CHAPTER III

RESULTS AND DISCUSSION

Although the chemical constituents from the stems and leaves of *C. fenestratum* have been reported, [13,14,18-21] the information involving the biological activity study has not been presented. Previously, a major compound in the class of protoberberine alkaloids has been isolated and characterized from the stems; nevertheless, various biological activities of these compounds have not been thoroughly studied. Thus, in this research, the information of chemical constituents and their biological activity from the stems of *C. fenestratum* would be rationalized.

3.1 The Result of Extraction

The stems of *C. fenestratum* were extracted following the procedures described in Chapter 2. The results of extraction are summarized as shown in Table 3.1.

Table 3.1 The extraction of the stems of *C. fenestratum* (2.6 Kg)

Extracts	Fraction	Weight (g)	%w/w
Ethanol	I	380	14.6
Precipitate	II	104	4.0
Dichloromethane	III	7	0.3
Ethyl acetate	IV	3	0.1
Butanol	V	152	5.8

The result of extraction indicated that the major constituents of this plant were in the polar parts: butanol extract and precipitate fraction. Especially, the precipitate was obtained when the ethanolic extract was treated with 1 N hydrochloric acid. It was possible that the precipitate (Fraction II) contained many compounds in the form

of salt or as a single compound, which were capable of precipitating in acidic condition. The other parts: dichloromethane and ethyl acetate extracts had a tiny amount compared with the butanol and precipitate portions.

3.2 The Results of the Biological Activity Screening Tests

3.2.1 Brine Shrimp Cytotoxic Lethality Test

The ethanolic extract of *C. fenestratum* was preliminarily screened for cytotoxicity against brine shrimp *Artemia salina* Linn. and *Vero cell* (*Vero cell* line ATCC CCL-81) according to the procedure elaborated in Chapter 2. The results are displayed in Table 3.2.

Table 3.2 The results of cytotoxicity against brine shrimp *Artemia salina* Linn. and *Vero cell* of ethanolic extract (Fraction I)

Cytotoxicity test	LC_{50} (μg/mL) [a]	IC_{50} (μg/mL) [b]
Brine shrimp	453	-
Vero cell	-	>50

[a] LC_{50} (μg/mL) Activity

<1000 Low activity

<100 Medium activity

<10 High activity

[b] non toxic concentration at >50 % inhibition

According to the above result, Fraction I exhibited low toxic activity against brine shrimp (LC_{50} < 1000 μg/mL). In addition, Fraction I displayed cytotoxicity against *Vero cell* with IC_{50} value more than 50 μg/mL. Therefore, it could be concluded that the ethanolic extract possessed less toxicity. This outcome was very interesting since if this extract showed remarkable activity for other bioactivities, it would exhibit low toxicity to cells.

3.2.2 Anti Cell Line Cytotoxicity Test

The *in vivo* cytotoxicity test against three human tumors was tested. The results are shown in Table 3.3.

Table 3.3 The results of anticell line cytotoxicity test of ethanolic extract (Fraction I)

Cancer cell lines	IC_{50} (μg/mL)[a]	Activity
KB	2.20	Strong
BC-1	3.50	Strong
NCl-H187	0.02	Strong

[a] IC_{50} (μg/mL) — Activity
- > 20 — Inactive
- >10-20 — Weakly active
- 5-10 — Moderately active
- <5 — Strongly active

The results from the above data revealed that Fraction I exhibited impressive anticancer activity against 3 strains of cell lines tested: KB (Human mouth carcinoma), BC-1 (Breast cancer) and NCl-H187 (Small cell lung cancer) with IC_{50} value less than 5 μg/mL (strong activity).

3.2.3 Antifungal, Antiviral, Antimalarial and Antituberculosis Test

Fraction I was screened for *in vivo* antifungal test against *Candida albicans* operated by using the NCCLS and antiviral test against herpes simplex virus type 1 ATCC-260 carried out by using microlitre colorimetric assay. In addition, antimalarial activity against *Plasmodium faciparum*, K-1 strain of Fraction I was also tested by using the *in vivo* antimalarial test as well as screening for anti-tuberculosis substances from Fraction I. All of the results are exhibited in Table 3.4.

Table 3.4 The results of *in vivo* antifungal test against *Candida albicans,* antiviral test against herpes simplex virus type 1 ATCC-260, *in vivo* antimalarial test against *Plasmodium faciparum,* K-1 strain and screening for antituberculosis activity of ethanolic extract (Fraction I)

Activity test	IC_{50} (μg/mL)[a]	EC_{50} (μg/mL)[b]	MIC (μg/mL)[c]	Activity
Antifungal	32.76	-	-	Weakly active
Antiviral	-	-	-	Inactive
Antimalarial	-	0.54	-	Active
Anti-tuberculosis	-	-	200	Active

[a] IC_{50} (μg/mL)	Activity	[b] % EC_{50} (μg/mL)	Activity
> 50	Inactive	> 20	Inactive
20-50	Weakly active	≤ 20	Active
10-20	Moderately active	[c] MIC (μg/mL)	
<10	Strongly active	> 200	Inactive
		≤ 200	active

According to the above results of screening test, Fraction I showed activity against *Candida albicans* with IC_{50} value of 32.76 μg/mL which could be interpreted that it exhibited weak activity. On the other hand, Fraction I was inactive against herpes simplex virus type 1 ATCC-260. However, Fraction I was active against *Plasmodium faciparum* with EC_{50} value of 0.54 μg/mL and showed antituberculosis activity with MIC value of 200 μg/mL. Consequently, it could be concluded that this plant exhibited interesting bioactivity against antifungal, antimalarial and antituberculosis.

From the above results involving the preliminarily screening test of Fraction I, it was found that this plant gave attractive bioassay results, especially, the bioactivities against anticancer cell lines, antimalarial, antifungal, and antituberculosis activity. In addition, this plant also displayed low toxicity against brine shrimp and *Vero cell.* The trend outline from preliminarily screening test pointed toward the presence of bioactive compounds in the stems of *C. fenestratum.* Therefore, this research emphasized the investigation of the chemical constituents and searching for bioactive compounds from the stems of this plant.

3.3 Chemical Constituents Studies of the Stems of *C. fenestratum*

3.3.1 Separation of the Precipitate (Fraction II)

The precipitate (Fraction II) as yellow-gray solid, 70 g was separated by quick column chromatography using silica gel 60G Art.7731 as an adsorbent. The column was initially eluted by hexane, followed by increasing polarity of solvent by the addition of dichloromethane. Eluting solvent was collected for each fraction approximately 1000 mL and then concentrated under vacuum to a small volume. Each fraction was monitored by TLC and the equivalent fractions were combined. The results of the separation of Fraction II are shown in Table 3.5.

Table 3.5 The separation of Fraction II by silica gel quick column chromatography

Eluents (% v/v)	Fraction	Remarks	Weight (g)
100% Hexane	IIA	white amorphous solid + fat + oil	trace
30% CH_2Cl_2-Hexane	IIB	pale yellow oil + white amorphous	1.95
50% CH_2Cl_2-Hexane	IIC	red brown viscous liquid	2.04
80% CH_2Cl_2-Hexane	IID	yellow viscous liquid	1.52
100% CH_2Cl_2	IIE	orange solid	2.04
10% EtOAc-CH_2Cl_2	IIF	white ppt + orange solid	3.12
		(Mixture 1)	
30% EtOAc-CH_2Cl_2	IIG	yellow brown viscous liquid	0.94
50% EtOAc-CH_2Cl_2	IIH	yellow orange solid	2.93
80% EtOAc-CH_2Cl_2	IIJ	orange brown solid	0.62
100% EtOAc	IIK	orange brown solid	0.38
10% MeOH-EtOAc	IIL	yellow brown mass	0.25
30% MeOH-EtOAc	IIM	yellow brown mass	0.87
40% MeOH-EtOAc	IIN	dark yellow solid	3.01
		(Compound 2, Compound 3)	
50% MeOH-EtOAc	IIO	dark yellow solid	4.52
100% MeOH	IIP	yellow ppt + dark brown solid	32.01
		(Compound 2)	

3.3.1.1 Separation of Fraction IIC

Fraction IIC was subjected to silica gel column chromatography using a mixture of dichloromethane and hexane as eluents. The results of separation are displayed in Table 3.6.

Table 3.6 The separation of Fraction IIC

Eluents (%v/v)	Fraction	Remarks	Weight (g)
100% Hexane	1-5	white amorphous solid + oil	trace
10% CH_2Cl_2-Hexane	6-9	orange viscous liquid	0.25
20% CH_2Cl_2-Hexane	10-13	red brown viscous liquid	0.31
30% CH_2Cl_2-Hexane	14-17	red brown viscous liquid	0.14
40% CH_2Cl_2-Hexane	18-21	yellow viscous liquid	0.21
50% CH_2Cl_2-Hexane	22-25	red brown viscous liquid	trace
70% CH_2Cl_2-Hexane	26-28	orange brown viscous liquid	0.15
100% CH_2Cl_2	29-32	yellow brown viscous liquid	trace
5% EtOAc-CH_2Cl_2	33-36	pale yellow viscous oil	trace
10% EtOAc-CH_2Cl_2	34-50	pale yellow viscous oil	trace

3.3.1.2 Separation of Fractions IIE and IIF

Owing to the fact that Fractions IIE and IIF displayed the characteristic pattern of TLC alike. Therefore, these fractions were integrated. A portion (5 g) of Fractions IIE and IIF were reseparated by silica gel column chromatography. The results of separation are displayed in Table 3.7.

Table 3.7 The separation of Fractions IIE and IIF

Eluents (%v/v)	Fraction	Remarks	Weight (g)
100% CH_2Cl_2	1-5	white oil	trace
3% EtOAc-CH_2Cl_2	5-10	white solid + colorless oil **(Compound 4)**	0.25
5% EtOAc-CH_2Cl_2	10-19	white ppt + brown solid **(Mixture 1)**	2.13
10% EtOAc-CH_2Cl_2	20-29	white solid + yellow oil	0.22
20% EtOAc-CH_2Cl_2	30-46	red brown oil	0.53
30% EtOAc-CH_2Cl_2	47-56	red brown oil	0.31
40% EtOAc-CH_2Cl_2	57-66	red brown oil	0.44
50% EtOAc-CH_2Cl_2	67-76	yellow orange viscous liquid	0.25
80% EtOAc-CH_2Cl_2	77-86	brown viscous liquid	0.17
100% EtOAc	87-105	brown viscous liquid	0.14

3.3.1.3 Separation of Fraction IIG

The thin layer chromatography of Fraction IIG presented at least two spots using 10% methanol-dichloromethane as solvent system. This fraction was reseparated by column chromatography using silica gel as an adsorbent. The results of separation are exhibited in Table 3.8.

Table 3.8 The separation of Fraction IIG

Eluents (%v/v)	Fraction	Remarks	Weight (g)
100% CH_2Cl_2	1-8	white oil	trace
10% EtOAc -CH_2Cl_2	9-14	pale yellow oil	trace
20% EtOAc -CH_2Cl_2	15-16	orange viscous liquid	trace
30% EtOAc -CH_2Cl_2	17-22	red brown solid	0.12
50% EtOAc -CH_2Cl_2	23-28	red brown solid	0.13
80% EtOAc -CH_2Cl_2	29-40	red brown solid	0.22
100% EtOAc	41-54	orange brown solid	0.15
10% MeOH-CH_2Cl_2	55-67	yellow brown solid	0.17
30% MeOH-CH_2Cl_2	68-72	red brown solid	trace

3.3.1.4 Separation of Fraction IIH

When Fraction II was eluted with 50% EtOAc-CH_2Cl_2, yellow orange solid mass (Fraction IIH) was gained. Thin layer chromatography indicated that there were at least two components in this fraction. This fraction was further separated by rechromatographed over silica gel using dichloromethane, a mixture of dichloromethane containing increased proportion of ethyl acetate and a mixture of ethyl acetate and methanol as eluents. The results of separation are displayed in Table 3.9.

Table 3.9 The separation of Fraction IIH

Eluents (%v/v)	Fraction	Remarks	Weight (g)
100% CH_2Cl_2	1-3	white blot	trace
3% EtOAc-CH_2Cl_2	4-7	pale yellow viscous liquid	trace
5% EtOAc-CH_2Cl_2	8-11	red brown viscous liquid	0.15
10% EtOAc-CH_2Cl_2	12-15	yellow viscous liquid	0.13
20% EtOAc-CH_2Cl_2	16-17	yellow viscous liquid **(Mixture 5)**	0.67
30% EtOAc-CH_2Cl_2	20-23	pale yellow solid	0.11
50% EtOAc-CH_2Cl_2	24-30	orange brown solid **(Compound 6)**	0.19
70% EtOAc-CH_2Cl_2	31-42	brown solid **(Compound 7)**	0.92
90% EtOAc-CH_2Cl_2	43-50	brown solid	0.15
100% EtOAc	51-58	gray brown viscous liquid	trace

3.3.1.5 Separation of Fractions IIJ and IIK

Fractions IIJ and IIK were combined due to their TLC behavior alike and rechromatographed using silica gel as an adsorbent. The column was eluted with gradient solvent mixture of EtOAc and CH_2Cl_2. The results of separation are displayed in Table 3.10.

Table 3.10 The separation of Fractions IIJ and IIK

Eluents (%v/v)	Fraction	Remarks	Weight (g)
100% CH_2Cl_2	1-12	yellow blot	trace
10% EtOAc-CH_2Cl_2	13-29	yellow oil+ wax	trace
30% EtOAc-CH_2Cl_2	30-54	red brown viscous liquid	0.10
50% EtOAc-CH_2Cl_2	55-71	brown viscous liquid	0.13
80% EtOAc-CH_2Cl_2	72-92	red brown viscous liquid	1.46
100% EtOAc	93-113	red yellow viscous liquid	trace
1% MeOH-CH_2Cl_2	114-134	red yellow viscous liquid	trace
	135-153	yellow viscous liquid	trace
5% MeOH-CH_2Cl_2	154-176	white ppt + yellow viscous liquid **(Mixture 8)**	0.87
10% MeOH-CH_2Cl_2	156-183	orange brown solid	trace
20% MeOH-CH_2Cl_2	183-195	yellow brown blot	trace
30% MeOH-CH_2Cl_2	196-220	orange brown solid	0.13
50% MeOH-CH_2Cl_2	221-220	colorless ppt + dark brown viscous liquid	0.35

3.3.1.6 Separation of Fraction IIO

Fraction IIO contained yellow solid in dark yellow mixture (4.52 g, 4.35 % w/w of Fraction II). The examination of this fraction by TLC revealed that there were at least three components in this fraction. It showed the same R_f value as Compound 2. Thus, this fraction was reseparated by using silica gel column chromatography and eluted with a mixture of dichloromethane containing increasing proportions of methanol. The results of separation are shown in Table 3.11.

