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Table 3 The results of the preliminary screening of the ethanol crude extract for cytotoxicity.

cell line	Concentration (μg/ml)	Inhibition (%)	Estimation
Human Leukemia Carcinoma (HL-60)	1	-14.67	+
	10	19.01	
	100	84.45	
Human Nasopharyngeal Carcinoma (KB)	1	-2.26	-
	10	-	
	100	23.26	
Human Gastric Carcinoma (BGC-823)	1	-19.15	-
	10	-14.47	
	100	35.85	
Human Colon Carcinoma (HCT-8)	1	12.50	+
	10	12.50	
	100	97.72	
Human Hepatocellular Carcinoma (Bel-7402)	1	1.3	-
	10	2.5	
	100	35.27	
Proliferation of mouse (B) lymphocyte	1	9.25	++
	10	53.85	
	100	63.27	

*Note +++ : **High activity**

++ : **Medium activity**

+ : **Low activity**

- : **No activity**

The ethanol crude extract of the leaves of *P. betle* shown inhibitory effects against 3 cell lines : *Human Leukemia Carcinoma* (HL-60), *Human Colon carcinoma* (HCT-8), and proliferation of mouse (B) lymphocyte.

3.1.2 Brine shrimp cytotoxic lethality test

The ethanol crude extract of *P. betle* was preliminarily screened for cytotoxicity against brine shrimp (*Artemia salina* Linnaeus). The LC_{50} value of 16.36 μg/ml (medium activity) was obtained. The leaves of *P. betle* Linn. were extracted according the procedure described in Chapter II (No. 2.5, and Scheme 1). Anticell line cytotoxicity test and brine shrimp cytotoxic lethality test were reported in Tables 4 and 5, respectively.

Table 4 The results of the preliminary screening of each crude extract of *P. betle*

Sample	Primary Screening test for *Human Colon carcinoma*(HCT-8)		
	Concentration (μg/ml)	Inhibition (%)	Estimation
Fraction 1 EtOH crude extract	1	2.97	-
	10	12.91	
	100	44.34	
Fraction 2 H_2O crude extract	1	2.15	-
	10	2.66	
	100	6.68	
Fraction 3 $CHCl_3$ crude extract	1	-4.14	-
	10	13.31	
	100	39.39	
Fraction 4 EtOAc crude extract	1	7.98	-
	10	5.49	
	100	3.96	
Fraction 5 BuOH crude extract	1	8.49	-
	10	-2.32	
	100	-12.34	
Fraction 7 solid residue	1	0.88	-
	10	10.37	
	100	5.57	
Fraction 8 MeOH crude extract	1	1.06	+
	10	19.30	
	100	62.11	
Fraction 9 Hexane crude extract	1	-0.39	-
	10	15.50	
	100	37.26	

Table 4 (cont.)

Sample	Primary Screening test for *Human Leukemia carcinoma* (HL-60)		
	Concentration (μg/ml)	Inhibition (%)	Estimation
Fraction 1 EtOH crude extract	1	4.72	+
	10	7.18	
	100	75.66	
Fraction 2 H_2O crude extract	1	-5.54	-
	10	11.29	
	100	9.03	
Fraction 3 $CHCl_3$ crude extract	1	6.67	++
	10	65.40	
	100	73.71	
Fraction 4 EtOAc crude extract	1	6.61	+
	10	8.41	
	100	75.35	
Fraction 5 BuOH crude extract	1	-12.32	-
	10	10.98	
	100	40.04	
Fraction 7 solid residue	1	2.60	-
	10	-10.30	
	100	5.40	
Fraction 8 MeOH crude extract	1	7.70	+
	10	27.51	
	100	63.65	
Fraction 9 Hexane crude extract	1	17.14	+
	10	7.70	
	100	90.55	

Table 4 (cont.)

Sample	Primary Screening test for *Human Hepatocellular carcinoma* (Bel-7402)		
	Concentration (μg/ml)	Inhibition (%)	Estimation
Fraction 1 EtOH crude extract	1	25.69	+
	10	0.00	
	100	63.67	
Fraction 2 H_2O crude extract	1	-21.04	-
	10	-5.20	
	100	-3.21	
Fraction 3 $CHCl_3$ crude extract	1	-21.81	+
	10	2.87	
	100	90.58	
Fraction 4 EtOAc crude extract	1	-21.59	-
	10	-5.20	
	100	37.65	
Fraction 5 BuOH crude extract	1	-16.72	-
	10	-6.42	
	100	14.72	
Fraction 7 solid residue	1	-18.83	-
	10	-26.68	
	100	-15.98	
Fraction 8 MeOH crude extract	1	-22.25	+
	10	8.85	
	100	96.56	
Fraction 9 Hexane crude extract	1	-19.15	+
	10	3.87	
	100	75.41	

Table 5 The results of brine shrimp cytotoxic lethality test

Fractions	Crude extracts	LC_{50}	Activity
Fraction 1	EtOH	16.36	medium activity
Fraction 2	H_2O	39.64	medium activity
Fraction 3	$CHCl_3$	8.36	high activity
Fraction 4	EtOAc	38.83	medium activity
Fraction 5	BuOH	393.92	low activity
Fraction 6	solid residue	43.72	medium activity
Fraction 7	solid residue	49.72	medium activity
Fraction 8	MeOH	14.16	medium activity
Fraction 9	Hexane	0.61	high activity

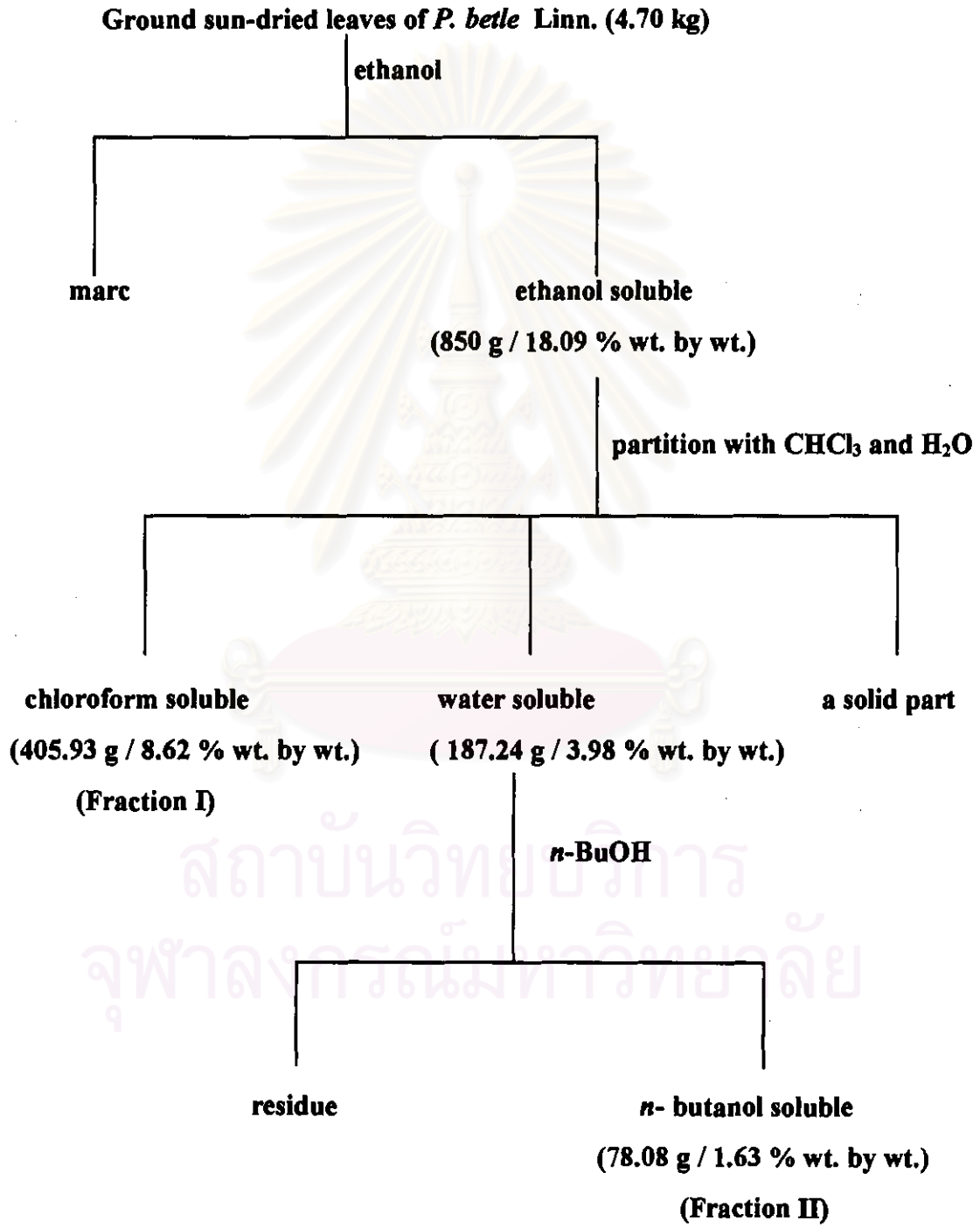
Note: High activity **(LC_{50} < 10 μg/ml)**

: Medium activity **(LC_{50} < 100 μg/ml)**

: Low activity **(LC_{50} < 1000 μg/ml)**

According to the preliminary screening tests of each crude extract of *Piper betle* Linn., the chloroform crude and *n*-butanol crude extracts were selected for further investigation.

solid part as a black tar. The water crude extract was reextracted with *n*-butanol to afford the *n*-butanol crude extract 78.08 g/1.63% wt. by wt. of dried leaves as a brown oil **(Fraction II)**. The results of extraction are summarized as shown in Scheme 3.



Scheme 3 The procedure of extraction

3.3 Separation of chloroform crude extract (Fraction I)

The chloroform soluble fraction (100 g) was mixed with silica gel (100 g) and applied to a silica gel column (6 cm. x 150 cm). The initial eluent was 10% chloroform in hexane. Approximately 250 ml of eluent was collected for each fraction. The fractions were concentrated on a rotary evaporator to a volume of about 10-15 ml and checked by TLC plate. The fractions which had the same components were combined. The results of the separation of the chloroform extract are presented in the Table 6.

Table 6 The results of separation of the crude chloroform extract by column chromatography

Eluents (% volume by volume)	Fraction No.	Code	Remarks	Weight (g)
10% $CHCl_3$ in Hexane	1-4	PB 1	dark yellow oil	0.59
	5-7	PB 2	solid in orange oil **(Mixture 1)**	3.51
	8-14	PB 3	orange oil	4.02
	15-17	PB 4	white solid in yellow oil **(Mixture 2)**	3.71
	18-20	PB 5	white solid in yellow oil **(Mixture 3)**	3.25
	21-22	PB 6	white solid in yellow oil	1.82
	23-25	PB 7	white solid in yellow oil	3.57
	26-30	PB 8	yellow-orange oil	2.52
	31-33	PB 9	light yellow oil	0.56
	34-36	PB 10	light yellow oil	1.19
30% $CHCl_3$ in Hexane	37-57	PB 11	orange-red oil	4.98
60% $CHCl_3$ in Hexane	58-70	PB 12	brown oil	3.62
	71-88	PB 13	brown oil	4.11
10% MeOH in $CHCl_3$	89-94	PB 14	brown-green oil	2.10
	95-101	PB 15	brown-green oil	1.50
	102-104	PB 16	brown-green oil	4.07
	105-110	PB 17	greenish oil	6.23
	111-113	PB 18	black brown oil	4.04
	114-119	PB 19	black brown oil	2.16
	120-124	PB 20	black brown oil	3.20
	125-127	PB 21	black brown oil	10.17
	128-130	PB 22	black brown oil	9.01
30% MeOH in $CHCl_3$	131-134	PB 23	black oil	23.00
	135-138	PB 24	black oil	6.77
	139-143	PB 25	black oil	5.31
	144-147	PB 26	black oil	6.41
100% MeOH	148-160	PB 27	black tar	23.97

Table 6 The results of separation of the crude chloroform extract by column chromatography

Eluents (% volume by volume)	Fraction No.	Code	Remarks	Weight (g)
10% $CHCl_3$ in Hexane	1-4	PB 1	dark yellow oil	0.59
	5-7	PB 2	solid in orange oil **(Mixture 1)**	3.51
	8-14	PB 3	orange oil	4.02
	15-17	PB 4	white solid in yellow oil **(Mixture 2)**	3.71
	18-20	PB 5	white solid in yellow oil **(Mixture 3)**	3.25
	21-22	PB 6	white solid in yellow oil	1.82
	23-25	PB 7	white solid in yellow oil	3.57
	26-30	PB 8	yellow-orange oil	2.52
	31-33	PB 9	light yellow oil	0.56
	34-36	PB 10	light yellow oil	1.19
30% $CHCl_3$ in Hexane	37-57	PB 11	orange-red oil	4.98
60% $CHCl_3$ in Hexane	58-70	PB 12	brown oil	3.62
	71-88	PB 13	brown oil	4.11
10% MeOH in $CHCl_3$	89-94	PB 14	brown-green oil	2.10
	95-101	PB 15	brown-green oil	1.50
	102-104	PB 16	brown-green oil	4.07
	105-110	PB 17	greenish oil	6.23
	111-113	PB 18	black brown oil	4.04
	114-119	PB 19	black brown oil	2.16
	120-124	PB 20	black brown oil	3.20
	125-127	PB 21	black brown oil	10.17
	128-130	PB 22	black brown oil	9.01
30% MeOH in $CHCl_3$	131-134	PB 23	black oil	23.00
	135-138	PB 24	black oil	6.77
	139-143	PB 25	black oil	5.31
	144-147	PB 26	black oil	6.41
100% MeOH	148-160	PB 27	black tar	23.97

Table 8 The results of the separation of fractions No. 21-22 by silica gel column chromatography.

Eluent	Fraction No.	Remark	Weight (g)
20% $CHCl_3$ in Hexane	1-4	yellow oil	0.91
	5-7	white solid in light yellow oil **(Mixture 5,6)**	0.75
	8-9	yellow oil	0.61

3.3.3 Separation of the eluted fractions No. 5-7 (Table 8)

The eluted fractions No. 5-7 contained white solid in light yellow oil. The white solid, Mixture 5 was purified by crystallization with ethyl acetate, yielding 4 mg. The melting point of Mixture 5 was 69-71 °C. Thin-layer chromatography of this portion showed at least 2 spots. (silica gel; solvent 5% ethyl acetate in hexane). The left solution was concentrated by rotary evaporator and was further purified by column chromatography (silica gel Art. 7734 10 g , 1 cm. i.d. x 30 cm column) . The eluent was 5% ethyl acetate in hexane. About 10 ml fractions were collected. The results are shown in Table 9.

Table 9 The results of separation of fractions 5-7

Eluent	Fraction No.	Remark	Weight (g)
5% EtOAc in Hexane	1	amorphous white solid in white oil **(Mixture 6)**	0.06
	2-5	pale yellow oil	0.05

3.3.4 Separation of the eluted fractions No. 26-30 (PB 8) (Table 6)

The eluted fractions No. 26-30 gave yellow-orange oil 2.52 g which was further purified by column chromatography (silica gel Art. 7734, 2 cm i.d. x 60 cm column). The eluent was 30% $CHCl_3$ in hexane. Fractions of about 30 ml were collected. Each fraction was checked by TLC and identical fractions were combined. The results are shown in Table 10.

