CHAPTER IV

RESULTS AND DISCUSSION

1. Extraction yield

The leaves of *C. hystrix, F. limonia*, *A. marmelos* and *C. aurantifolia* were extracted with hexane, dichloromethane, chloroform, ethanol and methanol respectively. The percentage yields of the crude extracts were calculated based on a dry weight (Table 4.1).

Table 4.1 The percentage yields of the extracts from Thai medicinal plants

Crude extract of medicinal plant	weight (g) and % yield
1. C. hystrix leaves 1.01 kg in various solvents :	
-Hexane extract	7.72 (0.76)
-Dichloromethane extract	3.74 (0.37)
-Chloroform extract	22.23 (2.19)
-Ethanol extract	40.73 (4.02)
-Methanol extract	21.83 (2.15)
2. F. limonia leaves 442.48 g in various solvents :	
-Hexane extract	10.02 (1.20)
-Chloroform extract	13.97 (1.68)
-Ethanol extract	27.96 (3.36)
-Methanol extract	28.04 (3.37)
3. A. marmelos leaves 355.19 g in various solvents :	
-Hexane extract	0.11 (0.03)
-Chloroform extract	4.49 (1.26)
-Ethanol extract	9.88 (2.78)
-Methanol extract	7.11 (2.00)
4. C. aurantifolia leaves 381.43 g in various solvents :	
-Hexane extract	2.22 (0.58)
-Chloroform extract	4.29 (1.12)
-Ethanol extract	18.64 (4.89)
-Methanol extract	11.51 (3.02)

The crude ethanol extract of *F. limonia* was partitioned with ethyl acetate and butanol respectively. Results are presented in Table 4.2.

Table 4.2 The percentage yield of ethyl acetate and butanol extracts from *F. limonia*

Partition solvents	weight (g) and % yield
Ethyl acetate	6.25 (22.35)
Butanol	7.76 (27.75)

1.1 Essential oil

The fresh leaves of *C. hystrix, F. limonia*, *A. marmelos* and *C. aurantifolia* were cut into small pieces and accurately weighed (~500 g), then the chopped leaves of each plant were subjected into the hydrodistillation to yield the essential oil of each medicinal plant. Results are shown in Table 4.3.

Table 4.3 The percentage yields of the essential oil from Thai medicinal plants

The essential oil from medicinal plant	weight (g) and % yield
C. hystrix leaves	3.78 (0.83)
F. limonia leaves	0.47 (0.10)
A. marmelos leaves	2.15 (0.46)
C. aurantifolia leaves	2.18 (0.53)

2. Screening method for total antioxidant activity

2.1 ABTS method

Three series of standard solutions containing: 0.05-2.50 mM of Trolox solutions, 0.50-2.50 mM of vitamine C solutions, and 0.10-0.50 mM of quercetin solutions were prepared. The absorbances of each series were measured at 734 nm. The % inhibition of each solution was calculation as shown in Table 4.4-4.6. Then the calibration curve of each standard was constructed (Figure 4.1). These calibration curves were used for ABTS screening method.

Table 4.4 Percentage inhibition of Trolox concentration series for ABTS screening method

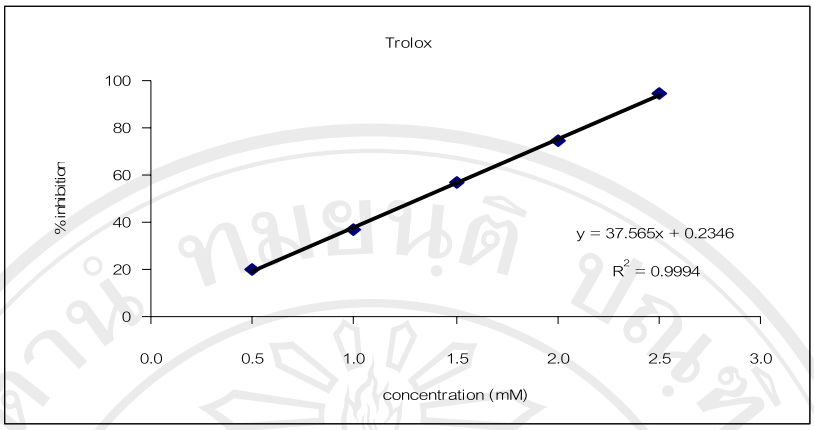
Concentation of Trolox (mM)	% inhibition
0.05	19.74
1.00	37.05
1.50	56.57
2.00	74.72
2.50	94.82

Table 4.5 Percentage inhibition of vitamin C concentration series for ABTS screening method

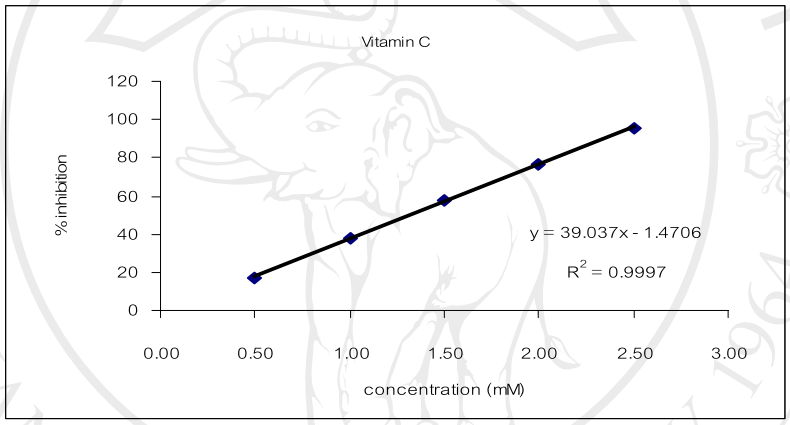
Concentation of vitamin C (mM)	% inhibition
0.50	17.42
1.00	38.06
1.50	57.53
2.00	76.74
2.50	95.68

Table 4.6 Percentage inhibition of quercetin concentration series for ABTS screening method

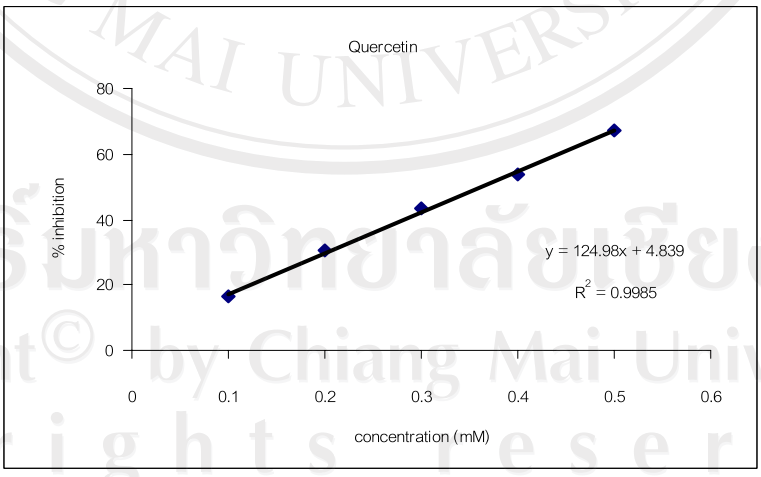
Concentation of quercetin (mM)	% inhibition
0.10	16.67
0.20	30.46
0.30	43.17
0.40	53.96
0.50	67.41



a



b



c

Figure 4.1 Concentration-response curve for the absorbance at 734 nm for $ABTS^{\bullet+}$ as a function of concentration of standard Trolox (a), vitamin C (b) and quercetin (c) solution.

The antioxidant activities of the essential oil and the crude extracts of *C. hystrix, F. limonia*, *A. marmelos* and *C. aurantifolia* leaves in various solutions: hexane, dichloromethane, chloroform, ethanol and methanol were determined by using ABTS free radical-scavenging method. The absorbance of each sample was measured at 734 nm and the percentage inhibition was calculated by reference to the calibration curve of standard Trolox, vitamin C and quercetin (Figure 4.1) respectively. Results are presented in Table 4.7.

Table 4.7 Antioxidant activities by ABTS assay

Scientific name	**ABTS (mM/mg extracts)**		
	Trolox	**Quercetin**	**Vitamine C**
1. C. hystrix leaves in various solvents :			
-Hexane extract	4.79	1.40	4.66
-Dichlorometane extract	7.21	2.13	6.98
-Chloroform extract	10.67	3.17	10.31
-Ethanol extract	15.15	4.52	14.62
-Methanol extract	14.41	4.30	13.91
- The essential oil from *C. hystrix* leaves	5.82	1.71	5.65
2. F. limonia leaves in various solvents :			
-Hexane extract	3.73	1.08	3.63
-Chloroform extract	7.85	2.32	7.60
-Ethanol extract	15.33	4.57	14.79
-Methanol extract	10.97	3.26	10.60
- The essential oil from *F. limonia* leaves	10.21	3.03	9.86
3. A. marmelos leaves in various solvents :			
-Hexane extract	2.90	0.84	2.84
-Chloroform extract	3.92	1.14	3.81
-Ethanol extract	8.77	2.60	8.49
-Methanol extract	7.58	2.24	7.34
- The essential oil from *A. marmelos* leaves	6.43	1.89	6.23
4. C. aurantifolia leaves in various solvents :			
-Hexane extract	2.98	0.86	2.91
-Chloroform extract	4.47	1.31	4.34
-Ethanol extract	10.95	3.25	10.58
-Methanol extract	10.13	3.01	9.79
- The essential oil from *C. aurantifolia* leaves	10.85	3.22	10.48

The results showed the crude extract of *C. hystrix* exhibited the highest antioxidant activity followed by *F. limonia*, *C. aurantifolia* and *A. marmelos* respectively. The ethanol extract showed the highest antioxidant capacity followed by the methanol, chloroform and hexane extracts of each plant respectively. The essential oil of *C. aurantifolia* showed the highest antioxidant activity followed by *F. limonia*, *A. marmelos* and *C. hystrix* respectively.

