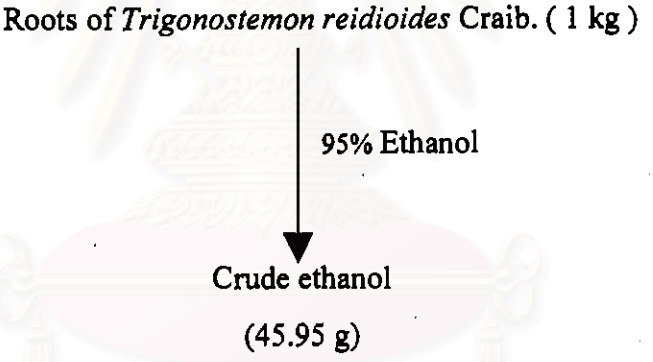
CHAPTER III
RESULTS AND DISCUSSION

The Results of Extraction

General Extraction for Preliminary Insect Antifeedant Activity Test

The sun-dried roots of *Trigonostemon reidioides* Craib. was ground and then extracted for preliminary insect antifeedant activity screening test according to the procedure described in Chapter II. The result of extraction is shown in Scheme 3.1.

Scheme 3.1 The result of extraction for preliminary insect antifeedant activity screening test



General Extraction

The ground sun-dried roots of *Trigonostemon reidioides* Craib. (28.5 kg) was extracted using the procedure described in Chapter II. The same procedure was repeated using the plant material 15.0 kg. The results of extraction are summarized as presented in Tables 3.1 and 3.2 and Schemes 3.2 and 3.3.

Table 3.1 The results of extraction by method I

Fraction (Solvent)	Weight (g) and Percentage (% wt/wt)	Feature
I (Hexane)	40.38 (0.14)	yellow-brown material
II (Methanol)	488.32 (1.71)	dark brown material
III (CH_2Cl_2)	57.06 (0.20)	yellow-brown material
IV (Ethyl acetate)	71.59 (0.25)	red-brown material
V (*n*-butanol)	5.36 (0.02)	red-brownish sticky material
VI (Water)	134.09 (0.47)	dark brownish sticky material

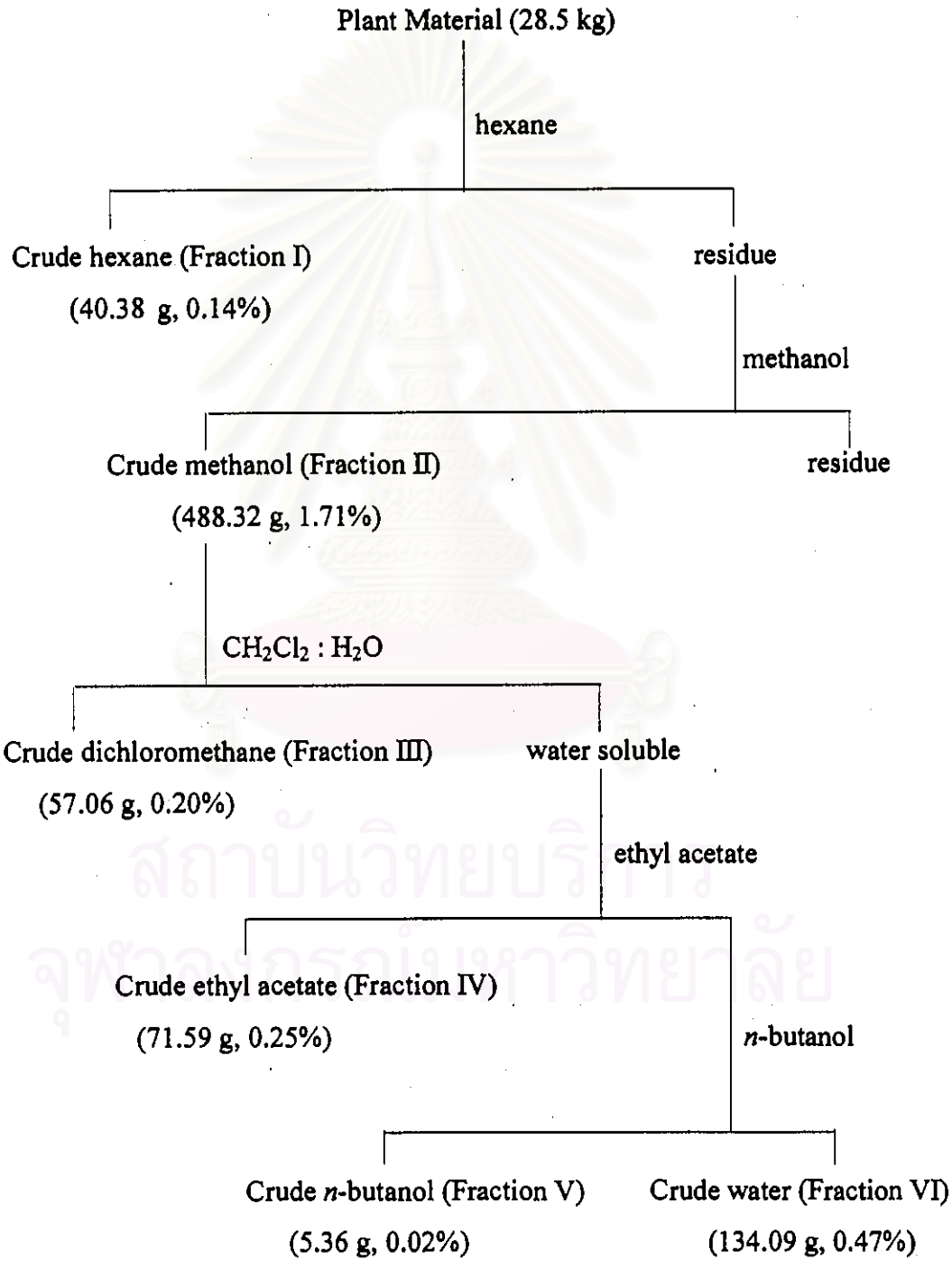
Table 3.2 The results of extraction by method II

Fraction (Solvent)	Weight (g) and Percentage (% wt/wt)	Feature
VII (Hexane)	29.85 (0.19)	yellow-brown material
VIII (CH_2Cl_2)	32.26 (0.21)	yellow-brown material
IX (Ethyl acetate)	21.35 (0.14)	red-brown material
X (Methanol)	240.81 (1.61)	dark brown material
XI (Water)	88.73 (0.59)	dark brownish sticky material
XII (Acetone)	3.91 (0.03)	yellow-brownish sticky liquid
XIII (*n*-butanol)	2.70 (0.02)	red-brownish sticky material

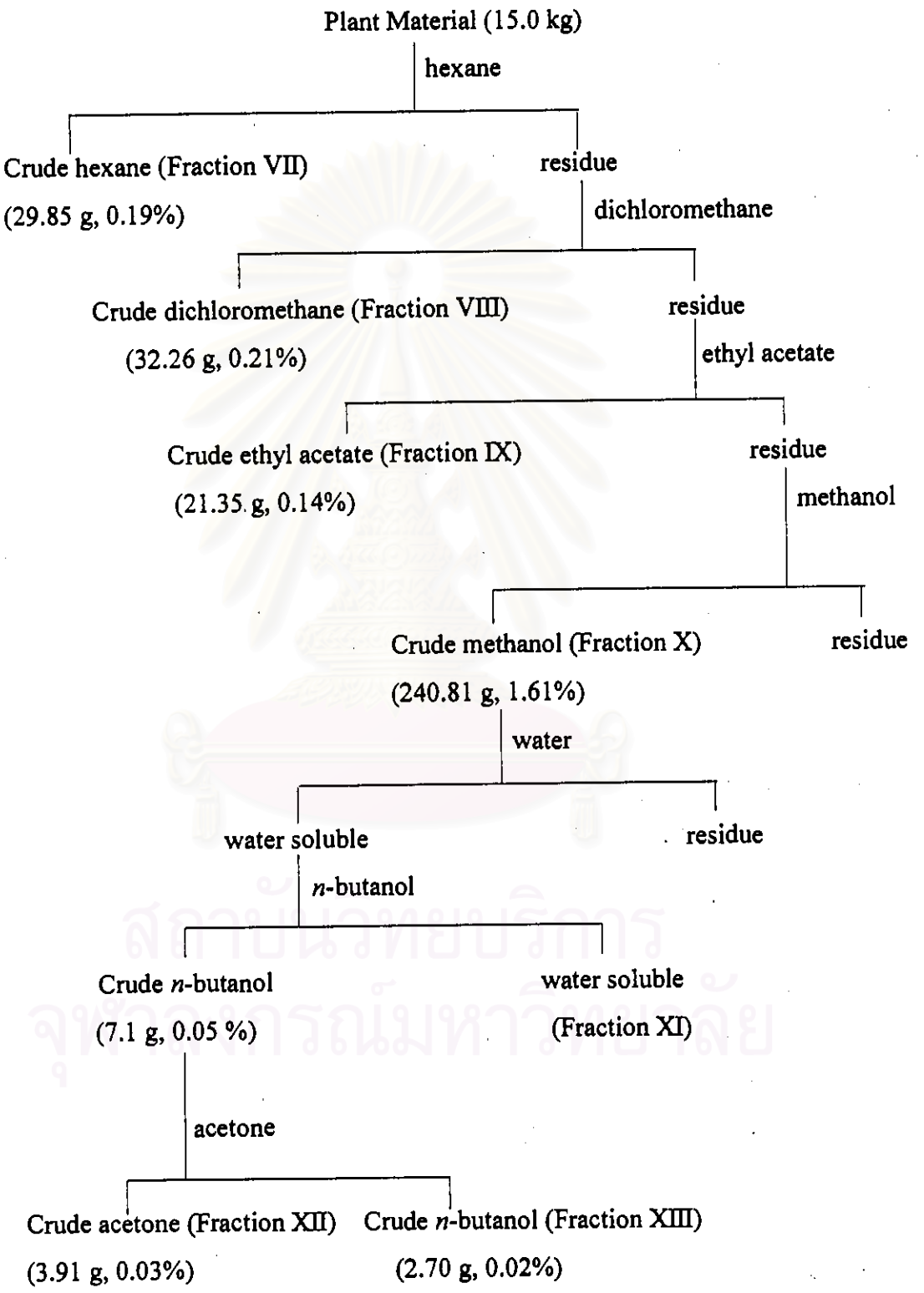
In order to compare the efficiency of two extraction methods described earlier, the percentage yields of derived crude extracts were determined. It was found that

these two extraction procedures for the roots of *Trigonostemon reidioides* Craib. did not show significantly difference in terms of percentage yield obtained.

Scheme 3.2 The results of extraction by method I



Scheme 3.3 The results of extraction by method II



The Results of Insect Antifeedant Activity Screening Tests

The ethanolic crude of the roots of *Trigonostemon reidioides* Craib. was preliminarily screened for insect antifeedant activity against the Greater Wax Moth larvae, *Galleria mellonella*. The result is displayed in Scheme 3.4.

Scheme 3.4 The preliminary insect antifeedant activity of the roots of *Trigonostemon reidioides* Craib.

The ground roots of *Trigonostemon reidioides* Craib.

↓ ethanol

Ethanol extract

% Insect antifeedant activity = 75

Notes:

71 - 100 = High activity
41 - 70 = Medium activity
11 - 40 = Low activity
0 - 10 = No activity

According to the preliminary result, it was found that the ethanolic extract of the roots of *Trigonostemon reidioides* Craib. possessed high antifeedant activity. Furthermore, the crude extracts from the first and second extraction procedures were subjected to insect antifeedant activity test. The results are shown in Tables 3.3 and 3.4, Schemes 3.5 and 3.6 and Figs. 3.1 and 3.2.