Table 3.11 The separation of Fraction IIO

Eluents (%v/v)	Fraction	Remarks	Weight (g)
80% Hexane -CH_2Cl_2	1-20	white gray blot	trace
100% CH_2Cl_2	21-45	orange brown viscous liquid	0.25
1% MeOH-CH_2Cl_2	46-82	yellow red viscous liquid	0.37
3% MeOH-CH_2Cl_2	83-119	yellow mixture solid	0.42
5% MeOH-CH_2Cl_2	120-145	yellow mixture solid	0.50
10% MeOH-CH_2Cl_2	146-167	yellow ppt + gray solid **(Compound 2)**	1.46
30% MeOH-CH_2Cl_2	168-206	yellow brown solid	0.51
50% MeOH-CH_2Cl_2	207-220	yellow orange solid	0.17

3.3.2 Separation of the Dichloromethane (Fraction III) and Ethyl acetate (Fraction IV) Crude Extracts

According to TLC analysis, Fraction III had the characteristic pattern of TLC similar to that of Fraction IV. Fraction III was then integrated with Fraction IV. Column chromatography over silica gel was conducted using hexane, dichloromethane, ethyl acetate and methanol and gradient solvent mixtures as eluents. On the basis of their TLC behavior, 20 fractions were combined to yield 10 portions (A-J). The results of separation are exhibited in Table 3.12.

Table 3.12 The separation of Fractions III and IV

Eluents (%v/v)	Fraction	Remarks	Portion	Weight (g)
50% CH_2Cl_2-Hexane	1-15	colorless oil	A	trace
100% CH_2Cl_2	16-18	colorless oil		
	19-21	pale red brown solid	B	0.46
25% EtOAc-CH_2Cl_2	22-27	red brown solid		
	28-31	red brown solid	C	0.39
30% EtOAc-CH_2Cl_2	32-36	pale brown solid		
40% EtOAc-CH_2Cl_2	37-42	yellow brown solid **(Compound 6)**	D	1.95
50% EtOAc-CH_2Cl_2	43-46	orange brown solid	E	0.65
60% EtOAc-CH_2Cl_2	47-49	yellow orange viscous liquid		
	50-51	yellow orange viscous liquid	F	0.27
70% EtOAc-CH_2Cl_2	52-57	yellow orange viscous liquid		
80% EtOAc-CH_2Cl_2	58-61	yellow brown solid	G	0.55
90% EtOAc-CH_2Cl_2	62-66	yellow brown solid		
100% EtOAc	67-71	pale yellow brown	H	1.22
5% MeOH-EtOAc	72-76	orange brown solid		
10% MeOH-EtOAc	77-81	red brown solid	I	2.80
20% MeOH-EtOAc	82-96	red brown solid		
30% MeOH-EtOAc	97-99	dark brown solid		
50% MeOH- EtOAc	100-125	dark yellow solid	J	1.39

3.3.2.1 Separation of Portion I

Portion I was rechromatographed over silica gel with dichloromethane and methanol using gradient solvent mixture. The results of separation are displayed in Table 3.13.

Table 3.13 The separation of Portion I

Eluents (%v/v)	Fraction	Remarks	Weight (g)
100% CH_2Cl_2	1-14	pale yellow oil	trace
1% MeOH-CH_2Cl_2	15-29	yellow solid + orange solid	trace
3% MeOH-CH_2Cl_2	30-44	yellow orange solid	0.23
5% MeOH-CH_2Cl_2	35-40	yellow ppt + yellow orange solid **(Compound 9)**	0.40
	41-49	white ppt + pale yellow solid **(Compound 9)**	0.61
10% MeOH-CH_2Cl_2	50-64	pale yellow solid	0.14
20% MeOH-CH_2Cl_2	65-79	pale yellow solid **(Compound 10)**	0.72
30% MeOH-CH_2Cl_2	80-99	yellow orange solid	0.11
40% MeOH-CH_2Cl_2	100-114	red brown solid	0.13
50% MeOH-CH_2Cl_2	115-135	brown solid	trace

3.4 Purification, Properties and Structural Elucidation of Isolated Substances

3.4.1 Purification, Properties and Structural Elucidation of Mixture 1

After recrystallized with a hot mixture of methanol-ethyl acetate for several times, white solid, m.p.. 157-161 °C, (192 mg, 0.18 % w/w of Fraction II) was obtained and designated as Mixture 1. It showed two spots overlapped on TLC with R_f value of 0.75 (10% methanol-dichloromethane). This mixture was soluble in dichloromethane and ethyl acetate but slightly soluble in methanol. It gave a positive test with Liebermann-Burchard's reagent but gave a negative test with Dragendroff's reagent.

The IR spectrum (Fig. 1) exhibited the absorption band belonging to OH stretching vibration at 3400-3500 cm^{-1} (br), C-O stretching vibration at 1048 cm^{-1} and 1020 (s) and C=C stretching vibration at 1652 cm^{-1}. In addition, the stretching and bending vibration of $-CH_3$ were detected at 2856-2949 cm^{-1} and 1300-1460 cm^{-1}. According to the color test result and the spectroscopic data, this mixture should contain steroidal moiety.

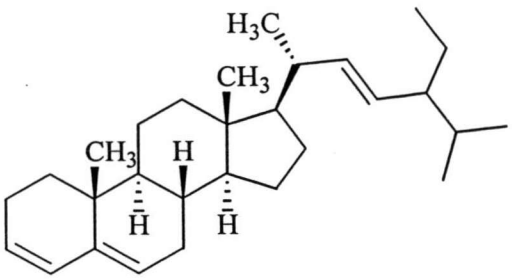
The GC-MS analysis (Fig. 2) displayed five peaks. Peak nos. 4 and 5 were major components in this mixture. According to the search on MS library database

(NIST) (Fig. 3), it was suggested that Mixture 1 consist of five steroids. The results of GC-MS analysis are shown in Table 3.14.

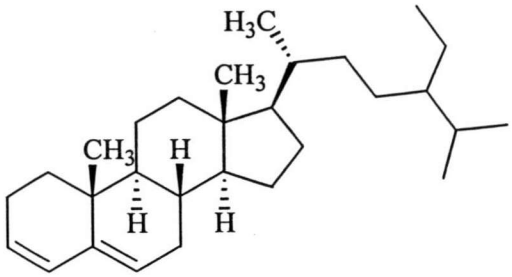
Table 3.14 The results of GC-MS analysis

Peak no.	Retention time, t_R (min)	% Composition	MW	Name
1	5.89	4.89	394	Stigmastan-3,5,22-triene
2	6.93	3.76	396	Stigmastan-3,5-diene
3	8.56	4.89	414	β-Sitosterol
4	8.92	44.74	400	Campesterol
5	9.87	41.73	412	Stigmasterol

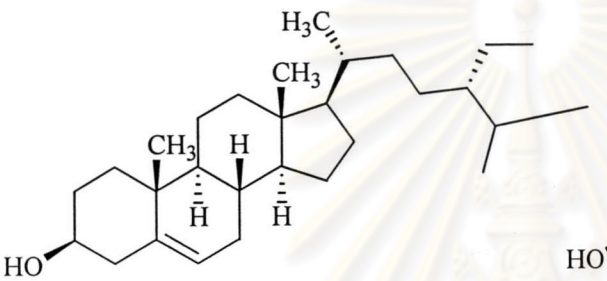
According to the above data, it could be concluded that Mixture 1 was composed of stigmastan-3,5,22-triene, stigmastan-3,5-diene, β-sitosterol, campesterol and stigmasterol. Campesterol and stigmasterol were found to be major components in this mixture.



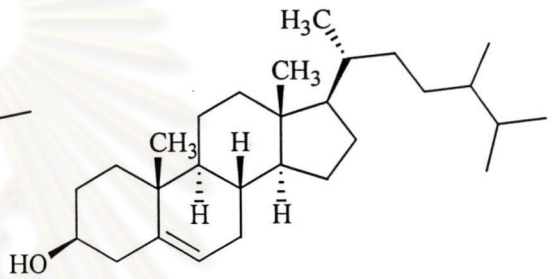
Stigmastan-3,5,22-triene



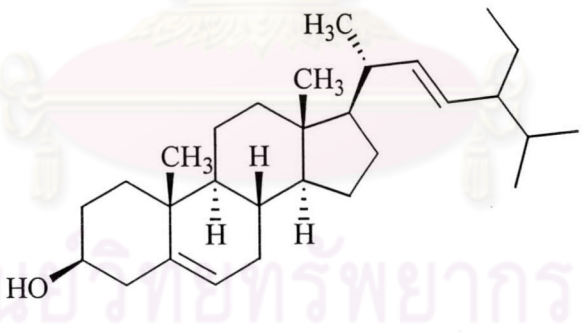
Stigmastan-3,5-diene



β-Sitosterol



Campesterol



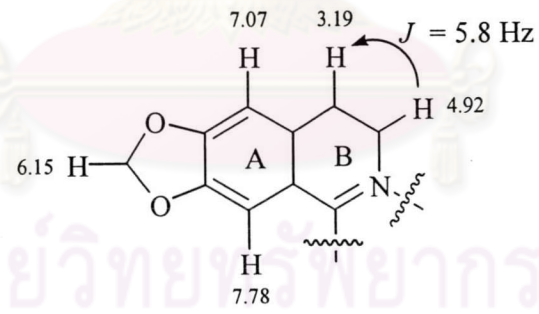
Stigmasterol

Mixture 1

3.4.2 Purification, Properties and Structural Elucidation of Compound 2

Compound 2 was obtained as yellow solid from Fractions IIN and IIP. After recrystallized from methanol-dichloromethane for several times, yellow needle, m.p. 221-222 °C, (19.85 g, 19.08 %w/w of Fraction II) was obtained. It exhibited a single spot on TLC with R_f value of 0.67 in 20% methanol in dichloromethane as a solvent system. Compound 2 was soluble in various solvents, such as chloroform, ethyl acetate and methanol but slightly soluble in dichloromethane. This compound gave a positive test with Dragendroff's reagent, which suggested the presence of alkaloid nucleus.

The ^{1}H NMR (DMSO-d_6) spectrum (Fig. 4) displayed the characteristic feature of protoberberine alkaloid signals for methoxy, methylene dioxy and four aromatic protons.[13,14] The one singlet signal at δ 6.15 ppm, corresponding to 2H, could be assigned for a methylene group of methylene dioxy moiety. The two singlet signals at δ 7.07 and 7.78 ppm also showed the feature of aromatic ring protons. The ^{1}H NMR spectrum further displayed the two triplet 2H proton signals at δ 3.19 and 4.92 ppm with the coupling constant 5.8 Hz. The splitting pattern and the coupling constant were indicative of two methylene groups of protoberberine.



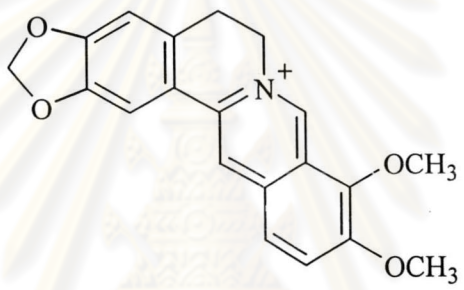
In addition, the two singlet signals at δ 8.93 and 9.88 ppm exhibited the feature of aromatic ring protons. The other aromatic protons were detected as the two doublet signals at δ 8.02 and 8.19 ppm with the coupling constant 9.2 Hz. The signals at δ 4.07 and 4.05 ppm were clearly indicated the presence of two methoxy groups.

The ^{13}C NMR (DMSO-d_6) spectrum (Fig. 5) displayed 20 carbon signals. The spectrum revealed the chemical shifts of carbons very close to those of protoberberine.[13,14] From the literature survey, it was found that protoberberine alkaloids are widely distributed in the *Coscinium* plants. The ^{13}C NMR chemical

shifts were assigned by comparison with those reported in literature. The ^{1}H and ^{13}C NMR chemical shift assignments of Compound 2 are shown in Table 3.15.

The mass spectrum (Fig. 6) also supported the above evidence. It gave the fragmentation ion at *m/z* 321, which was the base peak. However, from literature survey, it was reported that berberine exhibited the molecular ion peak at *m/z* 335.[13,24] The possible mass fragmentation pattern of this compound is elaborated in Scheme 3.1.

The structure of Compound 2 was confirmed by direct *co*-TLC with the authentic berberine. By means of TLC and spectral comparison, Compound 2 was obviously concluded to be berberine, the structure is depicted as shown below.



Compound 2: Berberine

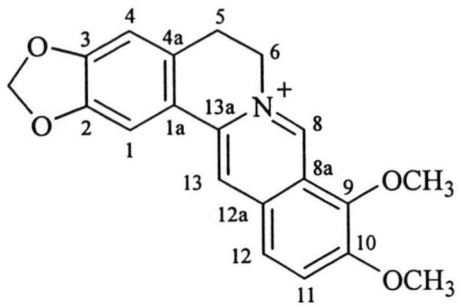
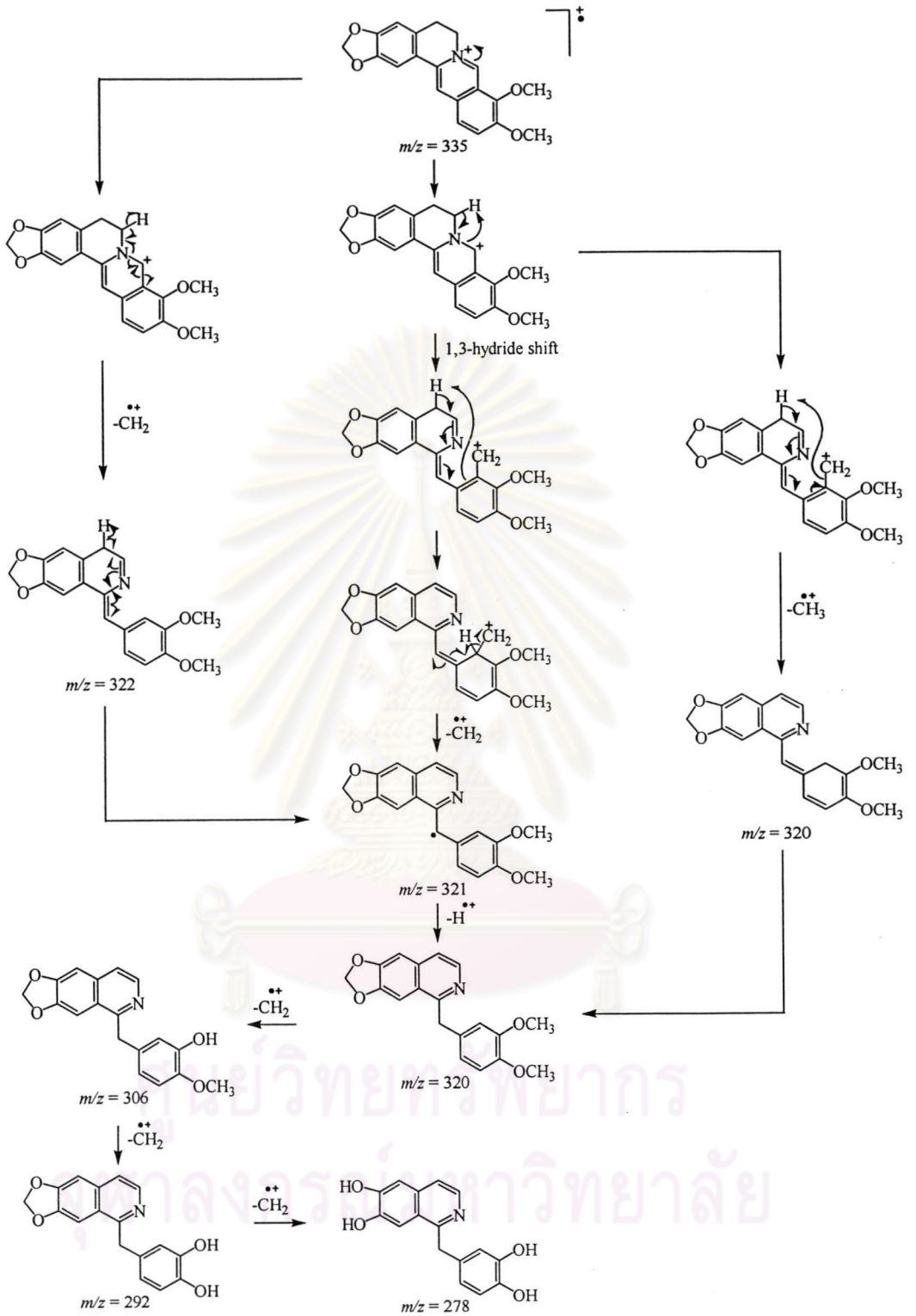