Table 10 The results of the separation of fractions No. 26-30

Eluent	Fraction No.	Remark	Weight (g)
30% $CHCl_3$ in Hexane	1-10	light orange oil	0.92
	11-18	white crystal in orange oil **(Compound 7)**	0.04
	19-25	light yellow oil	1.2

3.3.5 Separation of the eluted fractions No. 37-57 (PB 11) (Table 6)

The eluted fractions No. 37-57 gave an orange-red oil 4.98 g which were separated by column chromatography using the same procedure as that used for the eluted fractions No. 8-14 but the eluent was 10% chloroform in hexane. The results are shown in Table 11.

Table 11 The results of the separation of fractions No. 37-57

Eluent	Fraction No.	Remark	Weight (g)
10% $CHCl_3$ in Hexane	1-10	pale yellow oil	1.53
	11-14	light orange oil **(Compound 8)**	0.96
	15-20	yellow-orange oil	2.09

3.3.6 Separation of the eluted fractions No. 58-88 (PB 12 and PB 13) (Table 6)

TLC indicated that fractions No. 58-70 (PB 12) and No. 71-88 (PB 13), should be combined (brown green oil 7.73 g). The combined fractions were chromatographed on silica gel using the same procedure as that compound for the separation of the eluted fractions No. 8-14, but the column was eluted with 60% chloroform in hexane. The results of the separation of fractions No. 55-88 are shown in Table 12.

Table 12 The results of the separation of fractions No. 58-101

Eluent	Fraction No.	Remark	Weight (g)
60% $CHCl_3$ in Hexane	1-5	yellow oil	2.15
	6-12	white needle solid in yellow oil **(Mixture 9)**	4.53
	13-21	yellow oil	2.09

3.3.7 Separation of the eluted fractions No. 89-94 (PB 14) and No.95-101 (PB 15) (Table 6)

TLC indicated that fractions No. 89-94 and No. 95-101 should be combined. The combined fractions No. 89-101 were composed of a green oil 3.60 g and was chromatographed on silica gel. The column was eluted with chloroform. Approximately 50 ml of each fraction was collected and concentrated by rotary evaporator to a volume of about 10 ml. Each fraction was checked by TLC and identical fractions were combined. The results of separation of fractions No. 89-101 are shown in Table 13.

Table 13 The results of the separation of the eluted fractions No. 89-101

Eluent	Fraction No.	Remark	weight (g)
100% $CHCl_3$	1-7	green oil	0.25
	8-12	yellow-green oil	0.35
	13-17	yellow-green oil	1.23
	18-19	orange oil	0.97
	20-23	white solid in orange oil **(Mixture 10)**	3.12

3.3.8 Separation of the eluted fractions No. 105-110 (PB 17) (Table 6)

The eluted fractions No. 105-110 containede a green oil 6.23 g which were separated by column chromatography using the same procedure as that described for the separation of the eluted fractions No. 8-14, but the column was eluted with 100% chloroform. The results of the separation of fractions No. 105-110 are shown in Table 14.

Table 14 The results of the separation of fractions No. 105-110

Eluent	Fraction No.	Remark	Weight (g)
100% $CHCl_3$	1-5	dark green oil	2.11
	6-9	dark green oil	0.88
	10	semi-solid in green oil **(Compound 11)**	3.13
	11-12	green oil	1.73

3.4 Purification and properties of the substances from *Piper betle* Linn.

3.4.1 Purification and properties of Mixture 1

Mixture 1 was obtained from the separation of chloroform extract by elution with 10% $CHCl_3$-Hexane in fraction No. 5-7 as an orange oil. It was purified by recrystallization from the mixture of chloroform and methanol for several times to obtain 30 mg of orange solid (6.38 x 10^{-3}% wt. by wt. of dried leaves). This compound has m.p. 108-112 °C . The R_f value was 0.96 (silica gel/ 50% methanol-chloroform). It was soluble in chloroform and dichloromethane. This substance was absorbed by ultraviolet light.

FT-IR spectrum, ν_{max} (cm^{-1}) (Fig. 4): 3650-3100 (m), 3050-2850 (m), 1800-1650 (m), 1459(w), and 1372(w).

^{1}H-NMR ($CDCl_3$) δ (ppm) (Fig. 5) : 0.82-1.04, 1.12-1.34, 1.40-1.58, 1.66-1.98, 2.08-2.14, 2.24-2.40, 3.86-3.96, 5.64, 6.76-6.80 and 8.00

^{13}C-NMR ($CDCl_3$) δ (ppm) (Fig. 6) : 19.6, 24.1, 24.3, 27.3, 29.8, 40.0, 41.6 54.3, 56.0, 58.7 and 112.3.

3.4.2 Purification and properties of Mixture 2

Mixture 2 was a solid in yellow oil in fraction 15-17 (see Table 6, Fraction I), eluted by 10% $CHCl_3$-hexane from chloroform extract. After recrystallization from the mixture of ethyl acetate and methanol several times , a white amorphous solid, 369.7 mg (7.87×10^{-3}% wt. by wt. of dried leaves) with m.p. 78-80 °C was obtained. This substance dissolved in ethyl acetate, chloroform, dichloromethane, dissolved slightly in hexane and did not dissolve in methanol and ethanol. TLC exhibited R_f 0.67 (silica gel/ chloroform).

FT-IR spectrum, ν_{max} (cm^{-1}) (Fig.11) : 3500-3200 (O-H stretching), 2909,2850 (C-H stretching), 1465 (C-H bending), 1060 (C-O stretching), and 730,720 (C-H rocking).

^{1}H-NMR spectrum ($CDCl_3$) gave the proton signals at chemical shift (δ) : 0.88 (t), 1.26(s), 1.55(m) and 3.63(t) ppm (Fig. 12)

^{13}C-NMR spectrum ($CDCl_3$) δ (ppm) (Fig. 13) 14.1, 22.7, 25.8, 29.3, 29.4, 29.6, 29.69, 31.9, 32.9 and 63.1.

GLC chromatogram (Fig. 14) (conditions : column OV-1 2% , column temp. 240 °C , injection temp. 280 °C, carrier gas N_2 50 ml/min with FID detector). Chromatogram showed 11 peaks at retention time: 1.87, 2.93, 3.70, 4.84, 6.01, 7.78, 10.06, 22.95, 30.34, 37.72 and 47.13 min, respectively.

The GLC chromatogram of standard saturated long chain aliphatic primary alcohol showed 5 peaks of retention time at 1.07, 1.44, 2.06, 3.14 and 5.03 min which were corresponded to the number of carbons 14,16,18,20 and 22 respectively (Fig. 15). The linear standard correlation curve of logarithm of retention time *versus* the number of carbons in the authentic long chain alcohol sample was plotted in Fig. 10.

3.4.3 Purification and properties of Mixture 3

The solid in yellow oil was obtained after combination of fraction No. 18-20 (see Table 6) eluted by 10% $CHCl_3$-Hexane from chloroform extract. The solid was recrystallized from the mixture of ethyl acetate and methanol for several times to afford white amorphous solid designated as Mixture 3, 60 mg (1.28×10^{-3}% wt. by wt. of dried leaves), m.p. 80-81 °C. The TLC plate (silica gel) displayed R_f. 0.67 (solvent: chloroform).The compound was soluble in chloroform, dichloromethane, but slightly soluble in hexane.

FT-IR spectrum : ν_{max} (cm^{-1}) (Fig. 17) : 2915,2835 (s) ,1732 (s), 1465(m), 1273(m), 1118,1069 (m) and 746,728 (w).

3.4.4 Purification and properties of Compound 4

Compound 4 was a solid in orange oil in fractions No.8-14(see Table 6) eluted by 10% $CHCl_3$-hexane. Rechromatography on silica gel Art. 7734 eluted with 20% $CHCl_3$-hexane afforded solid in yellow oil in fraction 11-15 (see Table 7). It was purified with the mixture of ethyl acetate and methanol for several times to obtain bright white needle crystal, m.p. 215-216 °C , R_f 0.43 (silica gel/ chloroform), 159.5 mg ($3.39x\ 10^{-3}$ % wt. by wt. of dried leaves).

FT-IR spectrum , ν_{max} (cm^{-1}) (Fig. 18) : 3600-3200 (b), 3090 (w) , 2945-2800 (s), 1639(m) , 1450-1400 (s), 1383 (s), 1180 (m), 1040 (s) and 876 (s).

Mass spectrum (Fig. 19) : 426 (M^{+}), 315, 218, 207, 189, 135, 121, 109, 95, 91, 81, 69, 55, 45 and 44.

^{1}H-NMR ($CDCl_3$) δ (ppm) (Fig. 20) : 0.64-0.94, 1.01-1.66, 2.34, 2.37, 3.15, 4.53, 4.54, 4.55, 4.66, 4.67 and 7.24.

^{13}C-NMR ($CDCl_3$) δ (ppm) (Fig. 21) : 14.5, 15.4, 16.0, 16.1, 18.0, 18.3, 19.3, 21.0, 25.1, 27.4, 27.4, 28.0, 29.2, 34.1, 35.6, 37.2, 38.1, 38.7, 38.8, 40.0, 40.8, 42.8, 42.9, 47.9, 48.3, 50.4, 55.3, 78.7, 109.1 and 150.6.

DEPT-90 ^{13}C-NMR ($CDCl_3$) δ (ppm) (Fig. 22) :

CH signals 6 peaks: 38.8, 47.9, 48.3, 50.4, 55.3 and 78.7.

DEPT-135 ^{13}C-NMR ($CDCl_3$) δ (ppm) (Fig.23) :

CH_3,CH signals (down phase) 14.5, 15.4, 16.0, 16.1, 18.0, 19.3, 28.0, 38.1, 47.9, 48.3, 50.4, 55.3 and 78.7.

CH_2 signals (up phase) 11 peaks : 18.3, 21.0, 25.1, 27.4, (two signals), 29.9, 34.1, 35.6, 37.2, 38.1 and 109.1.

3.4.5 Purification and properties of Mixture 5

Mixture 5 was a solid in yellow oil in fractions No. 21-22 (Table 6) eluted by 10% $CHCl_3$ in hexane afforded solid in light yellow oil in fraction No. 5-7 (Table 8). It was recrystallized from ethyl acetate for several times to give white amorphous solid 4 mg (8.5×10^{-3}% wt. by wt. of dried leaves), m.p. 69-70 °C. The R_f value of this compound was 0.60 (silica gel/$CHCl_3$). It was soluble in chloroform, dichloromethane, but slightly soluble in hexane.

FT-IR spectrum, ν_{max} (cm^{-1}) (Fig. 26) : 3450-3200 (b, O-H stretching), 2929,2847 (s, C-H stretching), 1467 (m, C-H bending) , 1065 (m, C-O stretching), and 730, 725 (m, C-H rocking).

GLC chromatogram (Fig. 24) (conditions: column OV-1 2%, column temp. 240 °C, injection temp. 280 °C , carrier gas N_2 50 ml/min with FID detector). Chromatogram showed 2 peaks at retention time : 3.59 and 5.10 min

The GLC chromatogram of standard saturated long chain aliphatic primary alcohol showed 5 peak of retention time at 0.90, 1.20, 1.74, 2.76 and 4.42 min, which were corresponded to number of carbon 14, 16, 18, 20 and 22 respectively (Fig. 24). The linear standard correlation curve of logarithm of retention time *versus* the number of carbon in authentic long chain alcohol sample was plotted in Fig.25.

3.4.6 Purification and properties of Compound 6

Compound 6 was a solid in light yellow oil in fraction No. 5-7 (Table 8) which was obtained from the column chromatography of chloroform crude extract. It was eluted from the silica gel column with 20% $CHCl_3$ in hexane. Rechromatography on silica gel eluted with 5% EtOAc in hexane afforded amorphous white solid in white oil in fraction No. 1 (Table 9) . After recrystallization with methanol for several times, Compound 6 was obtained as white amorphous product, 61 mg ($1.3x10^{-3}$% wt. by wt. of dried leaves), m.p. 70-71 °C, R_f value 0.85 in 5% EtOAc in hexane. This compound was soluble in chloroform, dichloromethane but slightly soluble in hexane.

FT-IR spectrum ,ν_{max} (cm^{-1}) showed the absorption peak at 2923, 2848 (s, C-H stretching), 1734 (s, C=O stretching), 1460, 1400 (m, C-H bending), 1174 (C-O stretching) and 725 (C-H rocking) (Fig. 27).

^{1}H-NMR spectrum ($CDCl_3$) gave the proton signals at (δ) : 0.87 (t),1.26 (s), 1.54 (m), 2.29(t) , 4.01 (t) and 4.6 (d) ppm (Fig. 28).

Compound 6 was analyzed by gas chromatography. The chromatogram showed only one peak at retention time 17.45 min (Fig. 29)

Mass spectrum (m/e) is shown in Fig.30. Using library search software (NIST database), The mass spectrum of Compound 6 was found to be similar to that of 2-propenyl hexanoate ($C_9H_{16}O_2$) (Fig. 31).

3.4.7 Purification and properties of Compound 7

Compound 7 was a solid in yellow-orange oil in fraction No.26-30 (Table 6) eluted by 30% $CHCl_3$ in hexane afforded solid in orange oil in fraction No. 11-18 (Table 10) and recrystallized by a mixture of ethyl acetate and methanol for several times. It gave bright white crystal 70 mg ($1.49x10^{-3}$% wt. by wt. of dried leaves), m.p. 278-280 °C. TLC revealed only one spot at R_f 0.33 (silica gel /50% dichloromethane-hexane). This compound was soluble in dichloromethane and chloroform, but slightly soluble in hexane and methanol.

FT-IR spectrum, ν_{max} (cm^{-1}) (Fig.32) : 3550-3400 (m, O-H stretching), 3000-2850 (s, C-H stretching), 1444 (m, C-H bending), 1378 (m, C-H sym.) and 1173 (C-O stretching and O-H bending).

Mass spectrum (m/e) (Fig. 33) : 428 (M^+),413,304, 275, 276, 273, 248, 234, 231, 220, 207, 206, 205, 193, 179 and 165.

^{1}H-NMR spectrum ($CDCl_3$) δ (ppm) (Fig. 34) : 0.8-1.56, 2.2-2.6, 3.2, 3.6, 3.8 and 8.2

^{13}C-NMR spectrum ($CDCl_3$ + CD_3OD) δ (ppm) (Fig. 35) : 11.6-61.1 and 72.5

DEPT-90 ^{13}C-NMR spectrum ($CDCl_3$ + CD_3OD) δ (ppm) (Fig. 36) :

CH signals 5 peaks : 42.3, 48.8, 52.5, 61.1 and 72.5

DEPT-135 ^{13}C-NMR spectrum ($CDCl_3$ + CD_3OD) δ (ppm) (Fig. 36) :

CH_3, CH signals (up phase) 13 peaks: 11.6-19.6, 30.0-34.7, 48.8, 52.5, 61.1 and 72.5.

CH_2 signals (down phase) 11 peaks, 15.5-36.5 and 38.4-42.3.