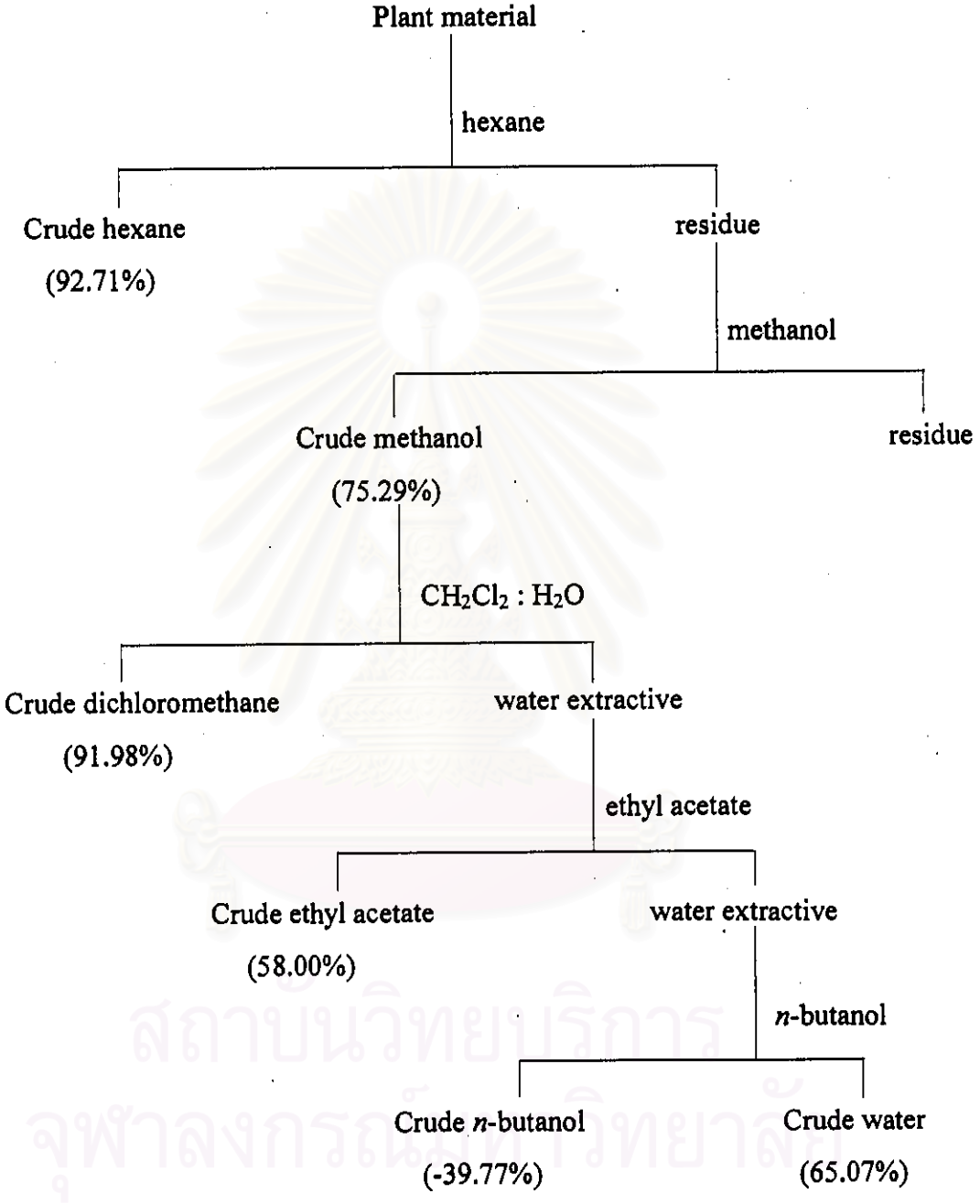
Table 3.3 The results of insect antifeedant activity of crude extracts from the roots of *Trigonostemon reidioides* Craib. following the first extraction procedure

Fraction (Crude extract)	Percentage of insect antifeedant activity	Level of activity
I (Hexane)	92.71	high
II (Methanol)	75.29	high
III (CH_2Cl_2)	91.98	high
IV (Ethyl acetate)	58.00	medium
V (n-butanol)	-39.77	attracted
VI (Water)	65.07	medium

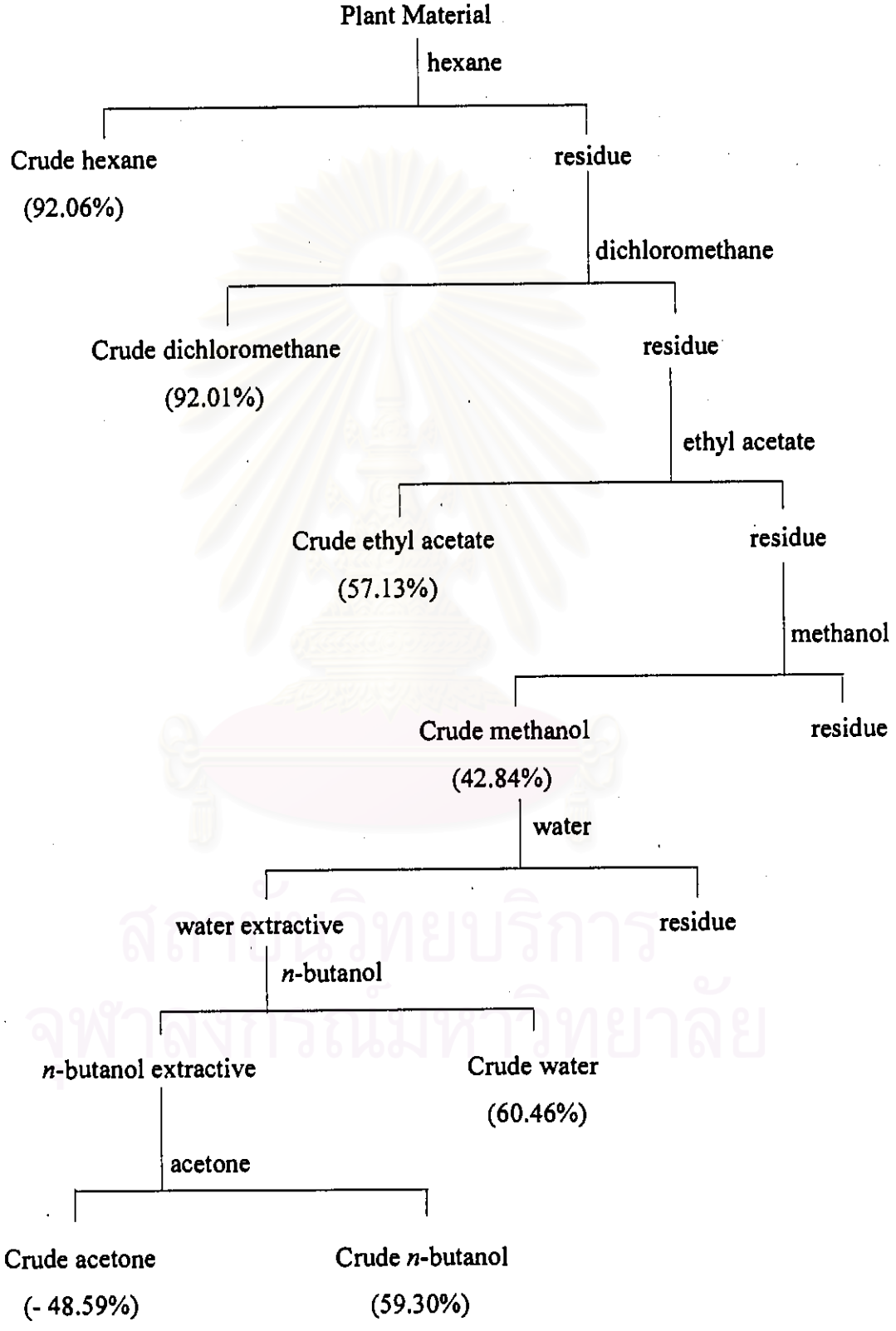
Table 3.4 The results of insect antifeedant activity of crude extracts from the roots of *Trigonostemon reidioides* Craib. following the second extraction procedure

Fraction (Crude extract)	Percentage of insect antifeedant activity	Level of activity
VII (Hexane)	92.06	high
VIII (CH_2Cl_2)	92.01	high
IX (Ethyl acetate)	57.13	medium
X (Methanol)	42.84	medium
XI (Water)	60.46	medium
XII (Acetone)	-48.59	attracted
XIII (*n*-butanol)	59.30	medium

Scheme 3.5 The insect antifeedant activity following the first extraction procedure



Scheme 3.6 The insect antifeedant activity following the second extraction procedure



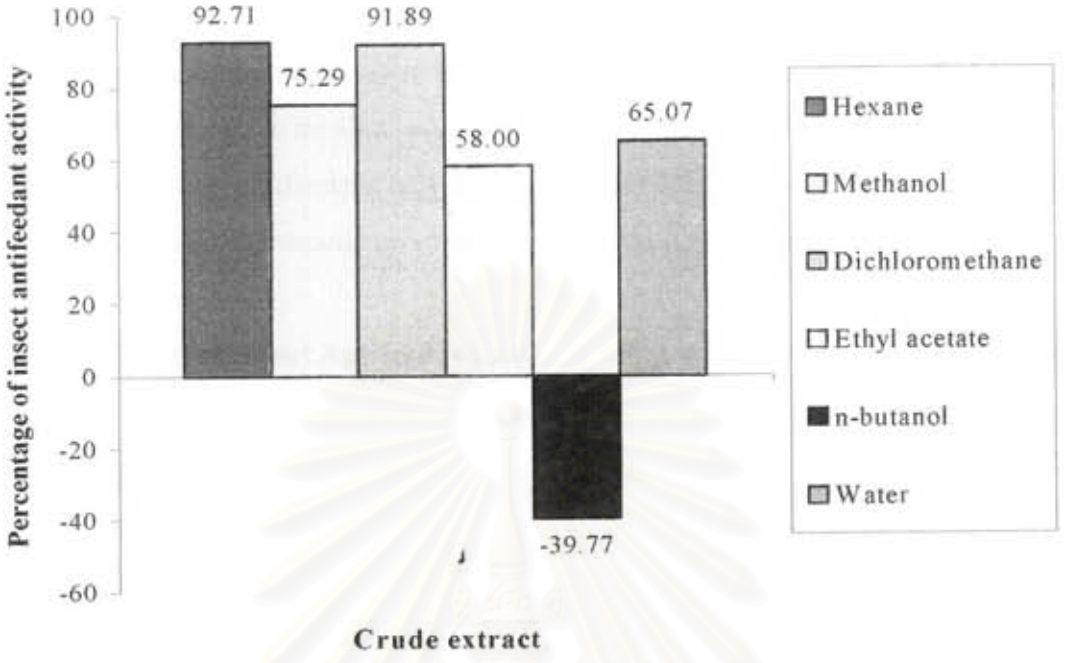


Fig. 3.1 The insect antifeedant activity of crude extracts by the first extraction procedure

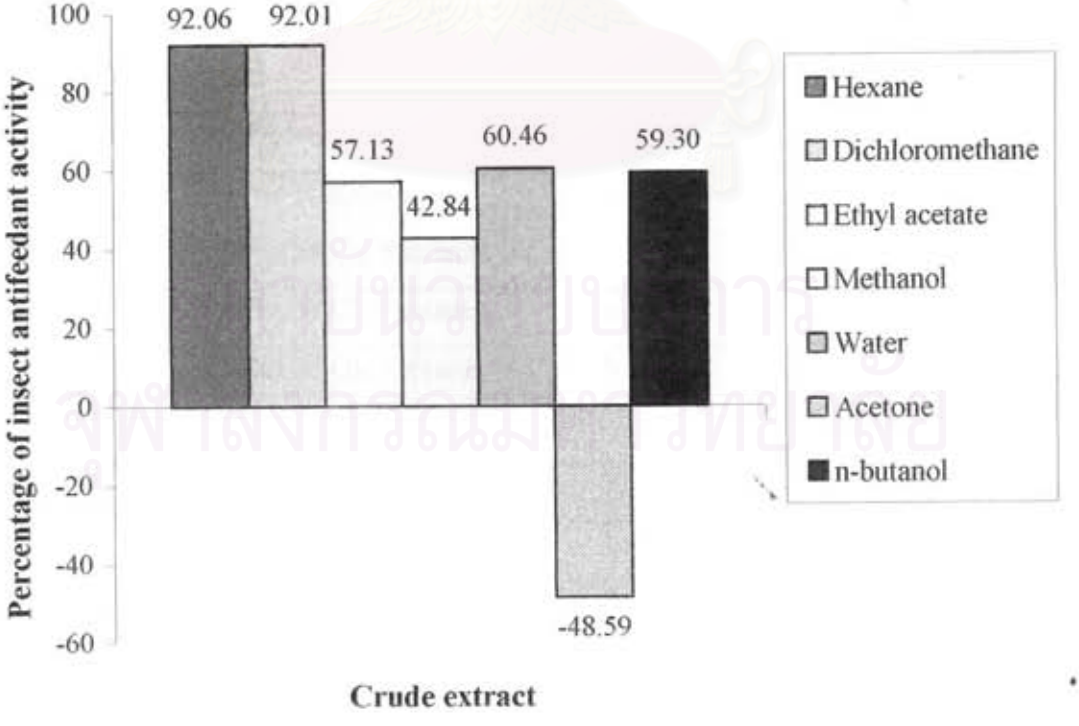


Fig. 3.2 The insect antifeedant activity of crude extracts by the second extraction procedure

From the results of insect antifeedant preliminary screening tests, the most tendency potent extracts were the crudes of hexane and dichloromethane. The other crude extracts gave medium activity results, except for *n*-butanolic extract which gave inverse insect antifeedant activity: low insect attractant activity. Thus, the crudes hexane and dichloromethane were selected for further examination.

