.9% based on dry weight of *B. biternata*'s powder) and named as F-004 and F-005, respectively.

4. The Isolation

4.1 The Isolation of BB-1 and BB-2

Fraction F-004 (150g) was dissolved in a small volume of chloroform/hexane mixture and triturated with silica gel (No. 7734).This mixture was dried under the vacuum. It, then, was fractionated by column chromatographic technique using a column of silica gel (1,000 g, 10x85 cm). The eluants were used in the order as shown below :

- hexane	3,000 ml	fractions # 1-2
- hexane : chloroform (4:1)	1,500 ml	fraction # 3
- hexane : chloroform (7:3)	1,500 ml	fraction # 4
- hexane : chloroform (1:1)	1,500 ml	fraction # 5
- hexane : chloroform (1:4)	33,000 ml	fractions # 6-27
- chloroform	15,000 ml	fractions # 28-37
- chloroform : methanol (98:2)	15,000 ml	fractions # 38-47
- chloroform : methanol (96:4)	10,500 ml	fractions # 48-54

Methanol was used to wash the column until the eluates were diluted and clear comparing to the former ones.

The eluates were examined by TLC using 20% hexane in chloroform and 2% methanol in chloroform as developing solvent. The fractions giving similar chromatographic pattern were combined and designated.

Table 3 The combined fractions from F-004

Fractions	Number of Eluates	Weight (g)
F-006	1-3	2.08
F-007	4-5	8.60
F-008	6-11	4.12
F-009	12-15	8.80
F-010	16-39	15.13
F-011	40-44	21.32
F-012	45-49	8.96
F-013	50-56	10.34
F-014	MeOH eluted	35.92

From F-007, the white amorphous compound was precipitated. It yielded 200 mg (2 x 10^{-3}% based on dry weight of *B.biternata*) and was named as BB-1. This compound was then isolated and identified by means of gas chromatography compared with authentic long chain alcohols. (Table 4 and Figure 4,8 and 9)

BB-1 was identified as mixture of $C_{26\text{-}30}$ long chain alcohols as followed

$C_{26}H_{53}OH$ (hexacosanol)	4.8%
$C_{28}H_{57}OH$ (octacosanol)	68.7%
$C_{29}H_{59}OH$ (nonacosanol)	2.9%
$C_{30}H_{51}OH$ (triacotanol)	23.6%

From F-008, the white compound was crystallized. It was recrystallized with hexane as white needles. It yielded 70 mg (7 x 10^{-4}% based on dry weight of *B.biternata*), was named as BB-2, and was identified as the mixture of stigmasterol and β-sitosterol (74.2 and 25.8% respectively) by means of gas chromatography compared with authentic steroids. (Table 5 and Figure 14-15).

Table 4 The relationship between log retention times and the numbers of carbons of the authentic long chain alcohols and BB-1

Chemical compound	Retention time (minute)	log Rt	NO. of C	Area under the curve	Relative amount (%)
tetradecanol	0.91	-0.0410	14		
hexadecanol	1.21	0.0828	16		
octadecanol	1.73	0.2380	18		
eicosanol	2.63	0.4200	20		
docosanol	4.19	0.6222	22		
triacotanol	32.39	1.5104	30		
BB-1	11.67	1.0671	26	23920	4.8
	19.2	1.2833	28	342523	68.7
	25.5	1.4065	29	14583	2.9
	32.57	1.5128	30	117733	23.6

Table 5 The retention times of the authentic steroids and BB-2 from gas chromatogram

Chemical compound	Retention time (minute)	Log Rt	Area under curve	Relative amount (%)
cholesterol	13.87	1.1421	136213	
campesterol	18.27	1.2617	143527	
stigmasterol	19.41	1.2880	367845	
β-sitosterol	21.97	1.3418	1006470	
BB-2	19.20	1.2833	445178	74.2
	22.07	1.3438	154840	25.8

4.4 The Isolation of BB-3

Fraction F-005 (80 g.) was divided into 4 g per portion. Each one was fractionated by gel filtration chromatography using column of Sephadex LH-20 (100 g , 3.5 x 60 cm). The eluant was a mixture of methanol : chloroform (1:2). The fractions were collected based on the color bands (approximately 15 ml per fraction). The eluates were examined by TLC and the fractions showing the same pattern were combined and designated as shown in Table 6.