3.4.8 Purification and properties of Compound 8

Compound 8 was a liquid in orange-red oil in fraction 37-57 (Table 6) eluted by 30% $CHCl_3$ in hexane. Rechromatography on silica gel eluted with 10% $CHCl_3$ in hexane afforded orange liquid in fraction 11-14 (Table 11), 956.2 mg (0.02% wt. by wt. of dried leaves) . TLC exhibited R_f 0.63 (silica gel/ chloroform).

FT-IR spectrum , ν_{max}(cm^{-1}) (Fig. 37) : 3550-3350 (m, O-H stretching), 3073, 2924, 2832 (C-H stretching), 1608 (C=C stretching), 1265, 1127 (C-H bending vibration of -CH_2, -CH_3) and 1019 (O-H stretching).

Mass spectrum (m/e) (Fig.38) : 164 (M^+),149,137,131,103,91,77,55

^{1}H-NMR ($CDCl_3$) δ (ppm) (Fig. 39) : 3.3, 3.9, 5.1-5.2, 5.7, 5.9-6.1, 6.7-6.9

^{13}C-NMR ($CDCl_3$) δ (ppm) (Fig. 40) : 39.6, 56.0, 110.7, 115.0, 115.5, 119.9, 133.4, 137.7, 145.0 and 145.6.

DEPT-90 ^{13}C-NMR ($CDCl_3$) δ (ppm) (Fig. 41) :CH signals 4 peaks : 110.7, 115.0, 119.9 and 137.8.

DEPT-135 ^{13}C-NMR ($CDCl_3$) δ (ppm) (Fig. 41) :CH_3, CH signals (up phase) 5 peaks : 56.0, 110.7, 115.0, 119.9 and 137.7.

CH_2 signals (down phase) 2 peaks : 39.6 and 115.5.

3.4.9 Purification and properties of Mixture 9

Mixture 9 was a solid in brown oil in fractions No. 58-70, 71-88 (Table 6) eluted by 60% $CHCl_3$ in hexane . Rechromatography on silica gel eluted with 60% $CHCl_3$ in hexane afforded solid in yellow oil in fractions No. 6-12 (Table 12) . After removing yellow oil by washing with methanol and recrystallizing the solid with hexane for several times , Mixture 9 as white bright needle, m.p. 134-136 °C , 450 mg. (9.57×10^{-3}% wt. by wt. of dried leaves) was gained. TLC (silica gel) exhibited only one spot at R_f 0.29 (solvent system: 60% $CHCl_3$-Hexane). It was soluble in chloroform, acetone and dichloromethane but slightly soluble in hexane, methanol and ethanol.

FT-IR spectrum, ν_{max} (cm^{-1}) (Fig. 42): 3416 (b, O-H stretching), 2945 (s, C-H stretching), 1644 (w, C=C stretching),1465,1378 (m, $-CH_3$, $-CH_2-$) and 1045 (m, C-O stretching), 950 (w, disubstituted vinyl, $R_1CH=CHR_2$) and 805 (w, trisubstituted vinyl, $R_1R_2C=CHR_3$).

Mass spectrum (m/e) (Fig.43) : gave the important fragmentation ion peaks at m/e 414, 412 and 400 (Calcd. for $C_{29}H_{50}O$, $C_{29}H_{48}O$ and $C_{28}H_{48}O$, respectively) together with other vital peaks at m/e 396, 394, 382, 381, 351, 329, 303, 273, 271, 255 and 213.

^{1}H-NMR ($CDCl_3$) (Fig. 44): revealed the significant signals at chemical shift δ (ppm): 0.68-2.3, 3.4-3.6 m, 5.00-5.06, 5.13-5.20 and 5.35 ppm (olefinic protons).

^{13}C-NMR ($CDCl_3$) δ (ppm) (Fig. 45) : 11.9 to 56.9, 71.8 (carbon attached to oxygen atom), 121.7 (olefinic carbons), 129.4, 138.3 and 140.8 ppm.

DEPT-90 ^{13}C-NMR ($CDCl_3$) δ (ppm) (Fig. 46) :CH signals 15 peaks : 29.3, 32.0, 36.6, 40.5, 45.9, 50.2, 51.3, 56.0, 56.1, 56.8, 56.9, 71.8, 121.7, 129.4, and 138.3.

GLC analysis (Fig.47) (Conditions: column OV-1 2% , column temp. 250 °C, injection temp. 280 °C , carrier gas N_2 50 ml/min with FID detector) gave 3 peaks on gas chromatogram at retention time 20.12, 20.99 and 23.96 min, respectively.

The results of GLC analysis of standard steroids namely campesterol, stigmasterol and β-sitosterol showed three peaks at retention time 19.77, 20.91 and 24.15 min, respectively (Fig. 47).

3.4.10 Purification and properties of Mixture 10

Mixture 10 was a solid in brown-green oil in fraction No. 89-101 (Table 6) eluted by 10% MeOH-$CHCl_3$. Rechromatography on silica gel eluted with chloroform afforded solid in orange oil in fraction No. 20-23 (Table 13) . After removing orange oil by washing with methanol and recrystallizing the solid with hexane for several times. Mixture 10 as white bright needle, m.p. 129-132 °C , 612 mg (13.02×10^{-3}% wt. by wt. of dried leaves) was gained. TLC (silica gel) exhibited only one spot at R_f 0.72 (solvent system: 30% EtOAc-Hexane) . It was soluble in chloroform, acetone and dichloromethane but slightly soluble in hexane and methanol.

^{1}H-NMR ($CDCl_3$) (Fig. 48) : revealed the significant signals at chemical shift δ (ppm) 0.67-2.35, 3.4-3.6, 5.0-5.2, 5.35 (olefinic protons) ppm.

^{13}C-NMR ($CDCl_3$) δ (ppm) (Fig.49) : 11.9-56.9, 71.3 (carbon attached to oxygen atom), 121.7 (olefinic carbons), 129.3, 138.3, and 140.8 ppm.

GLC analysis (Fig. 50) (Conditions : column OV-1 2%, column temp. 250 ° C, injection temp. 280 °C, carrier gas N_2 50 ml/min with FID detector) gave 3 peaks on gas chromatogram at retention time 20.55, 21.68 and 24.66 min, respectively. The results of GLC analysis of standard steroids namely campesterol, stigmasterol and β-

sitosterol showed three peaks at retention time 19.77, 20.91 and 24.15 min, respectively.

3.4.11 Purification and properties of Compound 11

Compound 11 was the semi-solid in green oil, which was eluted from silica gel column chromatography with chloroform in Fraction No. 105-110 (PB 17). Fraction 10 (Table 13) as the semi-solid in greenish oil showed a one spot at R_f 0.69 using 50% MeOH-$CHCl_3$ as a developing solvent, 3.11 g (0.07% wt. by wt. of dried leaves) . This compound was labeled as Compound 11.

FT-IR spectrum , ν_{max} (cm^{-1}) (Fig. 51): 3349 (b, O-H stretching), 1598 (w, C=C stretching), 1275 , 1101 (m, C-O stretching) 901 and 799 (w, C-H rocking)

Mass spectrum (m/e) (Fig. 52) : 150 (M^+), 135, 133, 131, 123, 104, 103, 91, 85, 83,77 and 51.

^{1}H-NMR ($CDCl_3$) δ (ppm) (Fig. 53) : 1.3, 3.2, 5.0, 5.8-6.0 and 6.2-6.8.

^{13}C-NMR ($CDCl_3$) δ (ppm) (Fig. 54) : 39.2, 115.5, 115.9, 116.1, 121.1, 133.4, 137.5, 141.4 and 143.3.

DEPT-90 ^{13}C-NMR ($CDCl_3$) δ (ppm) (Fig. 55) :CH signals 4 peaks : 115.9, 116.1, 121.1 and 137.5.

DEPT-135 ^{13}C-NMR ($CDCl_3$) δ (ppm) (Fig. 55) :CH_3 ,CH signals (up phase) 4 peaks : 115.9, 116.1, 121.1, and 137.5

CH_2 signals (down phase) 2 peaks : 39.2 and 115.5

3.5 Structural elucidation of the isolated substances from the leaves of *Piper betle* Linn.

3.5.1 Structural elucidation of Mixture 1

Mixture 1 was orange solid 30 mg (6.38 x 10^{-3} % wt. by wt. of dried leaves), m.p. 108-112°C. R_f value 0.96 (solvent system: 50% MeOH in $CHCl_3$).

The IR spectrum of Mixture 1 (Fig. 4) which is assigned in Table 15 indicated that this substance contained a carbonyl function group of ester at 1721 cm^{-1} (C=O stretching) and C-H bending vibration of gem-dimethyl group at 1372 cm^{-1}.

Table 15 The IR absorption assignments of Mixture 1

Wave number (cm^{-1})	Intensity	Tentative Assignments
3452	b, s	O-H stretching of alcohol
2939	m	C-H stretching of CH_3-,-CH_2-
1721	s	C=O stretching vibration of ester
1454	w	C-H asym. bending vibration of CH_3-,-CH_2-
1372	w	C-H sym. bending vibration of gem- dimethyl
1275	w	C-O-C stretching
1150-1000	w	C-O-H stretching

The ^{1}H-NMR spectrum (Fig. 5) exhibited proton signals of -CH_3 at chemical shift (δ) 0.95 ppm. The signals at chemical shift (δ) 1.09-1.57 ppm corresponded to the proton signals of methylene and methine protons. The signals at 5.66 ppm showed the characteristic of vinylic proton.

The ^{13}C-NMR spectrum (Fig. 6) showed the olefinic carbon signals at 112.4 ppm. The signal at 58.7 ppm should be the carbon signal adjacent to oxygen atom

bearing one proton (-CHOH). Other signals around 19.6-55.99 ppm ought to be methyl, methylene, methine and quarternary carbons.

The GC-MS data analysis indicated that Mixture 1 was not a single compound. The chromatogram (Fig. 7) showed many retention times, but major component showed retention times at 12.29 and 26.61 min.

The mass spectrum of each peak was compared with spectra through library search (NIST database).

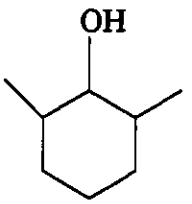
The experimental results are tabulated in Table 16.

Table 16 Retention time of Mixture 1

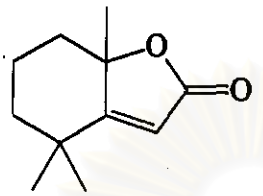
Retention time (min)	Molecular formula	M.W.	Compound
12.29	$C_8H_{16}O$	128	2,6-dimethylcyclohexanol
26.61	$C_{11}H_{16}O_2$	180	5,6,7,7A-tetrahydro-4,4,7A-trimethyl-2(4H)-benzofuranone

The mass spectrum of peak which has the retention time at 12.29 min is showed in Figure 8 and the mass spectra corresponding to the peak which has the retention times at 26.61 min is showed in Figures 9 and 10.

From GC-MS analysis, Mixture 1 might be a mixture of 2,6-dimethyl cyclohexanol and 5,6,7,7A-tetrahydro-4,4,7A-trimethyl-2(4H)-benzofuranone and their structures are shown below.



2,6-dimethyl cyclohexanol



5,6,7,7A-tetrahydro-4,4,7A-trimethyl-2(4H)-benzofuranone

Mixture 1

3.5.2 Structural elucidation of Mixture 2

Mixture 2 was white amorphous solid, 369.7 mg (7.87×10^{-3}% wt. by wt. of dried leaves), m.p. 78-80 °C . R_f value was 0.67 (solvent : chloroform).

The FT-IR spectrum of Mixture 2 shown in Fig. 11 which exhibited the absorption band at 3500-3200 cm^{-1} indicated that Mixture 2 should contain OH group as its functional group. The IR absorption band assignments of Mixture 2 is shown in Table 17.

Table 17 The IR absorption band assignments of Mixture 2

Wave number (cm^{-1})	Intensity	Tentative Assignments
3500-3200	m	O-H stretching vibration
2909, 2852	s	C-H stretching vibration of CH_3-, - CH_2-
1470, 1465	m	C-H asym. bending vibration of CH_3-, -CH_2-
1060	s	C-O stretching vibration of 1° ROH
730, 720	m	C-H rocking vibration of $(-CH_2-)_n$, n > 4

The ^{1}H-NMR spectrum (Fig. 12) showed the important triplet signals at 3.63 ppm which was the signal of the proton on the carbon attaching to oxygen atom (-$C\underline{H}_2$-OH), at 1.55 ppm was -CH_2-O$\underline{H}$. The high intensity singlet signal at 1.26 ppm revealed that there were several inter linking of methylene group (-CH_2)$_n$- in the molecule of this compound. The signal at 0.88 ppm was corresponded to the methyl group signal (-CH_3).

The ^{13}C-NMR spectrum (Fig. 13) showed the signal of carbon at chemical shift (δ) 14.1-32.9 ppm (CH_3-, -CH_2-) and the signal of carbon at 63.1 ppm which was the carbon adjacent to OH. The other signal of carbon at 29.7 ppm was the signal of (-CH_2-)$_n$. The structure of this mixture could be assigned below.

$$CH_3\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}(CH_2)_n\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}OH$$

14.1 22.7 31.9 29.3 29.7 25.8 32.9 63.1

The GLC data analysis indicated that Mixture 2 was not a single compound. The chromatogram (Fig. 14) showed 11 retention times at 1.87, 2.93, 3.70, 4.84, 6.01, 7.78, 10.06, 22.95, 30.34, 37.72 and 47.13 min. The standard chromatogram of long chain aliphatic alcohol (C = 14, 16, 18, 20 and 22) (Fig. 15) and calibration curve of log retention times and number of carbons of standard long chain aliphatic primary alcohol (Fig. 16) indicated that Mixture 2 was a mixture of 11 saturated long chain aliphatic primary alcohol with 17, 19, 21, 22, 24, 25, 26, 30, 31, 32 and 33 carbon atoms. The retention time of standard long chain aliphatic primary alcohol (C = 14, 16, 18, 20, 22) and Mixture 2 are shown in Table 18.