Separation of Insect Antifeedant Activity from Active Crude Fractions

Separation of Crude Hexane

The crude hexane, Fractions I and VII from the first and second extraction procedures, respectively was examined by TLC. It was found that both fractions displayed similar spots on TLC. Therefore, those two fractions are combined. The crude hexane, 26.42 g was then separated by quick column chromatography technique. The column was initially eluted by solvents following by their polarity to yield 6 fractions. The results are shown in Table 3.5.

Table 3.5 The results of separation of crude hexane by quick column chromatography technique

Fraction	Solvent system	Weight (g)	Feature
IA	Hexane, 10%CH_2Cl_2:Hexane	2.31	mixed yellow and white solid
IB	20%CH_2Cl_2:Hexane	0.87	solid in pale brown oil
IC	50%CH_2Cl_2:Hexane	6.14	mixed white and dark green solid
ID	75%CH_2Cl_2:Hexane	7.25	solid in dark green oil
IE	CH_2Cl_2	1.47	sticky brown material
IF	10%CH_3OH:CH_2Cl_2	8.34	sticky dark brown material

Each separated fraction was subjected to insect antifeedant activity against Greater Wax moth, *Galleria mellonella.* The results are displayed in Table 3.6 and Fig. 3.3.

Each separated fraction was subjected to insect antifeedant activity against Greater Wax moth, *Galleria mellonella.* The results are displayed in Table 3.6 and Fig. 3.3.

Table 3.6 The insect antifeedant activity of each fraction separated from crude hexane

Fraction	Solvent system	Percentage of insect antifeedant activity	level of activity
IA	Hexane, 10%CH_2Cl_2:Hexane	18.29	low
IB	20%CH_2Cl_2:Hexane	88.07	high
IC	50%CH_2Cl_2:Hexane	11.54	low
ID	75%CH_2Cl_2:Hexane	45.02	medium
IE	CH_2Cl_2	61.35	medium
IF	10%CH_3OH:CH_2Cl_2	84.33	high

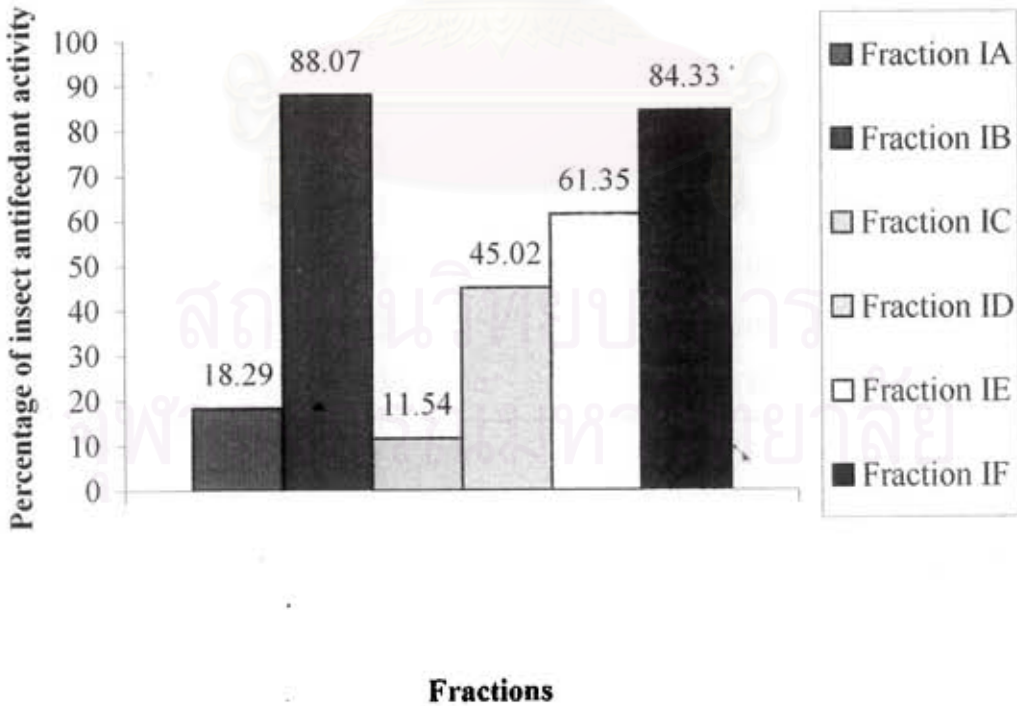


Fig. 3.3 The insect antifeedant activity of each fraction separated from crude hexane

From the activity results, Fractions IB and IF showed high antifeedant activity. These two promising fractions were further investigated. Other fractions combinding Fractions IA and IC had low activity while Fractions ID and IE displayed medium activity.

Separation of Fraction IB

Fraction IB which revealed high antifeedant activity (Fig. 3.3), 0.87 g was reseparated by using silica gel column chromatography. Hexane, a mixture of dichloromethane and hexane and dichloromethane were used as eluents. About 50 mL was collected for each fraction and then concentrated to a small volume approximately 10 mL. Each fraction was monitored by TLC and similar fractions were combined. The results of the separation of Fraction IB are recorded in Table 3.7.

Table 3.7 The results of the separation of Fraction IB

Eluent	Fraction No.	Remarks	Weight (g)
Hexane	1-2	yellow green oil	0.04
10%CH_2Cl_2:Hexane	3-6	yellow green oil	0.07
10%CH_2Cl_2:Hexane	7-9	green oil	0.02
20%CH_2Cl_2:Hexane	10-16	green oil	0.15
40%CH_2Cl_2:Hexane	17-22	green oil	0.18
60%CH_2Cl_2:Hexane	23-29	green oil	0.21
80%CH_2Cl_2:Hexane	30-35	green oil	0.18
CH_2Cl_2	36-38	green oil	0.06

From the results of separation, it could be grouped into six fractions by using TLC. In each fraction, there are many spots revealed on TLC plate. Unfortunately, each portion contained only small amount of substance, further purification was therefore impossible to conduct.

Separation of Fraction IF

Fraction IF was another promising fraction that displayed attractive antifeedant activity. This fraction, 8.34 g was reseparated by using silica gel chromatography. A mixture of dichloromethane and hexane, dichloromethane and a mixture of methanol and dichloromethane were used as eluents. About 50 mL of eluent was collected for each fraction and then concentrated to small amount of volume. Each fraction was monitored by TLC. The results of the separation of Fraction IF are shown in Table 3.8.

Table 3.8 The results of the separation of Fraction IF

Eluents	Fraction No.	Remarks	Weight (g)
60%CH_2Cl_2:Hexane	1-12	white solid with little	1.48
80%CH_2Cl_2:Hexane	13-30	green oil	0.97
CH_2Cl_2	31-41	white solid	0.49
2%CH_3OH:CH_2Cl_2	42-49	white material	3.51
10%CH_3OH:CH_2Cl_2	50-56	dark brown material	1.86

From the results of separation, fraction No. 1-12 was washed with hexane and the remained solid was further purified by recrystallization with dichloromethane-methanol several times, and white needle crystal (0.92 g) designated as Compound A was obtained. The solid containing in fraction No. 13-30 was purified by recrystallization with dichloromethane-methanol several times to afford white needle crystal (0.96 g). After monitoring by TLC, it was observed that this solid gave the same R_f value as that of Compound A. The solid material in fraction No. 31-41 was purified by first washing with methanol and then recrystallized with dichloromethane-methanol several times. The white plate solid (0.55 g), designated as Compound B was received.

Structural Elucidation of Compound A

Compound A had R_f 0.53 (5% methanol-dichloromethane), m.p. 298-300 °C, soluble in dichloromethane, ethyl acetate, acetone, ethanol and methanol; not soluble in hexane. This compound gave the same R_f value as an authentic acetyl aleuritolic acid. In addition, the Co-TLC of both compounds was also found to give the same R_f values in many solvent systems.

The IR spectrum of this compound (Fig. A1) showed the characteristic absorption peaks of carboxyl group at 3500-2500 (O-H) and 1690 (C=O) cm^{-1}, acetyl group at 1740 (C=O) and 1240 (C-O) cm^{-1}, gem-dimethyl group at 1375 cm^{-1} and trisubstituted vinyl group at 820 and 800 cm^{-1}, respectively. Other signals were tentatively assigned as shown in Table 3.9.

Table 3.9 The IR absorption band assignments of Compound A

Wave number (cm^{-1})	Intensity	Tentative assignment
3500-2500	broad	O-H stretching
3050	weak	C-H stretching of alkene
2950, 2880	strong	C-H stretching of CH_2, CH_3
1740	strong	C=O stretching of ester
1690	strong	C=O stretching of carboxyl
1480-1460	medium	C-H symmetric and asymmetric bending of CH_2 and CH_3
1375	medium	C-H symmetric bending of CH_3 (gem-dimethyl)
1300	medium	C-O stretching of acid
1240	strong	C-O stretching of acetate
820, 800	weak	C-H bending out-of-plane of trisubstituted vinyl

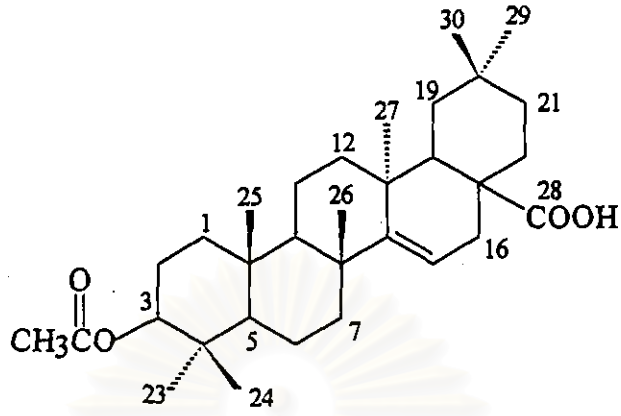
The ^1H NMR spectrum of Compound A (Fig. A2) showed signals of proton at δ 0.89-0.96 (21H, m, $7CH_3$), 1.14-2.03 (23H, m), 2.31 (3H, s, $COCH_3$), 4.47 (1H, m, H-3) and 5.53 (1H, m, H-14).

The ^{13}C NMR spectrum of Compound A (Fig. A3) exhibited carbon signals of two carbonyl groups at δ (ppm): 184.2 (COOH) and 171.9 ($COCH_3$). Other signals were assigned as shown in Table 3.10.

Table 3.10 The ^{13}C-NMR chemical shift assignments of Compound A

Position	Chemical shift (ppm)	Position	Chemical shift (ppm)
1	36.2	17	52.2
2	23.3	18	42.3
3	81.8	19	41.7
4	38.6	20	30.2
5	56.4	21	34.2
6	19.6	22	32.3
7	34.6	23	27.0
8	39.9	24	17.4
9	49.9	25	16.5
10	38.2	26	28.8
11	18.2	27	24.3
12	31.6	28	184.2
13	35.8	29	32.8
14	161.3	30	22.1
15	117.7	C-O-CO-Me	22.1
16	29.6	C-O-CO-Me	171.9

From the comparison of physical properties and all spectroscopic data with the reported acetyl aleuritolic acid in literatures[28,32], it was ascertained that Compound A was acetyl aleuritolic acid.



Compound A

Structural Elucidation of Compound B

Compound **B** had R_f 0.2 (dichloromethane), m.p. 199-200 °C. This compound was soluble in dichloromethane and ethyl acetate, slightly soluble in ethanol and methanol but not soluble in hexane and acetone. Compound **B** gave the same R_f value as an authentic 5α-stigmastane-3,6-dione in several solvent systems.

The IR spectrum of this compound (Fig. A4) showed the characteristic absorption peaks of ketone moiety at 1705, 1260 and 1240 cm^{-1}. Other signals were tentatively assigned as shown in Table 3.11.

Table 3.11 The IR absorption band assignments of Compound **B**

Wave number (cm^{-1})	Intensity	Tentative assignment
2950, 2860	strong	C-H stretching of CH_2, CH_3
1705	strong, broad	C=O stretching of keto group
1480	medium	C-H symmetric bending of CH_2 and asymmetric of CH_3
1420	medium	C-H symmetric bending of $-CH_2CO$
1380	medium	C-H symmetric bending of CH_3
1260, 1240	medium	C-C-C stretching and bending of ketone

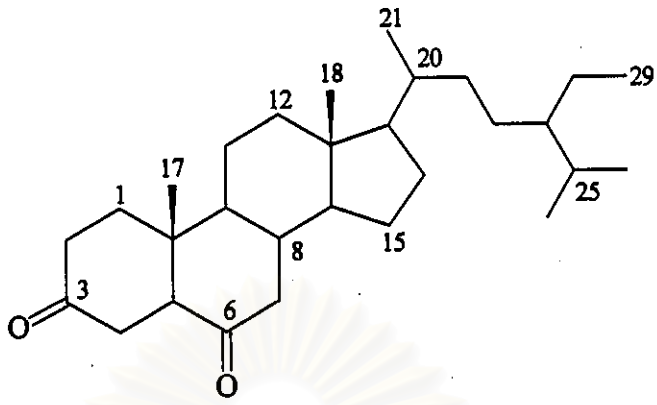
The ^{1}H NMR spectrum ($CDCl_3$) (Fig. A5) showed the proton signals at δ (ppm): 0.69 (3H, s, CH_3), 0.85 (12H, $4CH_3$), 0.96 (3H, s, CH_3), 1.19-1.56 (23H) and 2.01-2.65 (7H).