2.2 DPPH method

Three series of standard solution containing: 0.04-1.00 mg mL^{-1} of Trolox, 0.04-0.08 mg mL^{-1} of vitamin C and 0.02-0.06 mg mL^{-1} of quercetin solutions were prepared. The absorbances of each series were measured at 540 nm and the % inhibition of each solution was calculated. Results are presented in Table 4.8-4.10. The percentage inhibition were plotted against the each standard concentrations (Trolox, vitamin C and quercetin) as illustrated in Figure 4.2-4.4. Three calibration curves were used for DPPH screening method.

Table 4.8 The percentage inhibition of each concentration of the Trolox standard solution

Concentation of Trolox (mg/ml)	% inhibition
0.04	18.73
0.05	24.71
0.06	32.65
0.07	40.14
0.08	48.12
0.09	51.03
1.00	60.58

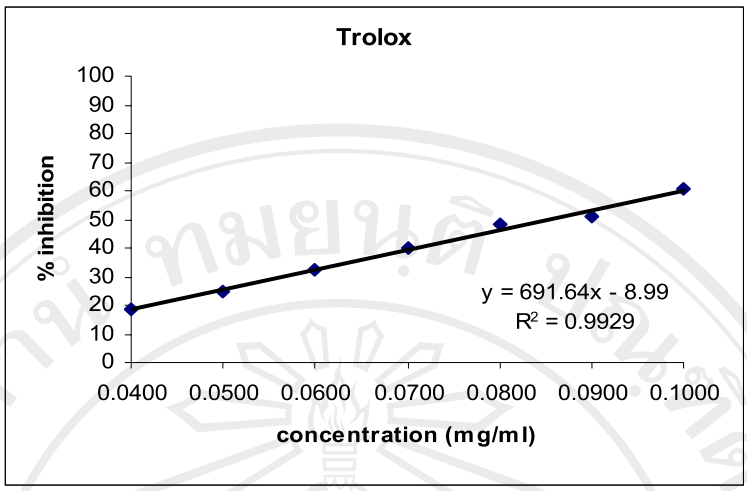


Figure 4.2 Calibration curve for the absorbance at 540 nm of DPPH method as a function of the concentration of Trolox standard solution.

Table 4.9 The percentage inhibition of each concentration of the vitamin C standard solution.

Concentation of vitamin C (mg/ml)	% inhibition
0.04	25.03
0.05	36.34
0.06	45.06
0.07	53.91
0.08	63.35

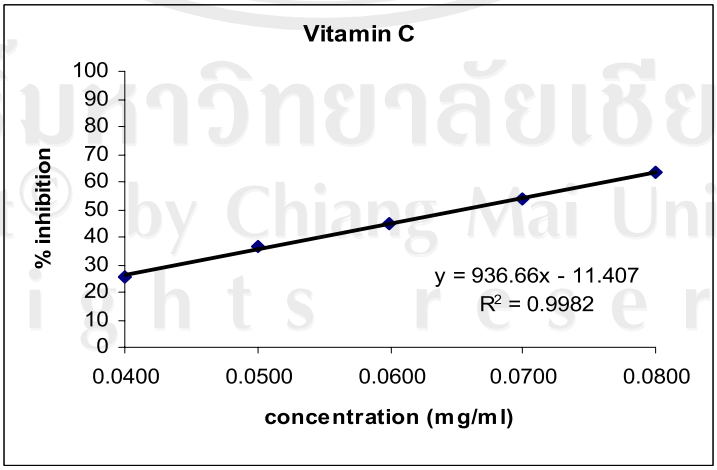


Figure 4.3 Calibration curve for the absorbance at 540 nm of DPPH method as a function of the concentration of vitamin C standard solution.

Table 4.10 The percentage inhibition of each concentration of the quercetin standard solution

Concentation of quercetin (mg/ml)	% inhibition
0.02	16.39
0.03	28.45
0.04	38.69
0.05	49.16
0.06	57.78

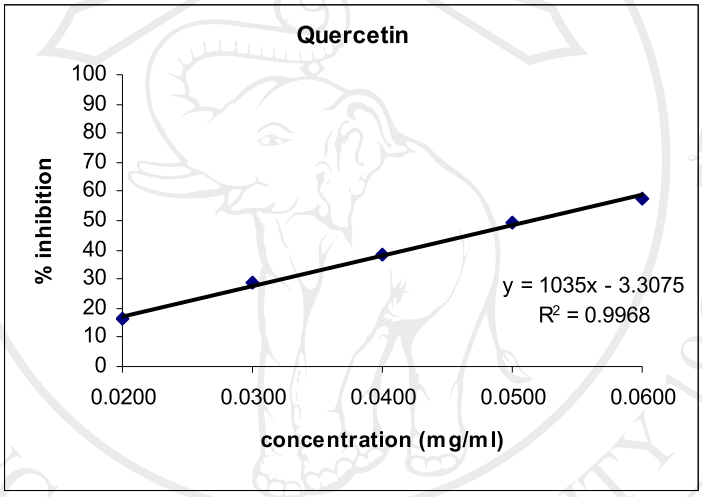


Figure 4.4 Calibration curve for the absorbance at 540 nm of DPPH method as a function of the concentration of quercetin standard solution.

The antioxidant activities of the crude extracts were assessed by different *in vitro* tests. Free radical-scavenging capacities of the oils were measured by the DPPH assay and the results are given in Table 4.11. In the DPPH assay, the ability of the examined crude extract act as donor of hydrogen atoms or electrons in transformation of DPPH$^{\bullet}$ into its reduced form DPPH-H was investigated. The examined crude extract could reduce the stable, purple-colored radical DPPH into yellow-colored DPPH-H. The IC_{50} of each crude extract was determined by reference to the calibration curve (Figure 4.2-4.4). The results were compared with three antioxidant standards, Trolox, vitamin C and quercetin. Results are shown in Table 4.11. The

antioxidant activity of the essential oil was also determined. But it gave only weak antioxidant activity.

Table 4.11 Antioxidant activities by DPPH assay

Crude extracts of medicinal plants	IC_{50} (mg mL^{-1})
1. C. hystrix leaves in various solvents :	
-Hexane extract	5.92
-Dichloromethane extract	3.56
-Chloroform extract	3.49
-Ethanol extract	3.26
-Methanol extract	3.91
2. F. limonia leaves in various solvents :	
-Hexane extract	7.18
-Chloroform extract	5.50
-Ethanol extract	2.14
-Methanol extract	3.44
3. A. marmelos leaves in various solvents :	
-Hexane extract	7.23
-Chloroform extract	12.82
-Ethanol extract	2.18
-Methanol extract	4.42
4. C. aurantifolia leaves in various solvents :	
-Hexane extract	4.76
-Chloroform extract	4.95
-Ethanol extract	3.01
-Methanol extract	3.95
5. Reference standards :	
- Trolox	0.09
- Vitamin C	0.07
- Quercetin	0.05

3 Screening method for antimicrobial activity

3.1 antibacterial activity

The antibacterial activities of the essential oil and crude extracts of *C. hystrix, F. limonia*, *A. marmelos*, *C. aurantifolia* were also investigated. Antibacterial studies were carried out *in vitro* against Gram-positive and Gram-negative organisms.

Results are presented in Table 4.12. The crude extract and the essential oil exhibited antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, using gentamicin as reference standard.

3.2 antifungal activity

The antifungal activities of the essential oils and crude extracts of *C. hystrix, F. limonia*, *A. marmelos* and *C. aurantifolia* were investigated by means of agar well diffusion method. The inhibitory zones were measured. Results are presented in Table 4.13. The essential oil also showed antifungal activity against *Candida albicans*, *Aspergillus flavas* and *Trichophyton mentagophyte* but the crude extract of the plants inhibited only *Candida albicans* and *Trichophyton mentagophyte*, using ketoconazole as reference standard.

4 Screening method for anticancer activity

The anticancer activity of the crude *C.* hystrix, *F. limonia* extracts and the essential oils of *C.* hystrix, *F. limonia*, *A. marmelos*, *C. aurantifolia* were performed using the resazurin microplate assay (REMA). All crude extracts of the leaves of *C. hystrix* and the hexane and chloroform extracts of *F. limonia* showed anticancer activity against NCI-H187-human, small cell lung cancer. Breast cancer (MCF7) was also inhibited by chloroform and dichloromethane extracts of *C. hystrix*. The essential oils of *F. limonia* and *A. marmelos* presented anticancer activity against NCI-H187-human and Breast cancer (MCF7) but the essential oils of *C. hystrix* and *C. aurantifolia* did not show anticancer activity. Ellipticine and doxorubicine were reference standard for determination. Results are shown in Table 4.14.