Table 3.15 The comparison of the ^{1}H and ^{13}C NMR chemical shifts of Compound 2 with berberine.[13,14]

Position	^{1}H NMR		^{13}C NMR	
	Compound 2	Berberine	Compound 2	Berberine
1	7.78 (1H, *s*)	7.81 (1H, *s*)	111.2	105.5
1a	-	-	126.2	120.5
2	-	-	153.4	147.7
3	-	-	155.5	149.8
4	7.07 (1H, *s*)	7.10 (1H, *s*)	114.2	108.5
4a	-	-	136.4	130.7
5	3.19 (2H, *t*, J = 5.8 Hz)	3.22 (2H, *t*, J = 6 Hz)	32.1	26.4
6	4.92 (2H, *t*, J = 5.8 Hz)	4.95 (2H, *t*, J = 6 Hz)	60.9	55.2
8	9.88 (1H, *s*)	9.91(1H, *s*)	151.1	145.5
8a	-	-	127.1	121.4
9	-	-	149.4	143.7
9-OCH_3	4.08 (3H, *s*)	4.11 (3H, *s*)	67.7	62.0
10	-	-	156.1	150.4
10-OCH_3	4.06 (3H, *s*)	4.08 (3H, *s*)	62.8	57.1
11	8.19 (1H, *d*, J = 9.2 Hz)	8.22 (1H, *d*, J = 9 Hz)	132.4	126.7
12	7.99 (1H, *d*, J = 9.2 Hz)	8.01 (1H, *d*, J = 9 Hz)	129.3	123.6
12a	-	-	138.7	132.9
13	8.93 (1H, *s*)	8.96 (1H, *s*)	125.9	120.3
13a	-	-	143.2	137.5
OCH_2O	6.16 (2H, *s*)	6.19 (2H, *s*)	107.8	102.1



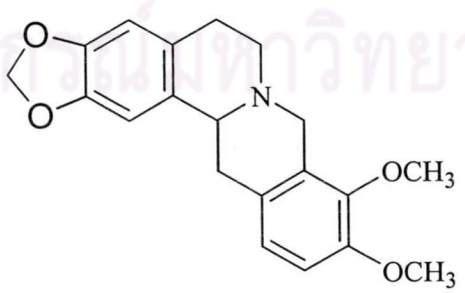
Scheme 3.1 The possible mass fragmentation pattern of Compound 2

3.4.3 Reduction of Compound 2

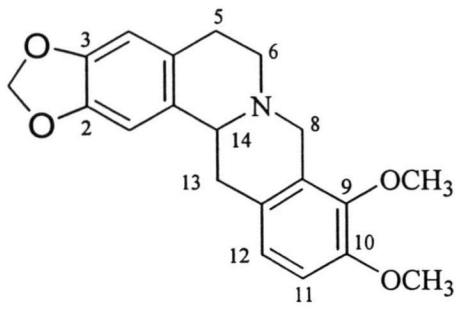
Reduction of Compound 2 (100 mg) with $NaBH_4$ afforded 75 mg of Compound 2A as pale yellow solid, m.p. 234-237 °C (dec.), (75 % w/w of Compound 2). It exhibited a single spot on TLC with R_f value of 0.73 in 4% methanol in dichloromethane as a solvent system. Compound 2A was soluble in various solvents, such as chloroform, methanol and dichloromethane.

The ^{1}H NMR ($CDCl_3$) spectrum (Fig. 7) displayed the singlet signal at δ 5.96 ppm, corresponding to 2H, which could be assigned for a methylene group of methylenedioxy moiety. The two singlet signals at δ 6.63 and 6.77 ppm and the two doublet signals at δ 6.83 and 6.90 ppm with the coupling constant 8.4 Hz also showed the feature of aromatic ring protons at C-1, C-4, C-11 and C-12, respectively.[13,14] The ^{1}H NMR spectrum displayed the two doublet of doublet signals at δ 3.23 and 4.28 ppm with the coupling constant 15.2 Hz which could be designated for 8-H*ax* and 8-H*eq* at C-8. The doublet signal (J = 16.0 Hz) at 3.57 ppm was also identified for 1H at C-14. The proton (13-H*ax*) at C-13 emerged at δ 3.26 ppm with the coupling constant 16.0 Hz and 2.8 Hz. The doublet signal at δ 3.57 ppm, which overlapped with 13-H*ax*, was indicated for equatorial proton (6-H*eq*) at C-6. The other axial proton (6-H*ax*) appeared at δ 2.86 ppm (multiplet). The multiplet signal, which appeared at δ 2.69 ppm, 3H, could be assigned for 5-H*pseudoax*, 5-H*pseudoeq* and 13-H*ax*.

From the ^{1}H NMR spectroscopic data, the complete assignment of Compound 2A (tetrahydroberberine) obtaining from the reduction of Compound 2 is shown in Table 3.16, its structure is exhibited below.



Compound 2A: Tetrahydroberberine



Compound 2A: Tetrahydroberberine

Table 3.16 The comparison of the ^{1}H NMR chemical shifts of Compound 2A with Compound 2

Position	^{1}H NMR	
	Compound 2A	Compound 2[a]
1	6.77 (1H, *s*)	7.78 (1H, *s*)
2	-	-
3	-	-
4	6.63 (1H, *s*)	7.07 (1H, *s*)
5*pseudoae*	2.69 (2H, *m*)	3.19 (2H, *t*, *J* = 5.8 Hz)
5*pseudoeq*		
6*ax*	2.86 (1H, *br t*, *J* = 12.4, 14.8 Hz)	4.92 (2H, *t*, *J* = 5.8 Hz)
6*eq*	3.57 (1H, *d*, *J* = 16 Hz)	
8*ax*	4.28 (1H, *d*, *J* = 15.2 Hz)[b]	-
8*eq*	3.23 (1H, *d*, *J* = 15.2 Hz)[b]	-
9-OCH_3	3.88 (3H, *s*)	4.08 (3H, *s*)
10-OCH_3	3.88 (3H, *s*)	4.06 (3H, *s*)
11	6.96 (1H, *d*, *J* = 8.4 Hz)	8.19 (1H, *d*, *J* = 9.2 Hz)
12	6.82 (1H, *d*, *J* = 8.4 Hz)	7.99 (1H, *d*, *J* = 9.2 Hz)
13*ax*	3.26 (1H, *dd*, *J* = 16.0, 2.8 Hz)	8.93 (1H, *s*)
13*eq*	2.69 (1H, *m*)	-
OCH_2O	5.96 (2H, *s*)	6.16 (2H, *s*)
14	3.57 (1H, *d*, *J* = 16 Hz)	-

[a]Chemical shifts in DMSO-d_6, [b]Assignment may be interchanged

3.4.4 Purification, Properties and Structural Elucidation of Compound 3

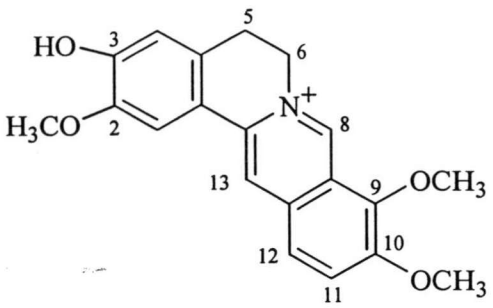
Compound 3 was obtained as yellow solid in mixed yellow-gray solid from Fraction IIN. After recrystallized by using a mixture of methanol-dichloromethane for several times, Compound 3 as yellow needle, m.p.. 240-243 °C, (14.6 mg, 0.014 % w/w of Fraction II) was gained. TLC analysis of Compound 3, using 20% methanol in dichloromethane as a solvent system, exhibited one spot with R_f value of 0.78. Compound 3 was soluble in various solvents, such as chloroform, ethyl acetate and methanol but slightly soluble in dichloromethane. This compound gave a positive test with Dragendroff's reagent representing the presence of alkaloid nucleus.

The ^{1}H NMR (DMSO-d_6) spectrum (Fig. 8) showed the characteristic feature of protoberberine alkaloid signals for methoxy, methylene dioxy and four aromatic protons like Compound 2. Its spectrum displayed the two singlet signals at δ 6.83 and 7.67 ppm, the two doublet signals at δ 7.99 and 8.18 ppm with the coupling constant 9.2 Hz and the two singlet signals at δ 8.95 and 9.83 ppm which could be assigned for aromatic ring protons. The two triplet 2H proton signals at δ 3.12 and 4.88 ppm with the coupling constant 5.9 Hz of two methylene groups of protoberberine were still observed. In addition, the three singlet signals at δ 3.92, 4.05 and 4.07 ppm were clearly indicated the presence of three methoxy groups.

When comparing the ^{1}H NMR spectroscopic data of Compound 3 with that of Compound 2 as displayed in Table 3.17, it was found that the singlet signal at δ 6.15 ppm (two protons of methylene dioxy) was not detected in the ^{1}H NMR spectrum of Compound 3. Three methoxy proton signals were observed in the the ^{1}H NMR spectrum of Compound 3 whereas Compound 2 displayed only two methoxy signal protons in its ^{1}H NMR spectrum. Hence, the methylene dioxy bridge in Comound 2 was not exist in Compound 3. The rest parts of Compound 3 exhibited similar feature as Compound 2.

The identification of Compound 3 was conducted by direct comparison of the ^{1}H NMR data with those of jatrorrhizine reported in literature as shown in Table 3.17.[13,14]

From all of the above information, Compound 3 was concluded to be jatrorrhizine. Its structure is showed below.



Compound 3: Jatrorrhizine

Table 3.17 The comparison of the ^{1}H and ^{13}C NMR chemical shifts of Compound 2, jatrorrhizine and Compound 3

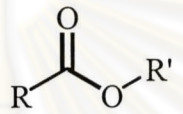
Position	Chemical shifts (ppm)		
	Compound 2	Jatrorrhizine	Compound 3
1	7.78 (1H, *s*)	7.45 (1H, *s*)	7.67 (1H, *s*)
2-OCH_3	-	3.94 (1H, *s*)	3.92 (3H, s)
3-OH	-	-	-
4	7.07 (1H, *s*)	6.65 (1H, *s*)	6.83 (1H, *s*)
5	3.19 (2H, *t*, J = 5.8 Hz)	-	3.12 (2H, *t*, J = 5.9 Hz)
6	4.92 (2H, *t*, J = 5.8 Hz)	-	4.88 (2H, *t*, J = 5.9 Hz)
8	9.88 (1H, *s*)	9.58 (1H, *s*)	9.83 (1H, *s*)
9-OCH_3	4.08 (3H, *s*)	4.17 (3H, *s*)	4.07 (3H, *s*)
10-OCH_3	4.06 (3H, *s*)	4.07 (3H, *s*)	4.05 (3H, *s*)
11	8.19 (1H, *d*, J = 9.2 Hz)	8.02 (1H, *d*, J = 9.2 Hz)	8.18 (1H, *d*, J = 9.2 Hz)
12	7.99 (1H, *d*, J = 9.2 Hz)	7.88 (1H, *d*, J= 9.2 Hz)	7.99 (1H, *d*, J = 9.2 Hz)
13	8.93 (1H, *s*)	8.56 (1H, *s*)	8.95 (1H, *s*)
OCH_2O	6.16 (2H, *s*)	-	-

3.4.5 Purification, Properties and Structural Elucidation of Compound 4

White solid in colorless oil was eluted from Fraction Nos. 5-10 with 3% ethyl acetate-dichloromethane of the combined extracts of Fractions IIE and IIF. After recrystallization with methanol-dichloromethane for several times, Compound 4 as white solid, m.p. 65-66 °C, (4.30 mg, 0.004 % w/w of Fraction II) was obtained. It showed one spot on TLC with R_f value of 0.66 in 10% methanol in dichloromethane as a solvent system. Compound 4 was soluble in dichloromethane, ethyl acetate and

methanol but slightly soluble in hexane. This compound gave a negative test with Dragendroff's and Liebermann-Burchard's reagents.

The IR spectrum (Fig. 9) revealed the absorption band belonging to C-H stretching vibration of CH_3- and -CH_2- at 2911 and 2851 cm^{-1} (s). The absorption band corresponded to C=O stretching vibration of ester was detected at 1707 cm^{-1} (s). The C-H bending vibration of –CH_2- and –CH_3 was perceived at 1462 and 1320 cm^{-1} (m). The additional absorption peak at 1299 cm^{-1} (m) was due to C-O stretching vibration. From this spectroscopic information suggested that Compound 4 be a long chain aliphatic ester, its structure is displayed below.



Compound 4: a long chain aliphatic ester

3.4.6 Purification, Properties and Structural Elucidation of Mixture 5

Mixture 5 was isolated from Fraction IIH eluted with 20% ethyl acetate-dichloromethane. After recrystallization for several times using a mixture of dichloromethane and ethyl acetate, Mixture 5 as white solid, m.p. 260-263 °C, (7.2 mg, 0.007 % w/w of Fraction II) was obtained. It showed one spot on TLC with R_f value of 0.58 in 10% methanol in dichloromethane as a solvent system. Mixture 5 was soluble in dichloromethane, ethyl acetate and methanol but slightly soluble in hexane. This mixture gave a violet color with Liebermann-Burchard's reagent representing the triterpene skeleton.