The ^{13}C NMR spectrum ($CDCl_3$) (Fig. A6) showed 27 carbon signals from 29 carbon atoms. The intensity of signals at δ 12.0 and 38.0 ppm was found around twice as other signals. In addition, there were characteristic signals at δ 211.3 (C=O) and 209.2 (C=O) ppm. Other signals are assigned as shown in Table 3.12.

Table 3.12 The ^{13}C-NMR chemical shift assignments of Compound **B**

Position	Chemical shift (ppm)	Position	Chemical shift (ppm)
1	38.0	16	28.0
2	37.4	17	56.0
3	209.2	18	12.0
4	37.0	19	12.6
5	57.5	20	36.0
6	211.3	21	19.0
7	45.8	22	33.8
8	38.0	23	29.1
9	53.5	24	46.6
10	41.2	25	26.0
11	21.7	26	18.7
12	39.3	27	19.8
13	43.0	28	23.0
14	56.4	29	12.0
15	24.0		

From the comparison of physical properties, IR, ^{1}H-NMR, ^{13}C-NMR spectra with an authentic sample, it could be obviously concluded that this compound was 5α-stigmastane-3,6-dione.[28]



Compound **B**

Compounds **A** and **B** were then subjected to insect antifeedant activity test. The results are displayed in Table 3.13.

Table 3.13 The results of insect antifeedant activity of Compounds **A** and **B**

Compound	% Insect antifeedant activity	Level of activity
A (acetyl aleuritolic acid)	83.15	high
B (5α-stigmastane-3,6-dione)	53.54	medium

It was found that Compound **A** (acetyl aleuritolic acid) showed high activity while Compound **B** (5α-stigmastane-3,6-dione) exhibited medium activity.

Compound **A** was further studied for insect antifeedant activity test by varying the qauntity of substances. The results are displayed as shown in Table 3.14 and Fig. 3.4.

Table 3.14 The results of insect antifeedant activity of Compound **A** (acetyl aleuritolic acid)

% Concentration (wt/wt)	% Insect antifeedant activity
0.10	39.61
0.15	47.40
0.20	68.71
0.25	83.15

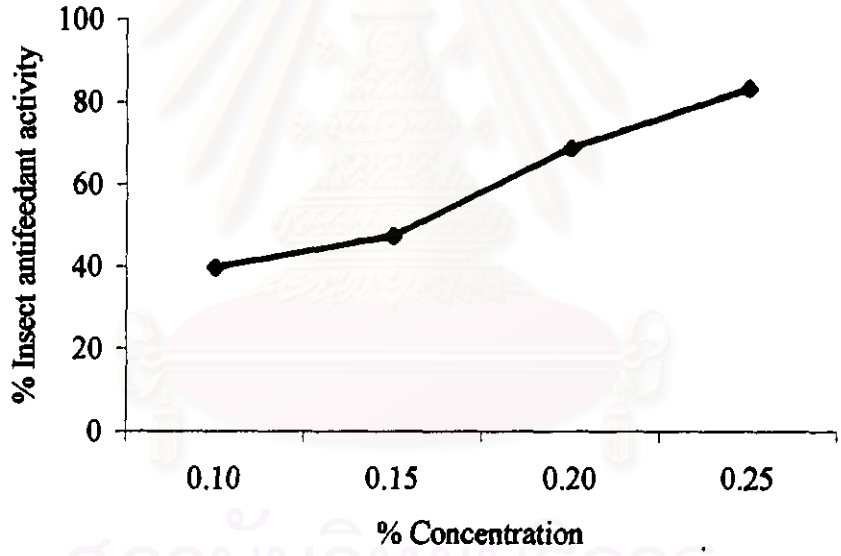


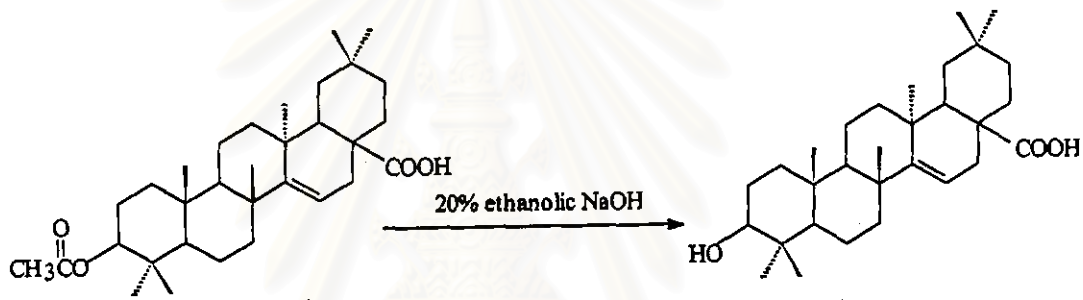
Fig. 3.4 Insect antifeedant activity of Compound **A** (acetyl aleuritolic acid)

From Fig.3.4, it was found that the effective concentration (EC_{50}) of this compound showed insect antifeedant activity approximately 0.16 % wt/wt.

Insect antifeedant activity of related triterpenoids

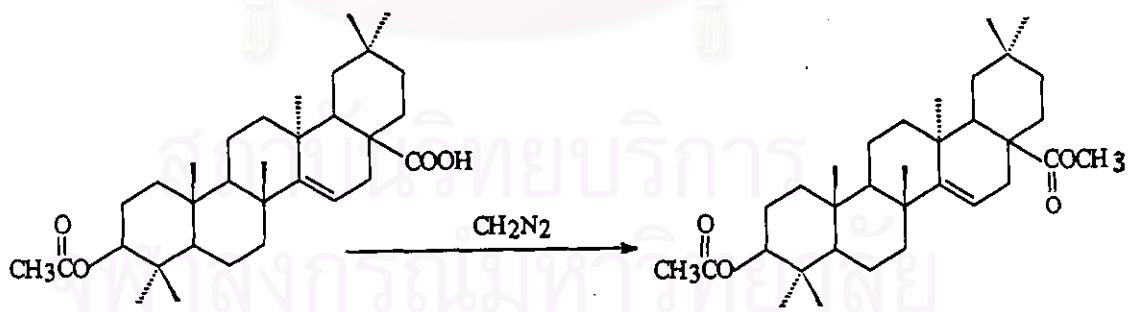
Stemmed from the results of insect antifeedant activity, conceivable Compound A or acetyl aleuritolic acid exhibited high activity. It was therefore worth considering what part of the molecule of this active triterpenoid affect this activity. The hydrolysis and methylation reactions of Compound A as shown below were set up. The derived products were then subjected to antifeedant activity. The outcome of this study would provide preliminary structure-activity relationship.

The hydrolysis of acetyl aleuritolic acid



acetyl aleuritolic acid — aleuritolic acid (**A1**)

The methylation of acetyl aleuritolic acid



acetyl aleuritolic acid — acetoxy methyl aleuritolate (**A2**)

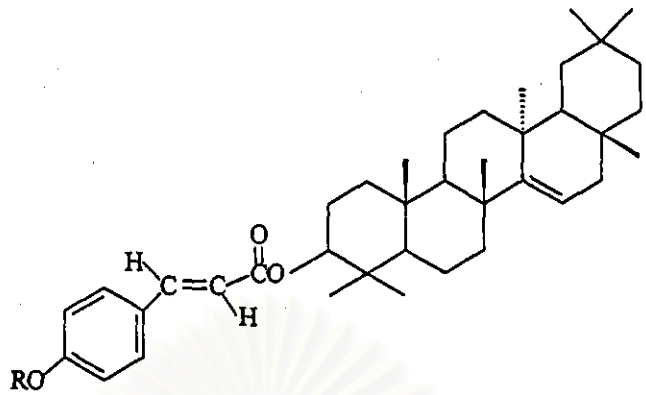
These two synthesized Compounds **A1** and **A2** (aleuritolic acid and acetoxy methyl aleuritolate, respectively) were subjected to insect antifeedant activity test. The results are shown in Table 3.15.

Table 3.15 The results of insect antifeedant activity of Compounds **A1** and **A2**

Compound	% Insect antifeedant activity	Level of activity
A1 (aleuritolic acid)	-14.73	attracted
A2 (acetoxy methyl aleuritolate)	40.43	medium

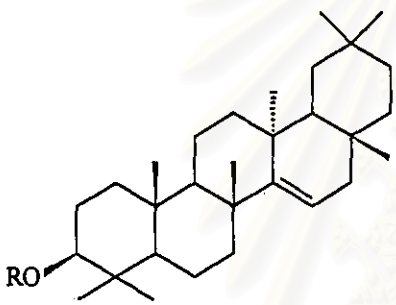
From the results of activity, Compound **A1** (aleuritolic acid) showed inverse insect antifeedant activity but Compound **A2** exhibited medium activity. These results implied that the acetyl group at carbon position 3 is virtually essential and affected the activity more than the carboxyl group at carbon position 17. From this hypothesis, several known triterpenoids* containing an acetyl group at carbon position 3 and those without this group were collected and subjected to the activity test. They are careaborin, careaborin acetate, taraxerol, taraxeryl acetate, lupeol, lupeol acetate, friedelan-3-β-ol, friedelan acetate, a mixture of steroid acetate and betulinic acid. The results are displayed in Table 3.16.

* Careaborin, creaborin acetate, taraxerol, taraxeryl acetate, lupeol, lupeol acetate and a mixture of steroid acetate were obtained from *Rhizophora apiculata* Bl.
Friedelan-3-β-ol and friedelan acetate was isolated from *Bridelia ovata* Decne.
Betulinic acid was obtained from *Sphenoclea zeylanica* Gaertn.
All triterpenoids are belonging to Natural Products Research Unit, Department of Chemistry, Chulalongkorn University.



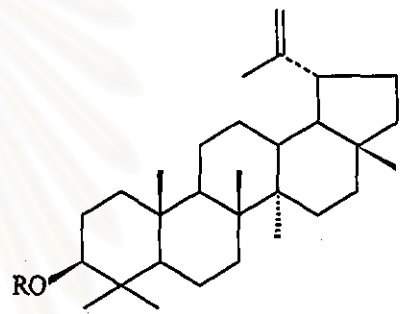
R = H ; Careaborin

R = Ac ; Careaborin acetate



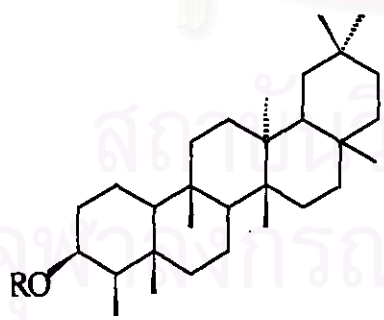
R = H ; Taraxerol

R = Ac ; Taraxeryl acetate



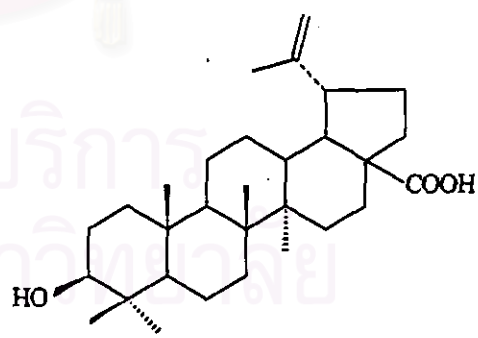
R = H ; Lupeol

R = Ac ; Lupeol acetate



R = H ; Friedelan-3-β-ol

R = Ac ; Friedelan acetate



Betulinic acid

Table 3.16 The results of insect antifeedant activity of triterpenoids

Compound	% Insect antifeedant activity	Level of activity
Careaborin	61.07	medium
Creaborin acetate	-38.15	attracted
Taraxerol	-47.57	attracted
Taraxeryl acetate	44.72	medium
Lupeol	16.29	low
Lupeol acetate	-28.49	attracted
Friedelan-3-β-ol	-1.36	attracted
Friedelan acetate	61.14	medium
Betulinic acid	-49.73	attracted
mixture of steroid acetate	65.34	medium

From the results of activity test, it was found that there was a correlation as above mentioned hypothesis. The acetyl group at carbon position 3 of pentacyclic six-membered ring triterpenoid, are affected to the insect antifeedant activity whereas lupane-type triterpenoid and steroids did not reveal this tendency. This means that a triterpenoid acetate showed higher activity than a triterpenoid without an acetyl group at carbon position 3 such as taraxerol and friedelan-3β-ol. Both of them exhibited inverse insect antifeedant activity while taraxeryl acetate and friedelan acetate showed medium activity.

