CHAPTER III

RESULTS AND DISCUSSION

Three species of Euphorbiaceae weeds were selected for preliminary screening on rice growth inhibition activity.

3.1 The Results of Extraction.

The air-dried samples were milled to course powder and extracted with organic solvent according to the procedure described in Chapter II . The results of extraction are shown in table 3.1

Table 3.1 Yield of crude extract by various solvent of studied species.

Plant	Plant part	Weight of Plant (g)	solvent	Yield (g)
E. hirta	aerial	1,000	hexane	10.71
			ethanol	83.20
			dichloromethane	7.30
			ethyl acetate	0.48
			butanol	0.44
			residue	49.79
E. heterophylla	leaves	1,000	hexane	17.50
			ethanol	121.91
			dichloromethane	24.12
			ethyl acetate	0.91
			butanol	7.33

Table 3.1 (Cont.)

Plant	Plant part	Weight of Plant (g)	solvent	Weight of crude extract(g)
			residue	40.17
E. heterophylla	stem	1,000	hexane	21.26
			ethanol	67.27
			dichloromethane	6.41
			ethylacetate	0.91
			butanol	1.71
			residue	8.26
E. heterophylla	root	500	hexane	6.75
			ethanol	16.93
			dichloromethane	1.46
			ethylacetate	0.11
			butanol	2.56
			residue	9.82
E. thymifolia	aerial	1,000	hexane	25.32
			ethanol	130.22
			dichloromethane	12.31
			ethylacetate	2.88
			butanol	5.75
			residue	20.94

3.2 The Results of Biological Activity Screening Tests with Carcinoma Cell lines

3.2.1 Ethanolic Crude Extract

The preliminary screening test of the ethanolic crude extract of *E. hirta* and *E. heterophylla* on seven carcinoma cell lines were presented in Tables 3.2 and 3.3.

Table 3.2 Inhibitory effect of ethanolic crude extract of *E. hirta* on carcinoma cell lines

Cell lines	Concentration (μg/ml)			Estimation
	1	10	100	
BEL-7420	10.72	0.66	17.91	-
BGC-823	15.51	16.57	51.51	+
HCT-8	-16.68	13.72	60.13	+
HL-60	-1.38	-4.31	24.57	-
KB	18.71	27.54	56.52	+
B	14.52	25.24	70.46	+
T	4.66	24.92	44.98	-

Note : BEL-7402 Human Hepatocellular Carcinoma

BGC-823 Human Gastric Carcinoma

HCT-8 Human Colon Carcinoma

HL-60 Human Leukemia Carcinoma

KB Human Nasopharyngeal Carcinoma

B Proliferation of Mouse (B) Lymphocyte

T Proliferation of Mouse (T) Lymphocyte

Table 3.3 Inhibitory effect of ethanolic crude extract of *E. heterophylla* on carcinoma cell lines

Cell lines	Concentration (μg/ml)			Estimation
	1	10	100	
BEL-7420	8.46	-4.10	6.40	-
BGC-823	2.26	7.19	18.37	-
HCT-8	-4.57	33.33	59.47	+
HL-60	2.99	-4.97	46.81	-
KB	26.88	28.85	15.15	-
B	20.46	34.48	62.21	+
T	4.66	24.92	44.98	-

Note : BEL-7402 Human Hepatocellular Carcinoma

BGC-823 Human Gastric Carcinoma

HCT-8 Human Colon Carcinoma

HL-60 Human Leukemia Carcinoma

KB Human Nasopharyngeal Carcinoma

B Proliferation of Mouse (B) Lymphocyte

T Proliferation of Mouse (T) Lymphocyte

The ethanolic crude extract of both *E. hirta* and *E. heterophylla* showed activity against some carcinoma cell line (table 3.1 and 3.2). Then they were further studies by extracting with various solvent, then test with Human Hepatocellular Carcinoma.

3.2.2 Various Crude Extract on Human Hepatocellular Carcinoma

The extracts from various solvent were tested with Human Hepatocellular Carcinoma and the results was showed in Tables 3.4-3.9.

Table 3.4 Percent inhibition of Human Hepatocellular Carcinoma (Bel-7402)

Plant	Solvent	Concentration (μg/ml)			Estimation
		1	10	100	
Euphorbia hirta	CH_2Cl_2	-14.66	-15.88	-13.54	-
	EtOAc	-7.63	-5.19	-5.60	-
	BuOH	-15.37	-10.18	-12.42	-
E. heterophylla	CH_2Cl_2	-8.45	3.56	54.88	+
	EtOAc	-11.50	8.96	61.96	+
	BuOH	-16.29	-6.21	22.19	-
	H_2O	-21.78	-16.15	19.70	-

Table 3.5 Percentage inhibition of Human Nasopharyngeal Carcinoma (KB)

Plant	Solvent	Concentration (μg/ml)			Estimation
		1	10	100	
Euphorbia hirta	CH_2Cl_2	-2.74	-4.03	7.03	-
	EtOAc	-2.79	-4.65	-2.84	-
	BuOH	-6.36	-3.51	-0.05	-
E. heterophylla	CH_2Cl_2	-8.98	-9.21	43.60	-
	EtOAc	-6.80	3.76	49.41	-
	BuOH	-5.52	-2.52	25.65	-
	H_2O	-9.57	-0.29	15.08	-

Table 3.6 Percentage inhibition of Human Gastric Carcinoma (BGC-823)

Plant	Solvent	Concentration (μg/ml)			Estimation
		1	10	100	
Euphorbia hirta	CH_2Cl_2	-26.26	-21.81	-12.50	-
	EtOAc	7.96	-5.63	-9.90	-
	BuOH	-9.51	-13.79	-7.38	-
E. heterophylla	CH_2Cl_2	3.11	-1.36	17.09	-
	EtOAc	-26.83	-30.49	-9.76	-
	BuOH	-3.073	-19.02	-22.20	-
	H_2O	-30.00	-28.29	0.73	-

Table 3.7 Percentage inhibition of Human Leukemia Carcinoma (HL-60)

Plant	Solvent	Concentration (μg/ml)			Estimation
		1	10	100	
Euphorbia hirta	CH_2Cl_2	-9.90	-16.30	-13.70	-
	EtOAc	-7.20	-12.60	-3.30	-
	BuOH	0.64	-12.80	2.30	-
E. heterophylla	CH_2Cl_2	4.48	3.00	27.10	-
	EtOAc	-1.50	-6.20	19.40	-
	BuOH	-20.30	-15.80	9.30	-
	H_2O	-32.34	-22.63	-14.26	-

Table 3.8 Percentage inhibition of Human Colon Carcinoma (HCT-8)

Plant	Solvent	Concentration (μg/ml)			Estimation
		1	10	100	
Euphorbia hirta	CH_2Cl_2	-22.36	4.92	4.21	-
	EtOAc	-15.36	6.28	10.32	-
	BuOH	-3.79	-2.43	-2.72	-
E. heterophylla	CH_2Cl_2	5.69	5.33	6.70	-
	EtOAc	4.44	-11.15	-0.29	-
	BuOH	-3.73	-6.04	-30.24	-
	H_2O	12.04	9.04	15.07	-

Table 3.9 Percentage inhibition of Human Erythroleukemia Carcinoma (K-562)

Plant	Solvent	Concentration (μg/ml)			Estimation
		1	10	100	
Euphorbia hirta	CH_2Cl_2	8.95	13.65	57.81	+
	EtOAc	13.65	18.15	47.99	-
	BuOH	12.84	7.59	40.95	-
E. heterophylla	CH_2Cl_2	13.31	13.68	58.98	+
	EtOAc	16.27	14.16	69.97	+
	BuOH	-3.80	6.34	92.60	+
	H_2O	9.09	74.63	82.24	++

Almost of all solvent of *E. hirta* showed negative inhibitory effect on all carcinoma cell lines, except Human Erythroleukemia carcinoma (K-562). The inhibitory effect of dichloromethane, ethyl acetate and butanol extract show strong effect at higher concentration. But only dichloromethane extract showed more than 50% inhibitory effect (+ estimation) (Table 3.9).

All various solvent extract of *E. heterophylla* showed positive inhibitory effect on almost all carcinoma cell lines. The inhibitory effect is increasing by the higher concentration. But only dichloromethane and ethyl acetate extract showed more than 50% inhibitory effect on Human Hepatocellular carcinoma. And Erythroleukemia carcinoma was positive estimation by all solvent extracts of *E. heterophylla*.