Table 18 Retention time of standard long chain aliphatic primary alcohol (C = 14, 16, 18, 20 and 22) and Mixture 2

Substances	Retention Time (min)	Log Retention Time	Number of carbon
$CH_3(CH_2)_{12}CH_2OH$	1.07	0.03	14
$CH_3(CH_2)_{14}CH_2OH$	1.44	0.16	16
$CH_3(CH_2)_{16}CH_2OH$	2.06	0.31	18
$CH_3(CH_2)_{18}CH_2OH$	3.14	0.50	20
$CH_3(CH_2)_{20}CH_2OH$	5.23	0.70	22
Mixture 2	1.87	0.27	17
	2.93	0.47	19
	3.70	0.57	21
	4.33	0.68	22
	6.01	0.78	24
	7.78	0.89	25
	10.06	1.00	26
	22.95	1.36	30
	30.34	1.48	31
	37.72	1.58	32
	47.13	1.67	33

Various types of saturated long chain aliphatic primary alcohol in Mixture 2 is shown in Table 19. Standard sample are tetradecanol ($C_{14}H_{29}OH$), hexadecanol ($C_{16}H_{33}OH$), octadecanol ($C_{18}H_{37}OH$), icosanol ($C_{20}H_{41}OH$), and doicosanol ($C_{22}H_{45}OH$)

Table 19 Saturated long chain aliphatic primary alcohols found in Mixture 2

Name of substances	Molecular Formula	Structural Formula	Molecular Weight	% composition
Heptadecanol	$C_{17}H_{36}O$	CH_3-$(CH_2)_{15}$-CH_2OH	256	1.00
Nonadecanol	$C_{19}H_{40}O$	CH_3-$(CH_2)_{17}$-CH_2OH	284	0.24
Eicosanol	$C_{20}H_{42}O$	CH_3-$(CH_2)_{18}$-CH_2OH	298	0.44
Docosanol	$C_{22}H_{46}O$	CH_3-$(CH_2)_{20}$-CH_2OH	326	4.22
Tricosanol	$C_{23}H_{48}O$	CH_3-$(CH_2)_{21}$-CH_2OH	340	0.63
Pentracosanol	$C_{25}H_{52}O$	CH_3-$(CH_2)_{23}$-CH_2OH	368	0.52
Hexacosanol	$C_{26}H_{54}O$	CH_3-$(CH_2)_{24}$-CH_2OH	382	0.30
Triacontanol	$C_{30}H_{62}O$	CH_3-$(CH_2)_{28}$-CH_2OH	438	0.08
Hentriacontanol	$C_{31}H_{64}O$	CH_3-$(CH_2)_{29}$-CH_2OH	452	0.07
Dotriacontanol	$C_{32}H_{66}O$	CH_3-$(CH_2)_{30}$-CH_2OH	466	85.25
Tritriacontanol	$C_{33}H_{68}O$	CH_3-$(CH_2)_{31}$-CH_2OH	480	8.31

The composition of each compound is presented in Table 19. The major component in this mixture is dotricontanol ($C_{32}H_{66}O$).

The structure of Mixture 2 is shown below:

CH_3-$(CH_2)_n$-CH_2OH

n = 15, 17, 18, 20, 21, 23, 24, 28, 29, 30 and 31

Mixture 2

3.5.3 Structural elucidation of Mixture 3

Mixture 3 was white amorphous solid from chloroform extract separation. This substance was purified by crystallized with ethyl acetate and methanol yielding 60 mg (1.28×10^{-3}% wt. by wt. of dried leaves). R_f value of this compound was 0.67 (solvent : chloroform) and the melting point was 80-81 °C.

The IR spectrum exhibited a strong absorption band at 1732 cm^{-1}, which is characteristic of an ester carbonyl group and long chain hydrocarbon at 2915 cm^{-1}, 2835 cm^{-1}. The spectral data indicated this compound might be long chain esters.[165]

Table 20 The IR absorption band assignments of Mixture 3

Wave number (cm^{-1})	Intensity	Tentative Assignments
2915, 2835	s	C-H stretching of $-CH_2-$, $-CH_3$
1732	s	C=O stretching of ester
1465	m	C-H bending of $-CH_2-$, $-CH_3$
1273, 1118, 1069	m	C-O stretching
746, 728	w	CH_2 rocking in $C-(CH_2)_n-C$ $(n > 4)$

The IR spectrum indicated that Mixture 3 was a mixture of long chain esters.

3.5.4 Structural elucidation of Compound 4

Compound 4 was isolated by silica gel chromatography of the crude chloroform extract and further purified by recrystallization (ethyl acetate : methanol) .

This compound, m.p. 215-216 °C was obtained as bright white needle crystal , 159.5 mg (3.39×10^{-3}% wt. by wt. of dried leaves). R_f of this compound was 0.43 using chloroform as a developing solvent.

The IR spectrum (Fig. 18) which is assigned in Table 21 revealed the characteristic absorption band of two groups at 3525-3250 and 1040 cm^{-1} and additional bonds of a vinylidine group (CH_3-C=CH_2) at 3090, 1639 and 876 cm^{-1} together with gem dimethyl group at 1383 and 1180 cm^{-1}

Table 21 The IR absorption bond assignments of Compound 4

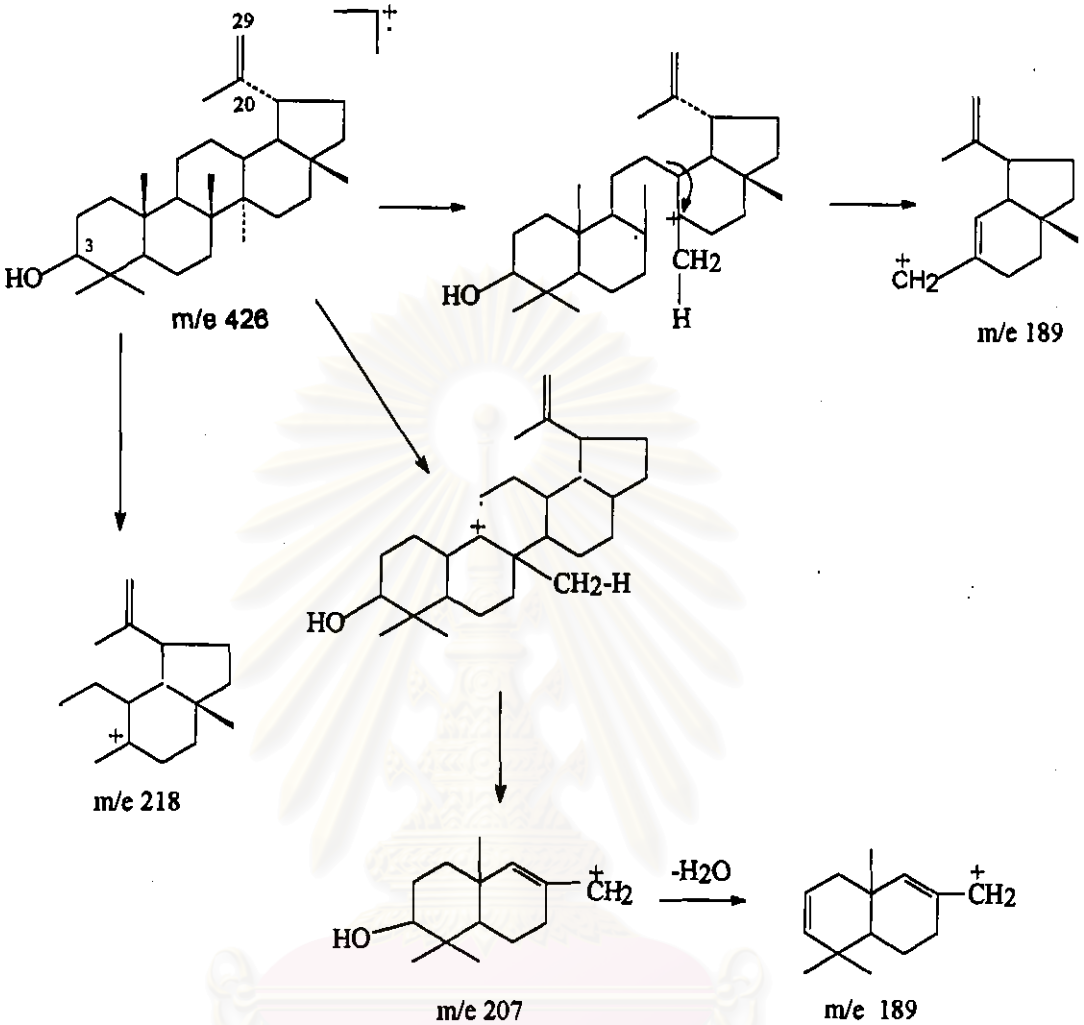
Wave number (cm^{-1})	Intensity	Tentative Assignments
3525-3250	very b.	O-H stretching vibration
3090	w	C-H stretching vibration of asymmetric $R_1R_2C=CH_2$
3000-2880	s	C-H stretching vibration of -CH_3, -CH_2-
1639	m	non-conjugated C=C stretching vibration
1470-1450	s	C-H bending vibration of -CH_3 , -CH_2-
1383	s	C-H bending vibration of gem- dimethyl
1180	m	skeleton vibration of $(CH_3)_2$-C< with no free hydrogen atom on the central carbon
1040	s	C-O stretching vibration of 3β - OH (equatorial)
876	s	C-H out of plane bending vibration of =CH_2

The ^{1}H-NMR spectrum (Fig. 20) gave good agreements with the IR spectrum information , *i.e.*, it exhibited the singlet signals of six methyl protons at δ 0.76, 0.80, 0.86 , 0.94, 1.00 and 1.05 ppm (3H each), the broaden singlet of vinylic methyl proton at 1.68 (3H), the broad multiplet of a methine proton attached to a carbon atom nearing a hydroxyl group (-C<u>H</u>-OH) at δ 3.16 ppm (1H) and the two signals in olefinic region which should be a terminal methylene proton (CH_2=C-) at 4.67 and 4.66 ppm (2H) . The ^{1}H-NMR chemical shift assignments of Compound 4 are given in Table 22.

Table 22 The ^{1}H-NMR chemical shift assignments of Compound 4

Compound	Chemical shifts (ppm)			
	olefinic protons	carbinol proton	vinyl methyl proton	methyl protons
lupeol	4.68, 4.78	3.13	1.70	0.78, 0.80 0.84, 0.98 0.98, 1.05
compound 4	4.66, 4.67	3.16	1.68	0.76, 0.80 0.86, 0.94 1.00, 1.05

The mass spectrum (Fig. 19) displayed the molecular ion (M^+) at m/e 426 (Calcd. for $C_{30}H_{50}O$: MW 426) together with other abundant fragmentation ions at m/e 315, 218, 207, 189 and 95. The series of fragmentation pattern implied that this triterpenoid compound should belong to a lupane series. The presence of the ion at m/e 207 indicated the hydroxy group ought to locate at C-3.[166] The possible mass fragmentation pattern of Compound 4 is presented in Scheme 4.



Scheme 4 The possible mass fragmentation pattern of Compound 4

The ^{13}C-NMR spectrum (Fig. 21) exhibited the olefinic carbon signals at 150.6 and 109.1 ppm. A singlet signal at the former position suggested that there was no hydrogen attached on that carbon, whereas the latter gave a triplet signal which was indicative the presence of a structure like $=CH_2$. The singlet signal at 78.7 ppm which was shown a doublet signal in the ^{13}C-NMR spectrum suggested the structure like >CH-OH. Besides, there were other signals of quaternary carbons around 55.37 to 14.56 ppm

Moreover, the ^{13}C-NMR chemical shift assignment of lupeol and Compound 4 were corresponded to that of reported lupeol[167]. The comparison of the ^{13}C-NMR chemical shifts of lupeol is presented in Table 23.

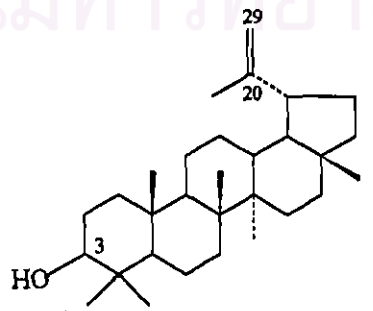
Table 23 The ^{13}C-NMR chemical shift assignment of lupeol and Compound 4

Carbon	Chemical shifts (ppm)	
	lupeol	Compound 4
1	38.7	38.7
2	27.4	27.4
3	78.8	78.7
4	38.8	38.8
5	55.2	55.4
6	18.3	18.3
7	34.2	34.2
8	40.8	40.9
9	50.4	50.5
10	37.1	37.2
11	20.9	21.0
12	25.1	25.1
13	38.0	38.1
14	42.8	42.8

Table 23 (cont.) The ^{13}C-NMR chemical shift assignment of lupeol and Compound 4

Carbon	Chemical shifts (ppm)	
	lupeol	Compound 4
15	27.4	27.4
16	35.5	35.7
17	42.9	42.9
18	48.2	48.3
19	47.9	47.9
20	150.6	150.6
21	29.8	29.9
22	39.9	40.0
23	28.0	28.0
24	15.4	15.5
25	16.1	16.2
26	15.9	16.0
27	14.5	14.6
28	18.0	18.0
29	109.2	109.2
30	19.3	19.4

The above spectral evidence supported that Compound 4 had to be lupeol.



Compound 4 : Lupeol

3.5.5 Structural elucidation of Mixture 5

Mixture 5 was white amorphous solid , 4 mg (8.5×10^{-5}% wt. by wt. of dried leaves) , m.p. 69-71 °C . R_f value was 0.60 (solvent system: chloroform).

The IR spectrum of Mixture 5 as shown in Fig. 26 which exhibited the absorption band at 3450-3200 cm^{-1} indicated that Mixture 5 should contain OH group as its functional group. Other absorption bands were similar to that of Mixture 2 . Thus, this compound ought to be a saturated long chain aliphatic primary alcohol. The IR absorption band assignments of Mixture 5 are shown in Table 24.

Table 24 The IR absorption band assignments of Mixture 5

Wave number (cm^{-1})	Intensity	Tentative Assignments
3450-3200	m	O-H stretching vibration
2915,2856	s	C-H stretching vibration of CH_3- , -CH_2-
1467,1460	m	C-H asym. bending vibration of CH_3-,-CH_2-
1065	s	C-O stretching vibration of 1° ROH
730,720	m	C-H rocking vibration of $(-CH_2-)_n$, n > 4

Mixture 5 ought to be long chain aliphatic alcohol when comparison with standard long chain aliphatic alcohol, they were similar.

The GLC analysis indicated that Mixture 5 was not a single compound. The chromatogram (Fig. 24) showed 2 retention times at 3.59 and 5.10 min. The standard chromatogram of long chain aliphatic alcohol (C = 14, 16, 18, 20, 22) (Fig. 24) and calibration curve of log retention times and number of carbons of standard long chain aliphatic primary alcohol (Fig. 25) indicated that Mixture 5 was a mixture of 2 saturated long chain aliphatic primary alcohols with 21 and 23 carbon atoms, respectively The retention time of standard long chain aliphatic primary alcohol (C = 14, 16, 18, 20, 22) and Mixture 5 are shown in Table 25.

Table 25 Retention time of standard long chain aliphatic primary alcohol (C=14,16,18,20,22) and Mixture 5

Name of subtances	Retention time (min)	Log retention times	Number of carbon
$CH_3(CH_2)_{12}CH_2OH$	0.90	-0.04	14
$CH_3(CH_2)_{14}CH_2OH$	1.20	0.08	16
$CH_3(CH_2)_{16}CH_2OH$	1.74	0.24	18
$CH_3(CH_2)_{18}CH_2OH$	2.76	0.44	20
$CH_3(CH_2)_{20}CH_2OH$	4.42	0.65	22
Mixture 5	3.59	0.56	21
	5.10	0.71	23

Various saturated long chain aliphatic primary alcohols in Mixture 5 is shown in Table 26

Table 26 Various saturated long chain aliphatic primary alcohols found in Mixture 5

Name of substances	Molecular Formula	Structural Formula	Molecular Weight	% Composition
Heneicosanol	$C_{21}H_{44}O$	CH_3-$(CH_2)_{19}$-CH_2-OH	312	1.53
Tricosanol	$C_{23}H_{48}O$	CH_3-$(CH_2)_{21}$-CH_2-OH	340	98.47

From Table 26 , Mixture 5 was the mixture of 2 saturated long chain aliphatic alcohols; heneicosanol ($C_{21}H_{44}O$) and triacosanol ($C_{23}H_{48}O$). Triacosanol was the main component 98.47% while heneicosanol was a trace component. The structure of Mixture 5 is shown below.