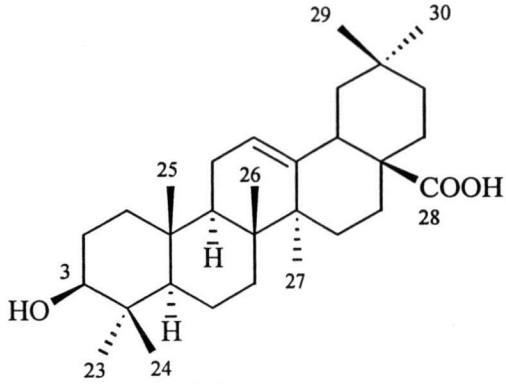
The IR spectrum (Fig. 10) indicated the characteristic features of triterpene skeleton,[30] corresponding to a color test. Its IR spectrum revealed the absorption band at 3400 cm^{-1} due to -OH functional group. The C-H stretching vibration and C-H bending vibration of –CH_2- and –CH_3 were observed at 2950 and 2850 cm^{-1}, respectively. The absorption bands originated from the C=O stretching vibration and C-H bending vibration of –CH_3 were detected at 1690 and 1460 cm^{-1}. The absorption band of the C-O stretching vibration and C-H bending vibration of gem-dimethyl were observed at 1190 and 1380, 1280 cm^{-1}. The additional absorption peaks at 1000 and 1032 cm^{-1} were due to C-OH vibration.

The ^{1}H NMR (DMSO-d_6) spectrum (Fig. 11) exhibited the triplet peak at δ 5.26 ppm (J = 3.6 Hz) which indicated an olefinic proton attached to a methylene group (-CH_2C<u>H</u>=C-). Two signals belonged to proton attached to a carbon bearing a hydroxy group could be visualized. A doublet signal at δ 3.20 ppm (J = 4.3 Hz) should be the chemical shift of hydroxy proton in the alcoholic portion of the molecule (-CH-O<u>H</u>), which –OH might be oriented at equaterial position.[30, 31] The other was also a doublet signal at δ 2.81 ppm (J = 4.3 Hz) which could be assigned for the chemical shift of α-proton on the adjacent carbon atom. In addition, a triplet signal of α-proton in the acidic part was detected at δ 2.32 ppm (J = 7.3 Hz). Other signals around δ 0.50 to 2.00 ppm ought to be the signals of methyl, methylene and methine protons.[31, 32]

The ^{13}C NMR (DMSO-d_6) spectrum (Fig. 12) showed two sets of carbon signals of totally 50 carbon signals. The major set displayed the carbon signals at δ 182.8, 143.5, 122.6, 79.0, 55.2, 47.6, 46.5, 45.8, 41.6, 41.0, 39.2, 38.7, 38.4, 37.0, 33.8, 33.0, 32.6, 32.4, 30.7, 28.1, 27.6, 27.2, 25.9, 23.6, 23.4, 22.9, 18.3, 17.1, 15.5, and 15.3 ppm, respectively, which implied the presence of triterpenoid skeleton.[32]

From the ^{13}C NMR spectroscopic data of Mixture <u>5</u>, it could be indicated that this mixture was consisted of two triterpenes. However, the ^{13}C NMR spectrum showed methine and quaternary Csp^2 signals (δ 122.6 and 143.5 ppm), which were the characteristic of oleanene triterpene.[32]This observation was supported by the presence of an additional methyl (δ 15.3 ppm) and of acyl group (δ 182.5 ppm). In addition, the carbon signal at δ 79.0 ppm, which was the specific feature of equatorial hydroxy allocated at C-3 of oleanene triterpene, was detected.

Mixture <u>5</u> was further identified by direct comparison of the ^{13}C NMR data with those reported in the literature[32] as presented in Table 3.18. It could therefore be concluded that Mixture <u>5</u> comprised oleanolic acid as a major component. Its structure is shown below.



Mixture 5: Oleanolic acid (a major component)

Table 3.18 The comparison of the ^{13}C NMR chemical shifts of oleanolic acid with Mixture 5[32]

Position	Chemical shift (ppm)	
	Oleanolic acid	Mixture 5
1	38.5	38.4
2	27.4	27.2
3	78.7	79.0
4	38.7	38.7
5	55.2	55.2
6	18.3	18.3
7	32.6	32.6
8	39.3	39.2
9	47.6	47.6
10	37.0	37.0
11	23.1	22.9
12	122.1	122.6
13	143.4	143.5
14	41.6	41.6
15	27.7	27.6
16	23.4	23.4
17	46.6	46.5
18	41.3	41.0
19	45.8	45.8
20	30.6	30.7

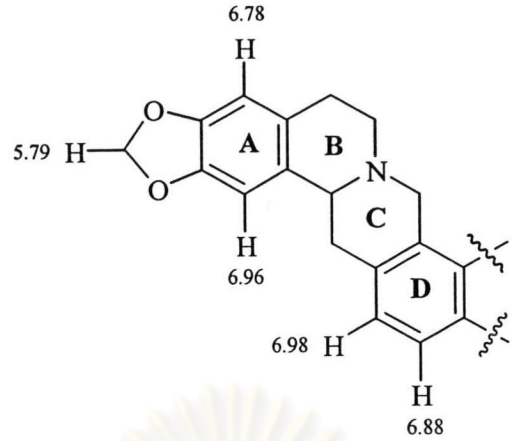
Table 3.18 (cont.)

Position	Chemical shift (ppm)	
	Oleanolic acid	Mixture 5
21	33.8	33.8
22	32.3	32.4
23	28.1	28.1
24	15.6	15.5
25	15.3	15.3
26	16.8	17.1
27	26.0	25.9
28	181.0	182.8
29	33.1	33.0
30	23.6	23.6

3.4.7 Purification, Properties and Structural Elucidation of Compound 6

A pale yellow solid derived from Fraction Nos. 25-30 which were eluted with 40-50% ethyl acetate in dichloromethane was recrystallized with a mixture of dichloromethane and methanol to colourless rod solid (m.p. 218-210 °C, 3.4 mg, 0.003% w/w of Fraction II). This compound revealed a single spot on TLC with R_f value of 0.54 by using 20% methanol in dichloromethane as solvent system. Compound 6 was soluble in dichloromethane, ethyl acetate and methanol. Moreover, this compound gave a positive test with Dragendroff's reagent indicating that this compound was alkaloid.

The ^{1}H NMR (DMSO-d_6) spectrum (Fig. 13) displayed the unique characteristics of protoberberine,[13, 14] *i.e.*, signals for methoxy, methylene dioxy and four aromatic protons were observed. The two singlet signals at δ 6.78 and 6.96 ppm (one proton each) for aromatic ring A and the two doublet signals at δ 6.88 and 6.98 ppm of one proton each for aromatic ring D exhibited distinctly. The spectrum also showed one doublet signal at δ 5.79 ppm of two protons, corresponding to methylene dioxy and displayed one singlet signal at δ 3.76 ppm of methoxy protons. The presence of one hydroxy group was indicated by one singlet signal at δ 9.25 ppm.

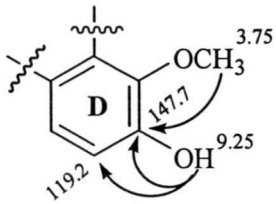


The ^{13}C NMR (DMSO-d_6) spectrum (Fig.14) and DEPT spectrum (Fig.15) showed 19 carbon signals. The signals belonging to quaternary carbons at δ 161.5, 150.1, 147.7, 146.1, 145.9, 129.6, 129.3, 128.4 and 122.7 ppm, five methine carbons at δ 122.6, 119.2, 108.2, 106.6 and 54.5 ppm, four methylene carbons at δ 100.8, 38.0, 37.7 and 29.2 ppm and the methoxy carbon at δ 60.7 ppm were visualized. The characteristic features of the ^{13}C NMR spectrum of this compound were similar to that of Compound 2. However, the signal position of Compound 6 differed from those of Compound 2 in such a way that a methoxy carbon at C-10 (δ 62.8 ppm) and two methine protons at δ 9.88 and 8.93 ppm of Compound 2 disappeared from the ^{1}H and ^{13}C NMR spectra of Compound 6, respectively.

From the above spectroscopic data, Compound 6 was suggested to be tetrahydroberberine, a protoberberine derivative, bearing one methoxy group and one hydroxy group. The position which was substituted by a hydroxy group could be assigned by the ^{1}H NMR spectrum. Moreover, this compound could be confirmed through long-range ^{1}H-^{13}C coupling deduced from the HMBC spectrum (Fig. 16).

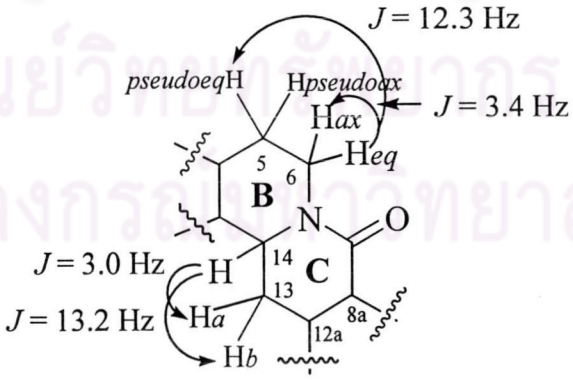
As the absence of signal for methoxy group at δ 62.8 ppm, the hydroxy group should therefore be located at C-10. This observation was endorsed from the HMBC spectrum by correlation of chelated hydroxy proton at δ 9.25 ppm, C-10 and C-11 at δ 147.7 ppm and 119.2 ppm, respectively. Furthermore, the methoxy protons (δ 3.75) were found to have a close relationship with aromatic carbon at C-10 (δ 147.7). The ^{1}H NMR spectrum further exhibited the aromatic doublet signals at δ 6.97 and δ 6.89 ppm with coupling constant 8.0 Hz, which could be properly located at C-11 and

C-12. This splitting pattern and the coupling constant were signified that those observed characteristic features were close to those of Compound 2.



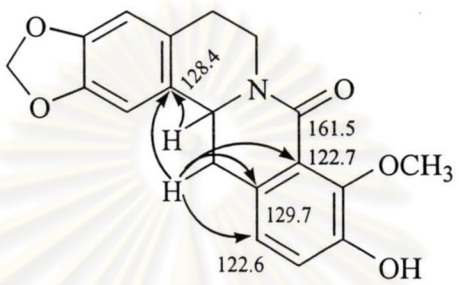
When considering the proton position of rings B and C, it was found that the coupling constants 3.0 and 13.2 Hz of doublet of doublet signal at δ 4.67 ppm should be located at C-14 (δ 54.6 ppm) which had the correlation with two methylene protons at C-13 (H-13a, δ 3.13 ppm, J = 3.1, 15.2 Hz and H-13b, δ 2.61 ppm, J = 13.8, 14.2 Hz). This assumption was supported by COSY spectrum (Fig. 17). From the coupling constant, it could be obviously concluded that the proton on C-14 should be oriented in the same direction as the H-13a.

The COSY spectrum pointed out that the splitting pattern of H-6*eq* (triplet of triplet signal with the coupling constant of 3.4, 3.9 and 12.3 Hz) displayed the correlation with the axial proton (H-6*ax*, δ 2.81 ppm) at C-6, a pseudoequatorial proton (H-5*pseueq*, δ 2.73 ppm) and a pseudoaxial proton (H-5*pseuax*, δ 2.81 ppm) at C-5.



Hence, the C-8 of Compound 6 should be the quaternary carbon which was a carbonyl group.[19,20] Furthermore, not only was the doublet of doublet at δ 3.13 ppm correlated with C-12 (δ 122.6 ppm), C-12a (δ 129.7 ppm), C-8a (δ 122.7 ppm), and

C-14a (δ 128.4 ppm) but also the doublet of doublet at δ 4.67 ppm was also correlated with C-14a (δ 128.4 ppm). The rest parts had the same features as those in Compound 2. The perfect structural elucidation of Compound 6 was confirmed by comparing the ^{13}C-NMR chemical shift data of Compound 6 with the data reported in the literature.[20] The complete assignment of ^{1}H and ^{13}C NMR chemical shifts of Compound 6 is shown in Table 3.19.



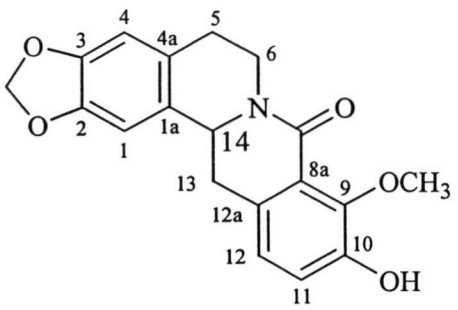
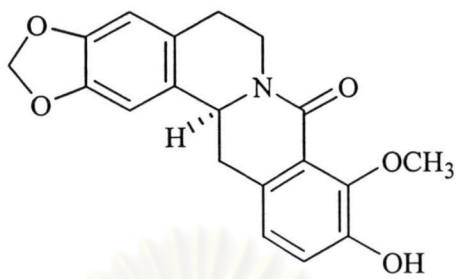


Table 3.19 The chemical shift assignment of Compound 6 and the comparison of the ^{13}CNMR chemical shifts of Compound 6 with 8-oxotetrahydrothalifendine

Position	Chemical shift (ppm)		
	^{1}H NMR	^{13}C NMR	
		Cpd. 6	8-oxotetrahydrothalifendine[a]
1	6.96 (1H, *s*)	106.6	106.0
1a	-	128.4	128.7
2	-	146.1	146.7
3	-	145.9	146.6
4	6.79 (1H, *s*)	108.2	108.6
4a	-	129.3	128.7
5*pseudoax*	2.81 (1H, *m*,)	29.2	29.8
5*pseudoaq*	2.73 (1H, *m*, *J* = 3.5, 4.1, 4.9, 12.6 Hz)		
6*ax*	2.73 (2H, *m*, *J* = 3.5, 4.1, 4.9, 12.6 Hz)	37.7	38.2
6*eq*	4.72 (2H, *tt*, *J* = 3.4, 3.9, 12.3 Hz)		
8	-	161.5	162.4
8a	-	122.7	not assigned
9	-	150.1	149.1
9-OCH_3	3.76 (3H, *s*)	60.7	62.4
10	-	147.7	147.4
11	6.89 (1H, *d*, *J* = 8.0 Hz)	119.2	118.2
12	6.97 (1H, *d*, *J* = 8.0 Hz)	122.1	122.8
12a	-	129.7	130.5
13a	3.13 (1H, *dd*, *J* = 3.1, 15.2 Hz)	38.0	39.0
13b	2.61 (1H, *m*, *J* = 13.8, 14.2 Hz)		
14	4.67 (1H, *dd*, *J* = 3.0, 13.2 Hz)	54.6	55.3
OCH_2O	5.98 (2H, *d*, *J* = 7.0 Hz)	100.8	101.1
10-OH	9.25 (1H, *s*)	-	-

[a]Chemical shift in $CDCl_3$

According to the above spectroscopic data Compound 6 can be deduced as 8-oxotetrahydrothalifendine. Its structure is displayed below.



Compound 6: 8-Oxotetrahydrothalifendine

In addition, this structure was confirmed by X-ray crystallographic technique as shown in Fig. 18. This is the first X-ray crystallographic structure of Compound 6.