3.3 The Results of Preliminary Rice Growth Inhibition Bioassay

Each crude extract was preliminary screened for rice growth inhibition activity according to the procedure described in Chapter II. The bioassay results are presented in Table 3.10 and Fig. 3.1 and Fig. 3.2

Table 3.10 Preliminary test of the Crude Extract at 0.1 , 0.5 and 1.0 g per solvent 3 ml. on root and leaf sheath of rice (*Oryza sativa* cv RD 23)

Plant	plant part	solvent	rice part	%Inhibition of difference concentration*		
				1.0	0.5	0.1
E. hirta	aerial	hexane	root	80.35	72.46	42.11
			leaf	-9.24	-35.33	-28.80
		ethanol	root	87.34	70.23	50.66
			leaf	78.84	89.93	30.51
E. heterophylla	stem	hexane	root	51.61	38.77	23.26
			leaf	-16.30	-2.72	-28.26
		ethanol	root	100	-	95.30
			leaf	100	-	-176.9
E. heterophylla	leaf	hexane	root	48.80	53.21	30.75
			leaf	28.26	-17.93	-11.41
		ethanol	root	-	-	89.11
			leaf	-	-	-107.6
E. heterophylla	root	hexane	root	84.49	47.46	4.01
			leaf	-22.83	-16.85	-33.69
		ethanol	root	-	-	80.69
			leaf	-	-	-184.6
E thymifolia	aerial	hexane	root	60.23	36.24	20.57
			leaf	-3.53	-10.98	-25.56
		ethanol	root	-	-16.68	-24.53
			leaf	-	-19.37	-36.85

* g/3ml

Note : - rice seeds have a fungi over and the growth cannot be determined.

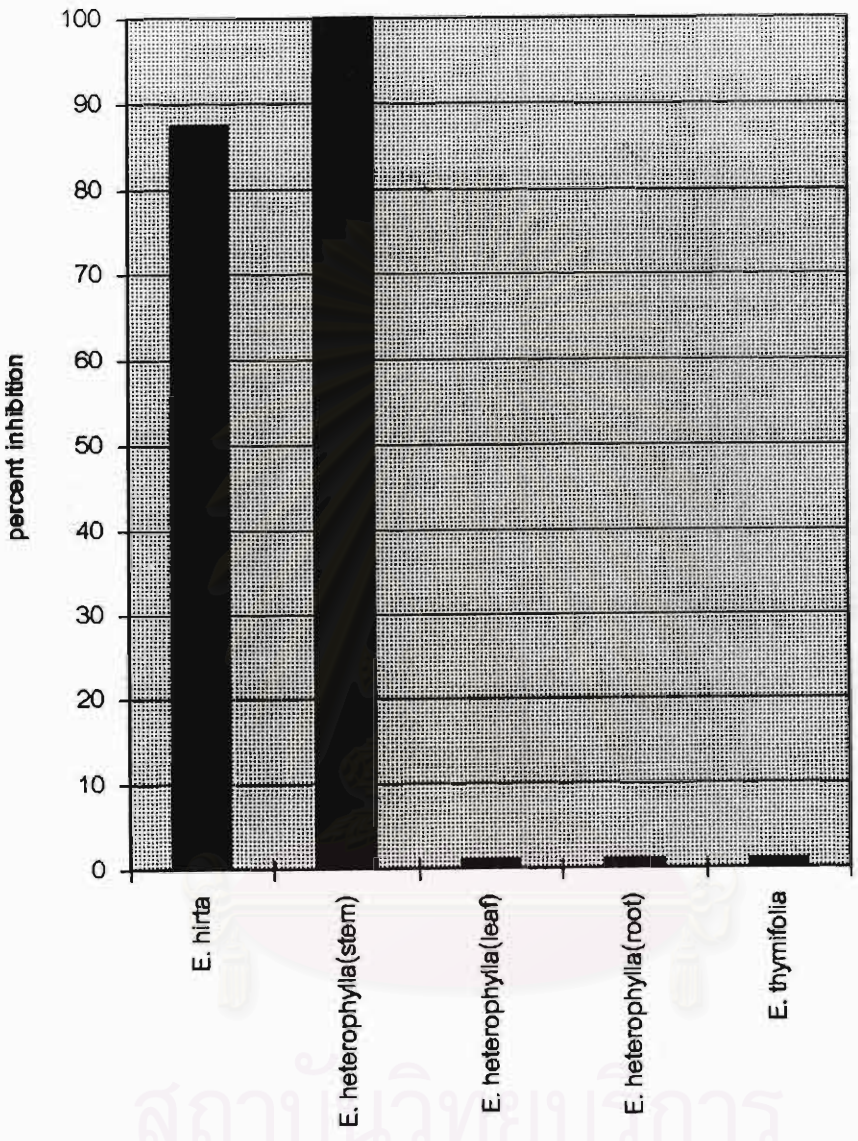


Fig 3.1 Inhibitory effect of ethanolic crude extract on rice root growth (at 1.0 g/3 ml)