CH_3-$(CH_2)_n$-CH_2-OH ; n = 19, 21

Mixture 5

3.5.6 Structural elucidation of Compound 6

Compound 6 was white amorphous solid , 61 mg (1.3×10^{-3} wt. by wt. of dried leaves). The melting point was 70-71 °C. This compound displayed only a single spot with R_f value 0.85 in 5% EtOAc in hexane. Compound 6 was soluble in dichloromethane, chloroform but not soluble in methanol.

The IR spectrum of Compound 6 as shown in Fig. 27 indicated that this compound should be of ester functional group at 1734 cm^{-1} (C=O stretching), and 1174 cm^{-1} (C-O stretching). Other absorption bands were observed at 2923, 2848, 1461, 730 and 720 cm^{-1} corresponded to long chain aliphatic compound. The IR absorption band assignments are tabulated in Table 27.

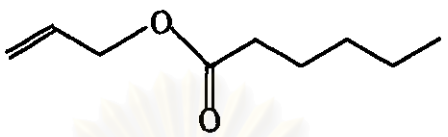
Table 27 The IR absorption band assignments of Compound 6

Wavenumber (cm^{-1})	Intensity	Tentative Assignments
2923, 2848	s	C-H stretching of -CH_2- , -CH_3
1734	s	C=O stretching of ester
1460	m	C-H bending of -CH_2-, -CH_3
1174	m	C-O stretching of ester
730, 720	w	C-H rocking in C-$(CH_2)_n$-C , n > 4

The ^{1}H-NMR spectrum (Fig.28) exhibited proton signals of -CH_3 at chemical shift (δ) 0.87 ppm. The signal at chemical shift (δ) 1.26 and 1.59 ppm exhibited proton signals of (-CH_2-)$_n$. Two signals were observed and could be assigned to be protons of ester as follows : 2.29 ppm (-C<u>H</u>$_2$-C=O) and 4.01 ppm (-C<u>H</u>$_2$-O-C=O).

Compound 6 was analyzed by gas chromatography. Chromatogram showed a single peak at retention time 17.45 min. (Fig. 29)

The mass spectrum of Compound 6 (Fig. 30) was found to be similar that of 2-propenyl hexanoate ($C_9H_{16}O_2$), M.W. 156 by using a library search software, NIST database (Fig. 31). The structure of Compound 6 is showed below.



Compound 6 : 2-Propenyl hexanoate ($C_9H_{16}O_2$)

3.5.7 Structural elucidation of Compound 7

Compound 7 was the bright white plate solid 70 mg (1.49×10^{-3}% wt. by wt. of dried leaves) , m.p. 278-280 °C. It displayed only one spot on TLC with the R_f value of 0.33 (silica gel / 50% CH_2Cl_2 - hexane). The IR spectrum (Fig. 32) absorption bands indicated in Table 28

Table 28 The IR absorption band assignments of Compound 7

Wave number (cm^{-1})	Intensity	Tentative assignment
3550-3400	m, b	O-H stretching vibration of R-OH
3000-2850	s	C-H stretching vibration of CH_3-, -CH_2-
1444	m	C-H bending vibration of CH_3-, -CH_2-
1378	s	C-H symmetric bending vibration of CH_3-
1173	m	C-O stretching and O-H bending vibration

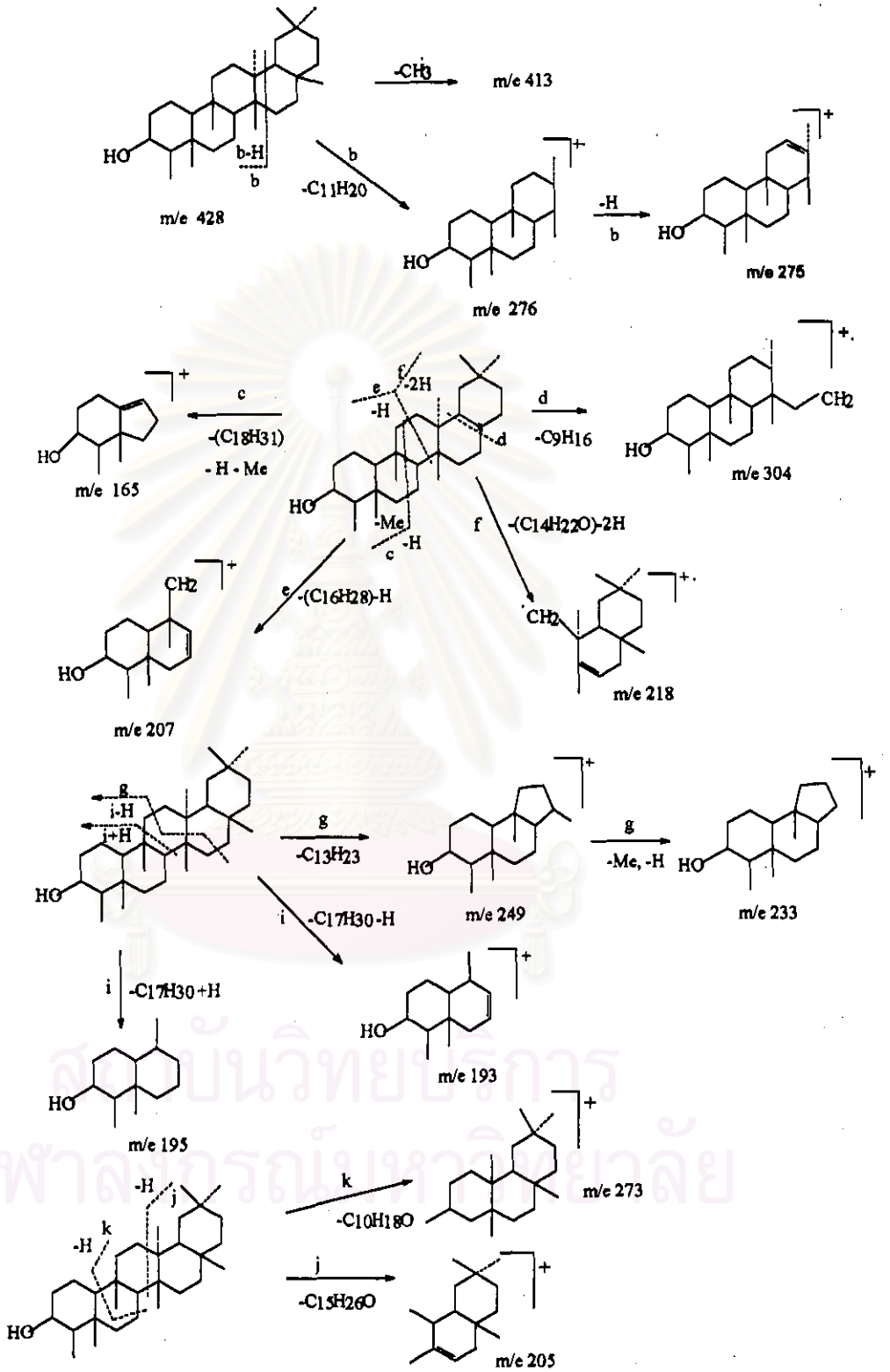
From ^{1}H-NMR spectrum ($CDCl_3$) δ (ppm) (Fig. 34), signals at 0.80-1.56 (m, could be assigned for -CH_3, -CH_2- and -CH. The signal at 3.82 (1H, s shouldbe C<u>H</u>-OH) and the signals at 0.85, 0.99 and 1.20 ppm (24 protons) indicated the present of CH_3 groups. Signals of CH_2,CH of triterpenoid substance displayed at 1.26-1.54 (27 protons).

From the ^{13}C-NMR spectrum ($CDCl_3$) δ (ppm) (Fig. 35) signal at 72.5 ppm exhibited -OH functional group linked with carbon. The ^{1}H and ^{13}C-NMR spectra of Compound 7 were similar to those of friedelan-3β-ol.[168] Therefore other signals in the range of 11.6-61.1 ppm were assigned as shown in Table 29.

Table 29 The assignment of the ^{13}C-NMR spectrum of Friedelan-3β-ol and Compound 7

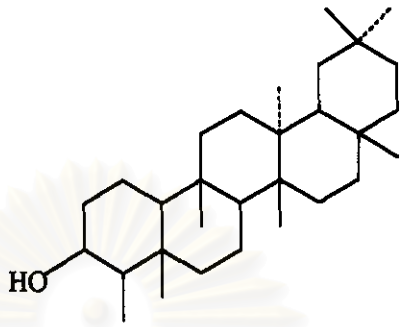
Position of carbon	Chemical shifts (ppm)	
	Friedelan-3β-ol	Compound 7
1	16.4	16.1
2	35.3	35.4
3	72.8	72.5
4	49.3	48.8
5	37.9	37.7
6	41.8	41.3
7	17.6	17.4
8	53.3	52.5
9	35.2	37.0
10	61.4	61.1
11	35.7	35.5
12	30.7	30.0
13	38.4	38.4
14	39.7	41.4
15	32.4	32.3
16	36.2	36.5
17	30.1	29.4
18	42.9	42.3
19	35.4	35.2
20	28.2	28.8
21	32.9	33.0
22	39.3	40.0
23	11.6	11.6
24	15.8	15.5
25	18.3	18.2
26	18.7	19.6
27	20.1	21.5
28	32.1	31.7
29	35.0	35.0
30	31.8	31.4

The mass spectrum (Fig. 33) displayed a molecular ion peak at m/e 428 and others fragmentation at m/e 413 ($M^+ - CH_3$), 304 ($M^+ - C_9H_{16}$), 275 ($M^+ - C_{11}H_{20} - H$), 273 ($M^+ - C_{10}H_{18}O$), 249 ($M^+ - C_{13}H_{23}$), 233 ($M^+ - C_{13}H_{23} - CH_3 - H$), 218 ($M^+ - C_{14}H_{22}O - 2H$), 207 ($M^+ - C_{16}H_{28} - H$) , 205 ($M^+ - C_{15}H_{26}O$) , 195 ($M^+ - C_{17}H_{30}$),193 ($M^+ - C_{17}H_{30} - H$) and 165 ($M^+ - C_{18}H_{31} - H - CH_3$). The possible mass fragmentation pattern of Compound 7 is presented in Scheme 5.



Scheme 5 Fragmentation of Compound 7

According to all spectral evidences, it was concluded that Compound 7 was friedelan-3β-ol ($C_{30}H_{52}O$). The structure of this compound is shown below :



Friedelan-3β - ol ($C_{30}H_{52}O$)

3.5.8 Structural elucidation of Compound 8

Compound 8 was an orange liquid, 956.2 mg (0.02 % wt. by wt. of dried leaves). This compound showed a single spot at R_f value was 0.63 (silica gel / chloroform).

The IR spectrum of Compound 8 (Fig.37) gave the characteristic absorption band of alcohol at 3503 cm^{-1} (b, O-H stretching vibration) and at 1019 (C-O stretching vibration), addition bands of C=C stretching vibration of aromatic ring at 1608 cm^{-1}. The IR absorption band assignments of Compound 8 are shown in Table 30.

Table 30 The IR absorption band assignments of Compound 8

Wavenumber (cm^{-1})	Intensity	Tentative Assignments
3503	s	O-H stretching vibration
3073,2924	w	C-H stretching vibration of -CH_2-
2832	w	OCH_3 stretching vibration
1608,1501	s	C=C stretching vibration of aromatic
1265,1127	s	C-O stretching vibration asymmetric of C-O-C
1019	m	C-O stretching vibration symmetric of C-O-C
820,780	m	=C-H out of plane bending of aromatic

The ^{1}H-NMR spectrum (Fig. 39) showed a methoxy group at 3.86 ppm (3H, s), a hydroxy group at 7.24 ppm (1H,s), aromatic protons at 6.71 ppm. A multiplet signal at 5.14 ppm was corresponded to the vinylic proton.

The ^{13}C-NMR spectrum exhibited 10 signals at δ 30.6, 56.0, 110.7, 115.0, 115.5,119.9, 133.4, 137.7,145.0 and 145.6 ppm (Fig. 40). DEPT 90 and DEPT 135 experiments (Fig. 41) showed a methoxy carbon at 56.0 ppm, three quarternary carbons at 133.4, 145.0 and 145.6 ppm , and two methylene carbons at 39.6 and 115.5 ppm. The spectrum showed that the protons of methoxy group at 3.86 ppm were attacked to the carbons at 56.0 ppm.

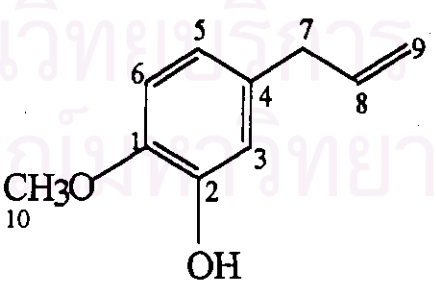
The mass spectrum of Compound 7 (Fig. 38) showed the molecular ion peak at (m/e) 164 and an M+1 at 165. The second most abundant ion is m/e 149, demonstrating the characteristic loss of 15 for an aromatic methyl ether and other fragmentation peaks at m/e, 137, 131, 121, 103 and 91.The molecular formula of Compound 7 was proposed as $C_{10}H_{12}O_2$.

Comparing IR , ^{1}H, ^{13}C-NMR and mass spectra of Compound 7 with those of many phenylpropanoid compounds, it colud be indicated that Compound 7 should be chavibetol. Therefore, the tentative assignments of carbon chemical shift of Compound 7 are proposed by comparing to those of the reported chavibetol[169] which are shown in Table 31.

Table 31 The comparison of ^{13}C-NMR spectrum of chavibetol and Compound 8

Position of Carbon	Chemical shifts (ppm)	
	Compound 8	Chavibetol
C_1	145.0	145.0
C_2	145.6	145.6
C_3	110.7	110.7
C_4	133.4	133.4
C_5	119.9	119.9
C_6	115.0	114.9
C_7	39.6	39.6
C_8	137.7	137.7
C_9	115.5	115.5
C_{10}	56.0	56.0

According to all spectral evidences, Compound 8 was 1-methoxy-2-hydroxy-4-(2-propenyl)-benzeze (chavibetol)[169]. The structure is show below :



Compound 8 : chavibetol

3.5.9 Structural elucidation of Mixture 9

Mixture 9 was bright white needle crystal 450 mg (9.57 x 10^{-3}% wt. by wt. of dried leaves), m.p. 134-136 °C . This compound showed a single spot at R_f value 0.29 (solvent system :60% $CHCl_3$ in hexane).

The IR spectrum of Mixture 9 (Fig. 42) gave the characteristic absorption bands of 2° ROH (probably equatorial) at 3416, 1045 and 1036 cm^{-1}, absorption band of unsaturation at 1644 cm^{-1} and disubstituted and trisubstituted vinyl at 950 and 805 cm^{-1}, respectively. The IR absorption band assignments of this compound are presented in Table 32.