3.4.8 Purification, Properties and Structural Elucidation of Compound 7

Compound 7 was obtained as pale yellow solid derived from Fraction Nos. 32-39 of ethyl acetate and dichloromethane extracts with 50-60% ethyl acetate in dichloromethane, m.p. 210-212 °C, 5.6 mg (0.005 %w/w of Fraction II). This compound was soluble in dichloromethane, ethyl acetate and methanol. Compound 7 revealed only one spot on TLC with R_f value of 0.48 using 20% methanol in dichloromethane as a solvent system. This compound gave a positive test with Dragendoff's reagent as Compound 6.

The ^{1}H NMR (DMSO-d_6) spectrum of Compound 7 (Fig.19) exhibited the doublet aromatic proton signal at δ 7.16, 7.03 ppm (1H each, *d*, J = 8.0 Hz) and the singlet aromatic proton signal at δ 6.95 and 6.78 ppm. The signal of methylene dioxy protons appeared at 5.97 ppm and those of two methoxy groups emerged at δ 3.79 and 3.76 ppm. Moreover, the doublet signal at δ 3.16 ppm of two protons was corresponded with methylene group. The triplet proton signal at δ 2.64 and multiplet proton signals δ 2.73 and 4.67 ppm were observed.

The ^{13}C NMR (DMSO-d_6) spectrum (Fig. 20) and DEPT 90, 135 spectra (Fig. 21) showed 20 carbon signals. The signals of quaternary carbons were observed at δ 161.3, 152.5, 149.0, 146.2, 145.9, 129.1, 31.0, 129.2, 128.5 and 123.0 ppm, five

methine carbons at δ 122.5, 115.6, 108.3, 106.6 and 54.5 ppm, four methylene carbons at δ 100.9, 38.0, 37.8 and 29.2 ppm and two methoxy carbons at δ 60.8 and 56.0 ppm.

According to spectroscopic evidence and color test, it could be concluded that Compound 7 was believed to be 8-oxoberberine with a methylenedioxy group and two methoxy groups (-OCH_3 signals at δ 3.76 and 3.79 ppm) distributed over positions 2, 3, 9 and 10 of the protoberberine skeleton. Allocation of the methylenedioxy group to ring A was clearly preferred because of the coincidence of ^{13}C NMR signals of the ring A with those of Compound 6 (see Table 3.20).

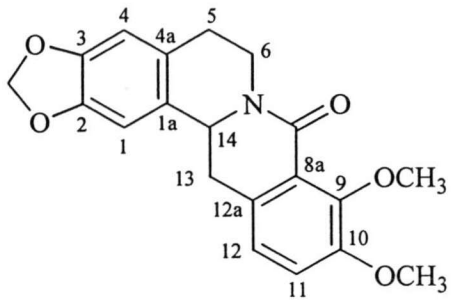
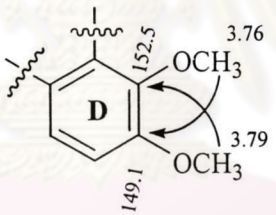


Table 3. 20 The comparison of the ^{13}C NMR chemical shifts of Compound 7 with Compound 6

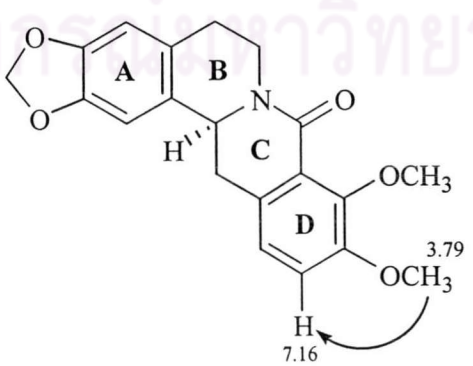
Position	Chemical shift (ppm)	
	Compound 6	Compound 7
1	106.6	106.6
1a	128.4	128.4
2	146.1	146.2
3	145.9	145.9
4	108.2	108.3
4a	129.3	129.2
5	29.2	29.2
6	37.7	37.8
8	161.5	161.3
8a	122.7	123.0
9	150.1	152.5
9-OCH_3	60.7	60.8
10	147.7	149.1
10-OCH_3	-	55.6
11	119.2	115.6
12	122.6	122.5
12a	129.7	131.0
13	38.0	38.0
14	54.6	54.50
OCH_2O	100.8	100.9

The ^{13}C NMR chemical shifts of Compound 7 were very close to those of Compound 6. To illustrate this, in Compound 6 the methoxy signal at δ 55.6 ppm could not be detected whereas the other compound displayed the chemical shift signal at this position. Besides, the hydroxy proton signal at δ 9.25 ppm of Compound 6 did not appear in the ^{1}H NMR spectrum of Compound 7.

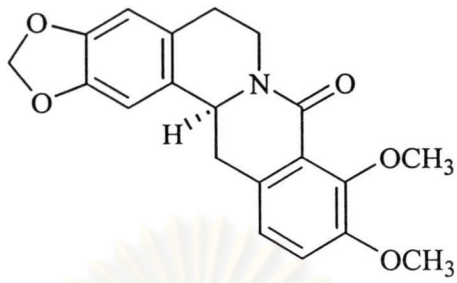
Therefore, it was possible to draw a conclusion that the hydroxy group in Compound 6 was replaced by the methoxy group. The position, which was substituted by a hydroxy group, could be assigned by the ^{1}H NMR spectrum. Moreover, this compound could be confirmed through long-range ^{1}H-^{13}C coupling deduced from the HMBC spectrum (Fig. 22). Since the signal for methoxy group was observed at δ 55.0 ppm, the hydroxy group should therefore be located at C-10. This assignment was supported by the correlation of the proton signal at δ 3.79 ppm and the carbon signal at δ 152.5 ppm as well as the correlation of the proton signal at δ 3.76 ppm and the carbon signal at δ 149.1 ppm.



Moreover, the observed NOE correlation in the NOESY spectrum (Fig. 23) between the aromatic proton signals at δ 7.16 ppm and 3.79 ppm suggested the complete structure of Compound 7.



From the above spectroscopic data, it could be concluded that Compound 7 was 8-oxoberberine. Its structure is exhibited below.



Compound 7: 8-Oxoberberine

3.4.9 Purification, Properties and Structural Elucidation of Mixture 8

Mixture 8, which was isolated form Fraction Nos. 154-176 of Fractions IIJ and IIK, was obtained by eluting with 5% methanol-dichloromethane as white solid, decomposed at 157-161 °C, (13.3 mg, 0.013 %w/w of Fraction II) after recrystallization with a hot mixture of methanol-dichloromethane for several times. It showed one spot on TLC with R_f value of 0.58 (10 % methanol-dichloromethane). This mixture was soluble in dichloromethane, ethyl acetate and methanol. Giving the positive test with Liebermann-Burchard's reagent implied that this mixture had a steroid nucleus.

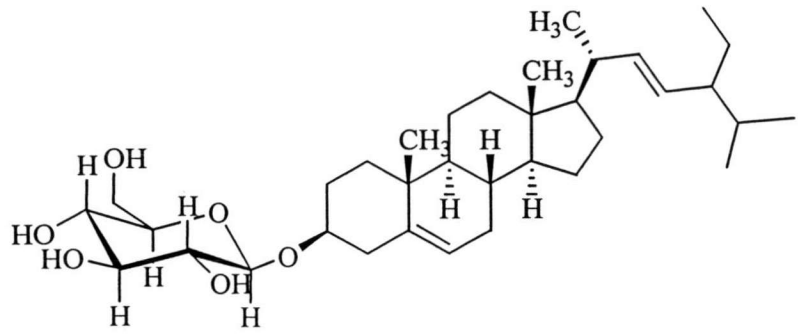
The IR spectrum (Fig. 24) showed vividly the absorption band at 3390 cm^{-1} (br), 1075 and 1021cm^{-1} (s), and 1640 cm^{-1} because of OH stretching vibration, C-O stretching vibration of glycosidic linkage and C=C stretching vibration, respectively. In addition, the C-H bending vibration of $-CH_2$ and $-CH_3$ at 1462 and 1375 cm^{-1} was detected.

The ^1H NMR (DMSO-d_6) spectrum (Fig. 25) indicated the characteristic of steroid according to the signals around δ 2.38 to 0.62 ppm. This spectrum showed the chemical shift at δ 5.31 ppm which could be an olefinic proton.[30,33] Moreover, the signals between δ 2.80 to 4.90 ppm could be assigned for protons on a sugar moiety. The doublet signal at δ 4.21 ppm (J = 8.0 Hz) was synonymous with an anomeric proton of a sugar. From the color test, IR and ^1H NMR spectra implied that this mixture was comprised a sugar moiety and steroidal part joining by glycosidic linkage at C-3 position of steroidal part and hydroxy group at an anomeric carbon of a sugar.[33]

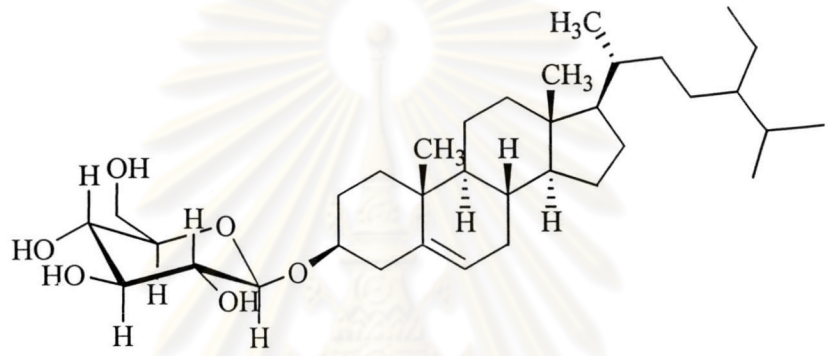
The ^{13}C NMR (DMSO-d_6) spectrum (Fig. 26) showed the distinguished pattern of steroids linked to a sugar with glycosidic linkage clearly. The signals at δ 140.4 and 121.1 ppm could be assigned for a double bond of β-sitosterol and/or stigmasterol. The carbon signals at δ 138.0 and 128.8 ppm were found which should be due to one double bond of stigmasterol. Therefore, it was found the mixture of β-sitosterol and stigmasterol in Mixture 1, Mixture 8 should be possible to compose of these steroids, which were linked with sugar. The carbon signals of sugar emerged at δ 100.8, 76.7, 76.7, 73.4, 70.1 and 61.1 ppm, which were the characteristic of D-glucose.[30]

The comparison of the ^{13}C NMR spectroscopic data between Mixture 8, stigmasterol and β-sitosteryl-3-O-β-D-glucopyranoside reported in the literature is shown in Table 3.21.

From the above information, it could be concluded that Mixture 8 was a mixture of steroid glycosides; stigmasteryl-3-O-β-D-glucopyranoside and β-sitosteryl-3-O-β-D-glucopyranoside. The structures are shown below.

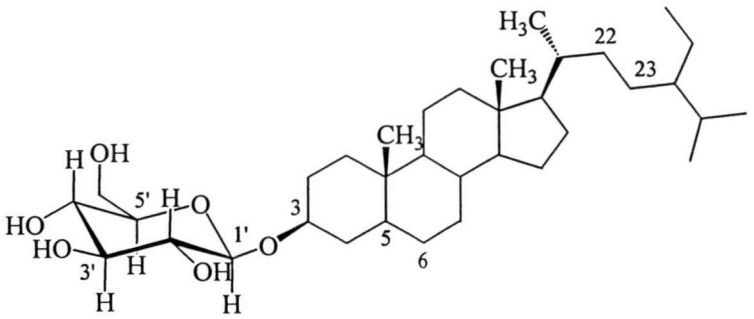


Stigmasteryl-3-O-β-D-glucopyranoside



β-Sitosteryl-3-O-β-D-glucopyranoside

Mixture 8



Δ^5 and Δ^{22}-Stigmasteryl-3-O-β-D-glucopyranoside
Δ^5-β-Sitosteryl-3-O-β-D-glucopyranoside

Table 3.21 The comparison of the ^{13}C NMR chemical shifts of Mixture 8, stigmasterol and β-sitosteryl-3-O-β-D-glucopyranoside[33]

Position	Chemical shift (ppm)			
	stigmasterol	β-Sitosteryl-3-O-β-D-glucopyranoside	Mixture 8	
			Stigmasteryl-3-O-β-D-glucopyranoside	β-Sitosteryl-3-O-β-D-glucopyranoside
1	37.4	37.6	36.7	38.3
2	31.8	30.3	31.2	31.2
3	71.8	78.5	76.9	76.9
4	42.3	39.3	45.1	45.1
5	140.8	140.9	140.4	140.4
6	121.7	121.9	121.1	121.1
7	32.0	32.1	31.3	31.3
8	32.0	32.1	31.3	31.3
9	50.3	50.4	50.5	50.5
10	36.6	37.0	36.7	36.1
11	21.1	21.4	22.6	22.6
12	39.8	40.0	41.7	41.8
13	42.3	42.5	49.6	49.6
14	56.9	56.9	56.2	56.1
15	24.4	24.6	24.8	24.8
16	28.9	28.5	28.6	28.6
17	56.1	56.3	55.4	55.3
18	12.2	12.0	12.1	11.8
19	19.5	19.4	19.7	18.9
20	40.5	36.4	27.7	27.7
21	21.1	19.2	21.1	18.6
22	138.3	34.3	138.0	35.4
23	129.4	26.5	128.8	23.5
24	51.3	46.1	45.1	45.1
25	32.0	29.5	29.2	27.7
26	19.0	19.4	18.8	20.9
27	21.1	20.0	21.1	20.5
28	25.5	23.5	25.5	25.5
29	12.1	12.2	11.7	11.6
C-1′	-	102.6	100.8	100.8
C-2′	-	75.3	73.4	73.4
C-3′	-	78.5	76.7	76.7
C-4′	-	71.7	70.1	70.1
C-5′	-	78.5	76.7	76.7
C-6′	-	62.8	61.1	61.1

3.4.10 Purification, Properties and Structural Elucidation of Compound 9

Compound 9 was obtained from Fraction Nos.77-98 of dichloromethane and ethyl acetate extracts. After recrystallization, white needle was precipitated (m.p. 234-236 °C, 61.30 mg, 0.61% w/w of Fractions III and IV). This compound revealed one spot on TLC with R_f value of 0.70 in 20% methanol in dichloromethane as a solvent system. Compound 9 was soluble in ethyl acetate, chloroform and methanol but slightly soluble in dichloromethane.

The IR spectrum (Fig. 27) displayed the absorption band due to O-H stretching vibration at 3400-3500 cm^{-1} and C-H stretching vibration at 2965 cm^{-1}. The additional band of α,β-unsaturated C=O at 1630, 1268 and 949 cm^{-1} were also perceived.

The EI mass spectrum (Fig. 28) of this compound gave a molecular ion at *m/z* 476 in accordance with a steroid containing six hydroxy groups, five methyl groups and one carbonyl group corresponding to the molecular formula $C_{27}H_{40}O_7$.