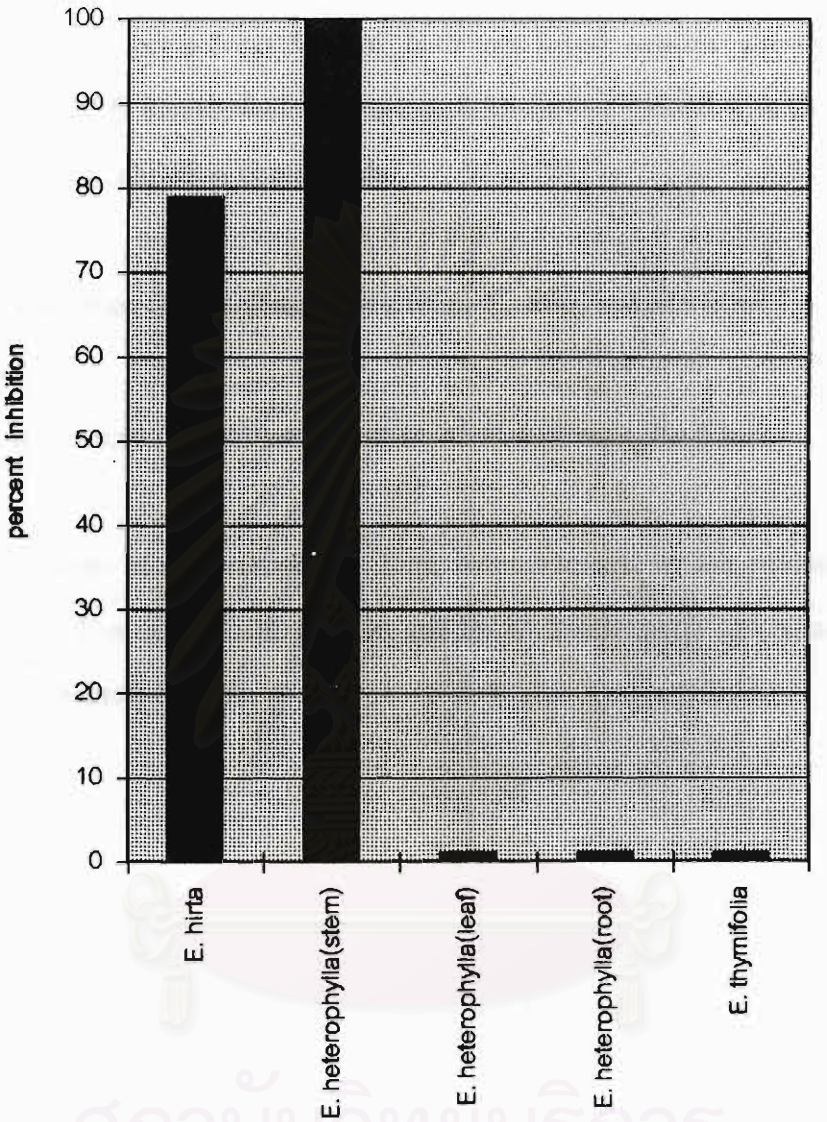


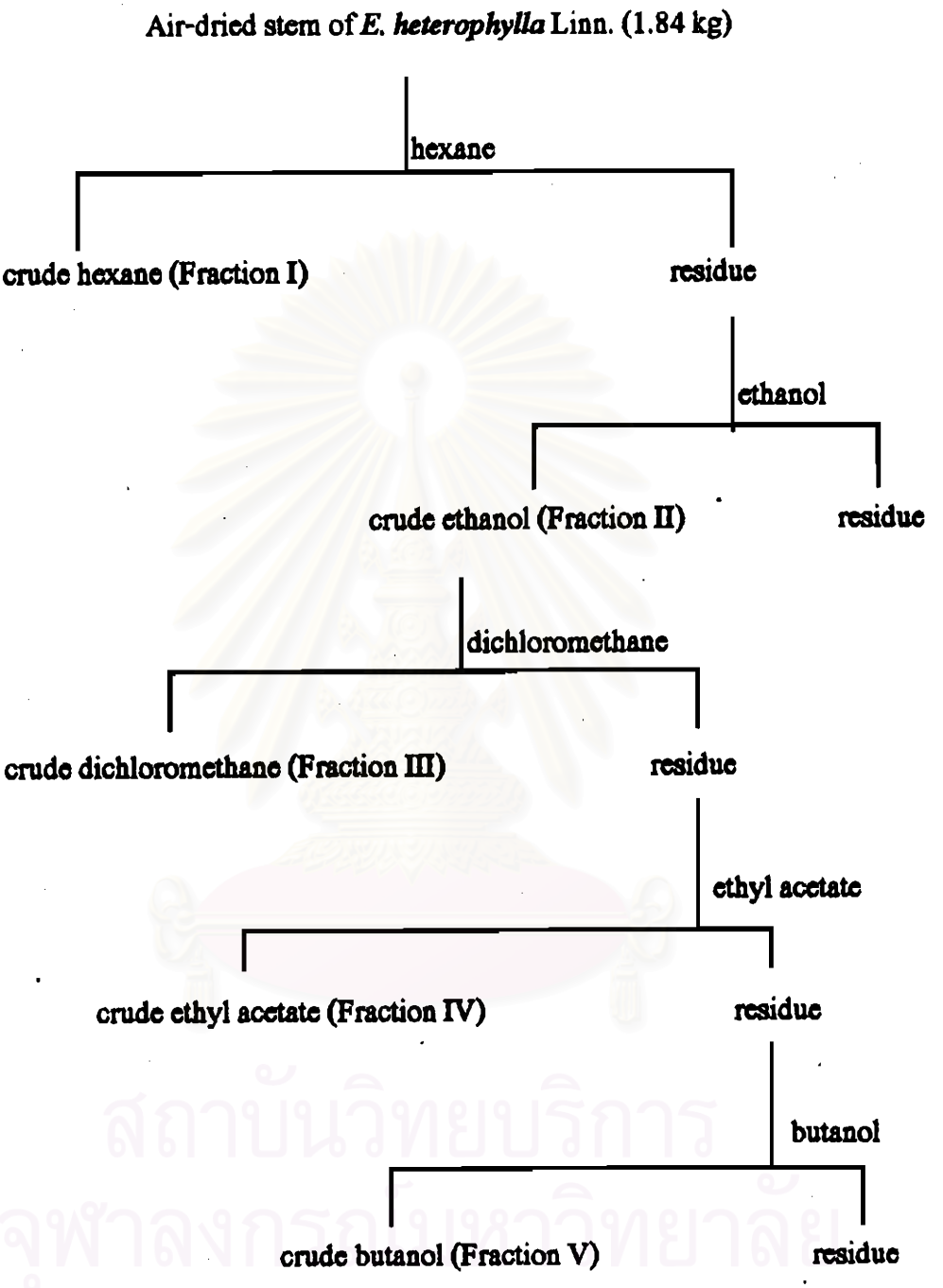
Fig 3.2 Inhibitory effect of ethanolic crude extract on rice leaf sheath growth (at 1.0 g/3 ml)

The ethanolic crude extracts of the stem of *E. heterophylla* showed 100% inhibition at 1.0 g per solvent 3 ml of both root length and leaf sheath of rice seeds. Then the stem of *E. heterophylla* was selected for further studied with the aim to search for plant growth inhibition compounds.

Searching for Rice Growth Inhibition from *E. heterophylla* Linn.

3.4 Extraction and Initial Fractionation of *E. heterophylla* Linn.

The stems of *E. heterophylla* Linn. were extracted by the procedure described in Chapter II. The results of extraction and initial fractionation can be summarized as showed in Scheme 3.1



Scheme 3.1 Extraction and fractionation of ***E. heterophylla*** Linn.

3.5 Plant Growth Inhibition Activity Test.

Each crude extract of the stems of *E. heterophylla* (from 3.4) were preliminarily bioassayed for plant growth inhibition activity on rice (*Oryza sativa* cv. RD 23) by the procedure described in Chapter II except fraction V because the butanol crude extract was too small in quantity for this bioassay. The results are showed in Table 3.11 and Fig 3.3 and 3.4.

Table 3.11 The growth inhibition activity on rice (*Oryza sativa* cv. RD 23)

Fraction (solvent extract)	rice part	% inhibition of difference concentration*		
		1.0	0.5	0.1
I (hexane)	root	51.60	38.77	23.26
	leaf	-16.30	-2.72	-28.26
II (ethanol)	root	100	-	95.30
	leaf	100	-	-176.92
III (dichloromethane)	root	100	100	93.63
	leaf	100	100	5.84
IV (residue)	root	-	-	88.73
	leaf	-	-	25.15
V (butanol)	root	n	n	n
	leaf	n	n	n

* g/3 ml

Note :- rice seeds have a fungi over

n not tested

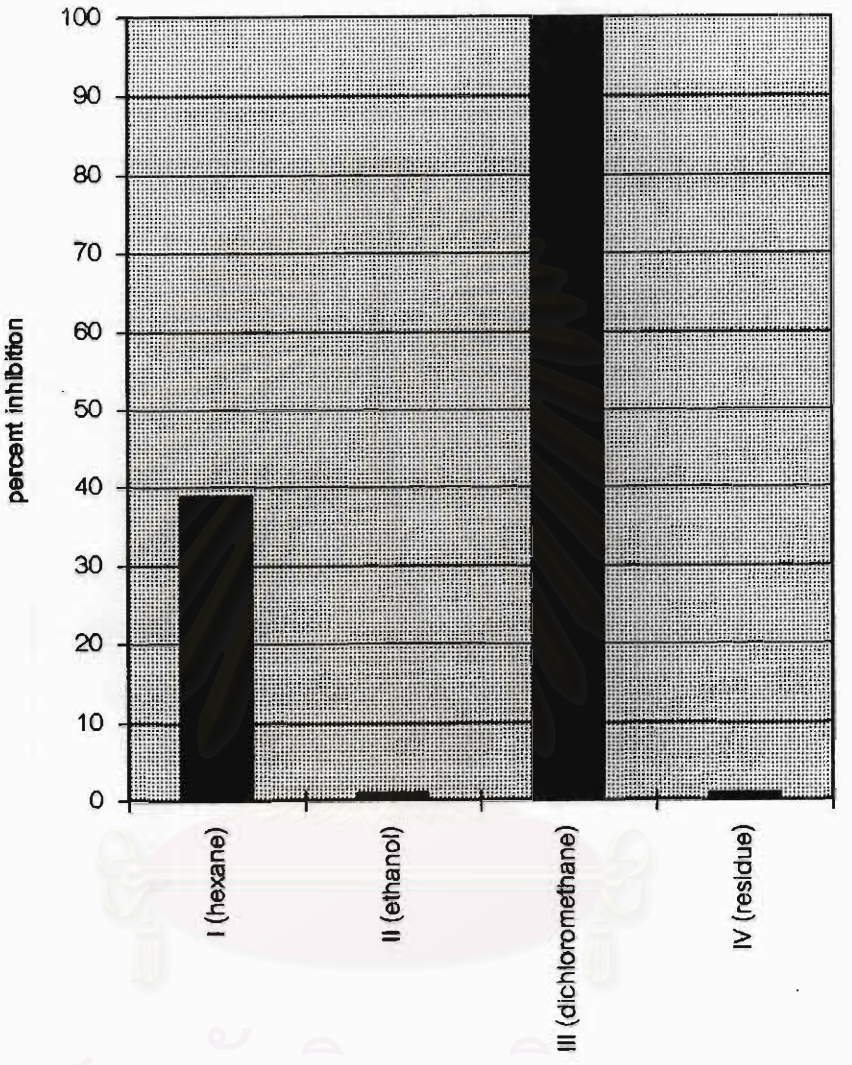


Fig. 3.3 Inhibitory effect of solvent extraction of *E. heterophylla* on root growth of rice (at 0.5g/3 ml.)