Table 32 The IR absorption band assignments of Mixture 9

Wavenumber (cm^{-1})	Intensity	Tentative Assignments
3416	b, m	O-H stretching vibration
2945	s	C-H stretching vibration of -CH_3, -CH_2-
1644	w	C=C stretching vibration
1465,1378	m	C-H bending vibration of -CH_3, -CH_2-
1045	m	C-O stretching vibration of 3β-OH equatorial position
950	w	C-H out of plane bending vibration of disubstituted vinyl
805	w	C-H out of plane bending vibration of trisubstituted vinyl

The ^{1}H-NMR spectrum ($CDCl_3$) of Mixture 9 (Fig. 44) showed that the signals of protons at chemical shift 0.68-2.30 ppm corresponded to those of methyl, methylene and methine protons, (-C<u>H</u>$_3$, -C<u>H</u>$_2$-, -C<u>H</u>- respectively). The proton adjacent to a hydroxy group (-C<u>H</u>-OH) was shown as the multiplet signal at 3.52 ppm . While the multiplet signal at 5.35 ppm was assigned to be the signal of vinylic proton (-CH=C-).

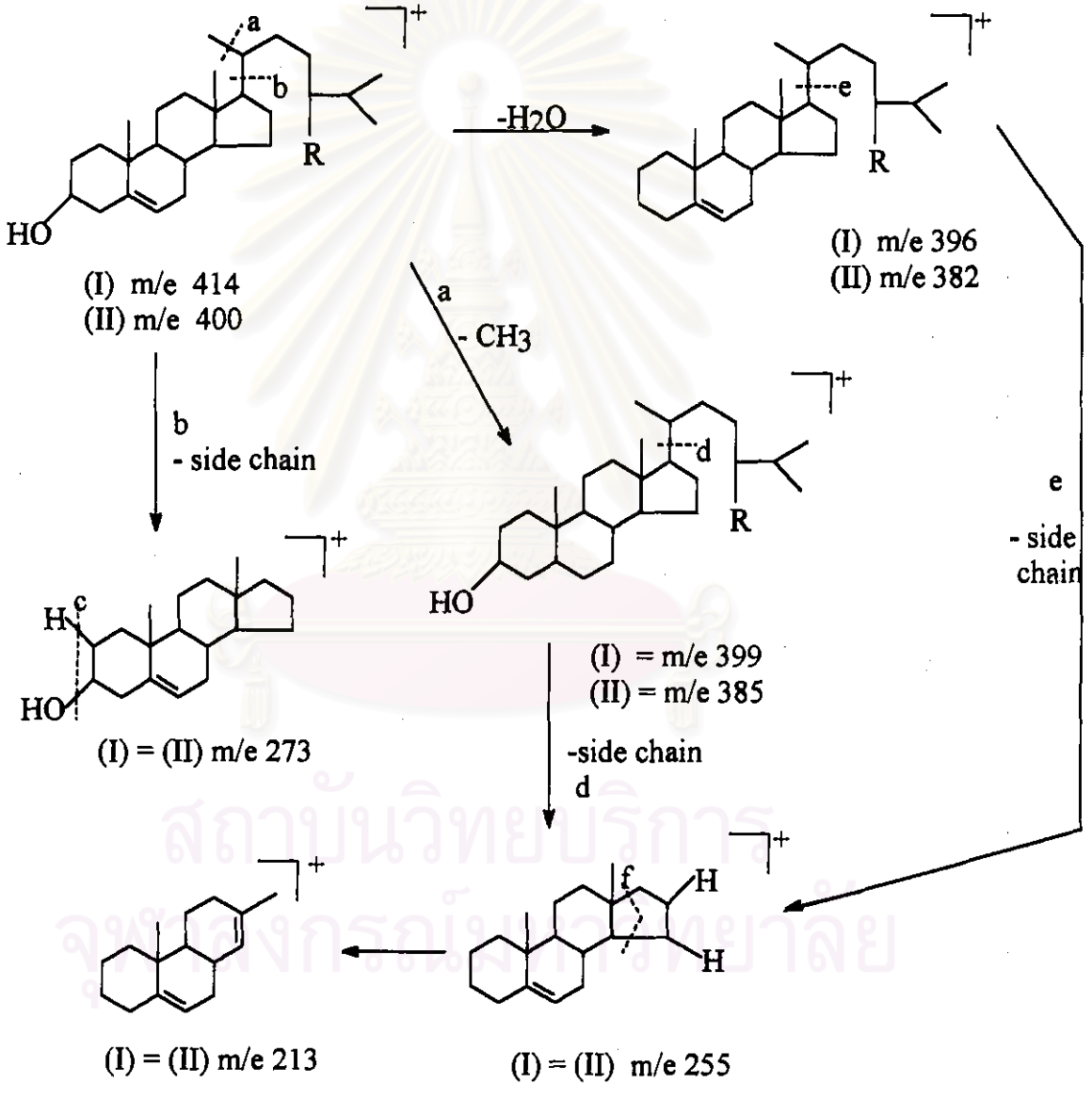
The ^{13}C-NMR , DEPT-90 and DEPT-135 spectrum in $CDCl_3$ (Fig.45) exhibited 43 carbon signals which were the olefinic carbon signals at 121.7 , 129.4 , 138.3 and 140.8 ppm, while the signal at 71.8 ppm was characteristic of a carbon adjacent to a hydroxy group (-C<u>H</u>-OH). Other signals at 11.9-56.9 ppm were not completely assigned at this point. The ^{13}C-NMR chemical shift assignments of Mixture 9 are presented in Table 33.

Table 33 The ^{13}C-NMR chemical shift assignments of Mixture 9

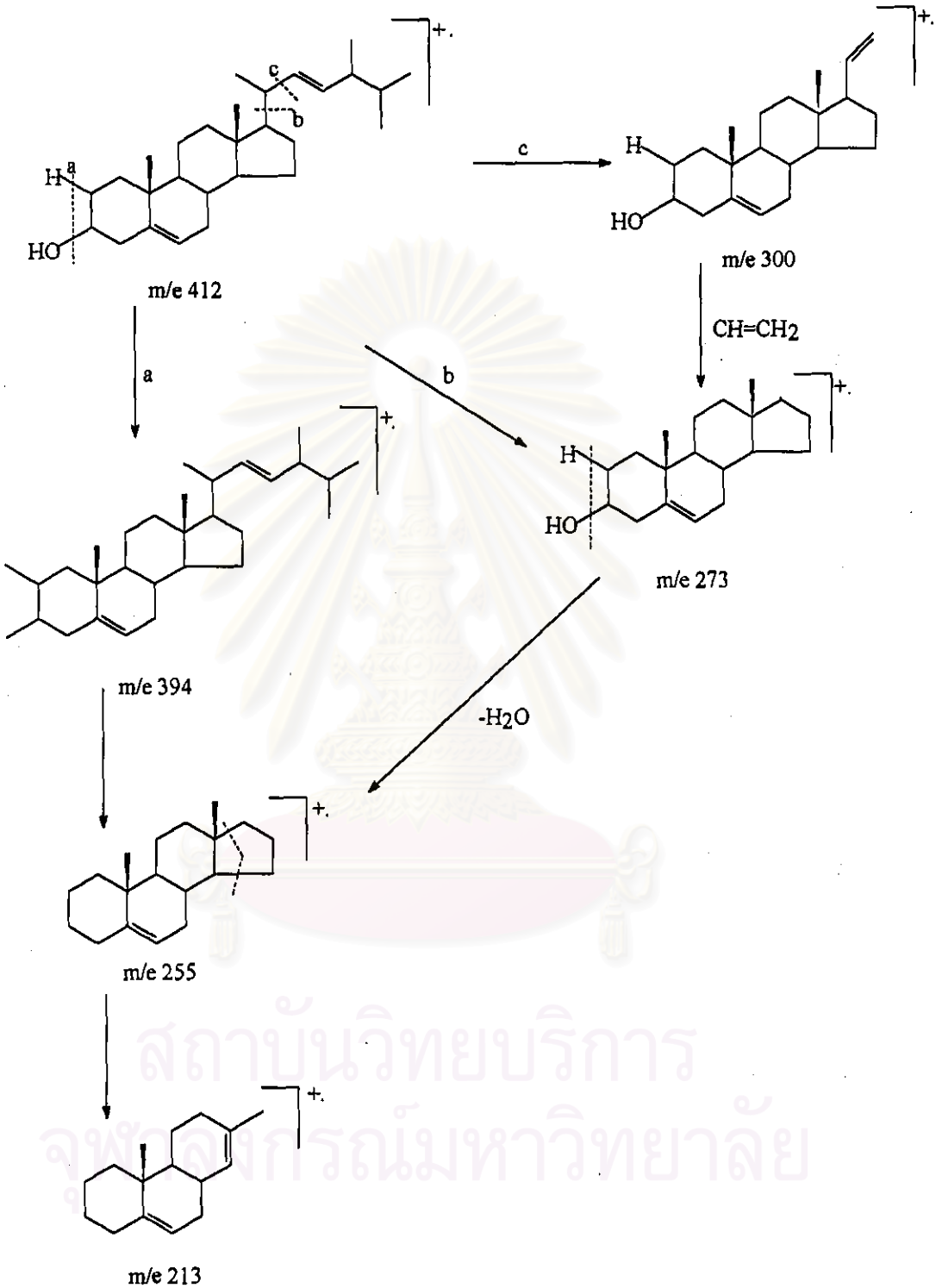
Carbon	Chemical shifts (ppm)			
	Stigmasterol	β-sitosterol	Mixture 9	
			Stigmasterol	β-sitosterol
1	37.4	37.3	37.3	37.3
2	31.8	31.7	31.7	31.7
3	71.8	71.8	71.8	71.8
4	42.3	45.8	42.4	45.9
5	140.8	140.7	140.8	140.8
6	121.7	121.6	121.7	121.6
7	32.0	32.9	32.0	32.0
8	32.0	32.9	32.0	32.0
9	50.3	50.1	50.2	50.2
10	36.6	36.5	36.6	36.6
11	21.1	19.4	21.1	19.4
12	39.8	39.8	39.7	39.8
13	42.3	45.8	42.4	45.9
14	56.9	56.8	56.9	56.8
15	24.4	24.3	24.4	24.3
16	28.9	39.8	28.9	39.7
17	56.0	56.1	56.0	56.0
18	12.2	12.0	12.2	11.9
19	19.5	19.0	19.4	19.1
20	40.5	34.0	40.1	34.0
21	21.1	18.8	21.1	18.8
22	138.3	36.2	138.3	36.2
23	129.4	23.1	129.4	23.1
24	51.3	42.3	51.3	42.4
25	32.0	28.2	32.0	28.2
26	19.0	21.1	19.1	21.1
27	21.1	19.8	21.1	19.8
28	25.5	26.1	25.4	26.2
29	12.1	12.0	12.1	11.9

*The ^{13}C-NMR signals of campesterol were superimposed on those of β-sitosterol.

The mass spectrum (Fig. 43) showed a molecular ion peak corresponding to campesterol, stigmasterol and β-sitosterol at m/e 400 ($C_{28}H_{48}O$), 412 ($C_{29}H_{48}O$) and 414 ($C_{29}H_{50}O$), respectively. The ion fragmentation pattern in the mass spectrum in this mixture indicated that it was a mixture of steroids. The possible mass fragmentation of Mixture 9 is shown in Scheme 6.



Scheme 6 The possible mass fragmentation pattern of Mixture 9 for β-Sitosterol ($R = C_2H_5$) and Campesterol ($R = CH_3$)



Scheme 6 (cont.) The possible mass fragmentation pattern of Mixture 9 for stigmasterol

The information obtained from all spectral evidences of Mixture 9 suggested that it might be a steroid compound.

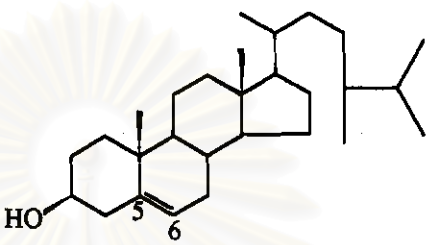
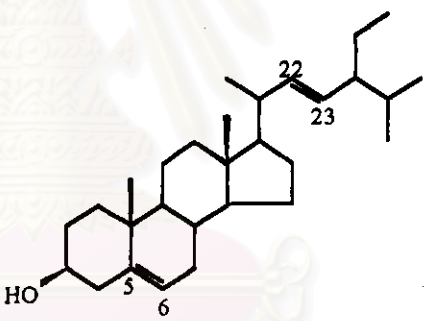
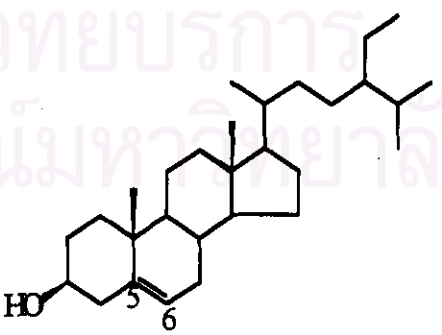
The structure of Mixture 9 was confirmed by GLC technique by comparison to its chromatogram with the chromatogram of the mixture of standard steroids (Fig. 40). The GLC analysis data showed that Mixture 9 was not a single compound. The 3 peaks at retention time 20.12 , 20.99 and 23.96 min indicated that Mixture 9 was a mixture of campesterol, stigmasterol and β-sitosterol, respectively. Retention time of the mixture of three standard steroids and Mixture 9 are shown in Table 34.

Table 34 Retention time of the mixture of three standard steroids and Mixture 9

Name of substances	Retention time (min)	% Composition
Campesterol	19.77	7.91
stigmasterol	20.91	62.72
β-sitosterol	24.15	29.36
Mixture 9	20.12	2.18
	20.99	36.54
	23.96	61.28

From all spectral data (IR, ^{1}H and ^{13}C-NMR spectra, GLC chromatogram and mass spectrum), it was concluded that Mixture 9 was a mixture of campesterol, stigmasterol and β-sitosterol. The major component in this mixture was β-sitosterol. The structures of steroids are shown in Table 35.

Table 35 The structure of campesterol stigmasterolβ-sitosterol

steroid compound	formula	structural	molecular weight
campesterol	$C_{28}H_{48}O$		400
stigmasterol	$C_{29}H_{48}O$		412
β-sitosterol	$C_{28}H_{50}O$		414

3.5.10 Structural elucidation of Mixture 10

Mixture 10 was bright white needle crystal 612 mg (13.02×10^{-3}% wt. by wt. of dried leaves), m.p. 129-132 °C. This compound showed a single spot at R_f value 0.72 (solvent system: 30% EtOAc in hexane)

The IR, mass spectrum , ^{1}H- NMR and ^{13}C-NMR spectra($CDCl_3$) of Mixture10 are similar to those of Mixture 9.Mixture 10 is therefore suggested that it might be a steroid compound.

The structure of Mixture 10 was confirmed by GLC technique by comparison of its chromatogram with the chromatogram of the mixture of three standard steroids (Fig. 50). The GLC analysis data showed that Mixture 10 was not a single compound.

The GLC analysis indicated three peaks on the chromatogram at retention times show in Table 36.

Table 36 The retention time from the gas chromatogram of Mixture 10 compared with standard steroids.

Name of substance	Retention time (min)	% Composition
Campesterol	19.77	7.91
Stigmasterol	20.91	62.72
β-sitosterol	24.15	29.36
Mixture 10	20.55	4.69
	21.68	41.25
	24.66	53.89

From all of the spectral data, it was concluded that Mixture 10 was a mixture of campesterol, stigmasterol and β-sitosterol. The structure is shown in Table 35.