Table 4.12 Antibacterial activities

Crude extracts of medicinal plants	Conc. (mg mL^{-1})	Diameter of inhibition zone (mm)[a]		
		E. Coli (mm)	*P.aeroginosa* (mm)	*S.aureus* (mm)
1. C. hystrix leaves in various solvents :				
-Hexane extract	10	11.0	16.0	11.0
-Dichloromethane extract	10	11.0	18.0	12.0
-Chloroform extract	10	12.0	17.0	12.0
-Ethanol extract	10	11.0	17.0	12.0
-Methanol extract	10	12.0	18.0	12.0
- The essential oil	50	14.0	17.0	19.0
2. F. limonia leaves in various solvents :				
-Hexane extract	10	0.0	17.0	11.0
-Chloroform extract	10	0.0	17.0	12.0
-Ethanol extract	10	11.0	17.0	11.0
-Methanol extract	10	11.5	15.0	11.0
- The essential oil	50	13.0	17.0	12.0
3. A. marmelos leaves in various solvents :				
-Hexane extract	10	10.0	14.0	11.0
-Chloroform extract	10	12.0	14.0	14.0
-Ethanol extract	10	11.0	14.0	12.0
-Methanol extract	10	11.0	15.0	11.0
- The essential oil	50	13.0	18.0	18.0
4. C. aurantifolia leaves in various solvents :				
-Hexane extract	10	11.0	11.0	11.0
-Chloroform extract	10	10.0	10.0	12.0
-Ethanol extract	10	10.0	16.0	10.0
-Methanol extract	10	0.0	16.0	12.0
- The essential oil	50	18.0	19.0	32.0
5. Gentamicin (positive control)	75 µg mL^{-1}	27	27	35

[a] 24-14 mm = significant activity; 7-13 mm = moderate activity; <7 mm = weak activity; 0 mm = inactive

Table 4.13 Antifungal activities

Crude extracts of medicinal plants	Conc. (mg mL^{-1})	Diameter of inhibition zone (mm)[a]		
		C.albican (mm)	*A.flavas* (mm)	*T. mentagophyte* (mm)
1. C. hystrix leaves in various solvents :				
-Hexane extract	10	11.0	0.00	13.0
-Dichloromethane extract	10	11.0	0.00	15.0
-Chloroform extract	10	11.0	0.00	12.0
-Ethanol extract	10	11.0	0.00	13.0
-Methanol extract	10	11.0	0.00	15.0
- The essential oil	50	27.0	15	19.0 (1 mg/ml)
2. F. limonia leaves in various solvents :				
-Hexane extract	10	11.0	0.00	14.0
-Chloroform extract	10	10.5	0.00	13.0
-Ethanol extract	10	11.0	0.00	15.0
-Methanol extract	10	11.5	0.00	12.0
- The essential oil	50	20.0	11.0	28.0
3. A. marmelos leaves in various solvents :				
-Hexane extract	10	11	0.00	12
-Chloroform extract	10	12	0.00	18
-Ethanol extract	10	12	0.00	17.5
-Methanol extract	10	11	0.00	11
- The essential oil	50	16	17	35
4. C. aurantifolia leaves in various solvents				
-Hexane extract	10	11	0.00	12
-Chloroform extract	10	10	0.00	13
-Ethanol extract	10	11	0.00	12
-Methanol extract	10	12	0.00	12.5
- The essential oil	50	38	21	20 (1 mg/ml)
5. Ketoconazone (positive control)	250 µg mL^{-1}	37	25	16

[a] 24-14 mm = significant activity; 7-13 mm = moderate activity; <7 mm = weak activity; 0 mm = inactive

Table 4.14 Anticancer activities

Crude extracts of medicinal plants	KB-Oral cavity cancer		MCF7-Breast cancer		NCI-H187-Small cell lung cancer	
	Activity	IC_{50} (µg/ml)	Activity	IC_{50} (µg/ml)	Activity	IC_{50} (µg/ml)
1. *C. hystrix* leaves in various solvents :						
- Hexane extract	inactive	-	inactive		active	13.31
- Dichlorome-thane extract	inactive		active	34.66	active	8,52
- Chloroform extract	inactive	-	active	50.00	active	13.41
- Ethanol extract	inactive	-	inactive		active	31.54
- Methanol extract	inactive	-	inactive		active	46.10
- The essential oil	inactive	-	inactive		inactive	-
2. *F. limonia* leaves in various solvents :						
-Hexane extract	inactive	-	inactive	-	active	30.14
-Chloroform extract	inactive	-	inactive	-	active	29.31
- Ethanol extract	inactive	-	inactive	-	inactive	-
- Methanol extract	inactive	-	inactive	-	inactive	-
- The essential oil	inactive	-	active	50.00	active	40.92
3. *A. marmelos*						
- The essential oil	inactive	-	active	14.95	active	23.53
4. *C. aurantiforia*						
- The essential oil	inactive	-	inactive	-	inactive	-
5. Reference standards :						
- Ellipticine	active	0.475	active	-	active	0.441
- Doxorubicine	active	0.133	active	0.649	active	0.042

5. Analysis of the essential oil

5.1 The essential oil of *C. hystrix* leaves

Fresh leaves of *C. hystrix* were homogenized and hydrodistilled for 3 h to yield a pale yellow oil of 0.83 %. The essential oil was analysed by means of GC and GC-MS. Identification of oil constituents was performed by comparison of mass spectra with literature data (NIST, wiley7n.l). Thirty eight compounds were identified

and are listed in Table 4.15. A typical total ion chromatogram of the essential oil of *C. hystrix* is presented in Figure 4.5.

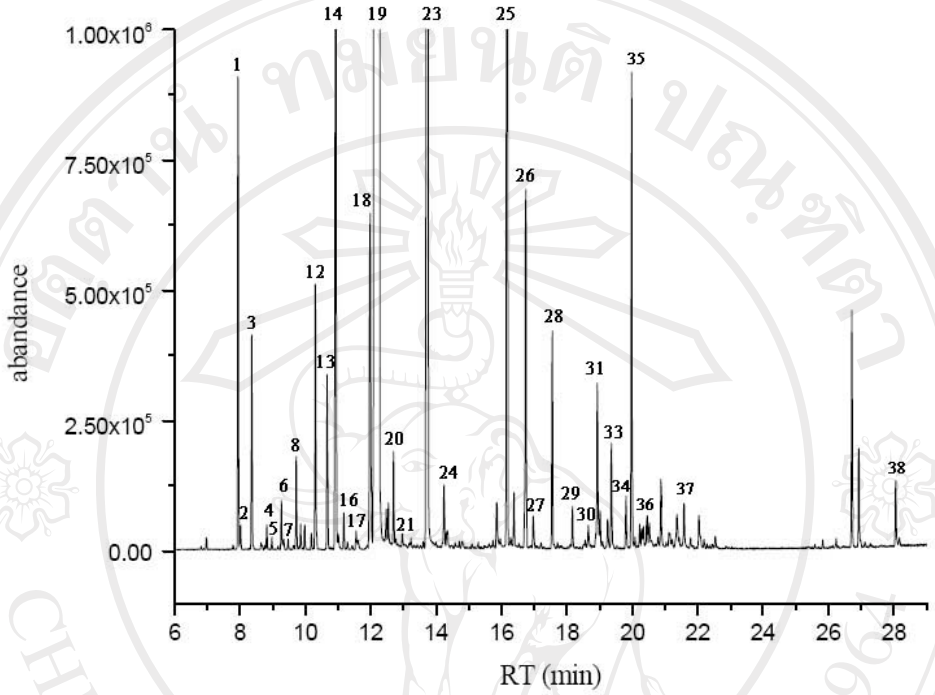


Figure 4.5 Total ion chromatogram of *C. hystrix* essential oil

Table 4.15 Volatile components in leaves of *C. hystrix*

Peak No.	Compound	R.T (min)	% RA
1	β-Thujene	7.95	0.77
2	α-Pinene	8.02	0.04
3	β-Myrcene	8.36	0.36
4	3-Carene	8.82	0.04
5	α-Terpinene	8.98	0.02
6	Limonene	9.27	0.09
7	Eucalyptol	9.34	0.02
8	*trans*-β-Ocimene	9.72	0.15
9	Melonal	9.86	0.05
10	γ-Terpinene	9.98	0.04
11	*cis*-sabinene hydrate	10.18	0.03
12	*cis*-linalool oxide	10.31	0.49

Table 4.15 Volatile components in leaves of *C. hystrix* (Continued)

Peak No.	Compound	R.T (min)	%RA
13	*trans*-Linalool oxide	10.67	0.30
14	Linalool	10.93	2.25
15	Nonanal	11.01	0.04
16	*cis*-Rose oxide	11.18	0.06
17	*trans*-Rose oxide	11.57	0.04
18	Isopulegol	11.98	0.88
19	Citronellal	12.26	77.74
20	4-Terpineol	12.7	0.18
21	α-Terpineol	12.97	0.06
22	Decanal	13.23	0.02
23	β-Citronellol	13.76	10.48
24	Geraniol	14.24	0.12
25	Citronellyl acetate	16.17	2.56
26	Geranyl acetate	16.74	0.65
27	β-Cubebene	16.97	0.08
28	Caryophyllene	17.55	0.40
29	α-Humulene	18.17	0.08
30	Germacrene-D	18.66	0.06
31	Bicyclogermacrene	18.93	0.37
32	α-Farnesene	19.01	0.07
33	*d*-Cadinene	19.36	0.21
34	Elemol	19.81	0.11
35	Nerolidol	19.98	0.84
36	Diethyl Phthalate	20.52	0.04
37	α-Cadinol	21.36	0.11
38	Phytol	28.07	0.14

RT, retention time
RA, retention area (peak area relative to total peak area)

5.2 The essential oil of *F. limonia* leaves

Fresh leaves of *F. limonia* were homogenized and hydrodistilled for 3 h to yield a pale yellow oil of 0.10 %. The essential oil was analysed by means of GC and GC-MS. Identification of oil constituents was performed by comparison of mass spectra with literature data (NIST, wiley7n.l) that yielded < 90% matches were

identified as unknowns or with those in the literature. Forty compounds were identified and Twenty-five compounds are listed in Table 4.16. A typical total ion chromatogram of the essential oil of *F. limonia* is presented in Figure 4.6.