Separation of Fraction IA

Fraction IA 2.31 g was reseparated by using silica gel column chromatography. Hexane, a mixture of dichloromethane and hexane and dichloromethane were used as eluents. About 100 mL of eluent was collected for each fraction and then concentrated to small amount of volume. Each fraction was monitored by TLC. The results of the separation of Fraction IA are shown in Table 3.17.

Table 3.17 The results of the separation of Fraction IA

Eluent	Fraction No.	Remarks	Weight (g)
Hexane	1-10	pale green oil	0.09
Hexane	11-42	white solid	0.95
Hexane	43-59	white solid with green oil	0.53
Hexane	60-65	green oil	0.10
5% CH_2Cl_2-Hexane	66-71	green oil	0.11
5% CH_2Cl_2-Hexane	72-85	green oil	0.25
5% CH_2Cl_2-Hexane	86-96	green oil	0.18
10% CH_2Cl_2-Hexane	97-108	green oil	0.20
10% CH_2Cl_2-Hexane	109-119	yellow-green oil	0.13
20% CH_2Cl_2-Hexane	120-131	pale yellow oil	0.15
CH_2Cl_2	132-144	pale yellow oil	0.15

Fraction Nos. 11-42 and 43-59 were combined and purified by recrystallization with hot hexane several times to give white amorphous solid, 1.09 g (Mixture **C**).

Structural Elucidation of Mixture C

Mixture **C** had melting point 85-87 °C and R_f 0.65 (40% dichloromethane-hexane). This mixture was soluble in dichloromethane and ethyl acetate but slightly soluble in hexane, acetone and methanol.

The IR spectrum showed characteristic absorption band of carbonyl group belonging to an ester at 1740 cm^{-1}, disubstituted vinyl group at 970 and 960 cm^{-1}, trisubstituted vinyl moiety at 800 and 790 cm^{-1} and long chain moiety at 725 cm^{-1}. Other signals were tentatively assigned as shown in Table 3.18.

Table 3.18 The IR absorption band assignments of Mixture C

Wavenumber (cm^{-1})	Intensity	tentative assignment
2920, 2850	strong	C-H stretching of CH_2 and CH_3
1740	strong	C=O stretching of ester
1465	medium	C-H symmetric bending of CH_2 and asymmetric of CH_3
1385	medium	C-H symmetric bending of CH_3
1200, 1180	medium	C-O stretching
970, 960	weak	C-H out of plane bending of disubstituted vinyl
800, 790	weak	C-H out of plane bending of trisubstituted vinyl
725	weak	CH_2 rocking

The ^{1}H-NMR spectrum showed characteristic peaks of steroid at δ 0.69, 0.85, 0.87 and 1.02 (18H, $6CH_3$), 4.63 (H-3) and 5.39 (H-6) ppm. The proton at δ 0.69 and 1.02 ppm were assigned for the signals of 2 angular methyl groups: C-18 and C-19, respectively while the signals at δ 0.85 and 0.87 ppm were the signals of methyl groups at side chain 4 groups, C-21, 26, 27 and 29. In addition, the triplet signal at δ 2.27 ppm revealed a proton of a carbon adjacent to a carbonyl group and a high intensity singlet signal at δ 1.26 ppm revealed a long chain compound composing of methylene groups.

The ^{13}C-NMR spectrum showed 37 signals of carbon including the carbonyl carbon signal of an ester at δ 173.3 ppm. Other signals were carbon signals of steroid and long chain acid as shown in Table 3.19.

Table 3.19 The ^{13}C-NMR chemical shift assignments of Mixture C

Position	Chemical shift (ppm)
1	37.1
2	27.8
3	73.7
4	38.2
5	139.8
6	122.6
7	31.9
8	31.9
9	50.1
10	36.6
11	21.1
12	39.8
13	42.4
14	56.7
15	24.3
16	28.3
17	56.1
18	11.9
19	19.3
20	36.2

Position	Chemical shift (ppm)
21	19.1
22	34.0
23	29.2
24	50.1
25	26.2
26	18.8
27	19.8
28	23.1
29	11.9
1′	173.3
2′	34.7
3′	25.1
4′	29.2
5′	29.5
6′-12′	29.7
13′	29.4
14′	31.9
15′	22.7
16′	14.1

By comparison physical properties and all spectroscopic data with steroidal ester reported in literature[28], this mixture can be concluded as a steroidal ester.

Mixture **C** was subjected to the insect antifeedant activity test. The result is displayed in Table 3.20.

Table 3.20 The result of insect antifeedant activity of Mixture **C**

Mixture	% Insect antifeedant activity	Level of activity
C (steroidal ester)	-33.52	attracted

It was found that Mixture **C** showed inverse insect antifeedant activity.

Separation of Fraction IC

Fraction IC 6.14 g was reseparated by using silica gel column chromatography. Hexane, a mixture of dichloromethane and hexane, dichloromethane and a mixture of methanol and dichloromethane were used as eluents. About 100 mL of eluent was collected for each fraction and concentrated to small amount of volume. Each fraction was monitored by TLC. The results of the separation of Fraction IC are shown in Table 3.21.

Table 3.21 The results of the separation of Fraction IC

Eluents	Fraction No.	Remarks	Weight (g)
20% CH_2Cl_2:Hexane	1-5	white wax	0.32
20% CH_2Cl_2:Hexane	6-8	yellow oil	0.11
40% CH_2Cl_2:Hexane	9-15	yellow oil with white material	0.70
40% CH_2Cl_2:Hexane and 60% CH_2Cl_2:Hexane	16-22	pale yellow-white solid	1.56
60% CH_2Cl_2:Hexane and 80% CH_2Cl_2:Hexane	23-27	yellow solid	0.54
80% CH_2Cl_2:Hexane and CH_2Cl_2	28-37	white crystal with yellow oil	1.64
CH_2Cl_2	38-41	white crystal	0.54
5% CH_3OH:CH_2Cl_2	42-44	pale brown material	0.61

Fraction Nos. 9-15, 16-22 and 23-27 were washed with hexane to gain white amorphous solid. After purification by recrystallization with hot acetone several times, Mixture **D** 2.39 g was obtained. In addition, Fraction Nos. 28-37 and 38-41 were combined and washed with hexane to remove yellow oil. After recrystallization several times with hot hexane, white amorphous solid, Mixture **E**, 0.79 g was obtained.

Structural Elucidation of Mixture D

Mixture **D** had m.p. 74-76 °C and R_f value 0.37 (40% CH_2Cl_2:Hexane). This mixture was soluble in dichloromethane and slightly soluble in hexane, ethyl acetate, acetone, ethanol and methanol. This mixture gave the same R_f value as authentic long chain acid. In addition, the Co-TLC of both mixtures were also found to give the same R_f values.

The IR spectrum of this mixture showed the characteristic absorption peak of carboxyl group at 3600-2500 and 1710 cm^{-1} and long chain at 730 and 720 cm^{-1}. Other signals were tentatively assigned as shown in Table 3.22.

Table 3.22 The IR absorption band assignments of Mixture **D**

Wavenumber (cm^{-1})	intensity	tentative assignment
3600-2500	broad	O-H stretching
2920, 2850	strong	C-H stretching CH_2, CH_3
1710	strong	C=O stretching of carboxyl group
1475, 1465	medium	C-H symmetric bending of CH_2 and asymmetric of CH_3
1410	medium	C-O-H bending in plane
1300	medium	C-O stretching
940	weak	O-H out of plane
730, 720	medium	CH_2 rocking

The ^{1}H-NMR spectrum showed the triplet signal at δ 0.88 and 2.35 ppm. The signal at δ 0.88 ppm was assigned for the signal of a methyl group while the signal at δ 2.35 ppm is the singlet signal proton on the adjacent carbon atom to the carbonyl group. In addition, there is the high intensity signal at δ 1.25 ppm compatible with several combined methylene groups.

The ^{13}C-NMR spectrum showed 11 carbon signals at δ 179.3 (C=O), 34.0, 32.0, 29.7, 29.5, 29.4, 29.2, 29.1, 24.7, 22.7 and 14.1 (CH_3) ppm.

From the comparison of physical properties and all spectroscopic data with an authentic sample of a mixture of long chain acid, it was found that both of them are corresponded. Thus, Mixture **D** should be a mixture of long chain acid.[28]

Structural Elucidation of Mixture E

Mixture **E** had m.p. 135-137 °C and R_f value 0.48 (50% CH_2Cl_2:Hexane). This mixture was soluble in dichloromethane, ethyl acetate, acetone, ethanol and methanol and slightly soluble in hexane. Mixture **E** gave the same R_f value as authentic mixture of steroid. In addition, the Co-TLC of both mixtures were also found to give the same R_f value.

The IR spectroscopic data is shown in Table 3.23.

Table 3.23 The IR absorption band assignments of Mixture **E**

Wavenumber (cm^{-1})	Intensity	Tentative assignment
3600-2500	broad	O-H stretching
2950 and 2850	strong	C-H stretching of CH_2 and CH_3
1640	weak	C=C stretching
1460 and 1380	medium	C-H symmetric and asymmetric bending of CH_2 and CH_3
970 and 960	weak	C-H out of plane bending of disubstituted vinyl
840 and 800	weak	C-H out of plane bending of trisubstituted vinyl

The ^{1}H-NMR spectrum showed the characteristic signal of steroid at δ 0.68-1.06 ppm which were the signals of two angular methyl groups at C-18 and C-19 and methyl groups at side chain, C-21, 26, 27 and 29. The proton signal at δ 1.54-2.31 ppm was the signal of methylene (CH_2) groups and methinic (CH) groups of steroid. The multiplet signal at δ 3.54 ppm was the signal of a hydroxyl proton. The doublet of doublet signal at δ 5.09 ppm was the signal of disubstituted vinyl protons (H-22 and H-23) while the signal at δ 5.37 ppm could be the signal of trisubstituted vinyl proton (H-6).

The ^{13}C-NMR spectrum showed 32 carbon signals. The tentative assignment are shown in Table 3.24.

Table 3.24 The ^{13}C-NMR chemical shift assignments of Mixture E

Position	Chemical shift (ppm)	Position	Chemical shift (ppm)
1	37.3	16	29.2
2	31.7	17	56.1
3	71.8	18	12.0
4	42.3	19	19.4
5	140.8	20	36.2, 40.5
6	121.7	21	19.0, 21.1
7	31.9	22	34.0, 138.3
8	31.9	23	28.3, 129.3
9	50.2	24	51.2
10	36.5	25	26.2, 31.9
11	21.1	26	18.8, 19.0
12	39.8	27	19.8, 21.1
13	42.3	28	23.1, 25.4
14	56.8	29	11.9, 12.0
15	24.3		

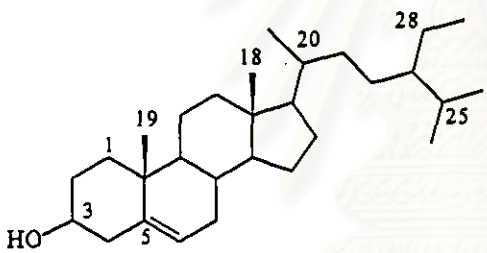
According to the information of this mixture, it was suggested that this mixture be close to those of steroids. The analysis method selected for further study of this mixture was GLC (condition: column temperature 260 °C, injection temperature 290 °C and flow rate of carried gas (N_2), 50 mL/min) compared with standard steroids, namely cholesterol, campesterol, stigmasterol and β-sitosterol. The results of GLC analysis of standard steroids showed the retention time at 13.36, 17.31, 18.36 and 20.91 min, respectively.

The results of GLC analysis of this mixture was showed retention time at 17.00, 18.16 and 20.66 min, respectively which were in fact corresponded to the authentic sample of campesterol, stigmasterol and β-sitosterol, respectively. The composition of steroids in this mixture is shown in Table 3.25.

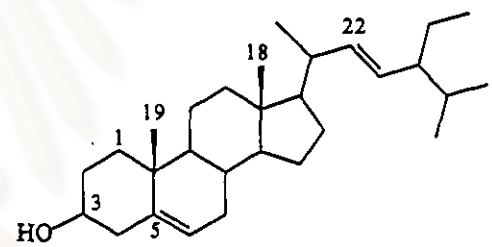
Table 3.25 The composition of steroids in Mixture **E**

Name	Retention time (min)	% Composition
Campesterol	17.00	3.13
Stigmasterol	18.16	41.05
β-Sitosterol	20.66	55.82

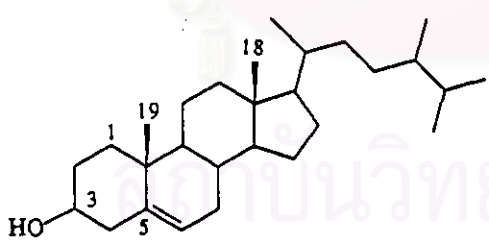
From the comparison of physical properties and all spectroscopic data including GLC analysis, there was no doubt to conclude that Mixture **E** is a mixture of steroid: campesterol, stigmasterol and β-sitosterol.