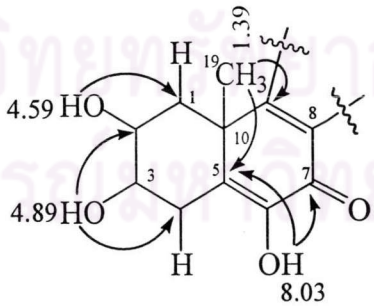
The ^{1}H-NMR (DMSO-d_6) spectrum (Fig. 29) exhibited the presence of five singlet signals at δ 1.39, 1.12, 1.06, 1.03 and 0.97 ppm corresponding to 3H protons of methyl group. Moreover, the ^{1}H-NMR spectrum also showed three secondary hydroxy groups at δ 4.89, 4.59 and 4.36 ppm, three tertiary hydroxy groups at δ 8.03, 4.09 and 3.67 ppm, and a vinylic proton at δ 6.76 ppm. The doublet of doublet at δ 2.90, 2.36, 2.24 and 1.83 ppm and the doublet of triplet at δ 1.65 ppm corresponding to 1H proton were observed. The other multiplet signals of at δ 3.17, 2.61, 2.54, 2.15, 2.10, 1.44, 1.41, 1.25 and 1.21 ppm matching 1H proton were also perceived.

The ^{13}C NMR (DMSO-d_6) spectrum (Fig. 30) and DEPT 90, 135 spectra (Fig. 31) showed 27 signals of carbons as follows: the signals of quaternary carbons at δ 179.5, 163.8, 142.6, 141.3, 132.6, 122.8, 75.0, 68.8, 46.2 and 40.8 ppm, five methine carbons at δ 125.9, 76.6, 72.0, 68.1 and 54.6 ppm, seven methylene carbons at δ 41.5, 41.4, 35.0, 31.3, 26.8, 26.0 and 23.7 ppm and five methyl carbons at δ 29.9, 29.0, 27.0, 20.1 and 17.7 ppm.

According to the above spectroscopic data, Compound 9 was suggested to be an ecdysone steroid with five hydroxy groups (-OH signals at δ 4.59, 4.89, 8.03, 3.67, 4.36 and 4.09 ppm) distributed over positions 2, 3, 6, 20, 22 and 25 of the ecdysone steroid skeleton. Furthermore, five methyl groups (-CH_3 signals at δ 0.97, 1.39, 1.12, 1.03 and 1.06 ppm) were observed at positions 18, 19, 21, 26 and 27. The methyl

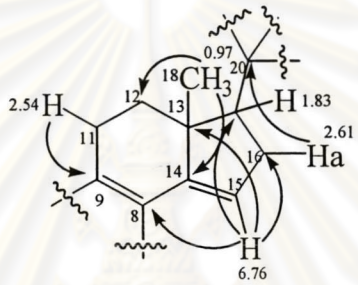
groups were allocated at positions 18 and 19, which is the characteristic feature of general steroid. Compound 9 had also an α,β-unsaturated carbonyl group in its molecule indicated by IR spectrum.

The structure of this compound was confirmed through long-range ^{1}H-^{13}C coupling deduced from the HMBC spectrum (Fig. 32) From this spectrum, the correlation between the doublet of doublet signal at δ 2.24 ppm with carbons at δ 68.1 (C-2), 72.0 (C-3), 132.6 (C-5), 40.8 (C-10) and 27.0 ppm (C-19) could be assigned for 1-Hb. The doublet of doublet signal at δ 2.90 ppm could be assigned for 4-H*ax* or 4- H*eq*, which correlated with the carbon signals at δ 68.1 (C-2), 72.0 (C-3), 132.6 (C-5), 142.6 (C-6) and 40.8 ppm (C-10). The signal for hydroxy group at δ 4.6 ppm was correlated with δ 41.5 (C-1) and δ 68.1 ppm (C-2) and the signal for hydroxy group at δ 4.59 ppm was also correlated with δ 68.1 (C-2), 72.0 (C-3) and 26.8 ppm (C-4). Thus, the hydroxy singlet signal at δ 4.59 ppm and the two hydroxy doublet of doublet signals at δ 4.89 ppm could be assigned for 2-OH and 3-OH of ring A of ecdysone steroid. Moreover, the methyl singlet signal detected at δ 1.39 ppm was assigned for C-10 of ring A. This was because it could couple with carbons at δ 142.6 (C-6), 40.8 ppm (C-10) and 163.8 ppm (C-9), respectively. The hydroxy singlet signal at δ 8.03 ppm was found to have a close relationship with carbons at δ 132.6 (C-5), 142.6 (C-6) and 179.5 ppm (C-7). Thus, the hydroxy group should be located at C-6.

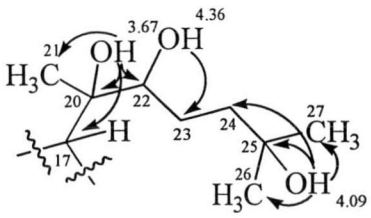


The HMBC spectrum further exhibited the correlation between the vinylic singlet proton signal at δ 6.76 ppm and carbons at δ 122.8 (C-8), 46.2 (C-13), 31.3 (C-16) and 54.6 ppm (C-17). Therefore, the vinylic proton was assigned for C-15. The multiplet signal at δ 2.54 ppm was found to correlate with C-9. The multiplet signal at δ 2.54 ppm could thus be assigned for 11-H*ax* or 11-H*eq*. With the aids of the

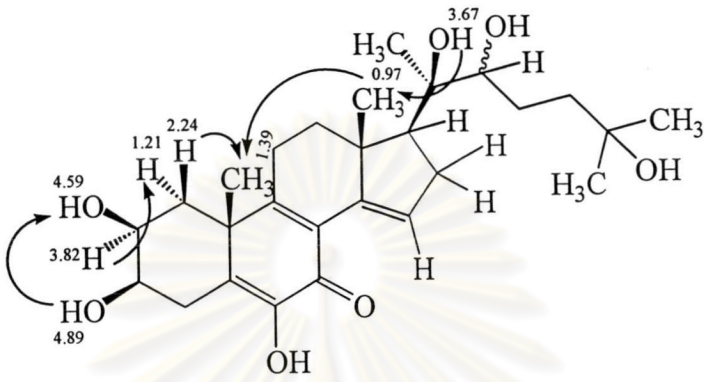
^{13}C-NMR and HMBC spectroscopic evidences, the conjugated alkene could be designated for the positions at C-8, C-9, C-14 and C-15. The multiplet signal at δ 2.61 ppm was appointed for 16-Ha as it was coupled with carbons at δ 125.9 ppm (C-15), C-17 and 75.0 ppm (C-20), respectively. The doublet of doublet signal at δ 1.83 ppm displayed the correlation with carbons at δ 35.0 (C-12), C-13, C-15, C-16, C-17 and 17.7 ppm (C-18), which could be deduced for 17-H. Moreover, the distinguished singlet signal at δ 0.97 ppm was also exhibited the correlation with carbons at C-12, C-13 and C-14, corresponding to the methyl group at C-18 of steroids.



Moreover, according to the above evidences, it was found that this ecdysone steroid had the side chains attributed as follows: the carbon position at δ 75.0 ppm (C-20) was assigned for quaternary carbon bearing the hydroxy group at δ 3.67 ppm, the methyl group at δ 1.12 ppm and long chain portion due to the correlation between a hydroxy singlet proton at δ 3.67 ppm and carbons at C-17, C-20, δ 1.12 ppm (C-21) and δ 76.55 ppm (C-22). The hydroxy doublet of doublet proton at δ 4.36 ppm was designated for 22-OH correlating with the carbon at δ 26.0 ppm (C-23). Finally, the doublet of doublet proton at δ 4.09 ppm correlated with carbons at δ 41.4 ppm (C-24), 68.8 ppm (C-25), 29.0 ppm (C-26) and 29.9 ppm (C-27), respectively. This could be assigned for 25-OH.



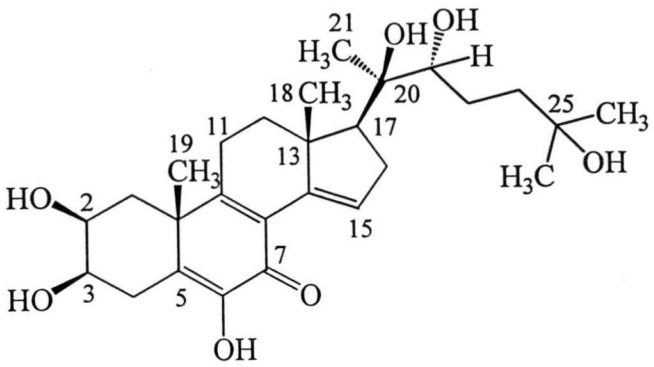
In addition, the observed NOE correlation in the NOESY (Fig. 33) found that the hydroxy proton signals at δ 4.59 and 4.89 ppm had the same stereochemistry. The correlations between proton signals at δ 3.82 and 1.20 ppm, 2.24 and 1.39 ppm, and 1.39 and 0.97 ppm suggested the structure of Compound 9.



However, the NOESY spectrum could not specify the stereochemistry of hydroxy group at C-22. Searching from the literature reviews[34-40] it was found that this compound had the structural feature similar to 2, 3, 6-trihydroxy-10, 13-dimethyl-17- (1, 2, 5-trihydroxy-1, 5-dimethyl-hexyl)-1, 2, 3, 4, 10, 11, 12, 13, 16, 17-decahydrocyclopenta [a] phenanthren-7-one or calonysterone which possessed a 22*R* configuration at C-22. The structure of Compound 9 was confirmed by direct *co*-TLC with the authentic calonysterone.

Even though there were many reports involving the isolation of Compound 9, the details of the assignment of ^{1}H and ^{13}C-NMR chemical shifts of Compound 9 were incomplete. The tentative ^{1}H and ^{13}C-NMR chemical shift assignments of this compound is displayed in Table 3.23.

Therefore, according to the above evidences, Compound 9 could be deduced as calonysterone. Its structure is exhibited below.



Compound 9: Calonysterone

Table 3.22 The ^{1}H and ^{13}C NMR chemical shift assignment of Compound 9

Position	^{13}C NMR (ppm)		Position	^{1}H (ppm)	
	Cpd 9	Steroid[b]		Cpd 9	Steroid[b]
1	41.5	-[c]	1-Ha	1.21, m	-[c]
		-[c]	1-Hb	2.24 (*dd*, J = 3 Hz)	-[c]
2	68.1	-[c]	2-H*ax*	3.82, s	-[c]
3	72.0	-[c]	3-H*eq*	3.32, s	-[c]
4	26.8	-[c]	4-H*ax*	2.90 (*dd*, J = 12 Hz)[a]	-[c]
			4-H*eq*	2.36 (*t*, J = 12 Hz)[a]	-[c]
5	132.6	-[c]	-	-	-[c]
6	142.6	-[c]	-	-	-[c]
7	179.5	-[c]	-	-	-[c]
8	122.8	-[c]	-	-	-[c]
9	163.8	-[c]	-	-	-[c]
10	40.8	-[c]	-	-	-[c]
11	23.7	-[c]	11-H*ax*	2.54, *m*[a]	-[c]
			11-H*eq*	not seen[a]	-[c]
12	35.0	-[c]	12-H*ax*	2.15, *m*[a]	-[c]
			12-H*eq*	1.41, *m*[a]	-[c]
13	46.2	84.2	-	-	-[c]
14	141.3	-[c]	-	-	-[c]
15	125.9	-[c]	15-H	6.76, *s*	6.78
16	31.3	-[c]	16-Ha	2.61, *m*	-[c]
			16-Hb	2.10, *m*	-[c]
17	54.6	-[c]	17-H	1.83 (*dd*, J = 10 Hz)	-[c]
18	17.7	-[c]	18-Me	0.97, *s*	1.00
19	27.0	-[c]	19-Me	1.39, *s*	1.41

Table 3.22 (cont.)

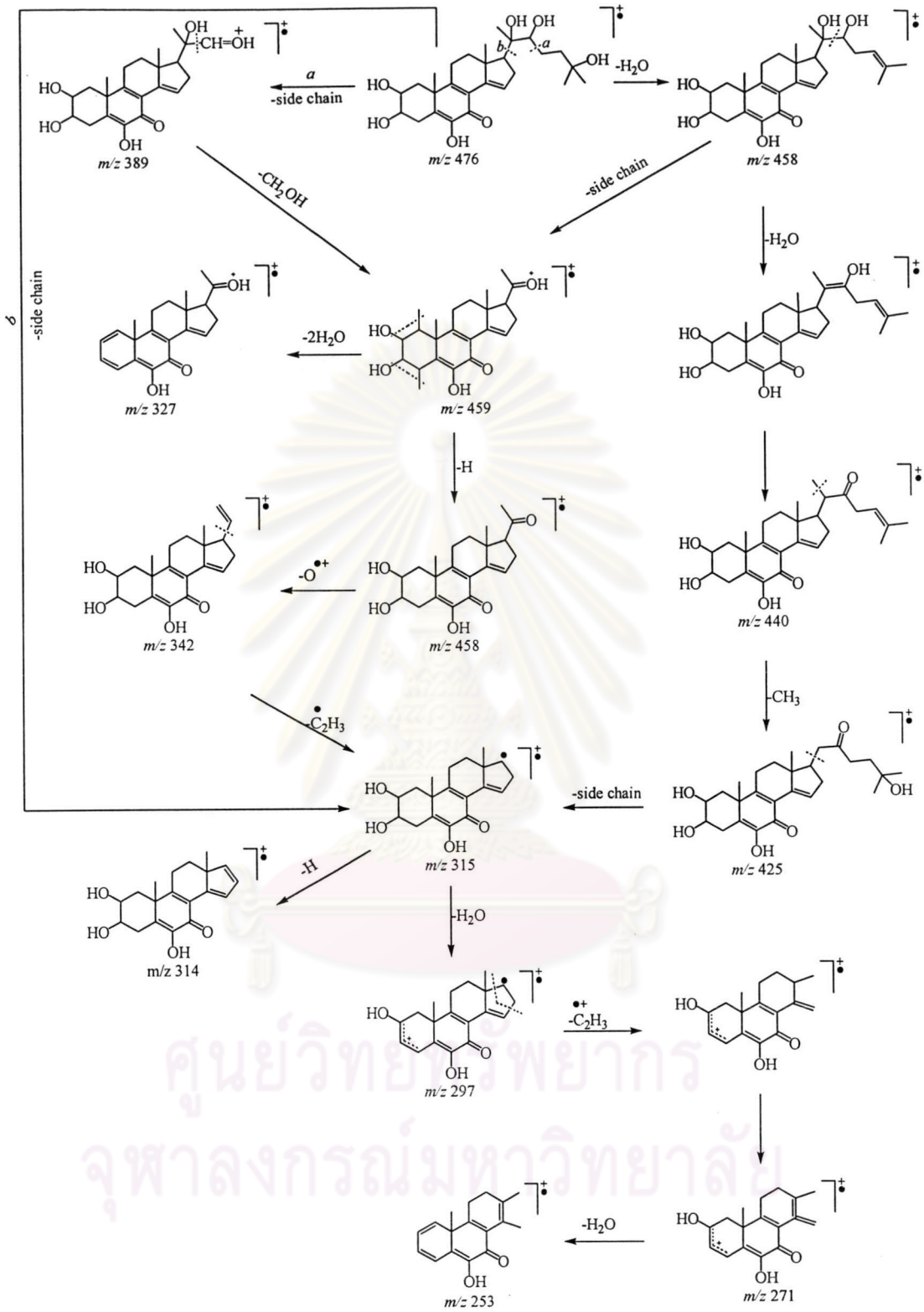
Position	^{13}C NMR (ppm)		Position	^{1}H (ppm)	
	Cpd 9	Steroid[b]		Cpd 9	Steroid[b]
20	75.0	74.9	-	-	[c]
21	20.1	-[c]	21-Me	1.12, *s*	1.16
22	76.6	76.4	22-H	3.17, *m*	-[c]
23	26.0	20.0	23-Ha	1.44, *m*	-[c]
			23-Hb	Not seen	-[c]
24	41.4	-[c]	24-Ha	1.25, *m*	-[c]
			24-Hb	1.65 (*dt*, *J* = 6 Hz)	
25	68.8	68.6	25-H	-	-[c]
26	29.0	29.8	26-Me	1.03, *s*	1.05
27	29.9	29.8	27-Me	1.06, *s*	1.07
			2-OH	4.59, *s*	-[c]
			3-OH	4.89, (*dd*, *J* = 5 Hz)	-[c]
			6-OH	8.03, *s*	-[c]
			20-OH	3.67, *s*	-[c]
			22-OH	4.36, (*dd*, *J* = 5 Hz)	--[c]
			25-OH	4.09, *s*	-[c]