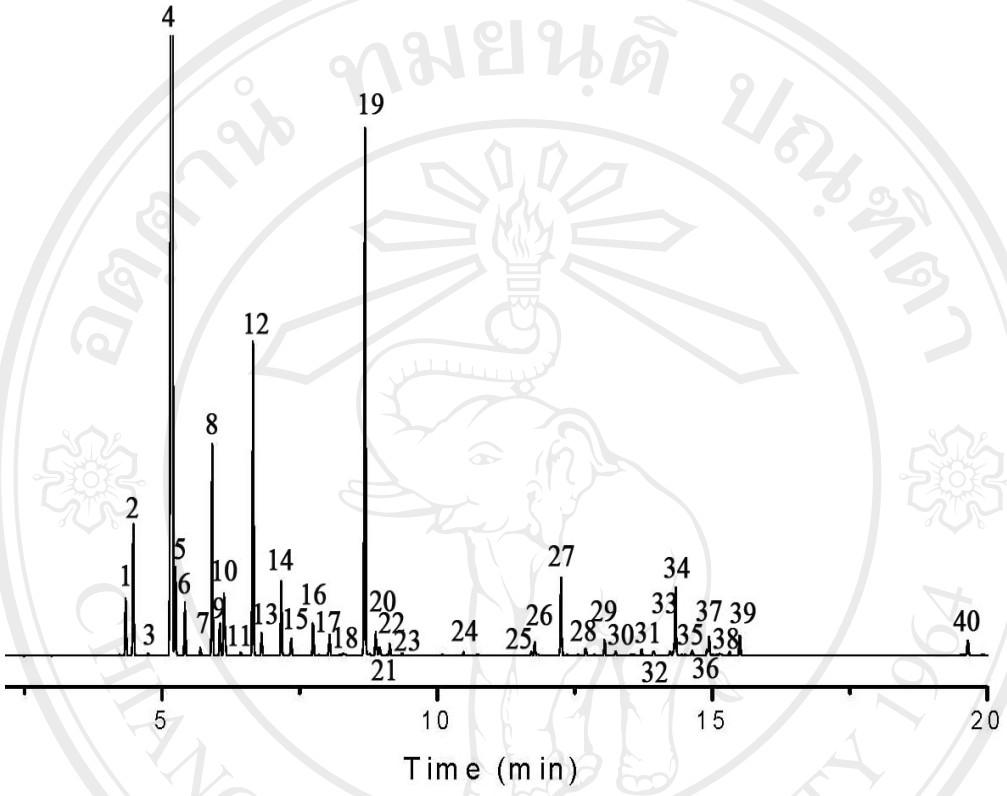


Figure 4.6 Total ion chromatogram of *F. limonia* essential oil

Table 4.16 Chemical composition of the essential oil of *F. limonia*

Peak No.	Compound	R.T (min)	%RA
1	α-Thujene	4.338	1.05
2	α-Pinene	4.479	2.39
3	Camphene	4.744	0.05
4	Sabinene	5.179	64.80
5	β-Pinene	5.237	1.44
6	β-Myrcene	5.414	0.95
7	l-Phellandrene	5.69	0.15
8	α-Terpinene	5.901	3.73
9	dl-Limonene	6.13	1.45
10	β-*trans*-Ocimene	6.43	0.06
11	γ-Terpinene	6.653	5.89

Table 4.16 Chemical composition of the essential oil of *F. limonia* (Continued)

Peak No.	Compound	R.T (min)	%RA
12	*trans*-Sabinene hydrate	6.806	0.41
13	α-Terpinolene	7.17	1.35
14	*cis*-Sabinene hydrate	7.347	0.36
15	1-Terpineol	8.04	0.40
16	4-Terpineol	8.686	10.32
17	α-Tepineol	8.88	0.53
18	β-Damascenone	11.706	0.07
19	β-Bourbonene	11.771	0.23
20	*trans*-Caryophyllene	12.253	1.47
21	α-Humulene	12.699	0.14
22	Germacrene-D	13.04	0.29
23	Caryophyllene oxide	14.333	1.43
24	Humulene oxide	14.638	0.12
25	Phytol	19.65	0.33

RT, retention time
RA, retention area (peak area relative to total peak area)

5.3 The essential oil of *A. marmelos* leaves

Fresh leaves of *A. marmelos* were homogenized and hydrodistilled for 3 h to yield a pale yellow oil of 0.46 %. The essential oil was analysed by means of GC and GC-MS. Identification of oil constituents was performed by comparison of mass spectra with literature data (NIST, wiley7n.l) that yielded < 90% matches were identified as unknowns or with those in the literature. Thirty-two compounds were identified and twenty-one compounds are listed in Table 4.17. A typical total ion chromatogram of the essential oil of *A. marmelos* is presented in Figure 4.7.

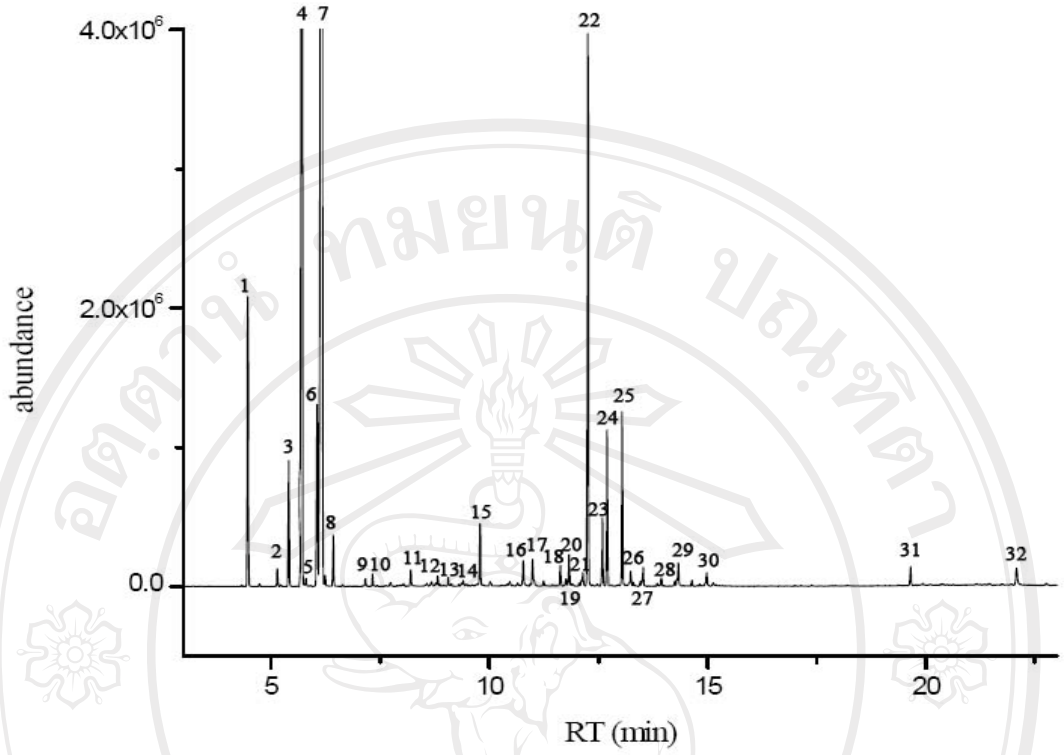


Figure 4.7 Total ion chromatogram of *A. marmelos* essential oil

Table 4.17 Chemical composition of the essential oil of *A. marmelos*

Peak No.	Compound	R.T (min)	%RA
1	α-Pinene	4.473	4.06
2	Sabinene	5.154	0.25
3	β-Myrcene	5.413	1.71
4	α-Phellandrene	5.719	25.15
5	*d*- 3-Carene	5.807	0.13
6	Limonene	6.165	44.85
7	*cis*-Ocimene	6.247	0.17
8	*trans*-Ocimene	6.435	0.67
9	Terpinolene	7.17	0.13
10	L-Linalool	7.334	0.18
11	Cryptone	8.827	0.15
12	α-Copaene	11.635	0.30
13	*trans*-Caryophyllene	12.264	8.22
14	β-Farnesene	12.599	0.95
15	α-Humulene	12.704	2.24

Table 4.17 Chemical composition of the essential oil of *A. marmelos* (Continued)

Peak No.	Compound	R.T (min)	%RA
16	Germacrene-D	13.051	2.43
17	Bicyclogermacrene	13.239	0.27
18	*d*-Cadinene	13.527	0.33
19	Nerolidol	13.944	0.11
20	Caryophyllene oxide	14.332	0.45
21	Phytol	19.649	0.28

RT, retention time
RA, retention area (peak area relative to total peak area)

5.4 The essential oil of *C. aurantifolia* leaves

Fresh leaves of *C. aurantifolia* were homogenized and hydrodistilled for 3 h to yield a pale yellow oil of 0.53%. The essential oil was analysed by means of GC-MS. Identification of the oil constituents was performed by comparison of mass spectra with the literature data (NIST, wiley7n.l) that yielded < 90% matches were identified as unknowns. Thirty compounds were identified and twenty-seven compounds are listed in Table 4.18. A typical total ion chromatogram of the essential oil of *C. aurantifolia* is presented in Figure 4.8.