Table 6 The combined fractions from F-005

Fractions	Number of Eluates	Weight (g)
F-025	1-2	14
F-026	3-4	25
F-027	5-8	15
F-028	9-13	4
F-029	14-17	7
F-030	17-20	6

The TLC chromatogram of fraction F-028 showed that there were three major yellow compounds. The isolation for one yellow compound was carried out by a three-step process.

This fraction (4 g) was equally divided into 2 portions, and fractionated by column chromatography using the column of silica gel (55 g, 5x7.5 cm) with a gradient system of 2-40% methanol in chloroform as an eluant. 40 ml of Eluates were collected based on the color band. The eluates No. 38-61, that showed one yellow spot in TLC, were combined and named as F-031. This fraction (1.06 g.) was further isolated using a column of sephadex LH-20 (2x77 cm.) with 10% methanol in chloroform as an eluant. The yellow band was separated out and examine to combine. The combined fraction was purified using a column of sephadex LH-20 (2x70 cm) with 5% methanol in chloroform as an eluant. The eluates were collected depend on the color bands (approximately 15 ml per fraction).

One yellow compound was crystallized from fractions 4-6. It yield 8 mg (8 x 10^{-5}% based on dry weight of *B.biternata*) and was named as BB-3. This compound was identified as (*Z*)-6-O-(4,6-O-diacetyl-β-D-glucopyranosyl)-6,7,3',4'-tetrahydroxyaurone.

4.5 The Isolation of BB-4

Since the TLC chromatogram of F-008 showed yellow spots ,one of them was isolated and named as BB-3,the other one was further isolated by a 4-step method.

The fraction F-028 (4g) was fractionated by column chromatography as descriped in section 4.4. The eluates No. 62-112 that showed the required spot were combined and named as F-032. This fraction (1.3 g) equally divided into 2 protions, and further purified by the chromatographic technique using a column of sephadex LH-20 (2x70 cm) with 50% methanol in chloroform as an eluant. 20 ml-Fractions were collected base on the color bands. The fractions of yellow compound, after examined by TLC, were combined and isolated by using a column of sephadex LH20 (2x70 cm) with methanol as an eluant. The fractions of one yellow compound were again combined and purified by using column of sephadex LH-20 (2x70 cm.) with 5% water in methanol as an eluant 15 ml of Eluates were collected and examined.

An orange non-crystallized compound was obtained from eluate 3-7. It yield 23 mg (2.3 x 10^{-4}% based on dry weight of *B.biternata*) and was named as BB-4. This compound was identified as *(Z)*-6-O-(6-O-acetyl-β-D-glucopyranosyl)-6,7,3',4'-tetrahydroxyaurone.

5. Characterization of the Isolated Compounds

5.1 Characterization of BB-1

BB-1 was obtained as white amorphous compound. It was soluble in chloroform.

EIMS ; *m/z* (relative intensity); Figure 7
139 (7), 125 (16), 111 (28), 97 (44), 83 (72), 71 (76), 69 (89), 57 (100), 55 (70) and 139 (96), 125 (15), 111 (42), 97 (80), 85 (23), 83 (61), 71 (36), 69 (50), 57 (100), 55 (80)

IR ; υ cm^{-1}, KBr disc; Figure 5
3400-3200, 2918, 2849, 1473, 1464, 1062, 720

1H NMR ; δ ppm, 200 MHz, in chloroform-d; Figure 6
0.85 (t, *J* = 6 Hz), 1.23 (br.s), 1.55 (br.s), 3.62 (t, *J* = 6 Hz)

5.2 Characterization of BB-2

BB-2 was crystallized as white needle in hexane. It was soluble in chloroform.

EIMS ; *m/z* (relative intensity); Figure 12

414 (8), 412 (10), 394 (96), 329 (6), 271 (13), 213 (11), 119 (36), 83 (74), 55 (100)

IR ; υ cm^{-1}, KBr disc; Figure 10
3500-3200, 2960-2860, 1640, 1460, 1380, 1370, 1060, 970-960, 840, 800

^1H NMR ; δ ppm, 200 MHz in chloroform-d; Figure 11
0.65-2.3 (overlapping), 3.51 (m), 4.98 (dd, J = 15.1, 8 Hz) 5.14 (dd, J = 15.1, 8 Hz), 5.33 (br.d)

^{13}C NMR ; δ ppm, 125 MHz in chloroform-d; Figure 12
11.85, 11.97, 12.03, 12.23, 18.77, 18.97, 19.03, 19.38, 19.80, 21.07, 21.21, 23.07, 24.29, 24.36, 25.38, 26.09, 28.23, 28.90, 29.16, 29.69, 31.65, 31.87, 31.90, 33.95, 36.14, 36.51, 37.25, 39.68, 39.78, 40.47, 42.21, 42.30, 50.14, 50.16, 51.23, 55.96, 56.07, 56.86, 71.78, 121.69, 129.28, 138.30, 140.76

5.3 Characterization of BB-3

BB-3 was crystallized in methanol as golden yellow needle. It was soluble in pyridine and methanol.