[a]assignments may be interchanged

[b]steroid from ref. No. 34

[c]not assigned

The EI mass spectrum (Fig. 28) also supported the proposed structure of Compound 9. It revealed significant mass fragmentation ion, besides the molecular ion at *m/z* 458 [M-H_2O], 389 [M-(C_{23}-C_{27})], 315 [M-(C_{20}-C_{27})], 297 [M-H_2O]. The fragmentation ion patterns were proposed as shown in Scheme 3.2.



Scheme 3.2 The possible mass fragmentation pattern of Compound 9

3.4.11 Purification, Properties and Structural Elucidation of Compound 10

A pale yellow solid derived from Fraction Nos. 25-30 which were eluted with 40-50% ethyl acetate in dichloromethane was recrystallized with a mixture of dichloromethane and methanol to yield white needle (m.p. 235-237 °C, 45.2 mg, 0.45 % w/w of Fractions III and IV). Compound 10 revealed a single spot on TLC with R_f value of 0.51 in 4% methanol in dichloromethane. Compound 10 was soluble in methanol, chloroform, and ethyl acetate but slightly soluble in dichloromethane.

The IR spectrum (Fig. 34) displayed the absorption band at 3600 (w) and 3400 (w) cm^{-1} due to free -OH (sharp) and bonded -OH (br) stretching vibration. The absorption band at 1638 cm^{-1} stretching vibration indicated cyclohexenone.[37] The C-O stretching vibration, -OH stretching vibration and –OH out of plane of alcohol presented at around 1200-1450 cm^{-1}, 970-1260 cm^{-1} and 719 cm^{-1}, respectively. The C-O-C stretching vibration at 1066 ad 999 $cm^{-1}$was observed as the feature of cyclic ether. The additional band of α,β-unsaturated C=O at 1638, 1245 and 953 cm^{-1} were also perceived.

The EI mass spectrum (Fig. 35) of this compound gave a molecular ion at *m/z* 478 in accordance with the molecular formula $C_{27}H_{42}O_7$. In addition, the mass spectrum exhibited the fragment ion peaks at *m/z* 460, 442, 343, 318, 299, 175, 143, 125, 99 and 81.

The ^{1}H-NMR spectrum in pyridine-d_5 (Fig. 36) exhibited the presence of five singlet signals at δ 1.25 (d, J = 8 Hz), 1.27 (d, J = 8 Hz), 1.35 (s), 1.56 (s) and 1.75 ppm (s), corresponding to 15H protons of five methyl groups. The singlet signal at δ 6.43 ppm was assigned for a methine proton of α-carbon of α,β-unsaturated ketone.[30] Proton signals at δ 3.90 and 4.29 ppm displayed the characteristic of protons of ecdysone steroid at C-2 and C-3.[34-40] Some hydroxy proton signals were observed at δ 6.24 and 5.83 ppm. The multiplet proton signals around δ 1.37 to 2.30 ppm indicated the feature of steroid compounds.

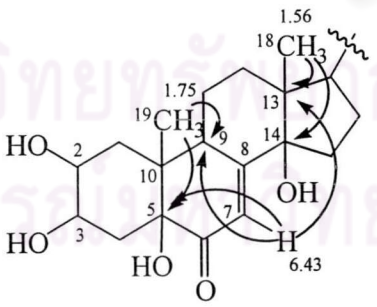
The ^{13}C NMR (DMSO-d_6) spectrum (Fig.37) and DEPT 90, 135 spectra (Fig. 38) exhibited 27 carbon signals, which supported that this compound was steroid. The eight quaternary carbon signals displayed at δ 41.2, 53.2, 58.8, 72.0, 80.0, 91.7, 169.7 and 200.1 ppm. The six methine carbon signals were detected at δ 47.1, 56.4, 67.2, 68.2, 87.0 and 124.5 ppm. The eight methylene carbon signals at δ 24.1,

25.0, 25.1, 26.2, 26.9, 28.2, 36.3 and 38.2 ppm were observed. Three methyl carbon signals exhibited at δ 20.3, 21.9, 26.2, 29.3 and 29.5 ppm.

According to the above spectroscopic data, Compound 10 was suggested to be an ecdysone steroid with five methyl groups assignable for C-18, C-19, C-21, C- 25 and C-26 of the ecdysone steroid skeleton. Moreover, the carbon signals at δ 124.5, 169.7 and 200.1 ppm indicated the presence of conjugated carbonyl group in this compound.[30] The carbon signals at δ 68.2 (C-2), 67.2 (C-3), 200.1 (C-6), 124.5 (C-7), 169.7 (C-8), 91.7 (C-14) and 80.0 ppm (C-20) were similar to those of 20-hydroxy ecdysone steroid.[35,40]

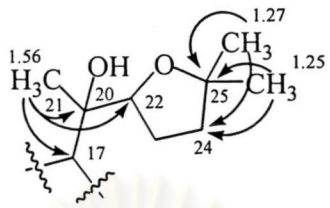
The structure of Compound 10 could be confirmed through long-range ^{1}H-^{13}C coupling (J = 4-12 Hz) deduced from the CIGAR (pridine-d_5) spectrum (Fig. 39). The correlation between the singlet proton signal at δ 6.43 ppm (methine proton at 124.5 ppm) with carbons at δ 72.4 (C-5), 169.7 (C-8), 47.1 (C-9), and 53.2 ppm (C-13) could be assigned for H-7. The methyl proton signal at δ 1.75 ppm could be assigned for C-19 due to correlation with δ 72.4 (C-5) and 47.1 ppm (C-9). The singlet signal at δ 1.56 ppm correlated with δ 53.2 (C-13) and 91.7 ppm (C-14) was identified that it was the methyl proton at C-18 of the ecdyson skeleton.

The above spectroscopic data indicated that at position of δ 72.4 (C-5), and 91.7 ppm (C-14) were quaternary carbons. In addition, from the data reported in literature supported that these positions should be assigned for tertiary alcohol.[35,40]

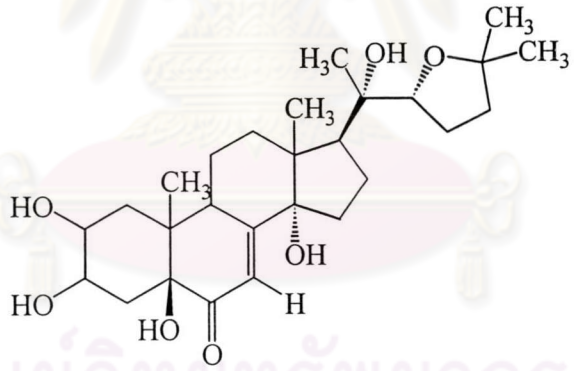


The CIGAR spectrum further exhibited the correlation of side chain steroid between the methyl proton singlet signal at δ 1.56 ppm and carbons at δ 56.4 (C-17), 80.0 (C-20) and 87.0 (C-22). This signal was assigned for the methyl at C-18. Both doublet of doublet signals at δ 1.25 and 1.27 ppm having the correlation with δ 41.2 (C-24) and 68.8 ppm (C-25) were identified as two methyl protons at C-26 and C-27. Moreover, the identity of the side chain was established by comparison with the data

reported in literature, which indicated that this side chain had tetrahydrofuran ring. It should be noted that the configuration at C-22 of Compound 10 was assigned as 22*R*.[37]



Therefore, from all the above evidence, it could be concluded that Compound 10 was 17-[1-(5, 5-dimethyl-tetrahydrofuran-2-yl)-1-hydroxy-ethyl]-2, 3, 5, 14-tetrahydroxy-10, 13-dimethyl-1, 2, 3, 4, 5, 9, 10, 11, 12, 13, 14, 15, 16, 17-tetradecahydrocyclopenta[a]phenanthren-6-one or ajugasterone D.[34] The chemical shift assignment of Compound 10 was displayed in Table 3.24 and its structure is shown below.



Compound 10: Ajugasterone D

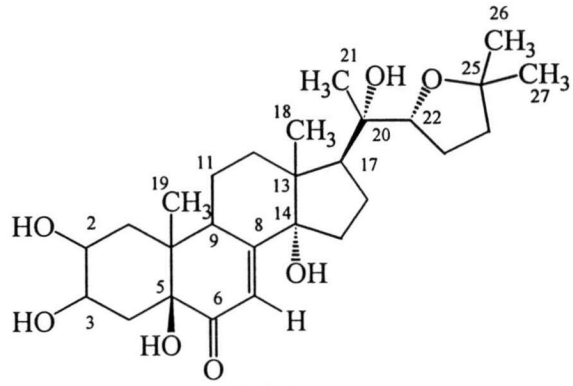
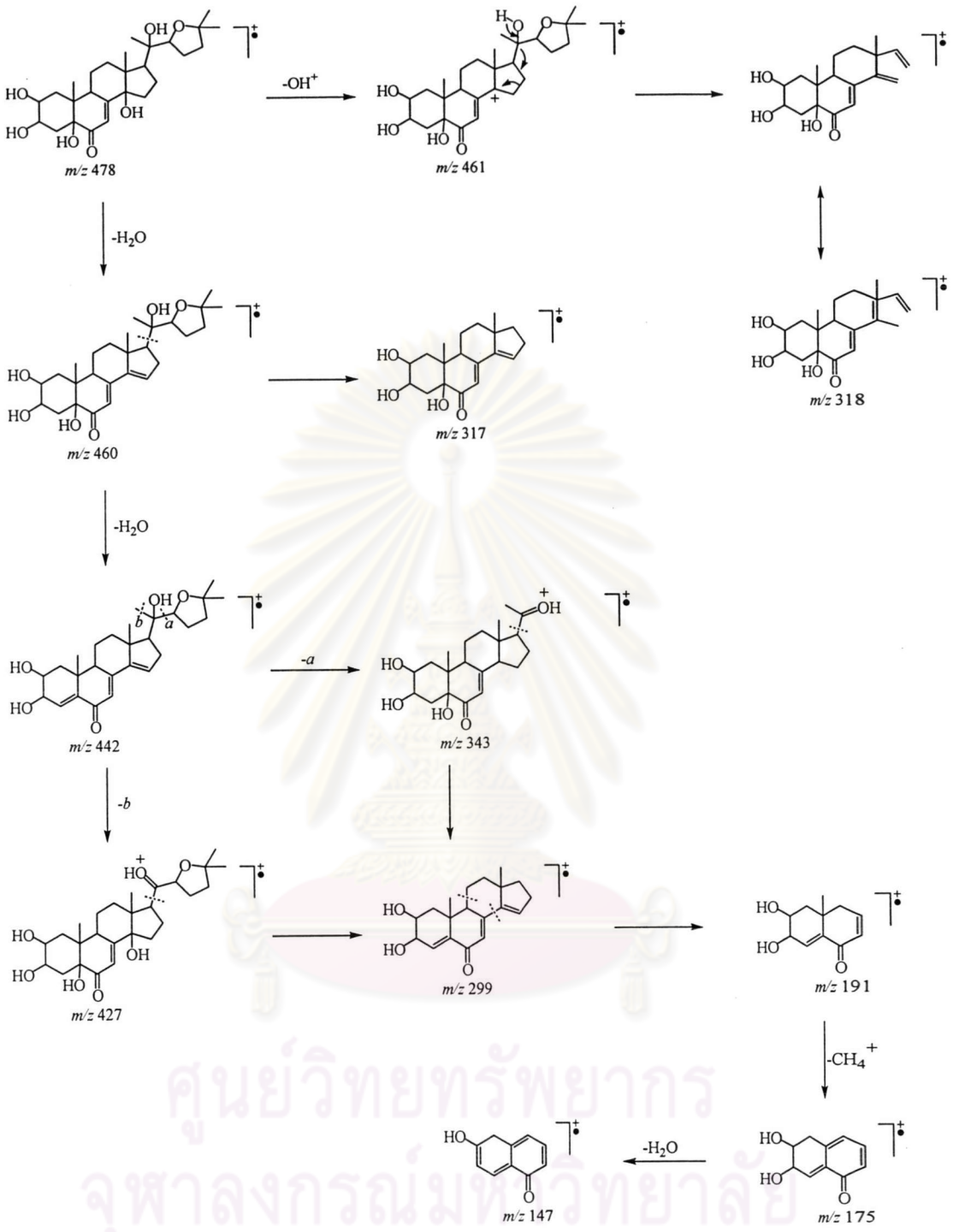


Table 3.23 The ^{1}H and ^{13}C NMR chemical shift assignment of Compound 10

Position	^{13}C NMR (ppm)[a]	Position	^{1}H NMR (ppm)[b]	
			Compound 10	Ajugasterone D[34]
2	68.2	2-Hax	4.29 (*br s*)	-[d]
3	67.2	3-H*eq*	3.90 (*m*)	-[d]
5	72.4	-	-	-[d]
6	200.1	-	-	-[d]
7	124.5	-	6.43 (*s*)	-[d]
8	169.7	-	-	-[d]
9	47.1	-	3.09 (*br s*)	-[d]
10	41.2	-	-	-[d]
13	53.2	-	-	-[d]
14	91.7	-	-	-[d]
17	56.4	17-H	2.67, *dd, J* = 4,10 Hz	-[d]
18	21.9	18-Me	1.35, *s*	0.94, *s*
19	20.3	19-Me	1.75, *s*	1.04, s
20	80.0	-	-	-[d]
21	26.2	21-Me	1.56, *s*	1.28, *s*
22	87.0	22-H	3.59, *m*	-[d]
24	41.4	24-Ha	-	-[d]
		24-Hb	-	-[d]
25	68.8	-	-	-[d]
26	29.3	26-Me	1.27, *d, J* = 8 Hz[c]	1.10, *s*
27	29.5	27-Me	1.25, *d, J* = 8 Hz[c]	1.10, *s*

[a]DMSO-d_6, [b]Pyridine-d_5, [c]Assignments may be interchanged, [d]not assigned

The EI mass spectrum (Fig. 34) also supported the proposed structure of Compound 10. It revealed significant mass fragmentation ion, besides the molecular ion at *m/z* 460 [M-H_2O], 442 [M-H_2O], indicated that Compound 10 had polyhydroxy groups in its structure. The fragmentation ion at *m/z* 427 [M-CH_3], 299 [M-(C_{20}-C_{27})] and 343 [M-(C_{22}-C_{27})] implied that this compound was steroid with tetrahydroxyfuran ring. Other fragmentation ion patterns were proposed as shown in Scheme 3.3.