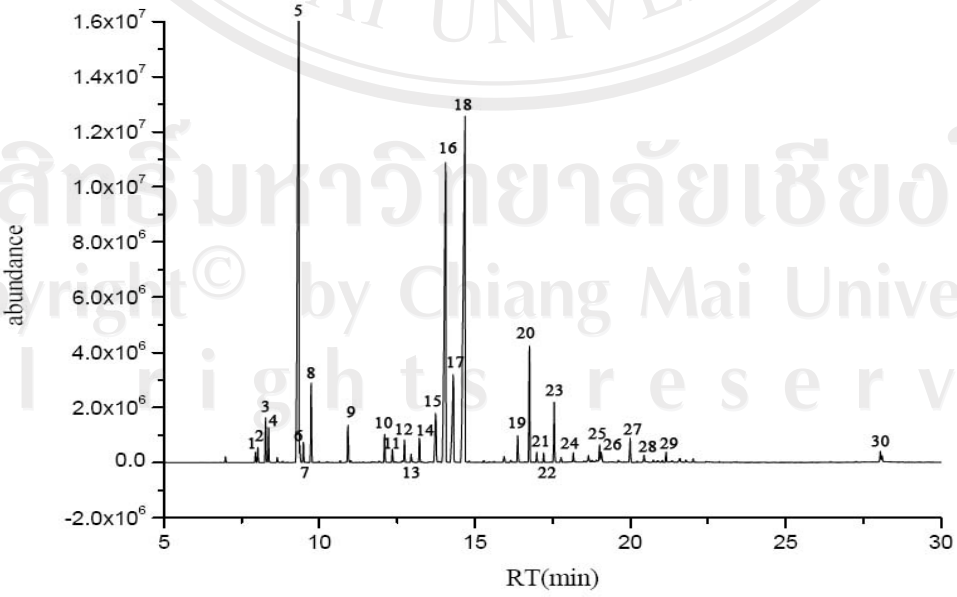


Figure 4.8 Total ion chromatogram of *C. aurantifolia* essential oil

Table 4.18 Chemical composition of the essential oil of *C. aurantifolia*

Peak No.	Compound	R.T (min)	%RA
1	Sabinene	7.946	0.32
2	β-Pinene	8.017	0.47
3	Methylheptenone	8.267	1.37
4	β-Myrcene	8.367	1.03
5	Limonene	9.335	29.49
6	Zineol	9.371	0.37
7	*cis*-Ocimene	9.49	0.58
8	*trans*-Ocimene	9.733	2.41
9	Linalool	10.921	1.18
10	Citronellal	12.103	0.89
11	α-Terpineol	12.952	0.28
12	Decanal	13.219	0.76
13	Neral	14.062	18.57
14	Geraniol	14.306	4.69
15	Geranial	14.686	23.98
16	Neryl acetate	16.384	0.75
17	Geranyl acetate	16.764	3.86
18	β-Elemene	17.002	0.33
19	Tridecanal	17.222	0.30
20	Caryophyllene	17.56	2.00
21	α-Humulene	18.172	0.30
22	α-Farnesene	19.015	0.50
23	β-Bisabolene	19.068	0.42
24	Germacrene B	20.001	0.82
25	Caryophyllene oxide	20.446	0.26
26	Spathulenol	21.159	0.34
27	Phytolisomer	28.053	0.25

RT, retention time
RA, retention area (peak area relative to total peak area)

5.5 Refractive index and Relative optical rotation of the essential oil

Refractive index of the oil was determined using a hand held refractometer (ATAGO N3, Atago Co. Ltd., Japan) at 28 °C. Results are shown in **Table 4.19**.

The optical rotation was measured with a Jasco Dip-370 Digital Polarimeter using sodium lamp as light source. The length of the Polarimeter cell was 1.0 dm and sample tube was 100 mn. About 0.1250 g of the essential oil was accurately weighed into volumetric flask and then the volume was made up to 25 mL with dichloromethane. The optical rotation of the essential oil (0.5g $100mL^{-1}$) was measured with Polarimeter at 29 °C. Results are shown in Table 4.19.

Table 4.19 Refractive index and relative optical rotation of the essential oil

The essential oil	Refractive index[a]	Relative optical rotation[b] (°C)
C. hystrix	1.4541	+54
F. limonia	1.4691	+26
A.marmelos	1.4750	+146
C.aurantifolia	1.4818	-56

[a] Refractive index was determined using a hand held refractometer at 28 °C
[b] The optical rotation was measured with Polarimeter at 29 °C

The essential oil from the leaves of each plant were isolated by hydrodistillation and analysed by using gas chromatography-mass spectrometry (GC-MS). The major constituents of *C. hystrix* leaves were citronellol, β-citronellal, citronellyl acetate and linalool (77.74, 10.48, 2.56 and 2.25% respectively). The major constituents of *F. limonia* leaves were sabinene (64.80%), 4-terpineol (10.32%), γ-terpinene (5.89%) and α-terpinene (3.73%). The major constituents of *A. marmelos* leaves were limonene, α-phellandrene and *trans*-caryophyllene (44.85, 25.15 and 8.22% respectively). The major constituents of *C. aurantifolia* leaves were limonene (29.49%), geranial (23.98%), neral (18.57%), geraniol (4.69%). Most of these compounds are used as a fragrance in the food, perfumery and cosmetic industries. Some can be used in medicine and aromatherapy. Citronellal, β-citronellol and β-myrcene are used extensively as a source of perfumery chemicals. But they are also highly valued as the intermediates for the preparation of flavor and fragrance chemicals. Citronellyl acetate is used in flavor and fragrance chemicals. Citronellyl acetate is used in floral perfumes (lavender, rose, lily and lergamot). Linalool is a naturally qecurring terpene alcohol. It is used as a scent in domestic products such as soap, detergent, shampoo and lotion, it is also used as a chemical intermediate. Trans-

caryophyllene is a natural bicyclic sesquiterpene. It is an FDA approved food additive and ingested dialy with food, it is the first, dietary cannabinoid. Isopulegol is a monocyclic monoterpene alcohol. It is a fragrance ingredient used in decorative cosmetics, fine fragrance, shampoos, toilet soap, and other toiletries as well as in non-cosmetic product such as household cleaners and detergents. Geraniol is a monoterpene alcohol. It is used in flavors and can be used in antitumoral therapy. Germacrene-D is a sesquiterpene which possesses antimicrobial and insecticidal properties, through they also play a role as insect pheromones. Alpha-humulene and caryophellene are sesquiterpene which possess anti-inflammatory properties. Nerolidol is used as a flavoring agent and in perfumery, soap, detergent, beauty care product and household product. It is an antiulcer (94) and also possesses antileishmanial activity. It is also currently under testing as a skin penetration enhancer for the transdermal delivery of therapeutic drugs (95). Bicyclogermacrene is a sesquiterpene hydrocarbon. It possesses antimicrobial and insecticidal properties. Alpha-cadinol is a bicyclic sesquiterpene. It is used as antibacterian against anaerobic bacteria such as streptococcus mutans. It may be incorporated into foodstuff, chewing gum, dentifrice or mouthwash. Phytol is an acyclic diterpene alcohol. It is a precursor for vitamins E and K.

6. Analysis of the mineral metal ions

The mineral metals ion in the leaves of *C. hystrix, F. limonia*, *A. marmelos* and *C. aurantifolia* were determined by atomic absorption spectrophotometry (AAS) after wet digestion with mixed acid. They were calcium, manganese and zinc present in young and old leaves of Leech lime, Wood apple, Bael-fruit and Lime. In addition, there were 4 mineral metals calcium, iron, magnesium and sodium present in the leaves. Results are shown in Table 4.20 and Figure 4.9-4.15.

Young and old leaves of each plant possessed the highest content of magnesium followed by calcium. Magnesium is an essential mineral for human nutrition. Excess magnesium in the blood is freely filtered at the kidneys, and for this reason it is difficult to overdose on magnesium from dietary sources alone. Calcium plays an important role in building stronger, denser bones early in life and keeping

bones strong and healthy later in life. The human body cannot produce calcium on its own. That is why it is important to try to get enough calcium through the foods we eat. When the diet does not have enough calcium for our body's needs, calcium is taken from the bones. Therefore these mineral metal ions play an important role for human health.

Table 4.20 Determination of mineral metal ions in *C. hystrix, F. limonia*, *A. marmelos*, *C. aurantifolia* leaves by AAS

Sample	Amount of mineral metal ions found (µg/g)						
	Ca	**Mg**	**Na**	**Fe**	**Mn**	**Zn**	**Cu**
C. hystrix young leaves	16.43	89.95	3.55	1.48	0.19	0.44	0.03
C. hystrix old leaves	33.91	96.68	3.94	2.65	0.22	0.45	0.08
F. limonia young leaves	12.04	90.63	2.30	2.67	0.67	0.78	0.14
F. limonia old leaves	30.69	96.72	1.88	3.06	0.89	0.75	0.07
A. marmelos young leaves	12.15	154.24	2.07	2.22	0.24	0.56	0.06
A. marmelos old leaves	41.92	128.63	2.21	1.96	0.46	0.88	0.05
C. aurantifolia young leaves	7.67	89.43	1.32	3.32	0.38	0.56	0.08
C. aurantifolia old leaves	25.17	85.64	5.18	5.45	0.32	0.54	0.05

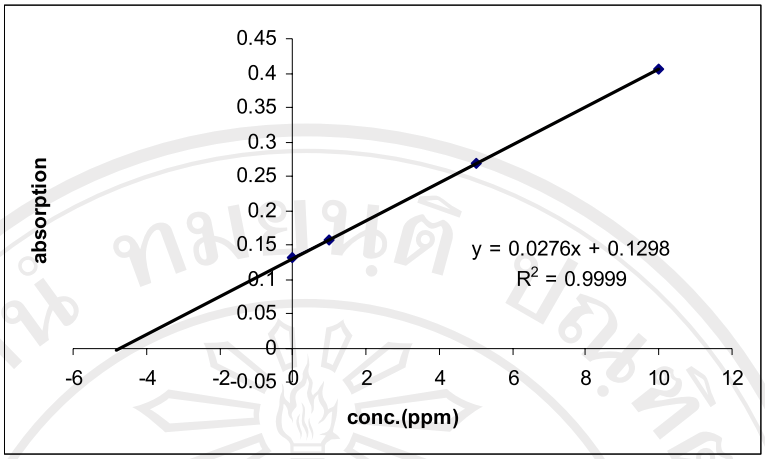


Figure 4.9 Standard addition curve for determination of calcium in old *C. hystrix* leaves