3.5.11 Structural elucidation of Compound 11

Compound 11 was the semi-solid in greenish oil, 3.11 g (0.07 % wt. by wt. of dried leaves) . R_f value 0.69 (solvent system : 50% MeOH in $CHCl_3$).

The IR spectrum of Compound 11 (Fig. 51) gave the characteristic absorption band of alcohol at 3349 cm^{-1} (b, O-H stretching) and at 1101 cm^{-1} (m, C-O stretching), addition bands of C=C stretching vibration of aromatic ring at 1598 cm^{-1}.

The IR absorption band assignments of Compound 11 is shown in Table 37.

Table 37 The IR absorption band assignment of Compound 11

Wave number (cm^{-1})	Intensity	Tentative Assignments
3349	b	O-H stretching
3000-2800	w	C-H stretching vibration of $-CH_3$, $-CH_2-$
1598, 1511, 1429	m	C=C stretching vibration of aromatic
1275, 1101	m	C-O stretching vibration
901	m	=C-H out of plane bending vibration of aromatic
799	m	C-H out of plane bending vibration of $=CH_2$

The ^{1}H-NMR spectrum (Fig. 53) showed two hydroxy groups at δ (ppm) 6.74 (1H,s) and 6.72 (1H, S). An aromatic proton appeared at 6.56 ppm and a multiplet signal at 5.08-5.90 ppm was correspond the vinylic proton.

The ^{13}C-NMR spectrum exhibited 9 signals at 39.2, 115.5, 115.9, 116.1, 121.1, 133.4, 137.5, 141.4 and 143.3 (Fig.54). DEPT 90 showed 4 peaks of CH signals at 115.9, 116.1, 121.1 and 137.5 (Fig. 55). DEPT 135 showed CH_3, CH signal (up phase) 4 peaks at 115.9, 116.1, 121.1 and 137.5 and CH_2 signals (down phase) 2 peaks at 115.5 and 39.2 (Fig. 55). From ^{13}C-NMR, DEPT-90 and DEPT-135 spectra exhibited three quarternary carbons at 133.4, 141.4 and 143.3 ppm.

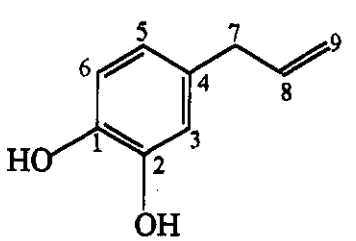
Mass spectrum of Compound 11 (Fig. 52) showed the molecular ion peak at (m/e) 150 and other fragmentation peaks at m/e 135, 133, 123, 104, 91, 85 and 83.

The molecular formula of Compound 11 ($C_9H_{10}O_2$) and spectral data indicated that Compound 11 should be allylpyrocatechol. Therefore, the tentative assignments of ^{13}C-NMR of Compound 11 are proposed by comparing to those of the reported allylpyrocatechol as shown in Table 38.

Table 38 The comparison of the chemical shift of the ^{13}C-NMR spectrum of allylpyrocatechol and Compound 11

Position of carbon	Chemical shift (ppm)	
	Compound 11	allylpyrocatechol
C_1	143.3	143.5
C_2	141.4	141.7
C_3	115.9	115.7
C_4	133.4	133.3
C_5	121.1	121.1
C_6	116.1	116.0
C_7	39.2	39.5
C_8	137.5	137.7
C_9	115.5	115.6

According to all spectral evidences, Compound 11 was 4-(2-propenyl)-1,2-benzenediol (allylpyrocatechol)[169] and the structure of this compound is shown below.



(Compound 11 : allylpyrocatechol)

Cardiac glycoside tests and saponin tests for *n*-butanol crude extract

The crude extracts of *n*-butanol were brought for screening cardiac glycoside tests.

CARDIAC GLYCOSIDE TEST[170]

About 5 cm^3 of the crude *n*-butanol extract was added 10% of lead acetate solution 40 cm^3. After heating for 15 minutes, the solution was cooled and filtered. The filtrate was extracted with dichloromethane for three times, about 20 cm^3 each time. Dichloromethane layer was added anhydrous Na_2SO_4 in order to remove water and the solution was evaporated to 1/10 of the original volume. The solution was divided into three test tubes for three cardiac glycoside tests.

Lieberman-Burchard Reaction (steroid test) : Dichloromethane solution in the first test tube was evaporated almost to dryness and 3 drops of acetic anhydride wass added with shaking . One drop of conc. H_2SO_4 was poured along the side of the test tube. The color change was observed immediately from pink → violet → blue → green.

Kedde's Reaction (α,β-unsaturated lactone test) : Dichloromethane solution in the second test tube was evaporated almost to dryness and then about 10 cm^3 of Kedde's reagent and 2-3 drops of 1 M. NaOH were added. The positive test giving blue or violet / pink color was observed.

Keller - Kiliani Reaction (deoxy sugar test) : Dichloromethane solution in the third test tube was added about 3 cm^3 of 5 % $FeCl_3$. After shaking, conc. H_2SO_4 at the junction between two layers and colour in the upper layer was observed.

The results of cardiac glycoside test are shown in Table 39

Table 39 The results of cardiac glycoside test of crude *n*-butanol extract.

Reaction	Observeation	result
Liebermann-Burchard reaction	pink → violet → blue → green	+
Kedd's reaction	no change	-
Keller-Kiliani reaction	green solution in upper layer and brown ring at interlayer	+

SAPONIN TEST[170]

Foam Test : The ground dried-leaves of Phluu about 0.1 g. were brought into test tube and distilled water was added about 5 cm^3 . These was heat on water baht for 5 minutes and observe the foam for 20 minutes, dil. H_2SO_4 was added about 1 cm^3 and heated. Shake the mixture for 1 minute.

Color Test : The crude *n*-buthanol extract about 5 cm^3 was added dil. H_2SO_4 10 cm^3 heated for 15 minutes, and then was extracted with dichloromethane about 15 cm^3. Dichloromethane layer was added anhydrous Na_2SO_4 and filtered. The filtrate was evaporated almost to dryness, acetic anhydride was added about 3 drops. About 1 drop of conc. H_2SO_4 was poured along the side of the test tube. The color change was observed within 1 hour. The positive test of steroid sapogenin was blue or blue-green color, the positive test of steroid sapogenin was blue or blue-green color, the positive test of triterpenoid sapogenin was red, pink or violet color. The results of saponin test are shown in table 40.

Table 40 The results of saponin test of crude *n*-butanol extract

Reaction	Observation	Result
Foam test	no change	-
Color test	no change	-

Note : + positive

■ negative

3.6 Separation of *n*-butanol crude extract (Fraction II)

The *n*-butanol soluble fraction (70 g) was mixed with silica gel (70 g) and applied to a silica gel column (6 cm i.d. x 150 cm column). The *n*-butanol fraction was chromatographed (using a solvent system of $CHCl_3$: CH_3OH : H_2O (78:20:2), $CHCl_3$: CH_3OH : H_2O (70:30:10, lower layer) and $CHCl_3$: CH_3OH : H_2O (65:35:10 , lower layer). Approximately 250 ml of eluant was collected for each fraction. The fractions were concentrated on a rotary evaporator to a volume of about 10-15 ml and checked by TLC plate. The fractions which had the same components were combined. The results of the separation of the *n*-butanol extract are presented in the Table 41.

Table 41 The results of the separation of crude *n*-butanol extract by column chromatography.

Eluents	Fraction No.	Code	Remarks	Weight (g)
$CHCl_3$: CH_3OH : H_2O (78:20:2)	1-2	Bu 1	red- brown oil	2.11
	3-7	Bu 2	red- brown oil	2.00
	8-19	Bu 3	red- brown oil	27.07
$CHCl_3$: CH_3OH : H_2O (70:30:10, lower layer)	20-28	Bu 4	red- brown oil	2.53
	29-34	Bu 5	red- brown oil	0.93
	35-58	Bu 6	red- brown oil	5.11
$CHCl_3$: CH_3OH: H_2O (65:35:10, lower layer)	59-73	Bu 7	brown oil	2.00
	74-78	Bu 8	brown oil	1.15

3.6.1 Separation of the eluted fractions No. 3-7 (Bu 2) (Table 41)

The eluted fractions No. 3-7, red-brown oil 2.00 g was further purified by column chromatography (silica gel Art. 7734 35 g , 2 cm i.d. x 60 cm column). The eluent was $CHCl_3$: CH_3OH : H_2O (78:20:2). About 50 ml fractions were combined. The results of the separation of fractions 3-7 are shown in Table 42.

Table 42 The results of the separation of the eluted fractions No. 3-7 by silica gel column chromatography

Eluent	Fraction No.	Remark	Weight (g)
$CHCl_3$: CH_3OH : H_2O (78:20:2)	1	brown oil	0.10
	2-5	brown oil	3.88
	6-11	brown oil	0.32
	12-15	brown oil	0.12

3.6.2 Separation of the eluted fractions No. 2-5 (Table 42)

The eluted fractions No. 2-5 (see Table 42), brown oil 3.88 g was further purified by column chromatography using the same procedure as for the separation of the eluted fractions No. 3-7 (Bu 2). Fractions of about 20 ml were collected. The results are shown in Table 43.

Table 43 The results of the separation of fractions No. 2-5 by silica gel column chromatography

Eluent	Fraction No.	Remark	Weight (g)
$CHCl_3$:CH_3OH:H_2O (78:20:2)	1	orange oil	0.71
	2-10	solid in brown oil (PBBu 1)	4.22
	11-12	brown oil	1.22
	13-23	brown oil	0.88

3.6.3 Separation of the eluted fractions No. 8-19 (Bu 3)

The eluted fractions No. 8-19 (Bu 3) (see Table 41), red-brown oil 27.07 g, was further purified by column chromatography (4 cm i.d. x 100 cm column). This fraction was chromatographed using a solvent system of $CHCl_3$:CH_3OH:H_2O (78:20:2). Fraction of about 50 ml was collected. Each fraction was checked by TLC and identical fractions were combined. The results are shown in Table 44.

Table 44 The results of the separation of fractions No. 8-19 (Bu 3)

Eluent	Fraction No.	Remark	Weight (g)
$CHCl_3$: CH_3OH:H_2O (78:20:2)	1-2	brown oil	0.46
	3-7	brown oil	0.91
	8-17	brown oil	15.61
	18-22	brown oil	2.01
	23-30	brown oil	0.81

3.6.4 Separation of the eluted fractions No. 8-17 (Table 44)

The eluted fractions No. 8-17, brown oil 15.61 g were separated by column chromatography (4 cm i.d. x 100 cm column). This fraction was chromatographed using a solvent system of $CHCl_3$:CH_3OH:H_2O (78:20:2). Fraction of about 30 ml were collected. Each fraction was checked by TLC and indicated fractions was combined. The results are shown in Table 45.

Table 45 The results of the separations of fractions No. 8-17

Eluent	Fraction No.	Remark	Weight (g)
$CHCl_3$: CH_3OH : H_2O (78:20:2)	1-5	brown oil	0.14
	6-7	brown oil	0.39
	8-9	brown oil (PBBu 2)	5.64
	10-22	brown oil (PBBu 3)	7.41

3.6.5 Separation of the eluted fractions No. 18-22 (Table 44)

The eluted fractions No. 18-22 (see Table 44), brown oil 2.01 g was further purified by column chromatography using the same procedure as for the separation of the eluted fractions No. 8-17, but about 20 ml for each fraction were collected. The results are shown in Table 46.

Table 46 The results of the separation of fractions No. 18-22

Eluent	Fraction No.	Remark	Weight (g)
$CHCl_3$:CH_3OH:H_2O	1-2	brown oil	0.11
(70:30:10, lower layer)	3-7	brown oil (PBBu 4)	5.69
	8	brown oil	0.90

3.6.6 Separation of the eluted fractions No. 20-28 (Bu 4) (Table 41)

The eluted fractions No. 20-28 (Bu 4), 2.53 g was further purified by column chromatography (silica gel Art. 7734). The eluent was $CHCl_3$:CH_3OH:H_2O (70:30:10, lower layer). Fractions of about 20 ml were collected. Each fraction was checked by TLC and identical fractions were combined. The results of the separation of fractions 20-28 are shown in Table 47.

Table 47 The results of the separation of fractions No. 20-28

Eluent	Fraction No.	Remark	Weight (g)
$CHCl_3$: CH_3OH : H_2O	1-2	brown oil	0.02
(70:30:10, lower layer)	3-8	brown oil	1.15
	9-29	brown oil	0.13

3.6.7 Separation of the eluted fractions No. 3-8 (Table 47)

The eluted fractions no. 3-8 (see Table 47 , brown oil, 1.15 g, was further purified by column chromatography (2 cm i.d. x 60 cm column). This fraction was chromatographed using a solvent system of $CHCl_3$: CH_3OH : H_2O (70:30:10, lower layer). Fraction of about 50 ml was collected. Each fraction was checked by TLC and indentical fractions were combined. The results are shown in Table 48.

Table 48 The results of the separation of fractions No. 3-8

Eluent	Fraction No.	Remark	Weight (g)
$CHCl_3$: CH_3OH : H_2O	1-2	brown oil	0.09
(70:30:10, lower layer)	3	brown oil	1.01
	4	brown oil (PBBu 5)	0.47
	5-15	brown oil	0.05

3.6.8 Separation of the eluted fractions No. 3 (Table 48)

The eluted fraction No. 3 (see Table 48), brown oil, 1.01 g, was further purified by column chromatography (1 cm i.d. x 30 cm column) using the same procedure as for the separation of the eluted fractions No. 3-8. But about 10 ml fractions were collected. The results are shown in Table 49.

Table 49 The results of the separations of fraction No. 3

Eluent	Fractions No.	Remark	Weight (g)
$CHCl_3$: CH_3OH : H_2O	1	brown oil	0.01
(70:30:10 , lower layer)	2-4	brown oil (PBBu 6)	0.10
	5-8	brown oil	0.02

Brine Shrimp Cytotoxic Lethality Test

Some fractions of *n*-butanol crude extract was further subjected to brine shrimp cytotoxic lethality test. The results of brine shrimp cytotoxic lethality test are shown in Table 50.

Table 50 The results of brine shrimp cytotoxic lethality test of various fractions derived from the separation of *n*-butanol crude extract.

Code	LC_{50} (μg/ml)	Activity
PBBu 2	> 1000	no activity
PBBu 3	> 1000	no activity
PBBu 4	> 1000	no activity
PBBu 5	> 1000	no activity
PBBu 6	> 1000	no activity

It could be clearly seen from the brine shrimp cytotoxic lethality test that the each fraction of *n*-butanol crude extract displayed no activity.

3.7.Purification, properties and structural elucidation for fractions of *n*-butanol crude extract.

3.7.1 Purification , properties and structural elucidation of PBBu 1

PBBu 1 was obtained from the separation of the *n*-butanol crude extract (Fraction II) (see Table 43). After multiple recrystallization from methanol,this compound was obtained as white crystals, m.p. 150-152 °C., 80 mg (1.70×10^{-3}% wt.by wt. of the dried leaves). This compound showed a single spot on TLC with R_f value 0.46 (silica gel, $CHCl_3$: CH_3OH : H_2O, 70:30:10, lower layer).