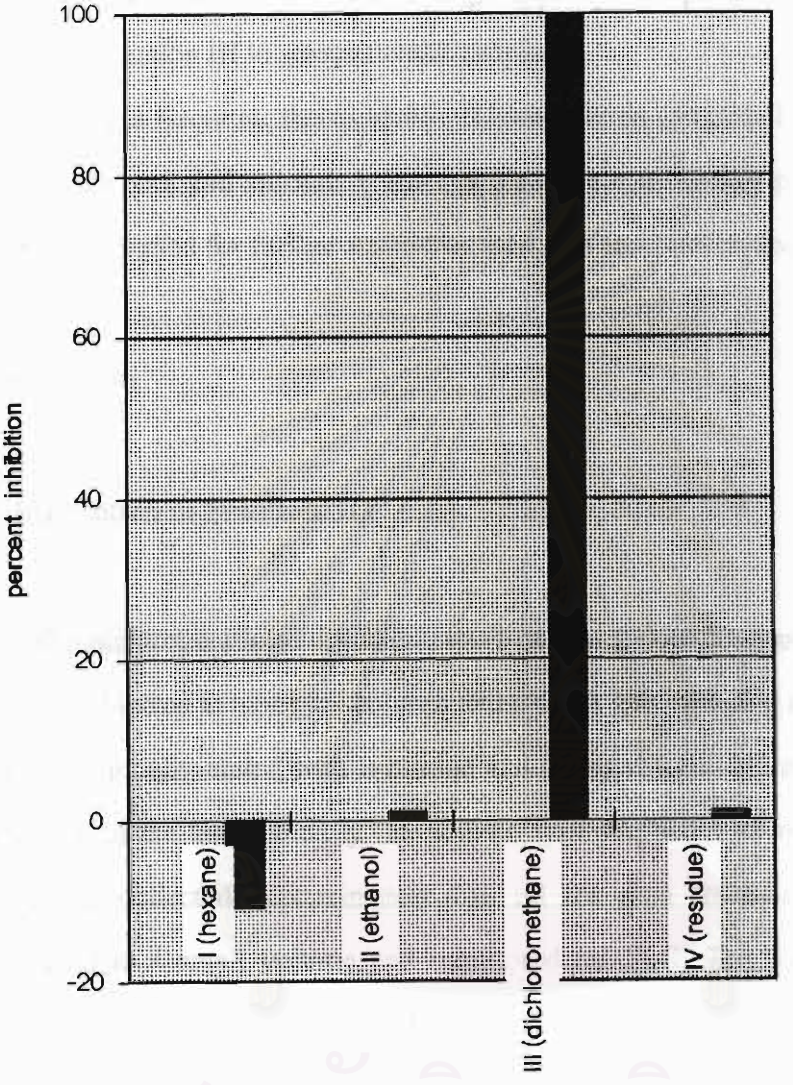


Fig. 3.4 Inhibitory effect of solvent extraction of ***E. heterophylla*** on leaf sheath growth of rice (at 0.5g/3 ml.)

From the result of rice growth inhibition Fraction III gave 100% inhibition of both root and secondary leaf sheath at 0.5g/ 3 ml. of crude extract. Then it was isolated and purified Fraction III to search active compound.

Among a fractions, the dichloromethane fraction (fraction III) showed strongest effect on both root and secondary leaf sheath growth of testing plant, 100%. So, this fraction was selected for further studied on isolation and purification.

3.6 Separation

3.6.1 Separation of Fraction III

The dichloromethane crude extract (Fraction III) 40.53 g as viscous dark green liquid was subjected to silica gel column using silica gel 486.0 g as an adsorbent. The column was initially eluted with *n*-hexane and changed to dichloromethane by gradual introduction of the latter. Finally the column was stripped with methanol. The eluted solution was collected approximately 250 ml for each fraction. Each portion was concentrated to a small volume and monitored by TLC. The fractions that showed similar components were combined. The results of separation of fraction III are showed in Table 3.12

Table 3.12 The results of the separation of Fraction III

Eluents	Fraction No.	Remarks	weight (g)
hexane	1-15 (III A)	viscous green liquid	1.48
5-30%CH_2Cl_2 in hexane	16-18 (III B)	white solid and pale yellow solid	2.42
30% CH_2Cl_2 in hexane	19-25 (III C)	white wax and white solid	2.01
30-60%CH_2Cl_2 in hexane	26-53 (III D)	dark green semisolid	10.14
60-80%CH_2Cl_2 in hexane	54-73 (III E)	viscous green liquid	9.89
80% CH_2Cl_2 in hexane	74-86 (III F)	viscous green liquid	3.22
100% CH_2Cl_2	87-95 (III G)	viscous green liquid	2.59
2% MeOH in CH_2Cl_2	96-103(III H)	viscous green liquid	1.15
5% MeOH in CH_2Cl_2	104-114(III I)	pale green liquid	1.04
20% MeOH in CH_2Cl_2	115-124 (III J)	pale green liquid and dark green oil	4.26

3.6.2 Rice Growth Inhibition Activity of Fraction III

Each small fraction derived from the separation of Fraction III was further subjected to rice growth inhibition bioassay experiments at dose level 10, 100, 1,000 and 10,000 ppm. The result of rice growth inhibition activity are reported as shown in Table 3.5, Fig. 3.5 and 3.6.

Table 3.13 Effect of various fraction from Fraction III (III A - III J) on rice growth

Fraction	rice part	% inhibition of difference concentration *			
		10,000	1,000	100	10
III A	root	44.55	-10.47	-21.63	-65.12
	leave sheath	-6.47	-7.42	-10.55	-21.69
III B	root	7.26	8.86	9.84	-2.49
	leave sheath	13.72	3.01	1.26	-5.91
III C	root	59.98	40.25	25.55	3.27
	leave sheath	63.32	25.59	10.05	1.12
III D	root	9.35	5.13	6.15	-9.90
	leave sheath	-5.87	-7.45	-10.55	-17.41
III E	root	31.20	11.27	-8.88	-11.15
	leave sheath	4.85	-8.61	-17.41	-17.58
III F	root	53.65	15.60	11.17	-7.50
	leave sheath	10.34	-2.74	-12.31	-23.07
III G	root	41.35	0.35	-0.90	-7.63
	leave sheath	15.40	-7.84	-10.97	-11.92
III H	root	36.67	18.99	14.68	8.65
	leave sheath	28.13	21.52	17.02	1.56
III I	root	31.54	7.82	7.56	5.21
	leave sheath	44.12	39.65	15.69	2.69
III J	root	42.03	34.69	28.68	8.99
	leave sheath	32.09	28.65	11.06	9.68

* ppm

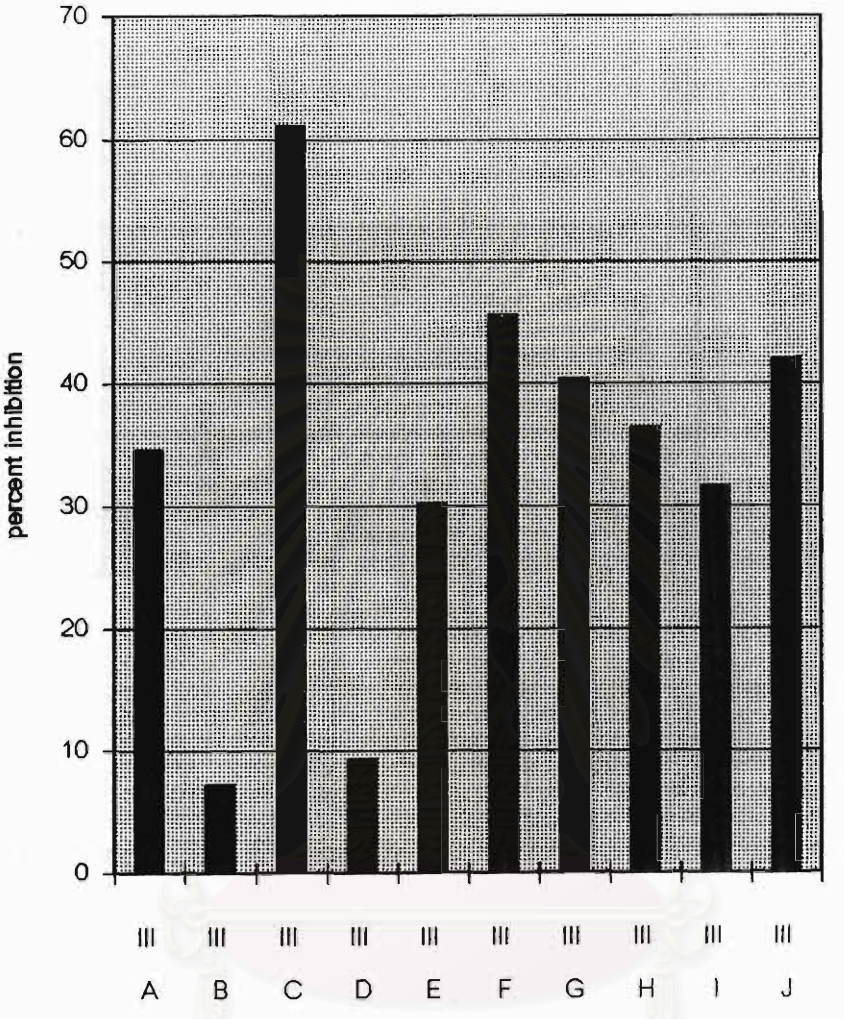


Fig. 3.5 Inhibitory effect of various fraction of dichloromethane extract on rice root growth (at 10,000 ppm)