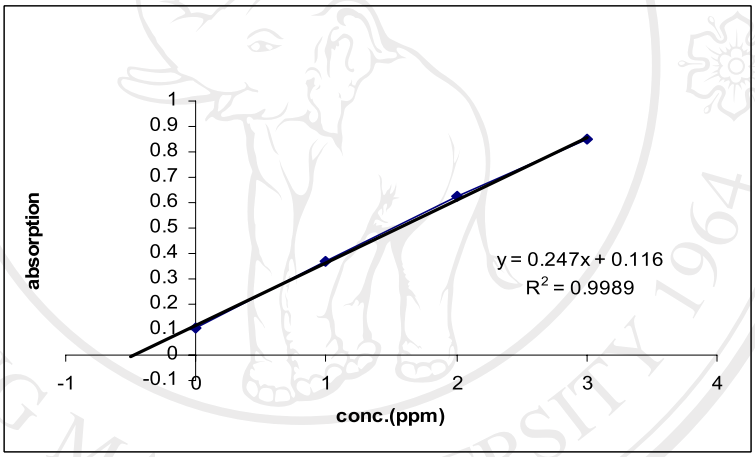


Figure 4.10 Standard addition curve for determination of sodium in young *A. marmelos* leaves

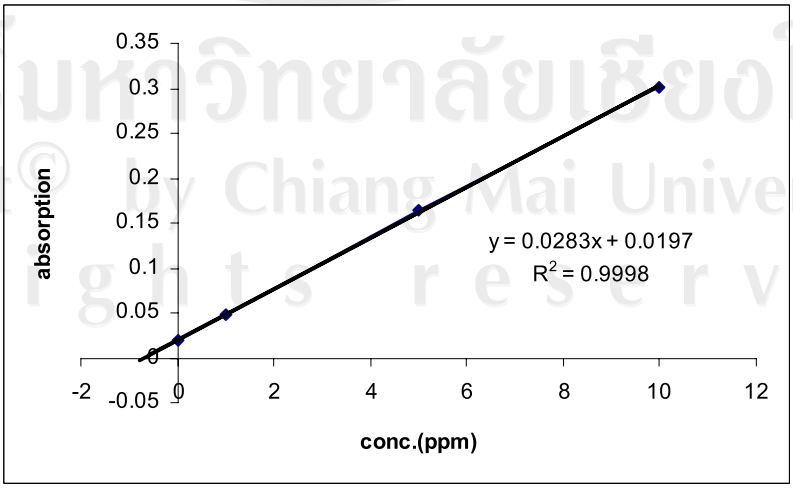


Figure 4.11 Standard addition curve for determination of iron in old *F. limonia* leaves

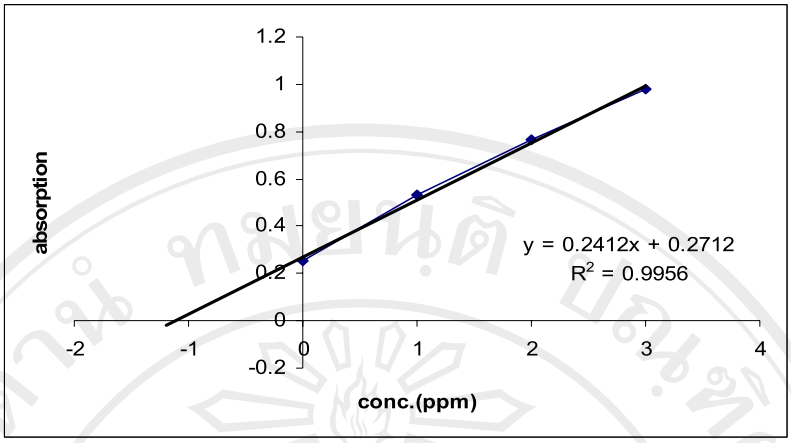


Figure 4.12 Standard addition curve for determination of magnesium in young *C. hystrix* leaves

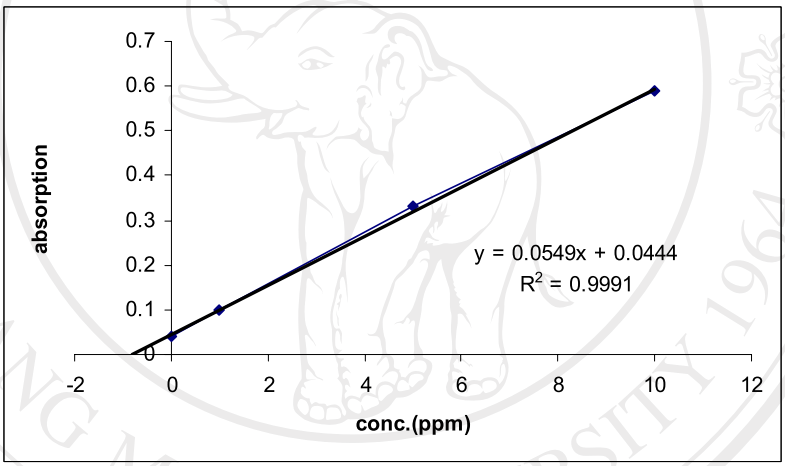


Figure 4.13 Standard addition curve for determination of manganese in old *C. aurantifolia* leaves

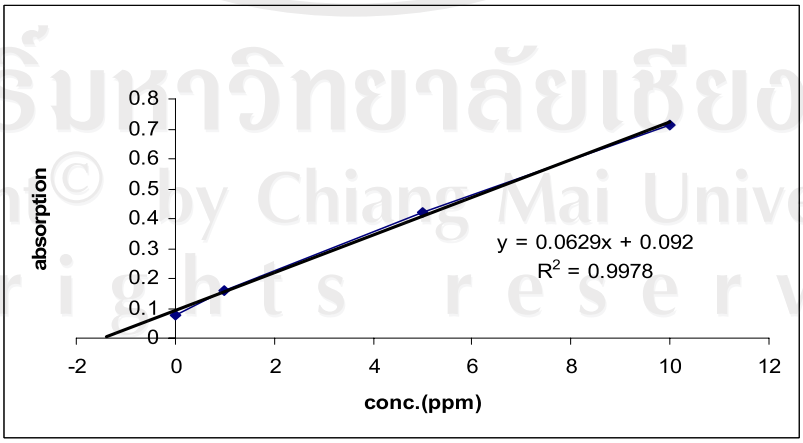


Figure 4.14 Standard addition curve for determination of zinc in old *A. marmelos* leaves

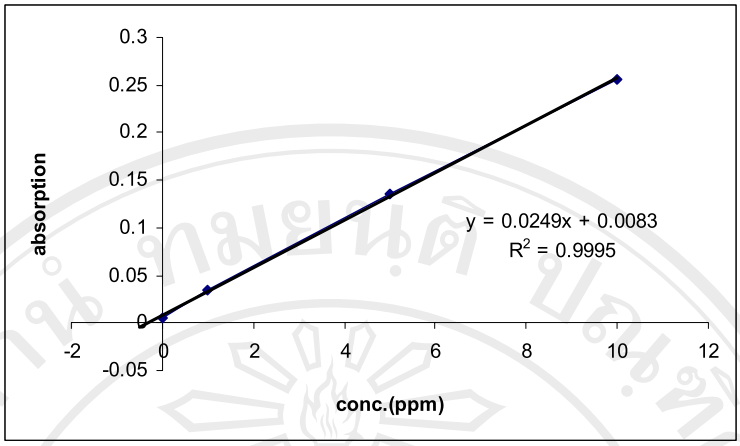


Figure 4.15 Standard addition curve for determination of copper in young *C. aurantifolia* leaves

7. Isolation of *C. hystrix* constituent

7.1 Isolation of CHAP (white crystalline 0.0325 g)

CHAP 0.0325 g was dissolved in chloroform and analysed by means of GC and GC-MS. Identification of CHAP fraction constituents was performed by comparison of mass spectra with the literature data (NIST, wiley7n.l), that yielded < 90% matches were identified as unknowns. Three compounds were identified and are listed in Table 4.21. A typical total ion chromatogram is presented in Figure 4.16.

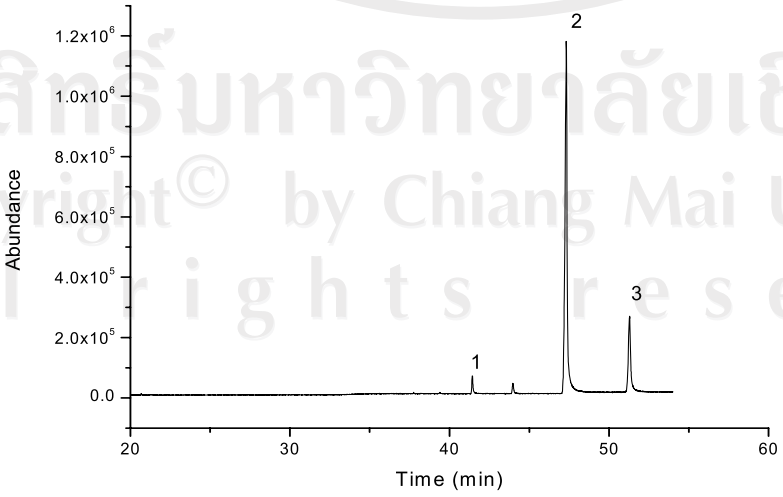


Figure 4.16 Total ion chromatogram of CHAP fraction

Table 4.21 Chemical composition of CHAP fraction

Peak No.	Compound	R.T (min)	%RA	MW
1	unidentified	41.41	2.18	-
2	Hentriacontane	47.28	78.51	436.50
3	Dotriacontane	51.29	17.83	450.52

7.2 Isolation of CHC4GP (white crystalline 5 mg)

CHC4GP 5 mg was dissolved in chloroform and analysed by means of GC and GC-MS. Identification of CHC4GP fraction constituents was performed by comparison of mass spectra with literature data (NIST, wiley7n.l), that yielded < 90% matches were identified as unknowns. Three compounds were identified and are listed in Table 4.22. A typical total ion chromatogram is presented in Figure 4.17.