The FT-IR spectrum of PBBu 1 (Fig. 56) revealed a strong absorption band at 3500-3200 cm^{-1} which was corresponded to the O-H stretching vibration of a hydroxy functional group and at 1115 and 1055 cm^{-1} (C-O stretching vibration). The C-H stretching vibration peaks of an aliphatic compound were observed at 2950 and 2850 cm^{-1} and the absorption peak at 723 cm^{-1} revealed the presence of saturated long chain of $(-CH_2-)_n$.

The ^{1}H-NMR spectrum of this compound (Fig. 57) exhibited a methyl group at 0.93 ppm (t,3H) and multiplet signals at 1.38, 1.55 and 3.51 ppm (6H) assigned for the methylene proton.

The ^{13}C-NMR spectrum (Fig. 58) exhibited a total of ten carbon signals at 13.5, 19.1, 13.8, 60.5, 62.2, 63.4, 69.2, 69.4, 70.17 and 99.9. The assigned carbon signals based on the ^{13}C-NMR spectrum were confirmed by the information obtained from the DEPT-90 and DEPT-135 spectra (Fig. 59). The DEPT-90 showed CH signals 3 peaks at 70.2, 69.4 and 62.2 ppm. The DEPT-135 showed CH_2 signals 5 peaks at 19.1, 31.8, 60.5, 62.2 and 63.5 and exhibited -CH_3 signal at 13.6 ppm. The remaining ten signals in the ^{13}C-NMR spectrum were compatible with quarternary carbons which were observed at 99.9 ppm.

From the above spectroscopic data of this compound was found to be similar to that of D-Fructose. The comparison of ^{13}C-NMR signals of D-Fructose[171] and PBBu 1 is presented in table 51.

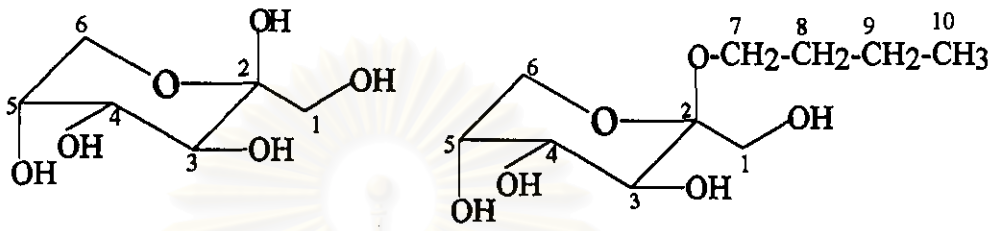
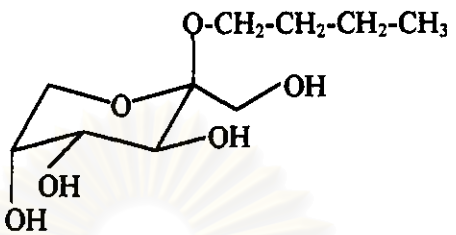


Table 51 The comparison of the ^{13}C-NMR signals of D-Fructose and PBBu 1

Position	Chemical shift (ppm)	
	D-Fructose	PBBu 1
C_1	64.7	63.6
C_2	99.1	99.9
C_3	68.4	69.4
C_4	70.5	70.2
C_5	70.0	69.2
C_6	64.1	62.2
C_7	-	60.47
C_8	-	31.81
C_9	-	19.11
C_{10}	-	13.55

The mass spectrum of PBBu 1 (Fig. 60) did not display the molecular ion peak at 236 (Calcd. For $C_{10}H_{20}O_6$: M.W. 236). Other significant fragmentation peaks were detected at m/e 205 (M^+ - CH_2OH) and, 163 (M^+- O-CH_2-CH_2-CH_2-CH_3)

According to all spectral evidence, the structure of this compound is 2-O-*n*-butyl-β-fructopyranose which is shown below.



2-O-*n*-butyl-β-fructopyranose

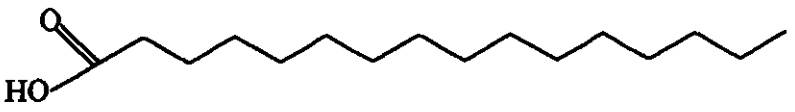
3.7.2 Purification, properties and structural elucidation of PBBu 2

PBBu 2 was obtained from the separation of *n*-butanol crude extract (Fraction II). It was brown oil in fractions 8-9 (see table 45) , 5.64 g.

The FT-IR spectrum of PBBu 2 (Fig. 61) gave the characteristic absorption band of alcohol at 3500-3150 cm^{-1} (b, O-H stretching vibration), 2970,2930 cm^{-1} (C-H stretching vibration of -CH_2-), at 1650 cm^{-1} (C=O stretching vibration) and at 1040 cm^{-1} (C-O stretching vibration).

The GC-MS (Fig. 62) indicated that PBBu 2 was not a single compound. The major component showed the retention time at 30.35 min and the mass spectrum of this retention time was found to be similar to the mass spectrum of hexadecanoic acid ($C_{16}H_{32}O_2$: M.W. 256) (by using library search software , (NIST database)) (Fig. 64). In addition, mass spectrum (Fig. 63) displayed molecular ion at m/e 256 corresponding to the molecular formula $C_{16}H_{32}O_2$.

From the above spectroscopic data, it may be concluded that PBBu 2 was a mixture of long chain carboxylic acids. The major component in this mixture was hexadecanoic acid. The structure is shown below.



Hexadecanoic acid

3.7.3 Purification, properties and structural elucidation of PBBu 3

PBBu 3 was obtained from the separation of *n*-butanol crude extract (Fraction II). It was brown oil in fractions 10-22 (see Table 45), 7.41 g.

The FT-IR spectrum of PBBu 3 (Fig.65) revealed the characteristic absorption peaks very similar to those of PBBU 2.

GC-MS indicated that PBBu 3 was not a single compound. The chromatogram (Fig. 66) showed 3 retention times at 29.00, 30.81 and 34.15 min. The major component appeared at retention time 30.81 min. The mass spectrum at retention time 30.81 min (Fig. 67) did not show the molecular ion peak. There is no fragmentation pattern corresponding to that of them.

3.7.4 Purification, properties and structural elucidation of PBBu 4

PBBu 4 was obtained from the separation of *n*-butanol crude extract (Fraction II) as brown oil in fraction 3-7 (see Table 46) , 5.69 g.

The FT-IR spectrum of PBBu 4 (Fig. 68) gave the absorption peaks close to those of PBBu 2 and PBBu 3 (ν_{max} 3570-3220 cm^{-1} (O-H stretching), 2929 cm^{-1} (C-H stretching vibration), 1654 cm^{-1}(C=O stretching) and 1040 cm^{-1} (C-O stretching).

GC-MS indicated that PBBu 3 was not a single compound. The chromatogram (Fig. 69) showed 2 retention times at 23.43 and 34.68 min. The major component showed retention time at 34.68 min. The mass spectrum at retention time 23.43 min (Fig. 70) and 34.68 min (Fig. 71) did not show the molecular ion peak. Therefore

molecular weight of these substances was not know, The structur of PBBu4 was therefore not be able to further elucidate.

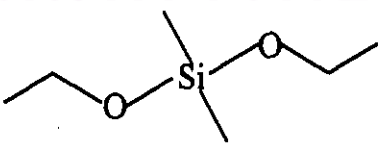
3.7.5 Purification, properties and structural elucidation of PBBu 5

PBBu 5 was obtained from the separation of *n*-butanol crude extract (Fraction II) as brown oil in fraction 4 (see Table 48), 0.47 g.

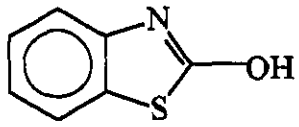
The FT-IR spectrum of PBBu 5 (Fig. 72) revealed the characteristic absorption peaks very similar to those of PBBu 4.

GC-MS indicated that PBBu 5 was not a single compound. The chromatogram (Fig. 73) showed 3 retention times at 13.00, 23.67 and 26.97 min.

The major component showed retention time at 23.67 min. The mass spectrum at retention time 13.00 min (Fig. 74) did not show the molecular ion peak. Fragmentation pattern of the component at Rt 13.00 had relative intensities nearly of the same as diethoxydimethyl silane ($C_6H_{16}O_2Si$: M.W. 148) (Fig. 75). The mass spectrum at retention time 23.67 min (Fig. 76) did not show the molecular ion peak. From the use of library search software (NIST database), there is no fragmentation pattern corresponding to that of compound at Rt 23.67. The mass spectrum at retention time 26.97 min displayed the molecular ion peak at m/e 151 (C_7H_5ONS : M.W. 151), (Fig. 77). Fragmentation pattern of compound at Rt 26.97 min had relative intensities nearly of the same as 2(3H)-benzothiazlone (C_7H_5ONS : M.W. 151) (by using library search software, (NIST database)),(Fig. 78). The structures are shown below.



Diethoxydimethyl silane



2(3H)-benzothiazolone

3.7.6 Purification, properties and structural elucidation of PBBu 6

PBBu 6 was obtained from the separation of *n*-butanol crude extract (Fraction II) . It was brown oil in fractions 2-4 (see Table 49) , 0.10 g.

The FT-IR spectrum of PBBu 6 (Fig. 79) revealed the characteristic absorption peaks very similar to those of PBBu 4.

GC-MS indicated that PBBu 6 was not a single compound (Fig. 80). The major component showed retention time at 35.15 min. The mass spectrum at retention time 35.15 min (Fig. 81) did not show the molecular ion peak. Therefore molecular weight of this substance was not known, since it did not exhibit molecular formulae.In addition, from the use of library search software (NIST database), there is no fragmentation pattern corresponding to that of compound at Rt 35.15. Unfortunately, this substance was obtained in such a limited amount that its structure can not be further elucidated. The aid of modern techniques of separation such as MPLC or HPLC may need to be applied for the better resolution of this separation.

3.8 Study on biological activity of isolated compounds

Literature survey of the biological activity of chavibetol showed the antifungal activity against *Cladosporium cucumerinum*,[157] hepatotoxic activity and glutamate-pyruvate transaminase stimulation [172]. For instance the active compound lupeol showed antibacterial activity against *escherichia* , *Salmonella typhosa*, *Shigella flexneri* and *Staphylococcus aureus*[173], antihyperglycemic activity [174], antiinflammatory activity[175-178], antileishmaniasis activity [179] against *Leishmania braziliensis*, *leishmania amazonensis* and *leishmania amazonensis donovani*,antimalarial activity[180] against plasmodium falciparum, antioxidant activity [181], antitrypanosomal activity [179] against *Trypanosoma cruzi*, antitumor activity against sarcoma-wm 256 cell line [182], antitussive activity [183], antiturolithiasis activity [184-185], calcium ion uptakeinhibition[186], chronotropic effect negative[186] cytotoxic activity against Osteosarcoma[187], leuk-L1210200[188], expectorant activity[183], farnesyl-protein transferase inhibition[189] hydroxysteroid (beta) dehydrogenase inhibition[190], hypocholesterolemic activity [191], hypotensive activity [192], kidney stone development inhibition[181], leukotriene b-4 production inhibition[187], ornithine decarboxylase inhibition[193], phorbol ester antagonist[194] and renal lithiasis inhibition[195].

The saturated long chain aliphatic alcohol are widely distributed in the waxy fraction of the plant extract. The triacontanol ($C_{30}H_{61}OH$) had been reported to be widely used as a plant growth regalator (PGR).[119,196]10-20 mg/L of triacontanol used as the plant growth stimulating agent on tomato plants had been illustrated[140].

In addition, the saturated long chain aliphatic alcohol and a mixture of steroids were reported to show the antifeeding activity to insect, boll weevil.[148]

3.9 Anticell line cytotoxicity test of isolated compounds

The isolated compounds, Mixture 2, Compound 4, Compound 7 and Mixture 9 were also tested for antitumor activity against *Human Bladder Carcinoma* (BIU) , *Human Erythroleukemia Carcinoma* (K562), *Human Gastric Carcinoma* (BGC-823), *Human Leukemia Carcinoma* (HL-60) and *Human Nasopharyngeal Carcinoma* (KB) and the results are shown in table 52 , table 53, table 54, table 55 and table 56 , respectively.

Table 52 The results of antitumor activity against *Human Bladder Carcinoma* (BIU)

Sample	Concentration (μg/ml)	Inhibition (%)	Estimation
Mixture 2	1	-10.28	+
	10	40.85	
	100	56.53	
Compound 4	1	1.76	-
	10	21.07	
	100	39.03	
Compound 7	1	11.53	-
	10	13.75	
	100	17.32	
Mixture 9	1	5.28	-
	10	3.18	
	100	15.51	

Table 53 The results of antitumor activity against *Human Erythroleukemia Carcinoma* (K562)

Sample	Concentration (μg/ml)	Inhibition (%)	Estimation
Mixture 2	1	-5.50	-
	10	-3.55	
	100	13.26	
Compound 4	1	17.79	-
	10	28.80	
	100	41.02	
Compound 7	1	11.53	-
	10	13.75	
	100	17.32	

Table 54 The results of antitumor activity against *Human Gastric Carcinoma* (BGC-823)

Sample	Concentration (μg/ml)	Inhibition (%)	Estimation
Mixture 2	1	-17.60	-
	10	46.76	
	100	49.21	
Compound 4	1	-22.96	+
	10	-24.87	
	100	78.56	
Compound 7	1	-2.46	-
	10	-20.15	
	100	-16.12	

Table 55 The results of antitumor activity against *Human Nasopharyngeal Carcinoma* (KB)

Sample	Concentration (μg/ml)	Inhibition (%)	Estimation
Compound 4	1	13.49	+
	10	12.79	
	100	59.33	
Compound 7	1	32.94	-
	10	12.30	
	100	11.56	

Table 56 The results of antitumor activity against *Human Leukemia Carcinoma* (HL-60)

Sample	Concentration (μg/ml)	Inhibition (%)	Estimation
Mixture 2	1	-	-
	10	-12.83	
	100	16.75	
Compound 4	1	-30.29	+
	10	-2.90	
	100	67.45	
Compound 7	1	-28.89	-
	10	-5.26	
	100	46.50	
Mixture 9	1	-	+
	10	-83.02	
	100	57.78	

3.10 The rsults of brine shrimp (*Artemia salina* Linnaeus) cytotoxic lethality test of isolated compounds

According to preliminary cytotoxicity screening test (see Table 5), the chloroform crude extract was selected for further study and searching for bioactive compounds were therefore submitted to the brine shrimp test in order to confirm that biological activity. The results are shown in Table 57

Table 57 The results of brine shrimp cytotoxic lethality test of isolated compounds

Sample	LC_{50} (at 24 hr)	Activity
Mixture 2	> 1000	no activity
Compound 4	> 1000	no activity
Compound 6	172.63	low activity
Compound 8	2.55	high activity
Mixture 9	> 1000	no activity
Compound 11	16.36	medium activity

From the results of brine shrimp cytotoxic lethality tests, it was found that Compound 8 (chavibetol) and Compound 11 (allylpyrocatechol) exhibited cytotoxicity activity against brine shrimp with LC_{50} values of 2.55 and 16.36 µg/ml at 24 hours, respectively.