$APCI^+$; *m/z* (relative intensity); Figure 32
533 (22), 293 (30), 279 (100), 274 (18)

EIMS ; *m/z* (relative intensity);Figure 33
286 (2), 152 (3), 116 (8)

UV ; λ max nm (logε), in methanol; Figure 16
250 ((logε 4.03), 418 (logε 4.39)

; λ max nm , in methanol with $AlCl_3$; Figure 17
250, 458

; λ max nm , in methanol with $AlCl_3$ + HCl; Figure 17
250, 408

^1H NMR ; δ ppm, 500 MHz, in pyridine-d5; Figure 18
2.01(3H, s),2.03 (3H, s) 4.12 (1H, dd, J = 8.8, 8.2 Hz), 4.24 (1H, m), 4.37 (1H, dd, J = 9.1, 9.2 Hz), 4.42 (1H, dd, J = 12.2, 2.4 Hz), 4.64 (1H, dd, J = 12.2, 5.6 Hz), 5.52 (1H, t, J = 9.8 Hz), 5.69 (1H, d, J = 7.9), 7.13 (1H, d, J = 8.2 Hz), 7.18 (1H, s), 7.36 (1H, d, J = 8.3 Hz), 7.46 (1H, d, J = 8.3 Hz), 7.60 (1H, dd, J = 8.2, 2 Hz), 8.09 (1H, d,J = 2 Hz)

^{13}C NMR ; δ ppm, 125 MHz, in Pyridine-d5;Figure 24
20.68, 20.81, 63.04, 71.56, 73.09, 74.56, 75.30, 103.2, 113.60, 113.84, 114.40, 116.95, 119.14, 119.35, 124.67, 125.62 ,133.54, 146.83, 147.49, 149.62, 153.06, 156.22, 170.55, 170.34, 184.00

5.4 Characterization of BB-4

BB-4 was obtained as an orange compound. It was soluble in water, methanol and pyridine.

APCI⁻ ; *m/z* (relative intensity); Figure 41
489 (100)

EIMS ; *m/z* (relative intensity);Figure 42
286 (12), 152 (12), 116 (25)

UV ; λ max nm (logε), in methanol; Figure 34
244 (logε 3.99), 268 (logε 3.9), 337 (logε 4.06), 409 (logε 4.26)
; λ max nm , in methanol with $AlCl_3$; Figure 35
291, 333, 452
; λ max nm , in methanol with $AlCl_3$ + HCl; Figure 35
275, 328, 413

IR ; υ cm^{-1}, KBr disc; Figure 36
3400, 1730, 1647, 1609, 1518, 1448, 1370, 1282, 1173, 1131, 1076, 1040

^{1}H NMR ; δ ppm, 500 MHz, in pyridine-d5 : methanol-d4 (1:5); Figure 34
2.05 (3H,s), 3.58 (1H, dd, *J* = 8.9, 9.1), 3.73-3.75 (2H, overlapping), 3.85 (1H, ddd, *J* = 9.6, 6.7, 2.1 Hz), 4.38 (1H, dd, *J* = 11.9, 6.4 Hz), 4.56 (1H, dd, *J* = 11.9, 1.9 Hz), 5.15 (1H, d, *J* = 7.4), 6.81 (1H, s), 6.93 (H, d, *J* = 8 Hz), 7.16 (1H, d, *J* = 8.3 Hz), 7.30 (1H, d, *J* = 8.6), 7.38 (1H, dd, *J* = 8,1.8 Hz), 7.67(H,d, *J* = 1.8 Hz)

^{13}H NMR : δ ppm, 125 MHz, in pyridine-d5 :methanol-d4 (1:5); Figure 36
20.8, 64.65, 71.55, 74.81, 75.80, 77.58, 103.18, 113.61, 115.32, 115.53, 116.89, 119.23, 119.44, 125.36, 126.65, 134.64, 147.02, 147.39, 149.94, 153.54, 156.21, 172.17, 185.07