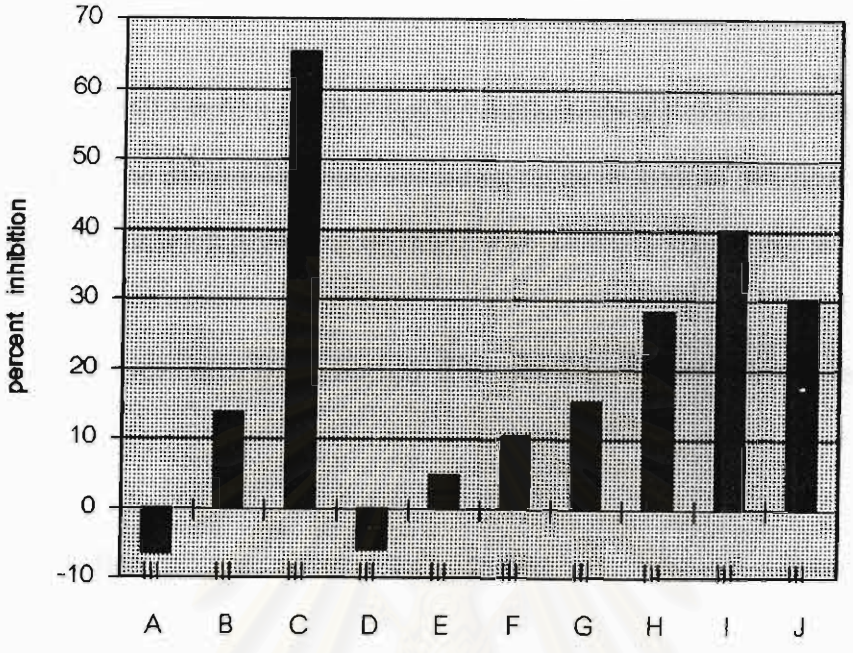


Fig. 3.6 Inhibitory effect of various fraction of dichloromethane extract on rice leaf sheath growth (at 10,000 ppm)

3.6.3 Brine Shrimp Bioassay Experiments of Fraction III

Each small fraction of the dichloromethane crude extract (Fraction III) was screened for Brine Shrimp Bioassay according to the procedure described in Chapter II. The bioassay results are presented in Table 3.14.

Table 3.14 The results of Brine Shrimp Bioassay Experiment of Fraction III

Fraction	LC_{50} µg/ml	Bioactivity
III A	nc	-
III B	45.37	medium activity
III C	9.09	high activity
III D	57.79	medium activity
III E	52.21	medium activity
III F	nc	-
III G	nc	-
III H	70.98	medium activity
III I	142.21	low activity
III J	211.07	low activity

Note : nc not calculated

LC_{50}	0 - 10	high activity
	11 - 100	medium activity
	more than 100	low activity

From the results of rice growth inhibition activity and brine shrimp cytotoxicity experiments of crude dichloromethane (Fraction III), Fraction III C showed the highest percent inhibition of both leaf sheath and root length of rice (63.32 and 59.98 %) at 10,000 ppm. and showed high activity of brine shrimp cytotoxicity test (LC_{50} 9.09 μg/ml). Then Fraction III C was continue on separation, purification bioassay.

3.6.4 Separation of Fraction III C

According to the rice growth inhibition (Table 3.5) and brine shrimp cytotoxicity test(Table 3.6). Fraction III C 1.80 g was subjected to TLC and exposed to UV light. There were 2 spots which absorbed UV light (Fig.3.7). This fraction had a mixture of white wax and pale yellow liquid. It was purified by crystallization with ethanol and hexane. The pale yellow liquid was soluble in ethanol. After recrystallization with ethanol and gave Compound 1 (41 mg). It was white powder with 203-205°C melting point. The white wax was soluble in hexane and recrystallization with hexane to gave Compound 2. In order to monitot the rice growth inhibition activity and brine shrimp cytotoxicity , Compound 1 and Compound 2 were resubjected to the bioassay experiments. The results of the rice growth inhibition bioassay of Compound 1 and Compound 2 were recorded in Table 3.15, Fig. 3.7 and 3.8,and the results on brine shrimp cytotoxicity test of Compound 1 and Compound 2 were showed in Table 3.16.

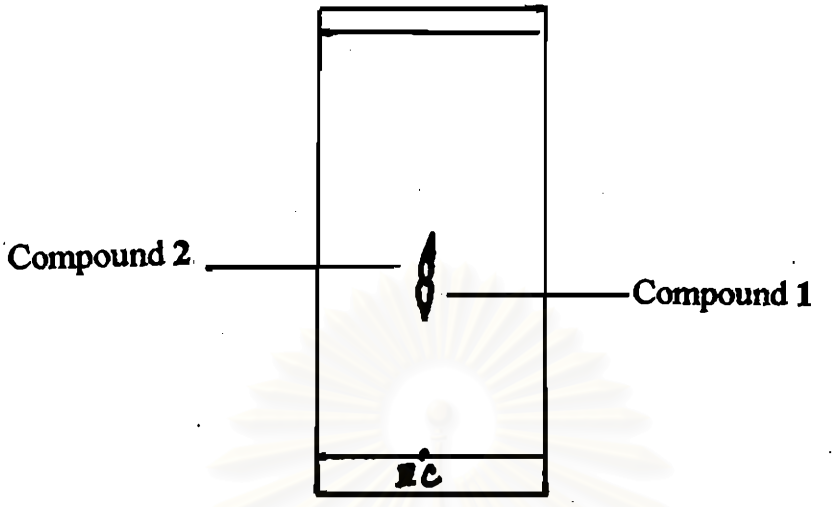


Fig. 3.7 TLC spots of fraction III C

Table 3.15 Effect of Compound 1 and Compound 2 on growth of rice.

Compound	rice part	% inhibition of different concentration*			
		10,000	1,000	100	10
Compound 1	root length	-23.00	-20.73	-12.25	-3.63
	leaf sheath	-22.29	-14.87	-12.28	-10.76
Compound 2	root length	55.54	9.27	-0.62	-7.04
	leaf sheath	39.64	19.33	14.83	7.17