Scheme 3.3 The possible mass fragmentation pattern of Compound 10

3.5 The Results of Biological Activities Test of Isolated Compounds

Compounds 2, 2A, 3, 9 and 10, isolated from the stems of *C. fenestratum,* were evaluated for biological activity. The interested activity could be classified into two categories;

1. Pharmaceutical-based activity, *i.e.*, cytotoxicity, anticancer, antimalarial, antifugal, and antituberculous tests.
2. Agrochemical-based activity, *i.e.*, plant growth regulator and antipathogenic fungi tests.

3.5.1 The Results of Biological Activities in the Class of Pharmaceutical-Based Activity

3.5.1.1 Cytotoxicity, Antimalarial and Antifungal Activity

Compounds 2, 2A, 3, 9 and 10 were subjectived to the above mentioned activity against *Vero cells*, *P. faciparum,* K-1 stain and *C. albicans.* The results are presented as shown in Table 3.25.

Table 3.24 The results of cytotoxicity, antimalarial and antifungal activity of isolated compounds and derivative.

Compound	Biological test (IC_{50} ; μg/mL)		Biological test (EC_{50} ; μg/mL)
	Cytotoxicity	Antifungal	Antimalarial
Compound 2	>50	13.0	0.11
Compound 2A	-	Inactive	-
Compound 3	>50	Inactive	-
Compound 9	>50	Inactive	Inactive
Compound 10	>50	Inactive	Inactive

- : not test

All the isolated compounds showed low toxicity against *vero cells* with IC_{50} value more than 50 μg/mL. In addotion, Compound 2 exhibited actively against *P. faciparum,* K-1 stain and *C. albicans.*

3.5.1.2 Anticancer Test

The results of anticancer activity of Compounds 2, 2A, 3, 9 and 10 are exhibited in Table 3.25.

Table 3.25 The results of anticancer activity of isolated compounds and derivative.

Compound	KB		BC-1		NCl-H187	
	IC_{50} (μg/mL)	Activity	IC_{50} (μg/mL)	Activity	IC_{50} (μg/mL)	Activity
Compound 2	0.48	Strongly active	0.95	Strongly active	0.30	Strongly active
Compound 2A	-	Inactive	-	Inactive	-	Inactive
Compound 3	-	Inactive	-	Inactive	0.08	Strongly active
Compound 9	-	Inactive	-	Inactive	-	Inactive
Compound 10	-	Inactive	-	Inactive	-	Inactive

From Table 3.25, it was found that Compound 2 displayed strong activity as anti-KB, anti-BC and anti-NCl-H187 with IC_{50} value of 0.48, 0.95 and 0.30 μg/mL, respectively. Whereas Compound 3 inhibited against NCl-H187 only with IC_{50} value of 0.08 μg/mL. The other compounds did not display significant activity as anti cell lines. This result was in good accordance with the preliminary screening test of Fraction I which could be concluded that the active ingredients in Fraction I for anticancer was Compounds 2 and 3. Considering the structure activity relationship of Compounds 2 and 3, it could be realized that the tetracyclic ring aromatic isoquinoline alkaloid might be the important part responsible for displaying the anticancer activity.

3.5.1.3 Antituberculosis Activity

Antituberculosis activity of isolated compounds: Compounds 2, 2A, and 3 using microplate alamar blue assay (MABA)[41] was conducted and the results are demonstrated as shown in Table 3.26.

Table 3.26 The results of antituberculosis activity of isolated compounds and derivative.

Compound	Results against TB at 200 μg/mL	MIC (μg/mL)
Compound 2	Active	100
Compound 2A	Inactive	-
Compound 3	Active	200

According to the result of the preliminary screening test of Fraction I which exhibited antituberculosis activity with MIC value of 200 μg/mL, Compounds 2 and 3 exhibited the same trend of inhibition as Fraction I. It could therefore be concluded that Compounds 2 and 3 were the active compounds revealing antituberculosis in *C. femestratum.* However, when comparing the activity of Compounds 2 with 2A, it was found that the activity was quite different. The difference in structure and activity of Compounds 2 and 2A, may lead to the conclusion that the aromatic ring C of the tetracyclic isoquinoline alkaloid was of importance for inhibiting tuberculosis.

3.5.2 The Results of Biological Activities in the Class of Agrochemical-Based Activity

3.5.2.1 Plant growth regulator of Compound 2

Compound 2, a major isolated compound, was evaluated for plant growth regulator against *Mimosa pigra* Linn. and *Echinochloa crus-galli* Beauv. The results are displayed in Table 3.27.

Table 3.27 The results of Compound 2 against the growth of *M. pigra* Linn. and *E. crus-galli* Beauv.

Concentration (μg/mL)	% Inhibition			
	M. pigra Linn.		*E. crus-galli* Beauv.	
	root	shoot	root	shoot
10	36	3	68	-20
100	51	-3	88	-29
1000	69	23	100	-14

The above results in Table 3.27 revealed that Compound 2 displayed inhibition against the root growth of *M. pigra* Linn. and *E. crus-galli* Beauv. with % inhibition of 69 % and 100 % at 1000 μg/mL and 51 % and 88 % at 100 μg/mL, respectively. This % inhibition indicated that Compound 2 possessed the activity for plant growth regulator.

3.5.2.1 Antipathogenic Fungal Activity

The effect of Compound 2 on antipathogenic fungal activity using the mycelial growth inhibition assay[29] is shown in Table 3.28.

Table 3.28 The results of Compound 2 against *Phytophthora sp.*572, *Alternaria sp.* 43-89 and *Fusarium oxysporum* 43-68.

Fungi	IC_{50} (μg/mL)
*Phytophthora sp.*572,	79
Alternaria sp. 43-89	83
Fusarium oxysporum 43-68.	1170

From the above data, Compound 2 exhibited high activity against *Phytophthora sp.*572 and *Alternaria sp.* 43-89 with IC_{50} value of 79 and 83 μg/mL, respectively. This compound however showed low activity against *Fusarium oxysporum.*

3.6 Discussion on Biological Activities of Isolated Substances from Both the Bioassay Results and Literature Survey

Mixture 1 was separated from the precipitate fraction of the stems of *C. fenestratum* and was elucidated its structure as a mixture of phytosterols: stigmastan-3,5,22-triene, stigmastan-3,5-diene, β-sitosterol, campesterol and stigmasterol. These phytosterols are widely distributed in plant kingdom. Pharmaceutical properties of steroids were well known for using as precursors for preparing steroid hormones.[33] In addition, these most abundant phytosterols: β-sitosterol, campesterol and stigmasterol have been used in medicine in recent years due to their relatively wide spectrum of therapeutic effects and non toxicity in humans and animals. The results of clinical studies have repeatedly shown that phytosterols taken as dietary supplements reduce serum or plasma total cholesterol and low density lipid cholesterol levels in normal and mildly hypercholesterolaemic subjects.[42,43]

Compound 2 was isolated from precipitate fraction of the stems of *C. fenestratum* as a major compound. This compound is a yellow quaternary protoberberine alkaloid occurring in many plant species.[13,14,19,20] Compound 2 is known as antimicrobial agent for treatment of eye infections, gastrointestinal

disorders, anticoagulant action and other diseases.[44-50] Bioassay results indicated that this compound did not exhibit cytotoxicity against brine shrimp and *vero cells* but displayed strongly activity against cell lines (KB cells, BC-1 cells and NCl-H187 cells). Compound 2 showed significant concentration dependent inhibitory effect against *P. faciparum,* K-1 stain, *C. albicants,* and antimycobacterial (*M. tuberculosis* $H_{37}Ra$) activity. Moreover, this compound exhibited the allelophatic property against the root of *M. pigra* Linn. and *E. crus-galli* Beauv as well as antifugal property against *Phytophthora sp.*572 and *Alternaria sp.* 43-89.

Compound 3 was separated from the precipitate fraction and was identified as jatrorrhizine. Biological activity of this compound was similar to those of Compound 2 such as antimutagenic activity [51] and other activities.[45-50] The results of biological activity test displayed inactively anticancer activity against KB cells and BC-1 cells but gave strong activity with NCI-H187 cells. Compound 3 also exhibited significant antituberculous activity.

Compound 4 was isolated from the precipitate fraction and its structure was established as a long chain ester. This compound was found in various plants but had no report concerning the biological activity.

Mixture 5 was separated form the precipitate fraction and was elucidated that it was a mixture of triterpene having oleanolic acid as a major component. Oleanolic acid is a pentacyclic triterpenoid compound which naturally occurs in a large number of vegetarian foods, medicinal herbs and plant.[52, 53] Pharmaceutical action of oleanolic acid is similar to ursolic acid. It was exclusively used as emulsifying agent in pharmaceutical, cosmetic and food preparations.[53]

Compound 6 is one of the protoberberine alkaloids, It was isolated and identified as 8-oxotetrahydrothalifendine. Due to the limit amount of this compound, the biological activity study did not investigate.

Compound 7 was separated from the precipitate fraction and was elucidated as 8-oxoberberine. This compound was reported as a minor alkaloid compound in *C. fenestratum* like Compound 6.

Mixture 8 was isolated from the precipitate fraction. Its structure was established as a mixture of steroid glycosides; stigmasteryl-3-O-β-D-glucopyranoside and β- sitosteryl-3-O-β-D-glucopyranoside. Literature surveys pointed out that this

mixture showed antitumor activity against P-388 leukemia and revealed inflammatory activity.[33]

Compounds 9 and 10 were separated from dichloromethane and ethyl acetate fractions and were elucidated as calonysterone and ajugasterone D. These compounds belong to ecdysteroid type showing physiological activities in insects and have been found in both invertebrates and plant species.[47,40] The results of biological test exhibited low toxic activity against *vero cells*. In addition, these compounds did not show the inhibitory effect against *P. faciparum* K1 and *C. albicants* as well as cell lines.

3.7 Structure Activity Relationship of Isolated Berberine and Its Salts

After the isolated compounds were evaluated for biological activity, it was found that Compound 2, a major compound, exhibited the remarkably significant biological activity against cell lines, *P. faciparum*, K-1 stain, *C. albicants,* and *M. tuberculosis* $H_{37}Ra$. In order to study the effect of counter anions to biological activities, four different salts were prepared (as described in Chapter II) and evaluated for biological activities.

3.7.1 Cytotoxicity, Antimalarial and Antifungal Activity

Four salts of Compound 2 was subjected to the above mentioned activity. The results of the biological test are expressed in Table 3.29.

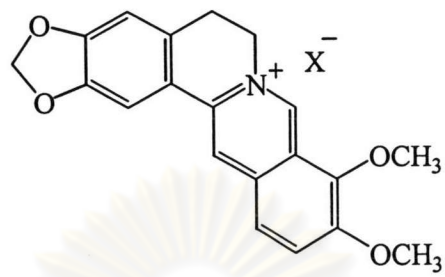


Table 3.29 The results of cytotoxicity, antimalarial and antifungal test of prepare salts of berberine

X^-	Biological test (IC_{50} ; μg/mL)		Biological test (EC_{50} ; μg/mL)
	Cytotoxicity	Antifungal	Antimalarial
[a]Cl^-	>50	13.0	0.11
PO_4^{3-}	>50	16.6	-
SO_4^{2-}	>50	9.36	-
CO_3^{2-}	>50	10.09	-
Picrate	>50	22.53	-

[a]Compound 2 was isolated as chloride salt

-: not tested

From Table 3.29, it was found that all salts displayed low toxicity against *vero cell* as Compound 2. In addition, sulfate and carbonate salts of Compound 2 showed the best significant antifungal activity whereas phosphate and picrate salts inhibited with less extent than Compound 2.

3.7.2 Anticancer Activity

The effect of prepared salts to anti cell lines was studied and the results are displayed in Table 3.30.

Table 3.30 The results of anticancer activity

Substance	KB		BC-1		NCl-H187	
	IC_{50} (μg/mL)	Activity	IC_{50} (μg/mL)	Activity	IC_{50} (μg/mL)	Activity
[a]Cl^-	0.48	Strongly active	0.95	Strongly active	0.30	Strongly active
PO_4^{3-}	-	Inactive	0.96	Strongly active	-[b]	-
SO_4^{2-}	-	Inactive	3.27	Strongly active	0.05	Strongly active
CO_3^{2-}	-	Inactive	3.61	Strongly active	0.06	Strongly active
Picrate	-	Inactive	6.62	Strongly active	0.07	Strongly active

[a]Compound 2 was isolated as chloride salt

[b]not tested

All prepared salts were inactive against KB cells and inhibited BC-1 cells less than the parent compound, Compound 2. However, all salts showed strong activity against NCl-H187 cells with IC_{50} value of less than 0.30 μg/mL. It could therefore be concluded that salts of Compound 2 had strong effect against NCl-H187 cells.

3.7.3 Antituberculosis Acitvity

The results of antituberculosis activity of all prepared salts were shown in Table 3.31.

Table 3.31 The results of anti tuberculosis activity

Substance	Results against TB at 200 μg/mL	MIC (μg/mL)
[a]Cl^-	Active	100
PO_4^{3-}	Active	200
SO_4^{2-}	Active	200
CO_3^{2-}	Active	100
picrate	Inctive	-

[a]Compound 2 was isolated as chloride salt

The results of antituberculosis activity exhibited that carbonate salt showed activity comparably equal to Compound 2. The others exhibited less activity than Compound 2. The carbonate salt however did not display antituberculosis activity against *M. tuberculosis* H37 *Ra.*

According to the all above result, it could be concluded that the water-soluble property of salt had no direct effect to biological activity. To illustrate this a phosphate salt reported in literature that it could completely be soluble in water[24] but it gave low inhibition activity against *M. tuberculosis* H37 *Ra.*