β-sitosterol



stigmasterol



campesterol

Mixtures **D** and **E** were subjected to the insect antifeedant activity test. The results of these mixtures are displayed in Table 3.26.

Table 3.26 The results of insect antifeedant activity of Mixtures **D** and **E**

Mixture	% Insect antifeedant actvity	Level of activity
D (mixture of long chain acid)	46.38	medium
E (mixture of steroid)	74.02	high

It was found that Mixture **D** (mixture of long chain acid) revealed medium insect antifeedant activity while Mixture **E** (mixture of steroid) presented high insect antifeedant activity.

Separation of Fraction ID

Fraction ID 7.25 g was separated again by column chromatography technique, using silica gel as an adsorbent. Hexane, a mixture of dichloromethane and hexane, dichloromethane and a mixture of methanol and dichloromethane were used as eluents. About 100 mL of eluent was collected for each fraction and then concentrated to small volume. Each fraction was monitored by TLC. The results of separation of Fraction ID are shown in Table 3.27.

Table 3.27 The results of the separation of Fraction ID

Eluents	Fraction No.	Remarks	Weight (g)
40% CH_2Cl_2:Hexane	1-3	pale blue-green wax	0.41
40% CH_2Cl_2:Hexane	4-6	blue-green liquid	0.15
40% CH_2Cl_2:Hexane	7-11	white-yellow material	0.62
40% CH_2Cl_2:Hexane	12-14	pale yellow solid	0.21
40% CH_2Cl_2:Hexane	15-18	white-yellow material	0.28
40% CH_2Cl_2:Hexane / 60% CH_2Cl_2:Hexane	19-20	pale yellow material	0.30
60% CH_2Cl_2:Hexane	21-25	crystal with green-yellow oil	0.43
60% CH_2Cl_2:Hexane	26-29	green crystal with green-yellow oil	0.47
60% CH_2Cl_2:Hexane	30-31	yellow-green oil	0.18
60% CH_2Cl_2:Hexane / 80% CH_2Cl_2:Hexane	32-37	little crystal with green oil	0.65
80% CH_2Cl_2:Hexane	38-40	green solid	0.26
80% CH_2Cl_2:Hexane / CH_2Cl_2	41-43	white crystal with green material	0.29
CH_2Cl_2	44-46	white crystal with yellow material	0.19
CH_2Cl_2 / 5% CH_3OH : CH_2Cl_2	47-49	white crystal with yellow material	0.26
5% CH_3OH:CH_2Cl_2	50-52	brown material	1.32
10% CH_3OH:CH_2Cl_2	53-54	brown material	0.38

Fractions No. 7-11, 12-14, 15-18 and 19-20 were combined and first purified by washing with hexane and then the receiving solid was further purified by recrystallization with hot hexane and the white-needle crystal (0.65 g) was obtained.

This compound possessed the same physical properties as those of Mixture **E** which can be concluded that this compound was the mixture of steroids.

Fraction Nos. 21-25, 26-29, 30-31, 32-37 and 38-40 were combined to give more target substance that need further purification. This combined fraction was washed with hexane and green solid was received (Compound **F**). This compound was further purified by recrystallization with hexane-dichloromethane several times, the green needle crystal (0.45 g) was obtained.

Structural Elucidation of Compound F

Compound **F** gave the R_f value 0.53 (2% methanol-dichloromethane) and revealed melting point 184-185 °C. This compound was soluble in dichloromethane, ethyl acetate, acetone, ethanol and methanol but not soluble in hexane.

The IR spectrum (Fig.3 A7) showed characteristic absorption peaks of α, β unsaturated carbonyl at 1670 and 1170 cm^{-1}, aromatic moiety at 1630, 1595, 1510 and 1460 cm^{-1}, ether at 1240 and 1095 cm^{-1} and gem-dimethyl group at 1395 and 1375 cm^{-1}. Other signals were tentatively assigned as shown in Table 3.28.

Table 3.28 The IR absorption band assignments of Compound **F**

Wavenumber (cm^{-1})	Intensity	Tentative assignment
3100	weak	C-H stretching of aromatic
3020	weak	C-H stretching of alkene
2980, 2840	weak	C-H stretching of CH_2, CH_3
2000-1680	summation	C-H bending of aromatic
1670	strong	C=O stretching of α, β unsaturated of carbonyl
1630, 1595, 1510, 1460	strong	C=C stretching of aromatic
1480	medium	C-H symmetric bending of CH_2 and asymmetric of CH_3
1395, 1375	medium	C-H symmetric bending of CH_3
1240	strong	C-O-C asymmetric stretching
1170	strong	C-CO-C bending
1095	strong	C-O-C symmetric bending
900, 850	medium	C-H bending of aromatic
830	medium	C-H out of plane bending of trisubstituted vinyl

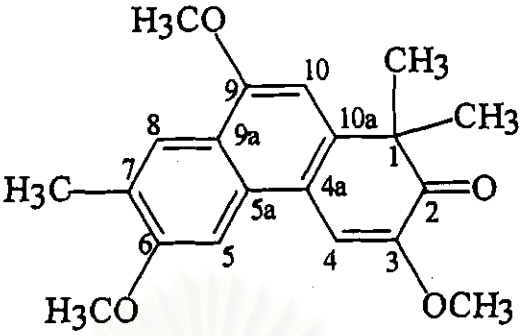
The ^{1}H-NMR spectrum (Fig. A8) showed 22 signals proton at δ 1.56 (6H, s, $2CH_3$), 2.39 (3H, s, CH_3), 3.93 (3H, s, OCH_3), 4.02 (6H, s, $2OCH_3$), 6.74 (1H, s), 7.28 (1H, s), and 8.04 (1H, s) ppm.

The ^{13}C-NMR spectrum (Fig. A9) showed 19 signals of 20 carbon atoms. That implied there were two equivalent carbon atoms. The signals exhibited at δ 199.3 (C=O), 157.8, 154.9, 147.4, 130.8, 130.7, 127.2, 123.4, 119.2, 114.3, 110.2, 100.6, 99.0, 55.5 (OCH_3), 55.4 (OCH_3), 55.1 (OCH_3), 49.5, 28.3 and 16.6 (CH_3) ppm. The carbon signals assignment is shown in Table 3.29.

Table 3.29 The ^{13}C-NMR chemical shift assignments of Compound **F**

Position	Chemical shift (ppm)
1	49.5
2	199.3
3	147.4
4	99.0
4a	130.7
5a	142.6
5	110.2
6	157.8
7	127.2
8	123.4
9a	114.3
9	154.9
10	100.6
10a	119.2
11	28.3
12	28.3
13	55.5
14	55.1
15	16.6
16	55.4

All spectroscopic data, melting point and the same R_f value comparing with 1,1,7-trimethyl-3,6,9-trimethoxy-2-phenanthrenone (Trigonostemone),[33] Compound **F** was ascertained to be this compound. The structure is shown below.



Compound **F**

This compound was subjected to insect antifeedant activity test. The result is displayed in Table 3.30.

Table 3.30 The result of insect antifeedant activity of Compound **F**

Compound	% Insect antifeedant activity	Level of activity
F (1, 1, 7-trimethyl-3, 6, 9-trimethoxy-2-phenanthrenone or Trigonostemone)	53.36	medium

It was found that Trigonostemone showed medium insect antifeedant activity.

Separation of Fraction IE

Fraction IE, 1.47 g was separated again by column chromatography technique using silica gel as an adsorbent. The column was initially eluted with a mixture of dichloromethane-hexane and gradually changed to dichloromethane and a mixture of methanol-dichloromethane. Eluting solvent was collected for each fraction approximately 100 mL and then concentrated to small amount of volume. Each one was investigated for the similarity by using TLC. The equivalent fractions were combined. The results of separation are shown in Table 3.31.

Table 3.31 The results of the separation of Fraction IE

Eluents	Fraction No.	Remarks	Weight (g)
60% CH_2Cl_2:Hexane	1-4	pale yellow wax	0.18
80% CH_2Cl_2:Hexane	5-10	pale yellow wax with little white solid	0.20
CH_2Cl_2	11-16	white solid with little yellow oil	0.31
5% CH_3OH:CH_2Cl_2	17-20	sticky brown material	0.71

Fraction Nos. 5-10 and 11-16 were purified by first washing with methanol and then recrystallization with dichloromethane-methanol several times. The white plate solid (0.09 g) was received. From melting point, TLC in several solvent systems and the IR spectrum of this compound, it was found to be close to the Compound B (5α-stigmastane-3,6-dione).

Separation of Crude Dichloromethane

The crude dichloromethane, Fractions III and VIII from the first and second extraction procedures, respectively was examined by TLC, it was found that they were almost the same. These two fractions were combined. The crude dichloromethane 24.36 g was separated by quick column chromatography technique. The column was initially eluted by solvents following by their polarity to yield 5 fractions. The results are shown in Table 3.32

Table 3.32 The results of the separation of crude dichloromethane by quick column chromatography technique

Fraction	Solvent system	Weight (g)	Feature
IIIA	30% CH_2Cl_2:Hexane	0.63	yellow oil and small solid granule
IIIB	50% CH_2Cl_2:Hexane	0.74	yellow sticky material
IIIC	75% CH_2Cl_2:Hexane	1.82	white solid in green sticky material
IIID	CH_2Cl_2	3.80	white crystal in brown solid
IIIE	10% CH_3OH:CH_2Cl_2	14.73	dark brown sticky material

Each separated fraction was subjected to insect antifeedant activity against Greater Wax moth, *Galleria mellonella.* The results are displayed in Table 3.33 and Fig. 3.5.

Table 3.33 The insect antifeedant activity of each fraction from crude dichloromethane

Fraction	Solvent system	Percentage of insect antifeedant activity	Level of activity
IIIA	30% CH_2Cl_2:Hexane	48.17	medium
IIIB	50% CH_2Cl_2:Hexane	57.61	medium
IIIC	75% CH_2Cl_2:Hexane	86.15	high
IIID	CH_2Cl_2	87.63	high
IIIE	10% CH_3OH:CH_2Cl_2	56.23	medium

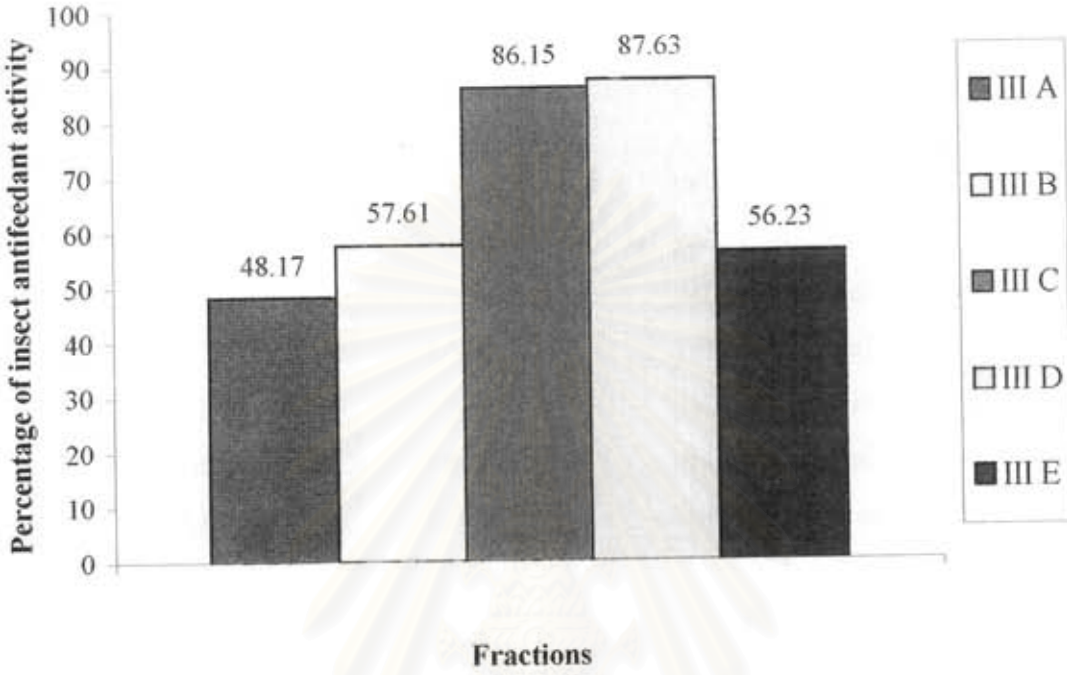


Fig. 3.5 The insect antifeedant activity of each fraction separated from crude dichloromethane

From the results of activity, Fraction IIID and IIIC showed high insect antifeedant activity. These two fractions were further investigated. Other fractions (IIIA, IIIB and IIIE) showed medium activity.

Separation of Fraction IIID

Fraction IIID which revealed high antifeedant activity, 3.80 g was reseparated using silica gel column chromatography. A mixture of dichloromethane and hexane, dichloromethane and a mixture of methanol and dichloromethane were used as eluents. About 50 mL was collected for each fraction and then concentrated to a small volume approximately 10 mL. Each fraction was monitored by TLC and similar fractions were combined. The results of the separation of Fraction IIIC are presented in Table 3.34.