* ppm

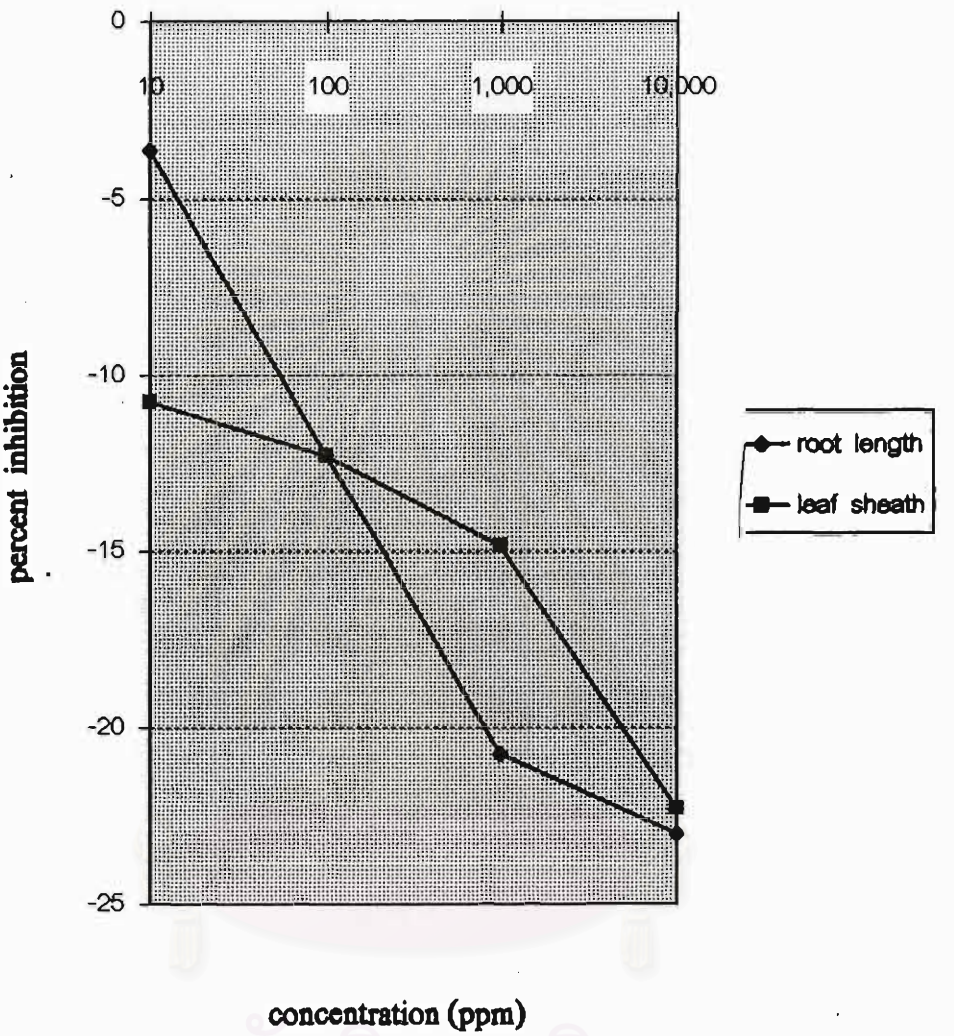


Fig. 3.8 Inhibitory effect of Compound 1 on root length and leaf sheath of rice

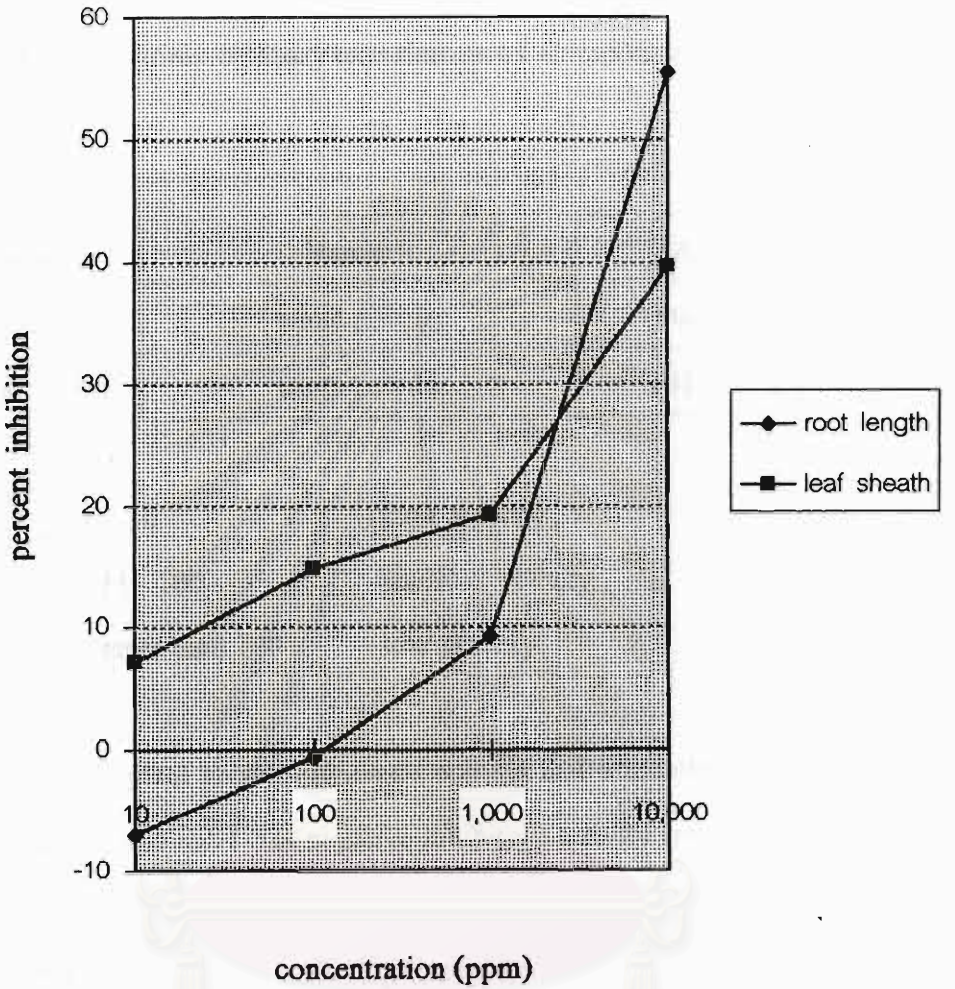


Fig. 3.9 Inhibitory effect of Compound 2 on root length and leaf sheath of rice

Table 3.16 Brine shrimp bioassay experiments of Compound 1 and Compound 2.

Compound	Remark	LC_{50}	Activity
Compound 1	white powder	40.37	medium
Compound 2	white powder	101.57	low

LC_{50}	0 - 10	high activity
	11 - 100	medium activity
	more than 100	low activity

From the plant growth inhibition activity and brine shrimp cytotoxicity test, it showed that Compound 1 has negative inhibitory activity (promotion) at the test dose (10,100,1,000 and 10,000 ppm.) on rice growth. However it showed moderately toxic to brine shrimp with LC_{50} = 40.37 μg/ml. But Compound 2 has positive inhibitory activity (inhibition) at the test dose (10,100,1,000 and 10,000 ppm.). And it showed slightly toxic to brine shrimp with LC_{50} = 101.57 μg/ml. So, Compound 1 and Compound 2 were further studied on structure elucidation.

3.7 Structure Elucidation of Compound 1

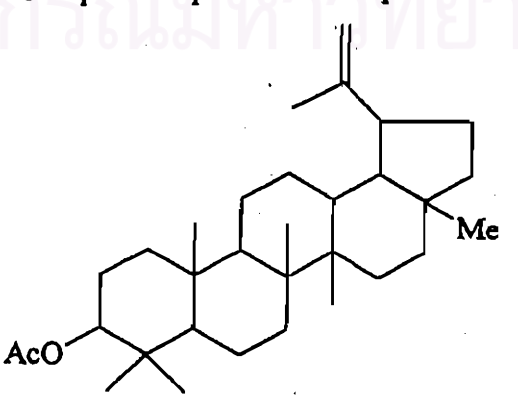
Compound 1 (41 mg) was isolated from dichloromethane crude extract by eluting with 30% dichloromethane in hexane. After recrystallization with ethanol a white powder of melting point 203-205 °C was obtained.

The IR spectrum (Fig. 3.10) of this compound gave 2850-3100 cm^{-1} of C-H stretching and 1730 (C=O stretching vibration of acetate) and 1250 cm^{-1}.

The ^{1}H NMR spectrum ($CDCl_3$) of Compound 1(Fig.3.11) displayed signals at δ 0.17-0.96 ppm (signal of methyl protons) ;1.20-1.60 (methylene protons); 4.55, 4.68(signals of 2H olefinic protons) and 2.01 (a methine proton attached to an acetyl group).

The ^{13}C NMR spectrum (Fig. 3.12) exhibited the carbonyl carbon signal at 170.97 ppm and the olefinic carbon signals at 150.91 and 109.35 ppm. There were other signals around 55.39 to 14.51 ppm which were the signals of methyl, methylene, methine and quarternary carbons. The comparison of the ^{13}C NMR chemical shifts of lupeol acetate (Warinthorn,1988) and those of Compound 1 are presented in Table 3.17.

Since all spectroscopic data of Compound 1 were agreeable to the reported spectrs of lupeol acetate. So Compound 1 proved to be lupeol acetate.



lupeol acetate

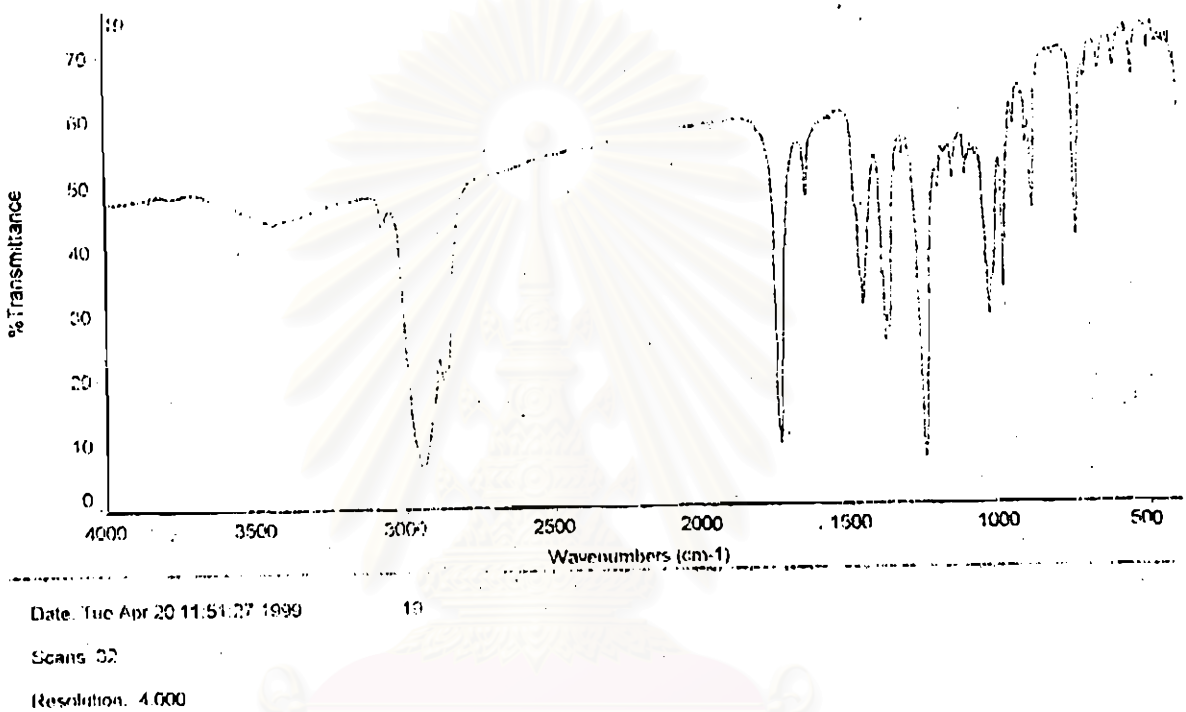


Fig. 3.10 IR spectrum of Compound 1.