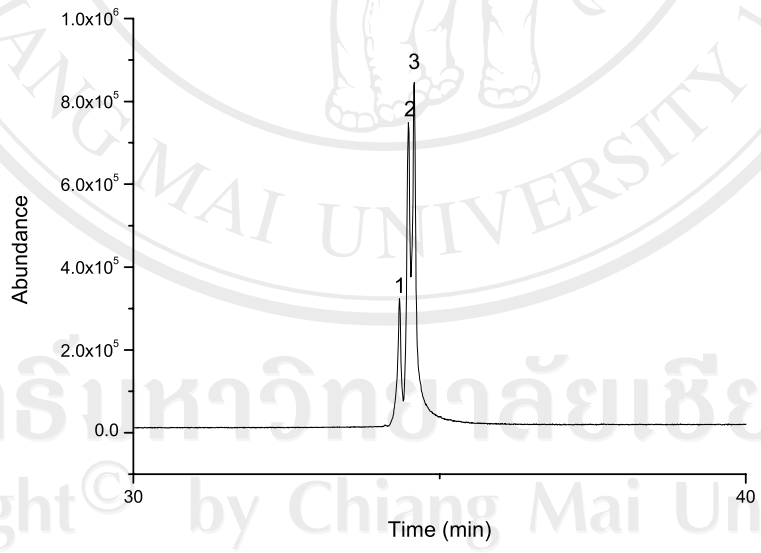


Figure 4.17 Total ion chromatogram of CHC4GP fraction

Table 4.22 Chemical composition of CHC4GP fraction

Peak No.	Compound	R.T (min)	%RA	MW
1	Tetratriacontane	34.34	15.87	478.55
2	Pentatriacontane	34.50	34.50	492.56
3	Hexatriacontane	34.59	49.63	506.58

7.3 Isolation of CHH3G (yellow sticky 2 mg)

The fraction was analysed by means of GC and GC-MS. Identification of CHH3G fraction constituents was performed by comparison of the mass spectra with literature data (NIST, wiley7n.l), that yielded < 90% matches were identified as unknowns. Three compounds were identified and are listed in Table 4.23. A typical total ion chromatogram is presented in Figure 4.18.

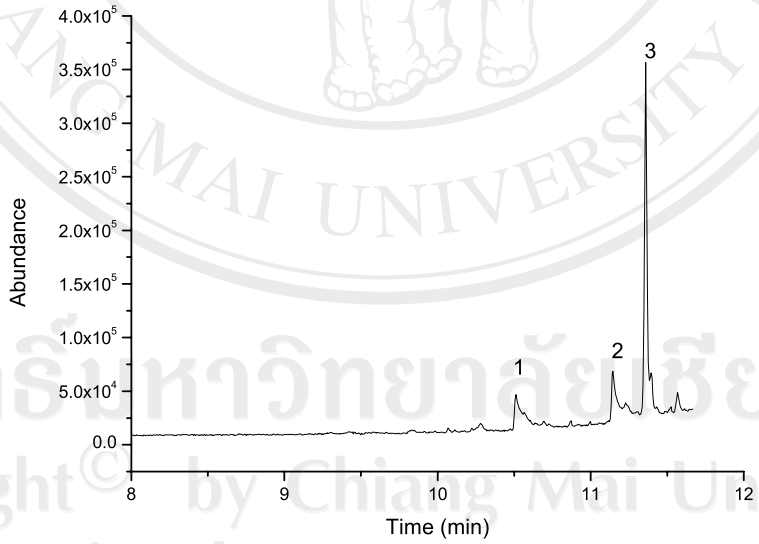


Figure 4.18 Total ion chromatogram of CHH3G fraction

Table 4.23 Chemical composition of CHH3G fraction

Peak No.	Compound	R.T (min)	%RA	MW
1	Hexadecanoic acid	10.51	15.22	256.24
2	Octadecanoic acid	11.15	15.89	284.72
3	Unidentified	11.36	60.14	-

7.4 Isolation of CDH (yellow sticky liquid 0.2273 g)

The fraction was analysed by means of GC and GC-MS. Identification of CDH fraction constituents was performed by comparison of the mass spectra with literature data (NIST, wiley7n.l), that yielded < 90% matches were identified as unknowns. Three compounds were identified and are listed in Table 4.24. A typical total ion chromatogram is presented in Figure 4.19.

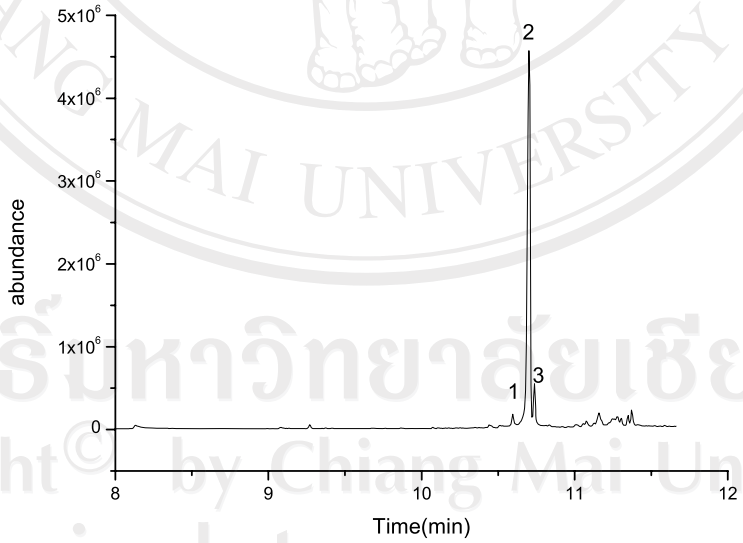


Figure 4.19 Total ion chromatogram of CDH fraction

Table 4.24 Chemical composition of CDH fraction

Peak No.	Compound	R.T (min)	%RA	MW
1	Ethyl palmitate	10.60	2.71	284.27
2	Unidentified	10.70	78.80	-
3	Unidentified	10.74	6.39	-

7.5 Isolation of CDO6B (0.0022 g)

The fraction was analysed by means of GC and GC-MS. Identification of CDO6B fraction constituents was performed by comparison of the mass spectra with literature data (NIST, wiley7n.l), that yielded < 90% matches were identified as unknowns. Five compounds were obtained, but only one compound was identified. Results are listed in Table 4.25. A typical total ion chromatogram is presented in Figure 4.20.

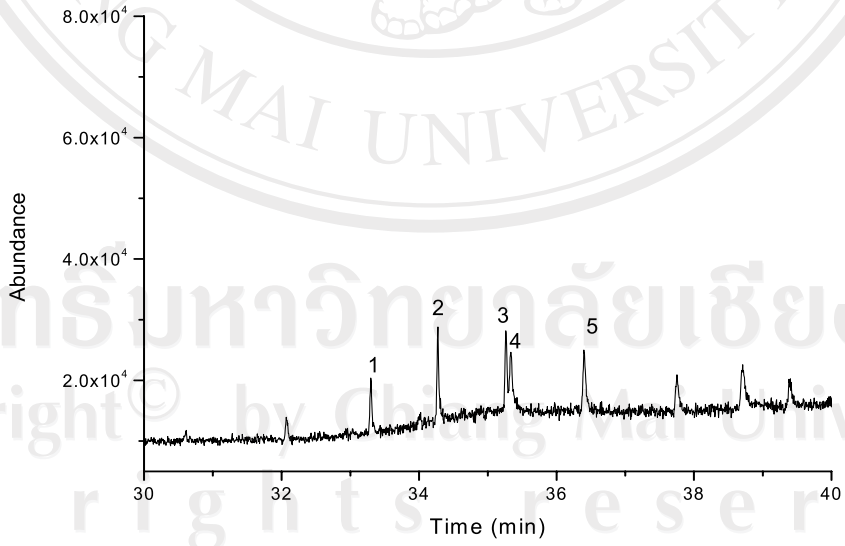


Figure 4.20 Total ion chromatogram of CDO6B fraction

Table 4.25 Chemical composition of CDO6B fraction

Peak No.	Compound	R.T (min)	%RA	MW
1	Unidentified	33.30	7.22	-
2	Unidentified	34.27	12.10	-
3	Eicosane	35.2	11.18	282.33
4	Unidentified	35.34	14.04	-
5	Unidentified	36.40	10.87	-

8. Isolation of *F. limonia* Constituent

8.1 Isolation of FAK1 (yellow sticky 0.0122 g)

The fraction was analysed by means of GC and GC-MS. Identification of fraction constituents was performed by comparison of the mass spectra with literature data (NIST, wiley7n.l), that yielded < 90% matches were identified as unknowns. Fourth compounds were identified and are listed in Table 4.26. A typical total ion chromatogram is presented in Figure 4.21.