Table 3.34 The results of the separation of Fraction IIID

Eluent	Fraction No.	Remarks	Weight (g)
60% CH_2Cl_2:Hexane	1-21	white wax	0.29
60% CH_2Cl_2:Hexane	22-31	yellow oil	0.36
60% CH_2Cl_2:Hexane	32-39	yellow material	0.45
80% CH_2Cl_2:Hexane	40-42	yellow material	0.07
80% CH_2Cl_2:Hexane	43-56	yellow material	0.47
80% CH_2Cl_2:Hexane	57-66	yellow material	0.47
80% CH_2Cl_2:Hexane	67-78	yellow material	0.40
CH_2Cl_2	79-80	yellow material	0.41
CH_2Cl_2	81-98	solid in yellow oil	0.67
2% CH_3OH:CH_2Cl_2	99-104	yellow material	0.27
2% CH_3OH:CH_2Cl_2	105-106	yellow-brown sticky material	0.35
2% CH_3OH:CH_2Cl_2	107-110	orange material	0.32

The yellow oil in fraction No. 81-98 was removed by washing with methanol to gain the pale yellow solid. After being recrystallized with dichloromethane-methanol for several times, pale yellow needle 0.52 g, designated as Compound **G**, was obtained.

Structural Elucidation of Compound G

Compound G is pale yellow needle, had the melting point 184-185 °C and R_f value 0.36 (5% methanol-dichloromethane). This compound was soluble in dichloromethane and acetone, slightly soluble in ethyl acetate, ethanol and methanol but not soluble in hexane.

The IR spectrum of this compound (Fig. A10) showed the characteristic absorption peak at 3500-3200 (O-H), 3040 (C=C), 1710 (lactone), 1620, 1565, 1500, 1470 (aromatic) cm^{-1}. Other signals are tentatively assigned as shown in Table 3.35.

Table 3.35 The IR absorption band assignments of Compound G

Wavenumber (cm^{-1})	Intensity	Tentative assignment
3500-3200	strong	O-H stretching of alcohol
3040	medium	C-H stretching of alkene and aromatic
2000-1650	summation	C-H bending of aromatic
1710	strong	C=O bending of lactone
1620, 1565, 1500, 1470	strong	C=C stretching of aromatic
1445	strong	C-H bending of ether
1400	medium	C-H bending of alkene
1360	strong	O-H bending in plane
1270, 1120, 1020	strong	C-O-C stretching of ether
1220	medium	C-O stretching of alcohol
1200, 1150	strong	C-O stretching of lactone
930, 850	medium	C-H bending of aromatic
665	medium	C-C out of plane bending
660	medium	O-H out of plane bending

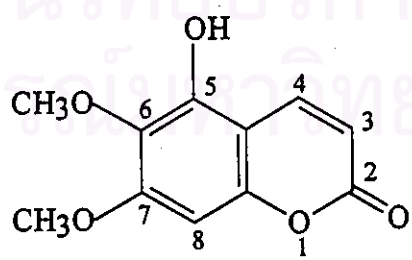
The ^{1}H-NMR spectrum of Compound G (Fig. A11) showed signals of proton at δ (ppm) 3.89 (3H, s, OCH_3), 3.92 (3H, s, OCH_3), 6.21 (1H, d, J = 9.8 Hz), 6.31 (1H, s), 6.44 (1H, s, OH) and 7.95 (1H, d, J = 9.5 Hz).

The ^{13}C-NMR spectrum (Fig. A12) revealed 11 signals of carbon atom at δ 56.0, 60.8, 91.3, 110.4, 132.4, 151.7, 156.5 and 161.2 ppm. The carbon signals at δ 56.0 and 60.8 ppm are the signals of methoxy carbon while the signal at δ 161.2 ppm is the carbon signal of carbonyl group of coumarin. Other assignments are shown in Table 3.36.

Table 3.36 The ^{13}C-NMR chemical shift assignments of Compound **G**

Position	Chemical shift (ppm)
2	161.2
3	110.4
4	139.4
5	147.0
6	132.4
7	151.7
8	91.3
9	156.5
10	103.4

From all spectroscopic evidence and the physical properties compared with those reported in literature[34], Compound **G** had no doubt to be 5-hydroxy-6,7-dimethoxy coumarin (tomentin) having the structure:



Compound **G**

Compound **G** (tomentin) was subjected to insect antifeedant activity test. The result of insect antifeedant activity test is shown in Table 3.37.

Table 3.37 The result of insect antifeedant activity of Compound **G**

Compound	% Insect antifeedant activity	Level of activity
G (5-hydroxy-6,7-dimethoxy coumarin or tomentin)	52.65	medium

From the result of insect antifeedant activity test, Compound **G** (5-hydroxy-6,7-dimethoxy coumarin) exhibited medium activity.

Separation of Fraction IIIC

This fraction 1.82 g showed high insect antifeedant activity was reseparated by column chromatography technique using silica gel as an absorbent. The eluents were a mixture of ethyl acetate and hexane, ethyl acetate and a mixture of ethyl acetate and methanol. About 100 mL of eluate was collected for each fraction and then concentrated to a small volume. Each fraction was monitored by TLC and similar fractions were combined. The results of the separation of Fraction IIIC are showed in Table 3.38.

Table 3.38 The results of the separation of Fraction IIIC

Eluents	Fraction No.	Remarks	Weight (g)
10% EtOAc:Hexane	1-4	pale yellow needle	0.25
10% EtOAc:Hexane	5-8	yellow green material	0.18
10% EtOAc:Hexane	9-12	yellow green material	0.17
10% EtOAc:Hexane	13-15	red pale brown material	0.15
10% EtOAc:Hexane	16-19	pale green material	0.12
20% EtOAc:Hexane	20-23	green material	0.20
20% EtOAc:Hexane	24-26	green material	0.13
20% EtOAc:Hexane	27-28	green yellow material	0.08
40% EtOAc:Hexane	29-30	red brown material	0.09
40% EtOAc:Hexane	31-32	red brown material	0.10
EtOAc	33-34	red brown material	0.08
10% MeOH:EtOAc	35	red brown material	0.19

Fraction No. 1-4 was washed with hexane and further recrystallized with dichloromethane-methanol several times to yield white needle crystal (0.20 g). This compound had the same physical properties as those of Compound **A**. Consequently, it can be concluded that this compound is acetyl aleuritolic acid. Fraction Nos. 20-23 and 24-26 were combined and first purified by washing with hexane to afford the green solid. The green solid was further purified by recrystallization with hexane-dichloromethane to obtain the green needle crystal (0.09 g). Its characteristic was the same as that of Compound **F**.

Separation of Fraction IIIE

The crude Fraction IIIE 14.73 g was reseparated by using column chromatography technique and using silica gel as an adsorbent. A mixture of ethyl acetate and hexane, ethyl acetate and mixture of methanol and ethyl acetate were used as eluents. The eluted solution was collected about 100 mL and then concentrated to a small volume. Each fraction was monitored by TLC and similar fractions were combined. The results of separation of Fraction IIIE are presented in Table 3.39.

Table 3.39 The results of the separation of Fraction IIIE

Eluent	Fraction No.	Remarks	Weight (g)
20% EtOAc:Hexane	1-5	yellow oil	0.53
20% EtOAc:Hexane	6-10	green-yellow oil	0.51
30% EtOAc:Hexane	11-14	green –yellow oil	0.48
30% EtOAc:Hexane and 40% EtOAc:Hexane	15-18	red-yellow oil	0.41
40% EtOAc:Hexane	19-25	red-brown oil	0.99
40% EtOAc:Hexane	26-29	dark brown material	0.48
50% EtOAc:Hexane	30-35	dark brown material	1.08
50% EtOAc:Hexane	36-38	pale brown material	0.48
60% EtOAc:Hexane	39-41	pale brown material	0.42
60% EtOAc:Hexane	42-48	orange material	1.10
80% EtOAc:Hexane	49-54	brown material	1.12
80% EtOAc:Hexane	55-60	white solid with brown material	1.15
EtOAc	61-65	white solid	0.99
EtOAc	66-70	white solid with brown material	0.98
EtOAc	71-74	dark brown material	0.55
10% MeOH:EtOAc	75-79	dark brown material	0.75
10% MeOH:EtOAc	80-83	dark brown material	0.66

The green-yellow oil containing in Fraction Nos. 6-10 and 11-14 was removed by washing with hexane to gain the pale brown solid. After being recrystallization with dichloromethane-methanol, the pale yellow crystal 0.10 g was obtained. The physical properties of this compound were found to be the same as those of Compound G. Fraction Nos. 36-38 and 39-41 were first purification by washing with methanol to remove the dark brown liquid, and white solid (Mixture H) was gained. This mixture was further purified by recrystallization with dichloromethane-methanol and white amorphous solid 0.2 g was received. Fraction Nos. 55-60, 61-65 and 66-70

were purified by washing with dichloromethane and white solid (Mixture I) was received. Mixture I was further purified by recrystallization with hot methanol several times and the white solid 0.98 g was obtained.

Structural Elucidation of Mixture H

Mixture H as white amorphous solid, had the melting point at 107-110 °C and R_f value 0.61 (5% methanol-dichlromethane). This mixture was slightly soluble in dichloromethane, ethanol and methanol but not soluble in hexane, ethyl acetate and acetone.

The IR spectrum showed characteristic absorption peaks of unsubstituted amide at 3400-3200 and 1520 cm^{-1} and long chain moiety at 710 cm^{-1} respectively. Other signals were tentatively assigned as shown in Table 3.40.

Table 3.40 The IR absorption band assignments of Mixture H

Wavenumber (cm^{-1})	Intensity	Tentative assignment
3400-3200	medium	N-H stretching
2900-2840	strong	C-H stretching of CH_2, CH_3
1660	strong	C=O stretching
1520	medium	N-H in plane bending
1460	medium	C-H symmetric bending of CH_2 and asymmetric bending of CH_3
710	weak	CH_2 rocking for methylene group > 4

The ^1H NMR spectrum showed the proton signals of unsubstituted amide at δ 7.5 ppm and the proton of carbon adjacent to an amide group at δ 4.1 ppm. The high intensity proton signal was belonged to methylene interlinking system at δ 1.25 ppm and the signal at δ 0.87 ppm was due to the proton of methylene group.

From all spectroscopic data and physical properties, Mixture H can be probably concluded to be a mixture of long chain amide.

Structural Elucidation of Mixture I

Mixture I as white amorphous solid had the melting point 280-283 °C and R_f value 0.23 (10% methanol-dichloromethane).

The IR spectrum showed characteristic absorption band of a hydroxyl group at 3410 cm^{-1} and glycosidic linkage at 1065-1020 cm^{-1}. Other signals were tentatively assigned as shown in Table 3.41.

Table 3.41 The IR absorption band assignments of Mixture I

Wavenumber (cm^{-1})	Intensity	Tentative assignments
3410	strong	O-H stretching
2940, 2850	strong	C-H stretching of CH_2, CH_3
1620	weak	C=C stretching
1475	medium	C-H symmetric bending of CH_2 and asymmetric bending of CH_3
1370	medium	C-H symmetric bending of CH_3
1065-1020	strong	C-O stretching
800	weak	C-H out of plane bending of trisubstituted vinyl

The ^1H NMR spectrum showed steroidal proton signals at δ 0.68-1.25 (m) and 5.35 (H-6) ppm. The signals of sugar protons at δ 3.41-3.78 (m) and 4.40 (d, J = 7 Hz) could be assigned for an anomeric proton.

The ^{13}C NMR spectrum showed the sp^2 carbon signal at δ 140.3 (C-5) and 121.3 (C-6) ppm and the signals of 6 carbon linking to oxygen of sugar at δ 101.0 (C_1), 73.5 (C_2), 70.2 (C_3), 61.4 (C_4), 56.3 (C_5) and 55.5 (C_6) ppm. The other signals exhibited between δ 49.7-11.7 ppm, were belonging to a steroid moiety.

Based on information obtained for Mixture I, it could be concluded that Mixture I was a mixture of steroidal glycoside.

Mixtures H and I were then subjected to the insect antifeedant activity test. The results are shown in Table 3.42.

Table 3.42 The results of insect antifeedant activity of Mixtures **H** and **I**

Mixture	% Insect antifeedant activity	Level of activity
H (mixture of long chain amide)	49.83	medium
I (mixture of steroidal glycoside)	48.25	medium

It was found that Mixtures **H** (mixture of long chain amide) and **I** (mixture of steroidal glycoside) exhibited medium insect antifeedant activity.

Separation of Fraction IIIA

Fraction IIIA 0.63 g was reseparated using silica gel column chromatography. Hexane, a mixture of dichloromethane and hexane, dichloromethane and a mixture of methanol and dichloromethane were used as eluents. About 100 mL was collected for each fraction and then concentrated to a small volume. Each fraction was monitored by TLC and similar fractions were combined. The results of the separation of Fraction IIIA are shown in Table 3.43.