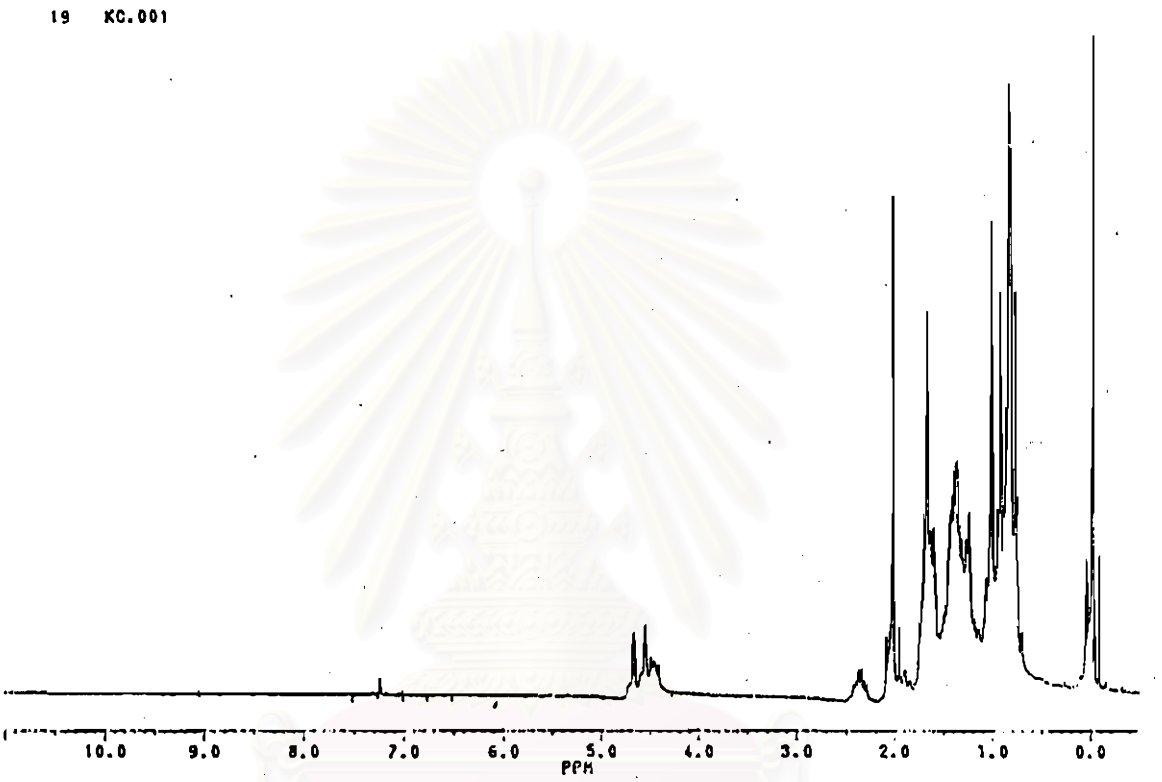


Fig.3.11 The ^{1}H NMR spectrum of Compound **1**.

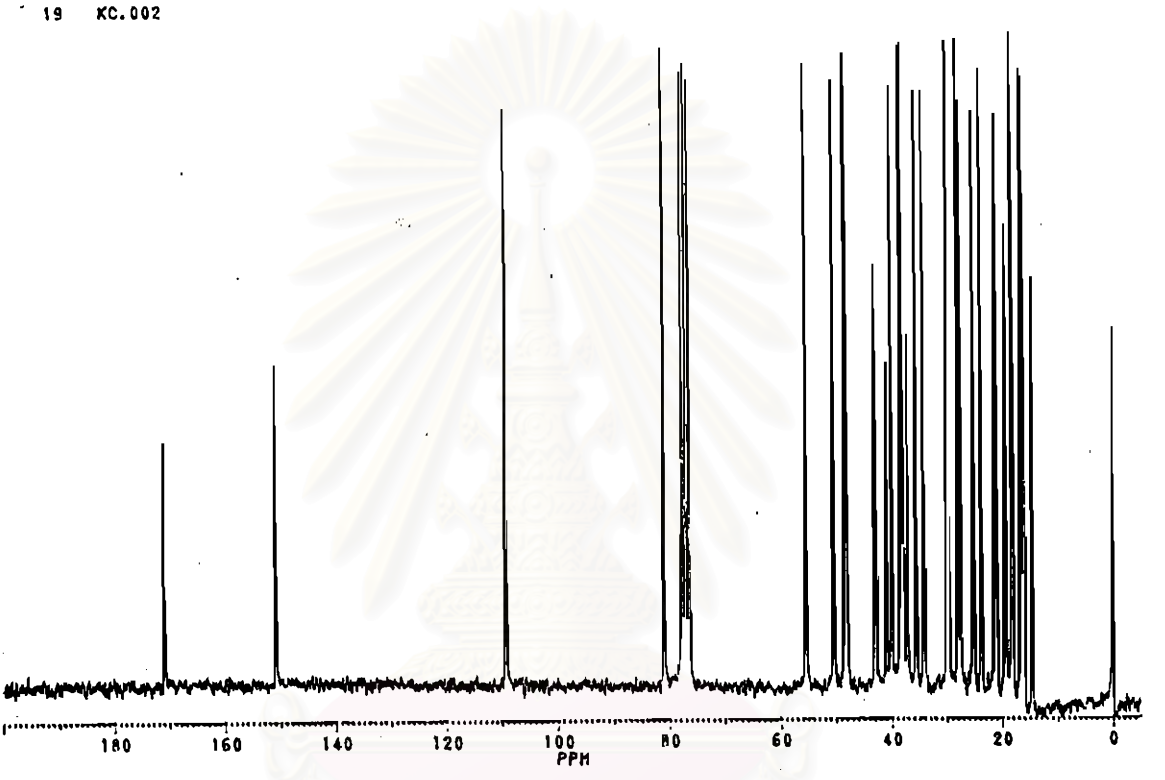


Fig. 3.12 The ^{13}C NMR of Compound 1.

Table 3.17 The ^{13}C NMR chemical shift assignments of lupeol acetate and Compound **1**

Carbon	Chemical shifts (ppm.)	
	lupeol acetate	Compound **1**
1	38.0	38.0
2	27.4	27.4
3	80.9	80.9
4	38.4	38.3
5	55.3	55.3
6	18.2	18.2
7	34.2	34.2
8	40.8	40.8
9	50.3	50.3
10	37.1	37.0
11	20.9	20.9
12	25.0	25.1
13	38.0	38.0
14	42.8	42.8
15	27.4	27.4
16	35.5	35.5
17	42.8	42.8
18	48.2	48.2
19	48.0	48.0
20	150.8	150.9
21	29.8	29.8
22	39.9	40.0

Table 3.17 (cont.)

Carbon	Chemical shifts (ppm.)	
	lupeol acetate	Compound 1
23	27.9	27.9
24	15.9	15.9
25	16.5	16.4
26	16.2	16.1
27	14.5	14.5
28	18.0	18.0
29	109.3	109.3
30	19.2	19.2
$-O_2C$	170.9	170.9
$CH3-CO_2$	21.2	21.3

3.8 Structure Elucidation of Compound 2

Compound 2 (48 mg) was isolated from dichloromethane crude extract by eluting with 30% dichloromethane in hexane. After recrystallization with hexane a white powder of melting point 168-170 °C was gained. This compound was tested with Liebermann-Burchard reagent and showed a red color, which is the characteristic of the presence of a triterpenoid structure.

The IR spectrum(Fig 3.13) of this compound showed characteristic absorption peaks at 2970 and 2830 cm^{-1} of C-H stretching vibration of CH_2 and CH_3, 1740 cm^{-1} of carbonyl (C=O) stretching of ester, 1480 and 1375 cm^{-1} of C-H bending of CH_2 and CH_3 and 1250 cm^{-1} of C-O stretching of acetate.

The ^1H NMR spectrum ($CDCl_3$) of Compound 2 (Fig. 3.14) exhibited the olefinic protons at δ 5.03 and 4.49 ppm. The proton signals of methyl group appearance at δ 0.75-1.05 ppm and a proton signal at δ 2.14 ppm should belong to an acetyl proton.

The ^{13}C NMR spectrum(fig. 3.15) of this compound showed about 32 carbon signals and displayed four olefinic carbons at δ 151.0, 139.6, 124.3 and 109.3 ppm. The carbon signals at δ 171.0 ppm should be the carbon of carbonyl belonging to an acetyl group. Other signals around 55.3 to 15.7 ppm ought to be methyl, methylene, methine and quarternary carbons.

From all spectroscopic data, this compound was proposed to be a triterpenoid with an acetyl group and two double bonds in the skeleton.