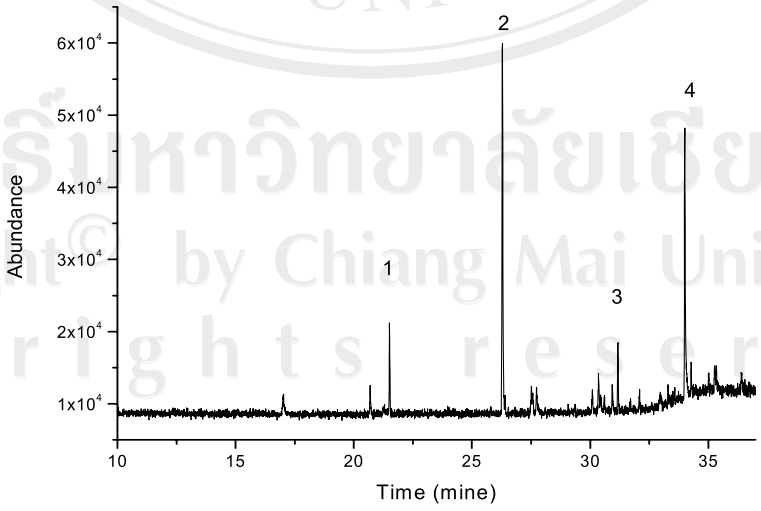


Figure 4.21 Total ion chromatogram of FAK1fraction

Table 4.26 Chemical composition of FAK1 fraction

Peak No.	Compound	R.T (min)	%RA	MW
1	Unidentified	21.51	8.75	-
2	2-Pentadecanone, 6,10,14-trimethyl	26.29	45.40	268.28
3	Unidentified	31.17	8.68	-
4	Hexanedioic acid, bis(2-ethylhexyl) ester	34.00	37.17	370.31

8.2 Isolation of FALP (white needles 12.2 mg)

FALP 12.2 mg was dissolved in dichloromethane and analysed by means of GC and GC-MS. The constituents were identified by comparison of the mass spectra with literature data (NIST, wiley7n.l), that yielded < 90% matches were identified as unknowns. Two compounds were obtained, but only one compound was identified as shown in Table 4.27. A typical total ion chromatogram is presented in Figure 4.22.

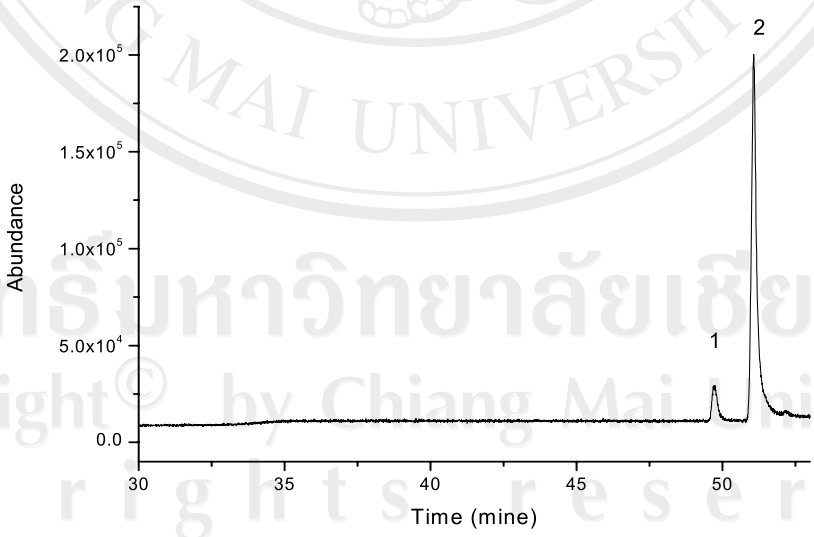


Figure 4.22 Total ion chromatogram of FALP fraction

Table 4.27 Chemical composition of FALP fraction

Peak No.	Compound	R.T (min)	%RA	MW
1	Unidentified	49.715	8.82	-
2	Stigmasterol	51.07	91.18	91.18

The isolated FALP compounds were characterized by NMR spectroscopy. The ^{1}H NMR and ^{13}C NMR spectra of FALP were analysed using a Bruker AVANCE 400 NMR spectrometer, operating at 400 and 100 MHz in deuterated chloroform ($CDDl_3$), respectively. The chemical shifts were recorded in ppm by reference to TMS signal.

In the ^{1}H NMR spectrum, the olefinic protons at δ 5.02 (1H, dd, J=15.2, 8.6 Hz) and δ 5.15 (1H, dd, J=15.2, 8.7 Hz) were assigned to H-22 and H-23 of stigmasterol and δ 5.35 (2H, d, J=5.18 Hz) was assigned to H-6 of both β-sitosterol and stigmasterol. The multiplet at δ 3.52 (1H) was assigned to the H-3 of both compound. Methyl groups of stigmasterol was deduced from the resonances at δ 0.69 (s), δ 1.01 (s). Due to the similarity in the structure of compound FALP, the methyl and methylene resonances for β-sitosterol were overlapped with those of stigmasterol. ^{13}C NMR analysis indicated the presence at δ 140.75 (C-5), 121.70 (C-6), 71.71 (C-3), 50.15 (C-9), 42.30 (C-13), 37.25 (C-1), 36.51 (C-10), 31.66 (C-2 and C-7), 21.20 (C-11), 19.39 (C-19) data as shown in Table 4.28. The signal corresponding to the molecular formula of $C_{29}H_{48}O$ (stigmasterol) and $C_{29}H_{50}O$ (β-sitosterol), which was identified by comparison with those published (96-98).

The final structural confirmation was accomplished by comparison of ^{1}H NMR and ^{13}C NMR spectral data with the previous reported in the literature allowing for assignment of the structure to that of mixture consisting of stigmasterol and β-sitosterol as shown in Figure 4.23-4.34.

Table 4.28 ^{1}H-NMR of compound FALP and reference

Position	Compound FALP	β-Sitosterol [96]	Stigmasterol[97-98]
	δ ^{1}Ha (Multiplicity, *J* in Hz)	δ ^{1}H (Multiplicity, *J* in Hz)	δ ^{1}H (Multiplicity, *J* in Hz)
1			
2			
3	3.53 (m)	3.56 (m)	3.52 (m)
4			
5			
6	5.35 (2H,d,J=5.18 Hz)	5.39 (m)	5.36 (d,J=5.1 Hz)
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18	0.69 (s)	0.72 (s)	0.695 (s)
19	1.01 (s)	1.05 (s)	1.01 (s)
20			
21		0.96 (d, J=6.5 Hz)	1.02 (d, J=6.4 Hz)
22	5.15 (1H,dd,J=15.2,8.7 Hz)		5.16 (dd,J=15.0,8.4 Hz)
23	5.02 (1H,dd,J=15.2,8.6 Hz)		5.02 (dd,J=15.0,8.4 Hz)
24			
25			
26		0.87 (d, J=6.7 Hz)	0.852 (d, J=8.0 Hz)
27		0.85 (d, J=6.7 Hz)	0.835 (d, J=5.3 Hz)
28			
29		0.89 (t, J=7.4 Hz)	0.82 (t, J=6.0 Hz)

Table 4.29 ^{13}C-NMR of compound FALP and reference

Position	Compound FALP δ^{13}C of		Stigmasterol[99] δ^{13}C of	
	β-Sitosterol	Stigmasterol	β-Sitosterol	Stigmasterol
1*	37.25		37.21	
2*	31.66		31.62	
3	71.80		71.80	
4*	42.21	42.30	42.18	42.30*
5*	140.75		140.72	
6*	121.7		121.71	
7*	31.66		31.87	
8*	31.89		31.87	
9*	50.15		50.08	
10*	36.51		36.48	
11*	21.20		21.07	
12	39.77	39.67	39.77	39.64
13*	42.30		42.29	
14	56.76	56.86	56.76	56.83
15	24.28	24.35	24.28	24.34
16	28.22	28.91	28.22	28.92
17	56.05	55.95	56.05	55.90
18	11.85	12.04	11.85	12.03
19	19.39		19.38	
20	36.14	40.48	36.14	40.50
21	18.91	21.07	18.91	21.05
22	33.71	138.30	33.71	138.32
23	25.78	129.27	25.78	129.22
24	45.83	51.23	45.83	51.21
25	29.15	31.89	29.15	31.87*
26	19.18	21.07	19.18	21.20
27	19.02	18.97	19.02	18.95
28	23.06	25.04	23.06	25.40
29	11.97	12.24	11.97	12.25*

* The carbons with signals overlapping either the same carbon of the two phytosterols (carbon nos. 1–3, 5–11, 13 and 19) or of different carbons (carbon nos. 4 and 25 of stigmasterol with carbon nos. 13 and 7 + 8 of both phytosterols, respectively).

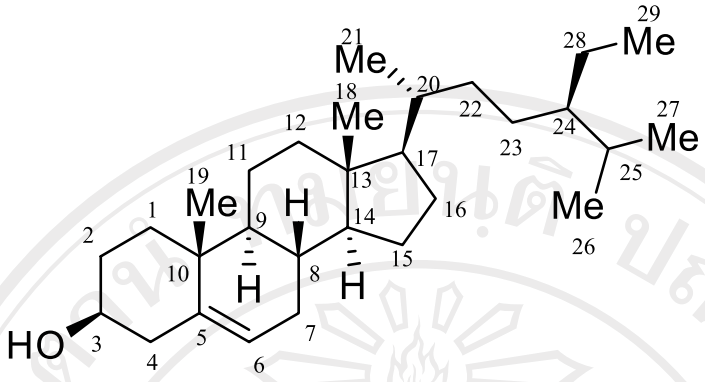


Figure 4.23 Chemical structure of β-sitosterol

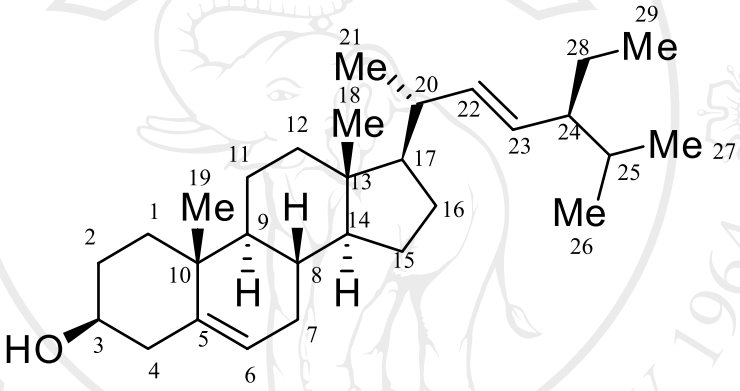


Figure 4.24 Chemical structure of stigmasterol