Table 3.43 The results of the separation of Fraction IIIA

Eluent	Fraction No.	Remarks	Weight (g)
Hexane	1-3	white wax	0.08
Hexane	4-6	yellow-green material	0.11
Hexane	7-10	white solid in yellow oil	0.10
10% CH_2Cl_2:Hexane	11-12	yellow oil	0.05
10% CH_2Cl_2:Hexane	13	yellow oil	0.04
CH_2Cl_2	14-15	yellow oil	0.05
CH_2Cl_2	16-17	orange material	0.10
10% MeOH:CH_2Cl_2	18	brown material	0.08

Fraction IIIA was separated into eight fractions. Each fraction contained small amount of substance. The TLC of each portion also revealed a mixture of at least 3

major spots. Since, each fraction was of small quantity, no further investigation will thus be conducted.

Separation of Fraction IIIB

Fraction IIIB 0.74 g was subjected to silica gel column using silica gel as an adsorbent. The column was initially eluted with hexane and changed to a mixture of dichloromethane and hexane, dichloromethane and a mixture of methanol and dichloromethane. The eluted solution was collected approximately 100 mL for each fraction. Each portion was concentrated to small volume and monitored by TLC. The results of separation of Fraction IIIB are shown in Table 3.44.

Table 3.44 The results of the separation of Fraction IIIB

Eluent	Fraction No.	Remarks	Weight (g)
Hexane	1-3	white wax	0.13
10% CH_2Cl_2:Hexane	4-7	yellow material	0.12
40% CH_2Cl_2:Hexane	8-11	yellow material	0.19
CH_2Cl_2	12-15	white solid	0.15
10% MeOH:CH_2Cl_2	16-18	dark yellow material	0.13

Fraction No. 12-15 was purified by recrystallization with dichloromethane-methanol and white needle crystal (0.09 g) was received. This compound had the same physical properties as those of Compound A. It can therefore be concluded that this compound maybe acetyl aleuritolic acid.

The Separation of Crude Ethyl Acetate

The crude ethyl acetate extract, Fraction IV from the first extraction procedure and Fraction IX from the second extraction procedure were comparatively examined using TLC, it was found that both of them contained almost the same components. The Fraction IX 21.35 g was further separated by column chromatography technique using silica gel as an adsorbent. The column was initially eluted with a mixture of hexane and dichloromethane and gradually changed to dichloromethane, a mixture of

ethyl acetate and dichloromethane, ethyl acetate and a mixture of methanol and ethyl acetate. Eluting solvent was collected for each fraction approximately 250 mL and then concentrated to about 20 mL. Each one was investigated for the similarity by using TLC. The equivalent fractions were combined. The results of the separation are shown in Table 3.45.

Table 3.45 The results of the separation of crude ethyl acetate

Eluent	Fraction No.	Remark	Weight (g)
50% CH_2Cl_2:Hexane	1-12	yellow-green material	0.95
75% CH_2Cl_2:Hexane	13-17	yellow material	0.47
75% CH_2Cl_2:Hexane	18-29	yellow material	0.97
CH_2Cl_2	30-33	brown material	0.48
CH_2Cl_2	34-36	brown material	0.49
CH_2Cl_2	37-38	brown material	0.30
10% EtOAc:CH_2Cl_2	39-42	red-brown material	0.35
10% EtOAc:CH_2Cl_2	43-45	red-brown material	0.49
10% EtOAc:CH_2Cl_2	46-48	red-brown material	0.41
20% EtOAc:CH_2Cl_2	49-52	red-brown material	0.53
20% EtOAc:CH_2Cl_2	53-57	red-brown material	0.52
20% EtOAc:CH_2Cl_2	58-62	red-brown material	0.62
40% EtOAc:CH_2Cl_2	63-69	red-brown material	1.39
60% EtOAc:CH_2Cl_2	70-78	red-brown material	1.06
80% EtOAc:CH_2Cl_2	79-83	red-brown material	0.42
80% EtOAc:CH_2Cl_2	84-90	red-brown material	0.69
EtOAc	91-101	red-brown material	1.12
EtOAc	102-105	brown material	0.83
EtOAc	106-112	brown material	0.89
10% MeOH:EtOAc	113-117	dark brown material	2.39
20% MeOH:EtOAc	118-120	dark brown material	2.29

Fraction Nos. 30-33, 34-36, 37-38 and 39-42 were first purified by washing with methanol and then was further purified by recrystallization with dichloromethane-methanol several times to yield yellow crystal (0.79 g). This compound had the same physical properties as those of Compound G which could be concluded as tomentin. Fraction Nos. 53-57 and 58-62 showed many small spots in long tail with one intensed spot on TLC, monitoring by UV. These fractions were further purified by washing with dichloromethane, white amorphous solid (Compound J) was then received. This compound was recrystallization with dichloromethane-methanol to yield Compound J 0.10 g. Compound J showed the same R_f value as the intensed spot as that appearred on TLC of the crude extract.

Structural Elucidation of Compound J

Compound J is pale yellow amorphous solid. This compound had melting point 223-224 °C and R_f value 0.31 (50% ethyl acetate:hexane). Compound J was soluble in methanol, slightly soluble in acetone but not soluble in hexane, dichloromethane and ethyl acetate.

IR spectrum (Fig. A13) showed characteristic absorption peaks at 3600-3200 cm^{-1} of a hydroxyl group and 1740 cm^{-1} of a carbonyl group. Other signals were tentatively assigned as shown in Table 3.46.

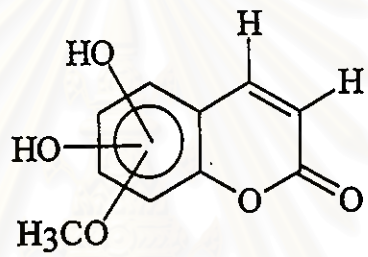
Table 3.46 The IR absorption band assignments of Compound J

Wavenumber (cm^{-1})	Intensity	Tentative assignment
3600-3200	broad	O-H stretching
3100	weak	C-H stretching of alkene and aromatic
1740	strong	C=O stretching of lactone
1620, 1565, 1500, 1470	strong	C=C stretching of aromatic
900-600	medium	C-H out-of-plane bending of aromatic

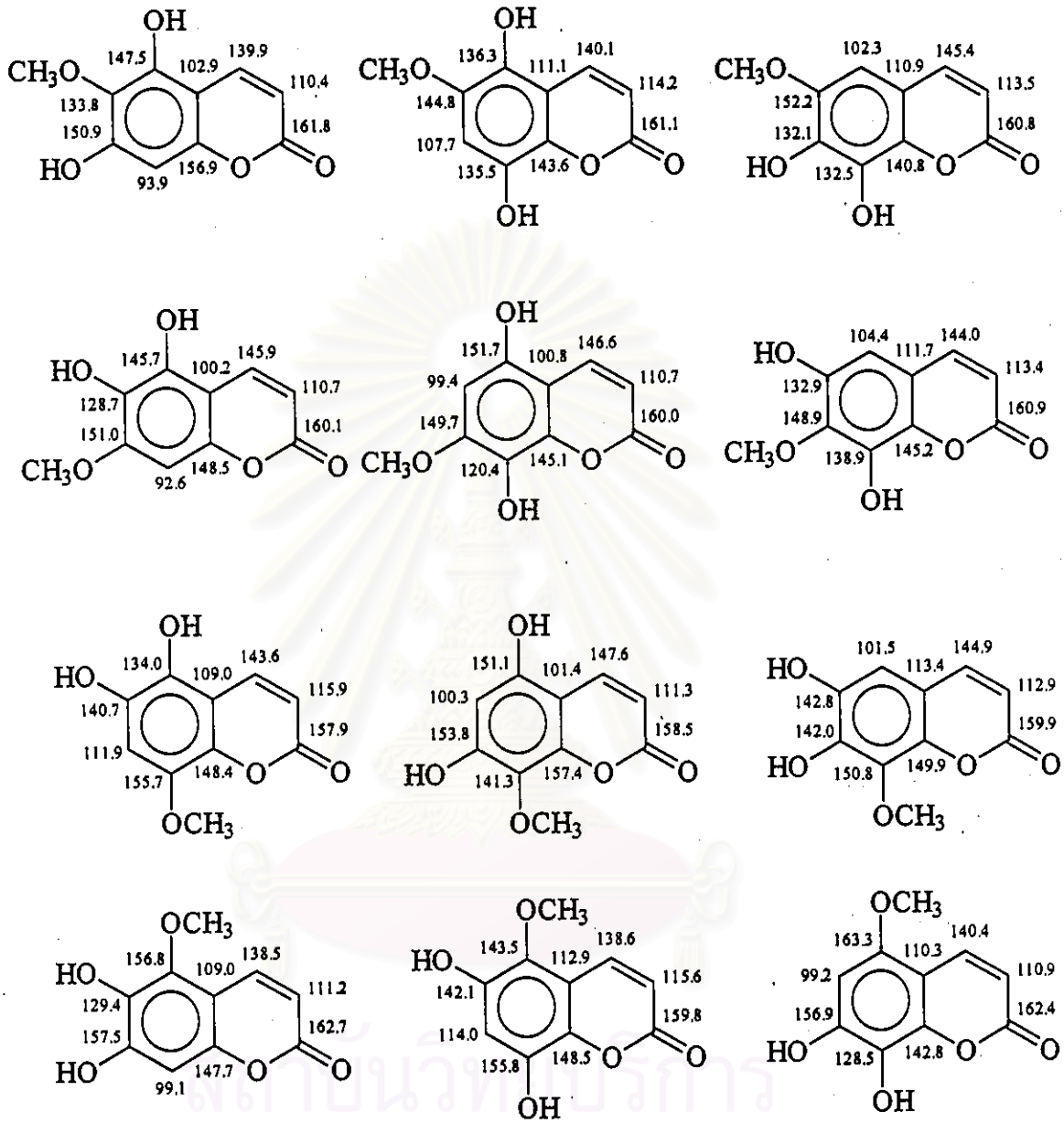
The ^{1}H-NMR spectrum (Fig. A14) showed the characteristic proton signals of coumarin at δ (ppm) 6.19 (1H, d, J = 9.4 Hz), 7.93 (1H, d, J = 9.5 Hz) and other signals at δ 3.85 (3H, s, OCH_3), 5.01 (1H, s, OH), 6.75 (1H, s, OH) and 8.57 (1H,s).

The ^{13}C-NMR spectrum (Fig. A15) showed characteristic carbon signals of coumarin at δ 160.7 ppm and other signals appearring at δ 56.2 (OCH_3), 92.8, 102.5, 111.4, 136.1, 139.3, 147.9, 148.9 and 152.1 ppm.

The spectroscopic evidence, particularly ^{1}H- and ^{13}C-NMR spectra provided informative data that this compound ought to be trisubstituted coumarin containing two hydroxyl groups and one methoxy group. In an α-pyrone ring there should not have any substituent because the ^{1}H-NMR spectrum manifestly exhibited the signals at δ (ppm) 6.19 and 7.93 which could be ascribed for the protons on C3 and C4, respectively. Therefore, these three substituents had to be in a benzene moiety.



From previous reports, the positions of substituents on a coumarin nucleus can be predicted by calculation the carbon chemical shift. This can be achieved by means of combination the parameter in each group of each substituent position.[28,35,36,37] By this way, all possible structures could be assigned as:

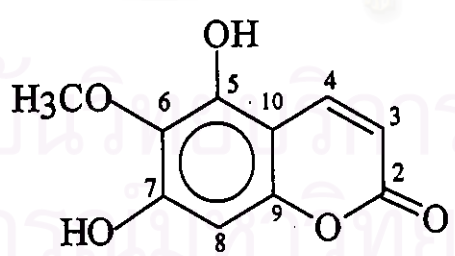


By comparing the carbon chemical shifts of above possible coumarins with those obtained for Compound **J**, it was found that the most appropriate structure for this compound was 5,7-dihydroxy-6-methoxy coumarin. The comparative assignments of carbon chemical shifts between those observed for Compound **J** and those derived from calculation are tabulated in Table 3.47.

Table 3.47 The comparative assignments of ^{13}C-NMR chemical shift of Compound **J** and those from calculation

Position	Chemical shift (ppm)	
	Observed	Calculated
2	160.4	161.8
3	111.4	110.4
4	139.3	139.9
5	147.9	147.5
6	136.1	133.8
7	148.9	150.9
8	92.8	93.3
9	152.1	156.9
10	102.5	102.9

Compound **J** can tentatively assigned as 5,7-dihydroxy-6-methoxy coumarin. According to chemical search, this compound has not been previously reported in literature. Thus, this compound should be a new trisubstituted coumarin. The structure is shown below.



Compound **J**

5,7-dihydroxy-6-methoxy coumarin