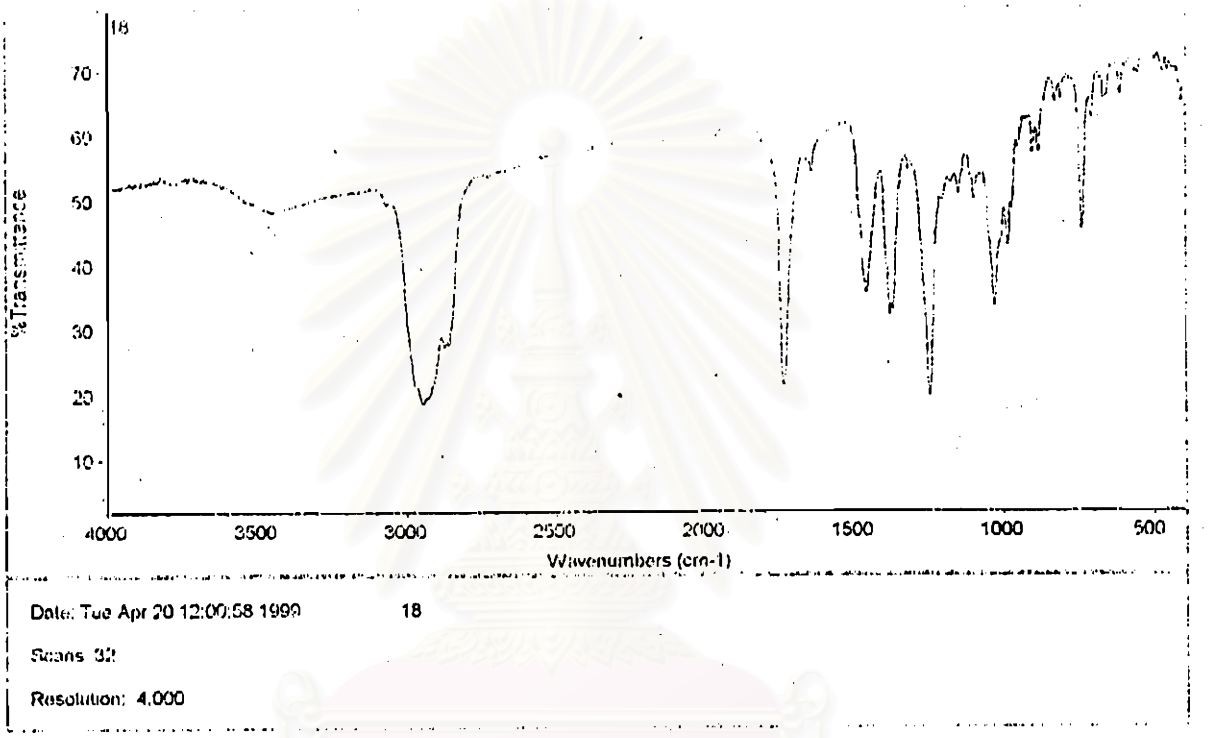


Fig. 3.13 IR spectrum of Compound 2.

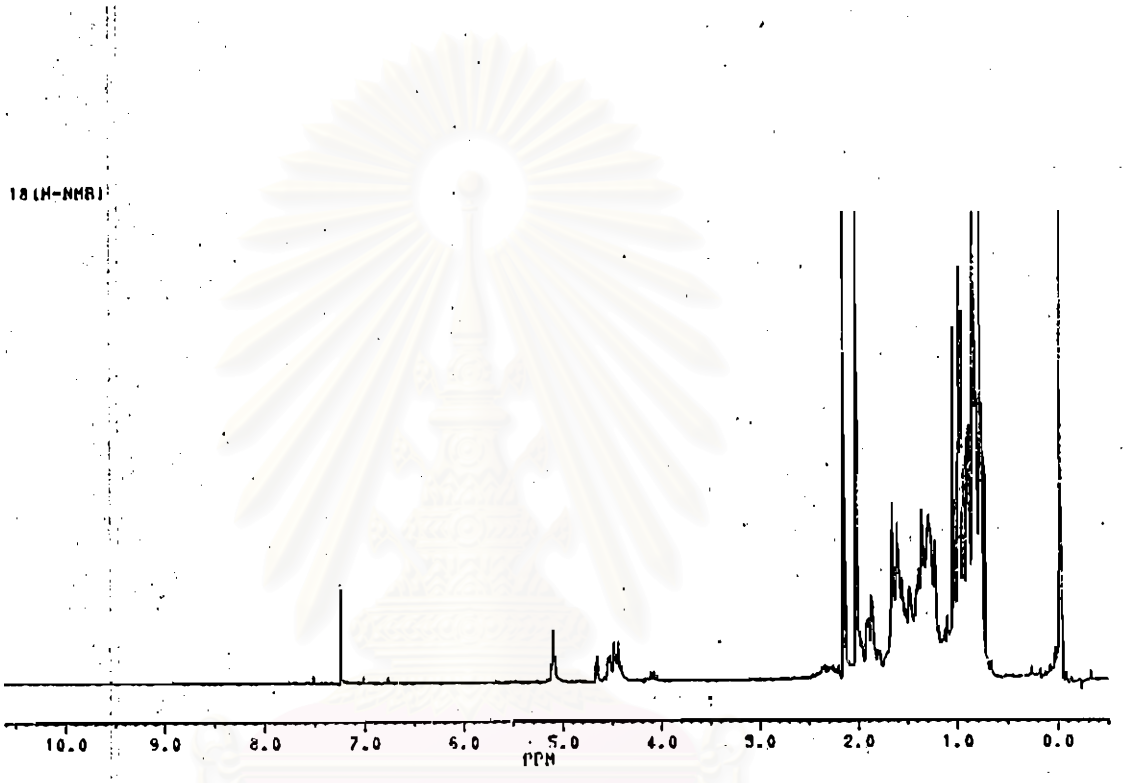


Fig.3.14 The ^{1}H NMR spectrum of Compound 2.

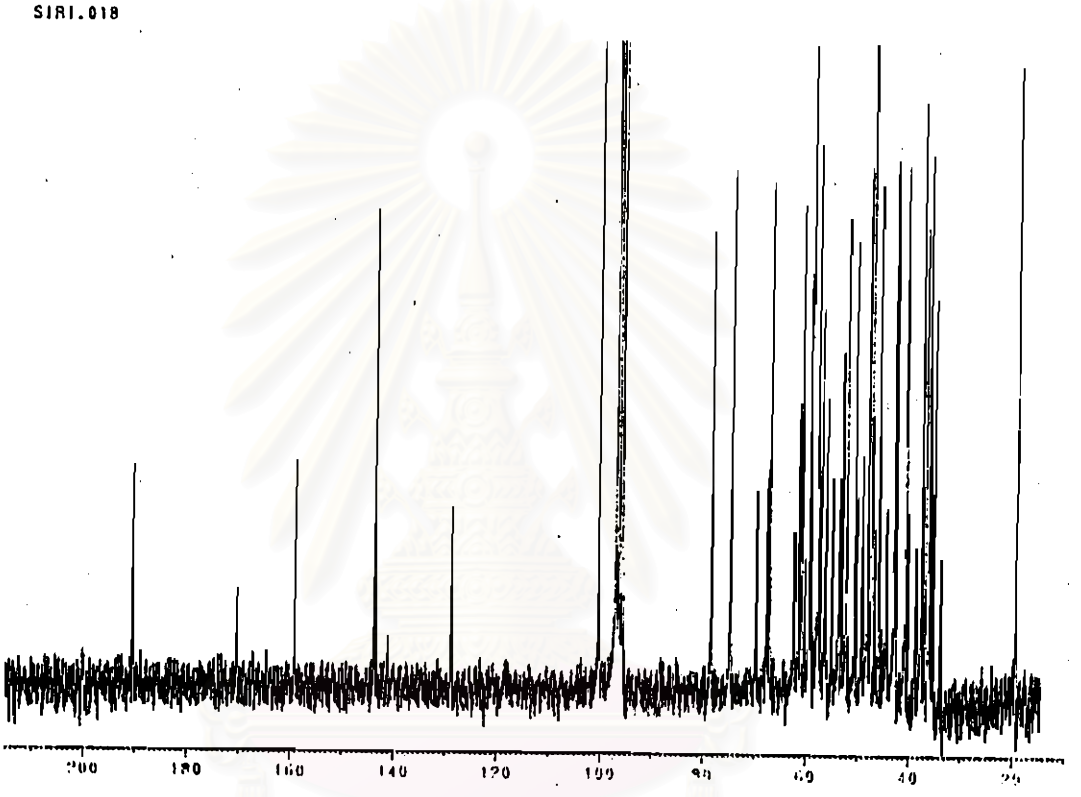


Fig. 3.15 The ^{13}C NMR of Compound 2.